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Fertilization of *Epichloë typhina* stromata by mycophagous slugs

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Abstract: *Epichloë typhina*, a fungal endophyte of cool season grasses, is heterothallic and an obligate out-crosser. In areas of endemism, its spermatia are moved between stromata of the two opposite mating types through egg-laying activities of *Botanophila* flies. In western Oregon, where the fungus was inadvertently introduced into seed-production fields of *Dactylis glomerata* (= orchardgrass, cocksfoot), flies do not appear to be the sole vectors for *E. typhina* fertilization. Here we examined the role of the common agricultural slug pest *Deroceras reticulatum* and mycophagous slug species *Prophysaon andersoni* and *Arion subfuscus* in *E. typhina* spermatia transfer. Frass from *P. andersoni*, *A. subfuscus* and *D. reticulatum* fed stromata of one mating type was transferred to stromata of the opposite mating type, resulting in 100%, 93% and 25% stromata fertilization respectively. An experiment designed to mimic field conditions examined stromata fertilization on *E. typhina*-infected plants of opposite mating type in the presence of slugs. Treatments with *P. andersoni* and *D. reticulatum* had greater stromata fertilization compared to the no-slug control, but the slug treatments were not different. This appears to be the first report of mollusks vectoring viable spermatia leading to the cross fertilization of stromata of different mating types.

Key words: choke disease, Clavicipitaceae, *Dactylis glomerata*, fungal endophyte, Gastropoda, mollusks

Introduction

The fungal endophyte *Epichloë typhina* (Pers.: Fr.) Tul. (Ascomycotina: Clavicipitaceae) causes significant yield loss in *Dactylis glomerata* L. (= orchardgrass, cocksfoot) in the Willamette Valley, Oregon, USA, (Pfender and Alderman 2006) and can dramatically shorten from decades to years the time a field is kept in production. *Epichloë typhina* first was recorded in Oregon in 1996 and likely was introduced from Europe where it is native (Alderman et al. 1997, Pfender and Alderman 1999). By 2000, approximately 90% of orchardgrass seed-production fields in Oregon were host to the fungus (Pfender and Alderman 2006). It appears that seed yield loss is proportional to the percentage of flowering tillers choked (Large 1954, Pfender and Alderman 2006).

During the vegetative growth phase of the host plant, growth of *Epichloë* hyphae is intercellular with little to no penetration of the host cell wall (Christensen et al. 2002). When the host grass enters the reproductive phase, branched hyphal masses (stromata) form epiphytically on and within grass culms and occasionally on vegetative tillers (Schardl 1996, Christensen et al. 2008). The developing inflorescence is encased in a dense fungal growth that develops into a stroma about 5–10 cm long. Growth of the stroma mechanically inhibits grass inflorescence development and production of viable grass seed; this syndrome is commonly known as choke (Kirby 1961, Bucheli and Leuchtmann 1996). Conidia produced on stromata function as spermatia and are of one of two mating types. It appears that only one mating type is found within a host plant (Schardl 1996). Transfer of spermatia to stromata of the opposite mating type is required for fertilization. After fertilization there is a proliferation of white fungal hyphae leading to a thickening of the stroma and eventual formation of perithecia and ascospore development.

Female flies in the genus *Botanophila* (Diptera: Anthomyiidae) transfer viable spermatia from one stroma to another during female feeding, defecation and egg laying (Kohlmeyer and Kohlmeyer 1974, Bultman et al. 1995, Bultman et al. 1998). This has been recognized as the primary means of fertilization in *Epichloë* under natural conditions and has been considered to be one of obligatory mutualism (Bultman et al. 2000). However recent studies in commercial seed-production fields have documented that most stromata in infested fields are fertilized even when the density of *Botanophila* flies are variable to absent (Rao and Baumann 2004; Górzyńska et al. 2010, 2011). Other mechanisms of stromata fertilization have been described recently, explaining this lack of relationship. Ascospores released from early maturing stromata can fertilize late emerging stromata (Alderman and Rao 2008). Spermatia dislodged from a stroma by air pressure-driven water mist, mimicking wind-blown rain, also can fertilize adjacent stromata (Rao et al. 2012). Spermatia are not carried by wind (Bultman et al. 1995).

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Another potential biotic vector of spermatia is mollusks. Speer and Dutin (1980) found that the conidia of the bark fungus *Dichaena rugosa* had to pass through the alimentary canal of slugs to germinate. Slugs are serious pests in the grass seed-cropping systems in the Willamette Valley, particularly on emerging grass seedlings (Gavin et al. 2008, Anderson et al. 2010, Mellbye et al. 2011). The introduced *Deroceras reticulatum* (Müller) (Agriolimacidae), known as either the gray garden slug or gray field slug, is the most common slug in Willamette Valley agronomic systems. Other slug species, particularly those in the genus *Arion* (Arionidae), are present in smaller numbers in many fields (Dreves and Fisher 2012).

When new stromata are being produced in *D. glomerata* seed-production fields, the native slug *Prophysaon andersoni* Cooper (reticulated taildropper) (Arionidae) and the introduced slug *Arion subfuscus* Draparnaud (dusky Arion) are found on stromata approximately 80% of the time during night feeding. Slug frass is seen occasionally on stromata. Frass from slugs collected from stromata always contained stromal material and usually spermatia. Stromal material and spermatia often were found in frass from slugs collected from other plant parts (Hoffman and Rao 2013). *Deroceras reticulatum* is found on stromata much less commonly, and spermatia were found only in 25% or less of the frass samples from slugs collected from stromata and other plant parts (Hoffman and Rao 2013).

The first objective of this study was to determine whether spermatia in the frass of *P. andersoni*, *A. subfuscus* and *D. reticulatum* were viable and could lead to fertilization of stromata. Two frass-transfer experiments explored this potential. Because spermatia also could be carried between stromata on the outside of a slug, we documented spermatia presence in their slime trails. The second objective was to examine the potential for stromata fertilization by slug vectors under field conditions. We mimicked a field situation in a whole-plant stromata-fertilization experiment. Preliminary results of one frass-transfer experiment were presented in a symposium (Rao et al. 2012).

MATERIALS AND METHODS

Identification of stroma mating type.—Thirty-five infected plants (var. Potomac) were transplanted from the field into 7.6 L pots in spring 2007; the crowns were split in 2008 and repotted. Plants were over-wintered outside and brought into the greenhouse in the spring when the first stromata appeared. Approximately 50% plants were identified as being infected with mating type MAT1-1-1 or MAT1-2-1 DNA sequences from Oregon (Chung and Schardl 1997), with the techniques described in Kaser (2009). The mating type of *E. typhina* in the remainder of the plants was determined via the transfer of spermatia from stroma of known mating type to the stroma on plants of unknown mating type. Spermatia were transferred with cotton applicators, which are 100% effective at transferring spermatia between stromata (Kaser 2009). The subsequent proliferation of hyphal growth or lack thereof determined whether the donor and recipient stroma were of the same or different mating types. For the mating-type test transfers and the frass-transfer experiments, we limited the possibility of accidental contamination/fertilization of a stroma by spermatia from sources other than from the specified donor stroma. Flies in the glasshouse were of primary concern. When new stromata first appeared they were covered with glassine bags to exclude potential biotic vectors. After inoculation, the stromata again were covered with glassine bags until fertilization assessments were completed.

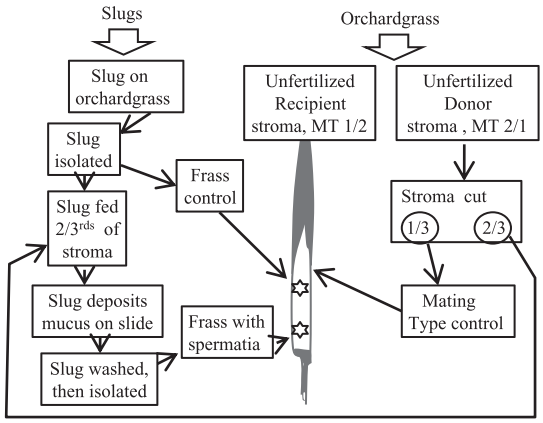

FIG. 1. Flow chart of the slug frass-stroma fertilization protocol. The protocol is begun with both the slug and orchardgrass (arrows). The donor and recipient stroma can be mating type 1 or 2 (MT). The three inoculations, frass with spermatia, frass control and mating-type control (opposite side of stroma from previous two), were made on the same recipient stroma. Inoculations were performed in the places indicated in diagram. Two-thirds of the donor stroma were fed to the slug and remaining piece held for mating-type control.

Prophysaon andersoni *and* D. reticulatum *frass-transfer experiment.*—This experiment compared the ability of *P. andersoni* and *D. reticulatum* to transfer viable spermatia in their frass. We ran 15 replicates with *P. andersoni* and 20 with *D. reticulatum*, the larger number for the later because of the greater variation in successful inoculations. Approximately half the replicates each were run in 2009 and 2010. The population of slugs was collected from orchardgrass field 1 (Hoffman and Rao 2013), held in a growth chamber at 7 C and fed on a combination of lettuce and wheat leaves for a week or longer. A replicate began with a slug isolated in a 9 cm diam Petri dish with a moist brown paper towel on the bottom and fed on orchardgrass leaves for 24 h. It was moved to a clean Petri dish and held without food for 12 h (FIG. 1). Frass deposited during this time was collected for

use as the control (frass without spermatia). The slug was allowed to feed on ⅔ of an unfertilized stroma from the donor plant for 12 h. One-third of stroma was held on moist paper towel for use in the mating-type control inoculation. After feeding the slug was placed on a glass slide where it deposited slime that potentially contained spermatia. The slime was stained with aniline blue lactoglycerin, and five observations for spermatia were made at 400× (number of spermatia per 0.152 mm^2). The slug was washed with a stream of distilled water and again isolated without food for 12 h. The frass deposited during this post stroma-feeding isolation was used to inoculate the recipient stroma (FIG. 1). The feeding sequence to obtain frass with spermatia occurred at room temperature, approximately 20 C. The stroma for the mating-type control and frass control were held at 7 C until inoculations.

The inoculation of a stroma on a plant occurred in a glasshouse. Material was transferred onto the recipient stroma with plastic coffee stirrers that were used for a single application. The frass control was in part a control for potential spermatia contamination during the frass-production and inoculation process. The mating-type control was used to confirm the previous mating-type determinations and ensure the donor spermatia and recipient stroma were viable. It was executed by scraping the saved donor stroma with the applicator and transferring the fungal material to the recipient stroma. If the post-feeding frass was large enough for material to remain after inoculation it was stained and the number of spermatia counted as above. A plant with stromata was used only once as a donor of spermatia and once as a recipient.

At greenhouse temperatures (18–25 C) initial hyphal growth indicating fertilization was visible 5–7 d post inoculation. We recorded the presence and extent of the spot of hyphal growth at approximately 7, 14 and 21 d post inoculation. Fertilization was categorized into three classes, strong, weak or lacking. Fertilization was considered strong if there was uniformly contiguous hyphal growth over the area where the frass was smeared. Weak fertilization was an initial spotty hyphal proliferation over this area. Hyphal growth usually extended no more than 2–3 mm beyond the area on the recipient stroma in contact with the applied frass and mating-type control. If the inoculation resulted in fertilization by 21 d the perithecia had begun to turn orange. In the one instance where the mating-type control showed no fertilization the replicate was repeated. Fisher's exact test (SAS 9.1) was used to compare the distribution of fertilization classes of the two slug species.

Arion subfuscus *frass-transfer experiment.*—After we found *A. subfuscus* feeding on stromata in 2010, we devised a stroma-fertilization test for this species that did not require using orchardgrass plants of known mating types in that these were being used in the previous experiment. The general protocol was similar to that illustrated (FIG. 1) with the following changes. Two centimeter-long pieces of stromata from four plants were used as donor stromata in each replicate. There were two recipient stromata, each from a different plant. The mating-type control came from scrapings of all four donor stromata. We did not look for spermatia in the slime of post-feeding *A. subfuscus*. There were 15 replicates of this design.

The theoretical proportion of recipient stromata, where both, one or neither were fertilized, was calculated based on the fact that a given donor or recipient stroma had a 0.5% chance of being either mating type 1 or 2. The total number of possible combinations of four donor stromata and two recipient stromata is 64. Assuming the slug frass contained sufficient spermatia from all four donor plants the probability that at least one of the recipient stroma would be fertilized is 0.969%. The probability of both of the recipient stromata being fertilized is 0.906%.

Whole-plant stromata fertilization experiment.—This test provides an estimate of the potential effect of slug-feeding behavior on stromata fertilization in a more natural situation. Orchardgrass plants of known *E. typhina* mating type, in 7.6 L pots, were over-wintered outside. As stromata began to form in the spring, plants were moved into a cold room to delay development and allow 10 replicates to be run over an extended period. Plants were moved to the glasshouse approximately 2 wk before the start of a replicate. Plants of the two mating types were kept in different glasshouse rooms as they developed stromata. Plants used had 15–20 stromata; those with an excess had stromata removed. A replicate (block) was composed of three fine-mesh cages (Bug Condo 4180, L45 × W45 × H90 cm; Hummert Internation, Earth City, Missouri 63045), with a plant of each mating type in each cage. Treatments were a cage with either six *P. andersoni*, six *D. reticulatum* or a no-slug control. The two pots in a cage were 8–10 cm apart. Tillers with stromata that could come in contact with stromata from the opposite mating type were removed.

Medium-sized (350–500 mg) *P. andersoni* and *D. reticulatum* slugs were held at 7 C and fed lettuce, young orchardgrass leaves and stromata in culture. One week before a replicate was initiated stromata were removed from the diet. Slugs were held without food at room temperature 12–24 h before the start of a replicate. Within a cage three slugs were placed in each of the two pots with plants for four nights. Slugs generally do not start climbing plants at night until water condenses on the leaves (G.D. Hoffman pers obs), so each evening the plants were misted with distilled water from above to minimize the potential of water-splash spermatia transfer (Rao et al. 2012). Cages were covered with black plastic until morning to maintain high humidity and to exclude light from adjoining rooms. After four nights the replicate was disassembled, the slugs collected and the soil surface treated with Durham metaldehyde granules 7.5 (AMVAC, Los Angeles, California) to kill slugs we could not find. Plants were segregated by mating type again and held 5–7 d at which time the percent of each stoma surface with hyphal proliferation (fertilization) was recorded for all stromata on each plant.

Because the number of stromata per plant varied, we used the sum of the percentage of stroma surface area fertilized on each stroma as the dependent variable (sum of the percentage of each stroma fertilized per plant). This variable could add up to greater than 100%. We did not

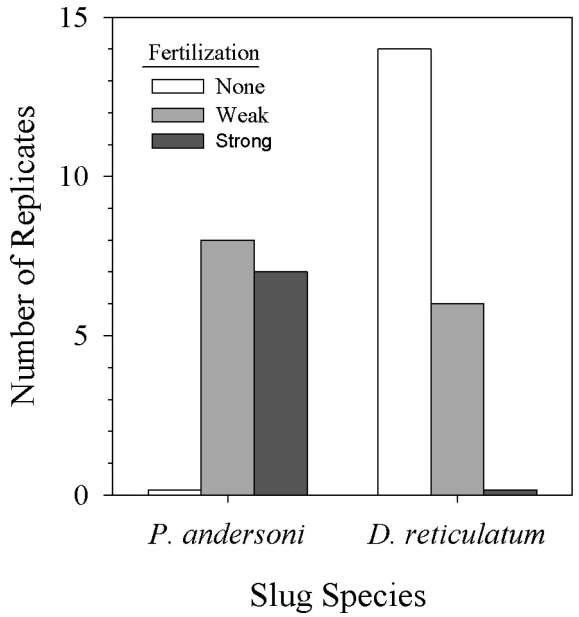

FIG. 2. Fertilization of *E. typhina* stroma by frass transferred from slugs feeding on stroma of the opposite mating type. Fertilization was categorized as: none = no evidence of fertilization, weak = initial spotty hyphal proliferation, or strong = uniformly contiguous hyphal growth. The slug species differences in fertilization was significant (Fisher's exact test, n = 35, $P < 0.0001$).

specifically design the experiment to test differences between *E. typhina* mating types, however the design we used fit a split-plot design, with slug species as the whole plot and mating type the subplot. Replicates were treated as blocks. We used a mixed model (SAS 9.1) for ANOVA, with variable*block as the error term. The data were square-root transformed for the analysis to normalize the residuals. The planned 10th replicate was not run because of poorly formed stromata. The eighth replicate was discarded because higher temperatures those 2 wk led to development of mature perithecia and potential for ascosporic fertilization of the stromata.

RESULTS

Prophysaon andersoni and *D. reticulatum* were able to fertilize *E. typhina* stroma via feeding and frass deposition (FIG. 2). *Prophysaon andersoni*, the more mycophagous species, had a greater proportion of replicates where the hyphal growth on the recipient stroma was ranked weak or strong and zero where there was no fertilization. Fertilization occurred in only six of the 20 *D. reticulatum* replicates. This between-slug species difference in fertilization was significant (Fisher's exact test, n = 35, $P < 0.0001$).

The prevalence and quantity of spermatia in the frass used to inoculate recipient stroma were much greater in the frass from *P. andersoni* than *D. reticulatum* (TABLE I). In the frass from *D. reticulatum*, spermatia were not detected in seven of the 17 samples examined. Slugs potentially can carry spermatia externally and effect spermatia transfer, however few spermatia were found in the mucus trial of slugs after feeding on stromata. Spermatia were found in low numbers in less than 50% of the slime-trail replicates from the *P. andersoni* and *D. reticulatum* experiment (TABLE I).

Using the modified frass-transfer protocol, *A. subfuscus* transferred spermatia and fertilized stromata in most replicates. In 11 of 15 replicates, both stromata were fertilized at the point of frass application and in three replicates one of the stromata was fertilized (TABLE II). The sum of the proportion of the single and both categories (0.933) was less than the theoretical value (0.969) (TABLE II, four donor plants column). We recalculated the theoretical probabilities of successful fertilization, assuming that the slugs ate only enough spermatia for effective fertilization from three of the four stromata. The distribution of these probabilities (TABLE II, 3 donor plants column) was not statistically different from the observed probabilities (Chi-square goodness of fit_2 = 0.92, $P = 0.6313$). Spermatia density in the frass of *A. subfuscus* cannot be compared statistically with that from the other two slug species because of the different protocols, but the density of spermatia was relatively higher (TABLE I).

In the whole plant experiment the main effect of slug treatment was significant ($F_{2, 14} = 6.96$, $P = 0.0080$). The back transformed variable, sum of the

TABLE I. Spermatia seen in frass after stroma-feeding and in the mucus trails

Species	Spermatia in frass			Spermatia in mucus trails		
	No. examined[a]	No. with spermatia	Spermatia counts[b]	No. examined	Mucus with spermatia	Spermatia counts[2]
D. reticulatum	17	10	6.0 ± 1.5	20	6	0.6 ± 0.2
P. andersoni	12	12	68.3 ± 7.1	14	6	0.3 ± 0.1
A. subfuscus	14	14	133.3 ± 23.3	0	—	—

[a] Replicates with frass remaining after inoculation of the recipient stroma.

[b] Mean ± SEM of the just the samples containing spermatia. Counts are number of spermatia per 0.152 mm^2.

TABLE II. Number and proportion of replicates where stromata were fertilized by frass from *A. subfuscus*

Fertilized stroma categories	Number observed	Proportion observed	Theoretical proportion if	
			spermatia from four donor plants	spermatia from three donor plants
Both (2)	11	0.733	0.906	0.812
Single (1)	3	0.200	0.063	0.125
Neither (0)	1	0.067	0.031	0.063

percentage of each stroma fertilized per plant, was 23.9, 115.5 and 205.7% respectively for the no-slugs control, *D. reticulatum* and *P. andersoni*. The two treatments with slugs were statistically different from the control (*D. reticulatum*: $t_{14} = 2.29$, $P = 0.0381$) and (*P. andersoni*: $t_{14} = 3.70$, $P = 0.0024$) but not from each other ($t_{14} = -1.41$, $P = 0.1815$). There was no difference in the extent of stromata area fertilized between the two mating types ($F_{1, 21} = 0.05$, $P = 0.8258$), and was the interaction between slug treatment and mating type was not significant ($F_{2, 21} = 0.28$, $P = 0.7555$).

DISCUSSION

In the two frass-transfer experiments, spermatia in the frass of *P. andersoni*, *A. subfuscus* and *D. reticulatum* that had fed on *E. typhina* stromata remained viable and capable of fertilizing other stromata. The two mycophagous slug species, *P. andersoni* and *A. subfuscus*, ate more of the donor stromata (G.D. Hoffman pers obs) and consequently had more spermatia in their frass. This led to a higher probability of cross mating-type fertilization of stromata. The data suggest that anytime the donor and recipient stromata were of opposite mating types, frass from these two species could affect fertilization. This appears to be the first report of mollusks vectoring viable spermatia leading to the cross fertilization of stromata of opposite mating types.

Fifty-nine percent of frass samples from *D. reticulatum* contained spermatia, while only 30 percent of stromata were fertilized. The lower fertilization rate and weak fertilization of some replicates is likely due to the low number of spermatia in the *D. reticulatum* frass samples. In addition, spermatia were not uniformly distributed in the frass but instead occurred within semidiscrete boluses of stromal material (Hoffman and Rao 2013). Small numbers of spermatia could have been in the applied frass but not in contact with the recipient stroma.

The frass-transfer experiment addressed the capacity of spermatia in slug frass to fertilize stroma of the opposite mating type. It did not elucidate the probably of this event happening in the field where slugs have to move from the stroma consumed to a stroma of the opposite mating type and perhaps defecate where the frass can come in contact with the stroma. The whole plant-stromata fertilization experiment explored this probability. In the context of the experimental design, the activities of *D. reticulatum* and *P. andersoni* led to higher fertilization than in the no-slugs control. However the large difference between these two species in the frass-transfer experiment was not seen in the whole-plant experiment, where stromata fertilization in the *P. andersoni* cages was not significantly greater.

The feeding and frass excretion of the slugs probably accounts for the differences between the frass application versus whole-plant experiments. During night observations we did not see slugs feeding on stromata or other aerial plant parts as often as expected. In addition, it was rare to see slug frass directly on stromata. Slug feeding could have been suppressed by the environmental conditions of the cages. However, the rare occurrence of frass on the stromata matches field observations. While it is not unusual to see slug frass on stromata in the field, its frequency was small relative to the number of slugs seen on the stromata (pers obs). Thus the rare frass-to-stromata contact in the whole-plant experiment probably accounts for most of the difference between it and the frass-application experiment.

The large number of the less discrete areas of spotted fertilization led us to reassess the role of spermatia shed in slug slime. A portion of this spotted fertilization, particularly that on the no-slugs control plants, was due probably to the activities of sowbugs (Isopoda) and small flies present in and around the potted plants. Misting also could have played a role. However, there was more of the spotted fertilization pattern seen on stromata from cages containing slugs and the spermatia that initiated this fertilization could have been in the slug slime trails.

Studies of *Epichloë*grass host systems suggested that there is an obligatory mutualistic relationship between *Botanophila* and *Epichloë* spp. (Bultman and White 1988, Bultman et al. 1995). However, Rao and Baumann (2004) and Górzyńska et al. (2010, 2011) found high stromata fertilization in the absence of

Botanophila flies and high populations of the fly were not associated with higher stromata fertilization. Some of the difference between these results might be due to lower densities of stromata in wild populations of *Epichloë* hosts (Williams 1971, Leuchtmann 1992), thus requiring a long distance dispersing vector for effective fertilization. However, even in similar situations, Górzyńska et al. (2011) found no association between the *Botanophila* spp. and stromata fertilization in wild populations of *Puccinellia distans*.

Ascosporic fertilization as seen in *E. typhina* (Alderman and Rao 2008) has not been documented as having a role in other fungal mating-type cross fertilization systems, however it could account for the uniform fertilization of stromata in the *Botanophila* fly exclusion experiment of Rao and Baumann (2004). *Epichloë typhina* ascospores are 150–200 µm long (SC Alderman pers comm), while mosquito netting has 1.2 mm openings. Thus ascospores can easily pass through the cages. Fertilization by spermatia in the frass of *Botanophila* flies (Bultman et al. 1998) and slugs leave characteristics patterns of perithecia development rather than the uniform development seen in Rao and Baumann (2004) and many other orchardgrass fields.

Rao and Baumann (2004) discuss the decoupling of egg laying by *Botanophila lobata* and the cross fertilization of *E. typhina* in Oregon, where endemic flies and the introduced fungus and *D. glomerata* have not coevolved. The discovery of yet another noncoevolved vector of spermatia transfer suggests the Oregon situation is not unique. Mechanisms of stromata fertilization through the movement of spermatia by slugs, by spray (Rao et al. 2012) or fertilization of *E. typhina* stromata by ascospores (Alderman and Rao 2008) are perhaps common ways to circumvent the obligatory mutualistic relationship between the *Botanophila* fly and *Epichloë* documented in other *Epichloë*-grass host systems. These noncoevolved agents of stromata fertilization also might be active within the native range of *E. typhina*. Because of the diversity of mechanisms by which stromata fertilization can occur, it is unlikely that the spread of the pathogen can be restricted by eliminating any one mechanism, such as spraying orchardgrass fields to kill *Botanophila* flies.

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