Differences in teliospore germination patterns of *Microbotryum violaceum* from European and North American *Silene* species

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Microbotryum violaceum (anther-smut) from *Silene caroliniana* and *S. virginica* produced promycelia that frequently appeared to contain more than three cells according to the number of septations. This is in contrast to samples from *S. latifolia* and to previous descriptions of this species. Apparent four-celled promycelia contained three nucleate cells and one anucleate zone. Anucleate zones were usually positioned between the first and third cells of the promycelium. Their presence was negatively correlated with the occurrence of intra-promycelial conjugation. Also, unlike isolates from *Silene latifolia*, isolates from *S. caroliniana* and *S. virginica* produced hyphae in the absence of an inducing agent.

INTRODUCTION

In the smut fungi, teliospore germination has been studied extensively because details of this process were considered to provide important taxonomic characters (Duran & Safeeulla 1968, Ingold 1983, 1999). In addition, there has been considerable interest in teliospore germination because events that occur during this process have important genetic and ecological consequences (Fischer & Holton 1957, Hood & Antonovics 2000). Because of this, smut fungi serve as model systems to investigate mating and sexual development in the basidiomycetes (Whitehouse 1951, Zambettakis 1977, Garber & Day 1985, Casselton & Olesnicky 1998).

A comprehensive series of papers on smut germination is presented by Ingold (i.e. 1983, 1984, 1989), and there are many additional descriptions from earlier decades (Kniep 1926, Hüttig 1931, Fischer & Holton 1957, Zambettakis 1977). In general, teliospore germination is characterized by the production of a septate promycelium (or basidium) in which meiosis occurs (Hood & Antonovics 1998, 2000). Each cell of the meiotic tetrad typically produces haploid sporidia (or basidiospores) that replicate by yeast-like budding. Mating between haploid cells of contrasting mating types is a prerequisite for infection, and this occurs in a variety of ways, but usually between cells of the promycelium or between sporidia (Fischer & Holton 1957).

Most studies of the anther-smut fungi have been carried out on samples from European hosts. We have investigated isolates that occur on the North American hosts, *Silene caroliniana* and *S. virginica*. Isolates from these hosts are clearly identified as *Microbotryum violaceum* according to the species key in Vánky (1998). While the isolates used in this study may not be separated using the criteria proposed by Vánky (1998) (e.g. host taxonomy, disease development, and spore morphology), future studies incorporating molecular biology methods are needed if taxonomic questions are to be addressed. However, we found that the teliospores germinate differently from previously described isolates of *M. violaceum* from Europe (Deml & Oberwinkler 1982, Ingold 1983, 1989, Hood & Antonovics 1998), and that this difference can influence mating behaviour. Here we describe germination of these two North American anther-smut fungi, and we compare them to the well-studied anther smut from the European host *Silene latifolia* (syn. *S. alba*).

METHODS AND MATERIALS

Anther-smut samples were obtained from two plants of *Silene caroliniana* subsp. *wherryi* (**USA**: *Kentucky*: Gilbert Creek) and two plants of *S. virginica* (**USA**: North *Carolina*: Durham, Duke Forest). Samples from two plants of an introduced population of *S. latifolia* were also included in this study (**USA**: *Virginia*: Giles Co., Clover Hollow). The collections were all made by M. E. H. and J. A. in June 1998. For purposes of clarity, these isolates are referred to here as special forms (i.e. *M. violaceum* f. sp. *caroliniana*, f. sp. *virginica*, and f. sp. *latifolia*). Little is known about host specialization of f. sp. *caroliniana* and f. sp. *virginica* on their respective hosts.

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All studies were carried out using fresh teliospores (stored under desiccation for no more than 1 week). Teliospores were sampled from diseased plants that had been collected from the field and were then grown under greenhouse conditions at Duke University (Durham, North Carolina) and the University of Virginia (Charlottesville, Virginia). Teliospore collections are stored and isolated sporidial lines preserved frozen in the University of Virginia's collections; selected sporidial lines have also been deposited in the American Type Culture Collection (ATCC).

To quantify cellular events during germination, teliospores were suspended in sterile deionized water plus a surfactant (Triton X-100) and incubated at 15 and 25 °C on 1.5% water agar and potato dextrose agar (PDA). Germination was observed after 24 h of incubation by adding a drop of lactophenol-cotton blue (Dhingra & Sinclair 1995). The number of apparent cells per promycelium (indicated by the number of septations) was recorded for 50 germinated teliospores per sample in random transects. Treatments were replicated three times with two smut samples from each host species. Nuclear distribution among the promycelial cells was determined using Giemsa stain (Dhingra & Sinclair 1995).

The position of anucleate zones within promycelia was determined for an isolate of f. sp. *caroliniana* and an isolate of f. sp. *virginica*. Anucleate zones were identified by their distinct morphology (see results below). Teliospores were incubated for 18 h at 25 ° in humid chambers on PDA-coated slides (*ca* 1 ml of PDA on each 75 × 25 mm glass microscope slides). A cover glass was placed on top of the spores prior to incubation so specimens could be observed under high magnification without disturbing the orientation of cells. Fifty promycelia with anucleate zones were identified and characterized in random transects.

The influence of anucleate zones on the frequency of conjugation between promycelial cells (intra-promycelial conjugation; Hood & Antonovics 1998) was determined for an isolate of f. sp. *virginica*. Teliospores were incubated on water agar for 16 h at 25 °, then for 8 h at 15 °. The frequency of intra-promycelial conjugation was determined in random transects for 50 promycelial with three cells and 50 promycelia with more than three cells. This test was replicated twice.

RESULTS

Promycelia of *Microbotryum violaceum* f. sp. *caroliniana* and f. sp. *virginica* frequently appeared to consist of more than three apparent cells (usually four, but rarely five or six), in contrast to f. sp. *latifolia*, which had no more than three cells. This pattern was more common under high nutrient and high temperature conditions. For f. sp. *caroliniana* on PDA at 25 °, 41% (standard error [SE] = 10.4, untransformed data; number of observations [*n*] = 300) of promycelia appeared to contain more than three cells, whereas on water agar at 25 °, 8% (SE = 1.2; *n* = 300) of promycelia appeared to contain more than three cells. For f. sp. *virginica* on PDA at 25 °, 20% (SE = 3.7; *n* = 300) of promycelia appeared to contain more than three cells, and on water agar at 25 °, 10% (SE = 1.2; *n* = 300) of promycelia appeared to contain more than three cells. At 15 ° on water agar or PDA, only two promycelia of f. sp. *caroliniana* or f. sp. *virginica* appeared to contain more than three cells. Promycelia from f. sp. *latifolia* were never observed with more than three cells under any of the conditions.

Table 1. Positions of anuncleate zones in promycelia of North American anther smut (*Microbotryum violaceum*) samples.

	4-celled promycelia			5-celled promycelia		
Special form	T-P	P-M	M-D	P-M&P-M	P-M&T-P	P-M&M-D
caroliniana	7	91	1	0	1	0
virginica	4	82	7	4	1	2

Positions are indicated as between nucleate cells that are labeled relative to the teliospore (T) as proximal (P), middle (M), and distal (D). Four-celled promycelia contained one anucleate zone. Five-celled promycelia contained two anucleate zones. Data are combined across replications ($n = 100$).

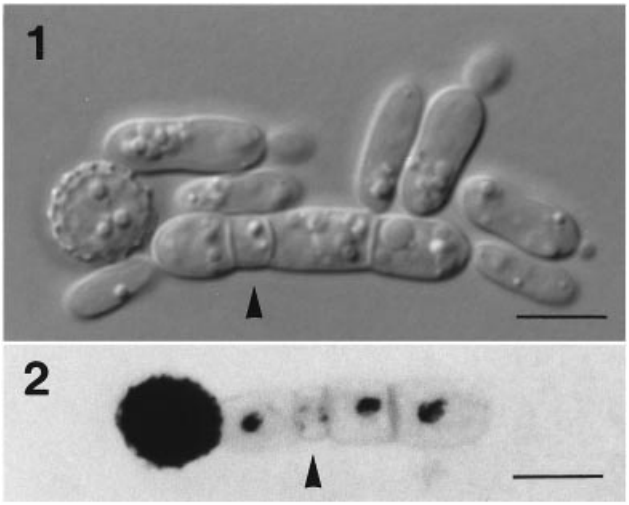
The three-celled promycelia of f. sp. *caroliniana* and f. sp. *virginica* were similar to three-celled promycelia of f. sp. *latifolia* and to previous descriptions of germination by *M. violaceum* (Harper 1900, Hüttig 1931, Deml & Oberwinkler 1982, Ingold 1983, 1989, Hood & Antonovics 1998). However, when more than three cells were present, the additional zones of the promycelium (separated by septa) were somewhat shorter and more narrow than the other cells (Fig. 1). Moreover, these zones did not produce sporidia or conjugate.

Distribution of the post-meiotic nuclei among the teliospore and promycelial cells was determined using nuclear staining. Only four post-meiotic nuclei were present regardless of the number of promycelial cells, reflecting the full meiotic tetrad (Hood & Antonovics 1998, 2000), and one of these nuclei was present within the wall of the teliospore. Therefore, three nuclei were retained in the promycelium, and any additional zones of the promycelium were anucleate (Fig. 2).

Anucleate zones were not randomly positioned within promycelia of f. sp. *caroliniana* (χ^2 [2, $n = 101$] = 152.32, $P < 0.001$) or f. sp. *virginica* (χ^2 [2, $n = 107$] = 138.45, $P < 0.001$) (Table 1). Considering that each promycelium contained only three cells with nuclei, these nucleate cells can be labeled relative to the teliospore (T) as proximal (P), middle (M), and distal (D) (Hood & Antonovics 1998). Among the possible positions for anucleate zones, they were most frequently positioned between P and M (Figs 1–2).

Promycelia containing an anucleate zone were less likely to contain an intra-promycelial conjugation (GLM procedure in SAS [SAS Institute, Cary, NC]; $P < 0.01$). Among three-celled promycelia, 76% contained an intra-promycelial conjugation, while only 31% of promycelia with more than three cells contained an intra-promycelial conjugation. When intra-promycelial conjugation occurred between cells on either side of an anucleate zone, the conjugation tubes resembled those between sporidia involved in distant mating (Day 1976) or between cells of the spore-like bodies of *Urocystis hypoxis* (Ingold 1999).

On water agar at 15 °, promycelia of f. *caroliniana* and f. sp. *virginica* were usually two-celled. In this way, they were similar to promycelia of f. sp. *latifolia* under these conditions. The two-celled promycelia often contained a conjugation between the cells. The pattern of conjugations in colonies



Figs 1–2. Germination of teliospores of *Microbotryum violaceum* f. sp. *caroliniana*. Teliospores were plated on potato dextrose agar and incubated at 25 °C for 18 h. Samples were viewed using differential interference contrast microscopy (Fig. 1) and Giemsa nuclear stain (Fig. 2). Promycelia contained more than three cells. Additional zones (arrowheads) were shorter, more narrow, and anucleate. Bars = 5 μm.

with three-celled promycelia was also consistent across all smut samples. There was usually a conjugation between the proximal and middle cells of the promycelium and a conjugation between the distal promycelial cell and a sporidium produced by the teliospore. This pattern has been described previously for f. sp. *latifolia* (Hood & Antonovics 1998). Conjugations between the middle and distal promycelial cells were not observed. Promycelia of f. sp. *caroliniana* and f. sp. *virginica* often detach from the teliospore, as did promycelia of f. sp. *latifolia*.

Sporidial colonies became visible to the unaided eye over the course of several days on PDA at 25 ° (*ca* 1 mm diam). In contrast to colonies of f. sp. *latifolia*, which were characteristically pink (Garber, Baird, & Weiss 1978), colonies of f. sp. *caroliniana* and f. sp. *virginica* were cream. Also, when grown on water agar at 25 ° for several days, cultures of f. sp. *caroliniana* and f. sp. *virginica* contained hyphae originating from nearly all of the colonies. Hyphal development in F. sp. *latifolia* was rare under these conditions and normally requires the addition of alpha-tocopherol (Day, Castle, & Cummins 1981, Kokontis & Ruddat 1986).

DISCUSSION

The anther-smut fungi, often subsumed under the name *Microbotryum violaceum*, are currently undergoing extensive revision (Vánky 1998). However, samples from *Silene caroliniana* and *S. virginica* have generally not been included in comparative studies. According to the most recent classification criteria (Vánky 1998), all samples used in this study are identified as *M. violaceum*. However, isolates from the North American hosts germinate differently from what was previously described for anther-smut fungi on other European hosts, including *S. latifolia* and the type specimen from *Saponaria officinalis* (Hüttig 1931, Day *et al*. 1981, Deml & Oberwinkler 1982, Ingold 1983, 1989, Hood & Antonovics 1998). Our results, and the genetic characterization of samples from *S. virginica* by Perlin *et al*. (1997), suggest the need for further studies using molecular methods before any taxonomic questions can be considered. However, our results reveal novel germination patterns for anther-smut fungi, which may influence other aspects of the life-cycle or disease interactions. F. sp. *caroliniana* and f. sp. *virginica* are distinct because they produce promycelia containing anucleate zones. They also differ in the production of hyphae in the absence of an inducing agent and in colony colour (Garber *et al*. 1978).

Hyphal development by f. sp. *caroliniana* and f. sp. *virginica* suggests that these North American samples may have different host interactions than smut fungi on other *Silene* hosts. Previously examined anther-smut fungi (Day *et al*. 1981) produce hyphae only when exposed to host plant-specific extracts, specifically alpha-tocopherol. This regulation of hyphal development has been suggested to be a mechanism for restricting host range (Day *et al*. 1981). F. sp. *caroliniana* and f. sp. *virginica* do not appear to regulate hyphal development in this way.

We also described some similarities between f. sp. *caroliniana*, f. sp. *virginica*, and f. sp. *latifolia*. These include the first division segregation of mating type (indicated by conjugation patterns within promycelia; Hood & Antonovics 1998) and the development of two-celled promycelia at low nutrient and temperature levels (Hood & Antonovics 1998). It is also known that sporidia of the anther smut from *Silene virginica* will conjugate with samples from *S. latifolia* (Antonovics *et al*. 1996), and this may suggest that these smut fungi are similar. However, these crosses do not result in normal disease expression (Antonovics *et al*. 1996), and f. sp. *virginica* and f. sp. *latifolia* are easily distinguished using a range of molecular phylogenetic approaches (Perlin *et al*. 1997). It should be noted that signals for inducing mating appear to be very highly conserved in smut fungi. Interspecific conjugations are possible among various monocot-infecting smut species and among dicot-infecting smuts, although not between these two groups (Kniep 1926).

Hood & Antonovics (2000) have demonstrated that the patterns of teliospore germination and hyphal development are likely to be very important to the disease ecology and mating system of the anther-smut fungi. However, germination patterns are greatly influenced by the environment (Hood & Antonovics 1998, 2000). In the current study, high nutrient levels or temperatures resulted in the production of promycelia containing anucleate zones. It remains to be determined under what conditions spores germinate in nature and whether anucleate zones are present under such conditions. It is commonly suggested that inoculum of anther-smut fungi are deposited in flowers of healthy plants by insect vectors (Jennersten 1983, Antonovics & Alexander 1992). There, teliospores germinate in the presence of nectar. They would therefore be expected to produce promycelia with anucleate zones at a high frequency due to the nutrient-rich environment. Even under low-nutrient conditions, promycelia contained anucleate zones at an appreciable frequency (*ca* 10%). Moreover, average daytime temperatures during the period of sporulation approach 25 ° where these samples were collected.

Therefore, such development is likely to be a normal part of the biology of f. sp. *caroliniana* and f. sp. *virginica*. However, the actual route of infection is not known for the North American anther-smut fungi, and caution is needed in extrapolating too much from the biology of the smut on *Silene latifolia*.

Where anucleate zones were present, it was observed that conjugations between promycelial cells were less frequent. The anther-smut fungi produce a linear tetrad during meiosis (Hood & Antonovics 1998), and the anucleate zones are most frequently positioned between the first-division products of meiosis. Because mating types segregate at the first meiotic division, the anucleate zones increase the distance between cells of opposite mating type within the tetrad (e.g. A1/A1/anucleate zone/A2/A2). It is therefore possible that the anucleate zone decreases the likelihood of conjugation. In this way, anucleate zones could influence the mating system of these anther-smut fungi by promoting outcrossing. Support for an adaptive function would require estimation of outcrossing frequencies in populations with and without individuals expressing anucleate zones. Alternatively, the formation of anucleate zones may be a residual trait of anther-smut fungi that produced four-celled promycelia. The occurrence of such promycelia under high nutrients and high temperatures suggest that rapid growth of the promycelium may also be responsible for additional septations, although it is unclear what nuclear behaviour would be associated with these cellular events (i.e. Wolkow, Harris & Hamer 1996).

From a developmental standpoint, it is not clear if the anucleate zones are formed during teliospore germination by a septation event without nuclear division, or if they result from a post-meiotic mitosis followed by degeneration of a nucleus. Both the production of anucleate sporidia and nuclear degeneration occur during teliospore germination of some *Tilletia* species. (Goates & Hoffman 1987), but these anucleate sporidia are not separated from the basidium by a septation and are only temporarily anucleate.

There is remarkable similarity in teliospore germination between the North American anther-smut fungi and the North American *Sphacelotheca hydropiperis* (Duran & Safeeulla 1968) causing smut disease of *Polygonum* species. It forms promycelia that appear very similar to ones containing anucleate zones. In addition, such development was observed in *S. hydropiperis* only under high nutrient concentrations. Although the nuclear condition is not described, the micrograph presented by Duran & Safeeulla (1968: fig. 9) shows a cell of reduced size in an analogous position to that observed in f. sp. *caroliniana* and f. sp. *virginica*. It also appears that this cell is not budding sporidia. Promycelia with more than four cells are described for two monocot-infecting smut fungi, *Ustilago ixophori* and *U. schroeteriana* (Piepenbring 1996). However, the nuclear condition of the cells and the distribution of the meiotic tetrad are not known. Interestingly, these smut species are members of a separate class (*Ustilaginomycetes*) from the dicot-infecting anther-smut fungi (*Urediniomycetes*) (Begerow, Bauer & Oberwinkler 1997), but development of anucleate zones in the transversely septate promycelia may be a feature common to these two groups (Bauer, Oberwinkler & Vánky 1997).

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