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Endophytic-host selectivity of *Discula umbrinella* on *Quercus alba* and *Quercus rubra* characterized by infection, pathogenicity and mycelial compatibility

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Abstract

The fungal endophytic–host relationships of *Discula umbrinella* and two oak species, *Quercus alba* and *Quercus rubra*, were characterized on the basis of endophytic infection, pathogenicity, and mycelial compatibility. Isolates of *D. umbrinella* were cultured from leaves of *Q. alba* and *Q. rubra* collected from a hardwood forest located in Patuxent Wildlife Research Center in Laurel, Maryland, USA. Endophytic infection was observed on sterile leaf discs and living 2-month-old seedlings of *Q. alba* and *Q. rubra*. Fungal-host reciprocal inoculations revealed the presence of conidiomata on both hosts but conidiomata production was more abundant on *Q. alba*. Isolates from *Q. rubra* produced milder disease symptoms on both oak species. Mycelial compatibility studies identified seven different MCG groups. MCG groups 1–3 contained isolates from both oak species whereas MCG groups 4–7 contained host specific isolates. Field studies monitored the seasonal appearance of the sexual fruiting structures, perithecia, as a possible source of new genetic variation that might alter host specificity/pathogenicity of the *D. umbrinella* isolates on *Q. alba* and *Q. rubra* hosts. Only 1–2% of the leaves contained perithecia throughout the sampling period (April–September). Isolates collected from *Q. alba* differed from those collected from *Q. rubra* in endophytic infection, pathogenic response, and perithecia production. The results of this study suggest that the endophyte–host relationship is one of host selective preference for *Q. alba*, but that the endophyte has the ability to maintain the endophytic/pathogenic life cycle on the less preferred host species, *Q. rubra*.

Introduction

Host range and specificity of fungal endophytes have been studied in grass, conifer and hardwood hosts (Petrini, 1986; 1995; Petrini et al., 1992; Sieber and Hugentobler, 1987; Sieber, 1989; Sieber-Canavesi and Sieber, 1991; Redlin and Carris, 1996). In 1995, Petrini reviewed the studies associated with fungal endophytes of tree leaves. In general, endophytic fungal communities demonstrated single host specificity at the plant species level but this specificity could be influenced by environmental conditions. Fisher et al. (1994) studied the endophytic community on *Quercus ilex* and found a higher degree of single host specificity within the plant's native geographic range. Endophytes are also able to colonize multiple host species of the same taxonomic family within the same geographic site. For example, *Discula umbrinella* isolates were collected from leaves of beech, oak and chestnut species, all members of the Fagaceae plant family (Hammerli et al., 1992) from the same geographic site. *D. umbrinella* populations isolated from these hosts could be subdivided into two host-selective groups of beech/oak or chestnut on the basis of genetic variation as determined by molecular studies. Additional evidence for host selective groups was provided by studies of host-related pectic enzyme patterns from oak/beech (Toti et al., 1991), differential conidia size (Toti et al., 1992a) and differential attachment of conidia to host/non-host surfaces (Toti et al., 1992b). These results suggest that

host specific groups could be detected at the plant genus level. However, endophytic host specificity at the plant species level has not been examined and is important to know in order to understand the co-evolution of fungus and plant host. In this paper, host specificity was evaluated on the basis of fungal endophyte infection, inoculations of plant hosts and mycelial compatibility.

Materials and methods

Fungal isolates

Isolates of *D. umbrinella* (ATCC 38311, ATCC 38312) were obtained from the American Type Culture Collection, Manassas, Virginia, USA. Both isolates were cultured from *Quercus alba* leaves but ATCC 38311 isolate was collected from Illinois, USA whereas ATCC 38312 was collected from Wisconsin, USA. All other isolates were collected from leaves of living *Q. alba* (white oak) and *Quercus rubra* (northern red oak) trees at Patuxent Wildlife Research Center, Laurel, Maryland, USA. Field sampling procedures were based on random selection of five leaves cut with a pole pruner from ten trees of each oak species. Both oak species were interspersed throughout the field site and each tree was selected randomly within the field site for sampling. Field studies, isolation and storage procedures used for *D. umbrinella* are previously described in Cohen (1999). Fungal cultures were maintained on potato dextrose agar plates for short term purposes and deposited in water for long term storage (Cohen, 1999).

Conidiomata leaf assay

Fungal cultures were tested for ability to produce conidiomata on both white and red oak leaf discs under conditions that excluded any other native fungal endophytes. Leaf discs were taken from 1-month-old oak seedlings using a 6 mm diameter hole puncher. Discs were then autoclaved and placed on 60 mm diameter petri plates (10 ml of distilled water agar per plate). Each water agar plate was inoculated with an agar disc of the fungal isolate in the center of the plate. Surrounding the inoculum to the left were three white oak leaf discs and on the right three red oak leaf discs. The plates were incubated for 3 weeks at 24 °C under cool-white fluorescent lights at 25 $\mu Em^{-2} s^{-1}$ with spectral range of 400–700 nm (Bickwood and Dunn, 1972). The number of conidiomata per leaf disc were counted for each species.

Pathogenicity tests

Acorns from *Q. alba* and *Q. rubra* (Schumacher, NY) were stored at 4 °C and periodically taken out and surface disinfected with 10% sodium hypochlorite + 0.1 ml Tween-80 and rinsed three times with deionized water. The pericarp of *Q. rubra* seeds was scored with a razor blade to facilitate water uptake for germination. Seeds were planted in vermiculite in plastic cups and watered from above. Seedlings were grown for 2 months under continuous fluorescent light at 25 $\mu Em^{-2} s^{-1}$ with a spectral range of 400–700 nm at 24 °C. Leaves were wounded causing a visible injury by touching them for 1–3 s with the base of a metal rod (6 mm diameter) preheated in a bunsen burner for 10–15 s and then leaves were allowed to cool for 15 min prior to inoculation. Each leaf was inoculated with an agar disc from a 2-week-old culture of each isolate. The disc was rubbed into the wound for maximum infection. Leaves were also inoculated with PDA discs not containing fungi. Inoculated seedlings were sprayed with deionized water, covered with plastic bags and placed in an incubator at 20 °C with 12 h daily fluorescent light at 25 $\mu Em^{-2} s^{-1}$ with a spectral range of 400–700 nm for 7 days. Leaves were then detached, incubated upside down in a moist chamber (100 mm diameter petri dish with moistened filter paper) at 24 °C with 12 h daily fluorescent light at 25 $\mu Em^{-2} s^{-1}$ with a range of 400–700 nm and observed for lesion and conidiomata development. After 10 days, the leaves were scored for the presence of disease symptoms and conidiomata. Leaves were then returned to an incubator at 10 °C for 4 months, removed, and scored for the presence of perithecia.

Mycelial compatibility tests

Fungal isolates were routinely transferred to PDA and stored in sterile distilled water at 24 °C. For mycelial compatibility studies (Cohen, 1998), the fungal isolates were initially grown on distilled water agar for 5 days at 24 °C. Water agar blocks (two per isolate) were taken from the margins of the fungal colonies and transferred to a 2 ml microcentrifuge tube containing 1 ml of distilled water. The agar blocks were smashed with a pestle and suspended in water. For pairing of mycelial isolates, 100 : 1 suspensions of each of the two isolates were placed 1 cm apart on oat agar plates, the plates were allowed to dry and then incubated at 24 °C. Oat agar was prepared by boiling 30 g of rolled oats in

500 ml distilled water for 10 min. The rolled oat suspension was then filtered through cheesecloth and diluted to 1000 ml with distilled water and 20 g of Bacto agar added (Cohen, 1998). All possible isolate pairings were included such as *Q. alba* × *Q. alba*, *Q. rubra* × *Q. rubra* and *Q. alba* × *Q. rubra*. Isolates were also paired with themselves. Mycelial interactions were judged to be compatible when mycelia of two isolates intertwined easily, no noticeable line formed and hyphal fusions were common. An incompatible reaction was characterized by a fine white barrier line formed between two isolates or by an empty zone where mycelia did not grow. A partial compatible reaction was defined if a small portion of the contact included either a white barrier line or an empty zone (Figure 2). Interactions of mycelia pairs on petri plates were observed over time and reactions noted at 2, 10 and 21 day intervals.

Mycelial interactions were also observed microscopically on slide cultures. Six isolates collected from white and red oaks were paired on slide cultures and observed for a range of hyphal interactions such as hyphal fusing, intertwining, coiling and rejection after 3, 7 and 14 days. Slide culture apparatus contained a glass slide elevated on two toothpicks on top of filter paper sitting in a glass petri dish. The apparatus was sterilized, 0.5 ml of molten 2.0% distilled water agar was dropped onto the slide and 5 ml of 5% glycerol was added to a filter paper in the petri dish to retard drying during the experiment. Two isolates were paired per slide by adding a drop of mycelial suspension from each culture 1 cm from each other. Mycelial interactions were observed and recorded photographically.

Field survey of perithecia on Q. alba *and* Q. rubra *fallen leaves*

Fallen leaves of *Q. alba* and *Q. rubra* were collected monthly from transect lines near Snowden Pond, Laurel, Maryland and the presence of perithecia was recorded. Selection of locations of the 30 m transect lines were randomized. For the first months collection, 10–1 m square frames were sampled from each transect line and 20 fallen leaves of each oak was collected per meter frame. Subsequent monthly collections were based on sampling from 5–1 m frames. An equal number of fallen leaves from both oak species were collected from each meter frame along all transect lines. Fallen leaves were examined microscopically and the presence of perithecia was recorded for each leaf.

Data analysis

Results were analyzed by a nonparametric statistical test, χ^2 contingency analysis. This statistical test was selected to test the independence of two or more attributes without regard to the distribution form of the data set. For example, number of perithecia per oak species and month of collection were analyzed for independent or dependent interaction. If the dependent interaction was verified, then a significant result was recorded. All statistics were performed with the Systat for Windows software program (Wilkinson et al., 1996). When sample sizes within frequency cells were insufficient for the analyses, the Mann–Whitney U-test was used for the analysis of the data.

Results

Discula umbrinella isolates derived from *Q. alba* and *Q. rubra* were able to produce conidiomata on autoclaved leaf discs of either oak species. The majority of *Q. alba*-derived isolates (8/12) produced a greater number of conidiomata on white oak leaf discs (Figure 1). *Q. rubra*-derived isolates (9/10) also produced a greater number of conidiomata on white oak leaf discs (Figure 1). Chi square contingency analysis revealed production of conidiomata to be significantly different when autoclaved white and red oak leaf discs results were compared for isolates derived from either oak species.

When living leaves of *Q. alba* and *Q. rubra* seedlings were challenged with *D. umbrinella* isolates, the white oak isolates were highly pathogenic on white oak and less so on red oak (Table 1, Figure 2). Leaves challenged with red oak isolates, except RO226, showed mild symptoms on both hosts (Table 1, Figure 2). All isolates tested produced abundant new conidiomata on white oak but rarely on red oak leaves (Table 1, Figure 3). Only isolate ATCC 38312 collected from white oak produced perithecia; this occurred only on one of six inoculated white oak leaves.

Mycelial compatibility studies showed that not all pairings of white and red oak isolates showed interspecific or intraspecific fungal-host compatibility (Table 2, Figure 2). Self pairings of all isolates generated compatible reactions. Mycelial pairings of WO122 with other white or red oak isolates resulted in incompatible reactions with the exception of a mixed reaction with WO812. Pairings of WO236 with WO546, WO655 and RO1035 resulted in compatible reactions. WO341

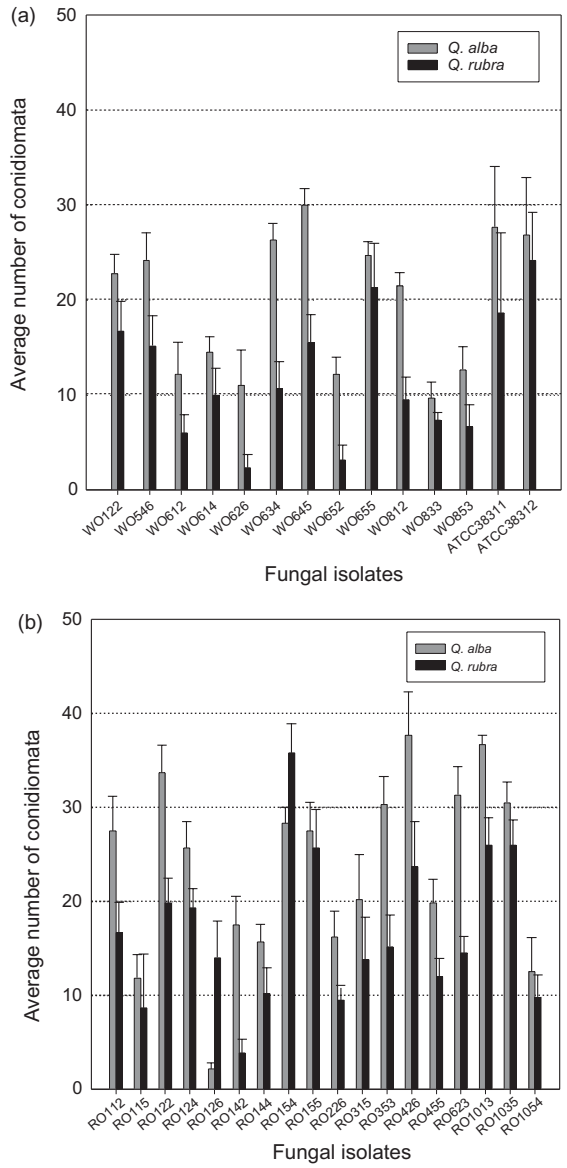

Figure 1. *Discula umbrinella* isolates collected from *Q. alba* and *Q. rubra* and production of conidiomata on autoclaved leaves of *Quercus* species (a) = Isolates from *Q. alba*, $\chi^2 = 65.265$, df = 13, $P = 0.00001$; (b) = Isolates from *Q. rubra*, $\chi^2 = 178.897$, df = 17, $P = 0.00001$.

and RO122 pairings also resulted in compatible reactions. WO655 paired with WO812, RO226 and RO623 gave compatible reactions. RO122 when paired with RO315 and RO315 when paired with RO623 gave compatible reactions. RO455 strain when paired with RO623 and RO1035 gave compatible reactions. Strain reactions were assigned to the following mycelial compatibility groups: MCG 1 (WO236, WO546, WO655, RO1035), MCG 2 (WO341, RO122), MCG 3 (WO655, WO812, RO226, RO623), MCG 4 (RO122, RO315), MCG 5 (RO315, RO 623), MCG 6 (RO455, RO623, RO1035) and MCG 7 (WO122). Mycelial compatibility groups 1–3 contained isolates derived from both *Q. alba* and *Q. rubra* and did not distinguish host-specific isolates. Groups 4–7 contained isolates derived from *Q. alba* or *Q. rubra* and could be identified as host-specific mycelial compatible groups.

Microscopic studies of hyphal interactions from mycelial pairings revealed a range of reactions including hyphal fusing, intertwining, coiling and rejection after 3, 7 and 14 days. No particular interaction could be consistently related to compatible or noncompatible reactions.

Field surveys for perithecia indicated that the sexual fruiting bodies do not occur frequently in nature on either white or red oak (Table 3, Figure 3). In the month of April, only 1% of the white oak or red oak leaves collected contained perithecia. In May, 2% of the white oak or red oak leaves collected contained perithecia. Microscopic examination of internal contents of perithecia revealed lack of ascospore development or immature ascospores in the April collection. Perithecia taken from later monthly collections of oak leaves did not provide viable ascospores.

Discussion

Neely and Himelick (1967) reported that it was rare to find oak anthracnose isolates from one oak species that would produce disease symptoms in another oak species. However, they were able to inoculate and produce disease symptoms in *Quercus macrocarpa*, *Quercus robur* and *Q. alba* with an isolate from *Q. alba*. Schuldt (1955) was able to cross-inoculate *Q. alba* and *Quercus borealis* using isolates from both of these oak species. Similar results were obtained in this study with white and red oak isolates collected from *Q. alba* and *Q. rubra*. Fungal isolates from each oak species were able to infect both oak species tested. However, isolates from both oak species rarely produced new conidiomata on *Q. rubra*. In addition, pathogenic symptoms were very slight on *Q. rubra*. These pathogenicity differences support evidence for separating these fungal populations into two host-selective groups, *Q. alba* and *Q. rubra*.

Mycelial compatibility was also used to screen for variation within and between populations derived from

Table 1. Reciprocal pathogenicity responses of *D. umbrinella* isolates on *Q. alba* and *Q. rubra* hosts

Isolates[1]	Number of leaves with disease symptoms[2,4]				Number of leaves containing new conidiomata[3,4]			
	Q. alba		*Q. rubra*		*Q. alba*		*Q. rubra*	
	Control	Isolate	Control	Isolate	Control	Isolate	Control	Isolate
ATCC38311	0	5	0	0	0	5	0	0
ATCC38312	0	5	0	1	0	5	0	1
WO122	0	6	0	6	0	2	0	0
WO612	0	4	0	6	0	0	0	0
WO614	0	6	0	3	0	6	0	0
WO623	0	3	0	6	0	3	0	0
WO645	0	6	0	6	0	3	0	2
WO655	0	6	0	3	0	1	0	0
RO112	0	4	0	3	0	1	0	0
RO122	0	6	0	6	0	6	0	0
RO151	0	3	0	4	0	1	0	2
RO154	0	5	0	6	0	4	0	0
RO226	0	6	0	5	0	5	0	1
RO315	0	4	0	6	0	1	0	0

[1]ATCC and WO isolates collected from trees of *Q. alba*, a susceptible host and RO isolates collected from trees of *Q. rubra*, a resistant host.
[2]Pathogenicity test: Six leaves were inoculated per isolate per oak species and the number of leaves with disease symptoms was counted. A leaf was considered infected if a lesion was present beyond the original inoculation site. Controls were inoculated with agar discs without fungal isolates.
[3]Number of leaves per oak species containing new conidiomata after inoculation with *D. umbrinella* isolates.
[4]When pathogenicity data of all isolates were grouped and species compared, no significant difference was noted with regard to infection on *Quercus* species. Mann–Whitney $U = 3738$, $P = 0.344$; when presence of conidiomata data of all isolates were grouped and species compared, a significant difference was noted in regard to *Quercus* species. Mann–Whitney $U = 4956$, $P = 0.00001$.

two oak species, *Q. alba* and *Q. rubra*. This technique has been used successfully to group isolates of *Sclerotinia sclerotiorum* (Kohn et al., 1990) and to describe genetic heterogeneity within the species. Assaying mycelial compatibility at the macroscopic petri plate level was more successful than at the microscopic level. A distinctive white line or barrage zone formed when isolates were incompatible. However, when mycelial interactions were examined at the microscopic level, no representative reaction of incompatibility could be identified. Kohn et al. (1990) also observed complex mycelial interactions microscopically with pairings of *S. sclerotiorum* and found many of the same interactions occurred in both compatible and incompatible pairings. The mycelial compatibility grouping (MCG) approach did not clearly separate *D. umbrinella* isolates by host-specific groups. Seven MCG groups were identified with groups 1–3 containing white and red oak isolates, groups 4–6 containing red oak isolates and group seven containing a single white oak isolate.

Isolates of the fungus collected from *Q. alba*, the susceptible host, differ from the isolates collected from the resistant host, *Q. rubra* in colony growth and appearance. Isolates from *Q. rubra* tended to grow slower, were isolated from leaves later in the season, and produced perithecia over a longer period of time. Characterization of isolates by MCG did not distinguish two host-specific groups but rather indicated a preference for one or the other host with some overlap. In a related study by Ragazzi et al. (2000), the authors were able to distinguish isolates from different oak species and different geographic regions of Italy using a vegetative incompatibility technique. Five VCG groups were identified and correlated with oak species type and geographic origin. The VCG group results from the *Discula quercina* populations derived from oaks in Italy may not be comparable to data collected on *D. umbrinella* using MCG groups. The MCG study examined isolates collected from two oak species, representing susceptible and resistant hosts, from only one study site and differed markedly in experimental design from the VCG study. The VCG study measures the actual contact of hyphal pairs and the formation of a heterokaryon whereas the MCG method records only the hyphal cell contact not nuclear contact and

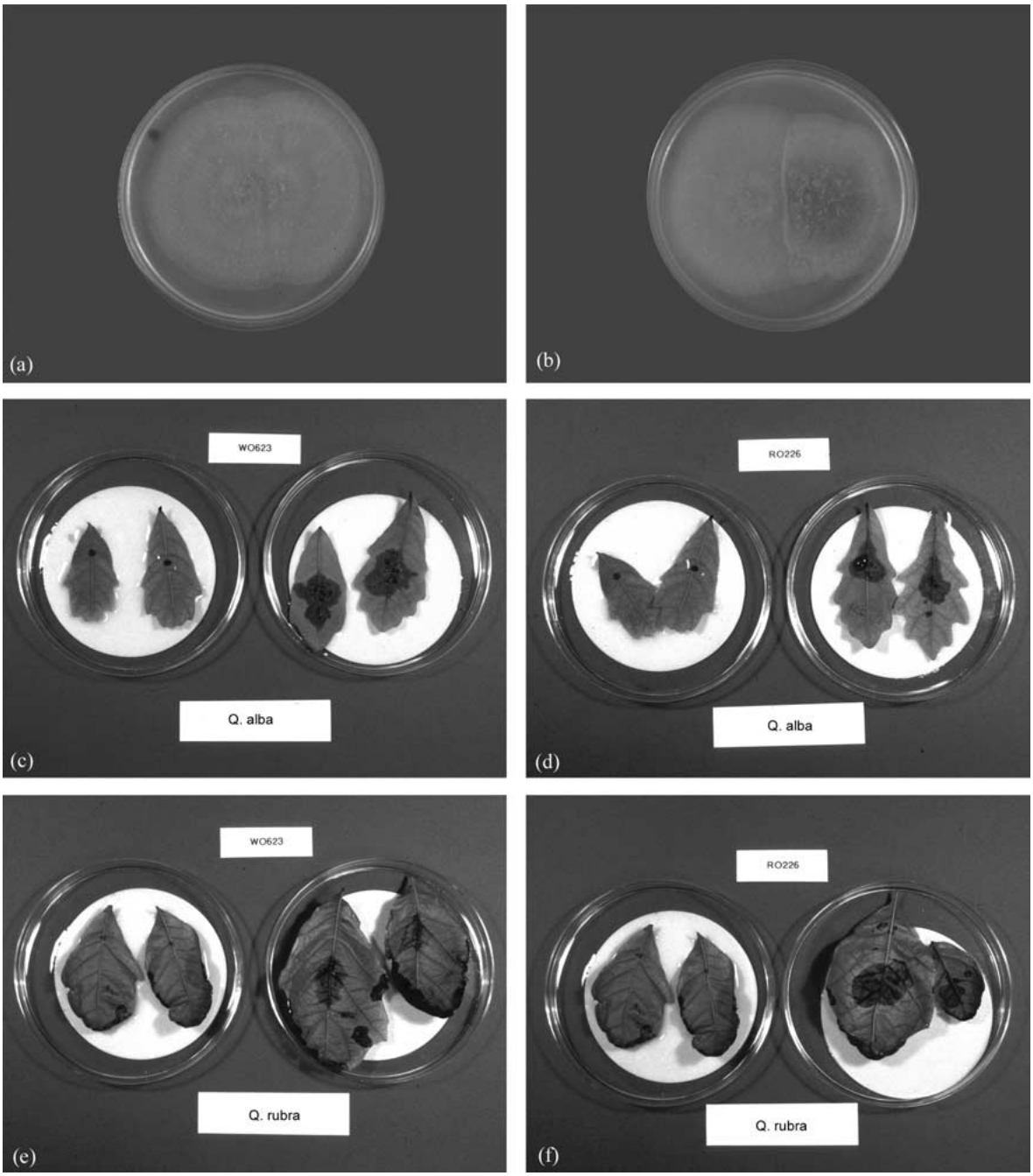

Figure 2. Mycelial compatibility and pathogenic responses of isolates of *D. umbrinella* in culture and on plant leaves. (a) Pairing of fungal isolates on agar medium demonstrating compatible mycelial interaction, (b) Incompatible mycelial interaction with a white barrage line at point of contact, (c) White oak isolate 623 symptoms on un-inoculated (left) and inoculated leaves (right) of *Q. alba*, (d) Red oak isolate 226 symptoms on un-inoculated (left) and inoculated leaves (right) of *Q. alba*, (e) White oak isolate 623 symptoms on un-inoculated (left) and inoculated leaves (right) of *Q. rubra* and (f) Red oak isolate 226 symptoms on un-inoculated (left) and inoculated (right) leaves of *Q. rubra*.

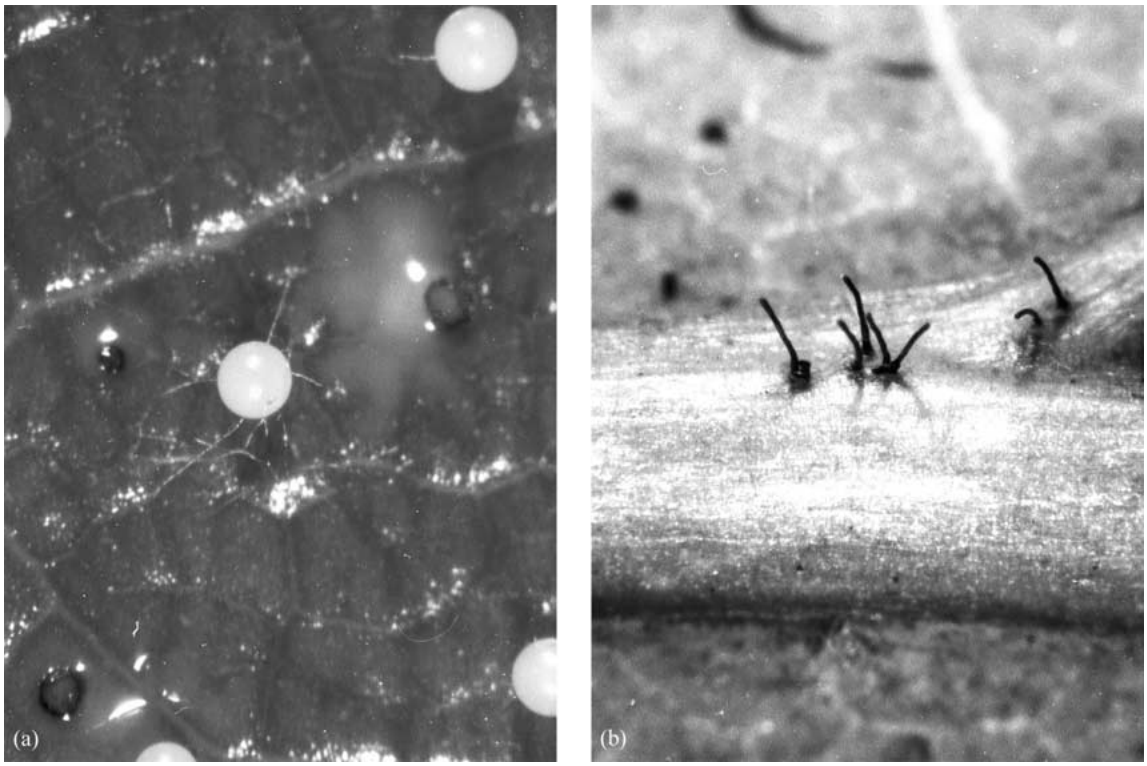

Figure 3. Asexual and sexual fruiting bodies of *D. umbrinella* on oak leaves. (a) Conidiomata, asexual fruiting bodies, on the underside of an oak leaf demonstrating release of droplets containing conidia. (b) Perithecia, sexual fruiting bodies, with characteristic long necks found embedded within the main vein located on the underside of an oak leaf.

Table 2. Mycelial pairings of *D. umbrinella* isolates collected from *Q. alba* and *Q. rubra* hosts[1]

	WO122	WO236	WO341	WO546	WO655	WO812	RO122	RO226	RO315	RO455	RO623	RO1035
WO122	+	−	−	−	−	±	−	−	−	−	−	−
WO236		+	±	+	+	−	−	−	−	−	±	+
WO341			+	−	±	−	+	−	−	−	−	−
WO546				+	±	−	−	−	−	−	−	±
WO655					+	+	−	+	−	−	+	−
WO812						+	−	−	−	−	±	−
RO122							+	−	+	−	−	−
RO226								+	−	−	−	−
RO315									+	−	+	−
RO455										+	+	+
RO623											+	±
RO1035												+

[1]Mycelial interactions: (+) = a compatible response in which a continuous mycelial mat is evident between two paired fungal isolates; (±) = interactions between two paired isolates may include a continuous mycelial mat and a barrage zone or a empty zone free of mycelia; (−) = an incompatible response occurs as a barrage zone or an empty zone free of mycelia between two paired fungal isolates.

fusion. The other issue associated with using either experimental method is that MCG or VCG testing may overestimate somatic/genetic variations. This could be verified by using an independent method of testing such as DNA sequencing of multiple gene regions. Future research studies with the *D. umbrinella* isolates collected from USA will analyze the genetic variation of these fungal isolates, compare molecular

Table 3. Number of perithecia on fallen leaves of *Q. alba* and *Q. rubra* collected at Patuxent Wildlife Research Center, Laurel, Maryland, USA[1,2,3]

	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.
Q. alba perithecia	0	0	0	0	118 (3)	94 (7)	25 (3)	0	0	0
Q. rubra perithecia	0	0	0	0	39 (3)	155 (7)	32 (2)	15 (2)	4 (2)	1 (1)

[1]Numbers in parentheses are the number of leaves containing perithecia.

[2]Procedures for leaf collection: During the month of December, two 30 m transect lines were run and 40 leaves of each oak species were collected per meter frame, 10-1 m frames were sampled along each transect line. During the months following December, three 30 m transect lines were run and 20 leaves of each oak species was collected per meter frame, 5-1 m frames were sampled along each transect line.

[3]The number of perithecia observed on leaves of *Q. alba* was significantly different from number of perithecia observed on *Q. rubra*. $\chi^2 = 70.191$, df $= 2$, $P = 0.00001$ based on the data from the months of April through June.

results with mycelial compatibility groups, and reexamine fungal-host selectivity based on pathogen–host relationships.

Sexual reproduction of *D. umbrinella* does not seem to play a part in the evolution of endophytic fungal–host selectivity. The production of perithecia on oak leaves in nature is extremely slight as only 1–2% of the leaves collected contained perithecia. It is quite possible that another host in the forest ecosystem may be more favorable for supporting large numbers of perithecia as beech and other oak species are considered hosts as well (Hammerli et al., 1992). However, these studies only represent indirect evidence of evolutionary impacts on fungal-host selectivity. Further research at the molecular level is needed to establish the genetic impacts of ascospore-derived fungal isolates on endophyte–host selectivity in native ecosystems.

Rodriguez and Redman (1997) describe host specificity of fungal endophytes as evolving differently in agricultural and native ecosystems. The authors propose that the two ecosystem types are significantly different from each other in plant diversity, fungal diversity and environmental conditions. For example, natural ecosystems tend toward higher diversity of plants and fungi than agricultural ecosystems. Endophytic fungal pathogens may have a greater opportunity to expand their host range within natural ecosystems either by maintaining a small reservoir of disease or surviving as endophytes. Natural ecosystems foster genetic variation and diversity of fungal pathogens that co-evolve with plant hosts. However, the potential exists for disease outbreaks when these fungal pathogens move into an agricultural ecosystem with less plant diversity and less genetic resistance. *D. umbrinella* may be an interesting example to study with regard to the possible expansion of the host range in a native environment and impacts on nearby urban forestry and horticultural landscape ecosystems.

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