Infection process of *Plectosporium alismatis* on host and non-host species in the *Alismataceae*

Wayne M. PITT1*, Eric J. COTHER2, Norma J. COTHER2 and Gavin J. ASH1

1Farrer Centre, School of Agriculture, Charles Sturt University, PO Box 588, Wagga Wagga, New South Wales, 2678, Australia.
2New South Wales Agriculture, Agricultural Research Institute, Forest Road, Orange, New South Wales, 2800, Australia.

E-mail: pittw@agr.gc.ca

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In Australia, the endemic fungus *Plectosporium alismatis* (syn. *Rhynchosporium alismatis*, *Spermosporella alismatis*) has potential use as a mycoherbicide for several species in the *Alismataceae*, a family of aquatic and semi-aquatic marsh herbs, which are considered to be important weeds in rice crops. Of five species identified in south-eastern Australia where rice is grown, two species, *Sagittaria graminea* and *Sagittaria montevidensis* are resistant (non-hosts), and no records of *P. alismatis* on these species have been reported. To better understand the interactions that lead to resistance in these pathosystems, the infection process of the fungus was studied on these species and also on the host *Alisma plantago-aquatica*, using light, fluorescent and scanning electron microscopy. On all three species both conidial germination and appressorium formation commenced within 6 h of inoculation with greater than 50% of conidia elongating to form germ tube structures and associated appressoria 12–18 h post inoculation. Germ tube elongation and appressorium formation occurred randomly over the leaf surface. Direct host penetration was facilitated by the production of penetration hyphae that emerged from beneath appressoria. Penetration sites were clearly identified by the presence of spherical holes 0.25–0.5 µm in diam, and were frequently accompanied by resistance reactions in non-host species. Visible symptoms of disease occurred 4–6 d after inoculation of susceptible (host) species.

INTRODUCTION

In Australia, the aquatic weed flora associated with rice cultivation includes several members of the *Alismataceae*, a small family of monoeocious marsh herbs comprising the natives, *Alisma plantago-aquatica* and *Damasonium minus* and three introduced species, *Alisma lanceolatum*, *Sagittaria montevidensis* and *Sagittaria graminea* (McIntyre & Newnham 1988, McIntyre et al. 1991).

At present, management strategies for these weeds rely almost entirely on the application of chemical herbicides and high rates and often multiple applications have been necessary to maximise yield potential. Unfortunately, the continued use of site specific herbicides has led to the development of herbicide resistance in the *Alismataceae* (Fowler & McCaffery 1994), and alternative forms of weed control are clearly required.

In 1994, the endemic fungal pathogen *Plectosporium alismatis*† (syn. *Rhynchosporium alismatis*, *Spermosporella alismatis*) was identified as the causal agent of necrotic lesions on the leaves, petioles and inflorescence stalks of several species in the *Alismataceae* (Cother, Gilbert & Pollock 1994). Symptoms begin as lens-shaped necrotic spots that soon increase in diam and coalesce to form elongated lesions. Under favourable conditions lesions become visible on mature leaves within three to four days after infection and are observed sporadically on petioles and inflorescence rachis (Cother et al. 1994). Due to its suppression of plant growth (Cother & Gilbert 1994) and limited host range (Cother 1999) *P. alismatis* was proposed as a potential mycoherbicide for alismataceous weeds in Australian rice crops.

Presently, *A. plantago-aquatica*, *A. lanceolatum* and *D. minus* are recognised hosts of the fungus. However, no records of the fungus on *S. graminea* or *S. montevidensis* have been reported and both species appear

* Present address: Ecological Pest Management, Agriculture and Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, Saskatchewan, S7N 0X2, Canada.
† See this issue (Pitt et al. 2004).
to be resistant to infection by *P. alismatis*. Because these species are currently some of the most invasive weeds of rice in Australia, the development of alternatives to chemical herbicides must be effective against these species.

The objectives of this study were to document the infection process of *P. alismatis*, chronicle the events that occur following inoculation of both resistant and susceptible species in the *Alismataceae*, identify modes of resistance and/or constraints to disease initiation in non-host species and to assess the suitability of *P. alismatis* as a broad spectrum mycoherbicide for *Alismataceae* weeds in Australian rice crops. The infection process of the fungus was examined using light, fluorescent and scanning electron microscopy. In addition, the rates of conidial germination and appressorium formation on resistant and susceptible species were also examined.

**MATERIALS AND METHODS**

**Sources and culturing of isolates**

Forty-two isolates of *Plectosporium alismatis* were obtained from various locations in south-eastern Australia during the period Jan. 1989–May 1999. Cultures were isolated from infected leaves of host plants, cultivated on lima bean agar (LBA; Difco Laboratories, Detroit, MI), lyophilised and deposited in the Agricultural Scientific Collections Unit, New South Wales Agriculture, Orange, NSW, DAR). Upon receipt of cultures from the herbarium, all isolates were passaged through their respective hosts, reisolated from cultures from the herbarium, all isolates were passaged through their respective hosts, reisolated from non-host species and to assess the suitability of *P. alismatis* as a broad spectrum mycoherbicide for *Alismataceae* weeds in Australian rice crops. The infection process of the fungus was examined using light, fluorescent and scanning electron microscopy. In addition, the rates of conidial germination and appressorium formation on resistant and susceptible species were also examined.

**Inoculum production**

Isolates were transferred to LBA and incubated at 25 °C for 5–10 d under a 12 h light regime provided by two 40-W, 120 cm long cool white fluorescent tubes situated 30 cm above the plates. Following sporulation, spores were harvested from each culture by adding 1 ml of sterile distilled water and scraping gently across the mycelium surface with a sterile glass rod. The resulting slurry of spores and mycelium were used to inoculate additional LBA plates to produce confluent stock spore cultures of each isolate. Plates were then incubated for an additional 4 d at 25 °C and spores harvested as described above. Spore suspensions were enumerated using a Weber haemocytometer (Crown Scientific, Burwood, Victoria), centrifuged at 10,000 g for 30 s, the supernatant discarded and the concentration of spores adjusted to 1 x 10^6 ml^-1 by the addition of sterile distilled water.

**Pathogenicity and selection for virulence**

**Plant growth and leaf material preparation**

Prior to the commencement of infection studies, a pathogenicity study was undertaken to assess the virulence of the isolates obtained from the herbarium. *Damasonium minus, Alisma plantago-aquatica, Sagittaria montevi densis* and *S. graminea* plants were collected from several field sites throughout southern New South Wales, transported to Charles Sturt University in plastic tubs and transplanted into 1100 mm diam stock water troughs (Riverina Cooperative Society, Wagga Wagga, NSW) maintained in a temperature controlled glasshouse (day/night, 30/25 °C) with a diurnal light regime. Fully expanded floating leaves of *D. minus* and erect emergent leaves of *A. plantago-aquatica, S. montevi densis* and *S. graminea* were excised from healthy adult plants, surface sterilised in a sodium hypochlorite solution (1 % available chlorine) for 45 s and rinsed with sterile distilled water. Leaves were cut into 2 cm diam discs, blotted dry with sterile filter paper and immediately transferred to 20 cm diam plastic Petri dishes containing 1.5 % Technical agar (Amyl Media) supplemented with 1 μg ml^-1 benzylaminopurine (Sigma-Aldrich). Benzylaminopurine prevents premature senescence of excised leaves for at least 2 weeks.

**Leaf disc inoculation and disease assessment**

Three leaf discs of each of the four plant species were inoculated with a 10 μl drop of a spore suspension (1 x 10^6 ml^-1) from each of 40 sporulating fungal isolates. Petri dishes were wrapped with Parafilm ‘M’ laboratory film (American National Can, Chicago) to avoid dehydration, and incubated at 25 °C. Control discs were inoculated with sterile distilled water. The percentage of leaf disc area affected by disease was determined visually 14 d after inoculation. The experiment was replicated three times for each plant/pathogen interaction and the scores were then averaged across the three experiments (data not shown). Disease severity scores (percentages) indicated that isolate RH97 (DAR 73151) was the most pathogenic of the 40 isolates with respect to all four plant species, and as a result this isolate was used for the infection study described in this paper. A duplicate culture of RH97 has been lodged with CBS under the accession no. CBS 112536.

**Infection studies**

**Leaf disc inoculation**

The adaxial surfaces of leaf discs of *Alisma plantago-aquatica, Sagittaria montevi densis* and *S. graminea* were inoculated with a 10 μl drop of a spore suspension of *Plectosporium alismatis* RH97 and incubated at 25 °C for 36 h. Control discs were treated with sterile distilled water. During both the ‘infection study’ and ‘pathogenicity’ components of this project, inoculum was
placed at the centre of leaf discs to maintain uniformity and to avoid the cut edges of the disc. The inoculated surface was not wounded. Damasonium minus, the fourth plant species used during the ‘pathogenicity’ experiment outlined above, was omitted from the ‘infection’ component of this study since experiments of this nature had already been conducted on this species (Jahromi, Cother & Ash 2002).

Light microscopy

Six leaf discs per species were removed after 6, 12, 18, 24, 30 or 36 h incubation, fixed and cleared in 1:2 acetic acid/ethanol solution, stained with lactophenol cotton blue (phenol 20% w/v; lactic acid 20% v/v; glycerol v/v; cotton blue 0.1% w/v) and mounted on 40 × 20 mm glass slides with glycerol. Observations were made at 100× and 400× magnification using a Nikon Labophot Episcopic microscope with EPlan objectives (Nikon Corporation; Nippon, Kogaku, K.K., Chiyoda-ku, Tokyo). 100 conidia were examined on each of the six leaf discs comprising each treatment. The number of conidia that had germinated, the number of germinated conidia that had formed appressoria and the number of un-germinated conidia were determined. Areas where conidia were clustered in large concentrations were avoided to ensure consistency during the counting process. Conidia were considered to have germinated if the length of the germ tube was equal to, or greater than, half the length of the conidium, or if an appressorium was present. Appressorium were recognised by their globose, sometimes slightly lobed structure formed terminally on a germ tube (Emmett & Parberry 1975). The periodic acid-Schiff (PAS) reagent (Sigma-Aldrich) was used to test for appressorial melanisation. The method of Craig & Beaton (1996). Sites of penetration by Plectosporium alismatis were examined by removing the inoculum from the leaf surface with Scotch brand tape prior to observation. Observations were made at 15 kV using a JEOL 6400 Scanning electron microscope equipped with a Bio-Rad E7400 cryotrans system (Department of Entomology, CSIRO, Canberra). Several non-inoculated leaf discs were also prepared for SEM in order to view leaf surface structure differences between plant species.

Electron microscopy

Several leaf discs from each plant species were inoculated with a drop of spore suspension of RH97 as described above. Following 24–36 h incubation, samples were prepared for electron microscopy via the method of Craig & Beaton (1996). Sites of penetration by Plectosporium alismatis were examined by removing the inoculum from the leaf surface with Scotch brand tape prior to observation. Observations were made at 15 kV using a JEOL 6400 Scanning electron microscope equipped with a Bio-Rad E7400 cryotrans system (Department of Entomology, CSIRO, Canberra). Several non-inoculated leaf discs were also prepared for SEM in order to view leaf surface structure differences between plant species.

Data analysis

Replications consisted of 6 leaf discs per time interval for each of the three plant species, and experiments were repeated in triplicate. Data were analysed as a split plot design, with replicates and species as main effects. Because variance for all experiments was homogeneous, data for each plant species were pooled before analysis. Because residuals were normally distributed, transformation of data was unnecessary, and an analysis of variance (ANOVA) was performed at each time interval for both germination and appressorium formation. The means at each interval were separated using the least significant difference (LSD) method. The computer package Genstat 5.0 (Rothamsted Experimental Station, Harpenden) was used for all statistical analyses.

RESULTS

On all three plant species both conidial germination and appressorium formation by Plectosporium alismatis commenced within 6 h after inoculation. Although greater than 50% of conidia elongated to form germ tube structures after 12 h, equivalent levels
of appressorial formation were not observed on any species until 18 h post inoculation. Differences in the numbers of germinating conidia (P=0.05) and germinated conidia that formed appressoria (P=0.05) were significant between the three species at the majority of examination times. However, the rates of conidial germination and appressorium formation did not differ significantly between the three species (P=0.05), even though the total number of conidia that formed appressoria on *A. plantago-aquatica* was only about half of that observed on *Sagittaria graminea* and *S. monteviendensis* (Figs 1–2).

Characteristically, conidia of *P. alismatis* are cellular separated by an internal septum (Fig. 3), and germination results in the production of a single unbranched germ tube that elongates to produce a terminal club-shaped appressorium of variable size (Fig. 4). In this study, however, multiple germ tubes were frequently observed originating from single conidia. In these instances, all of which occurred on *S. monteviendensis*, germ tube elongation was often lengthy and frequently progressed into highly branched networks where appressorium formation was less frequent and appeared to be associated with terminal and intercalary branches (Fig. 6). In contrast to germ tube production, multiple appressoria were observed infrequently, with never more than two per conidium being observed. Occasionally, sessile appressoria were observed (Fig. 11).

Germ tube elongation and appressorium formation occurred randomly on the leaf surface. No indication of directional growth or common stimulus for appressorium formation was observed. Appressoria formed at a variety of locations on the leaf surface including the junctions between adjacent epidermal cells and near the edges of stomatal guard cells (Fig. 12). On occasion appressoria were associated with stomata (Fig. 13). However, few stomatal infiltrations were observed (Fig. 14) and the majority of germ tubes that passed over stomata did so without forming infection structures (Fig. 12).

Development of appressoria and direct penetration of the host cuticle appeared to be the only method of infection by *P. alismatis*. Sites of appressorium formation were frequently accompanied by changes to the leaf surface structure. Fluorescent micrographs showed evidence of necrosis in epidermal cells of *S. graminea* beneath sites of appressorium formation (Fig. 7) and light micrographs revealed the presence of blue, disc-shaped zones or ‘haloes’ (Fig. 8). Haloes first appeared approximately 18 h after inoculation, were approximately 10–15 μm in diam and were visible by SEM as areas of increased electron density (Fig. 15). Darkly staining centres and margins of haloes suggested papilla formation (Fig. 8 inset). Occasionally fungal structures and infection sites were accompanied by evidence of a fungal exudate (Figs 13 and 15). Mela

![Fig. 1. Percentage of *Plectosporium alismatis* conidia that germinated on the leaves of *Alisma plantago-aquatica* (—●—), *G. monteviendensis* (— ▲—), *S. monteviendensis* (— x—), and *S. graminea* (— △—). Error bars represent LSD of means at each time interval (P=0.05).](image1)

![Fig. 2. Percentage of *Plectosporium alismatis* conidia that formed appressoria on the leaves of *Alisma plantago-aquatica* (—●—), *G. monteviendensis* (— ▲—), *S. monteviendensis* (— x—), and *S. graminea* (— △—). Error bars represent LSD of means at each time interval (P=0.05).](image2)
an association between penetration sites and fungal structures (Figs 19–20). Fluorescent microscopy enabled both structures to be observed simultaneously without prior removal of the inoculum (Fig. 10).

Successful invasion of the plant cuticle was reflected by the collapse of conidia and germ tubes and was mirrored by the migration of cytoplasmic contents from the conidium to the appressorium, evident in light microscopy.
Figs 11–22. Scanning electron micrographs of Alismataceae species following inoculation with Plectosporium alismatis. Fig. 11. Sessile appressoria. Fig. 12. Germs tubes passing over open stoma on Sagittaria montevidensis 48 h after inoculation. Fig. 13. Appressorium formation above open stoma on S. montevidensis 60 h after inoculation, including appearance of extracellular matrix. Fig. 14. Stomatal infiltration on Alisma plantago-aquatica 24 h after inoculation. Fig. 15. Appressorium formation on S. graminea 48 h after inoculation, including ‘halo’ associated with increased electron density. Fig. 16. Appressorium and associated penetration hypha following attempted removal of inoculum from Alisma plantago-aquatica 48 h after inoculation, including penetration site and cuticular depression. Fig. 17. Penetration sites on S. graminea 42 h after inoculation, and depressions around penetration sites (inset). Fig. 18. Adherence of spores to the leaf surface of S. montevidensis 24 h after inoculation, including evidence of physical stress exerted on stomatal structures. Fig. 19. Penetration sites and remnants of partially removed appressorium on A. plantago-aquatica 42 h after inoculation. Fig. 20. Appressorium and damaged cuticle following attempted removal of inoculum from S. graminea 48 h after inoculation, and subcuticular penetration (inset). Figs 21–22. Leaf surface topography of un-inoculated leaves. Fig. 21. Non-host species, S. montevidensis. Fig. 22. Host species, A. plantago-aquatica including wax deposition. Bars = 10 μm, except Figs 16–17, 17 inset, 19–20 inset = 1 μm.
micrographs (Fig. 5). Symptoms of successful invasion were apparent on the leaves of *A. plantago-aquatica* and *S. graminea* within four to six days after inoculation. The first macroscopic symptoms of disease included pepper spotting, which was followed by the development of small brown necrotic spots with yellow haloes. This chlorotic appearance suggests that *P. alismatis* produces toxins during the infection process. As disease progression continued individual lesions coalesced to form larger lens-shaped lesions (Figs 23–24). No evidence of penetration or disease initiation was observed on leaves of *S. montevidensis*.

**DISCUSSION**

This study represents the first critical investigation of the infection process of *Plectosporium alismatis* on *Alisma plantago-aquatica*, *Sagittaria graminea*, and *S. montevidensis*. With three species identified as targets for weed biocontrol. Surprisingly, the leaf surface of the host species *A. plantago-aquatica* was the least conducive to conidial germination and the formation of infection structures. Nevertheless, propagules of *P. alismatis* attached, germinated and formed appressoria on the leaves of both susceptible and resistant species at similar rates, indicating that attachment, germination and appressorial formation *per se* do not represent barriers to infection, nor a means of resistance in *S. graminea* and *S. montevidensis*.

In contrast, the plant cuticle represented a significant barrier to infection. Topographical and/or hydrophobicity differences in the leaf surface of individual species affected the ability of the fungus to penetrate this structure. This was particularly evident following inoculation of leaves of *S. montevidensis* (Fig. 21), which unlike *S. graminea* and *A. plantago-aquatica* (Fig. 22), appeared devoid of wax deposits. Hence, the behaviour of inoculum following deposition on leaves of *S. montevidensis* varied greatly from the ‘beaded’ nature observed on other species and likely affected the capacity of the inoculum to ‘wet’ the leaf surface of this species. While this did not seem to affect the number of appressorial initials on *S. montevidensis*, which were greater in number on this species than on any other in the study, there was a distinct increase in the number and length of germ tubes during interactions with *S. montevidensis*.

Niks (1990) reported that increases in germ tube number and length decreased the amount of energy available for *Puccinia hordei* sporelings to reach stomatal entry points during infection of *Hordeum vulgare*. He believed that sporelings simply ‘ran out
of steam’ prior to completion of the infection cycle, largely due to the depletion of endogenous energy reserves. While stimuli such as nutrient status are known to be important for germination and appressorial formation (Parberry & Blakeman 1978, Staples & Hoch 1997) and may limit the growth of the hyphal tip, thus limiting the ability of the fungus to penetrate S. montevidensis, nutrient deprivation is also a potent stimulus for germ tube differentiation and appressorial formation (Blakeman & Parberry 1977, Howard, Bourrett & Ferrari 1991). Unfavourable stimuli of this nature induce ‘mistakes’ in locating or recognising appropriate sites for penetration and contribute to ‘avoidance’. Hence, high numbers of appressoria do not translate to comparable levels of infection. This type of non-conducive cue may have contributed to the large numbers of appressoria observed on S. montevidensis.

In contrast to S. montevidensis, cuticular penetration was observed frequently on the leaves of both A. plantago-aquatica and S. graminea. In the former, evidence of ‘specific accommodation’ (Heath 1981) was observed and the pathogen parasitised the host with no apparent resistance. In such cases infection vesicles may be surrounded by an interfacial matrix containing glycoproteins that separate the fungal cell wall from invaginated host plasma membranes (O’Connell 1987). This has been demonstrated in species of Colletotrichum, similar taxonomically to P. alismatis, and allows the fungus to establish itself within sufficient tissue such that host defences are avoided, suppressed or reduced (O’Connell & Bailey 1991, Heath & Skalamera 1997).

In the case of S. graminea, ‘incompatibility’ between the two organisms was observed and mycelial proliferation within the plant triggered strong resistance reactions. Pigmented appositions or ‘haloes’ were apparent at infection sites and underlying cells rapidly developed a necrotic appearance. The literature indicates that aggregations of this nature often become pigmented, lose their shape and release their contents into the cytoplasm killing the cells, during which fungal development is restricted and disease progression limited to the epidermal cells invaded by the fungus (Snyder & Nicholson 1990, Snyder et al. 1991).

These pigmented appositions or ‘haloes’ may represent papillae, cell wall thickenings facilitated by rapid lignification of cell wall materials (Heath 1984). Notably, some studies indicate that the processes of papilla formation and the appearance of haloes are metabolically related (Ride & Pearce 1979, Zeyen & Bushnell 1979), and the development of lignified papillae has been linked to resistance of epidermal cells to fungal invasion (Sherwood & Vance 1980). The deposition of phenolics and silica within papilla has also been shown to prevent fungal invasion (Heath & Stumpf 1986, Perumalla & Heath 1991). Hence, these structures may play a significant role in arresting disease progression subsequent to fungal penetration of S. graminea. The appearance of necrotic cells at infection sites may also be attributed to other mechanisms including the release of phenolics, phytoalexins or hypersensitive response (HR)-related reactions (Heath 1984). Common during ‘incompatible reactions’ the HR is characterised by rapid cell death around infection sites and restriction of pathogen ingress (Heath 2000).

Evidence of fungal exudate around infection sites suggests that the cuticle and epidermis of A. plantago-aquatica and S. graminea might, in part, be circumvented by chemical means. If so, structural changes in the leaf surface thought to represent papillae may in fact result from degradation of the leaf surface due to the presence of enzymes, known components of fungal extracellular materials (McRae & Stevens 1990). The depressions that were observed around infection sites also indicate that physical pressure plays a role in the infection process. However, few reports indicate that mechanical penetration is accomplished in the absence of appressorial melanisation (Muirhead & Deverall 1981).

To date, information on the infection process of P. alismatis continues to accumulate and research shows that the fungus is able to germinate, form infection structures and penetrate the cuticles of both host and some non-host species. Knowledge of the processes that contribute to recognition between P. alismatis and members of the Alismataceae however, remains limited. Nevertheless, this study suggests that germination and appressorium formation by P. alismatis are non-specific responses that require little or no stimulus from the host, a concept introduced by Emmett & Parberry (1975) that explains why fungal pathogens are able to germinate and form infection structures on a wide range of surfaces. Because cytoplasmic migration is evident in fungal propagules on the leaves of the resistant species, S. montevidensis, the differentiation of penetration hypha may also be non-specific, with ‘recognition’ occurring after penetration of the plant cuticle and resulting in basic compatibility, resistance or avoidance-type responses.

Although additional work is required, P. alismatis has the ability to penetrate the cuticle of the S. graminea, one of two species in this genus identified as targets for biological weed control in Australian rice fields. While symptom development was confirmed on intact plants, behaviour on detached leaves did not reflect that observed in the field where no prior records of the fungus have been reported on this species. Although controlled environmental conditions likely predisposed plants of this species to infection, thus broadening the host range of the fungus artificially, it is clear that P. alismatis has the capacity to incite disease on species of Sagittaria, and that constraints to disease initiation in the field may be purely environmental in origin. Hence, the feasibility of a broad spectrum mycoherbicide for weedy members of the
Alismataceae remains a viable option. Additional experiments are planned in an attempt to overcome these constraints.

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