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Short Communication

Plant-Pathogen Interaction between *Exserohilum monoceras* with *Oryza sativa* and *Echinochloa crus-galli*

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ABSTRACT

Exserohilum monoceras was isolated from infected *Echinochloa crus-galli*, and maintained in the dark under near visible ultraviolet (NUV) light at 30 °C on V8 (half-strength) agar. Conidia were collected from 14 day old V8 (half-strength) agar plates by washing the surface with sterile distilled water and using a rubber spatula to rub. Prior research has indicated that *E. monoceras* was pathogenic to *E. crus-galli* and other *Echinocloa* species under optimum greenhouse conditions. Rice was also infected by *E. monoceras,* but it exhibited a resistant reaction and the inoculated plants recovered over time. The aims of this study were to examine the physical aspects of infection by *E. monoceras* on the leaf surfaces of the resistant rice (*Oryza sativa*) and susceptible *E. crus-galli* plants, and to suggest ways to make the pathogen an effective bioherbicide agent. Observation of the infection was done by light microscopy which involved cross section and leaf clearing method and also by scanning electron microscopy (SEM). Formation of appressorium indicated that an infection on the susceptible plant was clearly seen on both methods used. Germination of the conidia was always associated with appressoria formation on the leaf and formation of appressoria was significantly higher (98%) on *E. crus-galli* leaves as the target plant compared to rice leaves (20%). This research also shows the germ tubes rarely ever penetrated *via* the stomata but through the cuticle (direct penetration) instead.

Keywords: *Exserohilum monoceras, Echinochloa cruss-galli,* appressoria, bioherbicide

ABSTRAK

Exserohilum monoceras telah diperolehi dari *Echinochloa crus-galli* yang dijangkiti dan disimpan di bawah cahaya ultralembayung hampir nampak (NUV) pada suhu 30 °C di atas agar V8 (separuh kekuatan). Konidia diperolehi dari piring petri agar V8 (separuh kekuatan) pada hari ke 14 dengan membasuh permukaan dengan air suling steril. Kajian sebelumnya menunjukkan bahawa *E. monoceras* adalah patogenik kepada *E. crus-gali* dan spesis *Echinochloa* yang lain di bawah keadaan optimum dalam rumah hijau. Pokok padi turut dijangkiti oleh *E. monoceras* tetapi menunjukkan reaksi rintang dan seterusnya pulih mengikut masa. Matlamat kajian ini adalah untuk mengkaji aspek fizikal jangkitan

oleh *E. monoceras* di permukaan daun padi (*Oryza sativa*) yang rintang dan *E. crus-galli* yang rentan penyakit, serta mencadangkan kaedah untuk meningkatkan keberkesanannya sebagai agen bioherbisid. Pemerhatian terhadap proses jangkitan telah dilakukan dengan mikroskopi cahaya yang merangkumi keratan rentas (histologi) dan penyahwarnaan daun serta mikroskopi imbasan elektron (SEM). Pembentukan apresorium menunjukkan jangkitan di permukaaan daun yang rentan adalah jelas kelihatan dalam kedua-dua kaedah yang digunakan. Percambahan konidia selalunya berkait dengan pembentukan apresoria di permukaan daun dan pembentukan apresoria adalah tinggi secara signifikan (98%) pada permukaan daun *E. crus-galli* yang merupakan sasaran berbanding hanya 20% pada daun *O. sativa*. Kajian ini juga menunjukkan jangkitan pada keseluruhanya adalah melalui kutikel daun (jangkitan terus) dan jarang dijumpai melalui rongga terbuka seperti stomata.

Kata kunci: *Exserohilum monoceras, Echinochloa cruss-galli,* apresoria, bioherbisid

INTRODUCTION

The infection strategies of plant pathogenic fungi involves several stages such as attachment and germination of propagules, differentiation of the germ tubes into specialized pre-penetration structures, penetration of the host cell, and development of infection hyphae and colonization of plant tissue. At any of these stages, slight inherent or induced differences in morphology, biochemistry or physiology between plants can have a major effect on the establishment of a compatible interaction with a pathogen and hence expression of disease symptoms. Resistant reaction mechanisms restricting colonization of the resistant host may develop at any stage of the infection process, and act simultaneously or sequentially depending on the morphology and the biochemistry of the plants as well as the environmental conditions. Thus, detailed knowledge about the infection process may provide helpful information to carry out research towards optimizing the effectiveness of bioherbicides.

Prior research had indicated that *E. monoceras* was pathogenic to *E. crus-galli* and other *Echinocloa* species under optimum greenhouse conditions. Rice was also infected by *E. monoceras,* but it exhibited a resistant reaction and the inoculated plants recovered over time. Various mechanisms of infection have been explained in other pathosystems. The most closely related report was by Hau and Rush (1982), in which they studied the pre-penetration activities of *Helminthosporium oryzae* on susceptible rice cultivar. Scanning electron microscope observation suggested that seedlings and mature plants may have comparable resistance mechanisms in the pre-penetration period. However, limited information is available on the histological aspects of infection, particularly at the incipient stage for the *E. monoceras*-rice and *E. monoceras-E. crus-galli* pathosystems.

MATERIALS AND METHODS

Fungal Culture

Exserohilum monoceras was isolated from diseased *Echinochloa crus-galli*, and maintained in the dark under NUV light at 30 ^{o}C on V8 (half-strength) agar. Conidia were collected from 14 day old V8 (half-strength) agar plates by washing the surface with sterile distilled water and using a rubber spatula to rub and scrape the surface of the agar to dislodge the conidia. The conidia were counted with a hemacytometer and diluted to the required concentrations by adding sterile water.

Light Microscopy

Spore germination on leaves of different host

Detached leaves of *Echinochloa crus-galli* (as susceptible host), rice (as resistant plant) and water agar (as control) were inoculated with 10 µL of conidial suspension at a concentration of < 10^4 and placed in petri plates containing sterile moistened filter paper to maintain the humidity. The inoculated leaves were incubated at 30 ^oC for 24 hours in approximately 12 hour darkness and 12 hour natural light. After incubation, leaf sections were fixed on filter paper saturated with a formalin/alcohol/acetic acid (1:18:1 v/v/v) solution in plastic petri dishes sealed with Parafilm (American National Can, Greenwich, CT) for 2 hour. The leaf sections were soaked for 42 to 48 hours in a solution of chloral hydrate (200 g), distilled water (80 mL) and ethanol (250 mL) to decolorized the leaf sections. They were then preserved in scintillation vials containing 4 mL of lactophenol solution (20 g phenol, 20 mL lactic acid, 40 g glycerine, and 20 mL water). The sections were stained using cotton blue stain in lactophenol solution (100 mL lactophenol, 1 mL 1% aqueous cotton blue and 20 mL glacial acetic acid), and then mounted in glycerol.

The percentage germination and number of appressoria formed were determined by counting the germinated conidia and appressoria in five ocular views per leaf section through a light microscope. A conidium was considered to have germinated if the length of its germ tube was half the length of the conidium itself and the blue-staining germ tube was visible.

Cross section

Young detached leaves of the two hosts were inoculated with 10 uL conidial suspension with a concentration of $10^4/mL^3$ conidia. The inoculated leaves were incubated for 12 hours on moist filter paper. Leaf sections were fixed by soaking overnight at 4 °C in 3% glutaraldehyde in 0.05 M sodium phosphate, pH 6.8. After fixation, the sections containing conidial suspension were infiltrated with Xylene:Paraplast (75:25, 50:50, 25:75, 0:100 v/v) overnight in an oven at 75 ºC. The sections were then embedded in 100% wax and kept between 3 to 5 days in a freezer at 5 °C. The samples were sectioned with microtome and stained with 1% methylene blue.

Scanning Electron Microscopy

Leaves were inoculated with 10 µL conidial suspension with a concentration of $10^4/mL^3$ conidia and incubated at 30 ºC for 12 hours. Sections of the leaves were harvested for electron microscopic examination using standard techniques (Lee, 1993). The leaf sections were fixed with 2.5% gluteraldehyde in 0.05 M sodium phosphate, pH 6.8, for 4-6 hours at 4 ºC, washed with sodium cacodylate buffer three times (10 min each wash). They were finally fixed in 1% osmium tetroxide for 1 hour and washed with 0.1 M sodium cacodylate buffer as before.

The samples were dehydrated through a series of graded ethanol (10%, 20%, 30%, 40%, 50%, 70%, 80%, 90%, then 100%, 100% and 100% acetone). The samples were soaked for 10 min at each concentration except for 100% ethanol in which they were soaked for 15 min twice. Finally, they were soaked for 15 min in 100% acetone. The drying was completed by placing the samples in a flow of CO_2 in a Samdri-780-A critical point dryer (Tousimic Research Corp., Rockville, MD). The samples were mounted on aluminium stubs and coated with Au/Pd using Hummer V sputter coater (Techinc, Alexandria, VA), and viewed and photographed under a scanning electron microscope (Philips XL30).

RESULTS

Light Microscopy

The leaves used remained hydrated for the duration of the observation. After 8 hours of inoculation, brownish-black lesions formed on the leaves of *E. crus-galli* eventually causing necrosis to the entire leaf. Very few non-coalescing lesions developed on the rice leaves, indicating a resistance reaction. The conidia of *E. monoceras* germinated equally well on water agar, *E. crus-galli* leaves and rice leaves within 8 hours after inoculation (Table 1).

Table 1. Comparison of conidial germination and appressorium formation by *E. monoceras* on *E. crus-galli* (as susceptible plant) and *O. sativa* (as resistant plant).

Substrate	Time (h)	Germination (%)	Apressorium formation (%)
Water agar	8	97.50	0
O. sativa	8	91.25	1.25
E. crus-galli	8	98.25	92.75

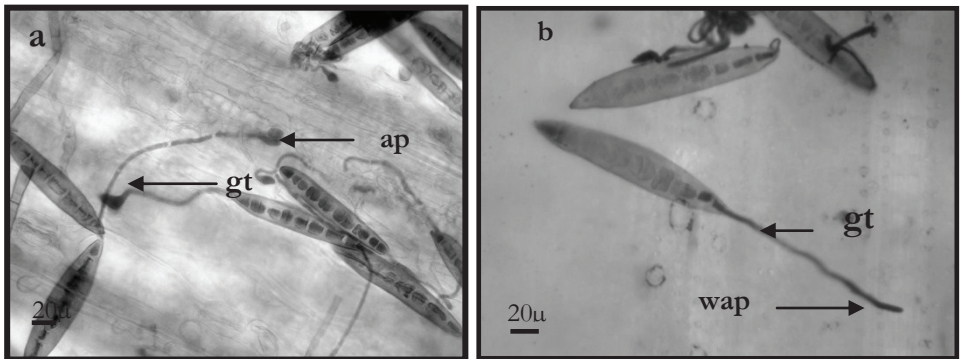

Fig. 1: Light micrograph of the infection process by *Exserohilum monoceras:* The fungus germinated and produced a germ tube (gt) on *Echinochloa crus-galli* (a) with appressorium (ap), and (b) without appressorium (wap) on *O. sativa*

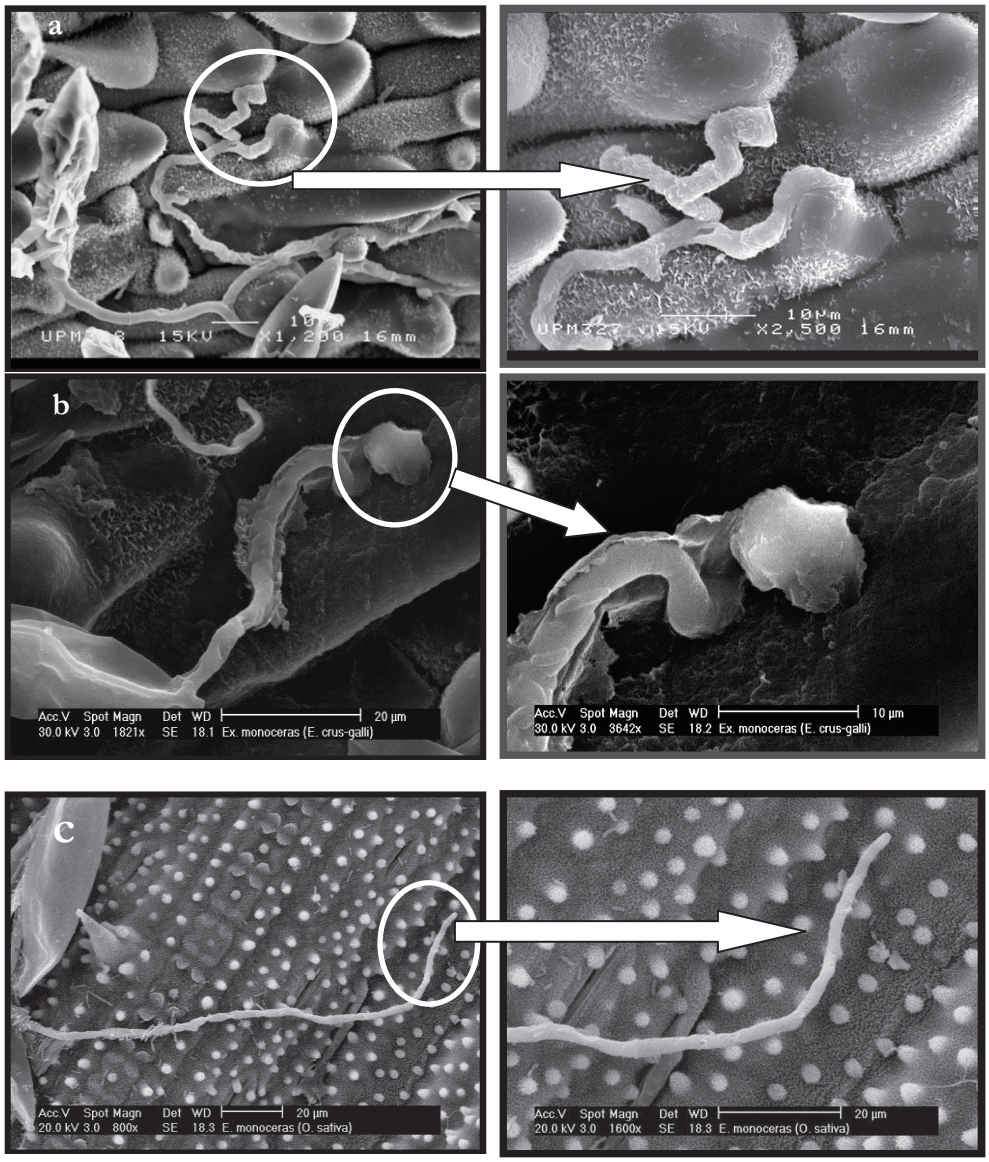

Fig. 2. Electron micrograph of *Exserohilum monoceras* infection on *Echinochloa crus-galli* (a and b) with appressorium, and (c) uninfected *O. sativa* without appressorium, proving that no infection process occurred

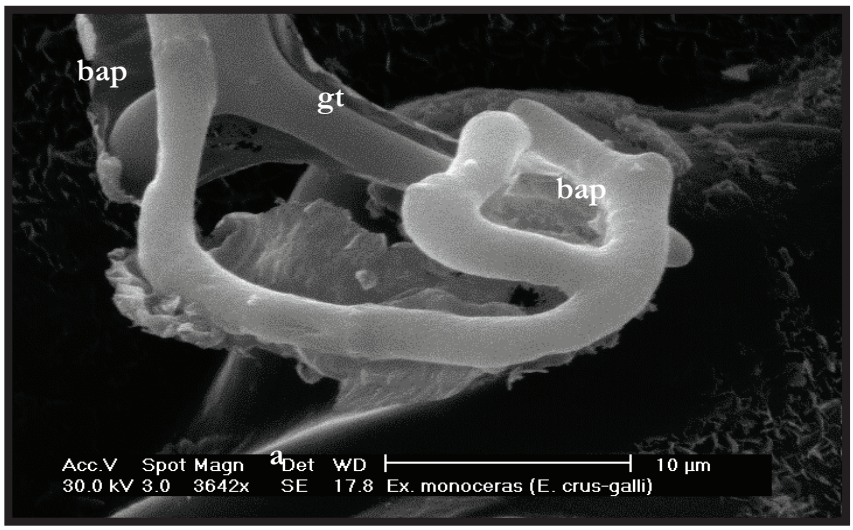

Fig. 3. Electron micrograph of the infection process by *Exserohilum monoceras* through stomata (a). The fungus germinated and produced a bulbous appressorium (bap) along the side, and especially at the end of the germ tube (gt)

Germination of the conidia was always associated with appressoria formation on the leaf. Appressoria formation was significantly higher (98%) on *E. crus-galli* leaves as compared to rice leaves (20%) (Table 1). The appressoria arising from the primary germ tube appeared slightly lobed to fully lobed on all the germinated conidia on *E. crus-galli* (Figure 1a). Branching of the primary germ tube and the emergence of a mycelium from the primary appressorium were common occurrences on the *E. crus-galli* leaves (Figure 1a).

The germination and pre-penetration of structures formed by *Exserohilum monoceras* on *Echinochloa crus-galli* and rice are depicted in Figures 1a and 1b, respectively. Germ tubes were produced on both rice and *E. crus-galli* leaf surfaces 4 hours after inoculation. Penetration occurred directly through the formation of appressoria 8-12 hours after inoculation in the cuticle and epidermal cell wall of both plants.

Although most of the conidia germinated on the rice leaves, they mostly failed to penetrate the leaf cuticle as evidenced by the unformed appressorium and thin elongated germ tubes (Figure 1b). On a susceptible host, the appressoria were predominantly sessile (Figures 2a and b). But in a resistant host, a septum developed between the appressorium and conidium or germ tube.

Appressoria could be produced anywhere on the leaf epidermis of *E. crus-galli*, irrespective of the surface topography. Sometimes the germ tubes even grew towards a stoma but the norm was to pass near to the stomata without appressorial formation. Penetration through the stomata was rarely observed (Figure 3), and extracellular matrix developed around the appressoria at the sites of attachment on *E. crus-galli* as indicated by bap in Figure 3.

The mesophyll cells beneath the appressoria sometimes appeared necrotic, although without any evidence of fungal invasion. The first signs of penetration on *E. monoceras* were observed 8 hours after inoculation and penetration occurred directly through the cuticle and epidermal cell wall (Figure 1a; Figures 2a and b). The fungus appeared not to produce extensive hyphae in the cuticle of the rice leaves. A few secondary hyphae were produced but restricted to around the infection sites (Figure 2a), indicating that a resistant reaction had inhibited the fungus from spreading beyond the infection sites. The successful infection on *E. crus-galli* often leads to the collapse of the infected and adjacent epidermal cells (Figure 5). Hyphal growth was vigorous and aggregated in the sub-epidermal chamber of *E. crus-galli*. This phenomenon was not observed in *Oryza sativa* (rice).

Leaf clearing and cross sectioning

The study of infection process can be described clearly by the evidence observed inside the cell. This study showed that fungus penetrated directly through the cells (intercellular) (Figure 4a) and intracellular movements (Figure 4b).

Cross-section of the *E. crus-galli* inoculated leaves showed the apparent growth of fungus inside the cells. The difference can be explained by the bulk of mycelium or infection structure observed within the susceptible *E. crus-galli* (Figures 5b and 5c) compared to the normal cell from the non-inoculated leaves (Figure 5a) and in rice.

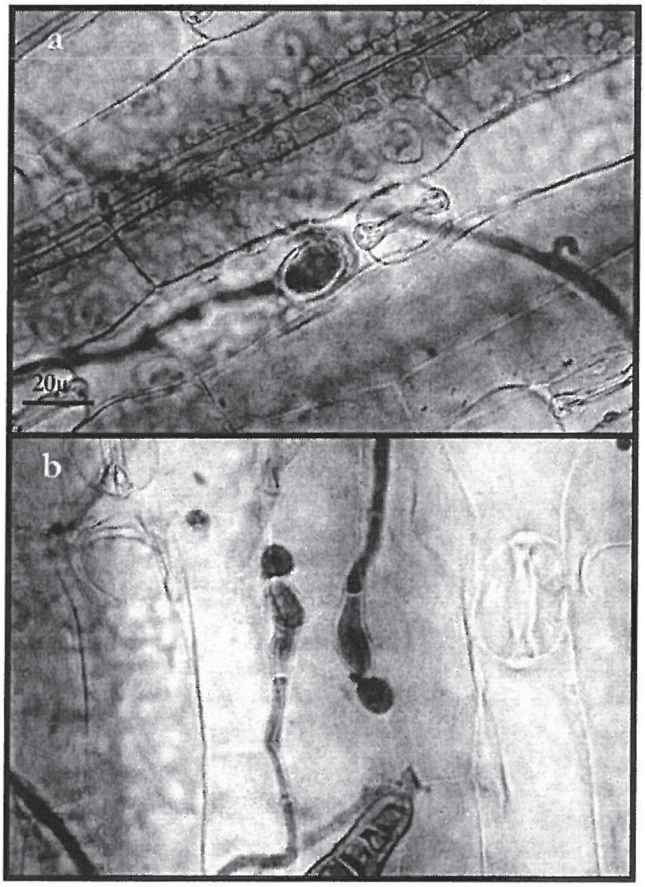

Fig. 4. Leaf clearing methods showed the mycelium intracells inside the *Echinochloa crus-galli* leaves viewed under light microscope

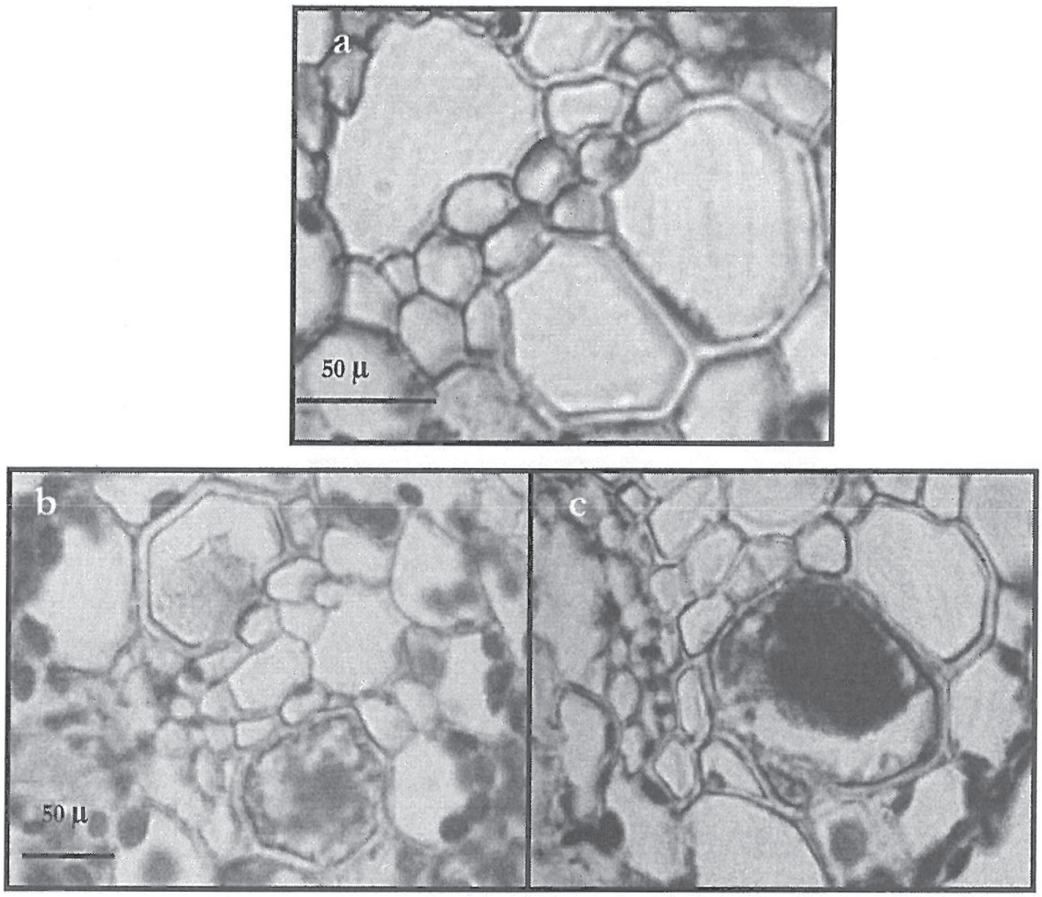

Fig. 5. Cross-sectional view of the infection process on leaves inoculated with *Exserohilum monoceras* at 24 hours after inoculation: (a) Non-infected cell (control), (b) and (c) are secondary hyphae compartmentalized in cells

DISCUSSION

This study compared the infection process of *Exserohilum monoceras* on susceptible and resistant hosts. The study of this pathogen has received limited attention especially at the microscopic level although the infection process of its group of fungi has been investigated in great detail on various hosts (Hilu and Hooker, 1963; Knox-Davis, 1974; Hau and Rush, 1982; Cromey and Cole, 1985; Morin *et al.*, 1996; Tsukamoto *et al.*, 1999).

In this study, conidia germination and production of appressoria was high on *E. crus-galli* and other susceptible *Echinochloa* spp. but relatively low on non-host plants such as rice, tomato and corn. This observation corroborated with the findings of Irwin *et al.* (1984), Vinijsanun *et al.* (1987) and Trevorrow *et al.* (1988) who reported significantly higher penetration which resulted in higher infection on susceptible hosts.

Under the light microscope, germinated conidia produced numerous germ tubes on the leaf surfaces of *E. crus-galli* (susceptible plant) but not on rice (resistant plant). The germ tubes rarely ever penetrated *via* the stomata but through the cuticle instead.

Within 12 hours of inoculation, *Exserohilum monoceras* had penetrated the epidermis of the susceptible host cells *via* a combination of chemical and mechanical means, after which it began to produce extensive secondary hyphae. The pathogen penetrated the susceptible plant and produced infection structures within, either intracellularly or intercellularly. In *E. crus-galli*, intracellular penetration of an epidermal cell resulted in the development of extensive hyphal branching which developed into a spherical vesicle, wholly colonizing the epidermal cell.

The deformation of the leaf from loss of physical pressure and the collapse of the germinated conidia were due to the outward transfer of cell contents through the appressoria to the infection sites of the hyphae (Roderick and Thomas, 1997). It can therefore be assumed that direct penetration starts soon after appressorium formation although further study is still needed on the presence of the penetration peg.

There were some surface constraints to the conidial infection. Staples and Hoch (1997) stated that the nature of the leaf surface is one of the most important factors affecting appressorium formation. Their furry leaf surface was a constraint, as most of the appressoria were formed in the non-furry leaf area. Staples and Hoch (1997) also stated that appressorium formation is regulated by different mechanisms with 'well defined' environmental conditions requirement for most species. They highlighted that these conditions are required for expression of the genotype controlling appressorium formation. The fact that not all of the conidia produced appressoria can be explained is caused by the likelihood that different conidia in a population may require different conditions for appressorium formation (Emmett and Parbery, 1975).

The mechanism for conidial attachment has not been established for phytopathogenic fungi, although Chandramohan (2000) reported that the attachment of *Phomopsis amaranthicola* and *Fusarium udum* to their hosts is induced by the presence of plant waxes, which serve as receptor sites. The sheath surrounding the germ tubes has been reported as aiding conidia attachment. This was supported by Hau and Rush (1992), who reported that a resistant plant produces more sheath for penetration than a susceptible plant.

The number of germ tubes produced by *Exserohilum* spp. and their ability to penetrate through the stomata or directly through the cuticle is not unusual. Whether these characteristics enhance pathogenicity in this group of fungi is still not clear. Hyphal penetrations were rarely observed prior to necrosis of the mesophyll cells, and many mesophyll cells beneath the appressoria were already necrotic even before penetration.

The death of these cells in advance of fungal penetration suggests action by one or more diffusible toxins. Studies on a wide variety of pathogenic species in the *Helminthosporium* group have shown that toxins (typically sesquiterpene) are produced by these fungi and are responsible for many of the disease symptoms on the host (Sugawara *et al.*, 1988; Yun *et al.*, 1988; Kenfield *et al.*, 1989).

Although some of the conidia were successful in penetrating the resistant host, infection progressed much slower than in highly susceptible barnyard grass. Moreover, extensive colonization was eventually stopped by what appeared to be a hypersensitive reaction of cells contiguous to those infected by the fungus. Indeed, recognition of incompatible reactions between *Colletotrichum gleosporioides* and moderately resistant hosts seemed to take place a few days after successful penetration of one of few epidermal cells (Morin *et al.*, 1990). The low number of appressoria of *E. monoceras* recorded on rice indicated that most conidia sprayed onto the plants were ineffective in initiating disease.

REFERENCES

Chadramohan, S. 2000. *Multiple-pathogen Strategy for Bioherbicidal Control of Several Weeds*. PhD Dissertation, University of Florida, Gainesville, FL. 199 pp.

Cromey, M. G. & Cole, A. L. J. 1985. Cytology of the host-pathogen interactions between *Lolium perenne* and *Drechslera dictyoides*. *Plant Pathology* **34**: 83-94.

Emmett, R. W. & Parbery, D. G. 1975. Appressoria annual review. *Phytopathology* **13**: 147-167.

Hau, F. C. & Rush, M. C. 1982. Preinfectional interaction between *Helminthosporium oryzae* and resistant and susceptible rice plants. *Phytopathology* **72**: 258-292.

Hilu, H. M. & Hooker, A. L. 1963. Monogenic chlorotic-lesion resistance to *Helminthosporium turticum* in corn seedlings. *Phytopathology* **53**: 909-912.

Irwin, J. A. G., Cameron, D. F. & Radcliff, D. 1984. Influence of environmental factors on the development of the anthracnose diseases of *Stylosanthes* spp. *Aust. J. Agric. Res*. **35**: 473-478.

Kenfield, D., Sugawara, F., Bunkers, G., Wu, Y., Strobel, G., Fu, Y. & Clardy, J. 1989. Gigantenome, a novel sesquiterpene activity of phytohormone mimic. *Experientia* **45**: 900-902.

Knox-Davies, P. S. 1974. Penetration of maize leaves by *Helminthosporium turticum*. *Phytopathology* **64**: 1468-1470.

Lee, R. E. 1993. *Scanning Electron Microscopy and X-Ray Microanalysis*. Prentice Hall, Englewood Cliffs, New Jersey. 458 pp.

Morin, L., Watson, A. K. & Reeleder, R. D. 1996. Effect of dew, inoculum density, and spray additives on infection of field bindweed by *Phomopsis concolvulus*. *Canadian Journal of Plant Pathology* **12:** 48-56.

Roderick, H. W. & Thomas, B. J. 1997. Infection of ryegrass by three rust fungi (*Puccinia coronata*, *P. graminis* and *P. loliina*) and some effects of temperature on the establishment of the disease and sporulation. *Plant Pathology* **46**: 751-761.

Staples, R. C. & Hoch, H. C. 1997. Physical and chemical cues for spore germination and appressorium formation by fungal pathogens. In *The Mycota a Comprehensive Treatise on Fungi as Experimental System for Basic and Applied Research Vol. V*. K. Esser and P. A. Lemke (eds.). Springer: Berlin. p. 27-40.

Sugawara, F., Takashi, N., Strobel, G., Strobel, S., Liu, H., Clardy, J., Triticones, A. & B. 1988. Novel phytotoxins from the plant pathogenic fungus *Drechslera tritici-repentis*. *J. Am. Chem. Soc.* **110:** 4086.

Trevorrow, P. R., Irwin, J. A. G. & Cameron, D. F. 1988. History compatible and incompatible interactions between *Colletotrichum gloeosporioides* and *Stylosanthes scabra*. *Transactions of the British Society* 90: 421-429.

Tsukamoto, H., Tsuda, M. & Fujimori, T. 1999. Mode of infection of *Echinochloa oryzicola* by *Exserohilum monoceras*. *Ann. Pathopathol. Soc. Jpn.* **65:** 553-556.

Vinijsanun, T., Irwin, J. A. G. & Cameron, D. F. 1987. Host range strains of *Colletotrichum gloeosporioides* from tropical pasture and comparative histological studies of interactions between typical producing strains and *Stylosanthes* (non-host) and *S. guianensis*. *Australian Journal of Botany* **35**: 665-677.

Yun, C., Sugawara, F. & Strobel, G. 1988. The phytotoxic ophiobolins produced by *Drechslera oryzae*, their structures and biological activity on rice. *Plant Science* **54**: 237-243.