

## Histological Study of the Interaction between *Exserohilum Longirostratum*, Barnyard Grass, and Rice var. MR219

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### ABSTRACT

The course of infection and the development of *Exserohilum longirostratum* (Subramanian) Sivanesan on barnyardgrass (*Echinochloa crus-galli* (L.) Beauv. spp. *crusgalli*) and rice (*Oryza sativa* L. var. MR219) were studied under light microscopy (LM) and scanning electron microscopy (SEM). Observation under SEM indicated similarity of the gross anatomy of both rice and barnyard grass leaves. Meanwhile, germination of the conidium of *E. longirostratum* was found to be not influenced by inoculation time as the conidia started to germinate 4 hours after the inoculation on both leaf surfaces. However, the patterns and number of germ tubes and appressoria formation were influenced by host plants. On barnyard grass, the primary infection process consisted of the conidial germination, elongation of the germ tube, formation of the appressorial initials, maturation of the appressoria, and formation of secondary hyphae. Successful penetration was followed by an extensive colonization of the invaded epidermal cell wall. Observation of the cross section revealed that the infection hyphae expanded into a spherical vessel and colonized the cells, causing the collapse of the epidermal cells and resulting in the formation of necrotic lesions of infected and adjacent tissue. Although fungus successfully grew and produced germ tube on rice, both the primary infection process and the successful penetration of the cuticle were not observed on rice. The conidia germinated and produced slender and thin germ tube, with occasional appressorium formation. Germ tubes and appressoria

formation on the barnyard grass (70% and 92%, respectively) were significantly higher as compared to rice leaves (51% and 10%). It was observed that the mycelium infected barnyard grass much faster (less than 24 h) than the conidia as it immediately formed

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appressorium without having the need, like the conidia, to germinate first. These results suggest that it may be possible to utilize *E. longirostratum* as a bioherbicide to control barnyard grass under rice production in Malaysia.

*Keywords:* Barnyard grass, biological weed management, *Exserohilum longirostratum*, rice

## INTRODUCTION

In Malaysia, wetland rice is facing increasing weed problems due to the change in its planting method, i.e. from transplanting to direct-seeding. Among the grassy weeds, barnyard grass (*Echinochloa crus-galli* complex) has become one of the most troublesome. Its genetic similarity and similar growth requirements to rice make it a formidable competitor to the crop causing untold losses in terms of yield by its competition. It has been reported that barnyard grass reduced rice yield by 21-40% (Azmi, 2000; Tjitrosemito, 1994; Tarif *et al.*, 2004). To compound the problem, the weed is also host to many insect pests and pathogens, which will indirectly cause yet more losses in the yield and produce quality.

The present method of weed control is by herbicides, but the method, which was once economic and effective, has now begotten its own problems. In particular, the continued use of chemicals has induced resistance in the weeds and the chemical residues wrought environmental havoc. Nevertheless, herbicides continue to be used for want of other choices. It is,

therefore, imperative that safer and more environmentally-friendly alternatives be developed for greater effectiveness and to minimize the wanton destruction of the environment.

*Exserohilum longirostratum* has been investigated as a bioherbicide to control barnyard grass (Juraimi *et al.*, 2006; Ng, 2007; Ng *et al.*, 2010), with a particular attention given to the influence of environmental factors on its bioherbicidal activities. However, the histology of the pathogen-host interaction has not been elucidated, especially to understand the resistance/susceptibility of the host to the pathogen. Slight inherent or induced differences in the morphology, biochemistry or physiology between the plants can have a major effect on their resistance/susceptibility to a pathogen. In addition, different inocula of *E. longirostratum*, conidia or mycelium, may have different modes of infecting the host plant.

The main pre-infection action of the fungus is to attach itself to the host – usually on the leaf surface - by forming a germ tube to penetrate the surface, before differentiating into infection structures. Stomata are the most important natural openings for the fungus to enter. However, direct penetration through an intact epidermis can also occur, and this usually happens through formation of an infection structure at the tip of the germ tube (appressorium) or arising from the mycelium (hypopodia or infection cushion) (Vandyke & Trigiano, 1987).

As fungi thrive in warm, moist conditions, the herbicidal activity is greatly

enhanced by the presence of dew on the surface to be infected. An oil emulsion can help to overcome the adverse effects of dry conditions by preserving any available moisture for the fungus propagules to germinate. Oil emulsions have therefore been evaluated for formulating bioherbicides (Shabana, 2005). Nevertheless, there remains a dearth of information at the ultra structural level on the fungal penetration and other early events in pathogenesis by *E. longirostratum*, such as whether more or less oil in the emulsion is better for infection. Understanding the interactions should shed light on the mechanism of host death and the effect of the oil emulsion on the infection process by *E. longirostratum*. The objectives of this study were, therefore, to examine the germination and growth of *E. longirostratum* on barnyard grass and rice and to assess the subsequent infection process through light (LM) and scanning electron microscopy (SEM).

## MATERIALS AND METHODS

### *Plant Production*

*Oryza sativa* var. MR219 (rice) was used in this experiment as this variety is widely planted in Malaysia. The seeds of rice and barnyard grass (*Echinochloa crus-galli* (L.) Beauv. spp. *crusgalli*) were collected from a rice granary in Tanjung Karang, Malaysia, and were produced in flats in the glasshouse, before they were transplanted after the emergence of coleoptiles into round pots (10 cm diameter x 10.6 cm height; 5 seedlings/pot) containing a potting medium (3:2:1 top soil:sand:organic matter). The plants

were watered to soil saturation twice daily and allowed to grow until the 3- to 4-leaf stage (LS).

### *Inoculum Production*

Inoculum of *E. longirostratum*, which was isolated from *Rottboellia cochinchinensis* (Lour.) W.D. Clayton (Kadir *et al.*, 2007), was produced using a biphasic culturing technique (Chandramohan *et al.*, 2002) with several modifications. Five mycelium plugs were transferred into 100 ml of V8 broth in 250 ml flask. The inoculated flask was then shaken (100 rpm) for 2 days at 28°C and then transferred into 500ml of V8 broth in 1-L flask. The culture was allowed to grow further in 1-L flask for 2 days. The contents of the flask were blended in a Waring blender at low speed for 30-60 sec and 25 ml of this suspension was poured onto a layer of V8 agar (250 ml) in trays measuring around 35 x 26 x 2.5cm. The trays were exposed continuously to 24 h light at 30±2°C. The conidia were gently scraped off with rubber spatula into sterile water and filtered through cheesecloth. The remaining conidia were then rinsed off the agar surface with sterile water. The conidial suspensions were pooled and the concentration of conidia was determined and adjusted to the required concentration with a hemocytometer. The inoculum in the form of mycelial suspension was produced using the above method with a slight modification. After blending the content of the flask, the suspension was stored in the refrigerator at 4°C before it was used for plant inoculation.

### *Plant Inoculation*

Groups of five seedlings each of rice or barnyard grass, at the three- to four-leaf stages, were inoculated with a suspension of either conidia ( $10^5$  conidia  $\text{mL}^{-1}$ , 20 % vegetable oil, 0.05% Maxigreen [nonionic spreader and sticker] v/v/v) or mycelia (1:5 ratio of 5 day-old mycelium:V8 juice, 20% vegetable oil, 0.05% Maxi green v/v/v) of *E. longirostratum*. Eight inoculated leaves were excised and placed on a moist filter paper in a glass humidity chamber (Mason jar) and incubated in an incubator (Memmert GTR0124) at  $28 \pm 2^\circ\text{C}$  for 4 hours. Similarly, other batches of eight leaves were incubated for 8, 16 and 24 hours, respectively. After the incubation, each leaf was trimmed to a disk (about 0.5 cm x 0.5 cm). Of the eight disks, four were examined under a light microscope (LM) at 40x magnification, while the other four under a scanning electron microscope (SEM) at 600x, 850x, and 2000x magnification, respectively.

### *Light Microscopy (LM)*

Four leaf disks were fixed on a filter paper saturated with a formalin/ alcohol/ acetic acid (FAA) solution (1:18:1 v/v/v) on a petri dish, which was then sealed with a parafilm and left for 2 hours. The leaf disks were cleared for 42-48 hours by soaking in a solution of choral hydrate (200 g),  $\text{dH}_2\text{O}$  (80 mL), ethanol (250 mL) and four drops of Tween-20 (Celio & Hausbeck, 1997). The leaf disks were transferred to glass slides, and a drop of lacto phenol (20% phenol, 20% lactic acid, 40% glycerol, and 20% water) containing 0.1% cotton blue was also

added (Bailey *et al.*, 2000). The percentages of the conidia germinated and appressoria formed were determined by counting 200 conidia per leaf (taken at random) under LM with a 4X objective. A conidium was considered germinated if its blue-staining germ tube was visible with the length of the germ tube at least equal to the width of the conidium.

### *Scanning Electron Microscopy (SEM)*

Four leaf disks were fixed in 3% glutaraldehyde buffer for 2 hours and post-fixed in 1% osmium tetroxide for 2 more hours and dehydrated by passing through a graded ethanol series (10%, 20%, 30%, 50%, 70%, 90% and 100%) prior to critical point drying with  $\text{CO}_2$  as the transition medium (Spencer, 2001). The samples were then mounted on stubs and coated with gold-palladium, as well as viewed and photographed with a JEOL 5610LV SEM.

### *Data Analysis*

All the percentage data were transformed to arcsine before analysis (Gomez & Gomez, 1984). Data points were curve fitted using linear regression. Analysis of variance (ANOVA) using the general linear model was also used wherever appropriate (SAS Institute, Cary, NC) to analyze the effect of each factor individually and their interactions. Mean separation was performed using Tukey's HSD test based on the variance if the treatments showed significant differences.

## RESULTS

### *Light Microscopy*

The conidia germinated on both the rice and barnyard grass leaves as soon as 4 hr after the inoculation. Most of the conidia germinated monopolarly, with occasional bipolar germination. All the germinating conidia initially formed appressoria, arising from the primary germ tube, which was slightly lobed to lobed.

After 4 hr of inoculation, about 25% of the conidia germinated on the barnyard grass (Fig.1). The most prolific production of

germ tubes occurred 8 hr after inoculation, presumably when suitable penetration sites on the leaf surface had prevailed. Few appressoria (12% of the germinated conidia) were formed by the conidia germinated between 4 and 8 hr after the inoculation; however after 8 hrs, more appressoria (79%) formed directly on the epidermal cells or over the junctions between them. The appressoria formation reached 92% after 24 hours (Fig.1).

On rice leaves, the initial germination (9%) of the conidia occurred 4 hr after the inoculation. The germination increased

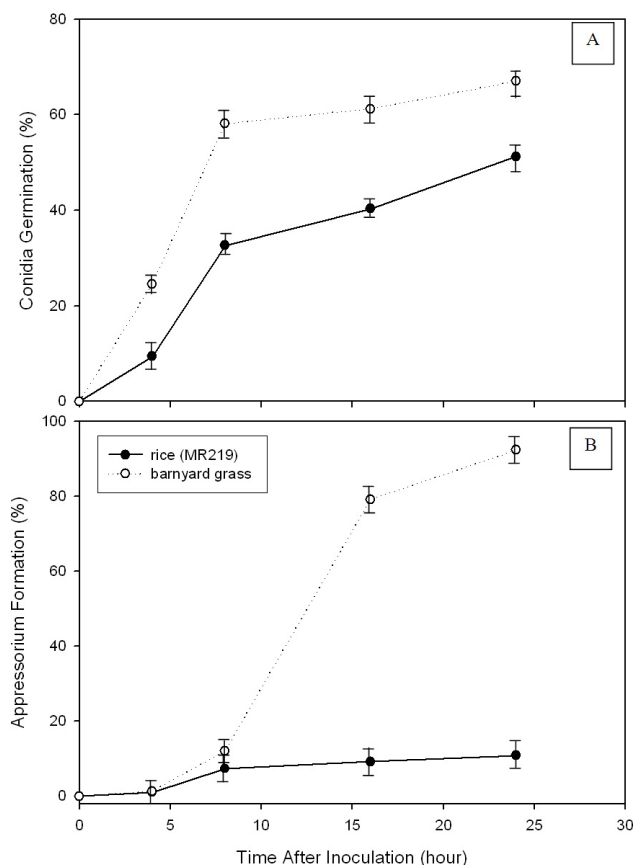


Fig. 1: The percentage of conidia germination (A), and percentage of appressorium formation (B) on rice and barnyard grass. Vertical bars represent standard errors of the means. Mean percentage of conidial germination were calculated based on 200 observations taken from a repeated experiment

quickly to reach 33% after 8 hrs, and then more gradually to 40% in 16 hrs, and finally 51% after 24 hrs (Fig.1). Fewer appressoria were formed on the rice leaves. Only 7% appressoria formed 4 hr after inoculation. The respective parallel figures for 16 and 24 hrs after inoculation were 9% and 11%. Earlier in the penetration period, there were no measurable differences in the numbers of appressoria formed on the rice and

barnyard grass leaves. Eight hours after the inoculation, a significant increase in the appressoria formation was recorded with preferential appressorium formation on the barnyard grass.

The relationship between spore germination and appressorium formation on barnyard grass is best described by a linear regression of the first order polynomial (see Fig.3). On the barnyard grass, the

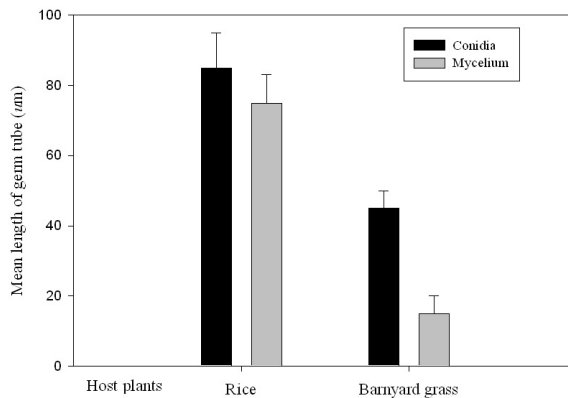


Fig.2: Length of germ tube of *E. longirostratum* on Rice var. MR219 and on Barnyard grass at 24 hrs after inoculation. Vertical bars represent standard errors of the means. All the experiments were repeated twice with 200 observations in each experiment

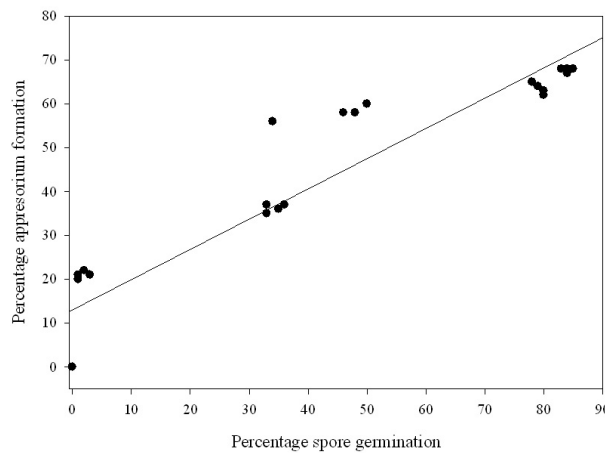


Fig.3: The correlation between percentage conidia germination and percentage appressorium formation of *E. longirostratum* on barnyard grass at 24 hrs after inoculation. The regression was fitted using percentage data. The regression obtained is  $Y = 13 + 0.69X$  ( $R^2 = 0.87$ ).

numbers of appressoria formed were positively correlated with the numbers of the germinating spores. Higher numbers of appressoria were formed after 24 hrs and this was found to be correlated with the higher numbers of germinating spores. This observation suggests that barnyard grass has many infection sites where the appressoria could penetrate the surface of barnyard grass leaves. Nonetheless, such a relationship was not manifested on rice leaves. These results indicate a random formation of appressorium on rice and is therefore not related to the number of germinating spores.

#### Scanning Electron Microscopy

The salient features of the anatomy of rice and barnyard grass leaves showed a similarity in both the leaves having vascular bundles in between thick-walled, non-living sclerotized sclerenchyma cells, most of which were fibrous. This observation has earlier been reported by Hau and Rush (1982). Both the leaves are characterized by the presence of papillae - epidermal cells

projected in rows along the axis of the leaf.

The primary infection process consists of conidial germination, germ tube elongation, formation of appressorial initials, maturation of appressoria, and formation of secondary hyphae. In the barnyard grass leaves inoculated with conidia, the infection hyphae were produced after the appressoria had formed over the bulliform cells. Germ tubes emerged mostly from the end cells of the conidia. Throughout this study, germ tube length refers to the first length of germ tube from the conidium to the first appressorium. The germ tube length varied with the host leaves. The length of the germ tubes formed by the conidia and the mycelia on rice was significantly longer (84 $\mu$ m and 78 $\mu$ m) as compared to the germ tubes on barnyard grass (42 $\mu$ m and 18 $\mu$ m), respectively (Fig.2). Many of the germ tubes extended on the surface along the junctions of the epidermal cells (Fig.4). In the intracellular penetration of the barnyard grass leaves, the infection hyphae distended into spherical vessels in colonizing the cells

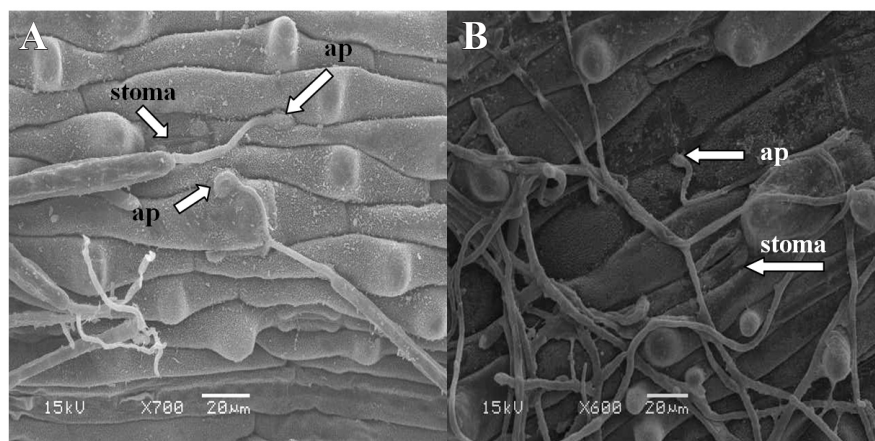


Fig.4: The SEM of the appressoria (ap) formed directly on the epidermal cells from conidial (A) and mycelium (B) based suspensions on barnyard grass

(Fig.5) leading to the collapse of the infected and adjacent epidermal cells and causing necrotic lesions.

In the mycelium inoculation, the formation of appressoria occurred before the formation of infection hyphae. The appressorium formed at the end of a massive germ tube deposited over the leaf surface. The infection hyphae then enlarged into

spherical vessels and colonized the cells (Fig.5). Colonization was more severe with the mycelial inoculation than with the conidial inoculation due to more infection hyphae being formed within 24 hrs of inoculation. It was very rarely that penetration was accomplished without a well-defined appressorium. The SEM images indicated that the fungus did not

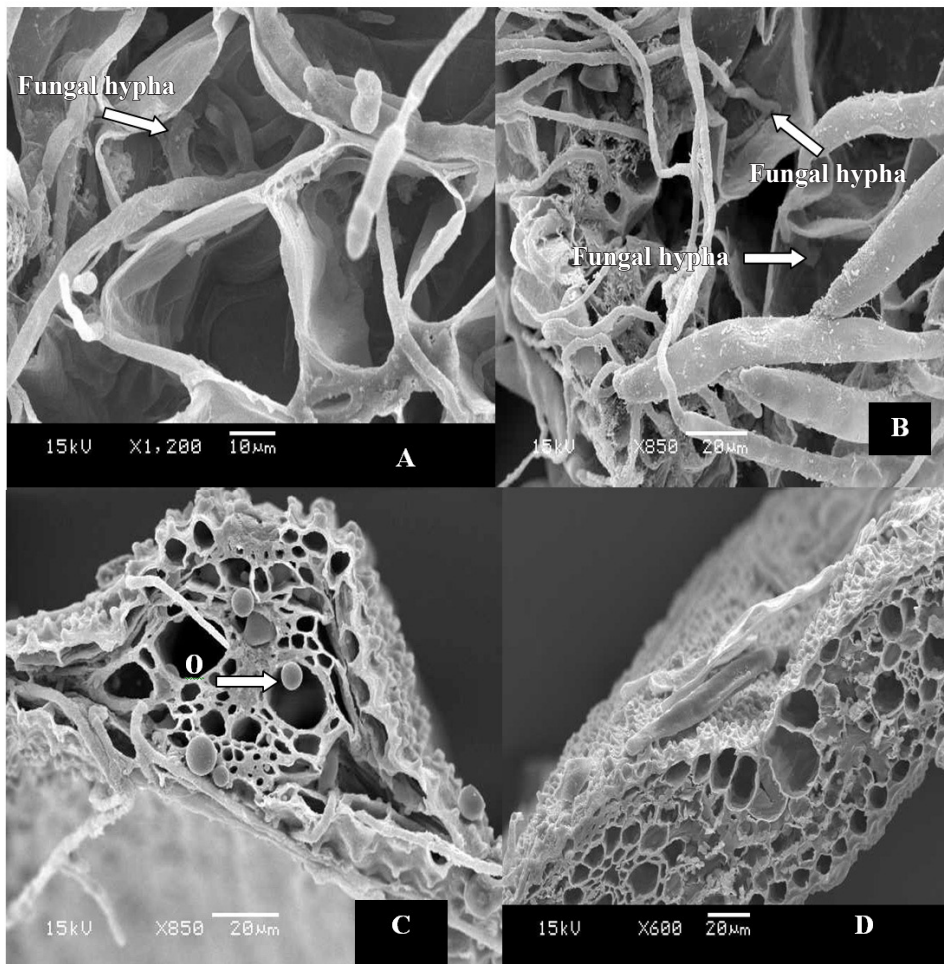


Fig.5: The cross-section of leaf showing infection process of *E. longirostratum* on the barnyard grass (A, B) and rice (C, D) leaves. In the barnyard grass, hyphae colonized the cells by mycelium (A) and conidia (B)-based suspensions. In rice, hyphae (C) and conidia (D); expansion and colonization of the cell did not occur even at 24 hr after inoculation. Some oil microdrops (O) are seen adhering to the rice cell



form appressoria over the stomata and trichomes nor entered the leaves through them.

The infection process in rice differed from that in barnyard grass, where the hyphae neither distended into vessels nor colonized the cells in rice, whether inoculated with conidia or mycelium. Meanwhile, the epidermal cells remained firmly rigid and did not collapse (Fig.5). Although the conidia were able to germinate on the rice leaves, they formed significantly

fewer appressoria compared to barnyard grass leaves. The orientation of the germ tubes was also random, suggesting that rice var. MR219 is not susceptible to *E. longirostratum*.

During the infection, the resistant host defends itself through a number of physical and chemical factors. *Exserohilum longirostratum* on the barnyard grass leaf surface is often associated with a sheath-like structure (Fig.6), mostly at the distal end of the appressoria in contact with the cuticle,

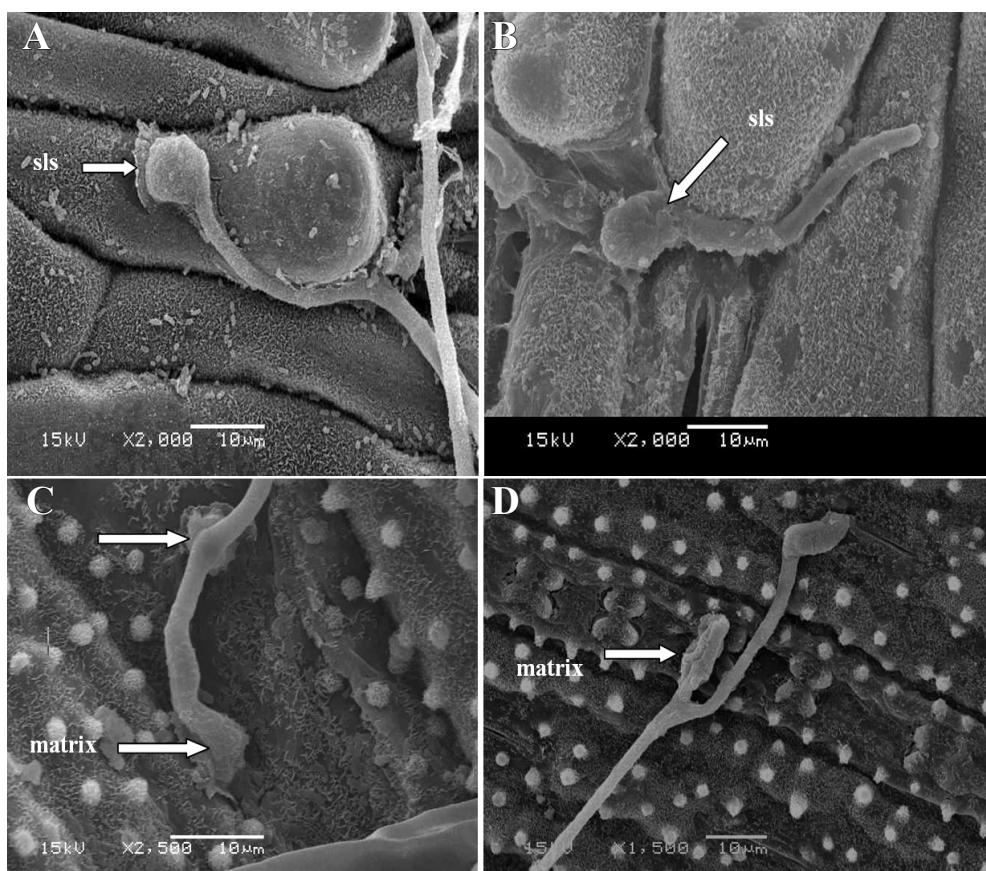


Fig.6: The appressorium of *E. longirostratum* adhering to the waxy surface of barnyard grass leaf by an extracellular sheath-like substance (A); The mycelium on the barnyard grass leaf surface often have sheath-like substances (sls) associated with them (B); The matrix formed outside the germ tube on rice (C and D)

suggesting the formation of the sheaths is induced by the presence of the pathogen. However, such sheaths were not observed on rice but instead a dense matrix of amorphous substance was formed outside the germ tube by the germinating conidia. This matrix accumulation may be the non-host response of rice to the pathogen conferring it (host) the resistance to infection.

## DISCUSSION

*Exserohilum longirostratum* has a similar infection process as other fungi. The conidium first attaches to a suitable site and then germinates in about 4 hrs after inoculation. The germ tube forms an appressorium, which in turn produces infection hyphae in the sub-epidermal cells. The appressorium forms directly on the epidermal cells or over the junctions between them so that the hyphae can directly penetrate the cuticle through the weak joints. Tsukamoto *et al.* (1999) observed that the appressoria of *E. monoceras* penetrated directly through the epidermal cells as well as through stomata of *Echinocloa oryzicola*. However, this observation was not noted by Hau and Rush (1982); instead, they reported that *Helminthosporium oryzae* infection process of susceptible rice plants started at the juncture between epidermal cells and penetrated the bulliform cells. This study concurs with both reports, with the exception that the penetration was never observed over the stomata as reported by Tsukamoto *et al.* (1999).

Barnyard grass was found to be infected faster by the mycelia than conidia inocula.

The mycelium of *E. longirostratum* infected barnyard grass much faster than the conidia as it immediately formed appressorium without having the need, like the conidia, to germinate first. Using mycelium, almost all the cells of the barnyard grass were colonized by infection hyphae within 24 hrs of inoculation. At the same time, with conidia, the infection hyphae had only just begun colonizing the host cells. The efficacy of mycelium, as a component of bioherbicidal formulation, was further justified by Shabana *et al.* 2010, who reported that the formulations containing 30% Sunspray 6E and *Drechslera gigantea* mycelium (10 g), causing 88 to 100% injury on tropical Signalgrass, Crabgrass, Smutgrass, and Torpedograss in greenhouse trials.

Rice var MR219 appeared to be resistant to both the conidia and mycelium of *E. longirostratum*. Longer germ tubes but with fewer appressoria and poor hyphal growth were observed on the rice – possible indicators of the resistance mechanism at work.

The percentages of germinating conidia and the fungus subsequently forming appressoria on barnyard grass inoculated with oil emulsion were higher than those reported by Chia (2005), who inoculated barnyard grass with *E. longirostratum* without oil emulsion. The oil emulsion was therefore effective in enhancing the efficacy of the inoculum. Similar results were also reported by Auld (1993) with vegetable-oil emulsions to formulate the fungus *Colletotrichum orbiculare* (Berk. and

Mont.) for controlling *Xanthium spinosum* L., and also by Daigle *et al.* (1990) who used invert emulsion formulations of *Alternaria cassiae* Jurair and Khan to control sicklepod [*Senna obtusifolia* (L.) Irvin and Barneby]. The oil emulsion not only retained water for the conidia to germinate, but might also have attached the conidia more strongly to the leaf surface. The oily phase of the emulsion rapidly penetrated the leaf surface into the intercellular spaces, and water diffused in from the neighbouring cells into the oil to form micro drops, and in effect, an invert emulsion. Greaves and Macqueen (1990) reported that water drops evenly diffused throughout the oily phase to form a water film on the leaf surface providing for the ideal environment for a microbial herbicide to function.

Bulliform cells have been reported as the usual infection point for *Pyricularia oryzae*, the causal agent of rice blast of rice (Hau & Rush, 1982). In Poaceae, bulliform cells are unusual epidermal cells, where the radial walls of these cells are thin and the outer walls remain in a pectic-cellulosic state long after the epidermal cells have become lignified (Clark & Lorbeer, 1976). Meanwhile, Whitney (1977) showed that a high degree of penetration in bulliform cells was correlated with low degree of mechanical toughness of the outer wall. In addition, Whitney (1977) also reported that lower concentration of chlorogenic acid (a fungitoxin) in the bulliform cells could explain the lower resistance of bulliform cells, which perhaps making them less resistant to penetration. The present

investigation showed that *E. longirostratum* preferentially formed appressoria over the bulliform cells of barnyard grass as compared to rice, especially over the junctions between them. Hau and Rush (1982) stated that the junctions between bulliform cells offer a chemical environment and groove-like topography that may stimulate the formation and maturation of appressoria.

An extracellular sheath was formed to cover the fungal structure on barnyard grass. This observation was first reported by Wheeler (1977), who had noticed that *Helminthosporium maydis* and *H. victoriae* have extra cellular sheaths associated with hyphae. The finding of the present study confirms the result of his study and also the observation by Locci (1969), who reported that under low resolution SEM, adhesive matrix associated with hyphae and appressoria and the absence of stomatal penetration was observed. The adhesive matrix adhered to the cuticle might have enabled the fungus to attach to the leaf surface and facilitated infection that caused the cell membranes of barnyard grass leaves to drastically alter and the ultra structure of cells to have severely deformed. Wheeler (1977) reported such sheaths to be common in the species within the genus *Helminthosporium* (*Exserohilum*) and this observation further concurred by the observation of Hau and Rush (1982), who reported that the sheath like substance was often associated with susceptible rice variety infected with *H. oryzae*. The absence of the extra cellular sheath on the rice var MR219

indicates a resistant reaction toward *E. longirostratum*. The extra cellular sheath is required for the appressorium to adhere to wax crystal and facilitate infections. Thus, this study has indicated that rice var MR219, a commonly cultivated rice variety in Malaysia, is resistant to *E. longirostratum*. In more specific, *E. longirostratum* has the potential to be used in controlling barnyard grass for rice production in Malaysia.

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