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Potential of *Exserohilum monoceras* as a Bioherbicide for Controlling *Echinochloa crus-galli* (Rumput Sambau)

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ABSTRACT

The fungus isolated from diseased *Echinochloa crus-galli* was identified as *Exserohilum monoceras*, based on the conidial morphology, germination characteristics and hilum morphology. The objectives of this study were to screen and isolate fungal pathogen of *E. monoceras* from infected *E. crus-galli* as well as to determine the pathogenicity of *E. monoceras* and the optimum conditions for growth and sporulation of *E. monoceras*. In repeated trials, the pathogenicity of this fungus to its host was confirmed by Koch's postulate. The pathogen caused a high level of disease on *E. crus-galli* where symptoms appeared within 24 h of inoculation. It induced eyespot lesions typical of infection by *Exserohilum* spp. and induced a severe 'burnt' appearance on the foliage of *E. crus-galli*. The leaf tissues in the vicinity of heavy infestation were completely killed. The disease did not develop on non-inoculated plants, which was indicative of localized infection and lack of significant secondary disease cycles. The laboratory study indicated that better growth and appressoria production can be attained when the fungus is exposed to temperature in the range of 25 to 30 ^{o}C. This study also indicated that Maxigreen®, which is an adjuvant, when mixed with sunflower in the ratio of 0:5:7 is ideal for formulating the conidia. This research has illustrated that solid media such as agar is an excellent growth media, but spore production has been influenced by the nutrient content of the media. The best medium for sporulation was half strength V8 juice agar.

Keywords: *Exserohilum monoceras, Echinochloa crus-galli,* Koch's postulate, infection, conidia, sporulation

ABSTRAK

Kulat yang dipencilkan dari *Echinochloa crus-galli* yang berpenyakit telah dikenalpasti sebagai *Exserohilum monoceras* berdasarkan morfologi konidia, ciri-ciri percambahan dan morfologi hilum. Objektif kajian ini adalah untuk mengenalpasti kulat patogen iaitu *E. monoceras* daripada *E. crus-galli* yang berpenyakit dan mengenalpasti kepatogenan *E. monoceras* dan kadar yang optimum untuk pertumbuhan dan sporulasi *E. monoceras*. Dalam percubaan ulangan, kepatogenan kulat ini pada perumah adalah terbukti dengan postulat Koch. Patogen ini menyebabkan penyakit pada kadar yang tinggi pada *E. crus-galli* dan simptom akan kelihatan dalam masa 24 jam. Selepas inokulasi, kelihatan lesion bintik mata yang merupakan simptom tipikal jangkitan oleh *Exserohilum* spp. Turut kelihatan

adalah keadaan 'terbakar' pada helaian daun *E. crus-galli*. Tisu daun pada kadar jangkitan yang teruk akan mati sepenuhnya. Jangkitan tidak berlaku pada pokok yang tidak diinokulasi, membuktikan jangkitan setempat dan tiada jangkitan kitaran kedua. Kajian makmal menunjukkan pertumbuhan dan penghasilan apresoria yang sesuai adalah pada suhu sekitar 25 hingga 30 °C. Kajian ini juga menunjukkan Maxigreen®, iaitu sejenis agen pelembap, adalah merupakan formulasi terbaik untuk penghasilan konidia apabila dicampurkan dengan minyak bunga matahari pada kadar 0:5:7. Kajian ini turut membuktikan bahawa media pepejal seperti agar adalah media pertumbuhan terbaik, tetapi penghasilan spora adalah dipengaruhi oleh kandungan nutrien di dalam media dan media terbaik untuk sporulasi adalah agar jus V8 separuh kekuatan.

Kata kunci: *Exserohilum monoceras, Echinochloa crus-galli,* postulat Koch, jangkitan, konidia, sporulasi

INTRODUCTION

Weeds in agrosystems have been managed through several strategies; including cultural, mechanical and chemical (Standifer *et al*., 1984; Teasdale *et al*., 1991). However, farm mechanization and effective weed management are recognized as two vitally important components of labor-saving technology in weed management in agrosystems. Chemical herbicides are the most cost-effective weed control technology currently available, however, these are becoming unpopular among members of the public. It is anticipated that environmental policies on the use of pesticides, including herbicides, will be strengthened, and become more restrictive than before.

Although chemical herbicides are a constant feature in modern agriculture, the recent confluence of factors is affecting the use of chemical herbicides as weed management strategies and consequently the agroecosystems in an unprecedented manner. The most notable changes include (1) the ban of the use of methyl bromide as a general purpose soil fumigant, (2) the phasing out of several older herbicides, (3) the high cost of developing and registering new chemical herbicides, (4) the impact of herbicide-resistant crops on the use of other weed-control methods, (5) the increasing problems with herbicide-resistant weeds and weed-shifting, (6) government-instituted mandates for reducing chemical pesticide usage, (7) consumer preference for non-chemical alternatives in food production, (8) consolidation of agrochemical companies, which affects the availability and marketing of certain chemical herbicides, and (9) shifts in the agricultural production from small and medium operations to large corporate operations and from high-cost to low-cost production areas of the world, following a pattern of globalization of agricultural production and marketing. Singly and collectively, these changes have a profound impact on weed management practices. This evolving situation creates a renewed interest and demand for biological controls. Such renewal is already evident in the United States, United Kingdom, Japan and Korea (Kadir and Charudattan, 2000) in the number of new opportunities for research and development of biologically based pest control alternatives. The potential of plant pathogens as weed control agents, both as conventional biocontrol agents, is too great for us not to continue our investment in this field of endeavor. The objectives of this study were to screen for and isolate fungal pathogen of *Exserohilum monoceras* from diseased *E. crus-galli,* to determine the pathogenicity of *E. monoceras,* as well as to determine the optimum conditions for growth and sporulation of *E. monoceras*.

MATERIALS AND METHODS

Isolation and Identification

Diseased leaves of *E. crus-galli* showing typical symptoms of *Exserohilum* spp. infection were collected from ricefields in Tanjong Karang, Selangor, and were brought to the laboratory for isolation of the

pathogen. The leaves were cut into 1-2 cm^2 pieces then soaked in 10% sodium hypochlorite solution for 60-80 s, washed with distilled water for 1 min, and air dried on sterile filter paper. Four pieces of tissue were placed on each petri plate containing water agar (WA) (granulated agar; Difco) or potato dextrose agar (PDA; Difco, Detroit, MI) on wet filter paper (Kadir *et. al.* 2008; Kadir and Charudattan, 2000). The plates were incubated in the dark at room temperature for 48 h, and observed for sporulating fungi under a light microscope. Any sporulating fungus was identified and a conidium was picked up with a needle for multiplication on PDA and V8 juice agar. Pure cultures of the recovered fungi were prepared from the single conidia and maintained on PDA slant as stock culture.

The fungi were confirmed to their genus based on their conidial morphology and growth characteristics on various growing media such as V8 juice agar and PDA. Further characterization was done on the isolates confirmed to be highly pathogenic to *E. crus-galli* by applying Koch's postulates. Cultures grown on PDA as well as the fungus sporulating on infected plants in the greenhouse were used for taxonomic characterization of their conidia.

Leaves from *E. crus-galli* plants that were naturally infected in the field were also included to determine the variability in conidia shapes and sizes. The infected leaves were sterilized in 5% sodium hypochlorite and incubated on moist filter paper in petri plates for two days in the dark at room temperature. Conidia and conidiophores were then scraped from the leaves with a scalpel and transferred onto glass slides for viewing under a light microscope. Measurements of the conidia sizes were taken using a calibrated ocular micrometer by measuring 200 conidia and conidiophores from each plate or infected leaf.

Pathogenicity Testing

Plant production

Seeds of *E. crus-galli* were germinated on wet filter paper in petri dishes. On the fifth day, five seedlings were transplanted to a plastic pot (10 cm diameter x 10.6 cm tall) containing a mixture of commercial soil conditioner (KOSAS peat) and top soil (3:1, v:v) and left in a greenhouse for nine to ten days to establish. They were watered daily to soil saturation.

Inoculum production

A small mycelial plug (5 mm diameter) from a seven day-old culture was aseptically transferred to a fresh modified V8 agar (100 mL V8 juice, 900 mL water, 18 g agar; Dinghra and Sinclair, 1995). The plates were incubated for two to three days (30 °C, 12 h/12 h light/dark) under near UV light (Philips TL 40W/08, F40 T12 BLB) until adequate colony growth was observed. Mycelia plugs from the margin of the young growing colony were transferred to fresh plates of V8 agar (four plugs per plate), and the new plates incubated for another 12 to 14 days before conidia were harvested. The conidia were harvested by flooding the agar plates with 15 mL sterile distilled water. The conidia were scraped off from the agar surface using a rubber spatula. Conidial suspensions were then sieved through a single layer of cheese cloth. The concentration of conidia was determined with Brite-line phase contrast hemacytometer (Reichert Scientific Instruments, Buffalo, N.Y., USA), and the spore suspension adjusted to the desired concentration by further dilution with water.

Plant inoculation

Seedlings at two to three leaf stages were used in these experiments. The seedlings were maintained in about 100% RH by frequent spraying with water. The seedlings were inoculated with a conidial suspension (6.5 x 10^5 spores/mL) containing 0.25% Maxigreen® (nonylphenol polyethylene glycol ether), a non-ionic surfactant, and 7 mL sunflower oil. Each pot of seedlings was sprayed with approximately 15 mL of the conidial suspension, containing about 9.7 million spores. Maxigreen®

was used to maintain leaf surface humidity and uniform conidia distribution on the leaves and to facilitate entry of the spores into the plants waxy leaf cuticle. The sunflower oil was used to maintain humidity on the leaf surface and to promote growth of the conidia.

The pots were arranged randomly in the greenhouse which was maintained at 31 ± 2 °C. The control pots with plants sprayed with only 0.25% Maxigreen® plus 7% sunflower oil was included. The disease incidence and disease severity were recorded 24 h after inoculation. Disease severity was recorded daily until the disease stopped progressing. Diseased leaves were collected and the fungus was re-isolated from symptomatic lesions to confirm Koch's postulate. The experiment was done with four replications.

Disease assessment

Disease assessment was based on the number of plants affected from the total number of plants inoculated (disease incidence), expressed as the percentage of diseased plants (James, 1974; Horsfall and Cowling, 1978; Kranz, 1988), and the disease severity was based on the area of plant tissue showing symptoms of the disease (Kranz, 1988).

The plants were scored for their severity of disease affliction on the scale: 0 = healthy; 1 = 10% of the plant diseased; 2 = 20% diseased; 3 = 30% diseased; 4 = 40% diseased; 5 = 50% diseased; 6 = 60% diseased; 7 = 70% diseased; 8 = 80% diseased; 9 = 90% diseased; and 10 = plant death (Kadir *et al.*, 2000a).

Effect of Media and Temperature

Fungus growth

Five mm plugs were taken from the margin of an actively growing culture (7 days old) and inoculated on PDA, PDA (half strength), V8 juice agar, V8 juice agar (half strength) and rice agar. The plugs were placed in the centre of each petri plate and the plates then sealed with parafilm and incubated at temperatures: 20 ºC, 25 ºC, 30 ºC and 35 ºC. Radial growth of the isolates was measured daily for seven days by using a pair of calipers.

Conidia germination

A 1 mL spore suspension containing ± 10^4 conidia was spread on the surface of the water agar medium in a petri plate with four replications in a completely randomized design (CRD). The petri plates were sealed with parafilm and incubated for 24 h at different temperatures; 20 ˚C, 25 ˚C, 30 ˚C and 35 ˚C. The percentage of germination of the spores was calculated based on random samples of 200 spores.

Sporulation

A 5 mm agar plug from a seven day old culture was placed in the center of a plate of PDA (Difco, Detroit, MI), PDA (half strength), V8 juice agar, V8 juice agar (half strength) and rice agar. The plates were sealed with parafilm and incubated at 30 ºC under NUV light, 12 h light and 12 h dark. The light duration was controlled by a 24 h regiment time controller (Theben-Werk Zeitantomatik Gmoh, Hohenbergstrabe 32, D-72401 Heigerloch, Germany). Conidia production was assessed 14 days after media inoculation. The conidia were harvested by flooding the medium with 10 mL sterile water and scraping the conidia off the agar surface with rubber spatula, followed by another 5 mL to flush any residual conidia from the medium. The conidia were counted using a Brite-line phase contrast hemacytometer.

Appressorium formation count

Four sterile cellulose tapes (2 x 3 cm) (dialysis tubing-visking, size 1-8"/32", Medical International Ltd., 239 Liverpool Road, London, N1 1LX) were placed on WA. A drop of spore suspension containing < 10^4 spores was dropped on each tape by using a micropipette. The plates were sealed with parafilm and incubated at 20 °C, 25 °C, 30 °C and 35 °C for 4 h, 8 h and 12 h. The conidial germination was then stopped by a few drops of lactophenol cotton blue (LCB) and the number of appressorium produced was counted under a light microscope.

Effect of Surfactant

Conidia germination and appressorium formation

One mL spore suspension containing ± 10^4 spores with 0.05%, 0.25%, 0.50% and 0.75% surfactant (Control (DW), T20, T80, Maxigreen®, Sapol and Polypol) was spread on the surface of WA in petri plates with four replications in a completely randomized design (CRD). The petri plates were sealed with parafilm and incubated for 24 h at 30 °C. The conidia activity was then stopped by using LCB, and the conidia germination and number of appressoria formed were counted under a light microscope.

Data Analysis

All the experiments were repeated twice by using a completely randomized design with four replications. All the percentage data were arcsine transformed before analysis (Gomez and Gomez, 1984). Data from both trials were pooled if a test of homogeneity by the Bartlett test (Gomez and Gomez, 1984) justified the pooling, and subjected to the standard SAS procedure (SAS Institute, Cary, NC). Means separation using SAS procedure was done if the treatments showed significant differences.

RESULTS

Isolate Identification and Characterization

Several fungi were consistently isolated from the diseased *Exserohilum crus-galli* var. *crus-galli*. However, only *Exserohilum monoceras* fulfilled Koch's postulate. This fungus was highly pathogenic on *E. crus-galli* with the symptoms produced in the greenhouse very similar to those seen in the field. The conidiophores emerged through the epidermal cells, and were slightly 0 to 1 septate and olivaceous brown, broadest around the central septum and narrowing towards both the basal and end septa. The end cells were often cut off by a dark thick septum often referred to as the hilum (Figures 1a). The conidia consisted of 5 to 10 disto septa (Table 1), and measured 15 to 20.48 μ wide x 92.16 to 133.12 μ long (Table 1).

Germination was usually from both ends of the conidium with the germ tube emerging from the first and last septa (Figure 1b), forming a dark brown colony on PDA, V8 agar and rice agar (Figure 2a). There were slight differences in the dimensions of the conidia obtained from this study compared to dimensions reported in a previous study (Sivanesan, 1987), but were all well within the range described for *Exserohilum monoceras* (Drechs) Leonard & Suggs. This study corroborated with descriptions by Chidambaram *et al.* (1973) (Table 1), thus this fungus was identified as *Exserohilum monoceras* (Drechs) Leonard & Suggs.

Table 1. Comparison of conidial dimensions (µm) of isolated fungus with those described in the literature.

Isolate	Width range	Width (min) (µm)	Length range	Length (min)
E. monoceras[a]	15-20.48	15.36	92.16-133.12	89.60
E. monoceras[b]	15-25	N. A.	60.00-150.0	N. A.
E. monoceras[c]	15.30-20.4	N. A.	85.00-161.5	N. A.
E. rostratum[b]	7-29	N. A.	15.00-190.0	N. A.
E. rostratum[c]	13.60-23.8	N. A.	13.60-153.0	N. A.

Note: [a] Isolated from diseased samples in this study
[b] Sivanesan (1987)
[c] Chidambaram *et al.* (1973)
N. A. – Not Available

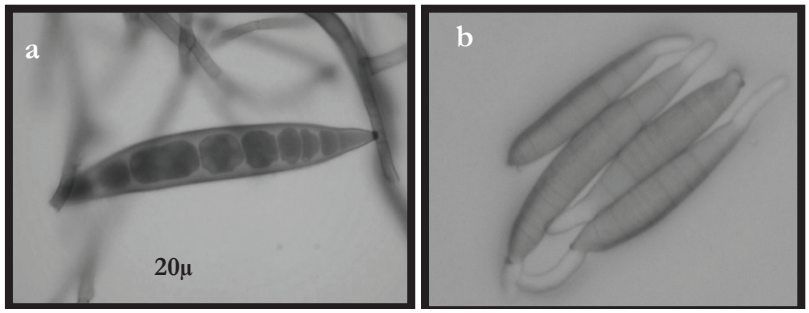

Fig. 1. Conidia of *E. monoceras*; (a) fixed with LCB and; (b) on the surface of distilled water. Viewed under a light microscope at 40x magnification.

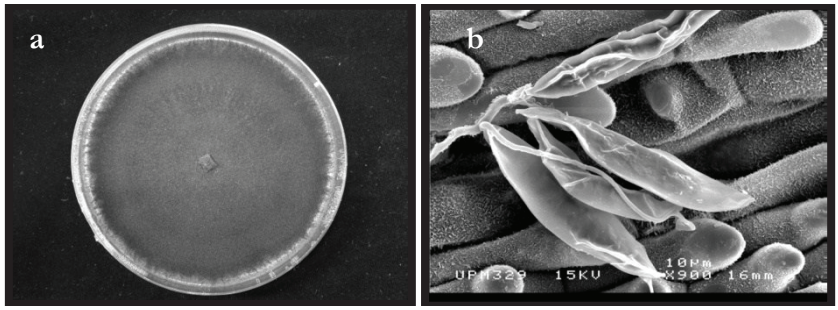

Fig. 2. Colony of *E. monoceras* on V8 agar; (a) at 7 days old and; (b) micrograph of *E. monoceras* on *E. crus-galli* leaf.

Pathogenicity Test

Exserohilum monoceras was very pathogenic to *Echinochloa crus-galli* in the greenhouse trials. The disease started with numerous specks which coalesced into discrete lesions. As the disease progressed, the margins around the lesions turned yellow and eventually the areas turned necrotic. The lesions did not expand (although some increased marginally; breadths from 0.5 to 0.8 mm and lengths by 0.2 to 0.5 mm). The infected leaves then turned dark green and brown, eventually shrinking and drying up.

The older, or lower, leaves were more afflicted with larger necrotic areas with yellow borders. Most of the leaf blade was blighted within 24 h of infection starting at the tip and edges. All the inoculated seedlings became infected with 30 to 40% severity within 24 h, 60% severity in 72 h and death in 168 hr (7th day) (Figure 3). No secondary spread was observed in the greenhouse. The control plants sprayed with 0.25% Maxigreen® remained healthy and asymptomatic throughout the trials.

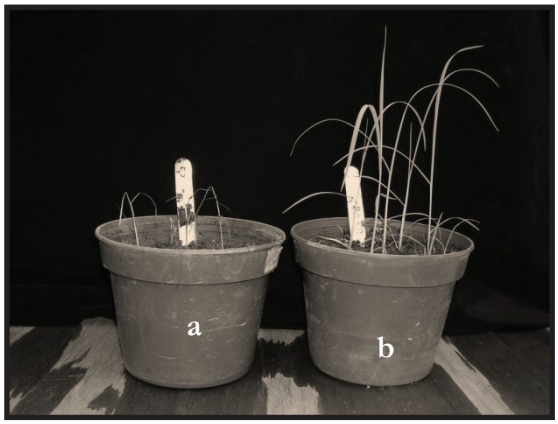

Fig. 3. Effect (infected plants) of *E. monoceras* on *E. crus-galli:* (a) diseased seedlings four days after inoculation with 2.1 x 10^6 spores/mL (10.5 million spores/pot) and, (b) healthy uninoculated seedlings (control).

Disease Progress

The disease progress of *E. monoceras* on *E. crus-galli* was measured by disease severity values (Figure 4a). It required at least 12 h for any symptoms to appear and the disease then displayed the typical sigmoid growth curve, slow in the beginning, then accelerating before plateauing off on the sixth day, with death of all the plants. The disease progress is best described by the logistic model (Figure 4b) with the average infection rate from two trials at $r_L = 1.736$ units/day (SE = 0.001, $R^2 = 0.932$; $P < 0.005$). In the greenhouse, the fungus was not capable of causing secondary infection as proved by the control, which remained disease-free.

Effect of Medium, Temperature and Surfactant

Fungus growth and conidia germination

Temperatures have a significant influence on the radial growth of *E. monoceras*. Radial growth was inhibited at temperature < 25 ˚C and 35 ˚C (Table 2). Radial growth of *E. monoceras* was visible after 24 h at all temperatures but with the higher rates occurring at 25 ˚C and 30 ˚C (Table 2). Based on the temperatures tested, 30 ˚C was the optimum temperature for fungal growth on all the media. Half strength V8 juice agar was the best of the medium with significantly higher growth ($P < 0.05$) (Figure 6), followed by full strength V8 juice agar (Figure 5), then PDA (Figure 7) and half strength PDA (Figure 8) both of which were not significantly different ($P > 0.05$) (Figure 9). The conidia germination test also showed that 30 ˚C was the optimum temperature for germination, which was significantly better ($P < 0.05$) than 25 ˚C, followed by 20 ˚C and 35 ˚C which gave similar results (Table 3).

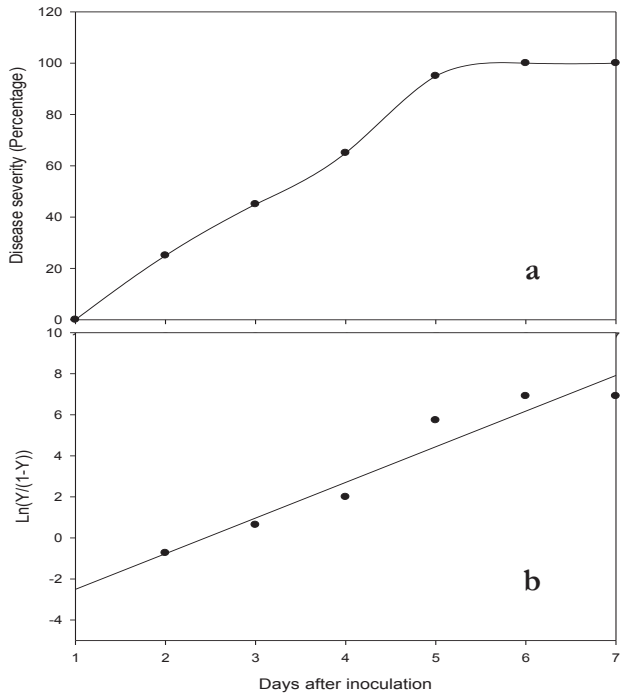

Fig. 4. Disease progress curve of seedling blight by *E. monoceras* on *E. crus-galli* seedlings: Untransformed diseased severity values (a), and Regression of the transformed disease severity values by using logistic model ln (Y/1-Y) (b), the equation for the line being $Y = 1.736x - 4.242$ ($R^2 = 0.932$)

Spore production

Spore production of *E. monoceras* was also influenced by the type of media used. Sporulation was significantly greater on the half strength V8 juice agar (3.105 x 10^5 spores/mL) compared to other media tested ($P < 0.05$) (Table 4). Although radial growth was fastest on PDAHs (4.83 cm/day), spore production (1.495 x 10^5 spores/mL) was very low, which unfortunately was the effective innoculum.

Table 2. Effect of incubation temperature on the mean radial growth of *E. monoceras* cultured on various media (the mean radial growth is expressed as the total area under the growth curve for the purpose of analysis)

Temperature	Total area under the progress growth curve[x]			
	Medium			
	V8J	V8J(Hs)	PDA	PDA(Hs)
20 °C	10.58[c]	13.95[c]	9.79[b]	5.13[c]
25 °C	15.30[b]	19.39[b]	12.57[a]	9.25[b]
30 °C	21.36[a]	24.50[a]	13.55[a]	14.70[a]
35 °C	10.52[c]	13.64[c]	9.61[b]	5.88[c]
Mean	14.44[B]	17.87[A]	11.38[C]	8.74[D]

Note: *data are the averages of two trials, each with eight replicates. Means followed by the same letter within a column are not significantly different according to Fisher's Protected LSD at $P < 0.05$. Lower case letters represent effects of temperature and upper case letters represent effects of media.

Table 3. Effect of temperature on the germination and appressorium formation by *E. monoceras*

Temperature mean (%)	Germination mean (%)	Appressorium
20 °C	64.30[c]	35.50[c]
25 °C	80.42[b]	50.28[b]
30 °C	86.37[a]	65.51[a]
35 °C	62.59[c]	32.37[d]

Note: *data are the averages of two trials, each with eight replicates. Means followed by the same letter within a column are not significantly different according to Fisher's Protected LSD at $P < 0.05$. Percentage values were transformed with arcsine before the analysis of variance.

Table 4. Effect of different media on the radial growth (cm) and sporulation (conidia/mL) of *E. monoceras*

Medium	Sporulation mean (conidia/mL)	Radial growth mean (cm/day)
V8aHs	310500[a]	2.74[c]
V8a	225500[b]	3.96[b]
PDA	150500[c]	3.54[b]
PDAHs	149500[c]	4.83[a]
RiceA	76000[d]	-

Note: *data are the averages of two trials, each with eight replicates. Means followed by the same letter within a column are not significantly different according to Fisher's Protected LSD at $P < 0.05$. Percentage values were transformed with arcsine before the analysis of variance.

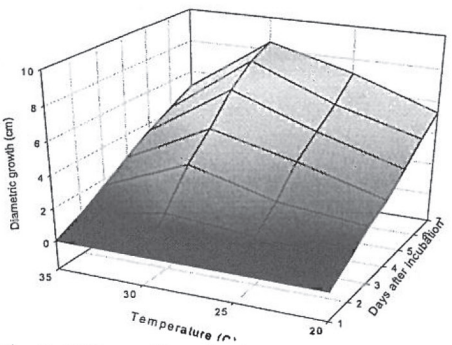

Fig. 5. Effect of incubation temperature on the radial growth of *E. monoceras* cultured on full strength V8 juice agar. Each point is the average from four replicates.

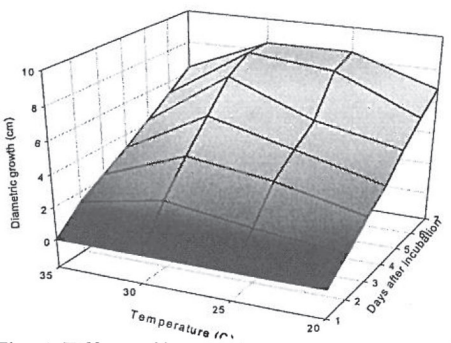

Fig. 6. Effect of incubation temperature on the radial growth of *E. monoceras* cultured on half strength V8 juice agar. Each point is the average from four replicates.

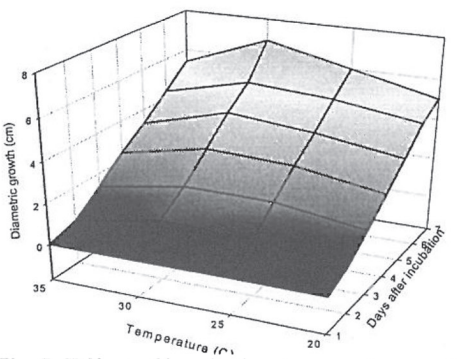

Fig. 7. Effect of incubation temperature on the radial growth of *E. monoceras* cultured on full strength PDA. Each point is the average from four replicates.

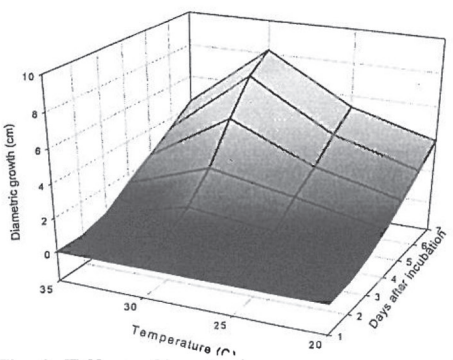

Fig. 8. Effect of incubation temperature on the radial growth of *E. monoceras* cultured on half strength PDA. Each point is the average of four replicates.

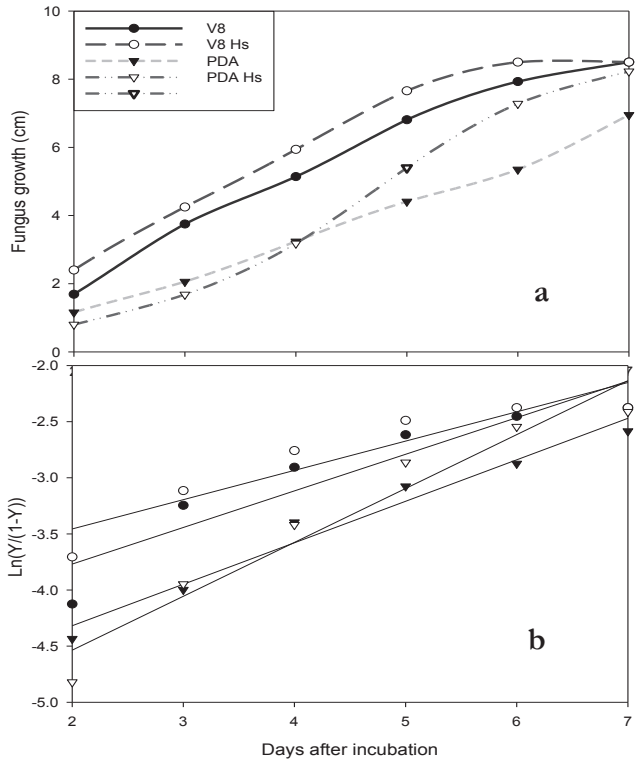

Fig. 9. Radial growth of *E. monoceras* on different culture media at 30 °C: Untransformed values (a). Regression of transformed data using the logistic model ln (Y/(1-Y)) (b). The equations for the line are Y = 1.736 - 4.421 + 0.33x (Curve 1, R^2 = 0.860); Y = 1.736 - 3.977 + 0.261x (Curve 2, R^2 = 0.860); Y = 1.736 - 5.058 + 0.370x (Curve 3, R^2 = 0.967); Y = 1.736 - 5.496 + 0.480x (Curve 4, R^2 = 0.940)

Appressorium Formation

For appressorium formation, 30 °C was the optimum temperature ($P < 0.05$) as with the other aspects of the fungal growth that include radial growth and conidial germination. However, the evaluation would have to be repeated *in vivo* on a more suitable host to reconfirm the results.

Surfactant Test

All surfactants tested supported growth of *E. monoceras*, however, the effect of these surfactants on growth and appressorial formation was very much affected by type of surfactants. Spore germination was significantly higher in Tween 20, Tween 80 and Maxigreen®, but was not significantly different to the control (Table 5). Polypol has the lowest ability to support spore germination. Formation of the appressoria was significantly high in Tween 20 and Maxigreen® ($P < 0.05$), and the lowest number of appressoria were formed in Polypol. Based on spore germination and appressorial formation Maxigreen® was selected for the subsequent study. This is due to the fact that Maxigreen®

is produced locally as compared to Tween 20. This study revealed that significantly high conidia germination was recorded in Maxigreen® at concentration of < 0.5% (Table 6), and was not significantly different from the control. At higher concentration (> 0.7%), conidia germination and appressoria formation was inhibited.

Table 5. Effect of surfactant on the germination and appressorium formation by *E. monoceras*.

Surfactant	Germination mean (%)	Appressorium mean (%)
Control (DW)	76.97[a]	40.09[a]
T20	71.87[ba]	35.73[ba]
T80	75.08[a]	21.50[d]
Maxigreen®	72.23[ba]	35.35[ba]
Sapol	66.58[bc]	34.37[b]
Polypol	60.71[c]	26.46[c]

Note: *data are the averages of two trials, each with four replicates. Means followed by the same letter within a column are not significantly different according to Fisher's Protected LSD at P < 0.05. Percentage values were transformed with arcsine before the analysis of variance.

Table 6. Effect of surfactant (Maxigreen®) concentration on the germination and appressorium formation by *E. monoceras*.

Concentration (surfactant)	Germination mean (%)	Appressorium mean (%)
0	76.97[a]	40.09[a]
0.25	72.51[a]	33.93[b]
0.50	70.17[ab]	33.23[b]
0.75	64.33[b]	21.62[c]

Note: *data are the averages of two trials, each with four replicates. Means followed by the same letter within a column are not significantly different according to Fisher's Protected LSD at P < 0.05. Percentage values were transformed with arcsine before the analysis of variance.

DISCUSSION

The fungus isolated from diseased *E. crus-galli* was identified as *E. monoceras*, based on the conidial morphology, germination characteristics and hilum morphology. This fungus was formerly classified under the genus *Helminthosporium* (Alcorn, 1998) but this genus is separated into *Drechslera*, *Bipolaris* and *Exserohilum;* with *Pyrenophora, Cochliobolus* and *Setosphoeria* as its telemorphs. The new classification is based mainly on conidial morphology, germination characteristics and hilum morphology.

Drechslera was established by Ito (1930) to accommodate fungi previously assigned to the subgenus *Cylindro-Helminthosporium.* Members of the genus are characterized as having 'cylindrical', not curved conidia, germinating from every cell and associated with *Pyrenophora. Drechslera tritici-vulgare* (Nisik) Ito (= *D. tritici-repentis* (Died) Shoem) was consequently designated under genus *Bipolaris* with the species placed in the subgenus *Eu-Helmitosporium.* Its conidium is described as fusoid, straight or occurred, germinating by one germ tube from each end. *Bipolaros maydis* (Nisik and Miyake) Shoem

was selected as the type species. Some species of *Bypolaris* are associated with *Cochliobolus* as telemorphs.

Leonard and Suggs (1974) established the genus *Exserohilum* for species in *Helminthosporium* with the conidial hilum strongly protuberant, thus providing the third segregate for graminicolous fungi once considered to belong wholly to *Helminthosporium*. The species described as *E. turcicum* (Pars.) Leonard & Suggs. In addition, a new ascomycate genus, *Setosphoeria*, was proposed to accommodate the telemorphs of *Exserohilum*.

In repeated trials, the pathogenicity of this fungus to its host was confirmed by Koch's postulate. The pathogen caused a high level of disease on *E. crus-galli*. It induced eyespot lesion, typical of infection by *Exserohilum* spp. and induced a severe 'burnt' appearance on the foliage of *E. crus-galli*. The individual lesions did not expand but coalesced, forming larger lesions which eventually caused blighting. The leaf tissues in the vicinity of heavy infestation were completely killed. Symptoms appeared within 24 h of inoculation. The disease did not develop on non-inoculated plants, which was indicative of localized infection and lack of significant secondary disease cycles. There was no re-growth of severely diseased plants even one month after inoculation. The disease symptoms were quite characteristic of leaf spot disease on graminicolous hosts caused by fungal species of *Exserohilum* (Sivanesan, 1987).

The blighting of infected plants may have been caused by phytotoxins which are involved in pathogenesis and rapid necrosis. *Helminthosporium* spp. fungi have been reported to produce a host-specific toxin in plant pathogenesis (Walton and Panacione, 1993). In addition to host-selective toxins, this group of pathogens is known to produce secondary metabolites which are believed to be important in plant-pathogen interactions (Ballio, 1991). Twelve eromophialines (sesquiterpenes) have been isolated from culture of *Drechslera gigantean*, and most of them have been found to be phytogenic to several grasses (Sugawara *et al*., 1988).

Currently, there is no commercial bioherbicide to control *E. crus-galli*. A few fungal pathogens have been evaluated for their potential to control this weed. Zhang *et al.* (1996) isolated six pathogenic fungi from naturally infected *E. crus-galli*. Among them, *E. monoceras* (Drechs.) Leonard & Suggs killed seedlings of *E. crus-galli*. Seedlings at one- and two-leaf stages were more susceptible than those at the three- and four-leaf stages. With an increase in inoculum density, the weed control efficacy of this fungus could be enhanced on the young as well as the old *E. crus-galli* seedlings (Zhang and Watson, 1997).

Temperature and dew period are two of the factors influencing the efficacy of fungal bioherbicidal agents. Both these factors affected the inherent capacity of the conidia to germinate and to form appressoria, which are apparently required for disease development. The laboratory study indicated that better growth and appressoria production can be attained when the fungus is exposed to temperature in the range of 25 to 30 °C. Many of the fungi developed as bioherbicides require an optimum dew period of 12 h and temperature in the range of 25 to 30 °C. *Alternaria cassiae* Jurair and Khan required a dew period of at least 6-8 h for 90 to 100% kill of seedlings of sicklepod (*Senna obtusfolia* (L.) Irwin & Barneby, under greenhouse and field conditions. Kadir and Charudattan (2000), reported that *Dactylaria higginsii* (Luttrell) M. B. Ellis was highly pathogenic to purple nutsedge (*Cyperus rotundus* L.) in a temperature range of 20 to 30 °C, while Ahmad (2004) reported that *Exserohilum longirostratum* (Subram) Subram can cause 100% mortality in a temperature range of 25 to 30 °C. The results from this study corroborated with these findings, and the temperature requirements are a common experience during the cropping season in warm regions.

The necessity for prolonged exposure to dew is a major limiting factor in the development of fungi as bioherbicides, however, in this study, the need for dew periods had been circumvented by the addition of emulsions. Surfactants were added to the emulsions to homogenate and stabilize the emulsion. There has been several reports which cited that the use of oil emulsion, invert emulsion (Egley and Boyette, 1995), and water in oil in water (WOW) (Auld *et al.,* 2003) have improved efficacy of bioherbicides in the absence of leaf wetness. This is because emulsion has the ability to retain high levels of water when sprayed at low application rates, which is essential for effective

disease establishment. Healthy conidia are directly related to viability and virulence of the bioherbicide (Kadir and Charudattan, 2000). Oil emulsion has also been reported to cause toxicity to the seedling, which may have enhanced activity of the conidia suspension (Egley *et al*., 1993).

The type of growth medium and the nutritional and physical environments of mass conidia production are of paramount importance because they greatly influence the number, type, stability, durability and virulence of the bioherbicide. For instance, conidia produced on solid substrate have been found to be more virulent and stable and have a longer half life than conidia produced in a submerged culture (Morin, 1993). This study has illustrated that solid media such as agar are excellent growth media, but spore production has been influenced by the nutrient content of the media. The best medium for sporulation was half strength V8 juice agar. This might be because fungal sporulation is induced by stress in their survival 'instinct' to disseminate widely before death, and reducing the medium strength could have provided the stimulus for sporulation. However, rice medium, a reportedly good medium for sporulation and radial growth (Zhang, 1997), gave disappointing results. The fungus remained largely vegetative with little sporulation. Thus, medium containing half strength V8 juice agar will be used in the consecutive experiments.

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