Effect of inoculum concentration, wetness duration and plant age on disease development by *Exserohilum prolatum* in itchgrass (*Rottboellia cochinchinensis*)

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ABSTRACT

Exserohilum prolatum, a fungus isolated from diseased itchgrass plants in Peninsular Malaysia, has been considered as a potential agent for biological control of this serious grass weed. Inoculum concentration, wetness duration and itchgrass age significantly influenced disease development by *E. prolatum* in itchgrass when evaluated in glasshouse experiments. Disease development increased with increasing inoculum concentration from $2x10^4$ to $2x10^7$ conidia/ml. As wetness duration increased, disease development increased. Complete kill of itchgrass at 3 to 4 leaf stage was obtained at $2x10^6$ conidia/ml and a 24 hr dew period. However, when formulated in 0.01% Tween 20 and 2% palm oil, *E. prolatum* reduced wetness duration requirement, and exposure to 12 hr dew period resulted in 80% reduction in itchgrass shoot dry weight. Susceptibility of itchgrass to the fungus decreased with increasing plant age. Itchgrass at two to six leaf stages were the most susceptible to fungus infection.

INTRODUCTION

Itchgrass is a serious annual grass weed causing severe yield losses in annual and perennial crops in the tropical and subtropical regions of the world. In Malaysia, the weed is a major problem in sugar cane plantations. Previous studies showed that the fungus *Exserohilum prolatum* isolated from diseased itchgrass plants has a potential to be developed as a mycoherbicide to this weed due to its effectiveness and safety to economically important crops in Malaysia (Alloub *et al*., 2004).

However, environmental, epidemiological and ecological factors may often limit the effectiveness of mycoherbicides. Artificial spray inoculation has been used in the application of bioherbicides enabling the pathogen to regulate the weed at very low population densities (TeBeest *et al*., 1992). In the field, a high number of infection sites of a bioherbicide are generally essential to ensure rapid and complete weed control (Yang and TeBeest, 1993). Watson and Wymore (1990) noted that low initial inoculum level contributes to the failure of the disease epidemic to develop and persist in weed population.

Moisture is the most restricting factor for disease development for mycoherbicides (TeBeest *et al*., 1992). Dew is important for germination of fungal propagules and subsequent infection (Kadir *et al*., 2000). Another important factor affecting disease development is changing in host resistance as plants develop (TeBeest *et al*., 1992). Determination of susceptible plant growth stage is important for understanding potential of bioherbicide (Watson and Wymore, 1990).

Therefore, studies to determine factors influencing the optimum inoculum concentration, environmental conditions and plant growth stage are required to achieve maximum control in the field to assure field efficacy. The objectives of the present study were to evaluate the effects of conidial concentration, wetness duration and itchgrass growth stage on disease development of *E. prolatum*.

MATERIALS AND METHODS

All experiments were carried out under glasshouse conditions at the Faculty of Agriculture, University Putra Malaysia, Serdang, Malaysia. In each treatment, ten seeds of itchgrass were planted in 10 cm diameter plastic pots in 2 : 1 : 1 mixture of soil, sand and peat. The soil mixture was steam sterilized for one hour at 120 ^{0}C. The pots were maintained in a glasshouse at a temperature range of 25-30 ^{0}C and 75-90% relative humidity. Emerging seedlings were thinned to 5 plants/pot.

Inoculum production

E. prolatum isolated from diseased itchgrass plants was grown on potato dextrose agar (PDA) slants and maintained under mineral oil at 4 ^{0}C as a stock culture. A small piece of agar mycelium from the stock culture of the fungus was transferred to PDA in 9 cm diameter petri dishes. The plate were sealed with parafilm and incubated at room temperature (25-28 ^{o}C) for seven days. Small pieces of mycelium (6 mm diameter) from the margins of the actively growing colonies were placed in the center of PDA petri dishes, sealed with parafilm and incubated at room temperature under a 12 hr light. Ten days after incubation, inoculum was prepared by adding 10 ml distilled water containing 0.01% Tween 20 to each petri dish and scraping the spores from the surface of the colonies with a glass slide. Resulting

suspensions were filtered through 2 layers of cheesecloth and desired conidial concentrations were adjusted with water containing 0.01% Tween 20 with a haemocytometer.

Effect of conidial concentration

Seedlings at 3-4 leaf stage were sprayed till run-off with 2 x 10^4, 2 x 10^5, 2 x 10^6 or 2 x 10^7 conidia/ml suspensions containing 0.01% Tween 20. A check with distilled water containing 0.01% Tween 20 was included. After inoculation, the plants were covered with clear polyethylene bags for 24 hrs and maintained in glasshouse at the conditions mentioned before. The treatments were arranged in a completely randomized design with 4 replications. Disease development was expressed as disease severity (DS) which was rated at two days intervals based on a scale of 0-10, in which: 0 = no symptom; 10 = 91-100% of leaf area affected.

$$DS\ (\%) = \sum \frac{\text{Severity rating x number of plants in that rating}}{\text{Total number of plants assessed x highest scale}} \times 100$$

Dry weight of living above ground biomass was also determined. Completely collapsed plants were considered dead. Living aerial plant parts at soil level, were cut, dried at 65 ^{0}C for 48 hr and weighed. Dead tissue was removed before drying. The dry weight data were expressed as percentage reductions in biomass relative to biomass of the uninoculated control.

Effect of wetness duration

Seedlings of itchgrass at 3 to 4 leaf stages were sprayed to run-off with either of two formulations of *E. prolatum* conidial suspension at 2 x 10^6 conidia/ml in 0.01 Tween 20, with or without 2% palm oil. The seedlings were covered with clear polyethylene bags and maintained in the greenhouse conditions mentioned previously. The polyethylene bags were removed after 0, 8, 12, 24 and 48 hr. The treatments were arranged in a completely randomized design with 4 replications. Disease severity was rated at two days intervals for disease development and a final rating was made after 10 days. Biomass reduction was obtained as previously described.

Effect of plant growth stage

Seedlings of itchgrass at 2-3, 3-4, 4-5, 6-7 and 9-10 leaf stages were sprayed to run-off with 2 x 10^6 conidia/ml suspension containing 0.01% Tween 20. Appropriate controls with distilled water containing 0.01% Tween 20 were included. After inoculation, the plants were covered with clear polyethylene bags for 24 hr. The treatments were arranged in a completely randomized design with 4 replications. Disease severity was rated at two days intervals and a final rating was made after 10 days. Biomass reduction was obtained as previously described.

Statistical analysis

Data were subjected to analysis of variance using General Linear Model (GLM) procedure. Dry weight data were transformed using $\log_{10}(x + 1)$. Percentage data were arcsine transformed. Tukey's test was used to evaluate differences between treatment means.

RESULTS

Effect of conidial concentration

Significant differences were observed between the four conidial conce-ntrations compared to the control. Increasing inoculum concentration increased disease severity (Figure1), while concentration of 2 x 10^4 conidia/ml resulted in only 40% disease severity. Concentration of 2 x 10^5 conidia/ml resulted in 68% disease severity and concentrations of 2 x 10^6 and 2 x 10^7 showed 100% disease severity. The same trends were also observed in shoot dry weight of itchgrass (Table 1). However, there were no significant differences in disease severity or dry weight between 2 x 10^6 conidia/ml and 2 x 10^7 conidia/ml.

Effect of wetness duration

Significant differences were observed between the two formulations under all wetness durations. Increasing wetness duration increased disease severity (Figures 2 and 3). Highest disease severity (100%) was observed with 24 and 48 hr wetness duration. Under shorter wetness durations (8 and 12 hr) disease severity was low. Seedlings which received zero hr wetness duration did not show any symptoms. Shoot dry weight decreased as wetness duration increased (Table 2). The combination of surfactant and palm oil increased disease severity and reduced shoot dry weight under 8 and 12 hr (Table 2). When itchgrass seedlings were sprayed with 2 x 10^6 conidia/ml in 0.01 Tween 20 and exposed to 12 hr wetness duration, disease severity percent and percent reduction in dry weight were 37 % and 37.3 %, respectively. However, when sprayed with 2 x 10^6 conidia/ml containing 0.01 Tween 20 in 2 % palm oil under the same wetness duration, disease severity percent and percent reduction in dry weight were 79 % and 80 %, respectively. The wetness duration for complete kill of itchgrass seedling for the two formulations was 24 hr.

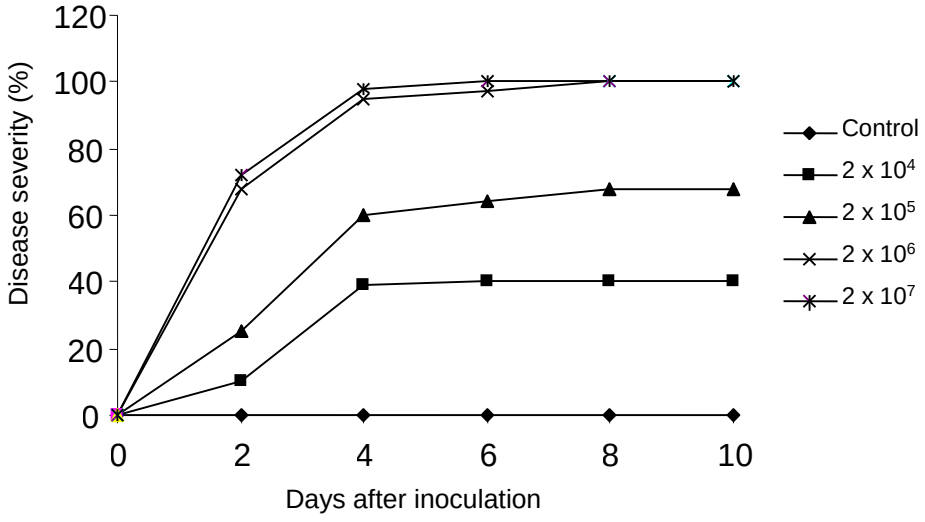

Figure 1. Effect of *E. prolatum* inoculum concentration on disease development in itchgrass seedlings.

Table 1. Effect of conidial concentration on itchgrass shoot dry weight.

Conidial concentrations	Shoot dry weight (g)	Reduction in dry weight (%)
0	0.21[a]	0.0 [d]
2 x 10^4	0.12 [b]	41 [c]
2 x 10^5	0.05 [c]	75 [b]
2 x 10^6	0.00 [d]	100 [a]
2 x 10^7	0.00 [d]	100 [a]
SE ±	0.003	1.83

Means in each column with same letters are not significantly different at 0.05% according to Tukey's test.

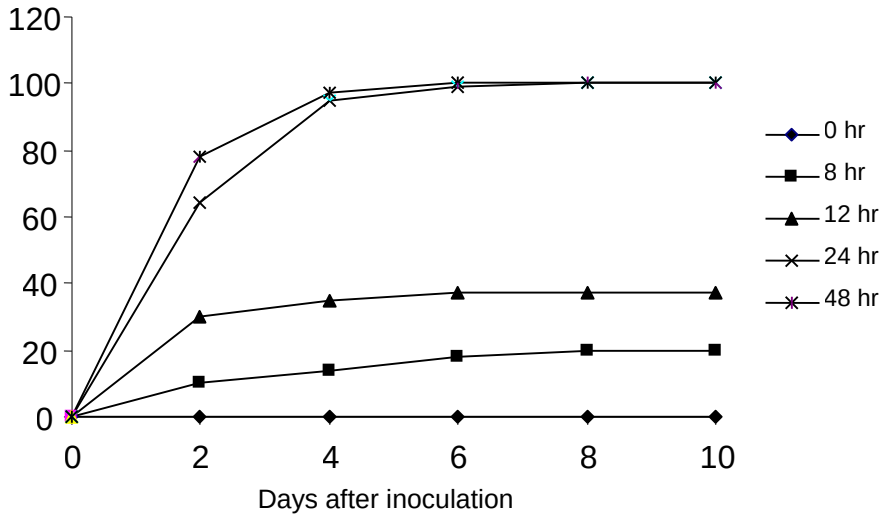

Figure 2. Effect of wetness duration on *E. prolatum* development in itchgrass seedlings inoculated with 2 x 10^6 conidia/ml suspension containing 0.01% Tween 20.

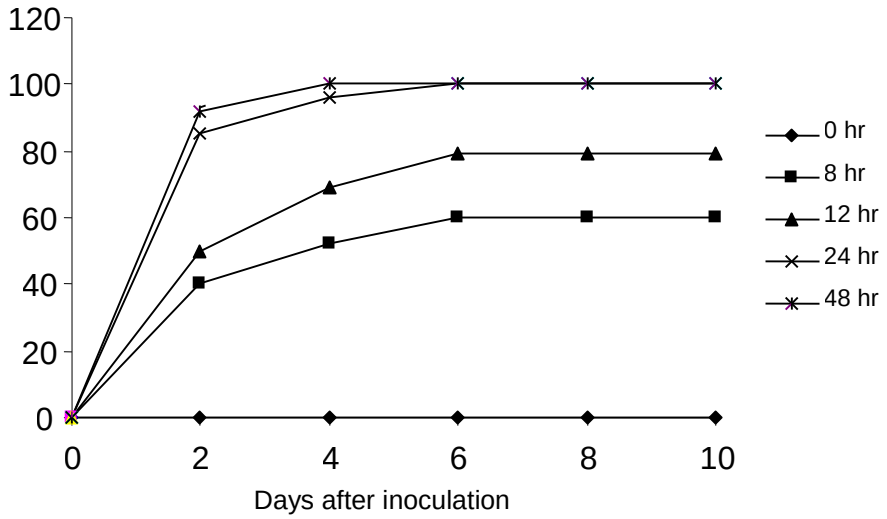

Figure 3. Effect of wetness duration on *E. prolatum* development in itchgrass seedlings inoculated with 2 x 10^6 conidia/ml suspension containing 0.01% Tween 20 and 2% palm oil.

Table 2. Effect of wetness duration on itchgrass dry weight.

Wetness duration (hr)	2 x 10^6 conidia/ml in 0.01 Tween 20		2 x 10^6conidia/ml in 0.01 Tween 20 and 2% palm oil	
	Shoot dry weight (g)	Reduction in dry weight (%)	Shoot dry weight (g)	Reduction in dry weight (%)
0	0.21[a]	0.00 [d]	0.21[a]	0.00 [d]
8	0.17 [b]	18.0 [c]	0.08[b]	60 [c]
12	0.13 [c]	37.3 [b]	0.004 [c]	80 [b]
24	0.00 [d]	100 [a]	0.00 [d]	100 [a]
48	0.00 [d]	100 [a]	0.00 [d]	100 [a]
SE ±	0.005	0.65	0.005	0.66

Means in each column with same letters are not significantly different at 0.05% according to Tukey's test.

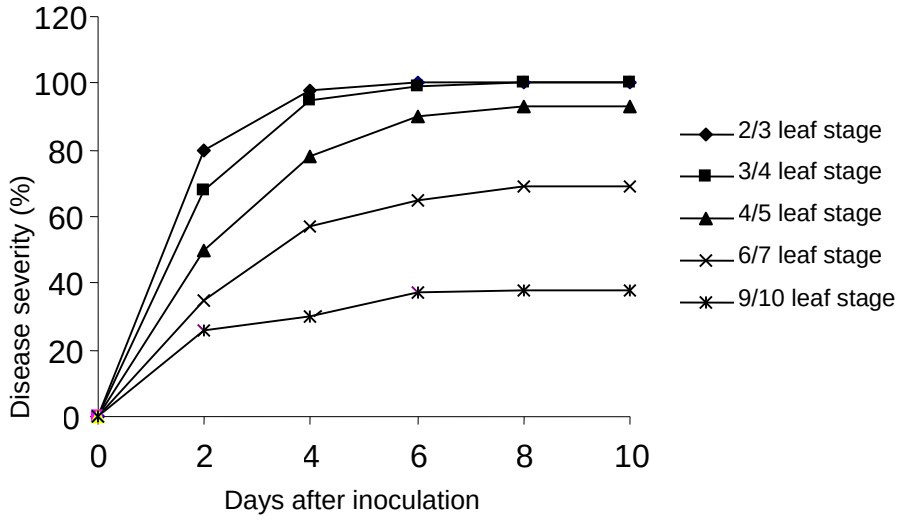

Figure 4. Effect of plant growth stage on *E. prolatum* disease development in itchgrass seedlings.

Effect of plant growth stage

Growth stage of itchgrass seedlings had a significant effect on disease development. Figure 4 showed that itchgrass seedlings at 2-3 and 3-4 leaf stages at the time of inoculation were completely killed by *E. prolatum* with 2 x 10^6 conidia/ml suspension containing 0.01% Tween 20 followed by 4-5 leaf stage seedlings (93%). The lowest disease severity was observed on 9-10 leaf stage seedlings (38%). Seedlings at 2-4 and 4-5 leaf stages showed 100 % and 90.8% reduction in shoot dry weight, respectively. However, dry weight increased with increasing plant growth stage (Table 3).

Table 3. Effect of plant growth stage on itchgrass shoot dry weight.

Growth stage (leaf)	Shoot dry weight (g)	% reduction in dry weight
2-3	0.00 ^{d}	100 ^{a}
3-4	0.00 ^{d}	100 ^{a}
4-5	0.03 ^{c}	90.8 ^{b}
6-7	0.21 ^{b}	68.6 ^{c}
9-10	0.73 ^{a}	34.2 ^{d}
SE ±	0.003	0.25

Means in each column with same letters are not significantly different at 0.05% according to Tukey's test.

DISCUSSION

The findings of this studyindicated that increasing inoculum concentra-ion of *E. prolatum* increased itchgrass control. *Exserohilum prolatum* at 2x10^6 conidia/ml appeared to be the optimum concentration for complete kill of itchgrass seedlings under glasshouse conditions, however, under field conditions higher concentration levels might be needed to achieve good control. Increasing weed control with

increased inoculum concentration has also been observed on *Exserohilum monoceras* for the control of *Echinochloa* spp. (Zang *et al*., 1996; Zang and Watson, 1997).

Pathogens have different temperature and moisture requirements for germination, infection and sporulation. Temperature generally has not been considered to be as critical as moisture for mycoherbicides since most pathogens studied infect over broad temperature ranges and pathogens can be applied under suitable conditions (TeBeest *et al*., 1992). The effect of moisture on infection by many weed pathogens have been well documented and most studies demonstrate a direct relationship between dew period and disease development (TeBeest *et al*., 1992; Auld and Morin, 1995). With appropriate formulation, moisture problem may be solved by delivering the fungus to the weed surface with sufficient water and to retain it for sufficient time to satisfy the dew period requirement of the fungus.

From this study, Requirement of 24 hr wetness duration appeared to be the most limiting factor to induce mortality by *E. prolatum* on itchgrass seedlings under field conditions. Requirement for longer dew periods has also been observed for *Alternaria angustiovoidea* in *Euphorbia esula* (Yang *et al*., 1990) and for *A. alernate* f. sp. *sphenocleae* in *Sphenoclea zeylandica* (Masangkay *et al*., 2000). However, addition of palm oil to the fungus formulation increased disease severity and decreased dry weight at 8 and 12 hr wetness duration. Oil on leaf surface was known to reduce evaporation (Quimby, 1985) and hence retain water and reduce dew period needed for fungus infection and development. The long wetness duration requirement for *E. prolatum* can be overcome by using Tween 20 surfactant and palm oil suspensions and by applying the isolate late afternoon near sunset to coincide with the high prevailing relative humidity.

Young itchgrass seedlings were more susceptible to *Exserohilum prolatum* than older plants. A 100 % reduction in shoot dry weight was observed on itchgrass seedlings at 2 to 4 leaf stages. Many studies have shown that foliar pathogens can completely kill seedlings at young stages, however they only reduce dry weight of older plants (TeBeest *et al*., 1992; Zang *et al*., 1996; Zang and Watson, 1997).

It could be concluded from this study that disease development is greatly influenced by inoculum concentration, wetness duration and plant age. Disease development increased with increasing inoculum concentration and wetness duration and decreased with increasing plant age. Formulation of the pathogen in 0.01 % Tween 20 and 2% palm oil significantly reduced dew period requirement and improved control efficacy and two to six leaf stages were the most susceptible stages to *E. prolatum* infection.

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تأثير تركيز اللقاح وفترة الرطوبة وعمر النبات على تطور المرض بواسطة (*Exserohilum prolatum*) في عشبة أم بليلة (*Rottboellia cochinchinensis*)

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الخلاصة

يعتبر فطر(Exserohilum prolatum) والذي تم عزله من عشبة أم بليلة في شبه جزيرة ماليزيا وسيلة مكافحة إحيائية فاعلة لهذه العشبة النجيلية الضارة. عوامل تركيز اللقاح ، فترة الرطوبة وعمر النبات أثرت معنوياً على تطور المرض بواسطة (E. prolatum) في عشبة أم بليلة عندما قيّمت بتجارب داخل صوبة زجاجية. وقد تلاحظ أن تطور المرض قد إرتفع إرتفاعاً معنوياً مع إرتفاع معدل تركيز اللقاح من104 2 x وإلى 107 2 x كونيديا/مل. من ناحية أخرى تلاحظ أن شدة الإصابة بالمرض إزدادات معنوياً بإزدياد فترة الرطوبة. هذا وقد حدت موت كلي للعشبة عمر 2-3 ورقات عندما تمت معاملتها بواسطة تركيز لقاح 2x106 كونيديا/مل وفترة رطوبة 24 ساعة. أدى إستخدام عامل إنتشار Tween 20 بنسبة 0.01% وزيت النخيل بنسبة 2% إلى تقليص الحاجة إلى فترة رطوبة ممتدة (24 ساعة) حيث إنخفض الوزن الجاف للمجموع الخضري بنسبة 80% عند معاملة العشبة بفترة رطوبة بلغت 12 ساعة. أبانت النتائج أن قابلية العشبة للإصابة بالفطر قد إنخفضت مع تقدم عمر النبات وأن الأعشاب عمر 2-6 ورقات كانت أكثر قابلية للإصابة.