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Full Length Article

Diversity of Fungal Pathogens Isolated from Diseased Goosegrass (*Eleusine indica*) as Potential Biological Control against Goosegrass

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Abstract

Several studies have been undertaken on the possible use of fungal pathogens for biological control of goosegrass (*Eleusine indica*), but the diversity of fungal pathogens isolated from diseased *E. indica* infesting various crops has received little attention. The objectives of this study were to 1) isolate and identify fungal pathogens from diseased *E. indica* that infected numerous food crops and 2) assess the pathogenicity of the isolated fungal pathogens against diseased *E. indica* in the greenhouse. On the basis of morphological characteristics and genetic analysis, *Fusarium chlamydosporum*, *F. proliferatum*, *Bipolaris bicolor*, *Curvularia senegalensis*, and *Lasiodiplodia theobromae* were successfully isolated and identified from diseased *E. indica* growing around mango, oil palm, wax apple, maize, and paddy fields, respectively. Within ten days of evaluation, the pathogenicity of the fungal isolates varied, with *B. bicolor*, *L. theobromae*, and *F. proliferatum* exhibiting high values of area under the disease progression curve (AUDPC) ranging from 274 to 339. These findings indicate that fungal pathogens are potential candidates for use as biological control agents against *E. indica*.

Keyword: *Eleusine indica*; Biological agent; Fungal pathogens

Introduction

Eleusine indica, also known as goosegrass, is an annual grass prevalent in farming areas (Randall 2012). *E. indica* is often found in the orchards, oil palm plantations, and vegetable farms of Malaysia (Barnes and Chan 1990). *E. indica*, one of the five most problematic weeds in the world, lowers the productivity of 46 crops in over 60 countries (Holm *et al.* 1977). Since *E. indica* has no roots at the nodes, it can be easily eradicated by hoeing in the early stages of growth. However, as *E. indica* matures, a robust root system develops in the soil, making physical removal more difficult. It could withstand a wide range of pH, salt, and water stresses (Ismail *et al.* 2002, 2003; Chauhan and Johnson 2008). Additionally, *E. indica* seed buried at a depth of 20 cm for two years retained about 75% of its viability (Chuah *et al.* 2004).

The evolution of herbicide-resistant *E. indica* biotypes (Dilipkumar *et al.* 2020; Franci *et al.* 2020) has contributed to the increased interest in alternate goosegrass control methods such as chemical, physical, or biological control (Dilipkumar *et al.* 2019, 2020; Chuah and Kent 2021a, b; Xiao *et al.* 2021; Fakri *et al.* 2022). To suppress weeds, biological control employs biotic agents of insect pests and microorganisms. Compared to insects, the use of fungi or bacteria as a biological control of weeds is a more recent notion that has proven effective in weed management (Harding and Raizada 2015). Biocontrol utilising microbes provides precise control over a specific weed, is environmentally benign, and minimises herbicide resistance in the weed biotype. Biological control is biodegradable and poses a minimal risk of contamination and phytotoxicity from spray spread, soil leaching, and non-target plants and animals (Maizatul-Suriza *et al.* 2017). *Phoma herbarum*, *Curvularia aeria*, and *Bipolaris sorokiniana* were successfully isolated from diseased goosegrass in oil palm (Ismail *et al.* 2020). Pathogenicity studies revealed that *B. sorokiniana* infected goosegrass five days after conidial inoculation and had the most severe disease compared to *P. herbarum* and *C. aeria* 40 days after conidial inoculation (Ismail *et al.* 2020).

In Malaysia, microorganisms are not currently used to control weeds in agricultural areas or plantations. Although several potential fungi have been screened to control several weed species such as *Mikania micrantha* (Barreto and Evans 1995) and *Chromolaena odorata* (Elango *et al.* 1993) in Malaysian oil palm plantations, there is limited research on the potential of indigenous plant pathogens for

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goosegrass control. In this paper, fungal pathogens isolated from infected *E. indica*, that infested a variety of crops were identified and pathogenicity of the isolates was determined. This is the initial step in developing a mycoherbicide for sustainable management of *E. indica*.

Materials and Methods

Isolation of pathogenic fungi

The putative fungal pathogens were isolated from various parts (leaf, stem, and seed) of diseased *E. indica* grown around ten agricultural crops, including mango, corn, oil palm, rambutan, wax apple, water spinach, papaya, okra, pineapple, and paddy, using a modified method from Maizatul-Suriza *et al.* (2017). Four metres apart, samples of infected *E. indica* were collected and cut into roughly 0.5 cm × 0.5 cm squares with a sterile scalpel in a predetermined area (2/3 contaminated area, 1/3 healthy tissue). The samples were surface sterilised with 10% sodium hypochlorite for 5 min before being rinsed with sterilised distilled water. The selected samples were subsequently air-dried on sterile filter paper. The samples of *E. indica* were cultivated on potato dextrose agar (PDA) and incubated at 27°C for 10 days. To obtain the pure culture, actively developing mycelium was sub-cultured using a 5-mm cork borer onto new PDA. The morphology of healthy mycelium was observed.

$$\text{AUDPC} = \sum_{i}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i).$$

Conidial identification of pathogenic fungi

The pure culture of these isolates was sub-cultured onto new PDA using a cork borer with a 5-mm diameter and incubated at 27°C. After 15 days of incubation, the developing mycelium was looped, placed on a microscope slide with 50 μL of sterile distilled water, and covered with a cover slip and the morphology of the spores was observed under 400 magnifications.

Molecular identification of pathogenic fungi

The total genomic DNA was extracted from overnight culture of fungal isolates using DNeasy Plant Mini kit (Qiagen, Hilden, Germany). Fragment of the gene of interest, was amplified using standard PCR protocol and the universal primers with Thermal Cycler machine. Then, the PCR products were analysed by electrophoresis on a 1% agarose gel, stained with SYBR Safe DNA gel stain. The bands were visualized under E-Gel Imager. The PCR products were then further analyzed by Apical Scientific Sdn. Bhd. Sequence similarity was estimated by searching the homology in the GenBank DNA database and the National Centre for Biotechnology Information (NCBI) using Basic Local Search Tool (BLAST).

Isolate inoculation and evaluation of disease severity

At the five- to six-leaf stage, *E. indica* seedlings were infected with conidial suspensions containing 1.0 x 10^8 spores/mL in 0.8% (v/v) Tween 80 and 10% cooking oil (Seri Murni). A control sample was sprayed with sterilised water containing 0.8% (v/v) Tween 80 and 10% frying oil. A total of 3 mL of suspensions were applied to the leaf surface using a hand sprayer until it was thoroughly saturated. After inoculation, samples were placed in a glasshouse with 70–80% relative humidity, 27–35°C temperature, and 800–1000 mol m^{-2} s^{-1} light intensity for a 12-h photoperiod. The progression and disease severity were recorded using a digital camera. Disease severity (DS) was assessed every two days for ten days after conidial inoculation. The severity was graded using a slightly modified version of the scale developed by Kadir and Charudattan (2000) (Table 1). The following formula was utilised to analyse the rating scale:

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

Overall disease levels were expressed as the area beneath the disease progression curve (AUDPC). The AUDPC was measured during the entire study period as follows:
Where,
y_i is an assessment of disease at the i^{th} observation,
t_i is time (in days) at the i^{th} observation,
n is the total number of observation.

Statistical analysis

The AUDPC data were checked for the normality and homogeneity of variance. Square root transformation was performed on the data before being subjected to one-way ANOVA, followed by Tukey's test to compare means at 5% level of significance.

Results

Identification of isolated fungi

Five fungal pathogens were successfully isolated from several affected parts of *E. indica* in five different crops. The MS2 strain was isolated from the *E. indica* stems that were growing near the mango plant. This strain formed a vigorous colony of floccose, moderately thick, off-white to slightly yellow mycelia after 10 days of incubation on potato dextrose agar (PDA). Conidia possessed singular, oval to obvate shapes. The conidia had one to two 20–30 µm diameter septa (Fig. 1). Based on GeneBank data, MS2 strain was identified as *Fusarium chlamydosporum*. The MS2 strain was identical to *F. chlamydosporum* strain VKM, which was isolated in India from *Trianthema*

Table 1: Disease severity scale

Disease scale	Leaf area damaged (%)
0	0
1	1-10
2	11-20
3	21-30
4	31-40
5	41-50
6	51-60
7	61-70
8	71-80
9	81-90
10	91-100

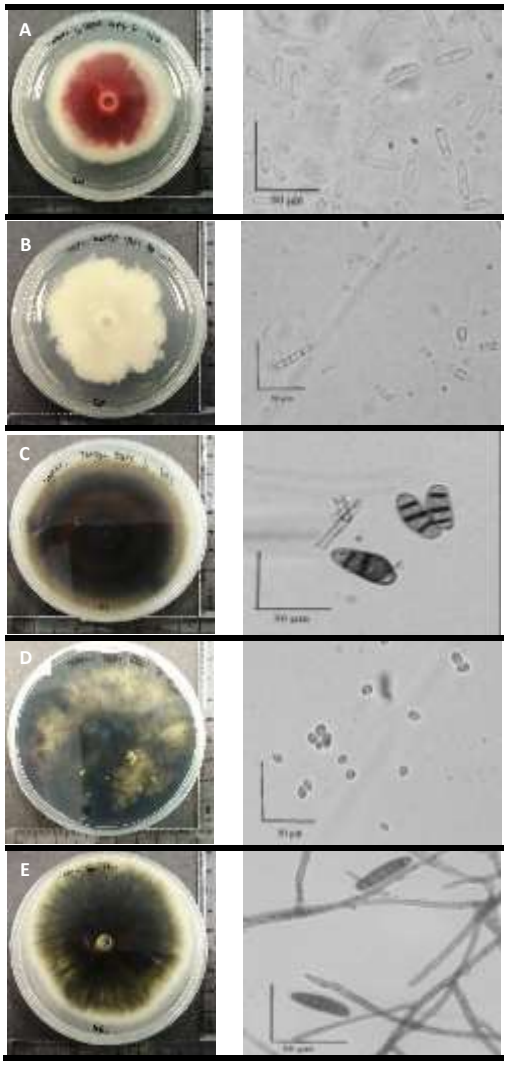

Fig. 1: Morphological characteristics of mycelia and conidia. A. *Fusarium proliferatum* (isolate KSL6), **B**. *Fusarium chlymydosporum* (isolate MS2), C. *Curvularia senegalenis* (isolate JS3), **D**. *Lasiodiplodia theobormae* (isolate PDS7) and **E.** *Bipolaris bicolor* (isolate BR). μm

portulacastrum (Table 2).

Strain KSL6 was isolated from *E. indica* leaves growing next to an oil palm tree. After 10 days of incubation, the colony of this strain had dense, cottony, white, concentric rings of aerial mycelium with purple pigments. The conidia were club-shaped with a flattened base and a diameter of 10 to 30 μm (Fig. 1). Based on the results of a BLAST search in GeneBank, the KSL6 strain was identified as *F. proliferatum*, with 100% similarity to the *F. proliferatum* strains 18S and E26F isolated from rice plants in China and India, respectively (Table 2).

The BR strain was obtained from *E. indica* leaves found in corn fields. Within ten days of incubation, the colony appeared flat and spreading, fluffy to cottony, grey or lead-coloured, and with tiny whitish borders. Conidia were smooth, straight, cylindrical, and occasionally obclavate, with a diameter between 25 and 35 m and five to eight septa. The core of the conidia was dark brown and the ends were lighter (Fig. 1). Based on GeneBank information, this strain was identified as *B. bicolor*, with a genetic makeup 95% similar to that of *B. bicolor* strain YB450101 isolated from bitter gourd plant in China (Table 2).

Meanwhile, the JS3 strain was isolated from the infected stem of *E. indica* infesting wax apple trees. Over time, the rapidly growing fungal colony of this strain on PDA produced velvety olive-brown to dark brown mycelia. Conidia were 30–35 μm in diameter, smooth, straight to slightly curved, predominantly ellipsoidal but sometimes clavate, with three to four dark brown septa. Using GeneBank data, it was shown that the JS3 strain shared a 99% identity with the *C. senegalensis* strain SC4.1 that was observed on sugarcane plants from India (Table 2).

It was interesting to learn that the PDS7 strain was obtained from *E. indica* seeds that grew near paddy plants. It took just two days for the isolate to completely colonise a Petri dish with a diameter of 60 mm in PDA, which was significantly faster than the other isolates (data not shown). Mycelia were either grey or lead-coloured, with conidial diameters ranging from 10–15 m. The conidia had an ellipsoid oval shape, thin wall thickness, and zero to one septum (Fig. 1). Based on a BLAST search, the PDS7 strain was identical to the sequences of *Lasiodiplodia theobromae* strains LYN-UPM S13 and NIBM-ABIJL obtained from papaya and wood in Malaysia, respectively (Table 2).

Pathogenicity test of isolated fungi

Fig. 2 depicts the progression of disease in *E. indica* inoculated with *F. chlamydosporum* (MS2), *F. proliferatum* (KSL6), *B. bicolor* (BR), *C. senegalensis* (JS3), and *L. theobromae* (PDS7). *B. bicolor*, *L. theobromae*, and *F. proliferatum* were more pathogenic to *E. indica* than *F. chlamydosporum* and *C. senegalensis*. During the assessment period of ten days, the disease severity percentage of *E. indica* inoculated with *B. bicolor*, *L. theobromae*, and *F. proliferatum* increased to 50–60%, and the area under the disease progress curve (AUDPC) registered 270–340 (Table 3). When *E. indica* was inoculated with *F. chlamydosporum* and *C. senegalensis*,

Table 2: Identification of fungal pathogen using DNA sequencing by depositing the sequences into GeneBank in NCBI

Strain	ID from NCBI	Accession No.	Origin	Source	Similarity (%)
MS2	*Fusarium chlamydosporum* strain VKM	KM076600.1	India	*Trianthema portulacastrum*	100
JS3	*Curvularia senegalensis* isolate SC4.1	MH087107.1	India	Sugarcane	99.29
KSL6	*Fusarium proliferatum* 18S	FJ040179.1	China	Rice	100
	Fusarium proliferatum strain E26F	KY425734.1	India	Rice	100
PDS7	*Lasiodiplodia theobromae* isolate LYN-UPM S13	MW157270.1	Malaysia	Wood	100
	Lasiodiplodia theobromae isolate NIBM-ABIJL	MN335222.1	Malaysia	Papaya	100
BR	*Bipolaris bicolor* strain YB450101	MH201400.1	China	Bitter Gourd	95

Table 3: Area under disease progress curve (AUDPC) of *Eleusine indica* inoculated with spores of different fungal pathogens during 10 days of experimental period

*Location of sample	Plant part of *E. indica*	Fungal pathogen	AUDPC
Mango	Stem	*Fusarium chlymydosporum*	208 c
Oil palm	Leaf	*Fusarium proliferatum*	274 b
Wax apple	Stem	*Curvularia senegalenis*	103d
Paddy	Seed	*Lasiodiplodia theobormae*	288 b
Corn	Leaf	*Bipolaris bicolor*	339 a
		Non inoculated plant	10 e

*Samples were collected from *Eleusine indica* plants which infested selected crops
Means within the same column followed by different letter are not different at 5% of significance level

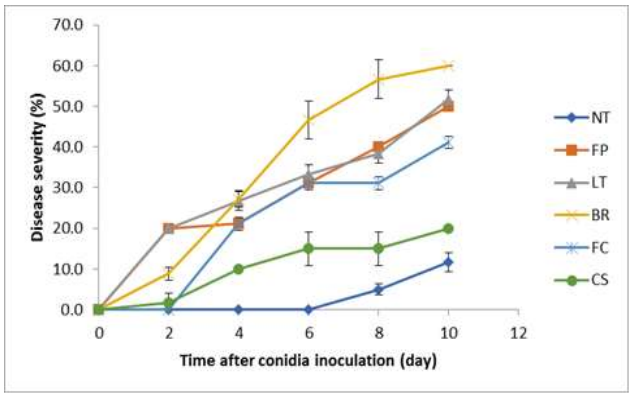

Fig. 2: Disease progress curve of *Eleusine indica* caused by *Fusarium proliferatum* (FP), *Lasiodiplodia theobormae* (LT), *Bipolaris bicolor* (BR), *Fusarium chlymydosporum* (FC) and *Curvularia senegalenis* (CS) and non-treated control (NT)

the percentage of disease severity increased to 20–40% and the AUDPC ranged between 100–210 (Table 3). These findings suggested that *B. bicolor*, *L. theobromae*, and *F. proliferatum* could be possibly developed as a mycoherbicide to control goosegrass (Fig. 2).

Four days after the leaves of *E. indica* were inoculated with conidial suspension of *F. proliferatum*, the symptoms of the disease were increasingly apparent on the leaves. The loss of chlorophyll on the leaf surface was indicated by a gradual decolorization from dark green to light green. Four days after *B. bicolor* inoculation, disease spots on the leaves of *E. indica* were blackish-brown in colour and surrounded by chlorotic tissue. Meanwhile, after four days of inoculation with *L. theobromae*, the infected leaves revealed a tiny, irregular black lesion surrounded by yellowish halos, and the lesion with yellowish area continued to spread (Fig. 3).

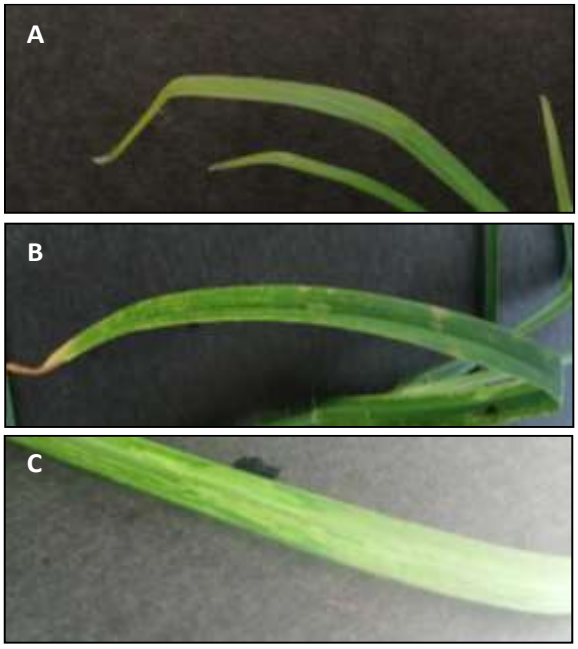

Fig. 3. Disease symptoms of goosegrass leaf on day six after conidia inoculation of A. *Bipolaris bicolor*; **B**. *Lasiodiplodia theobormae* and **C**. *Fusarium proliferatum*

Discussion

Siddiquee *et al.* (2010) isolated *F. chlamydosporum* from *Dendrobium crumenatum* with reddish pigmentation, whereas *F. chlamydosporum* strain MS2 exhibited off-white to slightly yellowish coloration in the current investigation. Nonetheless, both strains possessed the same spore morphology. Additionally, Baori and Vurro (2004) successfully isolated *F. chlamydosporum* from *Orobanche*

ramosa tissue and seeds. *F. chlamydosporum* isolate exhibited a low level of pathogenicity, with the isolate being primarily responsible for tubercle browning and delayed development in *O. ramose*. In the same manner, the MS2 strain of *F. chlamydosporum* was associated with a low infection rate, a disease severity of 40%, and a AUDPC of 208. The inoculation of this strain onto *E. indica* seedlings resulted in only mild discoloration that did not result in the plants' mortality within ten days.

Hawa *et al.* (2013) isolated and characterised *F. proliferatum* from diseased *Hylocereus polyrhizus* stems. The colour pigmentation of mycelia cultures obtained from Hawa *et al.* (2013) was identical to that observed in the present study, in addition to similar spore morphology. There have also been reports of *F. proliferatum* being isolated from *Abutilon theophrasti*, *Chenopodium album*, *Xanthium strumarium*, and *Rumex crispus* (Postic *et al.* 2012). Baori and Vurro (2004) isolated *F. proliferatum* from *Orobanche ramosa* seeds and tissue. The isolate *F. proliferatum* exhibited a moderate level of virulence, as it reduced tubercle growth and induced browning and necrosis in *O. ramosa*. In accordance with a previous finding, when infected with *F. proliferatum* strain KSL6, *E. indica* exhibited a mild infection with a disease severity of 50% and a AUDPC of 274. Within ten days of evaluation, the strain gave symptoms of decolourization that could gradually harm the seedlings. These data suggested that the *F. proliferatum* KSL6 strain is a potential option for use as a biological control agent.

Xiao *et al.* (2021) found *B. bicolor* strain SYNJC 2-2 from diseased *E. indica* in tea plantations. Comparing the strain SYNJC 2-2 to *B. bicolor* strain CPC28811 using Genbank information revealed a 99% match between the two. The mycelia culture obtained by Xiao *et al.* (2021) did not differ from the present investigation, in which the *B. bicolor* colony had a grey or lead colour and cylindrical and obclavate-shaped spores. Xiao *et al.* (2021) demonstrated that the strain SYNJC 2-2 did not harm Convolvulaceae, Amaranthaceae, Compositae, or Leguminosae plants, even when it was present at a density of 5×10^5 conidia/mL. The majority of weeds in the Poaceae family, including goosegrass, green bristlegrass, and *Microsregium vimineum*, were however susceptible to this fungus disease. At a spore density of 1.92×10^4/mL, the SYNJC-2-2 strain was extremely pathogenic to *E. indica* at the 3- to 4-leaf stage. Similar results were obtained in the present study, with *B. bicolor* strain having 60% disease severity and 339 AUDPC at the 5- to 6-leaf stage. Xiao *et al.* (2021) showed that conidial germination, hyphal development, and appressorial production of the *B. bicolor* strain SYNJC-2-2 occurred within 3 to 6 hours on *E. indica* leaves. Hyphae primarily entered leaf tissues through epidermal cell connections and fissures, causing cell death and necrotic lesions within two days on inoculated leaves.

Bandara *et al.* (2022) identified strain CWj of *C. senegalensis* from the leaf of *Zinnia elegans*. The mycelia culture and spore morphology described by the authors were consistent with the present study's findings. According to Tilley and Walker (2002), *C. senegalensis*-caused lesions on large crabgrass plants were elongated, grey to tan, and bordered by chlorotic tissue. Lesions occurred and hardened after three to seven days of inoculation, and leaf blades were frequently impacted. However, huge crabgrass plants were still able to survive seven days after being inoculated, although it exhibited significant stunting. *C. senegalensis* strain JS3 exhibited the lowest levels of disease severity and AUPDC, in the current study.

Urbez-Torres *et al.* (2008) discovered that *L. theobromae* strain PDS7 was identical to the strain of *L. theobromae* isolated from *Vitis vinifera*. The pigmentation and morphology of the author's mycelia cultures and spores were remarkably similar to those of the present study. On one-year-old cuttings and young shoots of grapevine, they also discovered dark-brown necrotic lesions that spread upwards and downwards from the inoculation site. *L.* theobromae rarely grew rapidly, but when it did, its new shoots, petioles, and leaves quickly shrivelled and died (Urbez-Torres *et al.* 2008). The *L. theobromae* strain PDS7, which had a AUDPC of 288 and a disease severity of greater than 50%, might gradually injure *E. indica* seedlings over a ten-day evaluation period. This fungal pathogen shows promising evidence that it can be used as a biological control agent against *E. indica*.

Conclusion

This study demonstrated that the pathogenicity of five fungi isolated from *E. indica* plants was variable, with *B. bicolor*, *L. theobromae*, and *F. proliferatum* showing potential as mycoherbicides against *E. indica*. More study is now underway to evaluate the host range and pathogenicity of these strains against different herbicide-resistant biotypes of *E. indica* at different growth stages.

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Author Contributions

ZS, MSAH and CTS designed the research flow. MAF performed the research and wrote the manuscript. MAF and CTS edited the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability

Data presented in this study will be available on a reasonable request.

Ethics Approval

Ethical approval is not applicable in this study.

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