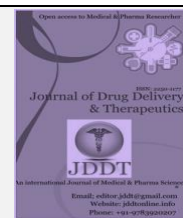
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Research Article

Efficacy of *Colletotrichum gloeosporioides*, potential fungi for bio control of *Echinochloa crus-galli* (Barnyard grass)

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ABSTRACT

A systematic field study was conducted in agricultural fields such as food crops, pulses, vegetable crops, oil crops and commercial crops to estimate infestation of *Echinochloa crus-galli* (barnyard grass), a common terrestrial weed belonging to family poaceae. The *in vitro* pathogenicity studies on barnyard grass were conducted using spore inoculum (8×10^7 /ml) of an indigenous fungus, *Colletotrichum gloeosporioides*. The pathogen was re-isolated from inoculated plants to fulfill Koch's postulates and confirmed its host specificity on barnyard grass. The disease by the isolate was critically analyzed and the results revealed that *Colletotrichum gloeosporioides* is a potential agent to biological control of barnyard grass. The results revealed that the pathogen causes significantly ($P < 0.05$) severe infection on host weed and destructs the weed population by leaf spot diseases. The findings of the research suggested that the isolate *Colletotrichum gloeosporioides* is highly virulent and host-specific, and recommended for further studies as a promising biocontrol agent against barnyard grass weed.

Keywords: Mycoherbicides, Barnyard grass, *Colletotrichum gloeosporioides*, Koch's postulates

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INTRODUCTION

Echinochloa crus-galli L., commonly known as cockspur or cockspur grass, water grass and barnyard grass belongs to the family poaceae, causes high yield loose in different agricultural crops. In recent years, herbicide resistant biotypes of barnyard grass were reported (Fu *et al.* 2001; Manechote 2003). It is essential to develop some new alternative tactics for the long term management of barnyard grass in the future. It reduces crop yields and cause forage to fail by removing up to 80% of the available soil nitrogen. Barnyard grass commonly occurs throughout tropical Asia and Africa in fields and along roadsides ditches, along railway lines and in distributed areas such as gravel pits and dumps. It occurs in all agricultural regions. *Echinochloa crus-galli* (Barnyard grass) is one of the most troublesome weed species in rice production. It is well adapted to wet soils, and is common in both temperate and tropical crops, being reported in 36 crops in over 60 countries (Holm *et al.*, 1977). Barnyard grass (*Echinochloa crus-galli*) is a major weed in lowland rice cultivation in many parts of the world. The most widespread and economically important species from the genus is *E. crus-galli*, or barnyard grass (Maun and Barrett 1986) which has been reported to reduce the rice yield by 5–72% (Kuan *et al.*, 1990).

In Malaysia, the grass is mainly controlled by chemical herbicides which have contaminated the environment (Leong *et al.* 2007), it also affected the microorganism communities (Chen *et al.*, 2009) and led to resistant biotypes of the weed (Sangakkara *et al.*, 2004). Biological control has therefore, been identified as an alternative method to avoid the negative impacts of chemical herbicides. *Echinochloa crus-galli* spreads only by seed, and its high capacity for seed production allows large populations to rapidly establish. Seeds can be dispersed by check, water or as a contaminant in soil, seed crops, and on agricultural machinery (CABI, 2015). The origin of *Echinochloa crus-galli* remains obscure but it probably native to tropical Asia (USBA-ARS, 2014).

According to (CAB 2015), *Echinochloa crus-galli* has a distribution extending from Northern Europe, to the subtropics and to tropical. The awareness on conservation of biodiversity and control of environmental pollution demands the exploitation of microorganisms as pets and weed control agents. Especially plant pathogenic fungi are now emerging as an effective and eco-friendly alternative to conventional methods of weed control (Charudattan 1991). Devine and Collego, are used commercially in the United States to control respectively the milk weed vine, *Morrenia odorata* in citrus groves and northern jointvetch, *Aeschynomene virginica*, in rice and soya bean fields

odarkansas and high borning states (Bowers 1986; Templeton *et al.*,1984). Zhang (1996) found that *E. monoceras*, at a rate of 10^7 spores/ ml, killed *E. crus-galli*.

Biological control of weeds has more advantages over mechanical and chemical methods, and can be specific to the target weed and does not lead to residue problems and accumulation of toxic pollutants in the soil or underground water (Tebeest *et al.*, 1992). Seeds with a positive tetrazolium test carsenal of phytotoxins which recovered only from were summed with germinal seeds to give viable seeds. Culture filtrates as Colletotrichnins by *C. nicotianae* and viable seeds were then expressed as a percent of total *C. capsici*; Colletopyrone produced by *C. nicotianae* seeds to arrive at a percent viability. In some cases, Aspergillomarasmine produced by *C. gloeosporioides* viability was also expressed as a percent of and Ferricrocin produced by *C. gloeosporioides* (Pajon *et al.*, 2003; Boyette *et al.*, 2007).

MATERIALS AND METHODS

Field study and samples collection

The field observation on invasion of barnyard grass was in different crop classifieds as food crops, pulses, and vegetable crops at delta District. Diseased leaves of *Echinochola crus - galli* weed were collected in delta districts of Nagapattinam, Thanjavur and Thiruvavur in the year 2017-2019. Infected plants were collected in sterile polythene bags and brought to the laboratory for the study of symptoms isolation, identification and pathogenicity test of the pathogens involved. Specimens were pressed, dried and kept as herbarium record, bearing details like name of the host location, date of collection etc. The disease symptoms on leaves and stems were examined as round to oval straw colored spots with maroon margins. The diseased plants and propagule were collected in sterilized polythene bags and brought to the laboratory for the study of symptoms, isolation and pathogenicity test of the organism (s) involved in leaf spot disease. The disease symptoms on leaves and stem were critically observed and studied in Microbiology laboratory, Department of Microbiology, Annamalai University, Annamalainagar.

Isolation Identification and Subculture of fungi pathogens

The diseased leaves were washed thoroughly in running tap water to remove soil particles, and the infected portions of the leaves were cut into 1.0-1.5 cm fragments. The pieces were surface sterilized by 70% ethyl alcohol for 1-2 min and then rinsed in sterile distilled water for three to four times. These fragments of leaf bits were transferred to Czapek dox agar (CDA) and potato dextrose agar (PDA) plates supplemented with 3.7 mg of streptomycin sulfate and 2.5 mg of chloramphenicol per liter of medium. The antibiotics wear added to prevent bacterial contamination of cultures, under sterile conditions in an inoculation chamber. After inoculation, plates were incubated at $27 \pm 2^\circ\text{C}$ for 21 days on a 12 h light/dark photoperiod. The isolates were purified by separation of small aerial mycelia fragments with inoculation loop on new culture plats in the initial growth of fungal colonies. The stock cultures isolates were prepared using mono culture (single conidial culture) and stored at room temperature as slant cultures on PDA media. The isolates were examined by the staining techniques, and diagnostic characteristics of the isolates were examined under light microscope. Identification and taxonomic features of the isolates were made with help of the relevant literature (Simmonse *et al.*, 1986; Mitchell *et al.*, 1988; Aneja *et al.*, 1998;; Aneja *et al.*, 2000; Akhtar *et al.*, 2013). The

isolates were properly sub-cultured on PDA and CDA for further research and maintained in PDA slants at $26 \pm 2^\circ\text{C}$ in Microbiology Laboratory, Department of Microbiology, Annamalai University.

Inoculum Production of Test Pathogen

The young and well sporulated isolates of *Echinochloa crus-galli* were used for the assay of disease intensity at *in vitro* conditions. After 12-14 d of incubation, the spore suspensions were harvested from each culture by adding 20 mL of sterile distilled water and scraping of mycelium surface with a sterile glass rod or spatula. The resulting slurry of spores and mycelia fragments was filtered with 3-4 folded cheesecloth, and filtrate was taken into 250 mL conical flasks. Spore suspensions were centrifuged at 3,500 rpm for 20 s for purification of filtrated spores, and the optimum spore concentrations were enumerated using a haemocytometer. An aqueous conidial suspension (8×10^7 spores/mL) of the fungus was prepared and standardized with haemocytometer. An optimum concentration of Tween-20 (0.02% v/v) was added as wetting agent to the conidial suspension before application of the spore treatment of test plants.

Inoculation of Test Plants

Inoculations were made on young plants growing under greenhouse conditions up to spore treatment. The plants with young leaves (at 6-8 leaf stage) were inoculated with an optimal conidial suspension (8×10^7 spores/mL spore inoculum + 0.02% Tween-20) of *Echinochloa crus-galli* by hand sprayer after sunset (between 6:00 pm and 7:00 pm) to avoid drying of the spores. After inoculation, leaves were enclosed with sterile polythene bags for 48 h to maintain 100% humidity for feasibility of the spore germination. Control plants were treated in the same manner, except that they were sprayed with sterile water + 0.02% Tween-20. The adjuvant

Tween-20 was applied as a wetting agent. After inoculation experimented, leaves were collected from both test plants (spore treated) and control plants (water treated) at 10 days of intervals to examine the virulence of pathogen. For *in vitro* experiments, both the control plants and test plants were maintained in five replicates, each contains 10 pots (Five plants per one pot). A total of 500 plants (250 control plants and 250 test plants) were maintained for each experiment.

Measurement of disease intensity

The intensity of disease was measured in terms of disease incidence (Wheder 1977) and disease severity (Chaube *et al.*, 1991). The disease severity was examined at 10 days of interval, and the leaf spot disease was evaluated using standard area diagram of infected leaves. The quantitative data on disease severity were calculated using the analysis of variance, as following Balyan *et al.*, (1986). For the estimation of leaf area diseased, the whole leaf surface area was considered as 100, and thereby the infected area was determined by eye estimation for percent of disease index (PDI). ie. Disease severity disease intensity and severity were rated by visual observation, and the infected leaves were scored using a 0.5 scale rating system Ray *et al.*, (2012). Using this rating system, a disease index (DI) was calculated per observation made at an interval of 10 days after treatment, for the assessment of disease severity individual leaf ratings were taken into an account until the death of weed

Disease intensity (DI)

Inoculum was applied onto the test plants of *Echinochloa crus-galli* within 2 hours of sunset to avoid drying and to allow for a natural dew period shortly afterwards. Plants were observed three days after treatment (DAT) for disease symptoms. The intensity of infection was determined visually, based on the initiation of disease and increase in disease area on the leaves, stems of test plants every day. The disease intensity of pathogen on test plants was determined using a score chart (-, no symptoms, a healthy plant; +, mild symptoms, a plant showing slight symptoms on $\leq 15\%$ of the leaf area; ++, moderate symptoms, a plant showing definitely bigger patches of diseased areas on 16 to 59% of the leaf area; and +++, severe symptoms, enlarged lesions covering 60 to 80% of the leaf area) (Ray and Hill, 2012).

RESULTS AND DISCUSSION

Identical characteristics of *C. gloeosporioides*

The identification features of the isolates, such as colony diameter, color, texture, sporulation, the shape and sizes of conidiophores and conidia, were carefully studied. The causal agent of leaf spot disease of *Echinochloa crus-galli* was isolated from wild plants and simultaneously from test plants inoculated with spore suspension. The pathogenicity of the isolate was confirmed by Koch's postulates, and the host specificity of the pathogen was tested using repeated spore treatments and re-isolation of causal agent. At maturity stage, the profuse radial growth and sporulation of a fungal pathogen were recorded, and the isolate was confirmed as *C. gloeosporioides* by microscopic study of mycelium, conidia and conidiophores. Inoculated leaf lesions yielded white colored colonies of *C. gloeosporioides* at initial stage on the surface of nutrient media. Subsurface mycelial growth was dense and dark on PDA. Sporulation was excellent at agar surfaces of CDA, and the moderate amounts of sporulation appeared on PDA. Significantly ($P < 0.05$) higher radial growth (mm) of the isolate was recorded on PDA (76.67 ± 1.76 mm), followed by on CDA (70.67 ± 1.76 mm) on the 12th day. *C. gloeosporioides* often proliferates by means of a secondary conidiophore that arises immediately below the apical cell of the existing conidiophores.

Mycoherbicidal efficiency of *C. gloeosporioides*

An optimum (8×10^7 spore/mL) spore suspension of *C. gloeosporioides* caused severe infection on host weed *in vitro*. The leaf spot disease was initiated as round to oval straw colored spots with maroon margins at initial stage of spore treated plants (test plants). The typical symptoms were examined on test plants at 3 day after inoculation. No symptoms were observed on control plants treated with sterile distilled water. After 24 h of incubation, small pinpoint maroon colored spots were examined on the surfaces of the leaves. These minute lesions on leaves were the result of the penetration of the germ tube or infectious structures on host tissue. An infection rate of ($79.41 \pm 1.98\%$) was observed at 30 days after treatment (DAT), and the epidemic increased timely with increase of incubation period. The significant ($P < 0.05$) virulence of pathogen in terms of PDI was determined as $55 \pm 3.57\%$, $76.66 \pm 3.17\%$ and $79.41 \pm 1.98\%$ on 7, 15 and 30 DAT, respectively. The values are represented mean of five replicates and standard error (Table-1). The environmental conditions, like 100% moisture, temperature, nutrients and incubation period, enhanced the rate of infection on host, and in addition the susceptibility of the host plant and the virulence of the pathogen significantly increased the rate of infection. The increase of incubation time offers the favorable conditions, such as spore germination, the development of long germ tubes and appressoria (infection structures) and the germination of mycelia fragments. *C. gloeosporioides* (host) pathosystem revealed the virulence of the pathogen as a promising mycoherbicide agent, which was highly potential to cause severe endemic on leaves, petioles, stem and other propagules of the target weed plant. Statistical analysis of the data on the inoculated plants revealed that percent infection was highly significant ($P < 0.05$) at various growth stages of the weed (Table-2). The early stage of the weed plant with 3-6 foliage favours the germination and penetration of the conidia of *C. gloeosporioides* the infective propagules of the pathogen. The destructive damage of leaves and stems was examined on susceptible stage of the weed and caused 100% mortality of the weed within short period

Table.1 Disease intensity on test plant inoculated with spore suspension (8×10^7 /ml) of *Echinochloa crus-galli*

Days after Treatment (DAT)	Disease intensity (DI)		DI Scale
	Control plants ^a	Test plants ^b	
5 rd Day	no symptoms	+, mild symptoms on 10%	1
10 th Day	no symptoms	+, mild symptoms on 20%	2
15 th Day	no symptoms	++, moderate symptoms on 35%	3
20 th Day	no symptoms	++, moderate symptoms on 59%	4
25 th Day	no symptoms	+++, severe symptoms, enlarged lesions covering 80%	5
30 th Day	no symptoms	Affected leaves became chlorosis and dried up causing severe defoliation and withering of leaf.	5

a: inoculated with cool sterilized distilled water, b: inoculated with spore suspension of isolate

Table.2 Disease intensity on test plants inoculated with *Colletotrichum gloeosporioides* inoculum at different growth stages of Barnyard grass.

Effect of Tween 20 on biocontrol efficacy of selected fungal biocontrol agents				
Fungus biocontrol agents	No. of Days	Without adjuvants	With adjuvant	Control (%)
<i>Colletotrichum gloeosporioides</i>	7 days	37 ± 3.07	55 ± 3.57	3.4 ± 4.5
	15 days	65 ± 2.03	76.66 ± 3.17	5.2 ± 6
	30 days	78 ± 1.25	79.41 ± 1.98	5.2 ± 6



Fig. 1. Effect (infected plants) of *colletotrichum gloeosporioides*, on *Echinochloa crus - gali*

The present data suggest that *Colletotrichum gloeosporioides* was approved as highly aggressive towards control of Barnyard grass and has certain characteristics suggested by various workers. Barnyard grass is one of most noxious limiting factors in rice production in the world. *Colletotrichum gloeosporioides* was highly pathogenic on this weed and very safe to rice. The results of this study indicated that this strain could be a potential mycoherbicide to control barnyard grass in the future. There are several biological and environmental limitations which obstruct the efficacy of a biocontrol agent. In the present days, advances in adjuvants formulation and delivery system have been used to overcome some of these limitations and improve the efficacy of a bio-control agent (Boyette *et al.*, 1996).

CONCLUSIONS

Microbial pesticides (bioherbicides/ mycoherbicides) for the management of weeds are host specific, inexpensive and eco-friendly approaches. There has been a great number of naturally occurring fungal strains investigated for possible use as mycoherbicides, but only a small proportion has been developed to commercial products. The findings in this paper enlightened the mycoherbicide properties of *Colletotrichum gloeosporioides* and concluded that it is an effective bio control agent to the target weed. The results also revealed that the Barnyard grass weed was controlled by the fungal pathogen *C. gloeosporioides* at field as well as in greenhouse conditions. The fungal pathogen *C. gloeosporioides* is highly virulent as well as host specific. The quantitative data on disease severity revealed the bio control potential of *C. gloeosporioides* as a successful mycoherbicide. The more extensive work is required to study pathogenicity, adaptability and dispersal and survival efficiency of the pathogen for the development of a commercial mycoherbicide. Type of strain and its virulence, variability in spore size and internal nutrients, spore hydration rate, spore adhesion to plant parts, the number of germ tubes per conidia, germ tube lengths, infection structures produced by germ tubes, the number of appressoria per conidia, penetration of plant parts, toxin productions, inoculum adjuvants such as surfactants, interaction with other phylloplane microbes, etc, can affect the successes of a particular fungal isolate. The research in the direction of the development of mycoherbicide based on several field applications should be needed, and also the exploration of different virulent strains of *C. gloeosporioides* is obligatory to develop a successful mycoherbicide.

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