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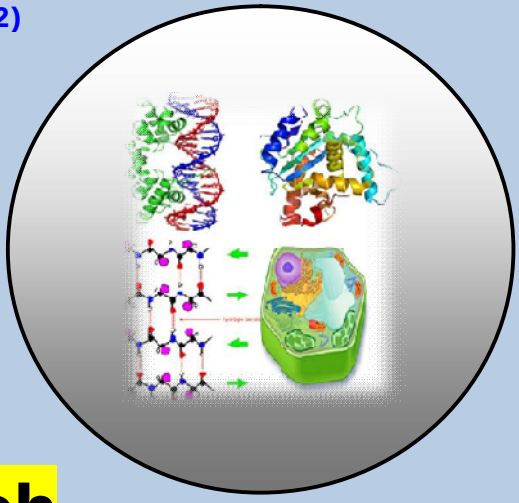

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Pathogenicity of Some Fungal Pathogens Isolated from Infested Weed Flora and *in vitro* Studies as Bio control Agents

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ABSTRACT

Weed infestation is one of the major problems affecting the production of many crops. Chemical herbicides have been heavily used for weed control but the weeds were highly herbicide resistant to them and chemical herbicides were contributed to environmental pollution. Biological control of weeds using plant pathogens is an effective, safe, selective and practical and environmentally sound method of weed management. The fungal pathogens showing pathogenic nature on some serious weeds were isolated and identified as bio control agents through in vitro studies. The test plants were inoculated with 5x 10^4 spores per ml and the disease intensity was determined three days after treatment (DAT). The isolates of Alternaria alternata, Ascochyta cypericola, Fusarium oxysporum, Curvularia lunata, Colletotrichum capsici and Colletotrichum spp. etc. were confirmed as bio control agents to some test plants.

Key words: Weeds, Bio control Agents, Fungal Pathogens, Spore suspension, Disease Intensity (DI), Days after treatment (DAT) and Potato Dextrose Agar (PDA).

INTRODUCTION

The plants growing in agricultural fields, having more negative values, and competing with the main crops for soil, water, nutrients etc. are known as weeds (Ali *et al.*, 2003; Muzik, 1970).Weeds are unwanted and undesirable plants growing usually with desirable plants having short vegetative phase, high reproductive output and capable of limiting the crop yields. Most of the crops infested with heavy weeds during the irrigation period and due to the adequate supply of nutrients. These factors like irrigation and supply of nutrients causes enormous growth of weeds. Weeds are genetically labile and phenotypically plastic; such characters enable them to pass through successfully in adverse habitats.

They easily invade crop fields which are favourite grounds for their quick growth. Weeds have higher contents of nutrients than crop plants; they grow faster and absorb nutrients more efficiently and thus limiting the availability of the same to crop plants (Prayaga Murty and Venkaiah, 2011).

Weeds are unwanted plants growing along with domesticated crops. They are non-indigenous plants that can invade or negatively alter native plant communities. Weed plants grow faster, spread rapidly, reproduce in high numbers and produce large quantity of seeds which enables them to establish a kingdom of their own within a short period of time (Dangwal *et al*., 2010).They always act as energy drains in the entire managed ecosystem such as agricultural fields, forestry, horticulture, and aquaculture etc. They decrease the yield of crops by competing for water, nutrients, space, CO_2 and sunlight. They provide habitat to harmful insects and may act as alternate host for pathogens and other organisms (Peters, 1955).Weeds show allelopathic effects on agricultural crops by secreting allele chemicals that inhibit the growth and germination of agricultural crops.

These weeds effectively compete with the crop for nutrients, water, and space; reduce the yield ranging from 12 to 51 % (Rao and Singh, 1998; Mukharjee and Singh, 2005; Halder and Patra, 2007). Weed flora and its composition in a crop is influenced by the type of cultivation, spacing, time or season of cultivation, soil type, soil PH, climatic conditions such as rainfall, temperature, cultivation practices like irrigation, tillage systems, application of fertilizer and weed management (Kiran and Rao, 2013). Weeds limit growth and yield of crops through becoming their partner with available moisture, nutrients, light, space and air; and escaping phytotoxic compounds in their environment (Zimdahl, 2007). The weeds influence the crop plants by releasing phytotoxins from their seeds, decomposing residues, leachates, exudates and volatiles (Narwal, 2004). The presence of different allelochemicals like caffeic, chlorogenic, and ferulic acids can inhibits the seed germination of other plants (Hussain *et al.,* 1987; Marwat *et al.,* 2008).

Weed infestation is one of major constraints affecting the production which is the most important for each crop. Although weeds have been eradicated using various cultural practices in current farming methods i.e., chemical herbicides have been heavily used for weed control as the most effective and immediate method. Some problems, however, have been emerged in association with heavy use of herbicides such as the appearance of herbicide resistant weeds and the contamination of soil and ground water by chemical residues that cause environmental pollution. Commonly used weed control strategies are water management, hand weeding, mechanical weeding and chemical herbicides. Water management can control certain weed species in irrigated lowland. Hand weeding is time-consuming and is becoming expensive, while the use of mechanical weeders is known to reduce yields. Chemical herbicides, on the other hand, not only are becoming more expensive, but also contribute to environmental pollution. Continuous use of chemical herbicides can result in the development of herbicide-tolerant weed populations (Bayot *et al.,* 1994).

Increasing awareness of the general public about the safety of herbicides and its influence on food crops and environment has encouraged researchers to develop alternative weed control approaches such as biological control (Charudattan, 2001).

Weed control using this approach can complement and be integrated with traditional cultural and chemical methods for weed control. Biological control of weeds using plant pathogens is a practical and environmentally sound method of weed management. A variety of herbaceous, woody, climbing, aquatic, and parasitic weeds have been shown to be capable of being controlled by plant pathogens (Charudattan, 1991). Biological control with plant pathogens is an effective, safe, selective and practical means of weed management that has gained considerable importance (Charudattan, 1986; Flint and Thomson, 2000; Pemberton and Strong, 2000; Bouda *et al*., 2001).

The study on pathogenicity of fungal pathogens on weeds can explores the biological control methods and the finding of new bio control agents for the control of some serious agricultural weeds commonly compete with agricultural crops. The investigation is useful to researchers, agronomists and formers to develop bio control agents for weed management as well as sustainable agriculture. The method biological control of weeds is eco-friendly and can enhance the soil fertility by avoiding herbicide pollution.

MATERIAL AND METHODS

Field Study

All the weeds encountered in the field sites of the crop fields were carefully collected and identified during the Kharif season (July - October 2013). The exploration of the area under study includes agricultural fields of Venkogipalem, Anandhapuram, Boyapalem, Thagarapuvalasa and PM Palem in Visakhapatnam District. The random sampling method was adopted for the collection of weed species among the crop fields. The collected weed plants were pressed, dried, preserved and properly identified with the help of available literature and monographs.

Identification of weeds

After completing the weed collection from the crop fields, the weed flora was identified by the help of floras, monographs and other relevant literature and consequently the correct scientific and common names were provided to each plant. Each plant was critically studied and identified using the 'Flora of British India' (Hooker, 1872-1897), 'Flora of Presidency of Madras' (Gamble and Fischer, 1915-1935), The grasses of Burma, Ceylone, India and Pakistan (Bor, 1960), 'Flora of Andhra Pradesh' (Pullaiah and Chennaiah, 1997), and district floras of Srikakulam (Rao and Sriramualu, 1986), Visakhapatnam (Rao and Kumari, 2002) and Vizianagaram (Venkaiah, 2004).

Sites and Sampling

Infested weeds were collected from some field crops such as *Oryza sativa* L. (Paddy), *Zea mays* L. (Maize), *Sorghum bicolour* (L.) (Sorghum), *Arachis hypogaea* L. (Ground nut), *Glycine max* (L.) Merr. (Soya bean), *Vicia faba* L. (Broad bean), *Amaranthus tricolor* L. and *Hibiscus cannabinus* L. etc. at agricultural fields of Venkogipalem, Anandhapuram, Boyapalem, Thagarapuvalasa and PM Palem in Visakhapatnam District. Diseased leaves, stems, roots, flowers and whole plants of the weed flora were collected randomly from various parts of infested fields; air dried in a paper press, stored in paper envelopes and brought to the laboratory.

Isolation and identification of fungal 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 sterilised by 70% ethyl alcohol for 1-2 minutes and then rinsed in sterile distilled water for six to seven times. Finally the leaf bits were rinsed in 0.01% mercuric chloride for 1 or 2 minutes fallowed by washing with sterile autoclaved double distilled water for 2 or 3 times.

These fragments were transferred on to Potato dextrose agar (PDA) plates supplemented with 1% streptomycin sulphate (antibiotic) under completely sterile conditions in an isolation chamber. After inoculation plates were incubated at 28 ±2°C for 21 days on a 12 h light/dark photoperiod. The petri dishes were incubated with artificial light supplied by fluorescent light. Pure cultures of fungi were maintained for the harvesting of spores in different growth media such as Potato Dextrose Agar (PDA) [Potatoes(peeled) 200 g; Dextrose 20 g; Agar 20 g; Distilled water 1L]; Czapek,s Dox Agar (CZA) [Sucrose 30g; $NaNO_3$ 2 g; K_2HPO_4 1g; $MgSO_4+7H_2O$ 0.5 g; KCl 0.5 g; $FeSO_4+7H_2O$ 0.01g; Agar 15 g; Distilled water 1L]; Sabouraud's Dextrose Agar (SDA) [Glucose 40g; Peptone 10g; Agar 15g; Distilled water 1L] supplemented with 1% Streptomycin (1gram of streptomycin was mixed thoroughly in 100 ml of sterilized distilled water). The pH of the culture media was maintained at 5.5 to 6.5 being optimal for the growth and sporulation of fungi. 1% Streptomycin was used as an antibiotic for the restrain of bacterial growth in culture. Fungi were maintained on half strength PDA slants in test tubes as stock cultures and stored at 28 ± 2 ^{0}C in a incubation chamber.

Identification of the fungal isolates were made with help of the relevant literature (Barnett, 1960; Barron, 1968; Booth, 1977; Domsch et al., 2007; Ellis, 1971, 1976; Gilman, 1959, 2001; Holliday, 1993; Nagamani et al., 2006; Sivanesan, 1987; Sutton, 1980). Fungal morphology was studied macroscopically by observing colony features (colony diameter, colour, texture and pigmentation) and microscopically by staining with lacto phenol cotton blue called as mounting fluid and observed under compound microscope for the observation of the conidia, conidiophores and arrangement of spores.

Test Plants

Seeds and seedlings of various weeds were collected from agricultural fields during the field study. The collected seeds were dried and maintained in healthy condition without any contamination. The plants for the studies were grown by sowing the seeds in 10 cm diameter plastic pots containing sterilized, black soil. The pots containing seed lings of weed plants were maintained in a green house with a 12 h light/dark photoperiod. For host–range studies, each weed was maintained in four replications along with control plant. The plants in the greenhouse were watered daily and fertilized farmyard manure when required.

Preparation of Spore inoculum

The isolated fungi were cultured on PDA in petri dishes and incubated for 14 days at 28 ± 2^0 C with a 12 h light/dark photoperiod. After that, conidia and mycelium were harvested with a sterilized spatula by flooding the plates with sterile distilled water and then scraping the mycelial mass slowly for conidial suspension. The suspension was then filtered through sterile, muslin cloth folded in four layers and the final inoculum was taken into 100 ml conical flasks containing sterile distilled water and 5 ml of 0.01% (v/v) Tween 20 (Merck).

The Inoculum concentration was adjusted to $5x10^4$ spores/ml using Improved Neubauer haemocytometer (Depth = 0.1mm).

Disease intensity (DI)

Inoculum was applied onto the plants within 2 hrs 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 and roots of test plants every day. The disease intensity of each fungal pathogen on test plans was determined using a score chart (-, no symptoms, a healthy plant; +, mild symptoms, a plant showing slight symptoms on ≤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

Agricultural weeds

All the weeds encountered in the field sites were carefully collected and identified. The fields were severely infested with weed species belonging to families of Asteraceae and Poaceae stood first and second followed by Amarantaceae, Aizoaceae, Euphorbiaceae, Rubiaceae Convolvulaceae, Cyperaceae, Fabaceae, Malvaceae, Acantaceae, Lamiacae,, Commelinaceae, Oxalidaceae, Portulacaeae and Solanaceae respectively.

Table 1. List of fungal pathogens isolated from diseased parts of different weeds.

S.No	Weed	Family	Diseased part	Fungi isolated
1.	***Commelina benghalensis* L.** (Benghal dayflower, Tropical spiderwort)	*Commelinaceae*	Leaf Leaf	***Alternaria alternata*** * *Ascochyta spp.*
2.	***Cyperus rotundus L.*** *(Purple nutsedge)*	Cyperaceae	Leaf Leaf Leaf, stem Root	***Ascochyta cypericola**** *Helminthosporium spp.* *Chaetomium globosum* ***Fusarium oxysporum****
3.	***Crotalaria verrucosa* L.** (Blue rattlepod)	Fabaceae	Leaf	***Alternaria alternata****
4.	***Digera muricata* (L.) Mart.** (False Amaranth)	Amaranthaceae	Leaf, Stem Leaf, Leaf,	*Bipolaris spp.* ***Curvularia lunata**** *Curvularia tuberculata*
5.	***Sida cordifolia L.*** (Country-mallow,Heart-leaf sida)	Malvaceae	Leaf	***Colletotrichum capsici****
6.	***Ipomoea pestigridis* L.** (Tiger foot morning glory)	Convolvulaceae	Leaf	***Colletotrichum spp.****
7.	***Trianthema portulacastrum* L.** (Horse purslane.)	Aizoaceae	Leaf, Stem	***Gibbago trianthemae****

***** Isolates were pathogenic to weeds and showing host specificity.

Cyperus rotundus was the most abundant weed followed by *Cynodon dactylon, Trianthema portulacastrum, Parthenium hysterophorus, Boerhavia diffusa, Merremia tridentata* and *Digera muricata* . *Cyperus rotundus* is one of the prominent weed of the present study. This weed is the native of India but has become cosmopolitan, spread over most of the tropic countries, and is treated as the world's worst weed. It is one of the weeds that appear immediately after sowing and may compete heavily with the crop plants for nutrients and water (Holm *et al.,* 1977).

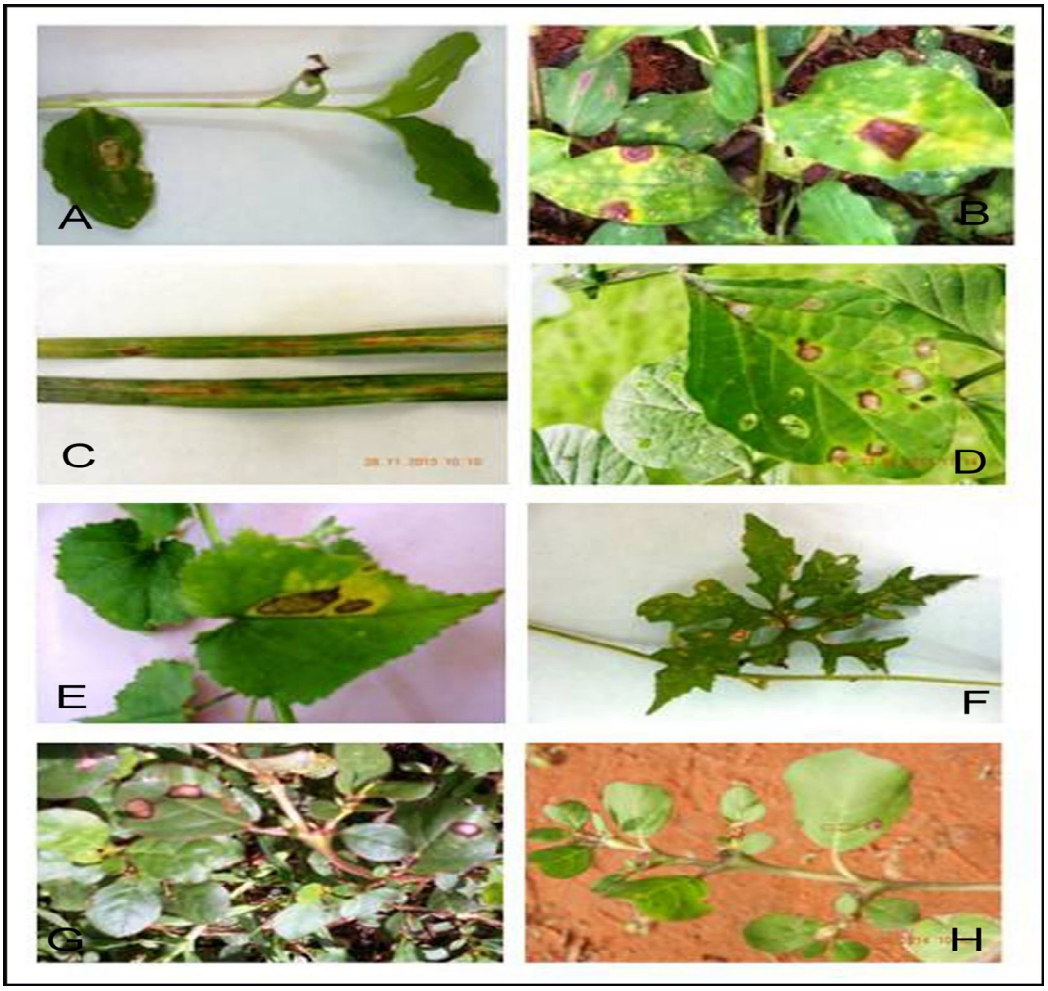

Figure 1. Agricultural weeds infested with symptoms of fungal diseases.
A& **B**.*Commelina benghalensis* L. **C**. *Cyperus rotundus L.* **D**.*Crotalaria verrucosa* L. **E**. *Sida cordifolia L.* **F**.*Ipomoea pestigridis* L. **G**&**H.** *Trianthema portulacastrum* L.

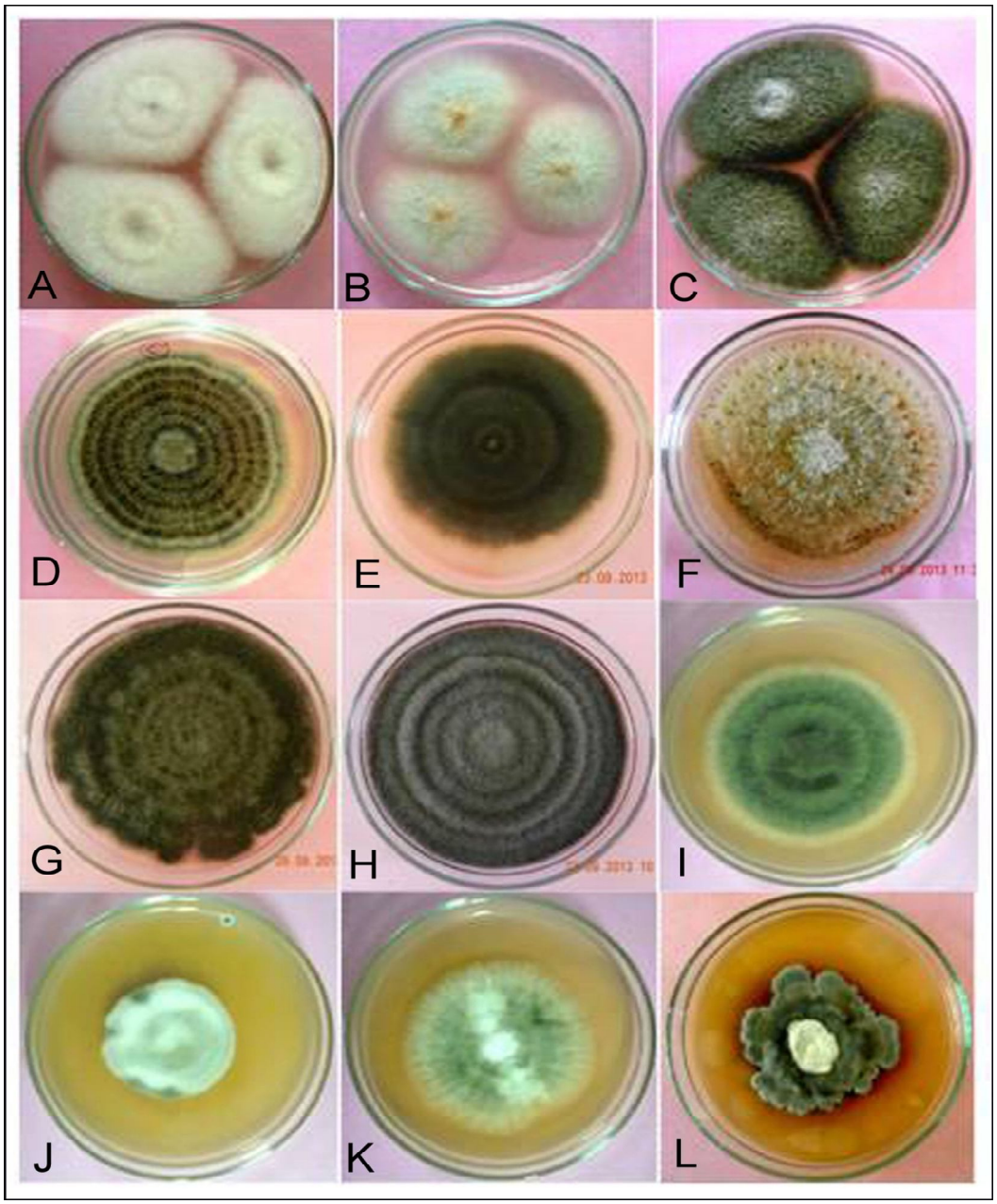

Figure 2. Pure cultures of some weed pathogens on Potato Dextrose Agar medium.

A. *Fusarium oxysporum* **B.** *Fusarium spp.* **C.** *Colletotrichum capsici*
D. *Colletotrichum spp* **E.** *Alternaria alternata* **F** *.Ascochyta cypericola*
G. *Curvularia tuberculata* **H**. *Curvularia lunata* **I.** *Helminthosporium spp.*
J. *Chaetomium globosum* **K**. *Bipolaris spp.* **L**. *Gibbago trianthemae*

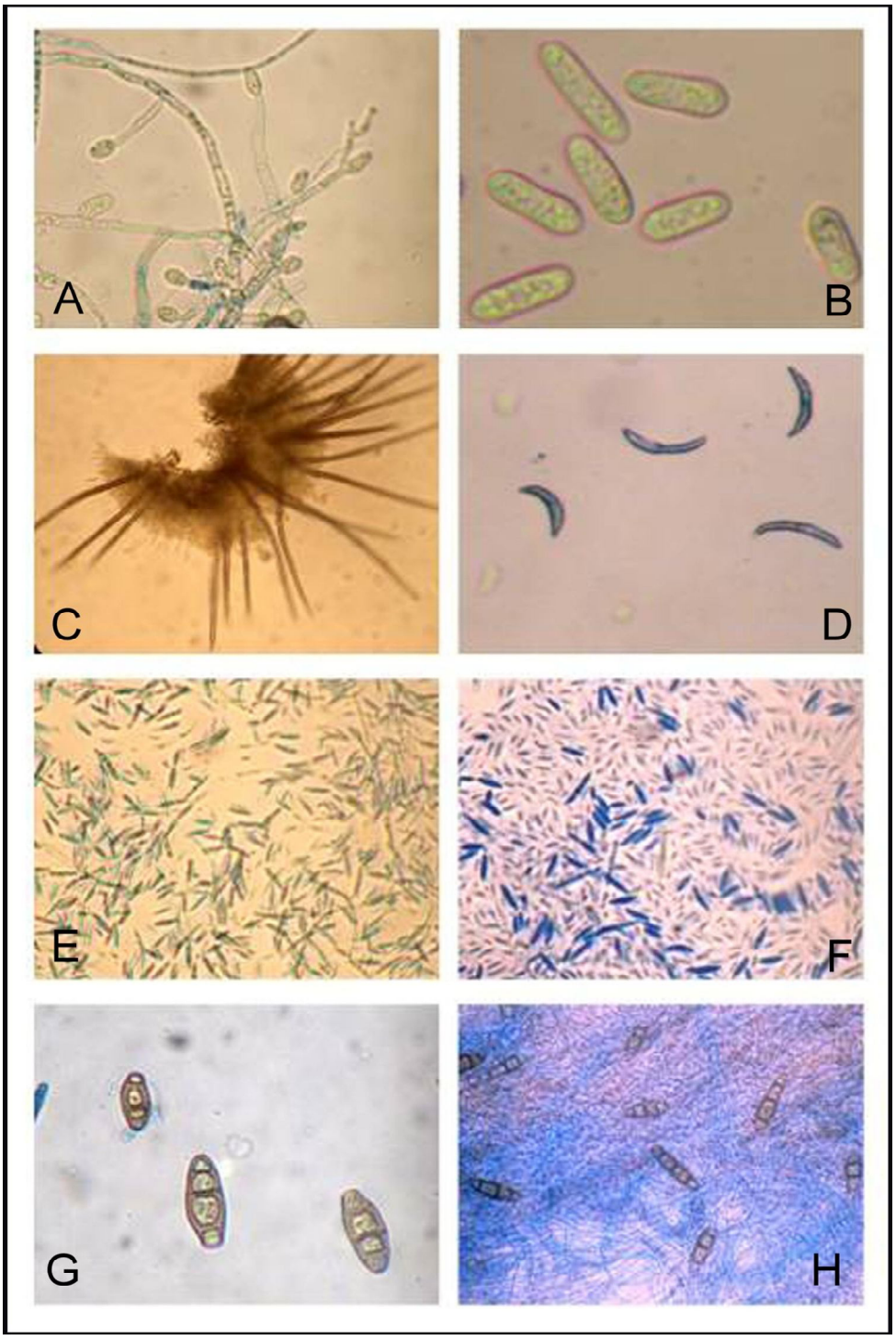

Figure 3. Microscopic features of weed pathogens isolated from diseased weeds (A-F).
A. *Ascochyta cypericola* **B**.*Ascochyta spores* **C.** *Colletotrichum capsici*
D. *Colletotrichum spores* **E. & F**. *Fusarium oxysporum* macro and micro conidia
G. *Helminthosporium .* **H.** *Bipolaris spp.*

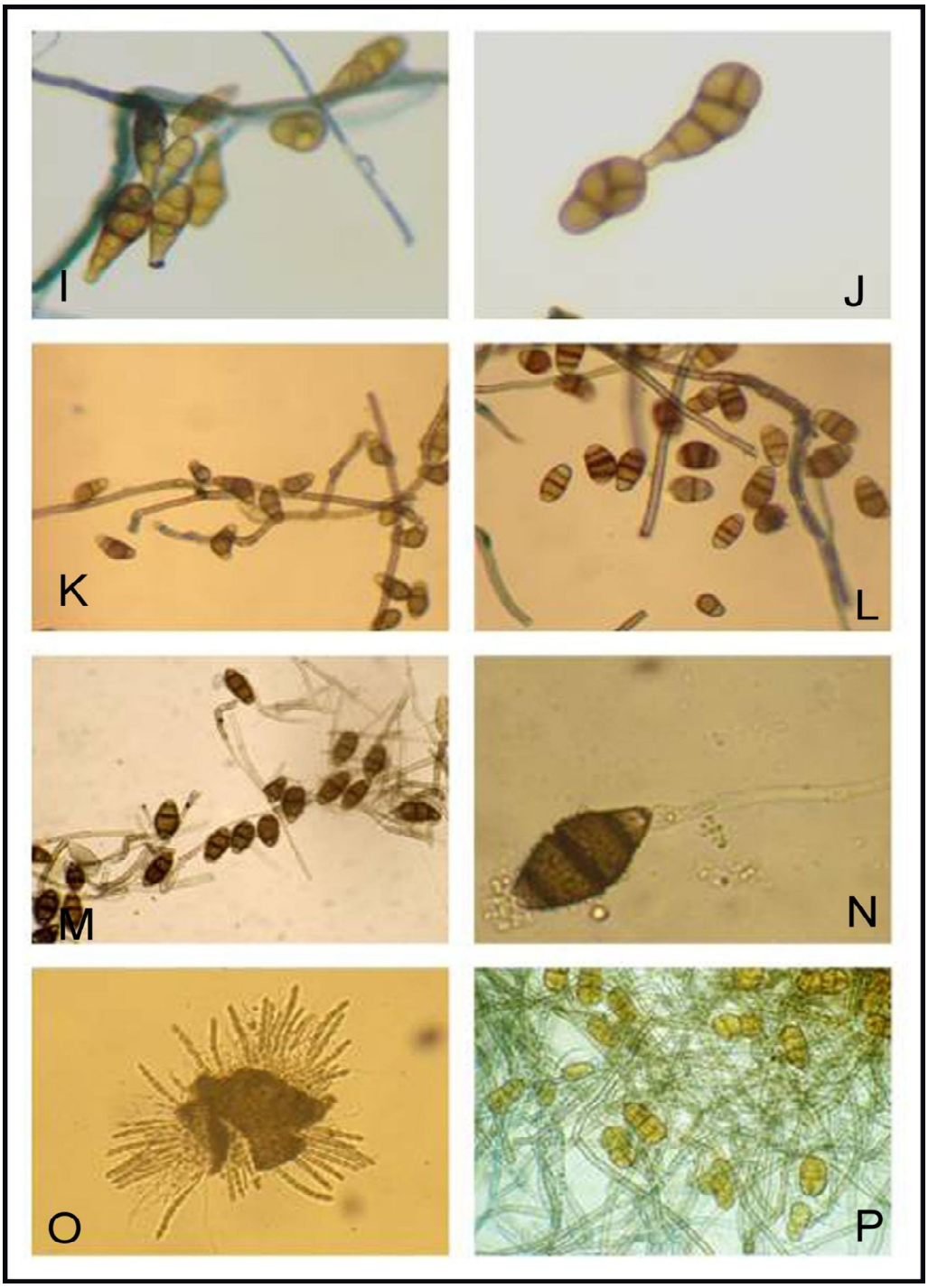

Figure 4. Microscopic features of weed pathogens isolated from diseased weeds (I-P).
I& J. *Alternaria alternata* **K**. *Curvularia lunata* **L**. *Curvularia spp.*
M. *Curvularia tuberculata* **N**.Spore germination of *Curvularia tuberculata*
O. *Chaetomium globosum.* **P.***Gibbago trianthemae*

Screening of the pathogenic fungi

Leaf blights, Leaf spots, root rot and anthracnose were the common symptoms caused by fungal pathogens observed on different plant parts of the weeds (Figure-1). Although all the stages of leaves showed infection, the mature leaves were more heavily affected. The parasitized fungi were isolated from some weeds infested with higher disease. The fungal isolates such as *Alternaria alternata , Ascochyta spp*., were isolated from diseased leaves of *Commelina benghalensis* L.(Commelinaceae); *Ascochyta cypericola, Helminthosporium spp*., were screened from diseased leaves of *Cyperus rotundus L.* (Cyperaceae), *Chaetomium globosum* was isolated from diseased stem and leaves of *Cyperus rotundus L. and Fusarium oxysporum* was isolated from infested root of *Cyperus rotundus L.*

Table 2. Disease intensity and host specificity of isolated fungal pathogens on weed flora (Test plants).

Fungal pathogen	Disease intensity and symptoms of test pathogens on weed plants						
	Commelina benghalensis L.	*Cyperus rotundus L.*	*Crotalaria verrucosa* L.	*Digera muricata* (L.)Mart.	*Sida cordifolia L.*	*Ipomoea pestigridis* L.	*Trianthema portulacastrum* L.
Alternaria Alternate	++	–	+++ Leaf blight	–	–	–	–
Ascochyta cypericola	–	+++ Leaf blight	–	–	–	–	–
Ascochyta spp.	++	–	–	–	–	–	–
Fusarium oxysporum	–	+++ Root rot	–	–	–	–	–
Bipolaris spp.	–	–	–	+	–	–	–
Chaetomium globosum		–	–	–	–	–	–
Colletotrichum capsici	–	–	–	–	+++ anthracnose	–	–
Colletotrichum spp.	–	–	–	–	–	+++ anthracnose	–
Curvularia lunata	–	–	–	+	–	–	–
Curvularia tuberculata				+++ Leaf spots			
Helminthosporium spp.	+	–	–	–	–	–	–
Gibbago trianthemae	–	–	–	–	–	–	+++ Leaf spots&blight

Intensity of infection: -, no symptoms; +, mild symptoms; ++, moderate symptoms; +++, severe symptoms.

The pathogenic fungi such as Alternaria alternate was isolated from diseased leaves of *Crotalaria verrucosa* L. (Fabaceae); *Bipolaris spp., Curvularia lunata Curvularia tuberculata* were screened from diseased leaf spots of *Digera muricata* (L.) Mart. (Amaranthaceae); *Colletotrichum capsici* was isolated from diseased leaves of *Sida cordifolia L.* (Malvaceae,); *Colletotrichum spp.,* was isolated from diseased foliage parts of *Ipomoea pestigridis* L. (Convolvulaceae) and *Gibbago trianthemae* was isolated from infested leaves and stems of *Trianthema portulacastrum* L. (Aizoaceae).Table-1showing the list of isolates of fungal pathogens isolated from the diseased weeds. The identification of fungi was made by macro and microscopic observation (Figure-3&4).

Pathogenic tests on selected weeds

Pathogenic nature of isolated pathogens as bio control agents was tested on some selected weeds. Some *in vitro* studies were conducted for the re-isolation and disease severity of pathogenic fungi. The isolates were maintained as pure and stock cultures for *in vitro* studies on different growth media (Figure-2). The spore suspension was prepared from the two week old pure cultures of isolates growing on PDA medium and the spore concentration was adjusted up to $5x10^4$ spores/ml. The healthy test plants were inoculated by spore concentration ($5x10^4$ spores /ml.) and the disease development on plants was observed after three days of treatment. The disease severity was determined by using a score chart. The results obtained by disease score chart confirmed that some of the test fungi were showing the host specificity and weed control properties by drying the plant and damage of leaves and some arial parts.

The fungi *Alternaria alternata* was showed sever pathogenic nature on *Crotalaria verrucosa* L. and causes some moderate pathogenic symptoms on *Commelina benghalensis* L. The isolate *Ascochyta cypericola* was pathogenic to *Cyperus rotundus L.* which causes leaf blight and the isolate *Fusarium oxysporum* causes root rot on *Cyperus rotundus L.* The isolate *Curvularia lunata* was showing leaf spots on *Digera muricata* (L.) Mart. and the species *Colletotrichum capsici* causes anthracnose on leaves of *Sida cordifolia L. and Ipomoea pestigridis* L. also infested with *Colletotrichum spp.,* which causes anthracnose. A phaeodictyoconidial hyphomycetes fungus identified as *Gibbago trianthemae* Simmons, causes leaf spots and leaf blight of *Trianthema portulacastrum* L. (Aizoaceae). Pathogens were re-isolated from diseased leaves of inoculated plants and found similar to the original isolates in both macro and microscopic characteristics thus confirmed the pathogenicity of various test fungi on selected weeds (Table-2).

Among the isolates species of *Alternaria alternata, Fusarium oxysporum,* and *Colletotrichum spp.* were well developed as bio control agents for the management of various problematic weeds in crop fields in world wide. Recently the isolates belonging to *Ascochyta spp*., *Helminthosporium spp*., *Chaetomium spp., Bipolaris spp.,* and *Curvularia spp.* and *Gibbago trianthemae* Simmons were used as weed control agents by spraying their spore inocula onto the some serious weeds. Pathogenicity and host-range tests of the study showed that a total of seven isolates were primarily screened as bio control agents through *in vitro* studies and the genus of *Alternaria , Ascochyta , Curvularia , Colletotrichum, Fusarium* and *Gibbago* having the biological control properties and the remain genus belonging to *Helminthosporium , Chaetomium* and *Bipolaris* were under study.

Several microorganisms have been studied or are under development as potential sources for microbial herbicides. Biological herbicides represent a means to reduce dependence on synthetic herbicides; focus on ecologically grounded methods of management; reduce weed seed bank populations through environmentally friendly practices; and potentially reduce costs of weed control in crop production (Kennedy & Stubbs, 2007).

CONCLUSION

During the extensive surveys conducted by the authors in the agricultures fields, the heavy weed infestation was observed in various crop fields. Several workers have earlier reported heavy infestation of weeds in agricultural crops of study area. Some of these weeds may be controlled by the fungal isolates, species belonging to *Alternaria alternata, Ascochyta cypericola, Fusarium oxysporum, Curvularia lunata, Colletotrichum capsici* and *Colletotrichum spp.* and *Gibbago trianthemae.* These biocontrol agents were showing more pathogenic nature on some serious weeds of many countries and released into market as commercial myco-herbicides. Recently the awareness of biological control methods was developed among the farmers and agronomists, researchers and plant breeders in India. The study on pathogenicity of fungal pathogens is more useful for the future steps and development of new methods in biological control of agricultural weeds by indigenous fungal pathogens.

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