Management of *Cyperus difformis* by using fungal pathogensas Mycoherbicide

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Abstract

The unwanted weed variable flat sedge (*Cyperus difformis L.*), which has become widely naturalized in tropical and subtropical areas of the world and its economically harmful to the field crops all around the world. However, this weed has been linked to yield losses of 20–40%.By using fungal pathogen as mycoherbicides is one of the eco –friendly approach to mitigate this problematic weed. In this study, the myco-herbicides were isolated from the Cyprus spp. infected parts under *invitro* condition. The preliminary identification of the pathogen was done through microscopic observation by using phase contrast microscopy under 40X magnification. The isolates were further confirmed through PCR analysis which yielded an amplicon size of 350- 400 bp and sequencing of amplified product confirmed the pathogen as *Epicoccum sorghinum* and *Rhizoctonia bicornis.* Among the two fungal pathogens *Rhizoctonia bicornis* (undiluted culture) recorded higher Percentage Disease Index (32.36) than the *Epicoccum sorghinum* (undiluted culture)

1.0 Introduction

Weed infestation is one of the major constraints affecting the crop production. (Oerke and Dehne, 2004). Among the different groups of weeds, *Cyperus* family weeds severely affect the food grains such as, *Cyperus rotundus*, *Cyperus esculentus*, *Cyperus iria* and *Cyperus difformis*. These weeds are widely naturalized all over the world, especially its very problematic in America, southern or central Europe, South Asia and Africa. The fast rise in the usage of chemical pesticides in agriculture around the world reflects the importance of weeds. Herbicide effectiveness has raised yields considerably in several cases, resulting in the creation of novel chemical herbicides. Weeds,

however, continue to inflict major losses despite tremendous efforts. These losses occur as a result of the selection and emergence of species that are resistant to currently available herbicides, the inability of a herbicide to selectively control particular weedy species without causing crop damage, or the establishment of resistant strains. Plant pathogens have been proposed as one of several potential methods for eliminating weeds that persist despite otherwise successful weed control programmes in intensive agriculture, or even as a alternative chemical.(Bouda *et al.*, 2001)

2.0 Materials and methods

2.1 Isolation and characterization of pathogens isolated from *Cyprus spp*.

Diseased *Cyperus difformis* and *Cyperus rotundus* plants showing symptoms like yellowing, chlorosis, leaf scorching, leaf blight, root rot leaf spot and necrosis were collected and brought to the laboratory. The collected plants were washed under running tap water and removed the surface debris. The diseased portions were dissected about 1-2cm along with healthy tissues using sterile blade. Then the dissected leaf pieces were placed in 10% sodium hypochlorite solution about 1min for surface sterilization and rinsed thrice with sterile distilled water. After surface sterilization, leaf tissues were placed on a blotting paper to become dry and aseptically transferred into the petriplates containing potato dextrose agar medium and incubated for 5-7 days under room temperature. The inoculated plates were regularly observed for appearance of fungal colonies**.**

2.2 Sub culturing and maintenance of the isolates

The fungal colonies observed in the isolation plates were aseptically transferred into fresh PDA plates for sub culturing and then the pure culture of fungal colonies were maintained by inoculating them into a sterilized PDA slants and stored at 4°C.

2.3 Cultural and morphological characterization of fungal species

The fungal isolates from the *Cyperus difformis* (variable flat sedge) and *Cyperus rotundus* (yellow nut sedge) were grown on PDA medium and observed the colony character of the fungus based on color, nature of growth and mycelial characters under microscope.

2.4 Molecular characterization by using ITS primers

The genomic DNA was extracted by using CTAB method (Chakraborty *et al.*, 2010). The PCR amplification of 18S rRNA using ITS 1 (sequence: 5’- TCCG ATGG TGAA CCTG CGG-3’) and ITS 4 (sequence: 5’-TCCT CCGC TTAT TGAT ATGC - 3’) was carried out according to White *et al.* (1990). The PCR cyclic conditions were an initial step at 95°C for 10 min; denaturation at 94°C for 30s; annealing at 60°C for 1 min; Extension at 72°C for 1 min; and final extension at 72°C for 10 min

followed by 35 cycles and hold at 10°C. A negative control was maintained using water instead of DNA. The PCR products were examined by 1 per cent agarose gel electrophoresis using ethidium bromide staining and documented for amplification of appropriate base pairs (Sambrook, 1989). For confirmation the PCR products were eluted using the QIA quick gel extraction kit (Qiagen, Inc., Chatsworth, California) and sequenced using the primer ITS1 and ITS4 by ABI 3730 XL sequencer at Biokart India Pvt.India. After sequencing of the isolates, forward and reverse nucleotide sequences were trimmed, joined and submitted in Gene Bank and the accession numbers of sequences were obtained.

2.5 Proving pathogenicity under *in vivo* condition

2.5.1Whole plant assay

The fungal isolates were tested for their ability to produce symptoms in healthy plants by using whole plant assay method. The isolated fungal pathogens is sprayed on the *cyperus difformis* infested field. The spraying area was completely covered by shade net to maintain humidity and temperature and the weeds were wounded artificially by pin prick method and the spore suspension was maintained at 1×10^6 /ml. After inoculation the plants were observed periodically for expressing the symptoms.

Disease severity of fungal isolates were observed for every 24h and intensity of infection was determined using the score chart developed by Freeman and Charudattan (1984) and designated as: – (no symptom: healthy plant), + (mild symptom: plant showing slight symptoms up to 15% of leaf area), ++ (moderate symptom: plant showing definite bigger patches of diseased areas from 16% to 59% of leaf area) and +++ (severe symptom: enlarged lesions covering 60–100% of leaf area). Disease was scored using a 0 to 5 scale rating system were 0 = no symptoms; 1 = 1-10%; 2 = 11-25%; 3 = 26-50%; 4 = 51-75%; 5 = >75% of leaf area covered by disease symptoms. Then the disease index was calculated by using the formula:

$$\text{Per cent Disease Index (PDI)} = \frac{\text{sum all of individual ratings x 100}}{\text{Total number of leaves scored x maximum score}}$$

3.0 Results

3.1 Cultural and morphological characterization of pathogen

Colony colour, growth pattern and growth showed great diversity in all the two isolates based on the colony pigmentation. Two isolates differ in their the colony color was yellowish brown, and pale brown in colour. *Epicoccum sorghinum* produce coenocytic and hyaline hypha and produce intercalary chlamydospore. The isolate *Rhizoctonia bicornis* produced hyaline and septate mycelium (Fig 1).

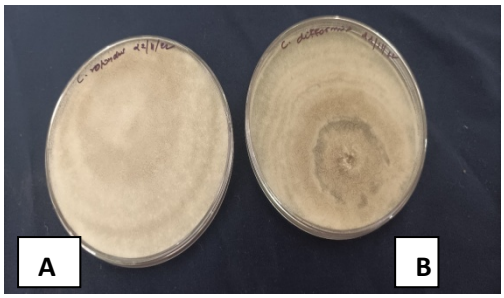

Figure 1. Cultural characters of fungal isolates A) *Rhizoctonia bicornis* B) *Epicoccum sorghinum*

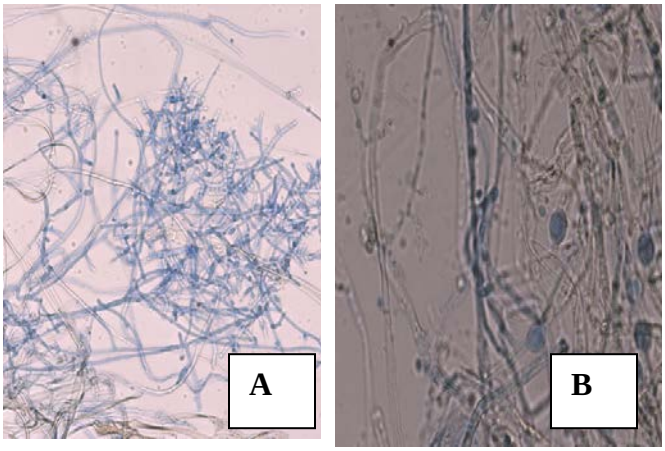

Fig 2. Spore images of fungal isolates A) *Rhizotonia bicornis* B) *Epicoccum sorghinum*

3.2 Molecular characterization of fungal isolates

PCR detection of *Cyprus* leaves infected with *Rhizotonia bicornis* and *Epicoccum sorghinum* were used for the extraction of DNA and subjected to PCR amplification using ITS 1 and ITS 4 primers to target the *Rhizotonia bicornis* and *Epicoccum sorghinum*. The PCR reaction yielded a product with an amplicon size of 350-400 bp (Fig 3)

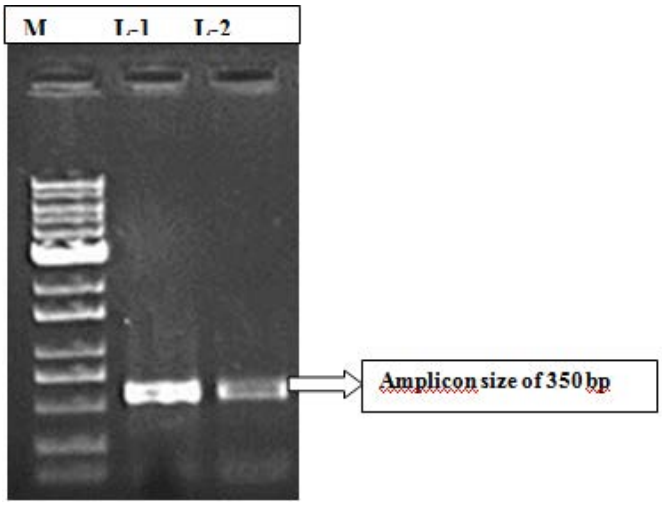

Figure 3.PCR amplification of fungus The amplicon size of the isolates amplified at 350 -400 bp

3.3Proving pathogenicity under *in vivo* condition

After10, 20 and 30 days inoculation (spore treatment) on pathogenicity of fungal species was recorded from each plants and random sample of leaves was taken to figure out the infection rate. The leaves were assessed using a disease rating scale based on the increase in disease area, and a percent disease index was calculated for various treatments. Test plants inoculated with *Rhizoctonia bicornis* (undiluted culture) has recorded high percent disease index values (32.36) than *Epicoccum sorghinum* (undiluted culture) (24.57) on 30 DAS Table1 and 2.

3.4 Seed Germination assay

Cyperus difformis seeds were treated with GA3 in order to break the dormancy. The treated seeds were inoculated with *Rhizotonia bicornis and Epicoccum sorghinum* and GA3 treated seeds alone taken as control. After 7th day of germination, the control recorded 96% germination whereas *Rhizotonia bicornis* (undiluted culture) *and Epicoccum sorghinum* (undiluted culture) treated seeds recorded the germination of 20 and 36 percent respectively. (Fig 4)

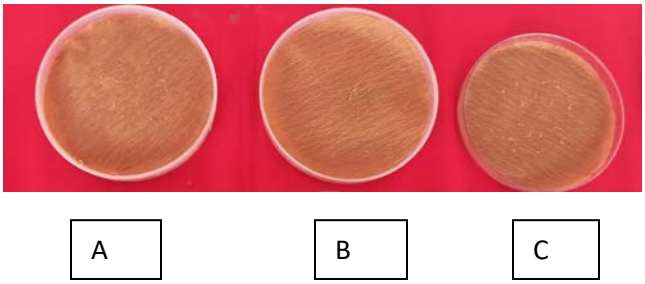

Figure 4. A. *Epicoccum sorghinum* B. *Rhizotonia bicornis* C. Control

Table1. Percentage Disease Index – *Rhizotonia bicornis*

Treatments *Rhizotonia bicornis*	**10 DAS**	**20 DAS**	**30 DAS**
T_1 - control	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T_2- Dilution of 2 x 10^{-8}/ml	3.31 (10.48)	5.64 (13.73)	10.76 (19.14)
T_3- Dilution of 3 x 10^{-6}/ml	3.83 (11.28)	7.05 (15.38)	13.52 (21.56)
T_4- Dilution of 4 x 10^{-4}/ml	5.30 (13.20)	8.19 (16.62)	17.17 (24.47)
T_5- Dilution of 5 x 10^{-2}/ml	7.81 (16.22)	10.26 (18.67)	23.78 (29.17)
T_6-Undiluted culture	12.51 (20.70)	18.66 (25.58)	32.36 (34.65)
Sed	**0.25**	**0.79**	**0.39**
CD(0.05)	**0.54**	**1.69**	**0.84**

Table 2. Percent disease index *Epicoccum sorghinum*

Treatments *Epicoccum sorghinum*	**10 DAS**	**20 DAS**	**30 DAS**
T_1 - control	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T_2- Dilution of 2 x 10^{-8}/ml	1.42 (6.84)	3.22 (10.32)	9.23 (17.68)
T_3- Dilution of 3 x 10^{-6}/ml	2.07 (8.27)	4.76 (12.60)	12.27 (20.49)
T_4- Dilution of 4 x 10^{-4}/ml	2.91 (9.82)	6.91 (15.23)	14.31 (22.22)
T_5- Dilution of 5 x 10^{-2}/ml	5.79 (13.91)	8.77 (17.22)	17.96 (25.06)
T_6-Undiluted culture	8.73 (17.18)	14.69 (22.52)	24.57 (29.70)
Sed	**0.12**	**0.23**	**0.32**
CD (0.05)	**0.26**	**0.50**	**0.70**

4.0 Discussion

4.1 Isolation and identification of mycoflora from *Cyperus difformis and Cyperus rotundus*

The two pathogens *Epicoccum sorghinum* and *Rhizotonia bicornis* were isolated from diseased leaf and root portions of variable flat sedge and yellow nut sedge.

4.2 Seed germination assay

In the present investigation, fungal pathogens isolated from the variable flat sedge and yellow nut sedge were assessed for their herbicidal activity against the *Cyperus difformis* germination.

The fungal isolates showed inhibition of weed seed germination at varying degrees. Further, these isolates were tested at the field level and found that the two isolates were caused symptoms such as necrosis, curling and wilting on the weeds. These results were supported by Dhanasekaran *et al*. (2012), who reported that actinobacterial isolates were found to inhibit the seed germination and shoot growth of *C. rotundus*.

Priyadharsini *et al.* (2013) screened actinobacterial isolates for herbicidal activity against *Cyperus rotundus*. Streptomyces sp. KA1-3 had shown excellent herbicidal activity against the weed. The culture filtrates severely affected seed germination and seedling growth of test weed.

4.3 Pathogenicity test

Cyperus difformis inoculated with *Rhizoctonia bicornis* (undiluted culture) showed maximum percent disease index values as compared with *Epicoccum sorghinum* (undiluted culture). This might be due to favorable environmental conditions like high humidity, low temperature and highly active spores of *Rhizotonia bicornis* (undiluted culture). This result is in accordance with Kumar *et al*.(2016) who concluded that after spore treatment with *Gibbago trianthemae* by using 5×10^4 spores/ ml inoculum, the pathogen infected the inoculated leaves and more symptoms appeared compared to other pathogens namely *Colletotrichum capsici*, *Alternaria alternata*, *Bipolaris maydis*, *Curvularia tuberculata* and *Curvularia lunata* Initially symptoms were pin-point, with brown margins. The lesions will increase in size at later stages and cause drying of weed.

5.0 Conclusion

There are immense possibilities for developing mycoherbicides using fungal pathogen to inhibit the growth of weed growth. In the present investigation, it was found that *Rhizoctonia bicornis* recorded higher percentage disease Index (32.36) than the *Epicoccum sorghinum* (24.57) on 30 DAS.

6.0 Reference

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