Biological Control of *Parthenium* Weeds using *Rhizopus oryzae*

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Abstract

The biological pollutant of recent times in India is a member of the sunflower family (*Compositae/Asteraceae*), botanically named as *Parthenium hysterophorus* Linn., and popularly called Congress Grass. The present study was carried out with plant pathogenic microorganisms isolated from the agricultural soil sample collected from Pudukkottai District, Tamilnadu, India. The isolated fungal colonies were confirmed as *Aspergillus niger*, *Penicillium chrysogenum* and *Rhizopus oryzae* based on microscopic and morphological characterization. The most efficient weeds germination control fungal isolates were confirmed by pot culture experiment were used for further study. The weeds germination control fungal strain was analyzed for molecular characterization. Based on the molecular characteristics and sequence alignments the isolated strains were conformed as *Rhizopus oryzae*. In this study, maximum enzyme productivity was recorded at pH range 5 in (0.086 U/ml/min) compared than other pH range. Lowest dehydrogenase productivity were noted in low pH range 3 (0.028 U/ml/min) and Highest pH range 7 (0.36 U/ml/min). In this study, maximum enzyme activity was recorded at temperature range 25°C (0.18 U/ml/min) compared than other temperature. The dehydrogenase activity was optimized using different inoculum concentration range from 20 – 100 mg/g. The maximum level of enzyme activity was present in 100.

Key words : *Parthenium* Weeds, *Parthenium hysterophorus, Rhizopus oryzae,* Biological Control

Introduction

Parthenium weed has now established itself almost throughout the Indian region and its high incidence in several areas has alarmed people to the extent that attracted Government's concern for its control. At several places in India efforts are, therefore a foot to discover suitable measures of its effective control as a short term target and to meet the long term objective of its eradication through biological means. But, despite a number of publications and discussions that have already been made on the control of this species in various parts of the country eversince 1956, it still continues to sprout at a furious pace and to invade the either to unexposed areas of this region. *Parthenium hysterophorus* invades disturbed land, including roadsides. It infests pastures and farmland, causing often disastrous loss of yield, as reflected in common names such as famine weed. In some areas, heavy outbreaks have been ubiquitous, affecting livestock and crop production, and human health. As an invader it first appeared as a contaminant in imported wheat. The plant produces Allelopathic chemicals that suppress crop and pasture plants, and allergens that affect humans and livestock. It also frequently causes pollen allergies. *Parthenium* plant contains chemicals, like parthenin, hysterin, hymenin, and ambrosin, and due to the presence of these chemicals, the weed exerts strong allelopathic effects on different crops (Gunaseelan, 1998). *Parthenium* weed is toxic to animals causing dermatitis with pronounced skin lesions on various animals including horses and cattles (Narasimhan *et al.*, 1977). Singh, (1997) considered use of biocontrol agents (insects and fungal pathogens) and exploitation of competitive plants (allelopathy), the most economic and practical way of managing *Parthenium*. In the last three to four decades, a great deal of emphasis has been given to control *Parthenium* through various biocontrol agents like microbial pathogens, insects, and botanicals (Ray and Gour, 2012).

MATERIALS AND METHODS

Sample collection

The soil samples were collected from agricultural field on area of Pudukkottai District, Tamil nadu, India. The collected soil samples kept were in sterile polythene bags and stored in refrigerator at 4˚C for further studies.

Isolation and Identification fungi from soil

Serial dilution plate technique was followed for the isolation of fungi using Potato dextrose agar medium.1 gm of soil sample was serially diluted upto 10^{-1} to 10^{-7} dilutions. The 10^{-2} to 10^{-4} diluted sample were pouried initially on potato dextrose agar(PDA) plates separately. The plates were incubated at 28˚C for 72 hours. The pure isolated fungal culture was identified based on cultural, morphological and molecular characterisation.

Selection of most efficient weed germination control fungal isolates

The most efficient seeds germination control fungal isolates were confirmed by pot culture experiment. The dominant fungal species was mixed with sterile soil and a control also maintained without inoculum then the *Parthenium* seeds were inoculated both treatment pot. The treatment pots were maintained in proper aeration, lighting and water.

Molecular characterization (Altschul *et al.*, 1997)

The most efficient fungal strain was characterised molecular level. The below steps were followed such as DNA Extraction**,** Polymerase Chain Reaction (PCR) **a**nd Composition of the Taq Master Mix**.**

Primer Details

Primer Name	Sequence Details	Number of Base
ITS1	TCCGTAGGTGAACCTGCGG	19
ITS4	TCCTCCGCTTATTGATATGC	20

Add 5 µL of isolated DNA in 20 µL of PCR reaction solution (1.5 µL of Forward Prime and Riverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). Perform PCR using the following thermal cycling conditions such as Denaturation, Annealing and Extension.

Purification of PCR Production

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).The PCR product was sequenced using the ITS1/ITS4 primers. Sequencing reactions were performed using a ABI PRISM Big Dye TM Terminator Cycle Sequencing Kits with Ampli Taq DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing protocol Single-pass sequencing was performed on each template using below 18 s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730 xl sequencer (Applied Biosystems).

Optimization of weeds germination control

The soil dehydrogenase is an indicator of soil quality and microbial activity. In the present study weed germination was controlled using pathogenic fungi. The isolated *Rhizopus oryzae* control weeds germination at high level so this was used for optimization study such as various pH, temperature and Inoculum Concentration.

Enzyme assay

Dehydrogenase activity was determined according to the modified method of Casida *et al.*, (1964), in which TTC (2,3,5-triphenyl tetrazolium chloride) serves as a terminal acceptor of protons and electrons from organic compounds being oxidized. 6 g of soil was placed in 50 ml conical flasks and 1 ml 3% aqueous solution of TTC, 120 mg $CaCO_3$ and 2-4 ml distilled water were added. The flasks were tightly stoppered and swirled for a few seconds. A small amount of free liquid was present at the surface of the soil. The soil samples were incubated for 20 h at 30°C. The 2,3,5-triphenyl formazan (TPF) product was extracted with 94% ethanol for 60 min in the dark at 20°C, and was immediately filtered through a filter paper and assayed at 485 nm.

Statistical analysis

The results obtained in the present investigation were subject to statistical analysis like Mean ($\bar{x}$) and Standard Deviation (SD) by Zar (1984).

RESULTS

In this study different fungal species were isolated from the agriculture field soil sample by using pour plate method. Among this study three different fungi from were *Aspergillus niger, Penicillium chrysogenum, and Rhizopus oryzae* isolated based on colony morphology and morphological characteristics and illustrated in Table. 1.

The three fungal isolates were analysed for weeds germination control ability using pot culture experiment the investigated results were presented in Fig. 1. Among the three isolates *Rhizopus oryzae* have maximum level of control on the of *Parthenium* weeds germination. The isolate was characterized by molecular level and used for optimization of weed germination control ability.

Table-1 Isolation of Fungi from Soil

Isolated colonies	Colony Morphology
ISF1	White to Black, fast growing; conidia in balls.
ISF2	Greenish or blue green colonies; Conidia in long chains on repeatedly branched condiophore resembling a brushlike head (Penicillus).
ISF3	White to Green, fast growing; Conidia in balls.

ISF1 – *Aspergillus niger;* ISF2 – *Penicillium chrysogenum*; ISF3 – *Rhizopus oryzae*

Fig. - 1 Selection of weeds germination control fungi

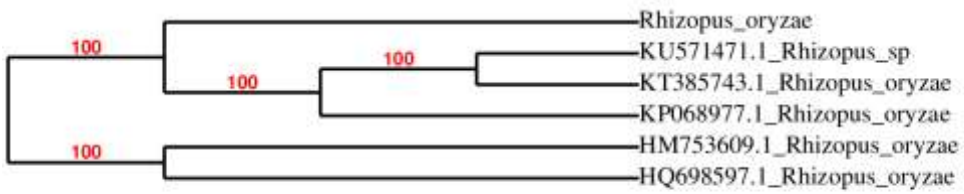
Rhizopus oryzae **Control**

The weeds germination control fungal species strain was analyzed for molecular characterization. In this study, DNA fragments ranging from 1 bp to 500 bp were separated on a 1.5% agarose gel. Extracted genomic DNA containing 18S rRNA from each isolate was amplified for gene sequencing using PCR. Partial sequencing of the genomic DNA of the test isolates revealed that the 18S rRNA portion contained base pairs is 380. The present study based on partial 18S rRNA sequencing of the sequences obtained from the effective isolates when subjected to BLAST (Fig. – 2). The dominant fungal alignment sequencing showed sequence similarities 99% with *Rhizopus oryzae*. The sequence has been deposited at GenBank Bethesda, MD, USA. After the alignment, the tree building option can be activated using Bioedit Software. The tree viewing software neighbour joining plot is used to generate a cladogram the bacterial isolates as shown in Fig. - 3. Based on the molecular characteristics and sequence alignments the isolated strain were conformed as *Rhizopus oryzae*.

Fig-2 Aligned Sequence of ISF3

AGACCTATCTGGGGTTTGATCGATGCCACTCCTGGTTTCAGGAGCACCCTTCATAAAAAC
CTAGAAATTCAGTATTATAAAGTTTAATAAAAAACAACTTTTAACAATGGATCTCTTGGTT
CTCGCATCGATGAAGAACGTAGCAAAGTGCGATAACTAGTGTGAATTGCATATTCAGTG
AATCATCGAGTCTTTGAACGCAGCTTGCACTCTATGGTTTTTCTATAGAGTACGCCTGCTT
CAGTATCATCACAAACCCACACATAACATTTGTTTATGTGGTAATGGGTCGCATCGCTGT
TTTATTACAGTGAGCACCTAAAATGTGTGTGATTTTCTGTCTGGCTTGCTAGGCAGGAAT
ATTACGCTGGTCTCAGGATCTTTTTCTTTGGTTCGCCCAGGAAGTAAAGTACAAGAGTAT
AATCCAGCAACTTTCAAACTATGATCTGAAGTCAGGTGGGATTACCCGCTGAACTTAAGC
ATATCAATAAGCGGAGGAA

Fig.-3 Tree Viewing – Neighbor Joining Plot



The soil dehydrogenase is an indicator of soil quality and microbial activity. Out of 3 isolation the *Rhizopus oryzae,* which showed maxinium weed germination was control used for optimization study such as various pH, temperature and Inoculum Concentration (Table 2,3 and 4). In this study, maximum enzyme productivity was recorded at pH range 5 in (0.086 U/ml/min) compared than other pH range. Lowest dehydrogenase productivity were noted in low pH range 3 (0.028 U/ml/min) and Highest pH range 7 (0.36 U/ml/min). The dehydrogenase activity was optimized using different temperature range from 25 °C to 45 °C. The maximum level of enzyme activity was present in 100 mg/g inoculated experiment compare then other treatments.

Table – 2 Dehydrogenase Activity of *Rhizopus oryzae* at Various pH

S. No.	pH range	Enzyme activity IU/ml/min
1	3	0.028 ± 0.002
2	4	0.068 ± 0.004
3	5	0.086 ± 0.002
4	6	0.072 ± 0.01
5	7	0.036 ± 0.004

Values are represented as Mean ± Standard deviation

Table – 3 Dehydrogenase Activity of *Rhizopus oryzae* at Various Temperature

S. No.	Temperature (ºC)	Enzyme activity IU/ml/min
1	25	0.18 ± 0.02
2	30	0.06 ± 0.002
3	35	0.08 ± 0.005
4	40	0.05 ± 0.004
5	45	0.02 ± 0.001

Values are represented as Mean ± Standard deviation

Table - 4 Dehydrogenase Activity of *Rhizopus oryzae* at Inoculum Concentration

S. No.	Inoculums Concentration (mg/g)	Enzyme activity IU/ml/min
1	20	0.06 ± 0.005
2	40	0.09 ± 0.002
3	60	0.10 ± 0.05
4	80	0.12 ± 0.004
5	100	0.14 ± 0.002

Values are represented as Mean ± Standard deviation

DISCUSSION

Parthenium is considered as the number one dangerous terrestrial weed because of its harmful effects both to humans and to biodiversity. From the soil sample, more number of the fungal colonies was isolated and identified, dominant species were used for the *Parthenium* weeds germination control. Screening of *Parthenium* weeds germination control was analysed. Dominant species among this study *Rhizopus oryzae* produced highly cleared weeds germination compared than other.

Saxena and Kumar, (2010) worked on the mycoherbicidal potential of *Alternaria alternata* ITCC (LC#508) in northern India to control *Parthenium* weed and reported 50% damage of plants *in vitro* detached leaf and whole plant bioassay at 96 hours after treatment at a concentration of 1×10^6 spores/mL.

Sclerotium rolfsii (teleomorph: *Athelia rolfsii*) incites a severe collar rot disease on *Parthenium* (Pandey *et al.,* 1998; Shukla and Pandey, 2006). Kauraw *et al.* (1997) reported *Fusarium pallidoroseum,* on *Parthenium* from Jabalpur. It was found to reduce seed germination, seedling vigour and height of plant, number of branches, and number of flowers and reported as a potential biocontrol agent for *Parthenium* management.

This behaviour was also observed by Nahas, (1988) for R. *oligosporu*s dehydrogenase and by Ferrer *et al.,* (2000) for *R. oryzae* dehydrogenase (Wahab, 2005). In the present study 18S rRNA gene sequences of the isolates with the reference 18S rRNA gene sequences confirmed the identity of the isolates already made based on morphological and cultural characterization. Based on the molecular characteristics and sequence alignments the isolated strains were conformed as *Rhizopus oryzae.*

Dehydrogenase is an enzyme that occurs in all viable microbial cells. These enzymes function as a measurement of the metabolic state of soil microorganisms (Watts *et al.,* 2010). Dehydrogenase activity (DHA) is one of the most adequate, important and one of the most sensitive bioindicators, relating to soil fertility (Wolinska and Stepniewska, 2012). According to Pettersson and Baath (2003), temperature is one of the most important environmental factors affecting soil bacterial community. In the present study the dehydrogenase activity was optimized using different temperature range from 25 °C to 45 °C maximum enzyme activity was recorded at temperature range 25 (0.18 U/ml/min) compared than other temperature.

CONCLUSION

The noxious *P. hysterophorus* grows in a wide variety of habitats and causes changes in above ground vegetation as well as in below ground soil nutrients. It is capable of out-competing native and non native palatable plants that are important to livestock. Furthermore, the changes in vegetation and soil nutrients could lead to ultimate changes in other trophic levels and alter the function of the ecosystem. Appropriate methods for the management of *P. hysterophorus* are necessary to avoid potential threats to biodiversity and economic losses. The fungus *Rhizopus oryzae* appears as small, circular, white powdery spots on the surface of leaves and spreads over the entire lamina on both the surfaces giving a powdery appearance to the plant. Severe infection leads to defoliation. It was found to reduce seed germination, seedling vigour and height of plant, number of branches, and number of flowers and reported as a potential biocontrol agent for *Parthenium* management.

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