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Research Article

BIOCONTROL OF *PARTHENIUM HYSTEROPHORUS* THROUGH DIFFERENT FUNGAL ISOLATES

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Abstract- The biological control through various isolated strains of fungi from naturally infected Parthenium plants collected from different locations of Punjab. Total nine fungal species were isolated *viz*., three *Alternaria* sp., four *Fusarium* sp., *Cladosporium* sp. and *Cephalosporium* sp. Isolated strain's pathogenicity was tested individually as well as in combination (both in lab and field conditions) against Parthenium with different control plants. Out of all the isolated strains, *Fusarium equiseti* followed by *Fusarium solani* and *Cephalosporium* sp. showed defoliation of leaves in laboratory conditions whereas less effective in the field conditions. In another approach, disc plate technique was used to investigate the spore germination time and fungus penetration route with a Scanning Electron Microscope. In addition, methods for fungal inoculum development with silica beads, wheat bran, CM-cellulose and Tween80 were also explored for spraying of fungal cultures in the field.

Keywords- *Biocontrol, Fungal isolates, Host specificity, Parthenium*

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Introduction

Parthenium hysterophorus L., member of the family Asteracae (Compositae) and in India commonly known as carrot weed or congress grass. The origin of *Parthenium* is considered to be Mexico, America, Trinidad and Argentina [1]. Within the last 100 years, it has found its way to Africa, Australia and Asia. In Australia and India *Parthenium* has achieved the status of "worst weed" [2]. *Parthenium* is a poisonous, pernicious, problematic, allergic and aggressive weed posing a serious threat to human beings and livestock. *Parthenium* weed causes many different types of diseases or allergies such as asthama, dermatitis, eczema, nasal-dermal and naso-bronchial diseases [3]. In addition to all these worse effects, this weed lead to obstruction in the orchards and common pathways and also diminish the aesthetic values of residential colonies, parks and gardens. In the last few years *Parthenium* has widely spread to the road sides and fallow lands under the forest cover to most of the valleys and hilly areas of Himachal Pradesh that solemnly posing the threat to human, animal and plant biodiversity as well as animal grazing areas. The invasive capacity and allelopathic properties have rendered *P. hysterophorus* with the potential to disrupt the natural ecosystems [4]. There are two approaches for biocontrol of *Parthenium*. Firstly, maintenance of naturally occurring biodiversity and the other is planting selected plant species in target areas. Elimination of *P. hysterophorus* through chemical herbicides, burning and eucalyptus oil opted from many years with very less effects [3,4}. Similarly, biological control with stem-galling moth, leaf-feeding beetle, stem-boring weevil and different types of fungal isolates were adopted worldwide with variable degrees of success [3-5]. The present work has been taken up for detailed study of biological control of *P. hysterophorus* with different species of fungal isolates. Naturally infected *Parthenium* plants collected from different regions of Punjab for isolation and identification of different fungi. Further severity of isolated fungal strain on *P. hysterophorus* plant was explored in lab (Leaf bioassay method and Disc plate technique) and field conditions. The effective fungal isolates identified through ITS primers and the fungal route of infection in the *Parthenium* plant revealed by Scanning Electron Microscopy.

Material and Methods

Isolation of fungus

The different fungus was isolated from infected *Parthenium* plant collected from different location of Punjab. The infected *Parthenuim* plant leaves, flowers and stem were washed with sterilize distilled water in order to remove adherent soil particles and then surface sterilized with 70% ethanol for 1-2 minutes and again rinsed in sterile distilled water for 1-2 minutes and then transferred to CDA media (Sucrose: 3g, $NaNO_3$: 0.2g, K_2HPO_4: 0.1g, KCl: 0.05g, $MgSO_4.7H_2O$: 0.05g, $FeSO_4.7H_2O$: 0.001g, pH: 5.5-6.0, ddH_2O: 100 ml) and Malt extract (Malt extract: 2g, Agar: 2g, ddH_2O:100 ml pH:5.8-6.0). Petriplates were incubated at 28-30°C for 5-7 days and then fungus was subcultured and maintained on both CDA and Malt extract media. The fungal incoulum was prepared by both submerged with CDA broth and solid-state fermentation with wheat bran method.

Identification of fungus

Isolated different fungus was identified first by microscopically (on the basis of fruity bodies) as well as by macroscopic features (colour of spores, growth pattern) and followed by ITS primer sequencing.

DNA was isolated from the mycelia of fungus by Sharma *et al*., [6] CTAB/NaCl method. ITS1 and ITS4 Primer sequence were used for the identification of fungi. The PCR product were resolved on 1.2% agarose having 0.5 µg/ml ethidium bromide (10 mg/ml) and prepared with 1X TBE buffer. The gels were visualized and photographed using Gel Documentation system (Syngene). DNA fragment of desired length obtained in PCR reaction were extracted from agarose gel by NucleoSpin®Gel and PCR clean-up kit by Macherey-Nagel and then sent for custom sequencing service at Chromus Biotech Bangalore.

Nucleotides Sequence

The resulting ITS sequences have been deposited in the GenBank under the accession numbers KT277307 (FPP), KT277308 (FCW).

Pathogenicity Test

The pathogenicity was determined both in lab and field conditions by using different methods discussed as follows:

***Parthenium* Leaf bio assay test**

In leaf bioassay test different parts of *Parthenium* plants were sterilized with 1% Sodium hypochlorite. Filter sheets were placed in the trays and moisture the filter paper properly with sterilize distilled water. Then, different parts of *Parthenium* plants including leaves, flowers and stems were placed in the trays and sprayed with different isolated fungus, grown on CDA broth for 3 days and approximate $1x10^2$ spores were sprayed over per leaf. Trays were covered by transparent polythene for maintaining moisture content as well as to avoid the contamination and kept it for 3 to 4 days at room temperature (25±5°C).

***Parthenium* Disc plate method**

Disc plate technique was also used in which route of fungus infection (with SEM) as well as spore germination and its pathogenicity were observed. The 2.0 mm discs from the 5 days old culture of each fungal strain were removed aseptically and then placed on the lid of the sterilized petri plates approximately 10-12 discs per plate and on the lowerside of the petriplates different parts of *Parthenium* plants includes leaves, flowers and stems (sterilized by 70% ethanol) on wet filter paper. After that petri plates were incubated at 28-30°C for 24 to 36 hrs. The dispersal of spores after 24 hrs from upper lid to leaves were observed by using the cellotape method: The sticky side of the cellotape was put over the leaves and pressed it carefully and attached spores over the cellotape then fixed over the glass slide and observed under the microscope. After 36 hrs of spore dispersal on leaves through disc plate technique, the leaves were fixed with Methanol-Ethanol fixation Protocol of Talbot and White [7] for Scanning Electron Microscopy.

Field condition for Fungal spray on the *Parthenium* plants grown inside the small Polyhouses

The *Parthenium* plants were grown in the two rows with control plants. The iron cage was used which were covered with plastic polythene sheet in order to maintained the humidity condition, so that proper growth of sprayed fungal spores and to avoid spread of the fungus to nearby vegetation. Isolated strains pathogenicity was tested individually as well as in combination against *Parthenium* plants along with different control plants in natural environmental conditions. Before fungal cultures sprayed over the *Parthenium* plants different fungal spray methods adopted *viz*., Wheat Bran, Silica beads, CM-cellulose, Tween 80 were mixed with fungal cultures which help for proper attachment of fungal spores and allows the growth of fungi over the *Parthenium* plants.

Evaluation of Host Specificity

Host specificity of the *F. equiseti, F. solani* was tested against 5 plant species belonging to the *Solanaceae, Brassicaceae, Poaceae, Asteaceae* and *Brassicaceae*. The crop plants were selected on the basis of their economic importance and growth. The plants tested for their host specificity were *Lycopersicon esculentum, Solanum tuberosum, Triticum aestivum, Helianthus annuus* and *Brassica campestris*. The crop plants were grown in rows among the *Parthenium* plants and sprayed with fungal cultures. Growth of *F. equiseti, F. solani* was monitored daily for 2 weeks for studying the symptoms.

Results and Discussion

Isolation and Identification of fungus

On the basis of morphological and microscopic features total nine fungi (4 *Fusarium* species, 1 *Cephalosporium* species, 1 *Cladosporium* species, 3 *Alterneria* species) have been isolated from the infected *Parthenium* plants from Sri Fatehgarh Sahib and Bathinda region of Punjab on CDA and MEA medium. Similarly, Jeyalakshmi *et al*. [8] during survey at Coimbatore recorded 21 pathogenic species on *Parthenium* and reported *Lasidiplodia theobromee*, a host specific blight which causes severe damage to *Parthenium* at 15-30 days stage. Author also reported *Oidium partheni* which also caused severe damage on *Parthenium* at flowering stage.

The species of *Alternaria, Drechslera Colletotrichum, Phoma, Curvularia, Acremonium, Cladosporium, Myrothecium*, causes leaf spots on the *Parthenium* whereas some species of *Aspergillus, Chaetomium,* and *Rhizopus* were found to associate with floral parts and seeds of the weed. Though, the researchers did not authenticate the pathogenicity of the fungal isolates.

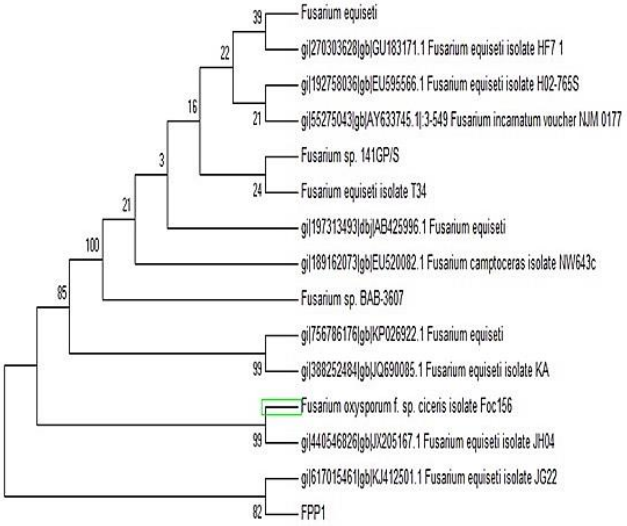

Fig-1 Evolutionary relationships of *Fusarium equiseti*

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 10.22993688 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 544 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

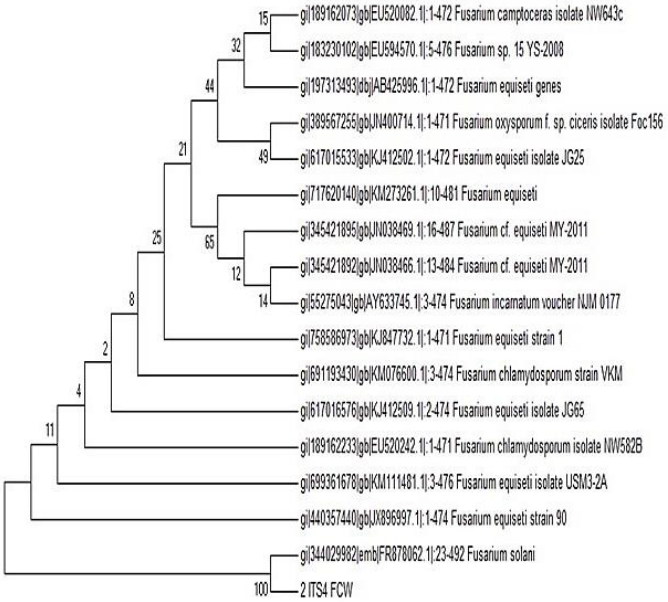

Fig-2 Evolutionary relationships of *Fusarium solani*

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.07139003 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 449 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Molecular identification of fungal strains

The isolated DNA from fungal strains was amplified by PCR using the primers ITS1 and ITS4. The resulting amplified PCR product showed the band size of 549bp (FPP) and 474bp (FCW) in the agarose gel electrophoresis run with 1000 bp ladder. Specific bands of FPP and FCW were eluted and then sent for custom sequencing service Chromus Biotech Bangalore. Two novel *Fusarium* species were identified as *Fusarium equiseti* (FPP) with Accession no. KT277307 and *Fusarium solani* (FCW) with Accession no. KT277308. The sequence analysis was done using NCBI database by employing BlastN algorithm. The sequence of FPP was used to BLAST against GenBank nucleotide database at NCBI and found maximum sequence identity of 82% with *Fusarium equiseti* isolate JG22 [Fig-1] and 100% sequence identity with *Fusraium solani* [Fig-2]. In the present study *Fusarium equiseti* (KT277307) which were isolated from infected *Parthenium* plant from Sri Fatehgarh Sahib (Punjab) reported first time and inhibited *Parthenium* weed significantly. Whereas, *Fusarium solani* (KT277307) was already reported by other authors earlier and explored for the *Parthenium* biocontrol [9-11].

***Parthenium* Leaf bio-assay**

The *Parthenium* leaves bioassay was performed with one-week old broth cultures of different fungal isolates. After 24 hrs fungal spores germinated over the *Parthenium* plant parts and maximum defoliation of leaves and flowers take place after 72 hrs at 25±5ºC with all fungal isolates but intense growth of fungi and damage was observed with the *Fusarium equiseti* followed by *Fusarium solani*, *Fusarium* species and *Cephlosporium* sp. [Fig-3]. The fungal strains grew on the stalk of the weed degrading its tissues and killing it eventually in *in-vitro* condition. Previously, Pandey *et al*. [12] assessed the pathogenicity of 19 fungi and concluded that *C. gleosporioides, Alternaria alternata. A. dianthi, A. macrosporus, Myrothecium roridum, Fusarium oxysporum, F. nioniliforme, Phoma herbamm* and *Bipolaris* sp. were able to cause considerable damage to the weed under laboratory conditions. Further in 1992 Pandey *et al*. [13] evaluated *Fusarium oxysporum* and *R. solani* and reported both the pathogens were found to be highly effective, causing severe infection and significant damage to the weed in laboratory conditions. In the present investigation the fungal attacks more on the flowers followed by young leaves as compared to the older leaves and stem parts.

Fig-3 (A) *Fusarium* species (B) *Fusarium solani* (C) *Fusarium equiseti* (D) *Cephalosporium* sp. infection on *Parthenium* leaves and flowers in-vitro conditions at 25±5°C.

Similar resistance of older *Parthenium* leaves was reported by Parker *et al*.[14] with *P. abrupt var. partheniicola* pathogen in which younger leaves were the most susceptible to infection. This considerable resistance to fungal infection might be associated with the surface texture of the leaves such as hairs and cuticle on the leaves that reduced the ability of the pathogen to penetrate to thicker tough cell walls of the plants [15]. Further in 2015, Kaur *et al*. [16] reported the herbicidal potential of cell free culture filtrate of *A. macrospora* MKP1 on the *Parthenium* leaves and seeds and a significant damage was exhibited by the cultural filtrate of pathogen to the *Parthenium* leaves and seeds. Recently, Biocontrol efficacy of the *Cercospora partheniphila* suspension was examined on young plants of *Parthenium* under injured, uninjured, covered and uncovered conditions and reported that the disease progressed with the increase of the incubation period and ultimately resulting in rotting of the complete plants within 22 days of artificial inoculation of the host [17].

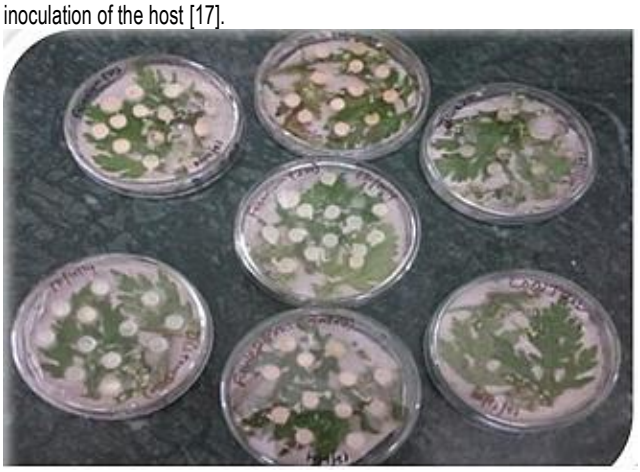

Fig-4 Disc plate technique for spore released from the *Fusarium equiseti* culture discs and penetration into the *Parthenium* leaves.

Field condition for Fungal spray on the *Parthenium* plants

The pathogenicity of isolated fungal species also investigated inside small polyhouses in the field conditions. Results indicated that *F. equiseti, F. solani,* and *Fusarium* species showing defoliation of leaves followed by flowers only in the younger *Parthenium* plants than the older plants while other fungal culture of *Cephalosporium* remains unaffected in the field conditions after 15 days of spray. The *F. equiseti* effectively suppressed the growth of flowers which ultimately decreased the seed viability. The most of the fungal strains were not affected in field conditions because of day and night temperature variations at the Himachal Pradesh region which were not favorable for the growth of fungus. Pandey *et al*. [18] reported *Sclerotium rolfsii* that controlled the 90-95% and 35-40% mortality of *Parthenium* seedlings in the greenhouse and field trials, respectively. Similarly, application of *P. abrupt var. partheniicola* urediniospores to the rosette and flowering growth stages resulted in significant reduction in height of the *Parthenium* plants as compared to the mature stage [15]. In controlled environment the fungal culture filtrates of *Alternana alternata, Penicillium granulatum, Trichoderma viride, Fusarium oxysporum* and *Curvularia lunata* investigated on seed germination, plant vegetative and reproductive growth. Among all only fungal culture filtrates of *Fusarium oxysporum* resulted in 63.3% germination reduction and 0.64 mg/plant dry weight accumulation in 50% concentration of culture filtrate [19]. Beside these, Patel and Patel [20], reported *Fusarium oxysporum* and *Fusarium solani* which showed enhanced mycoherbicidal potential against *Parthenium* seedlings in sandy soil at 25°C and 70% moisture and found host specific against *Parthenium* weed.

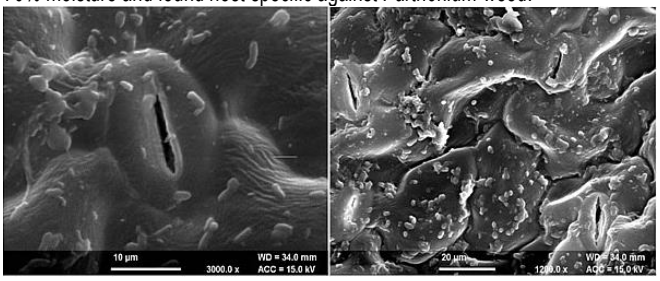

Fig- 5 A scanning micrograph of fungal structures of *Fusarium equiseti* on the leaf surface and spore's penetration through the stomata of leaf of Parthenium.

In the present investigation the tested plant species *Lycopersicon esculentum, Solanum tuberosum, Triticum aestivum, Helianthus annuus* and *Brassica campestris* were found resistance to *F. equiseti.* Whereas *F. solani* infected the leaves of Solanum tuberosum and minor symptoms occurred on the potato plant.

Therefore, *F. equiseti* clearly indicated as host specific in nature against the *Parthenium* and considered as suitable biological control agent against *Parthenium* weed biocontrol.

Disc plate technique indicated that fungal spores were released within 24 hrs on the surface of the leaves which was examined under the microscope using cellotype method [Fig-4]. Further leaves were fixed with methanol-ethanol for SEM analysis. The SEM showed that occurrence of the infection was through stomata and direct penetration. The spores of *F. equiseti* grew in the intercellular spaces of mesophyll tissue and damaged the cells and cell wall of the *Parthenium* [Fig-5].

The importance of fungal-substratum binding has rarely been critically investigated and no fungal adhesive compound that mediates attachment to *Parthenium* plants has been characterized. Fungal growth also collapsed when there was depletion of nutrients for spore growth. Therefore, the present study reported the fungal cultures sprayed over the *Parthenium* plants with different fungal spray methods adopted with Wheat bran, Silica beads, CM-cellulose and Tween 80. Adhesive substrates were mixed with fungal cultures that enhanced the proper attachment of fungal spores and allow the growth of fungi over the *Parthenium* plants. The fungal-plant attachment is complex because adhesion occurs at different stages of fungal morphogenesis; adhesion can be associated conidia, germlings, appressoria, zoospores and their cysts and infection cushions [21]. Among all the tested substrates wheat bran showed proper adhesion of fungal structures on the *Parthenium* leaves and flowers in the small polyhouses.

Conclusion

The present work aimed for the potential biocontrol agent which might be highly virulent, host-specific and emerges as an effective pathogen against the *Parthenium*. Among all the fungal isolates *F. equiseti* has the ability to suppress the weed in natural condition and also host specific in nature. *F. equiseti* sporulate within a week on the wheat bran which is cheap and simple culture media and explore for the mass production of the spores in a short duration. The host specificity of different economical important crops should be exploring more before *F. equiseti* used as biocontrol agent in the open fields.

Application of research: Eradication of *Parthenium* "a noxious weed" with host specific fungal isolates.

Research Category: Plant Pathology

Abbreviations: CM-cellulose: Carboxymethyl-cellulose; CDA: Czapek Dox Agar; MEA: Malt Extract Agar; ITS primer: Internal Transcribed Spacer; SEM: Scanning electron microscope

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University: Adesh University, Bathinda, 151109, Punjab, India
Research project name or number: MSc. Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Punjab

Cultivar / Variety / Breed name: *Parthenium hysterophorus* L.

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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