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Investigation on Biocontrol Efficacy of Fungal Metabolites Produced by *Alternaria alternata* against Invasive *Parthenium* Weed

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One of the most aggressive invasive weeds, *Parthenium hysterophorus* (Asteraceae) is generating serious difficulties for the environment, economy, and livestock in India and other parts of the world. The present study was conducted to isolate and identify the fungal pathogens from the leaves of *Parthenium* weed and evaluate their cultural filtrates against the growth of the weed. *Alternaria alternata* was isolated from the infected *Parthenium* leaves by using standard isolation techniques utilizing potato dextrose agar (PDA), during an extensive search for natural enemies of *P. hysterophorus*. After applying Koch's postulates and leaf bioassay, it was discovered that *A. alternata* was found pathogenic and can be used as biocontrol agent of the weed. Seed germination bioassay was performed to check the herbicidal potential of *A. alternata* metabolites against *Parthenium* plant. The fungal pathogen's metabolites caused severe harm to the leaves and seeds of *P. hysterophorus*. This research aims to explore the biological control efficacy of isolated fungus *A. alternata* to inhibit *Parthenium* growth.

Keywords: Biocontrol agent; herbicidal; invasive; livestock; *Parthenium*; postulates.

Parthenium hysterophorus, commonly called Congress grass or *Parthenium* weed is a herbaceous annual plant that belongs to the Asteraceae family. It originates from the Americas, particularly South and Central America, and has become a widespread invasive species in various regions across the globe, notably in Asia, Africa, and Australia[12]. Its rapid proliferation and ability to adapt to diverse environmental conditions have contributed to its status as one of the most problematic invasive plants. *P. hysterophorus* has spread unintentionally to new regions through contaminated agricultural produce, machinery, and vehicles. Its establishment and spread are facilitated

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by its capacity to colonize disturbed habitats like roadsides, agricultural lands, wastelands, and riverbanks[7]. *P. hysterophorus* is found in over 40 countries globally, posing significant challenges to agriculture, human health, and biodiversity[6]. A single *Parthenium* plant can produce thousands of seeds, contributing to its rapid spread and colonization. Its aggressive growth outcompetes native vegetation for resources such as water, nutrients, and sunlight, leading to decreased biodiversity and habitat degradation. Additionally, *P. hysterophorus* produces allelopathic compounds that suppress the germination and development of other plant species, exacerbating its impact on native flora[19]. *P. hysterophorus* poses significant economic and agricultural challenges. It infests agricultural fields, reducing crop yields and quality while contaminating fodder, resulting in livestock poisoning[21]. *P. hysterophorus* also poses health risks to humans and animals, causing allergic reactions and respiratory problems upon contact with or inhaling its pollen and leaf particles[20]. Symptoms such as skin rashes, asthma, and fever are common among individuals living in infested areas. Efforts to manage the spread of *P. hysterophorus* involve integrated approaches, including biological, chemical, and cultural methods. However, thus far no single method has been shown to be effective due to one or more drawbacks of each method. As a result, the focus of weed control needs to be switched toward non-chemical alternatives. Using traditional biological methods to control weeds is less expensive, more effective, and pollution-free[3]. There is increasing interest in eco-friendly alternatives due to growing social concerns about the adverse effects of chemical pesticides on livestock and the environment. Biological control agents, such as insects and pathogens specific to *P. hysterophorus*, have been introduced in some regions to suppress its growth[18]. Insects like leaf-feeding beetles, stem-boring weevils, stem-galling moths, and different fungal isolates like rust fungi were adopted worldwide with variable degrees of success. Fungi that have the potential to be utilized as bioherbicides or mycoherbicides cause a range of weed illnesses. For example, it was found out that the fungal metabolites of *Aspergillus niger* reduced *Parthenium* germination by 89% over control[5].

During the survey, naturally infected *Parthenium* plants were collected from different regions of Maharishi Markandeshwar University (Ambala) for isolation and identification of different fungi. Several different fungal strains were isolated from infected *Parthenium* plant leaves. The fungal strains were cultured under controlled conditions and checked for their biocontrol potential against *Parthenium* weed. It was observed that *A. alternata* showed remarkable results as compared to other fungal strains. As a result, this study was conducted using both leaf bioassay and seed germination bioassay to check the herbicidal potential of *A. alternata* against *Parthenium* weed in in-vitro conditions. Different growth parameters were examined in seed germination bioassay and the results showed that isolated fungal pathogen, *A. alternata* inhibited the growth of *Parthenium* plant.

MATERIALS AND METHODS

Isolation and maintenance of fungus

Infected leaves of *Parthenium* plant were gathered from nearby areas of Maharishi Markandeshwar (Deemed to be University), Ambala, Haryana. The surface sterilization of collected samples of *Parthenium* leaves was done by first washing under running tap water. To remove dirt and debris, plant leaves were sorted again and thoroughly rinsed using autoclaved distilled water. Following rinsing, the surface was disinfected with 70% ethanol for one minute and mercuric chloride (0.1%) for five minutes[2]. The plant leaves were thoroughly cleaned 4-5 times with autoclaved distilled water to eliminate any surface chemicals. Subsequently, the leaves were placed on autoclaved tissue paper and left for 30 minutes. Then, after proper drying and sterilization of leaves, they were used to isolate the fungus[17]. Following proper drying, the surface sterilized *Parthenium* leaves were sliced into smaller segments and placed on the petri plates containing PDA (Potato dextrose agar) medium enhanced with streptomycin (antibiotic). Then, the petri plates were incubated at 25 degrees Celsius for 5-7 days and subsequently, the fungus was subculture. The fungal culture was transferred to freshly prepared PDA plates to obtain pure culture. PDA slants were used to maintain the pure culture of the isolated fungus and preserved the slants at 4°C[9].

Morphological identification of isolated fungus

Fungal strain was first identified based on morphological characteristics of the spores and spore-producing structures by using lactophenol cotton blue mount with descriptions found in the literature and the isolates were initially assigned to a genus[16].

Molecular identification

The molecular characterization of the selected fungal pathogen was done by extracting DNA, and amplifying 18s rRNA by Polymerase Chain Reaction and sequencing. To confirm identification at the molecular level, the ribosomal ITS (Internal Transcribed Spacer) region was amplified using PCR (Polymerase Chain Reaction) with the help of ITS1 and ITS4 universal primers[15]. BLAST analysis was done followed by formation of phylogenetic tree and sequence similarities were determined.

Leaf bioassay

In vitro tests or leaf bioassay was performed to determine the pathogenicity of the isolated fungal strain. *Parthenium* leaves that were in good health were separated from the plant and used for inoculation. Four weeks old *Parthenium* plant leaves were cleaned with 70% alcohol and sterile distilled water. The enlarged healthy leaves were placed in sterilized petri plates with wet filter paper and were inoculated with identified fungal inoculum. Control plates were also maintained to compare the results. Later plates were incubated for one to three days at room temperature. The severity of leaf disease was evaluated with the help of a five-point scale after 72 hours of incubation[11].

Phytotoxin Production

The spore suspension was made from a 7-day-old PDA slant culture. The slant was scratched with an autoclaved glass rod in a solution of distilled water and 0.1% tween 20. To eliminate fungal residues, the suspension was filtered using cheese cloth and the filtrate was utilized as an inoculum. 1 litre of potato dextrose broth (PDB) was used to produce phytotoxin. Following sterilization, the medium was inoculated with a 1ml fungal inoculant that was found pathogenic to the weed. The inoculated flask was incubated for 21 days at 30 degrees Celsius under shaking condition at 250 rpm, and the culture filtrates were obtained after straining the media through three layers of cheesecloth. The filtrates were separated with ethyl acetate, using a volume three times greater than that of the filtrates. A flash evaporator was used to make the extracts completely dried. Following evaporation, the remaining residues were extracted and stored in sterile 10 milliliter screw-cap vials for future research[14].

***Parthenium* seed germination bioassay**

Parthenium seeds were collected at random and preserved in sterile polythene bags. The seeds were properly washed in tap water to remove dirt and dust, rinsed for 5 minutes in a mild detergent solution, surface sterilized for 10 minutes in 0.1% mercury chloride solution, and then washed 4–7 more times in sterilized distilled water. Following surface sterilization, the seeds were set onto moist filter paper inside a sterile petriplate. Then 7-15 days old fungal culture filtrate was added to the chamber containing moisture. A control with sterile water was also prepared and the petriplates were placed for incubation in B.O.D. at 25 degrees Celsius. The germination percentage of seeds was documented after 7-15 days[8]. The assay was performed in triplicate respectively.

RESULTS

Isolation and identification of fungal pathogen

During the survey, *Parthenium* infestation was recorded from nearby sites of Maharishi Markandeshwar University, Ambala and infected *Parthenium* leaves were collected for isolation of fungi. Several different fungal strains were isolated on PDA medium from infected parts of the leaves and checked their biocontrol potential against *Parthenium* weed. During the experiment, a fungal strain was found pathogenic to the weed and caused severe damage to the leaves as compared to the other fungal strains. On the basis of morphological features and microscopic studies isolated fungal pathogen was identified as *Alternaria sp.* (Fig. 1). The colony color changes from grayish white to greenish black or brown along a light border with age. Mycelium is dense, velvety or woolly with branching hyphae. Hyphae are septate, slight yellow or brown in color and 2-5 µm in diameter. Conidiophores are simple or branched, up to 10-50 µm in length and 2-5 µm in width. Conidia are ovoid to ellipsoidal, size ranged from 10-30 x 5-15 µm with 1-3 longitudinal septa and 3-5 transverse septa.

Molecular characterization

Molecular characterization of fungal strain was done by using the commercial service given by geneOmbio Technologies Pvt. Ltd. Pune, Maharashtra. Fungal pathogen was molecularly identified using the 18s rRNA gene sequencing technique. The DNA was extracted using pure culture of isolated fungal strain with the help of cetyl trimethyl ammonium bromide (CTAB) technique. Isolated fungal pathogen was amplified and sequenced with the help of universal primers ITS1 and ITS4. The fungal isolate was found as *A. alternata* (509 bp) according to a BLAST analysis of the resulted sequencing product. The fungal pathogen's gene sequence that has been submitted to NCBI is shown by its accession number (Table 1). A phylogenetic tree was created to show the evolutionary relationship between the isolated fungal pathogen and other biological organisms (Fig. 2).

Table 1. Detail of isolated fungal pathogen with its accession number

Sr. No.	Host plant	Plant part	Fungus	Symptom	Accession number	GenBank submission name
1.	*Parthenium hysterophorus*	Leaf	*Alternaria alternata*	Yellowing or browning of leaf with necrotic spots	PQ483096	*Alternaria alternata strain* PHMMU2

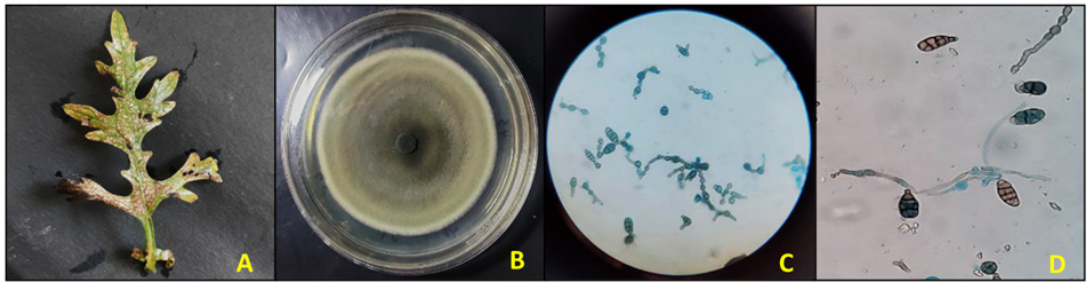

Fig. 1. *Alternaria alternata*: (A) symptoms on leaf; (B) fungal colony after 7 days of incubation at 25°C; (C) microscopic identification; (D) conidia on conidiophore

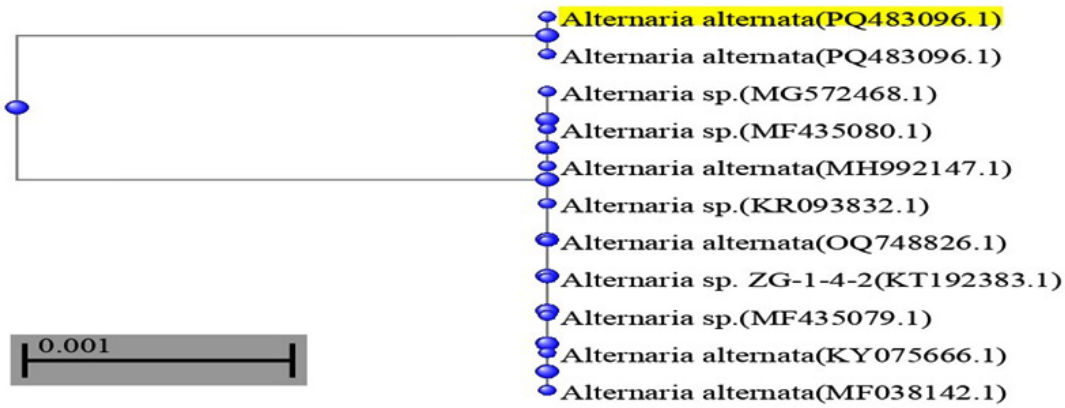

Fig. 2. Phylogenetic tree of the evolutionary relationship between the isolated fungal pathogen and other biological organisms

Leaf bioassay

The *Parthenium* leaf bioassay was conducted to evaluate the pathogenicity of the fungal isolate. Leaf surface inoculation and leaf puncture inoculation was done by applying one-week-old culture of the fungal isolate to healthy *Parthenium* leaves at 25°C. After 48 hours fungal spores germinated over the *Parthenium* leaves and resulted in defoliation of leaves. After 72 hours, the maximum amount of leaf defoliation was observed in case of puncture inoculation with 73.7% damage. Comparatively, 61.3% damage was observed in leaf surface inoculation (Fig. 4). Results indicated that the fungal pathogen grew on the leaves of the weed damaging its tissues and killed them in in-vitro conditions (Fig. 3). The experiment was replicated thrice having three petri plates.

***Parthenium* seed germination assay**

Parthenium plant seeds were treated with fungal culture filtrates of *A. alternata*. Germination percentage of the seeds was noted within 10 days of incubation at 25°C (Fig. 5). Seeds treated with the isolated fungal pathogen resulted in 70% reduction of germination percentage. There was negligible reduction in seed germination of control (distilled water) and 80% of the seeds germinated (Fig. 6). The results indicated that *A. alternata* treated seeds showed reduction in seed germination with respect to control. Root, shoot and total seedling length were also measured to check the effect of fungal culture filtrates on the *Parthenium* seedlings. The inoculation of *Parthenium* seeds with fungal culture filtrates resulted in a fluctuating alteration

Fig. 3. Effect of *Parthenium* leaves after 72 hours of incubation: (A) leaf surface inoculation; (B) leaf puncture inoculation; (C) control

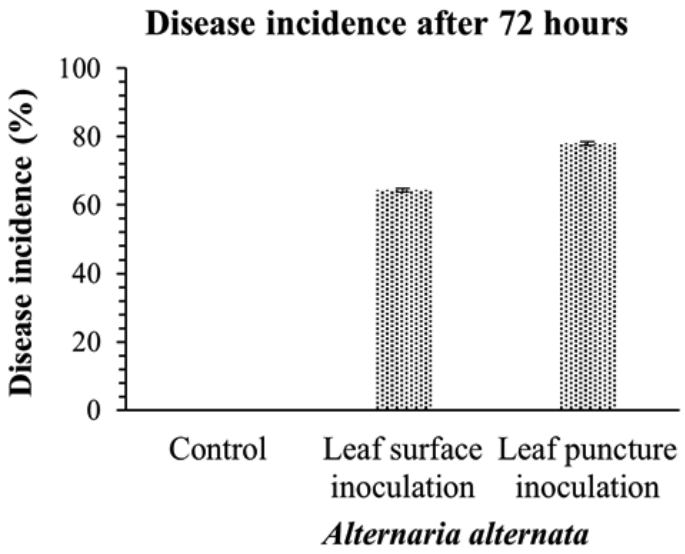

Fig. 4. Effect of isolated *A. alternata* on healthy *P. hysterophorus* leaves

in the root, shoot and total seedling length in comparison to the control group (Fig. 7).

DISCUSSION

The use of fungal pathogens to control weeds under the mycoherbicidal strategy stands out as one of the most effective methods and moreover, the idea of using phytotoxic metabolites from pathogenic fungi is an appealing option for *Parthenium* weed management[10]. For example, cultural filtrates of *Aspergillus* spp., *Alternaria* spp., and *Drechslera* spp. resulted in a significant decrease in the growth and germination of *Parthenium* plants[2]. Similarly, MEB and PDB synthesized metabolites of *Aspergillus niger* decreased *Parthenium* germination by 100% and 89%, respectively[5]. The present research aims to find a prospective fungal pathogen that exhibits phytotoxicity and could prove to be a powerful mycoherbicide against this weed. To achieve this, a fungal pathogen was isolated from *P. hysterophorus* which was identified as *A. alternata* through morphological and molecular characterization.

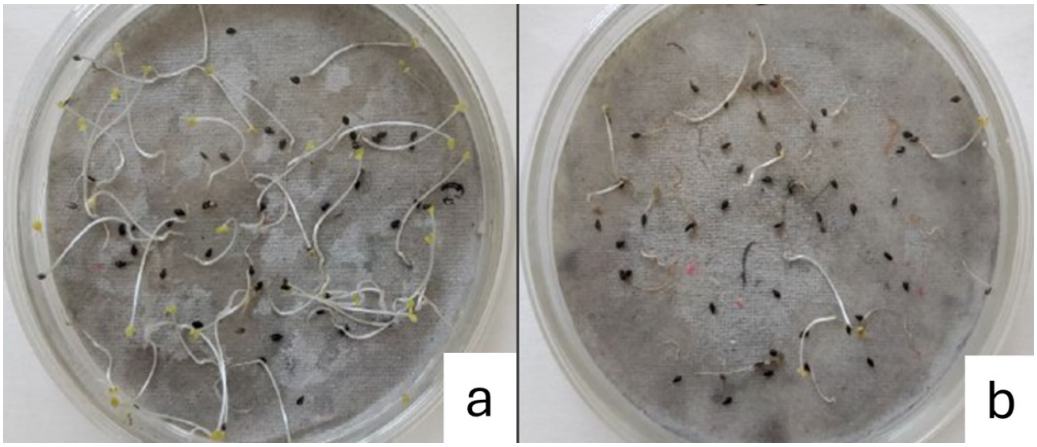

Fig. 5. *Parthenium* seed germination after 10 days of incubation at 25°C: (a) Control; (b) Seeds treated with *Alternaria alternata*

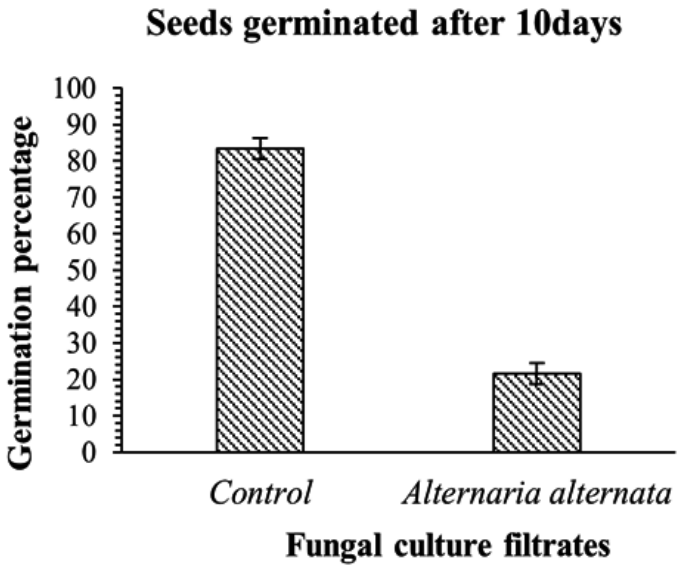

Fig. 6. Phytotoxic effect of fungal culture filtrates of *A. alternata* on seed germination of *Parthenium* with respect to control

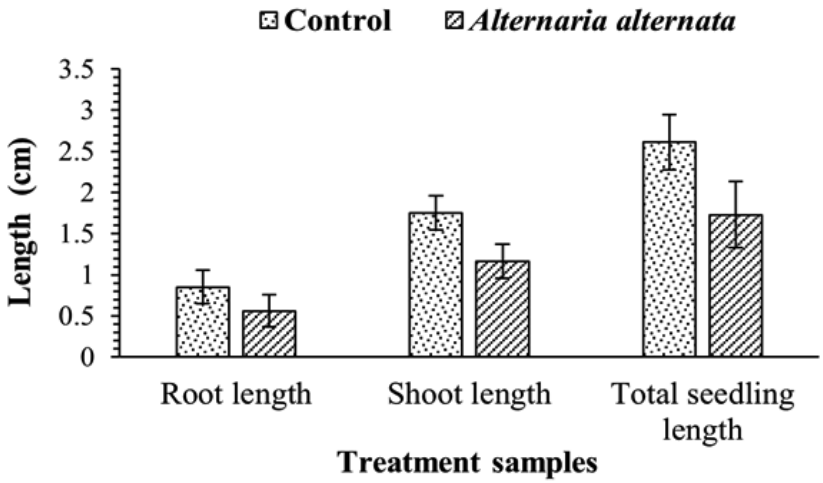

Fig. 7. Effect of fungal culture filtrates of *A. alternata* on *Parthenium* seedlings

It was reported that a mycoherbicide product containing spores of *A. alternata* was efficient against *Parthenium* seedlings[4]. Therefore, the pathogenicity of *A. alternata* was checked through leaf bioassay and culture filtrates of the pathogenic fungus were tested in laboratory bioassay to see its effect on the germination and seedling growth of *Parthenium*. In leaf bioassay the fungal pathogen resulted in defoliation of leaf with 61.3% and 73.7% disease incidence in leaf surface inoculation and leaf puncture inoculation respectively. In seed germination bioassay, culture filtrates of the fungal species showed 70% reduction in seed germination of the *Parthenium* weed and there was a considerable decrease in root, shoot and total seedling length also. According to a study, the crude toxins of *A. alternata* were found to have herbicidal activity against a variety of weeds and the chloroplasts, Golgi complex, mitochondria, nucleus, plasma membrane, etc. are the targets of these phytotoxins on plant cells[1,13]. Moreover, a tenuazonic acid and AAL-toxins were found in *A. alternata* which possess the herbicidal activity against *Parthenium*[8]. It was reported that the impact of *A. alternata's* secondary metabolites on plants varies depending on the kind of plant and increases as the concentration of secondary metabolites increases[22]. This article involves the use of in-vitro detached leaf bioassay and seed germination bioassay to evaluate the potential of cultural filtrates of *A. alternata* for use as a mycoherbicide for *Parthenium*.

CONCLUSION

The present study concludes that a fungal pathogen was isolated from infected *Parthenium* leaves which was identified as *A. alternata* and the isolated fungal species culture filtrates have the ability to suppress the weed. Further research is necessary to isolate, characterize and identify the bioactive compounds with herbicidal potential found in these fungal culture filtrates and ensure long-term effectiveness in weed management strategies.

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Conflict of interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials**.**

Author Contributions

Komal Sahu - collected the data and wrote the methodology and original draft of the manuscript; Vikas Kumar - designed the study and analysed the data; Raj Singh - assisted with data collection, project administration and manuscript revision. Urvasha Patyal and Anjali Kanwal - contributed to the literature review and editing the manuscript; Anuj Sahu and Galymbek Seralin - provided expertise in data interpretation and statistical analysis. All authors reviewed, edited and approved the final manuscript.

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