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Biocontrol Potential of Four Deadly Strains of *Alternaria macrospora* Isolated from *Parthenium* Weed

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ABSTRACT
Parthenium hysterophorus is a weed of global significance causing severe economic, environmental, human and animal health problems in Asia, Africa, Australia and the Pacific. A field survey and the systematic sampling were made during two years (November 2012 to October 2014) in KUK campus and its surrounding area. During a series of extensive surveys for natural enemies of *Parthenium hysterophorus*, leaf spot diseases creating epiphytotics were regularly observed on *P. hysterophorus*. The organism was isolated from the affected parts of the *Parthenium* by following the standard isolation techniques using Potato Dextrose Agar (PDA) and *Parthenium* Dextrose Agar (PeDA) medium. Koch's postulate was performed and found satisfactory for all the isolates and proved to be pathogenic to this weed. On the basis of cultural, morphological and molecular characteristics, these pathogens were identified as different strains of *Alternaria macrospora*. The literature survey indicates that *Alternaria macrospora* has never been reported on the *Parthenium* weed and it is the first report of occurrence of this pathogen causing leaf spot on *Parthenium* weed from the world. Further study will be conducted to develop mycoherbicide by using these fungal pathogens in combination or single.

Key words: *Alternaria macrospora*, mycoherbicide, *Parthenium hysterophorus*, pathogenicity

INTRODUCTION

The noxious weed, *Parthenium hysterophorus* L. (Asteraceae: Heliantheae), commonly known as *Parthenium*, white top, congress grass, American fever or carrot weed, is a devastating and hazardous, invasive weed (Ray and Gour, 2012). It has now naturalized in several tropical and subtropical parts of the world (Dogra *et al*., 2009; Kohli *et al*., 2009). It is one of the most troublesome weeds and figures among the list of invasive species in the GISD (2007). Rao in 1955 first of all reported its entry in India growing in the outskirts of Poona (Rao, 1956) and since then it has spread like a wildfire throughout the country (Aneja, 1991; Yaduraju *et al*., 2005). The weed now occurs in about 35 million ha of land (Sushilkumar and Varshney, 2007). *Parthenium* is presumed to have entered into India along with the wheat grains imported from the USA under the PL 480 scheme. This exotic neotropical annual weed has attained the status of number one terrestrial weed by virtue of its presence in varying dimensions in waste lands, cultivated fields, fallow lands, abandoned fields, residential areas, industrial areas, along roadsides and railway tracks (Sreeramakumar, 1998). With regard to its ecological amplitude 5 billion ha land in India is threatened by its spread (Bhan *et al*., 1997) and *Parthenium* can cause a yield decline of up to 40% in agricultural crops (Khosla and Sobti, 1981) and is reported to reduce forage production in grassland up to 90% (Nath, 1988). With the ever-increasing populations of the weed in both urban and rural localities, the associated problems like crop production, animal husbandry, human health and biodiversity are also phenomenally growing day by day (Sreeramakumar, 1998). To the weed scientist, *Parthenium* has proved a challenge because conventional methods have failed to suppress its growth and prevent its unchecked spread throughout the world and still efforts are being made to control this weed by all possible means. In this context, biological control with plant pathogens is an effective, safe, selective and practical means of weed management

(Aneja *et al*., 2013). Since 1979, considerable progress has been made towards practical use of plant pathogens as safe and selective agents of weed management (Charudattan and Walker, 1982; Aneja *et al*., 2000; Kaur *et al*., 2014). The biological control of this weed using fungal pathogens under the mycoherbicidal strategy has been suggested as one of the most efficient method, owing to its long lasting, less costly and eco-friendly nature. The objective of the present study was to search for fungal pathogens naturally occurring on *Parthenium* weed in northern India that could be used for reducing the plant population to economic levels.

MATERIALS AND METHODS

Isolation of the pathogen: Leaves collected from different regions with leaf spots symptoms were washed in running tap water to remove soil particles and keep for some time to remove water. The infected portions of the leaves were cut into small fragments with small portion of healthy leaves. Leaves fragments are surface disinfected in 70% ethyl alcohol for 1-2 min and then rinsed in sterile distilled water two to three times. These fragments were transferred to Potato Dextrose Agar (PDA) and *Parthenium* extract Dextrose Agar (PeDA) plates supplemented with streptomycin sulphate (10 mg L^{-1}) and were incubated at 25±2°C (Aneja *et al*., 2000). The PeDA medium (Fresh *Parthenium* leaves 200.0 g; dextrose 15.0 g; agar-agar 20.0 g and distilled water 1000.0 mL, pH 5.6) was prepared as follows: Fresh *Parthenium* leaves (200 g) were washed in running tap water and then in sterile distilled water. These were boiled for 20-25 min in 500 mL distilled water and filtered through cheese cloth for the collection of extract. *Parthenium* extract was mixed with the other constituents as in the preparation of PDA medium (Aneja and Singh, 1989).

Identification of pathogens

Morphological identification: Five millimeter diameter discs from 7 day old PDA cultures, taken from the advancing mycelial margins with a cork borer, were placed at the centre of PDA and maintained at 25±2°C under continuous darkness. Seven days later, the colony appearance and their diameters on PDA were determined. The morphological characteristics of the mycelium, conidia and perithecia of pathogens were observed and preliminarily identified by consulting monograms (Ellis, 1971, 1976).

Identification based on molecular characteristics: Fungal pathogen was molecular characterized by using the commercial service provided by Macrogen Inc., Advancing through Genomics, Korea. Fungus genomic DNA samples were extracted using a InstaGene^{tm} Matrix (BIO-RAD). The primers ITS1 primer (5-TCCGTAGGTGAACCTGCGG-3) and ITS5 (5-GGAAGTAAAAGTCGTAACAAGG-3) and ITS4 primer (5-TCCTCCGCTTATTGATATGC-3) were used for the PCR. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30 µL reaction mixture by using a EF-Taq (SolGent, Korea) as follows: Activation of Taq polymerase at 95°C for 2 min, 35 cycles of 95°C for 1 min, 55 and 72°C for 1 min each were performed, finishing with a 10 min step at 72°C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). The purified PCR products of approximately 600 bp were sequenced by using 2 primers as described (Table 1). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA) (Siddiquee *et al*., 2010; Satou *et al*., 2001).

Table 1: Universal primers used during amplification

Universal primer	Sequence	Bases
ITS1	TCCGTAGGTGAACCTGCGG	19
ITS 4	TCCTCCGCTTATTGATATGC	20

Pathogenicity test: *In vitro* pathogenicity of various isolates was assessed using a detached leaf bioassay techniques. Healthy leaves of *Parthenium* were washed with sterile distilled water and wiped with a cotton swab dipped in 70% alcohol. Under aseptic conditions in a laminar flow chamber, mycelial discs of 8 mm taken from 5 days old colony of all the four strains of *Alternaria macrospora* and placed on *Parthenium* leaves. The inoculated leaves were kept in a sterilized moist chamber and incubated at 25ºC. Regular observations for the appearance of symptoms were made after 3 days of incubation (Aneja *et al*., 2000; Aggarwal *et al*., 2014). The frequency of plant pathogenic fungi was expressed as the percentage of leaves in which the fungus was isolated and the pathogenicity was confirmed.

Effect of different media on growth and sporulation: To see the effect of different media, on the growth and sporulation of fungal pathogens, seven media namely Potato Sucrose Agar (PSA), Potato Dextrose Agar (PDA), Potato Dextrose Yeast Agar (PDYA), *Parthenium* Dextrose Agar (PeDA), *Parthenium* Dextrose Yeast Agar (PeDYA), Maltose Extract Agar (MEA) and Nutrient Agar (NA) were used. Inoculated plates were incubated at 25ºC for ten days. Three replicates were run per medium per condition for all the test fungi. Fungal growth was determined by measuring the diameter of the colony at two places at right angle to each other and an average of the cross diameter was considered as growth of the fungus (Aggarwal *et al*., 2014).

Conidial concentration of different pathogens on different media was measured by scraping the mycelial growth from the plate with distilled water and then homogenized on magnetic stirrer for 5 min, placed 1-2 drops of suspension on the hemocytometer slide and calculated the conidial concentration using microscope (Tuite, 1969).

Screening of antagonism: While antagonistic interactions act to decrease the effect of natural enemies acting at the same time, the term anti-antagonism was coined to describe interactions where natural enemies avoid each other with an end result of greater impact on host populations. To use the pathogens in combination, these were screened for antagonism.

For testing antagonism in dual culture method (Reddy *et al*., 2014) a mycelial disc (6 mm) was cut from the margins of actively growing region of 5 day old cultures and inoculated at both ends of the Petri plates (1 cm away from the edge of the plate) with sterilized Potato Dextrose Agar (PDA). For each treatment three replicates were maintained and were incubated at 25±1°C.

RESULTS

During the extensive surveys conducted in the Kurukshetra district of Haryana in October 2013, the infestation of *Parthenium* was recorded in crops, uncultivated areas and roadsides. A congress grass population was found affected by various leaf spot diseases at different parts of Kurukshetra. The spots on PDA (Fig. 1b) and PeDA yielded four different colonies of fungal pathogens and microscopic study revealed that the pathogens belong to the genus *Alternaria*. Molecular analysis of the ITS1-5.8S-ITS2 rDNA region was carried out to confirm the species identity of all these pathogens. Fungal pathogens were molecular characterized by using the commercial service provided by Macrogen Inc., Advancing through Genomics, Korea. The results of the molecular Identification (ITS rDNA sequence analysis) showed that isolates are the different strains of *Alternaria macrospora* (*A. macrospora* strain MKP1, *A. macrospora* strain MKP2, *A. macrospora* strain MKP3, *A. macrospora* strain MKP4). Sequence of the pathogens was compared for the genetic position in *Alternaria* spp., evolutionary phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method. The purified PCR products of approximately 1,400 bp were sequenced by using 2 Universal primers ITS 1 and ITS 4 (Table 1). These two primers amplified the non-coding spacer regions ITS 1 and 2 and the conserved 5.8S; included as well are the partial conserved coding regions of the 18S and 28S genes which are interspersed between the ITS 1 and 2 spacer regions.

When all the four strains were tested for pathogenicity on both injured and uninjured leaves the pathogens were re-isolated and found to be similar to the original isolates, thus confirming the pathogenicity of all the fungal isolates to *Parthenium* and proving of Koch's postulates. The symptoms and the cultural characteristics of the fungal pathogens regularly observed in nature on *Parthenium*. A survey of available literature reveals that this species of *Alternaria* has reported for the first time on *P. hysterophorus* from India.

***Alternaria macrospora* strain MKP1:** Colonies are grey in colour on PDA. The mycelium was septate, hyaline and branched. Conidia were solitary, dark brown, straight or slightly flexous, muriform and ellipsoidal with tapering long beak. The size of conidia ranged from 25-57.5×12.5-25 μm with 1-6 transverse septa and 0-2 longitudinal septa. Size of the beak ranged from 5-15×5-7.5 μm. The conidial morphology of *Alternaria macrospora* strain MKP1 is in agreement with those described by Ellis (1971). Culture has been identified from the Macrogen Inc., Advancing through Genomics, Korea and the sequence has been deposited to the NCBI gene bank with accession number KM186140. The *in vitro* pathogenicity of *A. macrospora* to *Parthenium hysterophorus* has been confirmed (Fig. 1).

***Alternaria macrospora* strain MKP2:** A pathogen was isolated on PDA media from infected leaves and it yielded grey colonies on PDA (Aneja *et al*., 2000). The morphological identification showed that mycelium was septate, hyaline and branched, conidia were solitary, dark brown, straight or slightly flexous, muriform and ellipsoidal with tapering long beak. The size of conidia ranged from 17.5-62.5×10-17.5 μm with 1-6 transverse septa and 0-2 longitudinal septa. Size of the beak ranged from 0-20×5-7.5 μm. Chlamydospores numerous in nature and in culture, dark brown; a chlamydospore initially may be one-celled, intercalary or terminal on a hypha, round with a thickened wall but often including several adjacent cells on hypha, eventually cells

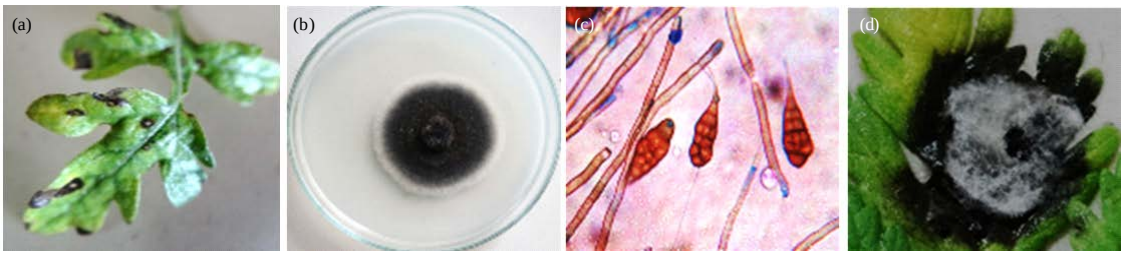

Fig. 1(a-d): *Alternaria macrospora* strain MKP1: (a) Symptoms of the pathogen on the leaf of *Parthenium*, (b) Colony of the pathogen after 5 days at 25°C on PDA, (c) Sympodially arranged conidia on conidiophore and (d) Infection on leave *in vitro* condition

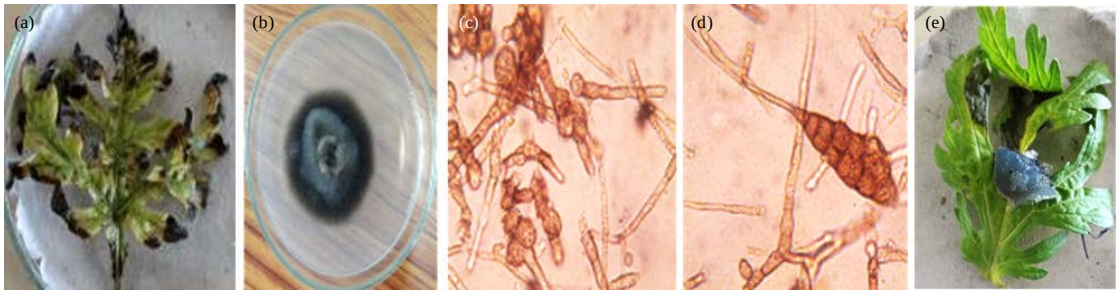

Fig. 2(a-e): *Alternaria macrospora* strain MKP2: (a) Symptoms of the pathogen on the leaf of *Parthenium*, (b) Colony of the pathogen after 5 days at 25°C on PDA, (c) Chlamydospores, (d) Conidia with conidiophore and (e) Infection on leave *in vitro* condition

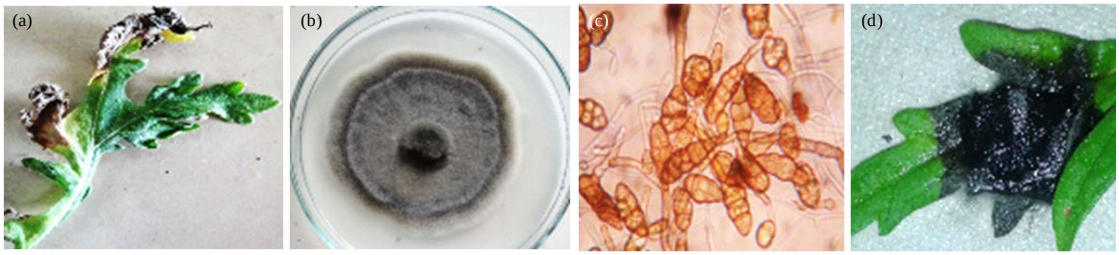

Fig. 3(a-d): *Alternaria macrospora* strain MKP3: (a) Symptoms of the pathogen on the leaf of *Parthenium*, (b) Colony of the pathogen after 5 days at 25°C on PDA, (c) Conidia and (d) Infection on leave *in vitro* condition

dividing in several planes and chlamydospores becoming many celled and irregular in shape. The identification of the pathogen has been confirmed from the CABI International Mycological Institute, UK with reference No. 503549 and the results showed that the top matches at 100% identity to two sequences of *Alternaria* species, neither of which relate to published strains. Thereafter matches were only at 98% identity to various members of the genus, including *A. macrospora*, *A. solani*, *A. dauci*, *A. crassa*, *A. porri* and others. Molecular analysis of the ITS1-5.8S-ITS2 rDNA region was carried out to confirm the species identity of the pathogen. Fungal pathogen was molecular characterized by using the commercial service provided by Macrogen Inc., Advancing through Genomics, Korea. The molecular identification (ITS rDNA sequence analysis) showed the similarity of isolate with *A. macrospora* strain B isolated from Cotton plant infected with leaf blight disease. Sequence has been submitted to NCBI with GenBank accession number KM213867. *In vitro* pathogenicity of the isolated pathogen was determined and the typical disease symptoms were observed on leaves in lab. The inoculated pathogen was re-isolated, thus confirming the pathogenicity of *A. macrospora* to *Parthenium hysterophorus* and usual Koch's postulates (Fig. 2).

***Alternaria macrospora* strain MKP3:** Colonies are dark grey in colour on PDA. The mycelium was septate, hyaline and branched. Conidia were solitary, dark brown, straight or slightly flexous, muriform and ellipsoidal with tapering long beak. The size of conidia ranged from 25-32.5×10-15 µm with 1-6 transverse septa and 0-2 longitudinal septa. Size of the beak ranged from 7.5-22.5×7.5 µm. Size of the conidiophore ranges from 25-67.5 µm. The conidial morphology of *Alternaria macrospora* strain MKP3 is in agreement with those described by Ellis (1971). Culture has been identified from the Macrogen Inc., Advancing through Genomics, Korea. and the sequence has been deposited to the NCBI gene bank with accession number KM514668. *In vitro* pathogenicity has been confirmed on *Parthenium* leaves (Fig. 3).

***Alternaria macrospora* strain MKP4:** Colonies are grey green in colour on PDA. The mycelium was septate, hyaline and branched. Conidia were solitary, dark brown, straight or slightly flexous, muriform and ellipsoidal with tapering long beak. The size of conidia ranged from 50-87.5×7.5-17.5 µm with 1-6 transverse septa and 0-3 longitudinal septa. Size of the beak ranged from 7.5-75×2.5-7.5 µm. The conidial morphology of *Alternaria macrospora* strain MKP4 is in agreement with those described by Ellis (1971). Culture has been identified from the Macrogen Inc., Advancing through Genomics, Korea and the sequence has been deposited to the NCBI gene bank with accession number KM514669. Koch postulates has been proven from *in vitro* pathogenicity test (Fig. 4).

Growth and sporulation on different media: Of the seven media tested for the growth and sporulation, all the strains of *Alternaria macrospora* showed excellent growth and

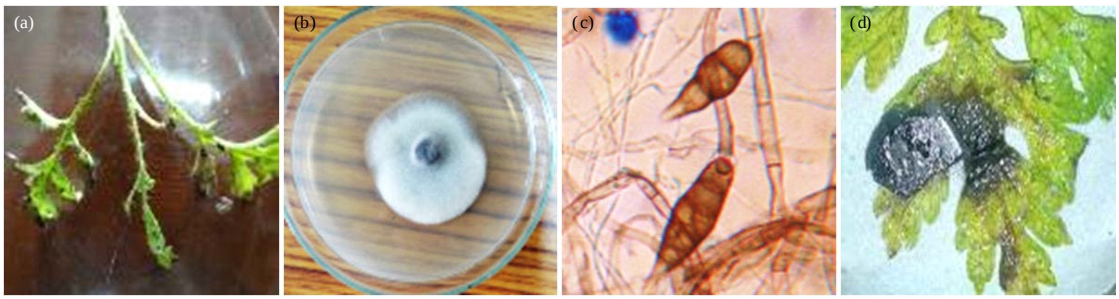

Fig. 4(a-d): *Alternaria macrospora* strain MKP4: (a) Symptoms of the pathogen on the leaf of *Parthenium*, (b) Colony of the pathogen after 5 days at 25°C on PDA, (c) Conidia and (d) Infection on leave *in vitro* condition

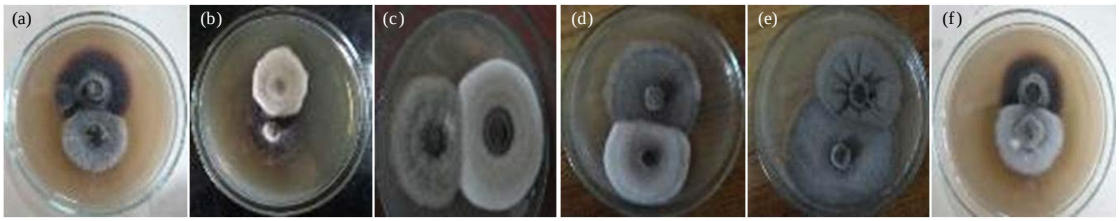

Fig. 5(a-f): Dual cultures between different strains of *A. macrospora* after 7 days on potato sucrose agar at 25°C, (a) MKP2 and MKP1, (b) MKP2 and MKP4, (c) MKP1 and MKP4, (d) MKP3 and MKP4, (e) MKP3 and MKP1 and (f) MKP2 and MKP3

Table 2: Colony diameter of different pathogens on different media

	Colony diameter on different media (cm)						
Pathogens	PDA	PDYA	PeDYA	PeDA	MEA	PSA	NA
Alternaria macrospora MKP1	6.72±0.21	6.95±0.18	6.84±0.03	6.59±0.45	5.68±0.08	6.97±0.28	3.11±0.17
Alternaria macrospora MKP2	5.88±0.08	6.95±0.22	6.39±0.28	6.45±0.28	6.87±0.28	7.15±0.28	3.05±0.28
Alternaria macrospora MKP3	7.35±0.51	7.53±0.31	6.14±0.28	6.05±0.28	7.06±0.28	8.19±0.28	3.20±0.28
Alternaria macrospora MKP4	6.78±0.16	6.95±0.04	7.10±0.28	6.95±0.28	6.79±0.28	7.81±0.28	3.86±0.28

Table 3: Effect of different media on the sporulation of pathogens

	Average spore count/mL (x10^5) on different media						
Pathogens	PDA	PDYA	PeDYA	PeDA	MEA	PSA	NA
Alternaria macrospora MKP1	18.00±0.46	17.55±0.81	16.34±0.53	15.69±0.45	14.68±0.68	19.97±0.05	7.11±0.17
Alternaria macrospora MKP2	24.88±0.56	24.95±0.43	24.39±0.14	23.45±0.39	22.87±0.26	28.15±0.58	3.95±0.68
Alternaria macrospora MKP3	16.51±0.16	18.95±0.04	10.10±0.06	12.95±0.28	10.79±0.28	15.81±0.18	4.86±0.17
Alternaria macrospora MKP4	17.85±0.25	19.55±0.32	15.40±0.20	13.29±0.28	19.31±0.28	21.39±0.20	6.25±0.33

sporulation on PSA, their growth and sporulation was good on PDA, MEA, PDYA, PeDA, PeDYA and poor on NA (Table 2, 3).

Screening for antagonism: The results of the antagonism test showed the mutual intermingling growth of one colony over another and there was no inhibition zone on the PDA plates which indicated that neither of mycelia growth of strains was affected the growth of others (Fig. 5). The results indicates that these different strains of *A. macrospora* could be used in combination or mixture as a part of multiple pathogen system to control this notorious weed.

DISCUSSION

Literature search revealed that a great deal of work has been done by the scientists to control this weed by fungal pathogens (Saxena and Kumar, 2007; Pandey *et al*., 1998; Shukla and Pandey, 2006; Satyaprasad and Usharani, 1981; Kauraw *et al*., 1997). But these pathogens suffered from one or the other disadvantages, so our work in this area aims for searching a potential pathogen which should be the host specific and emerges as an effective mycoherbicide against this weed. A total of twenty nine fungal pathogens have been reported on *P. hysterophorus* weed from various parts of the globe. A literature search reveals that this is the first report of

the occurrence of *Alternaria macrospora* causing leaf spot on *Parthenium* weed from the world. Looking into the severity of the disease and damage caused to the *Parthenium* weed during surveys in North India, the pathogens seem to offer great potential for development and exploitation as effective biocontrol agents for checking *Parthenium* growth. Further work on its host specificity and evaluation as biocontrol agents is in progress in our lab, which may leave to recognize the potential of these pathogens.

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