J. Crop Prot. 2015, 4 (Supplementary): 719-726

Research Article

First record of *Alternaria macrospora* MKP1 causing leaf spot disease on *Parthenium hysterophorus* from India

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Abstract: In the years 2012-14 a series of extensive surveys for natural enemies of parthenium were conducted in Haryana. During survey, a leaf spot disease has been regularly reported on congress grass at different parts of the Kurukshetra and its adjoining areas. The fungal pathogen was isolated from the affected parts of parthenium leaves and on the basis of cultural, morphological and molecular characteristics, was identified as *Alternaria macrospora* MKP1. Koch's postulate was performed and found satisfactory for the isolate and proved to be pathogenic to this weed. The growth of *A. macrospora* MKP1 was studied on eight different media and it exhibited varying degrees of growth on different media. The literature survey indicates that *A. macrospora* has never been reported on the parthenium weed and it is the first report of occurrence of this pathogen causing leaf spot disease on parthenium weed from the world.

Keywords: *Alternaria macrospora* MKP1, biological control, leaf spot, *Parthenium hysterophorus*

Introduction

Parthenium hysterophorus L. (Asteraceace: Heliantheae), commonly known as parthenium, white top, congress grass, feverfew or carrot weed, is one of the most aggressive invasive weeds, threatening natural ecosystems and agro ecosystems in over 30 countries worldwide (Adkins and Shabbir, 2014). It is one of the most troublesome weeds and figures among the list of invasive species in the global invasive species database (GISD, 2007). It causes losses of crops and pastures, degrading the biodiversity of natural plant communities, causing human and animal health hazards and resulting in serious economic losses to people and their interests in many countries around the globe. 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. (Aggarwal *et al.*, 2014). A great many chemical pesticides because of potential human health risks, environmental pollution, effects on non-target organisms and the development of pest resistance, have been or are being phased out (Kaur *et al.*, 2014). 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. Since 1979, considerable progress has been made towards practical use of plant pathogens as safe and selective agents of weed management (Aneja *et al.*, 2013). The biological control of this weed using fungal pathogens under the mycoherbicidal strategy, owing to its long lasting, less costly and eco-friendly nature has been suggested as one of the most efficient methods. The objective of the present study was to search for fungal pathogens naturally occurring on parthenium

Handling Editor: Naser Safaie

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Received: 06 October 2014, Accepted: 15 September 2015
Published online: 09 November 2015

weed in northern India that could be used for reducing the plant population to economic levels.

Materials and Methods

During the surveys conducted in the different districts of Haryana in years 2012 to 2014, the different crops, uncultivated fields, vacant lands, and road sides showed high infestation of *P. hysterophorus*. It mainly cause heavy loss in the crops of wheat, sugarcane, mustard, sorghum, onion and garlic. Diseased leaves were collected in polythene bags and brought to the laboratory for study of symptoms, isolation and pathogenicity test of the causal agent (Aneja *et al.,* 2014).

Isolation of the pathogen

Leaves collected from different regions with leaf spots were washed in running tap water to remove soil particles and kept 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 were surface disinfected in 70% ethyl alcohol for 1-2 minutes and then rinsed in sterile distilled water two to three times. These fragments were transferred to Potato dextrose agar (PDA) medium and Parthenium extract dextrose agar (PeDA) plates supplemented with streptomycin sulphate and were incubated at 25 ± 2 ºC (Aneja *et al.,* 2000). PeDA medium consisted of (Fresh parthenium leaves 200.0 g; Dextrose 15.0 g; Agar-agar 20.0 g and Distilled water 1000.0 ml).

Identification

Morphological identification

The isolates were grown on PDA at 25 ± 2 °C for seven days to study the morphological characters like size of conidia, number of transverse and longitudinal septa and size of beak. The size of conidia and beak were measured under light microscope at 40X using micrometry. Forty five observations were taken for conidial and beak measurements and mean values were calculated. (Ellis, 1976; 1971)

Molecular identification

Fungal pathogen was molecularly characterized by using the commercial service provided by Macrogen Inc., Advancing through Genomics, Korea. Fungus genomic DNA samples were extracted by using an InstaGene^{tm} Matrix (BIO-RAD.). The primers ITS1 (5'-TCCGTAGGTGA ACCTGCGG-3'), ITS5 (5'–GGAAGTAAAAGTC GTAACAAGG–3') and ITS4 (5'- TCCTCCGCTT ATTGATATGC-3') (White *et al.*, 1990) were used for the PCR. The PCR reaction was performed with 20 mg of genomic DNA as the template in a 30µl reaction mixture by using a EF-Taq (SolGent, Korea) as follows:-activation of Taq polymerase at 95 ºC for 2 minutes, 35 cycles of 95 °C for 1 minutes, 55 °C, and 72 °C for 1minutes, each were performed, finishing with a 10-minutes at 72 ºC. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). The purified PCR products of approximately 14,000 bp were sequenced by using 2 primers as described in 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 ºC for 5 min, followed by 5 minutes on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA) (Satou *et al.,* 2001).

Table 1 Universal primers used during amplification.

Universal Primer	Sequence	Bases
ITS1	TCCGTAGGTGAACCTGCGG	19
ITS 4	TCCTCCGCTTATTGATATGC	20

Phylogeny

The phylogenetic relationship was established using following softwares:- MUSCLE for multiple alignment, optionally Gblocks for alignment curation, PhyML for phylogeny and finally TreeDyn for tree drawing (Edgar, 2004; Castresana, 2000; Chevenet, 2006; Guindon, 2010). Gene sequences from different organisms that shared highest sequence similarity with 18S rRNA genes of the isolated pathogen were retrieved from NCBI database. The alignment

presented comprises the partial length nucleotide sequence of 18SrRNA gene.

Pathogenicity test

***In vitro* pathogenicity tests**

Healthy leaves of congress grass were washed with sterile distilled water and wiped with a cotton swab dipped in 70% alcohol. Some of the leaves before inoculation were injured on adaxial surface by pricking with a flamed needle. Mycelial discs of 8 mm were taken from 5 days old colony of isolated pathogen and placed on injured and uninjured portions. Then it covered with sterile moist cotton. The inoculated leaves were kept in sterilized moist chambers and incubated at 25 ± 2 °C. Observations for the appearance of symptoms were made after 3 days of incubation (Aneja *et al.*, 2014).

Effect of different media on the growth of isolated pathogen

To see the effect of different media on the growth of isolated pathogen, nine media including: Potato Sucrose Agar (PSA), Potato Dextrose Agar (PDA), Potato Dextrose Yeast Agar (PDAY), Parthenium Dextrose Agar (PeDA), Czapek's Dox Agar (CDA), Nutrient Agar (NA), Malt Extract Agar (MEA) and Sabouraud dextrose agar (SDA) were used. The inoculated plates were incubated at 25ºC for ten days. Three replicates were used per medium. 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 (Aneja, 2003).

Statistical analysis

The experimental results were repeated thrice in triplicate each time and expressed as mean ± SD and results were statistically evaluated using SPSS software version 16 at 5% significance level using Dunnett's test.

Results

During the extensive surveys conducted in the various districts of Haryana in year 2012 to 2014, infestation of parthenium was recorded in crops, uncultivated areas and roadsides. A congress grass population was found affected by leaf spot diseases at different parts of Kurukshetra. The symptoms on the leaves of Parthenium were characterized as dark brown, irregular and round spots with grey coloured centre distributed on all over the leaves. (Fig. 1 a). Also the results showed that young leaves had less infection than mature leaves in the field. This showed that young leaves are comparatively more resistant. Under severe conditions, the older diseased leaves are shed from the plant.

The leaf spot on PDA medium (Fig.1 b) yielded a fungal pathogen which was identified as *A. macrospora* MKP1 on the basis of microscopic and molecular characteristics. Typical disease symptoms were produced on both injured and uninjured leaves in *in-vitro* and the inoculated pathogen was re-isolated and found similar to the original isolate in cultural characteristics, thus confirming the pathogenicity of *A. macrospora* MKP1 to *P. hysterophorus* and proving the Koch's postulates (Fig.1 e). This species of *Alternaria* is reported for the first time on *P. hysterophorus* from the world.

Identification based on phenotypic characteristics

The colour of *Alternaria macrospora* MKP1 (Fig. 1 b-d) colonies were grey on PDA medium. 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 x 12.5-25 µm with 1 to 6 transverse septa and 0 to 2 longitudinal septa. Size of the beak ranged from 5-15 x 5-7.5 µm. The conidial morphology of *A. macrospora* MKP1 is in agreement with those described by (Ellis, 1976).

Identification based on molecular characteristics

The isolated pathogen has been identified by ITS rDNA sequence analysis using the FASTA algorithm with the Fungus database from EBI. 18S rDNA gene and internal transcribed spacer region (ITS) comprising the ITS1-5.8S-ITS2. rDNA gene cluster was amplified and sequenced by Macrogen Inc., Advancing through Genomics, Korea. The purified PCR products were sequenced by using 2

Universal primers ITS 1 and ITS 4 (Table 1) (White *et al*., 1990). 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. ITS 1 primer gave the product of 1401 bp and ITS 4 primer gave the product of 1422 bp. The removal of primer sequences and extraction of Consensus sequence resulted in the final product of 552bp. The Sequence has been submitted to NCBI with accession number KM186140 and was compared with other species of *Alternaria* spp. which are deposited in gene bank and phylogenetic tree is shown in Fig. 3. The evolutionary history was inferred using the Neighbor-Joining method.

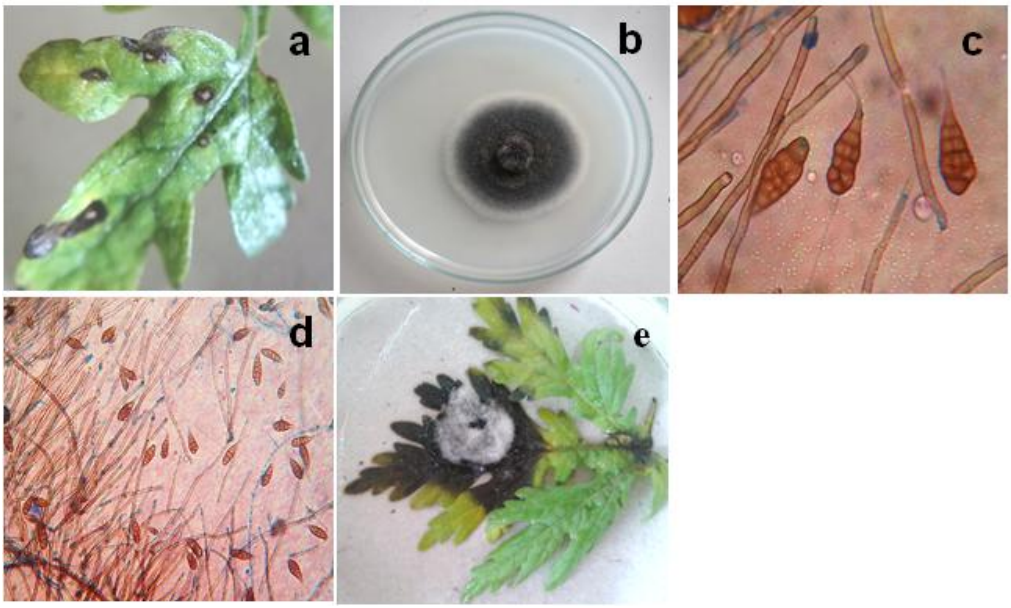

Figure 1 *Alternaria macrospora* MKP1: (a) symptoms of the pathogen on the leaf of parthenium; (b) Colony of the pathogen after 5 days at 25 ºC on PDA; (c) and (d) conidia with conidiophores ; (e) Infection on leaves *in vitro* condition**.**

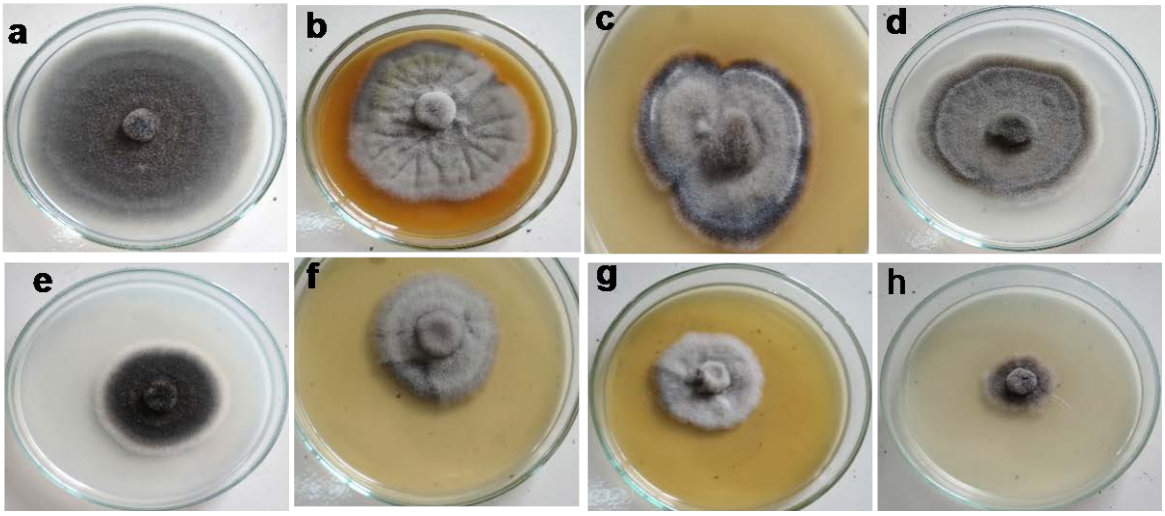

Figure 2 Growth of *Alternaria macrospora* MKP1 on selected media after 5 days at 25 ºC: (a) PSA; (b) PDAY; (c) PeDA; (d) PDA; (e) CDA; (f) MEA; (g) SDA; (h) NA.

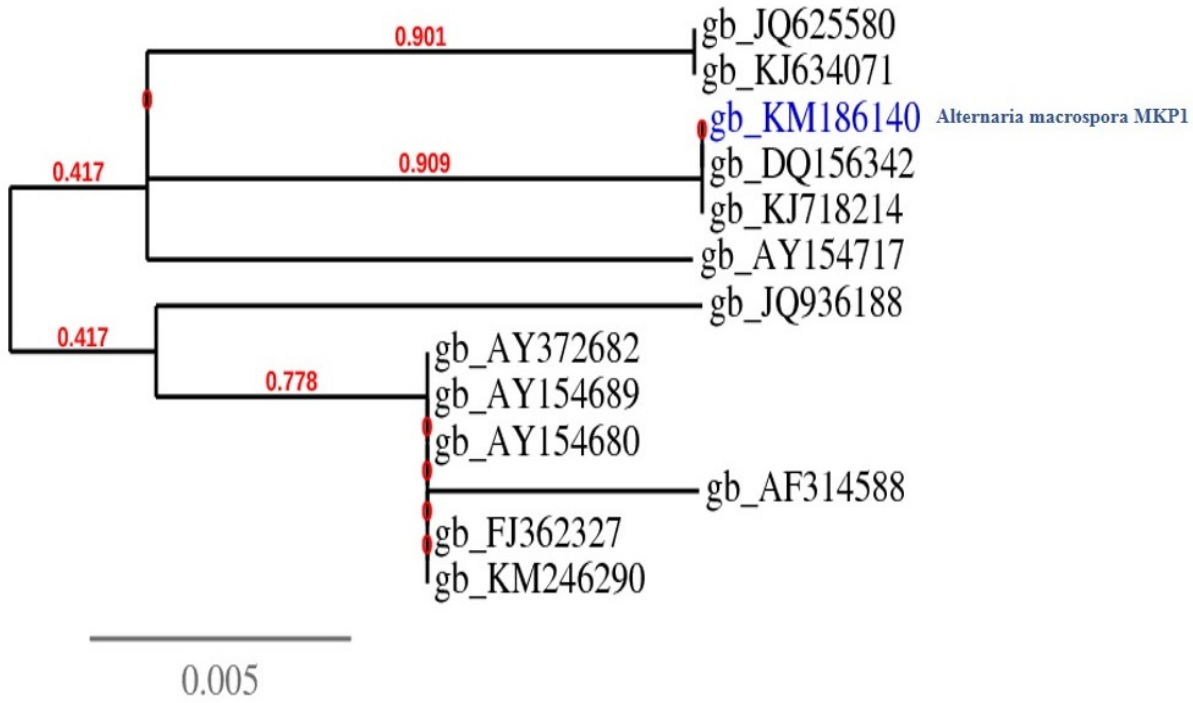

Figure 3 Phylogenetic tree using ITS sequences shows closest known relatives of *Alternaria macrospora* MKP1.

Growth on different media

The radial growth rates of mycelia on different nutritive solid culture media (PDA, PSA, PDAY, MEA, NA, SDA, CDA and PeDA) were studied. The active mycelial growth rates were observed after 3 and 5 days of inoculation on every medium. All the culture media tested for the growth of *A. macrospora* MKP1 supported the growth of test fungus to various degrees. *A. macrospora* MKP1 showed excellent growth on PSA (Fig. 2). PSA had the highest mycelial growth (8.23cm) after five days. The mycelia of *A. macrospora* MKP1 increased till the end of the experiment on PSA. The growth of pathogen was good respectively on PDAY > PeDA, PDA > CDA > MEA but lowest on SDA and NA (Table 2). Difference in surface and reverse colouration of fungal colonies was distinct on all the growth media.

Table 2 Colony diameter of *Alternaria macrospora* MKP1 on different media.

Serial number	Media	Growth diameter ± SD (cm)[1]	
		(A)	(B)
1	PDA	3.40 ± 0.10	4.75 ± 0.06
2	PDAY	4.90 ± 0.36	7.03 ± 0.09
3	PeDA	4.28 ± 0.08	6.40 ± 0.02
4	CDA	4.45 ± 0.11	6.16 ± 0.08
5	SDA	3.20 ± 0.19	4.42 ± 0.32
6	MEA	3.42 ± 0.15	4.43 ± 0.51
7	PSA	5.92 ± 0.05	8.23 ± 0.02
8	NA	2.51 ± 0.05	3.40 ± 0.13

[1] Values are means of three replicates.
[2] The data were analyzed by one way ANOVA followed by Dunnett's test at 5% significant level.
[A] After 3 days.
[B] After 5 days.

Discussion

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 host specific and emerge 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 (Kaur *et al.*, 2014). A literature search reveals that *A. macrospora* is known to be pathogenic to cotton and some malvaceous weeds (Walker and Sciumbato, 1981) but this is the first report of the occurrence of *A. macrospora* 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, this pathogen seems to offer great potential for development and exploitation as effective biocontrol agent for checking Parthenium growth. Further work on its host specificity and evaluation as biocontrol agent is in progress in our lab, which may lead to recognize the potential of these pathogens.

Acknowledgement

The authors are thankful to the University Grants Commission, New Delhi for providing financial assistance in the form of Maulana Azad National Fellowship for minority students.

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اولین گزارش از قارچ *Alternaria macrospora* MKP1 عامل بیماری لکه برگی روی *Parthenium hysterophorus* از هندوستان

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چکیده: طی سال‌های ۲۰۱۲ تا ۲۰۱۴ آزمایش‌هایی برای بررسی دشمنان طبیعی علف هرز *Parthenium* در شهر هاریانا هندوستان انجام شد. در این بررسی بیماری لکه برگی روی علف هرز *Parthenium hysterophorus* از مناطق مختلف کورکوشرتا و نواحی اطراف آن گزارش شد. قارچ عامل بیماری جدا شده از برگ‌های *Parthenium* براساس خصوصیات محیط کشت و خصوصیات شکل‌شناسی و مولکولی به‌نام *Alternaria macrospora* MKP1 تشخیص داده شد. بیماری‌زایی قارچ به‌روش کوچ روی این علف هرز به اثبات رسید. خصوصیات رشدی قارچ *A. macrospora* MKP روی هشت محیط کشت مطالعه شد و درجات مختلفی از رشد روی محیط‌های کشت مشاهده شد. مرور منابع نشان می‌دهد که *A. macrospora* به‌عنوان عامل بیماری لکه برگی برای اولین بار در جهان روی علف هرز *Parthenium* گزارش می‌شود.

واژگان کلیدی: *Alternaria macrospore* MKP1، کنترل بیولوژیک، لکه برگی، *Parthenium hysterophorus*