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Short communication

Enzymatic activities of pathogenic species of *Alternaria,* isolated from *Parthenium*

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Parthenium hysterophorus L. an exotic, pernicious weed has been considered as one of the most troublesome weeds for agricultural sector by virtue of its high ecological amplitude and adaptability (Kaur *et al*. 2014) besides environmental degradation, biodiversity depletion, yield loss and health related issue (Sushilkumar 2014). It is estimated that it has invaded about 35 million hectares of land in India (Sushilkumar and Varshney 2010). *P. hysterophorus* harbours a diversity of pathogenic fungi, as shown recently by Aggarwal *et al.* (2014). Enzymes play a fundamental role in host-parasite interaction and are involved not only in the initial entry of the pathogen and its spread within the plant tissue but also in the degradation of host tissue into metabolites which the parasite can utilise (Sarkar 2009). The characteristic feature of many phytopathogenic organisms is their ability to produce a variety of enzymes capable of degrading the complex polysaccharides of the plant cell wall and membrane constituents. Pathogenic organisms either continually secrete enzymes or upon contact with the host plant. The host cell walls are penetrated, tissues are colonized and permeability of host cells is altered. During the survey it was observed that isolates of *Alternaria* infected the *Parthenium* weed and caused leaf spot and leaf blight diseases. In virtue of the shortage of information about enzymatic activity of pathogenic fungi, this present study evaluated the potential of six isolates of *Alternaria* fungi for amylolytic, ligninolytic, pectinolytic and cellulolytic production.

The fungal pathogens were isolated from the infected leaf portion of the *Parthenium* weed. The leaves were cut into small portions and sterilized in 70% ethanol then washed in sterile distilled water for four to five times. Leaf portions were then placed on PDA medium plates supplemented with streptomycin sulphate. These were then incubated at ±25 °C for 7 days. Isolated fungi were aseptically transferred to PDA plates and the pure cultures were incubated at above conditions. The pure culture was maintained on PDA slants. Lacto phenol cotton blue mount was used to study the morphological characteristics of the mycelium, conidia and perithecia of fungal strain and preliminarily identification was done with the help of standard literature (Ellis 1976).

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Fungal pathogen was molecularly characterized by using the commercial service provided by Macrogen Inc., Advancing through Genomics, Korea. Fungus genomic DNA samples were extracted using a InstaGenetm 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 minutes, 35 cycles of 95°C for 1 minutes, 55°C, and 72°C for 1 minute each were performed, finishing with a 10-minutes 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 universal primers (ITS1 and ITS4). 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) (Satou *et al.* 2001).

The pathogenicity was determined *in vitro* and *in vivo* conditions. For *in vitro* pathogenicity test, healthy leaves of *Parthenium* were used for inoculation. The leaves were washed with sterile distilled water and wiped with a cotton swab dipped in 70% alcohol. Mycelial discs taken from 5 days old colony were placed on leaves. The inoculated leaves were kept in sterilized moist chambers and incubated at 25 °C. Regular observations were made for the

appearance of symptoms after 3 days of incubation as described by Aneja *et al*. (2000). For *in vivo* pathogenicity test, *Parthenium* plants were grown in earthen pots. Inoculum was prepared by growing the fungus on PDA plates, and mycelial and conidial mass was harvested by flooding the plates with sterile distilled water and then scraping the mass with a sterilized spatula. Inoculum concentration was adjusted to 6×10^4 spores/mL using hemocytometer and applied to the plants within two hours of sunset to avoid drying and to allow for a natural dew period shortly afterwards. Plants were observed weekly for the development of disease symptoms as described by Aneja *et al*. (2000).

For detection of extracellular enzyme production isolates from *P. hysterophorus* (4 isolates of *Alternaria macrospora* and two isolates of *Alternaria* sp.) were screened qualitatively for cellulase, pectinase, ligninolytic enzymes (laccase, lignin peroxidase, manganese peroxidase) and amylase production.

Screening of fungal extracellular enzymes was done by qualitative method *i.e*. agar plate method. The functional role of extracellular enzymes by fungal pathogens was assessed by growing them on PDA for 6-7 days, incubated at 25 °C and placing 5 mm mycelial plugs on the solid media. After incubation, at room temperature, the zone of enzyme activity surrounding the fungal colony was measured as described by Patil *et al.* (2015). Procedure followed for the qualitative estimation of amylolytic, Proteolytic, Cellulolytic and Ligninolytic activity is given below.

Amylase activity was assessed by growing the fungi on Glucose yeast extract peptone agar (GYP) medium with 0.2% soluble starch at pH 6.0. After incubation, the plates were flooded with 1% iodine in 2% potassium iodide (Sunitha *et al.* 2013).

A qualitative determination of cellulolytic activity, Yeast Extract Peptone Agar medium containing 0.5% Carboxy-methylcellulose (CMC) was used. After 3-5 days of fungal colony growth, the plates were flooded with 0.2% aqueous Congo red solution and destained with 1M NaCl for 15 minutes. Appearance of yellow areas around the fungal colony in an otherwise red medium indicated cellulose activity (Sunitha *et al.* 2013).

Pectinolytic activity was determined by growing the fungi in pectin agar medium (Pectin -5g, yeast extract-1g, agar- 15g pH 5.0 in 1L distilled water). After the incubation period, the plates were flooded with 1% aqueous solution of hexadecyl trimethylammonoium. A clear zone formed around the fungal colony indicated pectinolytic activity (Sunitha *et al.* 2013).

For Ligninolytic activity one cm diameter plug cut from the growing edge of PDA cultures of selected isolates, was centrally inoculated on the surface of azure B agar. The medium containing (g/L) glucose-0.2%, KH_2P0_4-1, yeast Extract- 0.01, diammonium tartarate-0.5, $CuS0_4.5H_2O$-0.001, $MgSO_4\cdot7H_2O$-0.5, $FeSO_4$- 0.001, $CaCl_2\cdot2H_2O$-0.01, $MnSO_4.H_2O$-0.001and agar agar-20; was supplemented with 0.01 % w/v Azure B. The plates were incubated at 30 °C for 7 day. The uninoculated plate served as a control. The production of lignin peroxidase and Mn peroxidase was recorded as clearance of blue coloured medium.

Laccase activity was also assessed by growing the fungi on solid medium with guaiacol as indicator. 0.01% guaiacol was added to the solid medium (PDA) and incubated at 25ºC. Guaiacol positive reaction was indicated by the formation of a reddish brown halo.

Morphological examination of pathogen

During the extensive surveys *Parthenium* was recorded in crops, uncultivated areas and roadsides which was found affected by various leaf spots and leaf blight diseases at different parts of Kurukshetra. The infected part on PDA yielded a fungal pathogens and microscopic study revealed that the pathogen belong to the genus *Alternaria*. Morphological examination by lacto phenol cotton blue mount and molecular analysis of the ITS1-5.8S-ITS2 rDNA region was carried out to confirm the species identity of the pathogen. The morphological examination (Ellis 1971) and molecular identification (ITS rDNA sequence analysis) confirmed its identity as *A. macrospora* strain MKP1, *Alternaria macrospora* strain MKP2, *A. macrospora* strain MKP3, *A. macrospora* strain MKP4, *Alternaria* sp. PMK1 and *Alternaria* sp. PMK2, which gene sequence of all the pathogens has been deposited to the NCBI gene bank with accession number KM186140, KM213867, KM514668, KM514669, KT192437 and KT192438, respectively. *In vitro* and *in vivo*, pathogenicity of the isolated pathogens was determined and the typical disease symptoms were observed on leaves in lab and in potted *Parthenium* plants. The inoculated pathogens were re-isolated, thus confirming the pathogenicity of *A. macrospora* to *Parthenium* by usual Koch's postulates.

***Alternaria macrospora* MKP1**: Colonies were 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* MKP1 is in agreement with those described by Ellis (1976). 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.

***Alternaria macrospora* MKP2**: A pathogen was isolated on PDA media from infected leaves and it yielded grey colonies on PDA (Aneja *et al*. 2000). 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.

***Alternaria macrospora* MKP3:** Colonies were dark grey in colour on PDA. 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 ranged from 25-67.5 µm.

***Alternaria macrospora* MKP4:** Colonies were grey green in colour on PDA. 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.

***Alternaria* sp. PMK1**: Leaf spot yielded a greenish grey fungal colony with abundant aerial mycelium on PDA and PeDA (Aneja *et al*. 2000). The identification of the pathogen has been confirmed from CABI, International Mycological Institute, UK (IMI accession No. 504470) and Macrogen Inc., Advancing through Genomics, Korea. Molecular analysis of the ITS1-5.8S-ITS2 rDNA region carried out at CABI UK confirmed the pathogen as *Alternaria* sp. Subsequently, however on submitting the pathogen isolate to Macrogen Inc., Korea sequence analysis did not show any similarity with published strains and the best match (99%) was with unpublished strain of *Alternaria alternata* strain S-f6 (HM165489). The Sequence has been submitted to NCBI with GenBank accession number KT192437.

Alternaria sp. PMK2: Leaf spot yielded a dark grey fungal colony with abundant aerial mycelium on PDA. The conidial morphology of *Alternaria* sp. PMK2 is in agreement with those described by (Ellis 1976). Conidiophores were dark brown, straight to geniculate, arises in clusters, scar present at the point of bearing conidia. Based on these characteristics, the fungus was identified as *Alternaria* sp.

Production of cell wall degrading enzymes

The use of simpler solid media permits the rapid screening of large populations of fungi for the presence or absence of specific enzymes. The six pathogenic *Alternaria* fungi were isolated from *Parthenium* plants. All isolates were sub-cultured routinely and maintained in the department. The isolates of filamentous fungi were screened for production of cellulose, starch, pectin and lignin degrading enzymes qualitatively on agar media containing specific substrates. Each isolate was able to produce one or the other extracellular enzymes (**Table 1**). Some of the pathogens were able to produce all the enzymes.

Table 1. Enzyme activities of fungal pathogens isolated from *Parthenium* weed

Pathogens	Qualitative enzymatic activities				
	Cellulase	Amylase	Pectinase	Laccase	LiP and Mn peroxidase
A. macrospora MKP1	+	+	+	+	+
A. macrospora MKP2	+	+	+	+	+
A. macrospora MKP3	-	+	+	+	+
A. macrospora MKP4	+	+	+	+	+
Alternaria sp. PMK1	+	+	+	-	+
Alternaria sp. PMK2	+	+	+	+	+

LiP: Lignin peroxidase; MnP: Manganese peroxidase; +: Positive; -: Negative

Cellulolytic enzyme assay was performed to test the cellulolytic activity of six pathogenic *Alternaria*. Zone production by the tested fungi indicate the presence of cellulase enzyme. All the test fungi showed positive result for the production of cellulolytic enzymes except *Alternaria macrospora* MKP3.

Results showed that all the fungal isolated except *Alternaria* sp. PMK1 exhibited an ability to oxidize guaiacol, halo of intense brown colour was formed under and around the fungal colonies (positive for guaiacol oxidation), indicating the presence of ligninolytic enzymes (laccase).

Lignin peroxidase and manganese peroxidase assay revealed decolourization of the dye Azure-B by fungi which was positively correlated with production of lignin peroxidase and Mn dependent peroxidase. In solid plate screening all the six pathogens decolorized the Azure B dye and exhibited an ability of producing lignin peroxidase and Manganese peroxidase enzymes.

All the tested fungi showed the positive results for amylase production by halo zone formation on

starch agar plates. The amylolytic potential of these pathogenic fungi may help them to degrade starch which is available in the host plant.

After 72 h of incubation, cultures of the tested fungi on pectin agar plates showed clear zones on treatment with iodine-potassium iodide solution indicating the pectinolytic ability of these fungi. The pectinolytic enzyme of these pathogenic fungi may help them to cause infection in the *Parthenium* plant.

In the present study, the fungal pathogens of *Parthenium* were screened qualitatively for cellulase, pectinase, amylase and lignin degrading enzymes production. The elaboration of an array of cell wall splitting enzymes helps the pathogen for easy penetration of the host cell wall and subsequent colonization. Cellulose is a major structural constituent of the cell wall of host plants. Many phytopathogenic fungi produce cellulases in culture adaptively which hydrolyse cellulose and its derivatives (Muthulakshmi 1990). The results obtained in the present study indicate that all the six isolates of *Alternaria* produced cellulase except *Alternaria macrospora* MKP3.

Several pathogenic ascomycetes and deutromycetes are known to produce lignin degrading enzymes. In our study the screened pathogens were able to produce one or the other ligninolytic enzyme which may contribute in the pathogenesis against *Parthenium*. A numbers of cell wall degrading enzymes have been shown to be produced by plant pathogens (Chenglin *et al.* 1996), which are known to facilitate cell wall penetration and tissue maceration in host plants. Since all the species of *Alternaria* are intercellular in the host, the productions of these enzymes appears to facilitate the dissolution of host cell wall and middle lamella and help entry and establishment of the pathogen in the host and are possibly responsible for playing a vital role in pathogenesis through cell wall degradation and disintegration of tissues.

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SUMMARAY

A common feature of pathogenic fungi to cause disease in plant is the necessity to pass through the plant cell wall, an important barrier against pathogen attack. To this end, fungi possess a diverse array of secreted enzymes to depolymerize the main structural polysaccharide components of the plant cell wall, *i.e.* cellulose, lignin and pectin. In the present investigation, six pathogenic fungal species such as *Alternaria* sp. PMK1, *Alternaria* sp. PMK2, *Alternaria macrospora* MKP1, *Alternaria macrospora* MKP2, *Alternaria macrospora* MKP3 and *Alternaria macrospora* MKP4 were isolated from diseased leaves of *Parthenium* plant and found to be pathogenic to this weed. Isolated fungi were examined for the presence of cellulolytic, pectinolytic, amylolytic and ligninolytic activity. Presence of enzymatic activities of these fungal indicating the importance of the cell wall degrading enzymes in pathogenicity against *Parthenium* weed.

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