Mycopath (2017) 15(1): 23-27 **Research Article**

Role of different substrates in mass production and pathogenicity of *Alternaria macrospora* for the management of parthenium weed

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Abstract

Contrary to chemical herbicides, the use of plant pathogenic fungi have been suggested as one of the best possible mean for controlling parthenium weed (*Parthenium hysterophorus* L.). Mass Production of mycoherbicides by solid substrate fermentation using four different strains of *Alternaria macrospora* Zimm. was standardized. Selection of suitable inexpensive and easily available agro-waste substrate for the mass production of mycoherbicidal agents was the main aim of the present study. Mycelial growth was recorded on seven of the inoculated solid substrates. The outcome of the study indicates that chopped parthenium leaves were the most suitable solid substrate for the mass production of *A. macrospora* MKP1 and *A. macrospora* MKP3 while wheat bran for *A. macrospora* MKP2 and *A. macrospora* MKP4. The highest sporulation was observed with wheat bran. Contrary to these, wood shavings failed to support the growth and sporulation of the pathogens. Parthenium leaves (chopped and powdered) and wheat bran colonized inocula incited severe infection and were responsible for significant mortality of parthenium plant.

Keywords: Agro-waste, *Alternaria macrospora*, mass production, mycoherbicide, sporulation.

Introduction

Parthenium, an exotic weed, is considered as one of the most troublesome weeds for agricultural sector by virtue of its high ecological amplitude and adaptability (Kaur and Aggarwal, 2015). 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 (Kaur *et al*., 2014). Thus, biological control of the weed especially with plant pathogenic fungi has attracted the attention of a large group of scientist world over. A local strain of *Sclerotium rolfsii* (FGCC#02) incited severe collar rot disease and showed very high pathogenic potential and satisfied most of the parameters required for consideration of any organism as mycoherbicide against parthenium (Pandey *et al*., 1996). However, difficulty in mass production of the agent constrained its application as mycoherbicide. Plant pathogens especially fungi possess great potential as controlling agent against this noxious weed (Pandey *et al.*, 2004; Chutia *et al*., 2007). Fungi are considered advantageous over other microorganisms because they are capable of developing epidemics, infection does not require a damaged or compromised host and spores are relatively stable (Jackson, 1997).

Mass production by solid substrate fermentation utilizes economically feasible, cost effective and easily available solid wastes as substrates for growth of the test strain which after fermentation become ready for direct application to the field with very little additional processing. There are world-wide resurgence of interest in the use of indigenous eco-friendly and host specific fungal pathogens as mycoherbicide and a significant advance in mass production and fermentation of some of them have been observed (Pfirter *et al.*, 1998; Eilenberg *et al.*, 2001). However, only few organisms have been commercially produced for large-scale field applications (Pandey *et al.*, 1999; Gressel, 2003). Non availability of low-cost mass production technology is one of the major hindrances in their application (Pandey *et al.*, 2001). This may be achieved by selecting a suitable substrate that is simple in composition, cheaper in price and available in large quantities and developing a production procedure that is easy to apply with minimum labor. Therefore, mass production of the test agent through solid substrate fermentation and its field potential have been determined and discussed in the present communication.

Materials and Methods

Isolation of the pathogen

During the surveys conducted in the different districts of Haryana in years of 2012 to 2014, the different crops, uncultivated fields, vacant lands, and road sides showed high infestation of parthenium. It mainly causes heavy loses in the crops of wheat, sugarcane, mustard, sorghum, onion and garlic (Kaur *et al.*, 2017). Diseased leaves were collected in polythene bags and brought to the laboratory for study of symptoms, isolation and pathogenicity test of the causal agents (Kaur and Aggarwal, 2015).

Leaves collected from different regions with leaf spots were washed under tap water to remove

soil particles. 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 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 °C (Kaur and Aggarwal, 2015). PeDA medium consisted of fresh parthenium leaves extract 200 g; dextrose 15 g; agar-agar-20 g and distilled water 1 L.

Morphological identification of pathogens

The isolates were grown on PDA at 25±2 °C temperature 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, 1971, 1976).

Molecular identification of pathogens

Fungal pathogen was molecularly characterized from the CABI International Mycological Institute and by 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 µg 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 at 95 °C for 1 min, 55 °C and 72 °C for 1 min each were performed, finishing with a 10-minute 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 2000 bp were sequenced by using 2 primers. 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).

***In vitro* pathogenicity tests**

Healthy leaves of parthenium were used for inoculation. These 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, taken from 5 days old colony, were placed on injured and uninjured portions. The inoculated leaves were kept in sterilized moist chambers and incubated at 25±2 °C. Regular observations for the appearance of symptoms were made after 3 days of incubation (Aggarwal *et al.*, 2014).

Mass production

For the fermentation process, solid substrates *viz.* gram bran, sugarcane bagasse, wheat straw, wheat bran, parthenium leaves (chopped and powdered) and wood shavings were employed.

Solid substrate fermentation

Each substrate of 250 g was soaked overnight in water and then kept for drying. These moistened substrates were dispensed in plastic bags and autoclaved at 121 ^{o}C for 15 min, inoculated with two discs of 8 mm diameter from 7 days old actively growing culture of the test fungi in sterilized conditions. These bags were closed with the help of cotton plugs in order to avoid contamination and were incubated in growth chamber for 21 days at 25±2 °C in a Biological Oxygen Demand incubator, as according to Pandey *et al.* (2001). Mycelial growth and coverage on each of the substrate was recorded after required period of incubation on the basis of visual observations, respectively. Conidia were harvested after 21 days by suspending the substrates in 250 mL sterilized distilled water and 0.02% Tween 80. The suspension was filtered through a double layered muslin-cloth and numbers of conidia were determined microscopically with a haemocytometer.

Bioactivity assessment

The viability of the mass produced conidia was assessed under pot house conditions. The seedlings of targeted weed were raised in plastic pots, filled with sterilized soil, sand and peat in the ratio of 1:1:1. Inoculum was prepared by growing the fungi on different substrates, mycelial and conidial mass was harvested with sterile distilled water and then scraping the mass with a sterilized spatula. Inoculum concentration was adjusted to 6×10^4 spores mL^{-1} using haemocytometer and 1 mL of Tween 80 was added as an adjuvant. Inoculum was applied to the plants within 2 h 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 (Aneja *et al*., 2000).

Results and Discussion

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 and PeDA yielded four different colonies of fungal pathogens and microscopic study revealed that the pathogens belong to the genus *Alternaria*. The results of the molecular identification (ITS rDNA sequence analysis) showed that isolates belonged to different strains of *Alternaria macrospora viz. A. macrospora* MKP1 (Accession no. KM186140), *A. macrospora* MKP2 (Accession no. KM213867), *A. macrospora* MKP3 Accession no. KM514668), *A. macrospora* MKP4 (Accession no. KM514669).

When all the four strains were tested for *in vitro* 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 been reported for the first time on parthenium from India.

Screening of solid substrates

The results showed that there was a significant variation in mycelial colonization of agro wastes. Extensive colonization was observed on parthenium leaves and wheat bran. The highest mycelial coverage of the MKP1 (85%) and MKP3 (82%) was recorded on wheat bran while MKP2 (82%) and MKP4 (88%) showed the highest growth on parthenium leaves (Fig. 1). Gram bran and wood shavings did not support any mycelial colonization for all the tested pathogen. Sporulation was maximum on wheat bran for all the tested pathogens and the highest sporulation was observed in MKP2 (24.15×10^4 mL^{-1}) followed by MKP1 (20.56×10^4 mL^{-1}), MKP3 (18.4×10^4 mL^{-1}) and MKP4 (17.4×10^4 mL^{-1}) (Fig. 2.). The highest percent infection was observed in wheat bran for MKP1 (71%), MKP3 (65%) and MKP4 (51%) and parthenium leaves for MKP2 (79%). The bioactivity assessment results showed that pathogenicity of the test pathogen was in proportion to sporulation i.e. both sporulation and seedling mortality was maximum with wheat bran and parthenium leaves (chopped) as shown in Fig. 3. Therefore, these two substrates were considered to be the most suitable for mycoherbicide production against parthenium weed.

During solid substrate fermentations, growth and sporulation of mycoherbicide was affected by the nature of substrates. Parthenium leaves and wheat bran followed by wheat straw and sugarcane bagasse showed successful colonization of fungal mycelia and the least mycelial growth was observed in gram bran and wood shavings. Whereas, wheat straw followed by parthenium leaves and sugarcane bagasse were found to be good substrates for spore production and gram bran and wood shavings did not support much sporulation. Pathogenicity was recognized on the basis of symptoms of infection appeared *viz.* wilting, chlorosis, severe necrosis and ultimately death of the seedlings. During the present study, it was also found that seedling mortality of the targeted weed was directly proportional to the sporulation of the test strain. Several low cost media have also been used earlier by many other workers. Morin *et al*. (1990) reported significant variations in growth and sporulation by solid substrate fermentation of *Phomopsis convolvulus* for field bindweed. Siddiqui and Bajwa (2008) used various agrowastes as substrates for the mass production of *Alternaria alternata* isolates effective against *Rumex dentatus* and *Chenopodium album*. Zambare (2010) used various agricultural residues for the solid substrate fermentation of *Aspergillus oryzae*. Weed bran has been a highly reported substrate promising results, among the various agro-industrial wastes used. Widespread suitability of wheat bran may be due to the presence of sufficient nutrient and its ability to remain loose even in moist conditions, thus providing a large surface area.

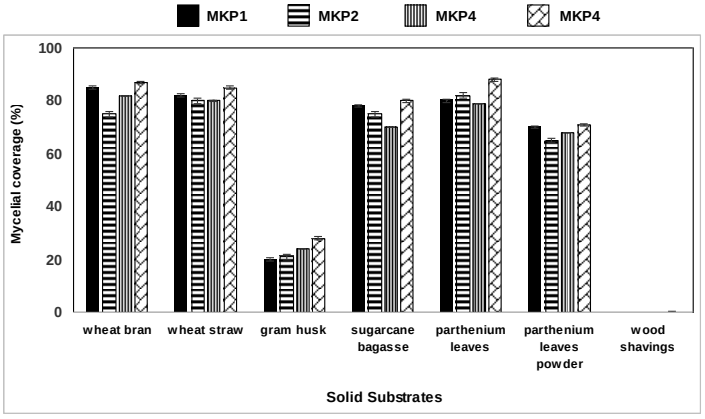

Fig. 1: Effect of various solid substrates on growth of *Alternaria macrospora* isolates.

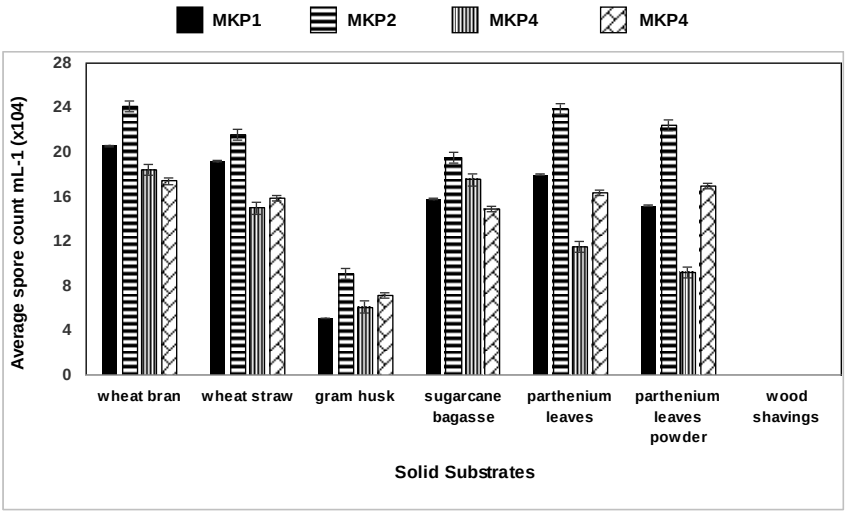

Fig. 2: Effect of various solid substrates on sporulation of *Alternaria macrospora* isolates.

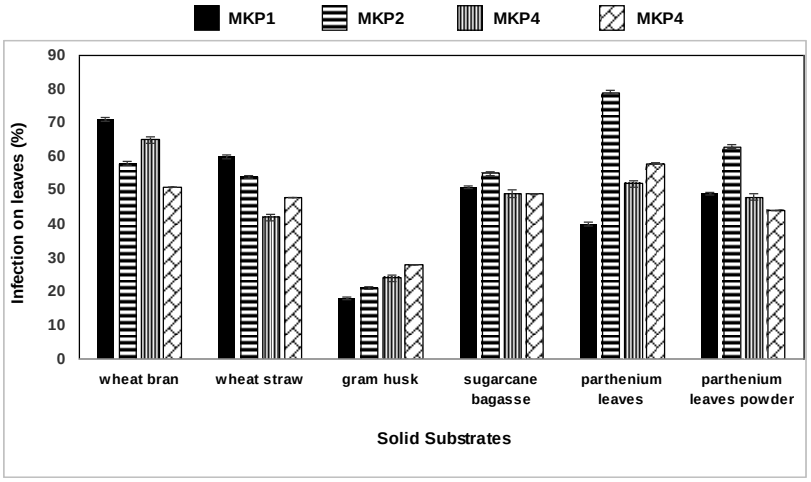

Fig. 3: Effect of various solid substrates on pathogenicity of *Alternaria macrospora* isolates.

Conclusion

The present work is the preliminary study of the screening of various agrowastes as substrates for production of cost effective mycoherbicide and it was found that wheat bran and parthenium leaves proved ideal substrates for growth, sporulation and percent infection of the test pathogens which has not been established by the other selected substrates. The bioactivity test of the mass produced mycoherbicide against parthenium revealed that the herbicidal efficacy of the test pathogens was not affected during mass culturing and causes significant mortality against the weed host.

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