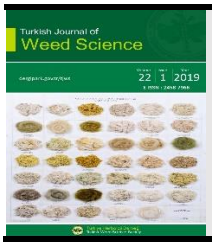

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Araştırma Makalesi / Research Article

Optimizing conditions for growth and sporulation of *Alternaria macrospora* MKP1: a biocontrol agent of Parthenium weed

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ABSTRACT

During a series of surveys for natural enemies of *Parthenium hysterophorus*, a leaf blight pathogen was isolated from the affected parts of the parthenium following the standard isolation techniques using potato dextrose agar (PDA) media. Koch's postulates were performed and found satisfactory for the isolate and proved to be pathogenic to this weed. On the basis of cultural, morphological and molecular characteristics, the pathogen was identified as *Alternaria macrospora* MKP1. The growth of the fungal pathogen is known to influence by environmental factors such as temperature, relative humidity and pH. Therefore, the main objective of the study was optimization of cultural conditions for the growth and sporulation of *A. macrospora* MKP1. The results of the present investigation indicated that physical factors greatly affected the growth and sporulation of the pathogen.

Key Words: *Alternaria macrospora* MKP1, Biocontrol, *Parthenium hysterophorus*, Relative humidity, Sporulation

INTRODUCTION

Parthenium hysterophorus L. (Asteraceace: Heliantheae), commonly known as parthenium, white top, congress grass, feverfew or carrot weed, is one of the worst weeds, threatening natural ecosystems and agro-ecosystems in over 30 countries worldwide (Adkins and Shabbir, 2014). Parthenium has proved a challenge, because the conventional means of its control have failed due to their innate drawbacks (Aggarwal et al., 2014). The use of native fungal pathogens as biological control agents is an alternative or complementary tactic to reduce herbicide inputs (Kadir and Charudattan, 2000). Much work has been carried out on the use of indigenous fungal plant pathogens as biological agents for weed control (Siddiqui et al., 2009; Kaur et al., 2014). Biological, technological and commercial perspectives of this concept are well documented in various publications (Singh et al., 2017). The mycoherbicidal potential of fungus depends on its best growth on synthetic media, which facilitates the mass production of infective stage of fungus, either spores or vegetative mycelium. It is essential to have a clear understanding of the conditions which influence growth and sporulation of the fungus. The fungal pathogen was cultured on different media and subjected to various regimes of pH, temperature and relative humidity to evaluate the best appropriate interactive conditions and to investigate the epidemiology of the pathogen. Present research depicts the role of different factors to understand ecological survival of pathogen which will be helpful in management of parthenium weed.

MATERIALS AND METHODS

Isolation and identification of the pathogen

Surveys were conducted to search naturally occurring fungal pathogens on *Parthenium hysterophorus* weed in different districts of Haryana. 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). Diseased leaves collected from different regions 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. The isolates were grown on PDA and PeDA 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). For molecular characterization fungus genomic DNA samples were extracted using an InstaGenetm Matrix (BIORAD). 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 test

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 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., 2000).

Optimization of cultural conditions

Optimization of cultural conditions was done using ‘one variable at a time’ approach. To determine the effect of various parameters on growth and conidia production, both solid and liquid media were used.

Effect of media

To see the effect of different media on the growth and sporulation of isolated pathogen, eight media include: 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 and broth of the same was used for liquid medium. The active mycelial growth rates were observed after 5 days of inoculation on every medium.

In solid media

15 ml of a prepared media was poured into each sterile Petri plates and allowed to solidify. The plates with solidified medium were kept in an inverted position for 24 hrs to remove the thin film of water from the surface. Mycelial discs of 8 mm diameter of the pathogen cut from the periphery of seven days old cultures and were placed in the center of each plate and were incubated at 25±1 ºC for 7 days. Fungal growth was determined by calculating the area of radial growth for each colony (Abbas et al., 1995). Conidial concentration was determined by using hemocytometer (Tuite, 1969).

In broth

25 ml of broth was taken in each 100ml Erlenmeyer flask and sterilized at 121 ºC for 15 minutes. Flasks were inoculated each with an inoculum disc of 8mm diameter and incubated at 25 ºC for 7 days. The experiments were conducted in triplicates. For measuring dry mycelial weight, mycelial mats were harvested on pre-weighed Whatman filter paper No. 1, dried at 40 ºC to constant weight. Dry weight of the mycelium was then calculated. Conidial concentrations at different broth were measured using hemocytometer.

Effect of temperature

PSA media and PS broth was used for the optimization of different physical factors on the growth and sporulation of fungus isolate. Experiments conducted in triplicates for each physical factor. The effect of temperature was studied in both solid and liquid media at different temperature conditions i.e., 5 ºC, 15 ºC, 25 ºC, 35 ºC and 45 ºC. The temperature was maintained in BOD incubator. The pH and relative humidity maintained at 6.5 and 100% respectively.

Effect of pH

The effect of pH was optimized by preparing the solid (PSA) and liquid medium (PSB) with the different pH ranges i.e. 3.5, 4.5, 5.5, 6.5 and 7.5 with relative humidity 100% and temperature 25 ºC.

Effect of Relative humidity

Moist chambers of different relative humidities were prepared using standard aqueous inorganic solutions. Different solutions used were: Na_2HPO_3 (93% relative humidity); KCl (85% relative humidity); NaCl (75% relative humidity); $Ca(NO_3)_2$ (50% relative humidity); and pure water (for 100% relative humidity) (Aneja, 2003). Plates of potato sucrose agar medium with pH 6.5 were prepared by pouring 15-20 ml medium into Petri plates. Saturated solutions of different relative humidity were poured aseptically into the lid of the PSA plates. Plates were incubated at 25 ºC for 2 days to allow the agar medium to equalize with desired relative humidity. After 2 days, agar plates were inoculated with the disc of the fungus and moist chambers were incubated for 5-7 days at 25 ºC.

RESULTS

The infected leaves after surface sterilization places on PDA media plates and yielded a fungal pathogen. The microscopic examination revealed that the pathogen belongs to the genus *Alternaria* species. Molecular analysis of the ITS1-5.8S-ITS2 rDNA region was carried out to confirm the species identity of the pathogen and the results of the molecular identification (ITS rDNA sequence analysis) confirmed its identity as *A. macrospora* strain MKP1. The gene sequence of the pathogen has been deposited to the NCBI gene bank with accession number KM186140 and was compared with other species of *Alternaria* spp. which were deposited in gene bank (Kaur et al., 2016).

Pathogenicity test

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 pathogens to *P. hysterophorus* and completing the Koch's postulates.

Growth and sporulation on different media

All the culture media tested for the growth of fungal pathogens supported the growth of test pathogen to various degrees. *A. macrospora* MKP1 showed excellent growth on PSA. PSA had the highest mycelial growth (6.68 cm) after five days. The mycelia of pathogens increased till the end of the experiment on PSA. The growth was good on PDAY, PeDA, PDA, MEA and CDA and lowest on SDA and NA (Table 1). Difference in surface and reverse coloration of fungal colonies was distinct on all the growth media. Similar results were observed in liquid media. Maximum dry weight of *A. macrospora* MKP1 (0.43 gm) was recovered at Potato Sucrose broth after five days.

Sporulation was best on PSA (19.43 $x10^4$/ml) followed by PDAY and PDA. The pathogens sporulate well on PeDA, MEA, CDA and SDA media. Poor sporulation was observed on NA (Table 1). If we consider both the parameters i.e., growth and sporulation which are the prerequisites of any mycoherbicide, for inoculum preparation, all the pathogens should be grown on PSA medium. Contrarily, in liquid media sporulation was nil in all tested broth.

Effect of Incubation temperature, pH and relative humidity on the growth/sporulation of selected fungal pathogens

The behavior and physiology of every pathogen during all developmental stages is largely determined by temperature, pH and relative humidity. Metabolic rate, nutrition and growth rate of pathogens can be correlated to physical factors like incubation temperature, incubation time, pH and relative humidity. Consequently, sporulation of pathogen occurs within a definite temperature range and a particular relative humidity, which can be experimentally determined. This serves as a basis from which models that estimate growth, development and reproduction of pathogen can be formulated. Such studies on temperature and relative humidity dependent are therefore important for understanding better pest management and biological control of weeds with fungal biocontrol agents. Temperature and relative humidity are the most important tools for determining the efficacy of a mycoherbicide because these two parameters govern the growth, sporulation and pathogenicity of any pathogen to be used as a biocontrol agent.

Table 1. Experimental conditions for optimization of growth and sporulation in *A. macrospora* MKP1

Parameters	***Alternaria macrospora* MKP1**							
Medium[1]	**PDA**	**PDAY**	**SDA**	**PeDA**	**MEA**	**PSA**	**CDA**	**NA**
Colony diameter (cm)	6.60*±0.21**	6.45±0.18	5.24±0.03	6.59±0.45	5.68±0.08	**6.68±0.28**	5.02±0.28	3.11±0.17
Sporulation (x10^4/ml)	15.72 ±0.01	17.95±0.18	10.24±0.13	16.59±0.45	15.68±0.08	**19.43±0.05**	7.02±0.12	5.11±0.17
Broth[1]	**PDB**	**PDBY**	**SDB**	**PeDB**	**MEB**	**PSB**	**CDB**	**NB**
Dry mycelial wt.(gm)	0.21±0.01	0.37±0.15	0.13±0.15	0.36±0.15	0.18±0.00	**0.43±0.15**	0.14±0.15	0.09±0.15
Temperature[2]	**5 ˚C**	**15 ˚C**	**25 ˚C**		**35 ˚C**		**45 ˚C**	
Colony diameter (cm)	3.11±0.03	4.07±0.02	**6.70±0.01**		3.07±0.02		0.00±0.00	
Sporulation (x10^4/ml)	1.11*±0.01	1.27±0.01	**19.49±0.05**		10.14±0.05		0.00±0.00	
Dry mycelial wt.(gm)	0.11±0.01	0.27±0.15	**0.44±0.15**		0.14±0.15		0.01±0.00	
pH[3]	**3.5**	**4.5**	**5.5**		**6.5**		**7.5**	
Colony diameter (cm)	4.56±0.53	5.71±1.17	6.63±0.44		**6.81±1.23**		6.54±0.87	
Sporulation (x10^4/ml)	6.56±0.53	9.71±1.17	9.63±0.44		**20.81±1.23**		17.54±0.87	
Dry mycelial wt.(gm)	0.31±0.53	0.36±0.15	0.35±0.15		**0.46±0.15**		0.31±0.53	
Relative humidity[4]	**100%**	**93%**	**85%**		**75%**		**50%**	
Colony diameter (cm)	4.28±0.53	**6.85±1.17**	3.50±0.44		2.86±1.23		1.94±0.87	
Sporulation (x10^4/ml)	19.62±0.65	**26.23±1.26**	12.12±0.61		11.24±1.19		7.36±1.23	

*Values, including diameter of the disc (8mm), are means of three replicates; **-Standard deviation; **Conditions**: [1] Temperature-25 ºC, pH-7, RH-100%; [2]Media- PSA and PSB, pH-7, RH-100%; [3]Media- PSA and PSB, Temperature-25 ºC, RH-100%; [4] Temperature-25 ºC, pH- 6.5; Media-PSA

Effect of Incubation temperature on growth and sporulation

In liquid medium

Maximum dry weight of *A. macrospora* MKP1 was recovered at temperature 25 ºC after five days. At this temperature highest biomass was shown by *A. macrospora* MKP1 was 0.44 gm. *A. macrospora* MKP1 can grow at temperature range in between 15 ºC to 25 ºC above and below the optimal range of temperature fungi shows poor growth and sometimes mortality may occur. The isolate showed maximum biomass production at 25 ºC therefore this temperature can be used for incubation of this organism (Table 1).

On solid medium

All the temperature regimes tested supported the growth of the fungus except at 45 ºC. It was observed that at 25 ºC the fungi attained maximum growth (6.70 cm) after 5 days of incubation. However, the growth of the fungus started to decline at 35 ºC and almost halted at 45 ºC, as these temperatures did not favor the growth of the fungus (Table 1).

The selected pathogen showed best sporulation (19.49 $x10^4$/ml) at 25 ºC on PSA medium and there was no sporulation at 45 ºC (Table 1). In broth media, sporulation was nil for all tested incubation temperatures. Thus, potential limiting factor for this pathogen is the inability to produce conidia in broth.

Effect of pH on growth and sporulation

Best sporulation (20.81 $x10^4$/ml) of the *Alternaria macrospora* MKP1 was observed at pH 6.5 in agar media whereas in broth culture, sporulation was nil at all tested pH ranges. Best growth of the *Alternaria macrospora* MKP1 was observed at pH 6.5 in both agar media (6.81 cm) and broth conditions (0.46gm).

Effect of relative humidity on growth and sporulation

Relative humidity also plays a key role on the growth and sporulation of fungus and infection of the host, so determining the successful use of fungal biocontrol agents to control weeds. Results revealed that the growth and sporulation were affected a lot with the change of relative humidity. The isolate was capable to grow and sporulate at different levels of relative humidity. Maximum growth (6.85 cm) and sporulation (26.23 $x10^4$/ml) of the pathogen was recorded when the relative humidity was between 93-100% because when pure water or disodium hydrogen phosphite were placed in a petri dish the atmosphere becomes saturated with water vapors providing 100%, 93%, relative humidity respectively (Table 1).

DISCUSSION

The biological control of weeds 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. *Alternaria macrospora* MKP1 was found to be highly aggressive towards parthenium weed and has most of the characteristics that make it a desirable candidate as biocontrol agent of this weed, such as: capable of limiting population of the weed; good sporulation capacity; narrow host range, fast growth rate and hence can be mass produced in a short time. Fungal nutritional requirements are important for successful cultivation. In this study, eight media were tested in order to achieve a favorable basal medium for the improvement of growth and sporulation of *Alternaria macrospora* MKP1. Results from this study have demonstrated that the radial growth rate, sporulation, cell mass production, and colony morphology were greatly influenced by different compositions of solid media. In general, radial growth of *Alternaria macrospora* MKP1 was enhanced when sucrose was present in the medium as a carbon source. The most suitable standard temperature, pH and relative humidity for growth and conidial production of *A. macrospora* was at 28 °C, 6.5, 93% respectively. Similar results for the effect of temperature on growth and sporulation of *Alternaria*, has been studied by several researchers. Neergaar (1945) observed that the optimum temperature for the growth of *Alternaria alternata* was 25 ºC. Similarly, Kamal, (1950) from India reported 25 ºC as optimum temperature for growth of *Alternaria alternata*. Whereas better growth and sporulation of *A. tenuis*, was observed by Tandon, (1961) at 26 ºC. Verma (1963) observed the optimum temperature of 25 ºC was essential for the growth and sporulation of *A. tenuis*. Verma, (1963) observed that optimum pH 6.60 was essential for the growth and sporulation of *A. tenuis*. Saad and Hagedorn (1970) were of the view that minimum, optimum and maximum pH for the growth and sporulation of *Alternaria alternata*, were 4.40, 6.50 and 7.60 respectively. Chettananavar et al. (1987) obtained maximum growth of *A. alternata* at pH 6.50. Our results confirmed the previous findings that temperature and relative humidity are important cultural parameters determining factor for growth and sporulation of fungal pathogen (Aneja and Kaushal, 1998; Aneja et al., 2000).

CONCLUSION

Alternaria macrospora MKP1 exhibited variable response in terms of growth and sporulation, to various employed ranges of media, temperature, pH and relative humidity. It may be concluded that optimal pH (6.5), temperatures (25 °C), and relative humidity (93%) is required for better growth and sporulation. Below or above it, both the

growth and sporulation are affected. The importance of interaction of temperature, pH and relative humidity will be helpful in mass culturing of *Alternaria macrospora* MKP1 and used as mycoherbicide for the management of *Parthenium hysterophorus* under field conditions. The findings of the present work will help in the research being carried out by different authors in the field of weed management.

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