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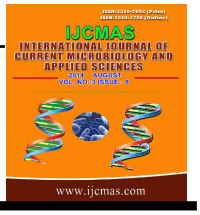

Original Research Article

Biocontrol and Environmental Studies on Paper Degrading Mycoflora Isolated from Sanganer Area, Jaipur, India

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A B S T R A C T

Culture media and temperature significantly affect the growth, sporulation and conidial discharge of any mycoflora. The colony diameter, cultural characteristics (texture, surface and reverse pigmentation) and sporulation of fungi were greatly influenced by the type of growth medium used. The survival period of fungi in relation to different temperature and pH conditions, media and natural habitats differs from species to species.Variation in mycelial growth and fungal sporulation was observed with media tested. Colony radial growth and sporulation of soil fungi was found to be excellent on Potato Dextrose Agar followed by Malt Extract Agar at optimal environmental conditions. In the present study *in vitro* antagonistic activity using the dual culture technique was carried out and showed that antagonist *Trichoderma sp.* grow faster than the isolated fungi and produced inhibition zones thereby limiting the growth of the fungi. Maximum growth inhibition by *Trichoderma sp* (biocontrol agent) were found in the following order i.e. *Fusarium sp*. (46.66%), *A. fumigatus* (42.85%), *A. flavus* (40.00%) and *A. niger* (24.44%), were found with the use of *Trichoderma sp* in dual culture. The present investigation was conducted to examine the effect of different temperature, pH and nutritional media on the mycelial growth and fungal sporulation of three soil fungi i.e. *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus*, isolated from paper and pulp industry and biocontrol studies were also carried out to develop antagonist *Trichoderma sp.* so as to prevent paper degradation by fungi.

Keywords

Mycelial; sporulation; culture media; *Trichoderma*; *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus* biocontrol.

Introduction

The growth of microorganisms in an artificial medium is influenced by several physical and chemical factors. A nutrient material prepared for the growth of microorganisms in a laboratory is called culture media and the nutrient composition of a culture medium plays a major role in microbial growth (Tortora and Funk, 1995). Fungi grow on diverse habitats in nature and are cosmopolitan in distribution requiring several specific elements for growth and reproduction. In laboratory, these are isolated on specific culture medium for cultivation, preservation,

microscopic examination and biochemical and physiological characterization (Northolt and Bullerman, 1982, Kuhn and Ghannoum, 2003, Kumara and Rawal, 2008). A wide range of media are used for isolation of different groups of fungi that influence the vegetative growth and colony morphology, pigmentation and sporulation depending upon the composition of specific culture medium, pH, temperature, light, water availability and surrounding atmospheric gas mixture (Northolt and Bullerman, 1982, Kuhn and Ghannoum, 2003, Kumara and Rawal, 2008). The most important environmental factors governing the growth and sporulation of fungi are temperature and hydrogen ion concentration. A little variation in these factors may induce marked difference in their morphological characters, growth and sporulation. It is an established fact that for each Fungus there is a minimum, optimum and maximum temperature for growth and sporulation (St-Germain and Summerbell, 1996). Due to hazardous effect of chemical fungicides, search for safer alternative to control the pathogen is better choice. This led to trials on the use of bioagents to control the pathogen.

Trichoderma is reported to be one of the most widely distributed soil fungi (Domsch and Gams, 1972). Biocontrol potential of *Trichoderma* has been studied extensively (Chung and Holtink, 1990). *Trichoderma* species produce both volatile and nonvolatile metabolites that adversely affect growth of different fungi. From several studies, it has been confirmed that *Trichoderma spp.* have antagonistic and biologically control potential against a diversity of soil borne pathogens.(Grondona *et al*., 1997; Hanson and Howell, 2004; Bajwa *et al*., 2004). Present study has been carried out to biologically control the isolated soil fungi by using *Trichoderma spp*. as biocontrol agent. With these perspectives, the present study was undertaken to observe the influence of different culture media on the mycelial growth, colony characters and sporulation patterns of fungi isolated from the soil of pulp and paper industry and biocontrol studies were conducted to prevent the degradation of paper.

Materials and Methods

Study Area

The study was carried out in the soil environment of Vedant Gyan Valley, Jharna village, Jaipur, Rajasthan State, India. Jharna is 48 km away from Jaipur and located on east longitude 75°27’38’’ and north latitude-26°49’34’’ and situated on altitude 450 to 500 m above sea level.

Sample collection

Soil samples of 200 g were collected from pulp and paper industry in Sanganer area, Jaipur. The samples were collected with small sterile shovels into sterile plastic containers. The soil samples were sent to the laboratory within 30 min for physical analysis. The pH and temperature of soil samples were determined using digital pH meter and thermometer respectively. Humidity and air pressure was determined by hygrometer and barometer respectively.

Isolation and identification of fungal isolates

Potato Dextrose Agar (PDA) and Sabouraud’s Dextrose Agar (SDA) media were employed for the isolation of fungi by spread plate method using serial dilution technique. All the plates were incubated at 30°C for 7 days. Fungal

isolates were identified by cultural and microscopic characteristics.

Test organisms

Aspergillus niger, A. flavus, A. fumigatus were used in our study to evaluate the effect of different temperature, pH and cultural conditions isolated from paper and pulp industry.

Effect of media

Different agar media namely: Potato Dextrose Agar (PDA), Yeast Extract Agar (YEA), Czapak Dox Agar (CDA), Malt Extract Agar (MEA) were prepared separately and used to evaluate the mycelial growth and sporulation of the fungi. The isolated fungi were inoculated separately in the respective media (triplicates) and incubated at 28^0C temperature for 9 days. Growth and sporulation was noted through macroscopic and microscopic method.

Effect of temperature

Potato Dextrose Broth was used for the study to evaluate the effect of temperature against *A. niger, A. flavus, A. fumigatus*. Isolated fungi inoculated into broth medium in triplicats and incubated at different temperatures 28^0C, 37^0C, 45^0C. After that, growing mycelia mats were harvested and dried to a constant weight. The degree of sporulation of fungi was determined according to Standard Methods (Sharma and Sharma, 2011).

Effect of pH

Potato Dextrose Broth used to evaluate the effect of pH on *A.niger, A. flavus, A. fumigatus*. Isolated fungi inoculated into broth medium (triplicats) and incubated at four different pH 4, 7, 9, 11 . After that, growing mycelia mats were harvested and dried to a constant weight. The degree of sporulation of fungi was determined according to Standard Methods (Sharma and Sharma, 2011).

Antagonistic studies of *Trichoderma* against isolated fungi of paper industry

Antagonism of *Trichoderma sp.* on *A. niger, A. flavus, A. fumigatus and Fusarium sp.* was studied by dual culture technique All the isolates were grown on sterilized standard PDA (Potato Dextrose Agar) at 25ºC in an incubator for 5 days in order to obtain juvenile colonies for the studies of antagonism. After the incubation period of 5 days, five millimeter diameter mycelial plugs of each isolated fungi were placed at the periphery of three different culture plates and on the same day Antagonist *Trichoderma sp*. was placed on the opposite side of the same previous petri- dishes, incubated for 7 days at 28 ± 2^oC. The growth of the pathogen in both the test and control experiments was recorded. The percent inhibition of the isolated fungi was calculated as follows:

The percentage of inhibition (I) = (R1-R2)/ R1 x 100

Where,
I = Percent inhibition
R1 = Radial growth of the isolated fungi in control (mm)
R2 = Radial growth of isolated fungi in Dual culture with antagonist (mm)

And the width of zone of inhibition (ZI) measured as the smallest distance between the colonies in the dual culture plate (Royse and Ries, 1977; Whips 1987;Reddy and Hynes 1993).

Data analysis

Results are given as mean ± standard error (S.E.) of N observations taken in four replicates (n = 4). Data sets were examined by one-way analysis of variance (ANOVA). P-value of less than 0.05 was considered significant.

Results and Discussion

All the four culture media i.e. PDA, MEA, YEA, CDA supported the growth of the three fungi i.e. *A. niger, A. flavus, and A. fumifatus* to various degree at optimal pH and temperature conditons. Colony diameter of fungi was estimated on different nutritional media. In the present study, different culture media and environmental factors affecting the growth and sporulation of the tested fungi under different conditions. In our findings, the diameter of *A. niger* colony on PDA (81.6 mm) followed by MEA, CDA and on YEA. Similarly on another test fungi *A.flavus* colony diameter on PDA was found to be highest among all the media tested. In *A. fumigatus*, excellent growth was reported on PDA media (Table 1) and the dry mycelial mat at different temperatures and pH against tested organisms were shown in Table 2 and Table 3. Among different media tested, *A.niger* and *A. flavus* shows excellent growth on Potato Dextrose Agar as compared to Malt Extract Agar after 9 days of incubation period, while *A.fumigatus* (80.0 mm) shows best growth on Malt Extract Agar (Table 1,Graph 1). In our result, all the three fungi grow on a wide range of temperatures and pH (Table 2, 3, Graph 2, 3). *A. niger* and *A. fumigatus* shows best growth at 45^0C and pH 4 while *A. flavus* best grown at 37^0C and pH 7. Result showed that *Trichoderma sp.* could restrict growth of isolated fungi on Potato Dextrose Agar medium in the dual culture (Table 4). The per cent inhibition of radial growth of tested isolates *A.niger* (24.44%), A. *flavus* (40%), *A. fumigatus* (42.85%) and *Fusarium sp.* (46.66 %) were reduced by *Trichoderma sp.* showed visible inhibition zone. The growth inhibition of pathogenic fungi by dual culture in this study could be due to its fast growing nature, *Trichoderma* species have been successfully used as biocontrol agents due to their high reproductive capacity, efficient utilization of nutrients, and strong aggressiveness against other pathogens. The suitability of a growth medium depends upon the specificity of a fungus under study and the purpose of the experiment (Lilly and Barnett, 1951). Physical and chemical factors have a pronounced effect on diagnostic characters of fungi. Hence, it is often necessary to use several media while attempting to identify a fungus in culture since mycelial growth and sporulation on artificial media are important biological characteristics (St-Germain and Summerbell, 1996). An individual medium shows great role to play in the growth and sporulation of fungi.(Jacques *et al*.,2002)**.** Our findings were similar to Chang *et al.*(2004), who stated that *Aspergillus fumigatus* grows optimally from 37 to 42^0C but can grow at temperatures up to 55^0C. In our result, *A.flavus* grows best at 37^0C, concides with Gadgile and Chavan (2010) who also reported the same results i.e. *A. flavus* grows at 37^0C. Devi *et al*.(2008) also reported that *A.niger* grows at 45^0C. The effect of pH on bioenzyme synthesis has been reported by many authors i.e. pH 4.0 to 5.5 was reported for *A. terreus* and *A. niger* (Garg and Neelakantan, 1981). The nutrient media, temperature and pH is a major factor that influences the growth and sporulation of fungi.

Table.1 Average colony diameter (mm) of fungal isolates on different agar media

S.No.	Agar media	*A. niger*		*A.flavus*		*A.fumigatus sp.*	
		Average fungal colony diameter	Sporulation	Average fungal colony diameter	Sporulation	Average fungal colony diameter	Sporulation
1	PDA	81.6	+4	50.0	+4	62.6	+3
2	MEA	79.3	+3	43.0	+3	80.0	+4
3	YEA	65.0	+2	34.0	+2	58.0	+3
4	CDA	57.6	+2	35.0	+1	50.0	+3

PDA- Potato dextrose Agar, MEA-Malt extract agar, YEA-Yeast extract agar, CDA-Czapek dox agar; +4 excellent growth,+3 good growth, +2 medium growth, +1 poor growth.

Table.2 Average dry weight and sporulation of fungi at different temperatures

S.No.	Temp-erature (^{0}C)	*A. niger*		*A.flavus*		*A.fumigatus*	
		Average Dry weight of mycelium (g)	Sporu-lation	Average Dry weight of mycelium (g)	Sporulation	Average Dry weight of mycelium (g)	Sporulation
1	28°C	10.526	+3	31.551	+3	10.002	+3
2	37°C	15.016	+4	45.197	+4	9.004	+2
3	45°C	16.226	+4	17.262	+2	14.281	+4

Table.3 Average dry weight and sporulation of fungal isolates at different pH

S.No.	pH	*A. niger*		*A.flavus*		*A.fumigatus*	
		Average Dry weight of mycelium (g)	Sporu-lation	Average Dry weight of mycelium (g)	Sporulation	Average Dry weight of mycelium (g)	Sporulation
1	4	11.778	+4	9.879	+2	12.541	+4
2	7	7.294	+3	12.128	+4	10.121	+3
3	9	9.172	+2	10.339	+3	8.969	+2
4	11	7.876	+2	11.58	+3	9.329	+2

Table.4 Radial growth(mm), inhibition (%) and zones of inhibition of pathogen in dual culture with test antagonists.

S.No.	Name of fungi	Radial growth of pathogen in control(mm) (R1)	Radial growth of fungi in dual culture with antagonist (R2)	Percentage of inhibition of radial growth (%)	Width Zone of Inhibition (mm) (ZI)
1	***A.niger***	45	34	24.44	2
2	***A.flavus***	55	33	40.00	4
3	***A.fumigatus***	35	20	42.85	5
4	***Fusarium sp***	60	32	46.66	5

Figure.1 Antagonistic test between *A. fumigatus and Trichoderma sp.*

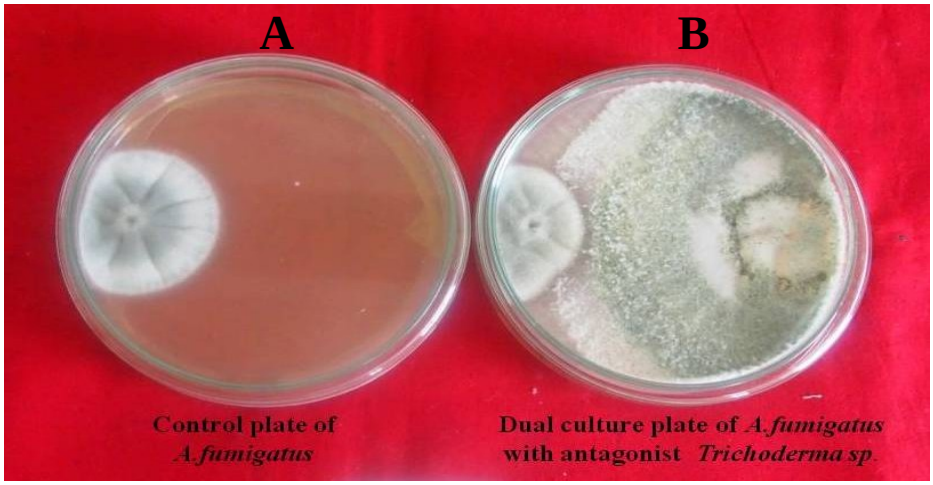


Plate A: control plate of *A. fumigates* and
Plate B: Dual plate culture of *A. fumigates* at the left side and *Trichoderma sp.* at the right side.

Figure.2 Antagonistic test between *Fusarium sp.* and *Trichoderma sp*

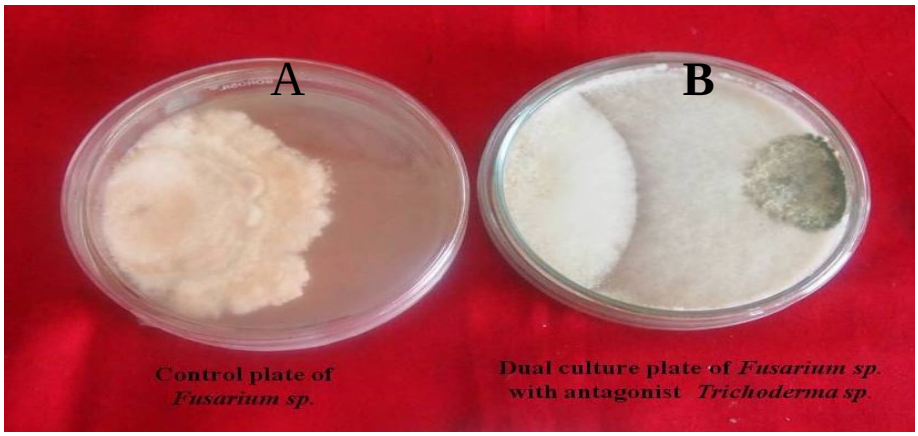
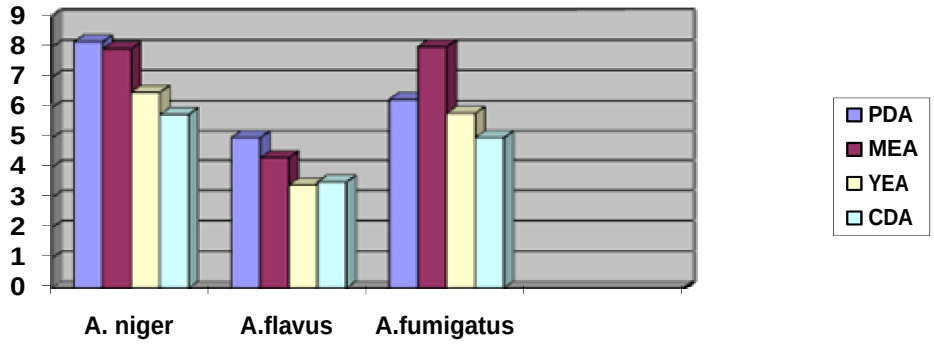
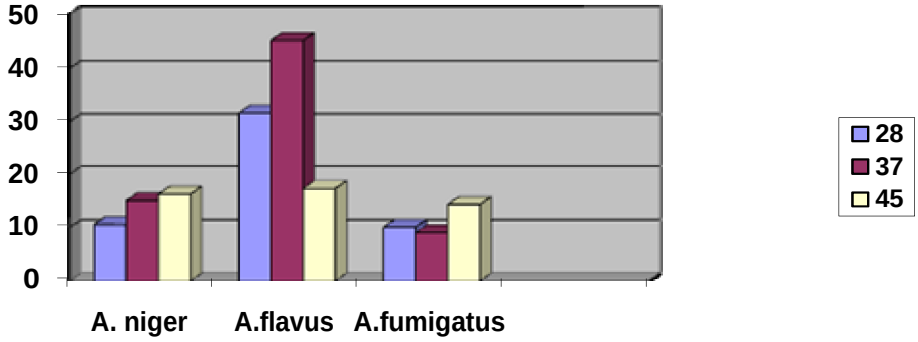


Plate A: control plate of *Fusarium sp* and
Plate B: Dual plate culture of *Fusarium sp* at the left side and *Trichoderma sp.* at the right side.

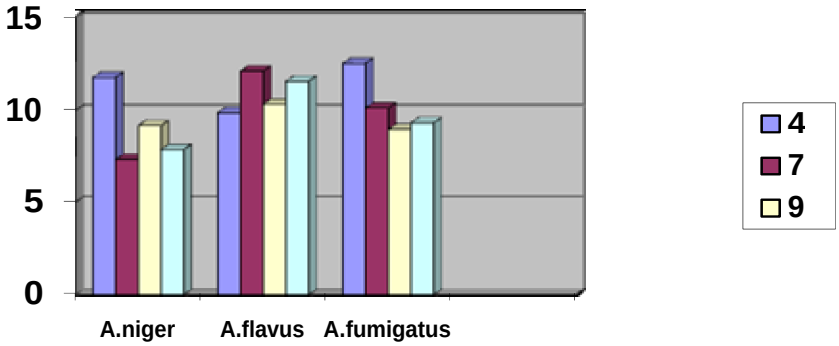
Graph.1 Graph shows the relationship between colony diameter(cm) of fungi and effect of different media



Graph.2 Graph shows the relationship between temperature(°C), and fungal growth(average dry weight in grams)



Graph.3 Graph shows the relationship between pH and fungal growth(average dry weight in grams



It was also found that pH and the temperature of the medium has marked effect on the growth and sporulation of fungi. The present study will help to maintain the fungus in the laboratory condition for preparation of inocula for different studies concerning control of the human pathogen. The study also concluded that the culture media are essential growing factor for controlling the growth and sporulation of human pathogenic fungi and effect of different temperature and pH studies provide the information about a optimum environmental condition necessary for excellent growth and sporulation of fungi.

Biological control of pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods (Baker and Paulitz, 1996). Agarwal *et al*.**,** (2011) also reported the antagonistic properties of *Trichoderma sp*. against *A. flavus* , and *A. fumigatus.* According to Baig *et al*., (2012) *Trichoderma sp*. act as biocontrol agent against the *A. flavus* and *A. niger* .The antagonistic activity of *Trichoderma* sp. against *Fusarium sp* also concides with the result reported by Morsy *et al.,* (2009).

The present study was aimed to determine the excellent media and cultural conditions for the growth and sporulation of paper degrading fungi. To control the degradation of paper, biocontrol agent i.e. *Trichoderma sp*. was used to control the paper degradation which has economic value and importance.

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