Nat. Volatiles & Essent. Oils, 2021; 8(4): 3599-3606

NVEO
Natural Volatiles &
Essential Oils

Isolation, Identification, Molecular And Antifungal Activity Of Aspergillus Flavus From Weed Plant Pergularia Tomentosa L.

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Abstract

Aspergillus flavus is a common filamentous soil fungus. Aspergillus flavus is saprophytic soil fungus that infects and contaminatesPreharvest and postharvest seed crops with the carcinogenic secondary Metabolite called aflatoxin. These common soil fungi can infect the wide range of agricultural crops fields by causing diseases. Due to my research work, I will isolate the Aspergillus flavus from diseased leaves of Pergularia tomentosa L. Weed plant in red gram. The aim of the present study was molecular analysis of the isolated Aspergillus flavus from Pegularia tomentosa L. In this Molecular analysis, The DNA barcoding is the major tool.

Key words: Aspergillus flavus, Aflatoxin, Pergularia tomentosa L., DNA barcoding, red gram.

Introduction

Aspergillus flavus was an opportunistic pathogen on both plants and animals and it's also a saprophytic molds found on worldwide. The disease caused by Aspergillus flavus was the second most frequently isolated pathogen (**David A. Stevens et al, 2021**). This common genus has been classified based on the morphology (Samson RA. 1992) and currently contains over 200 species. Aspergillus flavus was described by Link in 1809 and has been known as an asexual species that only produces asexual spores conidia and asexual fruiting bodies, sclerotia. Aspergillus flavus are present as a saprophytic in soils worldwide and cause diseases on several important agricultural crops such as maize (ear rot), peanut (yellow mold), and cottonseeds before and after harvest **(Klich MA. 2007;**

1. Michailides T. J and Thomidis T., 2007; Yu J et all., 2005). It shows pre and post harvesting infection on many crop fields to cause disease in certain environmental conditions. The Aspergillus flavus were present in many crop fields, seeds, fruits and food items are suspected to infection under certain environmental conditions, And the contamination may occur in storage also (**G. A. PAYNE,2006**). TheAspergilus flavus also cause a serious damage to stored food products such as wheat, rye grains, nuts, spices and peanuts (**Kurtzman et al.,1987; Moody& Tyyler,1990a; Samson et al.,2000; Rigo etal.,2002; Hedayati et al.,2007).** The species of Aspergillus have long been shows the common contaminants of human and animal feeds and first name the genus Aspergillus was given by Antonio Micheli in 1729**(Micheli, P. A., 1729**). Aspergillus produce the most potent naturally occurring carcinogens called as aflatoxins (**Niyo KA.,1990**). The diseased caused by Aspergillus species were most commonly caused by Aspergillus fumigates, and second most frequently was caused by **Aspergillus flavus.** Aspergillus flavus, classified as a separate species but genetically almost identical to A. oryzae, is not used for commercial applications mainly due to its capability of producing aflatoxins. Aspergillus flavus is thought to be predominately a saprophyte that grows on dead plant and animal tissue in the soil. Of all Aspergillus, it is the one most associated with preharvest contamination of certain crops. Because of its small spores and its ability to grow at 37 °C it can also be pathogenic to animals and humans. Infection by A. flavus has become the second leading cause of human aspergillosis (**Krishnan et al., 2009**). Aspergillusare common saprobic molds, which grow in a wide range of natural substrates and climatic conditions,Austwick reported that one conidial head may produce up to 50,000 spores (**Austwick, P. K. C. 1966**.). Although many Aspergillus species are considered pathogens or spoilage organisms, many others are beneficial. Some species are used to prepare fermented foods (**Hesseltine, C. W. 1965)**. The Aspergillus species may also can cause allergenic, toxigenic, and pathogenic to human and animals also,itrepresents a real hazard to many animals' health by produce numerous diseases such as avian aspergillosis and bovina mycotic abortion (**Ainsworth, G. C. et al., 1973**) Aflatoxins which are produced by some Aspergillusflavus species are considered potent carcinogens (Barnes, J. M.1964). The genus Aspergillus was first described by Florentine priest and mycologist P. A. Micheli in 1729 and named based on the structural similarityof its conidiophore structure to the aspergillum (**Badii, F.et al.,1986**).

Material methods

Sampling: The Aspergillus flavus was islolated from weed plant Pergularia tomentosa L.from study area. The Diseased leaves from the weed plant were taken for pathogen isolation. The leaves of the pathogenic plants were washed thoroughly in running tap water to remove unwanted dust or soil particles from the leaves. The infected portions of the leaves were cut into 1.0 to 1.5 cm fragments.

The diseased leaf bites were surface sterilized by 70% ethyl alcohol for 1-2 minutes for 2-3 times and then rinsed in sterile distilled water for three to four times. Then the leaf bits were rinsed in 0.01% mercuric chloride solution for 1 or 2 minutes followed by washing with sterile autoclaved double distilled water for 2 or 3 times. These leaves bites were transferred into Potato dextrose agar (PDA) plates supplemented with 1% streptomycin sulphate (antibiotic) under sterile conditions in an inoculation chamber. After inoculation plates were incubated at 25 ± 2°C for 21 days under a 12 h light/dark photoperiod. The mycelium growth was noticed with in 1 or 2 days after the isolation of the pathogens.The pathogenic genomic DNA isolation.

Genomic DNA isolation: The pathogenic genomic DNA Isolation were done by growing the fungal mycelium in Potato dextrose agar (PDA). For about 3-5 days. Then the growing mycelium was taken into inoculate in 20ml of potato dextrose broth (PDB), Then they are transfer into incubation chamber for about 5 days.

After 5 days of the incubation, the fully developed mycelium shows full development. And this fully developed mycelium was filtered by using the muslin cloth with distilled water. This filtered mycelium was used for the extracted the genomic DNA.The classical sets of primers for amplifying the targeted gene were selected, they are IST-1 ANS IST-4. The DNA amplification and sequencing were carried out with using a program according ZR fungal DNA MINI PRE. The PCR products were sequenced by MTCC gene bank Chandigarh. To avoid errors in sequencing, DNA strands were spliced using forward and reverse prime.

Preparation of the plant extracts:

The dried leaves were grinded into a fine powder and it was preserved in an ait tighter container for further use. About 50 grms of coarse powder of osmium sanctum was weighed and sued for cold maceration method by different solvents like ethyl acetate, chloroform, methanol, and distilled water. The coarse powder of plant extract was soaked in 250 ml in each solvent for 24 hrs at room temperature at shaking condition. The extract was filter by Whatman no.1 filter paper. The finally filtered extract collected separately. The extract was filtered extract of 100mg of each extract was weighed and dissolved in 1 ml of 5% DSMO and it can be used for further analysis.

Antifungal activity:

The Antifungal activity from extracts of plant was performed by agar well diffusion method. For this activity, the stock cultures of the fungi were maintained by inoculating in broth media and allow to grow for 72hrs at 27^{o}C. The potato dextrose agar was prepared and each Petri plate contain 10^3 (0.1 ml) spores/ml.The well with6mm diameter bored by using sterilized cork brer and then filled with

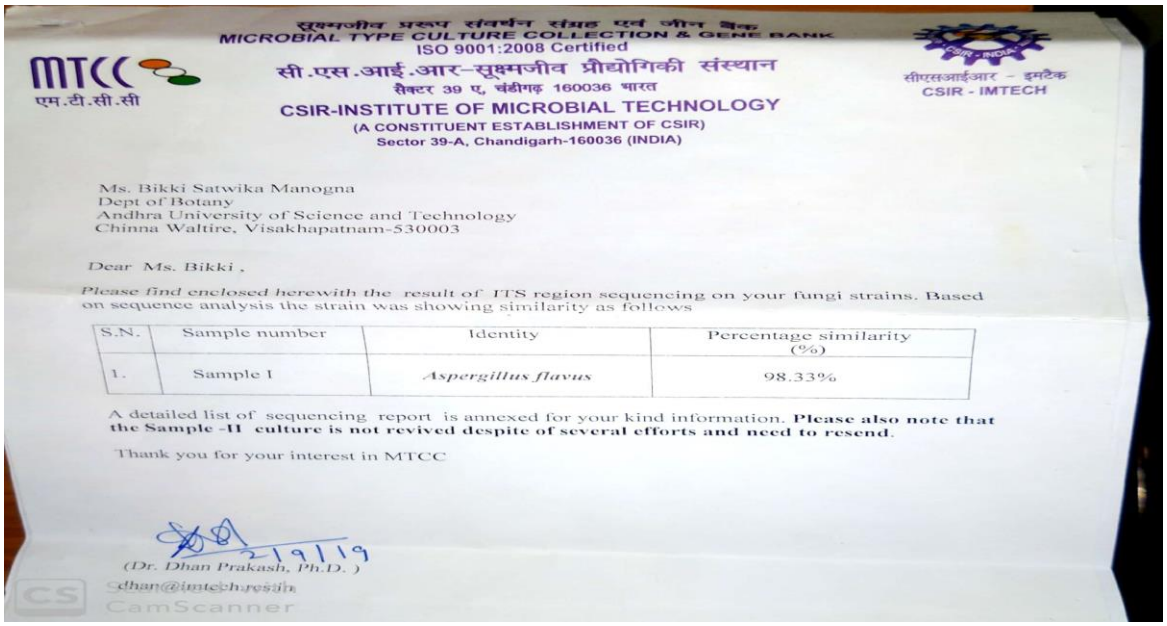
test samples of 100mg/ml concentration. The antifungal disc fluconazole (25mg concentration)and DMSO were also placed for positive and negative control. And all the Petri plates were incubated at incubation chamber for 5-7 days at 27^0C and then the diameter of the zone of inhibition were calucated.

Results

Isolation and Morphology of fungal pathogen

The sample collected from the diseased weed plant of Pergularia tomentosa L. and the isolated fungal pathogen was Aspergillus flavus. The cultured Petri plate shows the spores formation within 5-7 days after inoculation. The isolated pathogen was identified by MTCC gene Bank Chandigarh, and conformed pathogen was Aspergillus flavus. The isolated pathogen shows colonies of powdery yellow- green spores on the upper surface and reddish – gold on the lower surface. The hyphal growth was observed as thread -like branching, septate and hyaline and produces mycelia.

The isolated pathogen was identified by MTCC gene bank Chandigarh. The identified pathogens were Aspergillus flavus (Fig:1).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Dear Ms. Bikki ,

Please find enclosed herewith the result of ITS region sequencing on your fungi strains. Based on sequence analysis the strain was showing similarity as follows

S.N.	Sample number	Identity	Percentage similarity (%)
1.	Sample I	*Aspergillus flavus*	98.33%

A detailed list of sequencing report is annexed for your kind information. **Please also note that the Sample -II culture is not revived despite of several efforts and need to resend.**

Thank you for your interest in MTCC

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CamScanner

Fig : 1 The isolated pathogen Aspergillus flavus identified by MTCC gene bank Chandigarh.

Molecular and Phylogeny analysis results

The internal transcribed region (ITS) is the most frequently sequenced genetically marker of the fungi. The sequence of the ITS region of the cultured isolate Pergularia tomentosa were successfully amplified by using the ITS region of 16S rRNA sequence 55'GGAAGTAAAAGTCGTAACAAGG 3 respectively the size of the ITS regions should be between 500-600bp.For analysis of the molecular

analysis other species of the Aspergillus flavus will be taken from NCBI gene bank. After analysis of the tested pathogen is Aspergillus flavus with 98% similarity.

The ITS regions of the tested pathogen show the molecular sequence of ITS region of the 16 S rRNA is given below.

ITS4R +ITS5F SHOWS 98.33% sequence similarity to Aspergillus flavus

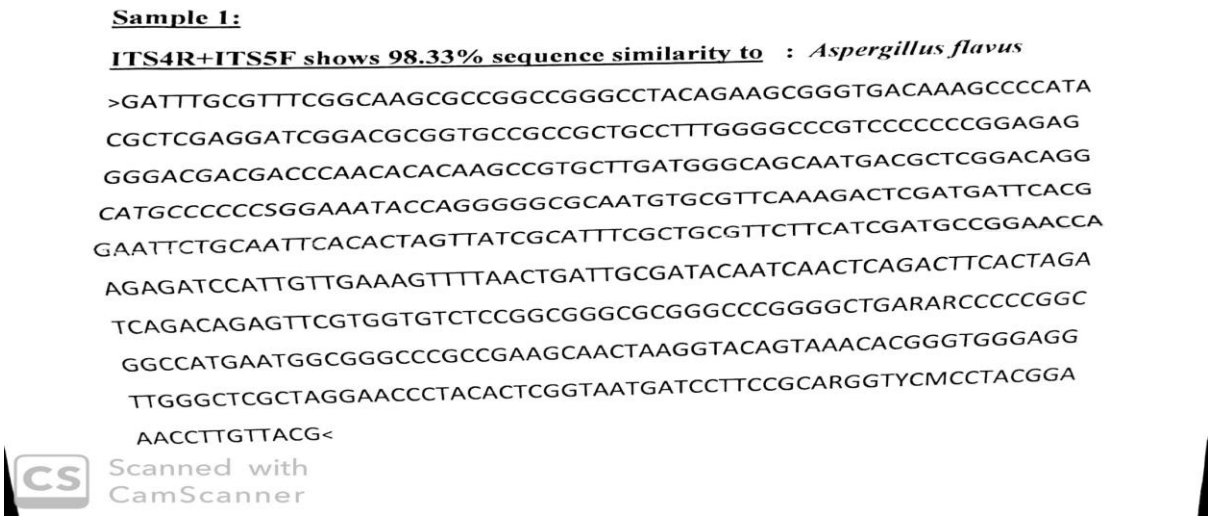

Fig : 2 ITS5F and ITS4R region sequence of the pathogen Aspergillus flavus

Antimicrobial result:

The antimicrobial activity was calucated by using Zone of inhibition. The Zone of inhibition is used to evaluated the antifungal activity of the plant extracts. The extracts of the medicinal plant Osmium sanctum L. Leaves showed effective antifungal activity to tested fungal organisms. In my study results showed that the Ethyl acetate extraction of the plant extracts shows the maximum inhibition ranges from12.05±0.3 fallowed from11.00±0.5mm and 10.11±0.2 in Chloroform and methanol extracts. The positive control of the Antifungal disc shows the highest zone of inhibition and negative control DSMO don't shows any zone of inhibition, they are listed in table :1

Extract	Con (mg/ml)	A. flavus
Methanol	100	11.00±0.5mm
Ethyl acetate	100	12.05±0.3
Chloroform	100	10.11±0.2

DMSO	100	-
Fluconazole	25mg /disc	15.78±1.72

Discussion:

Aspergillus Flavus species are present in soil and contaminate a wide variety of agriculture products in the field, storage area and processing plants and during distribution. A. flavus, A. parasiticus and A. nomius are the only molds they produce the alfatoxins.Along with aspergilli, the A. flavus genome has recently sequenced (Cleveland, T.E. et al,2009; Galagan JE, 2005). The genome sequence supports the view that A. flavus and A. oryzae are the same species with the latter representing a domesticated clade of A. flavus. In history, many documents shows that the medicinal plants and extracts like tea tree oil, clove, garlic and need shows the best antimicrobial properties (Hoffman, D.L.et al, 1987). Scientists have examined several pharmacological effects of Tulsi (Osmium sanctum) products which were obtained by different extractions method (Prakash and Gupta, 2005)

Conclusion:

flavus is the plant, animal and human pathogen causing yield losses and detrimental health impacts in animals and human. The fungus produces potent natural carinogenic compounds called alfatoxins. The genome sequence supports that the A. oryzae and A. flavus are the same species with latter representing a domesticated clade of A. flavus.

From the results of antimicrobial was concluded that Ocimum sanctum possesses sufficient antifungal properties towards aspergilus flavus, in these tested solvents the ethyl acetate shows the best results compared to the other solvents.

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