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Isolation and screening of aflatoxigenic *Aspergillus flavus* from the wheat rhizosphere of Birbhum, West Bengal and its growth inhibition through solvent extract of medicinal plants

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Wheat rhizosphere soil of Birbhum district was collected for isolation of *Aspergillus flavus* L. Ex. Fries and also analyzed for variations in pH, EC, and organic carbon content. Present study showed that soil pH, EC and organic carbon was found to vary in different sampling sites and a maximum was measured in Suri-1(6.02), Rampurhat-1 (0.43) and Suri-4 (0.61) sites respectively whereas minimum was noticed in Bolpur-3 (5.13), Bolpur-5 (0.2) and Rampurhat-4 (0.42) site. Altogether 125 *A. flavus* isolate was isolated from all sites of which maximum (46) were isolated from soil of Rampurhat sub-division followed by Suri sub-division (45) and minimum (34) from soil of Bolpur subdivision. During screening of aflatoxigenicity, only 55 were noticed as aflatoxin producer in SMKY medium. The most potent type of aflatoxin i.e., aflatoxin B_1 was quantify and found maximum (1891µg/l) in BAF-6 isolates and minimum (38µg/l) was noticed in SAF-13 isolates. Five medicinal plant extract was screened for antifungal efficacy of which ethyl acetate extract of *Mentha piperita* showed maximum (30 mm) zone of inhibition followed by *Justicia adhatoda* (23 mm) against highly toxigenic *A. flavus* (BAF-6) isolates in agar well diffusion plate. Ethyl acetate extract of other plant and other solvent of all selected plants showing negligible efficacy. MIC and synergistic efficacy of these two plants was also evaluated and find promising effects.

Key words : *Aspergillus flavus,* aflatoxin, wheat rhizosphere, medicinal plant extract, growth inhibition, minimum inhibitory concentrations.

INTRODUCTION

Soil contains diverse micro-organisms, of which fungi are one of the common microbes, and its diversity and relative abundance varied in different soil types as well as agro-climatic conditions (Prashar *et al.* 2014). Plant root produces various exudates in the rhizosphere regions, creates variations in microbial population than non-rhizosphere bulk soil (Bending, 2003). Due to specificity of root exudates of each plant, rhizospheric fungi and its population varied in different plants from germination to seed settings (Morgam *et al.* 2005). Out of several fungi occupied in wheat rhizosphere, *Aspergillus flavus* is one of the common fungi and is also acts as inoculums for pre-harvest aflatoxin contamination in wheat grains. It is well established facts, that all the isolates of *A. flavus* are not aflatoxigenic and only toxigenic strains are responsible for aflatoxin production. Several climatic factors as well as edaphic factors regulates fungal richness in soil as well as rhizosphere of which soil pH is one of the important abiotic factors influences microbial community and diversity (Kemmitt *et al.* 2006).

Out of several forms of aflatoxins, aflatoxin B_1 is most potent carcinogenic, mutagenic, and its quantity is also higher than other aflatoxins. Due to high frequency of aflatoxigenic fungi in soil, pre-harvest as well as storage contamination of foods with aflatoxin cannot be completely ruled out however recent past several scientist applied their ideas through the use of physical, chemical, botanical and biological methods and successfully achieves some goal (Dorner 2008; Ferreira *et al*, 2013; Nesci *et al.* 2016,). Moreover any single strategies may not suitable, cost effectives and sustainable for all the foods crops hence requires

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some more suitable approaches. Keeping the facts in mind, present investigation was focused to isolate *A. flavus* strains from wheat rhizosphere cultivated in different part of Birbhum district and also determine their relationship with soil pH, EC and organic carbon. Efficacy of different solvent extract of five medicinal plants was screened for growth inhibition of highly toxigenic *A. flavus* isolates as well as determines their minimum inhibitory concentrations (MIC) and synergistic effects.

MATERIALS AND METHODS

Isolation of A. flavus and determination of aflatoxigenicity

Wheat cultivated subdivisions of Birbhum district of West-Bengal (India) viz., Bolpur, Rampurhat and Suri were selected and rhizosphere soil sample was collected from five different sites of each subdivisions. Serial dilution of each rhizosphere soil was made and 10^{-4} dilution was spread on solidified PDA Petri-plates. After 7 days of incubation, cottony yellowish green colony of *A. flavus* was isolated, purified and identified with the help of manual of soil fungi (Gilman, 1957). Aflatoxin production ability of *A. flavus* was determined on SMKY medium (Diener and Davis, 1966). Qualitative analysis of aflatoxin was done using TLC methods (Reddy *et al.* 1970) however quantification of aflatoxin was made using spectrophotometry methods of Nabney and Nesbitt, (1965). Chemical confirmation of aflatoxin was done by the methods of Stack and Pohland, (1975).

Determination of pH, electric conductivity and organic carbon

Rhizosphere soil pH was determined in digital pH meter. For this 10gm of soil sample was taken in 100 ml of plastic beaker and 20 ml of double distilled water was added. After 1-2 min of stir with glass rod, pH was measured (Jackson, 1973). For determination of electrical conductivity (EC) additional 30ml of distilled was added in same pH set and kept in room temperature for over-night and EC was measured by using conductivity meter (Chopra and Kanwar, 1991). For determination of organic carbon (OC) of soil, method of Walkley and Black (1934) was followed. In this method, 0.5gm of soil sample was taken in 250ml of conical flask and added 5ml of 1N potassium dichromate as well as 10 ml of sulphuric acid. The conical flask is allowed to stand for 30 min in dark. After that 100ml of distilled water, 5ml of orthophosphoric acid (85%) and 0.5ml of diphenyl indicator was added in sequence. The colour of solution becomes changed to dark brownish and is titrating with 0.5N ferrous ammonium sulphate till colour becomes changes up-to bottle green colour. All analysis was made in triplicate and statistically analyzed.

Effect of medicinal plant extract against toxigenic A. flavus

In the present investigation, five well known, widely used, common plant having some medicinal properties such as *Justicia adhatoda*, *Moringa oleifera*, *Mentha piperita*, *Trigonella foenum-graecum* and *Paederia foetida* was selected and extracted with different organic solvent for determination of inhibition efficacy of toxigenic *A. flavus*. For this, 5 gm dry leaf/shoot powder of each plant was soaked about 48 hours in four different solvent such as ethyl acetate, chloroform, hexane and methanol thereafter filtrated with Whatman filter paper No-1. Separated solvent extract was completely evaporated in rotary evaporator and dry weight was determined (Owk *et al.* 2014, Owk and Lagudu, 2016). Desired amount of sterilizes Dimethyl sulfoxide (DMSO) was added on crude extract and make its final concentration 100 mg/ml. Agar well diffusion method of Fernandez-Garayzabal *et al.* (1992) was applied to check the inhibition efficacy. For this, 100µl of conidial suspension of *A. flavus* was spread on solidifying PDA petri-plates and with the help of cork borer wells are prepared. Each well was filled with 50µl of four different solvent extracted of same plants and one well filled with DMSO as control. After 3-4 days of incubation in incubator, radial growth inhibition in millimeter was measured. For determination of minimum inhibitory concentration (MIC), different dilution such as 10, 25, 50, 75, 100 mg/ml was prepared and same agar well methods was applied and inhibition zone was measured for each concentration . Similar methods were also applied for determination of combined efficacy of two plants by mixing of same concentration. The lowest concentration, inhibiting growth was recorded as MIC value of the extract.

RESULTS AND DISCUSSION

Wheat rhizosphere soil of Birbhum district was assessed in terms of quantity of pH, EC, and

Table 1: Collection of wheat rhizosphere soil and determination of soil pH, EC and OC contents.

	Sampling Site	pH	EC(mS)	OC (%)
Bolpur	Bolpur-1	5.61±0.006	0.28±0.007	0.53±0.013
Sub-division	Bolpur-2	5.58±0.005	0.23±0.005	0.46±0.015
	Bolpur-3	5.13±0.007	0.27±0.010	0.47±0.021
	Bolpur-4	5.34±0.010	0.25±0.009	0.55±0.011
	Bolpur-5	5.36±0.008	0.20±0.010	0.56±0.004
Rampurhat	Rampurhat-1	5.58±0.007	0.43±0.012	0.45±0.010
Sub-division	Rampurhat-2	5.78±0.009	0.40±0.010	0.53±0.015
	Rampurhat-3	5.84±0.008	0.25±0.011	0.43±0.010
	Rampurhat-4	5.49±0.001	0.22±0.012	0.42±0.011
	Rampurhat-5	5.76±0.010	0.23±0.009	0.58±0.012
Suri	Suri-1	6.02±0.002	0.21±0.004	0.56±0.009
Sub-division	Suri-2	5.30±0.004	0.21±0.005	0.59±0.010
	Suri-3	5.22±0.010	0.29±0.004	0.58±0.006
	Suri-4	5.58±0.009	0.31±0.006	0.61±0.008
	Suri-5	5.56±0.010	0.38±0.004	0.59±0.013

Table 2: Isolation of *A. flavus* from wheat rhizosphere and determination of aflatoxigenicity

Sample site	No. of *A flavus* isolated	No. of toxigenic isolates (a)	% toxigenic	Range of Afl B1 in (µg/lt)
Bolpur-1	10	6 (BAF 1-6)	60	480-1891
Bolpur-2	8	4 (BAF 7-10)	50	360-991
Bolpur-3	5	2 (BAF 11-12)	40	720-410
Bolpur-4	5	2 (BAF13-14)	40	371-505
Bolpur-5	6	3 (BAF 15-16)	50	520-1530
Rampurhat-1	10	4(RAF 1-4)	40	366-513
Rampurhat-2	11	5(RAF 5-9)	45	210-580
Rampurhat-3	8	4(RAF 10-13)	50	290-490
Rampurhat-4	6	2(RAF 14-15)	33	312-581
Rampurhat-5	11	5(RAF 16-20)	45	203-476
Suri-1	12	5 (SAF 1-5)	42	110-320
Suri-2	8	3 (SAF 6-8)	37	72-236
Suri-3	9	4 (SAF 9-12)	44	85-401
Suri-4	8	3 (SAF 13-15)	37	38-365
Suri-5	8	3 (SAF 16-18)	37	42-230

(a) In parenthesis denotes isolate numbers BAF=Bolpur isolates *A. flavus,* RAF=Rampurhat isolates, *A. flavus* SAF=Suri Isolates of *A. flavus*.

organic carbon. Birbhum district soil is lateritic type and its pH of was found varied in different sampling area and was ranged from minimum 5.13 to maximum 6.02 in Bolpur-3 and Suri-1 respectively and are come under acidic (pH 4.5-5.5) as well as slightly acidic (pH 5.6-6.5). Maximum (0.43mS) EC was recorded in Rampurhat-1 soil whereas minimum (0.2mS) was noticed in Bolpur-5 sites. Similarly maximum (0.61) OC was recorded in Suri-4 and minimum (0.42%) was noticed in Rampurhat-4 (Table-1). Altogether 125 *A. flavus* isolate was isolated from all sites of which maximum (46) isolates were isolated from soil of Rampurhat sub-division followed by Suri sub-division (45) and minimum (34) from soil of Bolpur subdivision. Individual sampling sites showing maximum (12)

number of *A. flavus* was recorded from Suri-1 where as minimum (5) was noticed in Bolpur-3 and 4 (Table 2) and their comparative study with soil pH, EC and OC was depicted in Fig. 1. Soil edaphic factors such as pH, EC and Organic Carbon not only the influence of microbial diversity but also determine their activity, dynamics and functionality of the micro-organism (Ranelli *et al.* 2015, Karolina and Anna 2019). Determination and comparing of microbial diversity in different sites is difficult because of several edaphic factors (soil texture, pH, salinity, soil temperature, and moisture) and various macro and micronutrients interacting in different ways and creates complexity. Several earlier workers also found that fungal diversity in rhizosphere is also varied in soil types as well as soil conditions and nature of growing plants (Furtak and Galazka, 2019). According to Rousk *et al.* (2009), low pH of soil can helps to increases upto fivefold of total abundance of fungi, whereas number of bacteria was reduced sharply and it was experimentally supported by soil Phospholipid Fatty Acid (PLFA) method.

Table 3: Antifungal efficacy of different solvent extract of medicinal plant against toxigenic *A. flavus* isolates

	Different solvent extract and Zone of inhibition in mm			
Medicinal plant	Ethyl acetate	Chloroform	Hexane	Methanol
Justicia Adhatoda	23	05	0	03
Moringa oleifera	06	02	03	08
Mentha piperita	30	10	04	06
Trigonella foenum-graecum	06	12	0	05
Paederia foetida	05	02	0	01

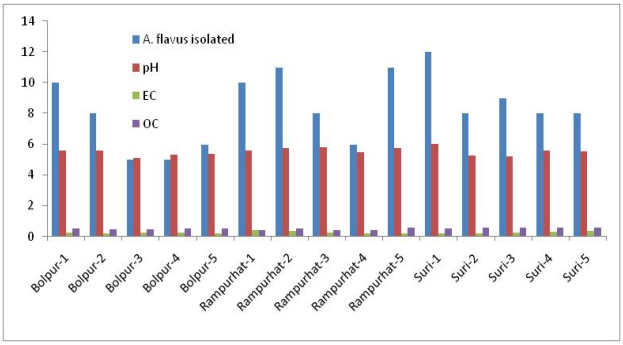

Fig. 1 : Comparative study of soil pH, EC and OC with incidence of *A. flavus* isolates

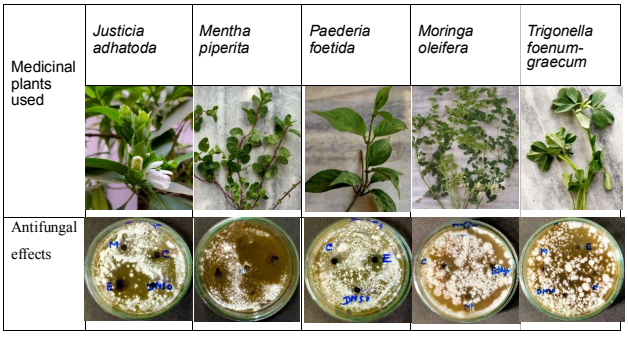

Fig. 2 : Medicinal plants and its inhibitory effects against toxigenic *A. flavus* isolates

During screening of aflatoxigenicity of all *A. flavus* isolates, only 55 were recorded as aflatoxigenic of which maximum (20) isolates recorded from Rampurhat soil followed by Suri sample (18) and minimum was noticed in Bolpur sites. Maximum percentage (60) toxigenic isolate was recorded in the sample of Bolpur-1 soil whereas minimum (33%) was noticed in Rampurhat-4. Individual sites having maximum (6) toxigenic isolates was recorded from Bolpur-1 whereas minimum (2) was noticed in Bolpur-3, Bolpur-4 and Rampurhat-4 (Table-2). All toxigenic *A. flavus* isolates was able to produced aflatoxin B_1 in varied amount and only 23 was able to produce Aflatoxin B_2 along with aflatoxin B_1 but in very lesser quantity. Of all types of aflatoxins, B_1 is more potent carcinogens, teratogens and mutagens and pose several health hazards to its consumers (Chu, 1991). Due to this reasons, present study was aim to quantified only aflatoxin B_1 and observed that their amount was found to varied in different sites. Maximum (1891µg/lt) aflatoxin B_1 was noticed in BAF-6 *A. flavus* isolate whereas minimum (38µg/lt) was recorded in SAF-13 isolate. In an exhaustive survey in Israel, among 1626 isolates of *A. flavus* from groundnut kernels and soils were tested, 90% of them were reported that produced aflatoxin B_1. Moreover, 21 isolates of *A. flavus* obtained from cotton, maize and wheat

Table 4: Determination of MIC of ethyl acetate fraction of two plants and their synergetic effects.

	Different concentration of Ethyl acetate extract in mg/ml and Zone of inhibition in mm					
Plant used	0 mg/ml	10 mg/ml	25 mg/ml	50 mg/ml	75 mg/ml	100 mg/ml
Mentha piperita	0	05	11	16	20	30
Justicia Adhatoda	0	03	08	10	16	23
Mentha piperita + *Justicia Adhatoda*	0	07	19	24	30	38

were screened of which only 16 isolates were recorded as toxigenic. This behaviour of different isolates for producing different types and quantity of aflatoxin might also be due to their genetically differentiation.

Earlier worker reported that medicinal plant contains different antimicrobial secondary metabolites plays a significant role for growth inhibiting of various kinds of micro-organisms (Webster *et al.* 2008; Dellavalle *et al.* 2011). Screening of antifungal efficacy of five medicinal plants extracted with different solvents (ethyl acetate, chloroform, hexane and methanol) are assessed and found that crude ethyl acetate extract of *Mentha piperita* showed maximum (30 mm) radial growth inhibition followed by *Justicia adhatoda* (23 mm) in agar well diffusion plate, against highly toxigenic *A. flavus* (BAF-6) isolate (Table-3; Fig. 2). Ethyl acetate extract of other plant and other solvent of all selected plants showing very less efficacy. MIC and combined efficacy of these two plants was also evaluated and find promising synergistic effects (Table-4). In all cases MIC was recorded as 10mg/ml but the efficacy in terms of radial growth inhibition was found to varied and was recorded maximum (5mm) inhibition by ethyl acetate extract of *Mentha piperita* at 10mg/ml where as minimum (3mm) was observed in *J. adhatoda*. During combination of both extract at 10mg/ml showing highest (7mm) radial growth inhibition and is due to synergestic effects of these two extracts. Synergistic results indicates that, may be two or more different phyto-chemicals responsible for such inhibitions. Singh and Singh (2000), while screening of antifungal activities of 50 different plants against *A. flavus* and *A. niger*, of which only four plant extracts viz., *Trachyspermum ammi, Allium sativum, Syzygium aromaticum* and *Plectranthus rugosus* were recorded as effective plant having potent antimicrobial compounds against both species of *Aspergillus*. Sharma and Sharma (2012) reported that alcoholic and aqueous extract of *Lawsonia inermis* and *Murraya Peniculata* showed promising inhibitory effects but more significant inhibition of mycelial growth as well as aflatoxin production was recorded in combined extract. The present study suggests that ethyl acetate extract of *Mentha piperita* and *Justicia adhatoda* posses some bioactive antifungal compounds responsible for inhibition of *A. flavus* growth.

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