The Pharma Innovation Journal 2022; SP-11(9): 3091-3096

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(9): 3091-3096

www.thepharmajournal.com
Received: 22-06-2022
Accepted: 25-07-2022

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Detection and identification of seed-borne fungal pathogens associated with seed discoloration of rice

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Abstract
The present investigation was undertaken to determine the nature of seed discoloration, detection and identification of seed borne fungal pathogens associated with discoloration of rice. In the present study, seven types of seed discolorations namely; light to dark brown dot like spots, dark purple discoloration, light pink discoloration, eye shaped spots with ash grey centers, ash grey discoloration marked by brown band, dark brown spots and black discoloration were observed. Twelve fungi namel; *Alternaria alternata, Fusarium- oxysporum, F. verticillioides, Aspergillus* niger, *A. flavus, A. ochraceus, Nigrospora Oryzae, Penicillium* sp. *Bipolaris tetramera, Curvularia lunata, Curvularia pallescens* and *Bipolaris Oryzae*, were found to be associated with different types of seed discolorations. Of the two incubation methods tested, agar plate method was found to be more efficient than standard blotter test. The study has shown that fungi associated with seed discoloration are major constraint in production of quality seeds of rice.

Keywords: Rice, seed discoloration, blotter paper, agar plate, see-borne pathogens

Introduction
Seed discoloration affects the quantity and quality of the produce (Sumangata *et al*., 2009, Tariq *et al*., 2012) [34, 36] leading to loss in crop yield. The seed discoloration has been shown to result in increase in chaffyness, loss in seed weight, deterioration in nutritional value, loss in viability, germination, reduction in market acceptability, etc. (Narain, 1992) [24]. Many types of seed discolorations are caused by several fungi in rice in different agro-climatic zones. The discoloration may be red, pink, orange, yellow, black or brown to dark brown depending upon the fungi. The extent of damage can also vary according to season, locality, seed microflora, host cultivar, their physiology, genetics and heavy nitrogenous fertilizer application. The fungi that are reported to be associated with discoloration of rice grains are Alternaria padwickii (ashy grey discoloration), Bipolaris *Oryzae* (dark brown dot like spots), Pyricularia *Oryzae* (black discoloration), *Fusarium* moniliforme (pink discoloration), *Fusarium graminearum*, Nigrospora *oryzae*, *Microdochium oryzae*, *Curvularia* spp. (eye shaped spots), *Phoma* sorghina, *Sarocladium oryzae* (light brown discoloration), and *Tilletia barclayana* (black discoloration) (Ou, 1985) [26]. The information available on seed discoloration of rice is mostly restricted to the seed-borne mycoflora associated with discoloration. Therefore, present investigation was carried out with the objective to detect and identify the causes of seed discoloration in rice.

Materials and Methods
The experiment was conducted in the Rice pathology lab, Department of Plant Pathology, College of Agriculture, Govind Ballabha Pant University of Agriculture and Technology, Pantnagar, Uttarakhand during 2017 to 2019.

Collection of seed samples
Freshly harvested seeds of most popular rice varieties in the region were collected from Norman E. Borlaug Crop Research Center of G. B. Pant University of Agriculture and Technology Pantnagar, Udham Singh Nagar, Uttarakhand. Nine varieties representing bold grain (Sarju 52, Pant Dhan 10 and Pant Dhan 21), medium grain (PR 121, HKR 47 and Govind) and fine grain/basmati (Pusa Sugandh 5 or Pusa 2511, Pusa Basmati 1509 and Pant Basmati 1 were used in experiment. A working sample of 200g of each variety was randomly collected from rice experiments as recommended by ISTA (1976) [15]. The samples were kept in cloth bags with proper labeling, and stored in the refrigerator at 5±1 °C until used for subsequent studies.

Detection and identification of seed-borne fungal pathogens associated with seed discoloration of rice

The incidence of fungi associated with discolored seeds of rice was detected by incubation methods *viz*., Standard Blotter paper Methods and Agar plate Methods following the rules of ISTA (ISTA, 2001) [14].

Standard blotter paper method

The standard blotter paper method as suggested by ISTA was used to determine the occurrence of seed-borne mycoflora on harvested rice seeds. Three blotter papers were kept on each other to form a layer at the bottom of 90 mm diameter glass Petri dishes. The blotter papers were soaked in sterilized distilled water. Representative samples from a seed lot were placed on a blotter @ 25 seeds per Petri dish with 15 seeds in the outer ring, nine in the middle ring and one in the center. A total of 400 seeds of each type of discoloration were tested with sixteen replications. Seeds were surface sterilized with 2 per cent sodium hypochlorite ($NaOCl_2$) for 5 minutes and washed thrice in sterilized distilled water. The Petri dishes containing seeds were incubated at 22±2 °C under alternate cycle of 12 hours near ultraviolet light (NUV) and 12 hours darkness for seven days. The incubated seeds were examined after seven days under a stereo-binocular microscope in order to record the incidence of different seed-borne fungi. Fungal growths on the seeds were aseptically mounted in lactophenol or cotton blue on slides using flamed-sterilized needle and examined under a compound microscope for identification of fungi. Pure cultures of fungi were obtained and all isolations were maintained on potato dextrose agar medium. A list of morphological characters on taxonomic importance such as spore size, shape, septation, color and their arrangement on the conidiophores, appearance of the spore masses, characters of the mycelium, density of the colony were compiled for each fungus. The observation on frequency of pathogen(s) associated with seeds was calculated by using the following Formula:

$$\text{Frequency of association (\%)} = \frac{\text{No. of seeds in which fungus is appeared}}{\text{Total no. seeds}} \times 100$$

Agar plate method

In the agar plate method for the detection of seed-borne pathogens 200 seeds were tested for each type of discoloration. The seeds were surface sterilized using 2 per cent sodium hypochlorite for 5 minutes, washed thrice with sterilized distilled water. Seeds were then placed on the PDA medium 9 in periphery one in center and the plated seeds were incubated for 5-7 days at 22-25 °C under 12h of alternate cycle of light and darkness. At the end of the incubation period, fungi growing out from the seeds on the agar medium were examined and identified. Identification was done based on colony characters and morphology of sporulation structures under a compound microscope. Once the fungus was identified, the frequency of each fungus was determined as above.

Isolation and maintenance of pure cultures

As soon as fungal colonies emerged from the rice seeds were picked up with the help of a sterilized inoculation needle, transferred aseptically on PDA slants and properly labeled. The isolates were further purified by hyphal tip culture method and preserved in refrigerator at 5 °C for further studies.

Identification of fungal pathogens

The fungal isolates were identified morphologically on the basis of culture color, spore shape, structure of fungus fruiting bodies and growth pattern of fungus (Dube, 2013; Ellis, 1971, Alexopolus *et al*., 1996) [5, 7, 1].

Results and discussion the data pertinent to fungi associated with different types of discoloration in rice is presented in (table 1). A total of 12 fungi namely; *Alternaria alternata,- Fusarium-oxysporum, F. verticillioides, Aspergillus-* niger, *A. flavus, A. ochraceus, Penicillium* sp. *Bipolaris tetramera, Curvularia lunata, Curvularia pallescens, Bipolaris Oryzae* and *Nigrospora Oryzae* were isolated by agar plate and standard blotter paper methods. In the present study, agar plate method was found to be more efficient than standard blotter test as number and frequency of fungi was more in agar plate method (Plate 1). Several workers (Signaboubo *et al*., 2016, Imolehin, 1983, Khan *et al*., 1988, 1999, Kim and Lee, 1989, Wahid *et al*., 1993, Javaid *et al*., 2002, Ibiam *et al*., 2006, Sharma and Kapoor, 2016) [33, 13, 17, 20, 39, 16, 11, 30] around the world have reported agar plate method as more efficient than standard blotter paper method for seed health testing.

Fungi associated with different types of seed discoloration in rice

Nine fungi namely; *Alternaria alternata, F. oxysporium, F. verticillioides, A. flavus, A.* niger, *A. ochraceus, Penicillium* sp., *Curvularia pallescens* and *Nigrospora Oryzae* were found to be associated with light to dark brown dot like spots (Table 1). Of the nine fungi, *A. alternata* was found to be most predominant (20.00%) fungi with the seeds showing light to dark brown dot like spots. It was followed by *F. verticillioides* (10.00%), *A.* Niger (6.68%), *A. flavus* (6.67) and *Curvularia pallescens* (3.75). These fungi were recorded only by agar plate method (Plate 1).

Whereas, six fungi namely; *F. oxysporum, F. verticillioides, A.* Niger, *A. flavus, Bipolaris tetramera,* and *Curvularia lunata* were found to associated with dark purple type of discoloration (Table 1). The incidence of these fungi ranged from 1.50% to 20.00%. Of the six fungi, *F. verticillioides* (20.00%) was found to be most predominant fungi. It was followed by *A. flavus* (13.34%), *Bipolaris tetramera* (10.00%) (Table 1 & Plate 2).

Similarly, seven fungi namely; *F. oxysporum, F. verticillioides, A.* Niger, *Penicillium* sp., *Curvularia lunata, C. pallescens* and *Bipolaris Oryzae* were isolated from the seeds showing light pink discoloration (Table 1). Of these, *Penicillium* sp. and *F. oxysporum* were found to be most predominant (13.33%) (Table 1 & Plate 2).

Likewise, six fungi *viz*., *F. verticillioides, A. flavus, Penicillium* sp., *Bipolaris tetramera, Curvularia lunata* and *C. pallescens,* were found to be associated with the seeds showing eye shaped spots with ash grey center (Table 1). Of these, *F. verticillioides* was found to be most predominant (16.78%), followed by *C. lunata* (16.67%) and *A. flavus* (16.00%) (Table & Plate 2).

Five fungi *viz*., *A. alternata, F. verticillioides, A. flavus, A. ochraceus,* and *C. lunata* were detected with seed having ash grey discoloration marked by brown band. Among these, *F. verticillioides* (23.33%) had maximum frequency followed by *C. lunata* (13.40%), *A. alternata* (6.67%), *A. flavus* (3.33%), and *A. ochraceus* (3.33%) (Table 1 & Plate 2). However,

eight fungi namely; *A. alternata, F. verticillioides, A.* Niger, *A. flavus, Nigrospora Oryzae, C. lunata, C. pallescens* and *Bipolaris Oryzae* were found to be associated with seeds showing dark brown spots (Table 1). Among these, *A. flavus* was found to be most predominant (13.34%) followed by *Bipolaris Oryzae* (13.33%), *C. lunata* (10%), *A. alternata, A.* Niger and *F. verticillioides* (6.67%) (Table 1 & Plate 1). A total seven fungi namely; *A. alternata, F. verticillioides, A.* Niger, *A. flavus, Bipolaris tetramera, B. Oryzae and Curvularia lunata* were found to be associated with seeds showing black discoloration in varied frequency (Table 1). *A.* Niger was found in maximum frequency (26.60%) followed by *Bipolaris tetramera* (13.30%), *Bipolaris Oryzae* (13.30%), *F. verticillioides* (6.67%) (Table 1 & Plate 2).

The results showed that a total of 12 fungi were associated with different types of seed discoloration, however, maximum number of fungi were recorded in the seeds showing light to dark brown dot like spots and black discoloration. *Fusarium verticillioides* and *Aspergillus flavus* were associated with all types of discoloration. *Aspergillus* Niger was associated with all types of discoloration except seed showing eye shaped spot with ash grey center and ash grey discoloration marked by brown band. However, *Aletrnaria alternata* was associated with light to dark brown dot like spots, ash grey discoloration marked by brown band, dark brown spots and black discolorations, *Fusarium oxysporum* was recorded in seeds with light to dark brown dot like spots, dark purple discoloration and light pink. *Penicillium* sp. was recovered from the seeds with light to dark brown dot like spot, light pink discoloration, eye shaped spots with ash grey center and black discoloration, *Aspergillus ochraceus* with light to dark brown dot like spots and ash grey discoloration marked by brown band, *Bipolaris Oryzae* with eye shaped spot with ash grey center, black and dark purple discoloration. Two species of *Curvularia* were associated with seed discoloration. One was identified as *C. lunata* however, another sp is *Curvularia pallescens. C. lunata* was found to be associated with all types of discoloration except light to dark brown dot like spots, however, other species was recovered with the seeds showing light to dark brown dot like spots, light pink discoloration, eye shaped spots with ash grey center and dark brown spots.

Similar pathogens have also been reported to be associated with rice seeds by various workers (Basak and Mridha, 1986; Riaz *et al*., 1995; Sharma and Chahal, 1996; Khan *et al*., 1999; Haque *et al*., 2007; Mandhare *et al*., 2008; Gopala krishnan *et al*., 2010; Archana and Prakash, 2013) [3, 41, 31, 18, 9, 21, 8, 2]. There are many reports indicating the association of various fungi with different types of seed discoloration in rice. *Alternaria alternata* was reported to be responsible for ashy grey and black discoloration and dark brown dot like spots (Ibrahim *et al*., 2014, Rawte, 2013, Sachan and Agrawal, 1995) [12, 27, 29], *Phyllostica waracola and Sporodesmium Oryzaecolus* for black dots on glumes (Hora *et al*., 1959) [10], *Alternaria padwikii, C. clavata and C. raggrostidis* for black glume discoloration (Martin and Altstatt, 1940) [22], *Alternaria padwikii* for dark brown, light pink, light brown, pale yellow (Ibrahim *et al*., 2014) [12] and also for general seed discoloration spotting (Shetty *et al.*, 1987) [32], *Bipolaris Oryzae* for black point, *Bipolaris tetramera* for light brown and black dots, *Curvularia Oryzae* for dark brown spots, *F. graminearum* for Pale yellow and light pink discoloration, *Sarocladium Oryzae* for light brown discoloration (Ibrahim *et al*., 2014) [12], *Fusarium roseum* and *H. Oryzae* for brown discoloration (Sung *et al.*, 1984, Merny 1987, Duraiswamy and Mariappan, 1983, Sachan and Agrawal, 1995) [35, 23, 6, 29], *Manascus purpureus* for red discoloration (Uyeda, 1901) [40], *Walkia decolorans* for yellow discoloration (Vanderwolk, 1913), *Bipolaris tetramera* for black, dark purple, dark brown spot, light to dark brown, brown dot like spots (Sachan and Agrawal, 1995) [38], *F. equiseta, F. graminearum* and *F. verticillioides* for light pink discoloration (Bateman and Kwasna, 1999, Khanzada *et al.*, 2002) [4, 19], *Cochliobolus miyabeanus* for black discoloration, dark brown spot and light to dark brown dot like spots. *Curvularia lunata* for ebony black to chocolate brown stains (Tullis, 1936) [37].

Rawte (2013) [27] in his study on causes of seed discoloration in rice have reported that the seed-borne inoculum of *Alternaria alternata* was responsible for ashy grey discoloration and *Bipolaris Oryzae* for black discoloration, dark brown spots and light to dark brown dot like spots, while, *Curvularia geniculatus* for eye shaped spots. Whereas, *Cochliobolus miyabeanus* was reported to be responsible for black discoloration, dark brown spot and light to dark brown dot like spots, *Sarocladium oryae* for light brown discoloration. Besides, *Fusarium equiseti, F. oxysporum F. verticillioides* have been reported for pink discoloration and *Sarocladium Oryzae* for light brown discoloration.

Table 1: Fungi associated with different types of discoloration

Types of discoloration	Incidence of different fungi (%)													
	A. alternata		***F. oxysporum***		***F. verticillioides***		***A.* niger**		***A. flavus***		***A. ochraceous***		***N. Oryzae***	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Light to dark brown dot like spot	20.00	0.00	3.00	0.00	10.00	3.75	6.68	0.75	6.67	0.00	3.00	0.00	3.00	0.00
Dark purple discoloration	0.00	0.00	6.67	0.00	20.00	3.75	3.30	0.00	13.34	1.50	0.00	0.00	0.00	0.00
Light pink discoloration	0.00	0.00	13.33	0.00	3.30	1.50	6.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Eye shaped spot with ash grey Center	0.00	0.00	0.00	0.00	16.68	0.00	0.00	0.00	16.00	0.00	0.00	0.00	0.00	0.00
Ash grey discoloration marked by brown band	6.67	0.00	0.00	0.00	23.33	7.50	0.00	0.00	3.33	0.00	3.33	0.00	0.00	0.00
Dark brown spot	6.67	0.00	0.00	0.00	6.67	2.25	6.67	0.00	13.34	0.75	0.00	0.00	3.30	0.00
Black discoloration	6.68	0.00	0.00	0.00	6.67	3.00	26.60	0.00	6.67	0.00	0.00	0.00	0.00	0.00

Types of discoloration	Incidence of different fungi (%)									
	***Penicillium* sp.**		***Bipolaris tetramera***		***Curvularia lunata***		***Curvularia pallescens***		***Bipolaris Oryzae***	
	A	B	A	B	A	B	A	B	A	B
Light to dark brown dot like spot	2.76	0.00	0.00	0.00	0.00	0.00	3.75	0.00	0.00	0.00
Dark purple discoloration	0.00	0.00	10.00	0.00	6.67	3.00	0.00	0.00	0.00	0.00
Light pink discoloration	13.33	0.00	0.00	0.00	3.30	3.75	6.67	0.00	6.67	0.00
Eye shaped spot with ash grey Center	3.34	0.00	3.33	0.00	16.67	6.75	3.33	0.00	0.00	0.00
Ash grey discoloration marked by brown band	0.00	0.00	0.00	0.00	13.40	0.00	0.00	0.00	0.00	0.00

Dark brown spot	0.00	0.00	0.00	0.00	10.00	0.00	3.35	0.00	13.33	0.00
Black discoloration	0.00	0.00	13.30	0.00	0.00	2.25	0.00	0.00	13.30	0.00

A= Agar plate method
B=Standard Blotter paper method

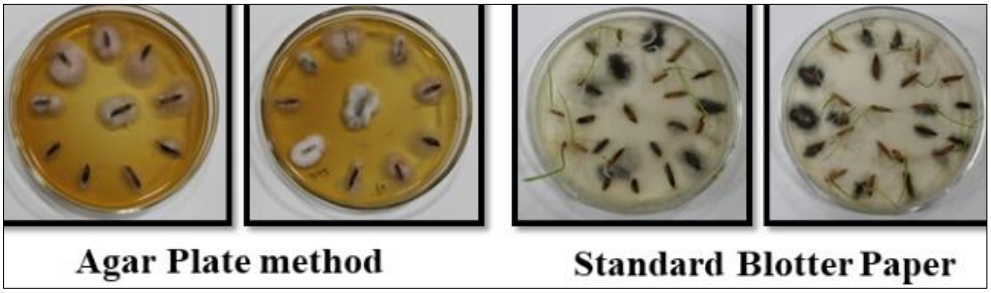
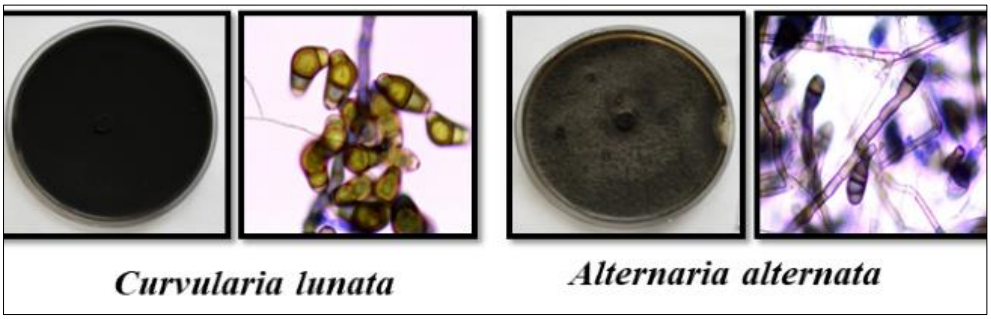
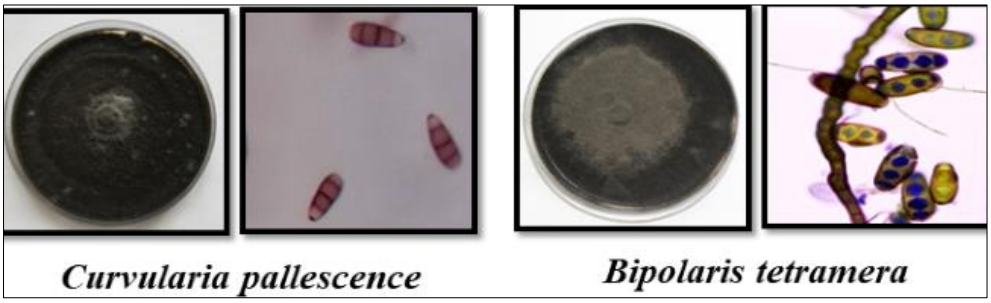
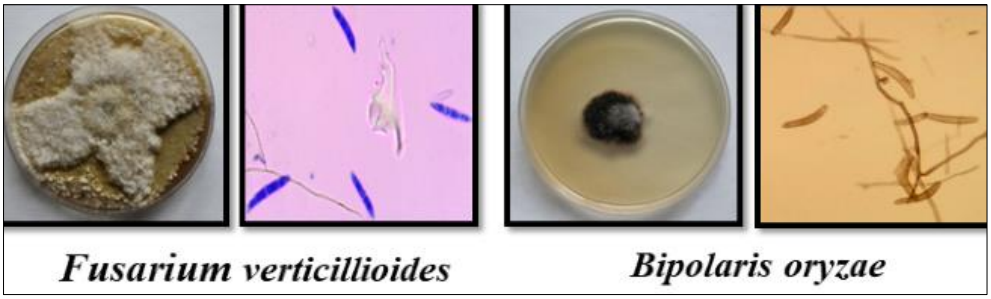
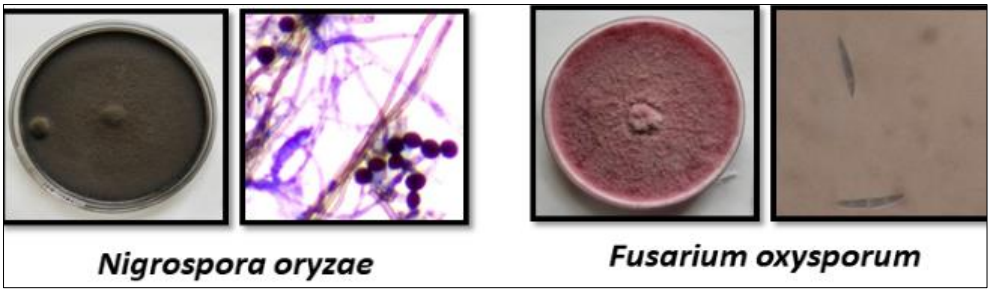


Plate 1: Isolation of seed-borne Mycoflora









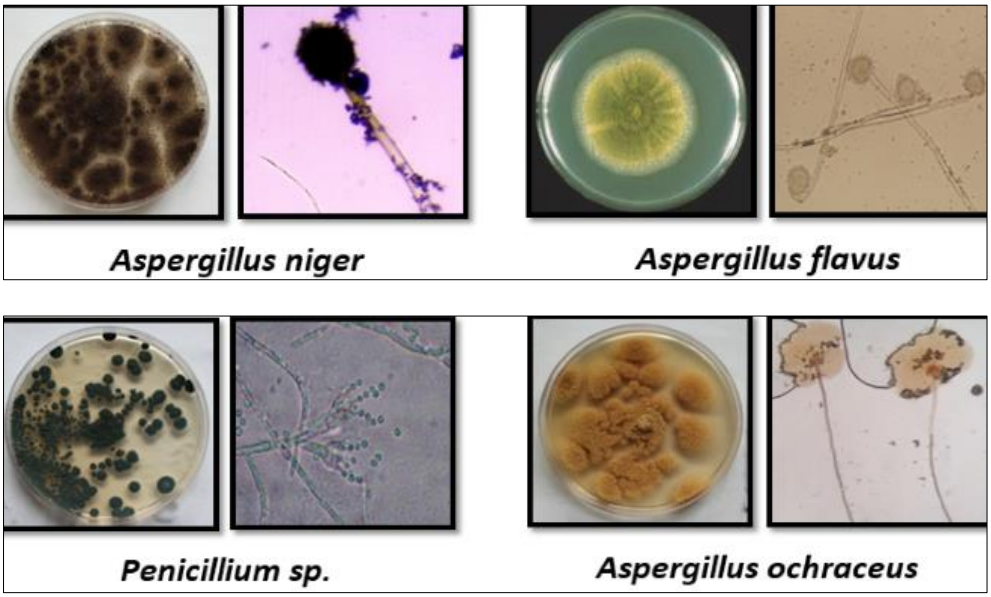


Plate 2: Culture plate of different isolated fungi and their microscopic characters

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