

Full Length Research Paper

Identification of some *Aspergillus* species isolated from Dal Lake, Kashmir by traditional approach of morphological observation and culture

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The current study on some species of genus *Aspergillus* revealed that any of the general purpose media like potato dextrose agar (PDA) or Rose-Bengal agar is only useful for identification of the molds upto genus level; however, the use of differential culture media like Czapek dox agar (CZ), Czapek yeast agar (CYA) and Malt extract agar (MEA) for their growth to study various macroscopic features like colony color (both conidium and reverse), colony diameter and microscopic features like conidia length, width, shape, ornamentation, stipe length, width, shape, ornamentation and branching Pattern is a key factor in the identification of these molds to species level. Different species of genus *Aspergillus* isolated from the lake water samples showed slightly too large variations in their micro and macro morphological features leading to their species level identification. This study further revealed that follow up of the micro and macro morphological feature of the molds isolated from soil, water, damaged buildings, damp walls, paper or other surfaces is a reliable and simple technique for their identification.

Key words: *Aspergillus*, morphological observation, culture, Dal Lake, Kashmir.

INTRODUCTION

Aspergillus is a large genus composed of more than 180 accepted anamorphic species, with teleomorphs described in 9 different genera (Pitt and Samson, 2000). The genus is subdivided in 7 subgenera, which in turn are further divided into sections (Klich, 2002). *Aspergillus* mold species are found throughout the world and are the most common type of fungi in our environment. About 16 species of these molds are dangerous to humans, causing diseases and infections. *Aspergillus* molds have a powdery texture. However, the color of the mold's surface differs from species to species and can be used to identify the type of *Aspergillus*. The rate of growth can also be used to identify *Aspergillus*, with most species growing quite quickly. After 1 week of growth at around 25°C, an *Aspergillus* colony will generally be 1 to 9 cm in

diameter; however, *Aspergillus glaucus* and *Aspergillus nidulans* grow more slowly and will generally be 0.5 to 1 cm after the same time. As with fungi in general, *Aspergillus* taxonomy is complex and ever evolving. The genus is easily identified by its characteristic conidiophore, but species identification and differentiation is complex, for it is traditionally based on a range of morphological features. Macromorphological features which are considered include conidial and mycelial colour, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia. Micromorphology characterization is mainly dependent on seriation, shape and size of vesicle, conidia and stipe morphology, presence of Hülle cells, and morphology of cleistothecia and ascospores (Klich, 2002). Furthermore, all these morphological features have to be determined under standardized laboratory conditions (Okuda et al., 2000) by trained mycologists, in order to obtain an accurate

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identification. Several *Aspergillus* taxonomic keys and guides are available (Klich, 2002; Raper and Fennell, 1965).

Generally, identification of the fungal species is based on the morphological characteristics of the colony and microscopic examinations. Although, molecular methods continue to improve and become more rapidly available, microscopy and culture remain commonly used and essential tools for identification of the fungal species like *Penicillium* and *Aspergillus*. A survey by the American Society for Microbiology (ASM, 2004) documented that 89% of laboratories performing mycological examinations (morphology based), 16% of them use serologic tests and fewer than 5% use molecular tests for identification of microbial pathogens. Only 3% of reporting laboratories use 'home-brew' molecular testing for microbial pathogens.

MATERIALS AND METHODS

Location and site description

The Dal Lake, lying between geographical coordinates 34° 07' N, 74° 52' E, 1584 m a.s.l in Srinagar, Jammu and Kashmir, India- a multi-basined lake with Hazratbal, Bod Dal, Gagribal and Nageen as its four basins, having two main inlets as Boathall Nallah and Tailbal Nallah and two main outlets as Dal Lock Gate and Pokhribal Nallah, was taken up for the current study. Sixteen (16) sites viz., Hazratbal Open, Hazratbal littoral, Nageen Open, Nageen near Houseboats, Gagribal Open, Gagribal near Houseboats, Nishat Open, Near Centeur, Boathall Nallah-I, Boathall Nallah-II, Tailbal Nallah-I, Tailbal Nallah-II, Dal Lock Gate-I, Dal Lock Gate-II, Pokhribal Nallah-I and Pokhribal Nallah-II with 8 sites from the 4 basins, 4 sites from two inlets and 4 other sites from two outlets were selected for present study.

Collection of water samples

Water samples were collected at different sites of the lake in white plastic containers, which were previously sterilized with 70% alcohol and rinsed with distilled water. At the lake, the containers were rinsed thrice with the lake water before being used to collect the samples.

Isolation of fungi

Water samples obtained were serially diluted five folds and then spread plate technique was followed for isolation of *Aspergillus* species in the study, spreading 0.1 ml inoculum from the serial dilution tubes on the Petri dishes containing Rose-Bengal streptomycin agar medium.

Inoculation and incubation

Three-point inoculation on 80 mm Petri dishes, an accepted standard technique for cultivation for the morphological identification of *Penicillium*, *Aspergillus* and other related genera as followed in one of our studies (Bandh et al., 2011) was followed in the present study for the morphological features of *Penicillium*. The three-point inoculation was done by using glass Petri dishes inoculated with very low quantities of conidia using glass needles, incubated at different temperatures upside down for 7 days to

prevent spread of conidia all over the plate and growth of the colonies.

Culture and identification

Aspergillus isolates were identified upto genus level on potato dextrose agar (PDA). To improve the sensitivity and specificity of routine culture approach for identification of species level, we used some differential media including, Czapek dox agar (CZ) [czapek concentration 10.0 ml, K₂HPO₄ 1 g, sucrose 30 g, agar 17.5 g, distilled water (DW) 1 liter], Czapek yeast agar (CYA) [czapek concentration 10.0 ml, K₂HPO₄ 1 g, powdered yeast extract 5 g, sucrose 30 g, Agar 15 g and DW 1 liter] and Malt extract agar (MEA) (powdered malt extract 20 g, peptone 10 g, glucose 20 g, agar 20 g, DW 1liter]. Morphological features of *Aspergillus* cultures were studied, the major and remarkable macroscopic features in species identification were the colony diameter and color (conidia and reverse). We used Riddle's classic slide culture method (Riddle, 1950) for microscopic study of the isolates. Microscopic characteristics for the identification were conidia length, conidia width, conidia shape, conidia ornamentation, stipe length, stipe width, stipe ornamentation, phialide shape and branching pattern. Fungal morphology was characterized by using a semiautomatic image analysis system consisting of an Olympus microscope (Olympus, New Hyde Park, NY, U.S.A.) operated as phase contrast, a charge coupled device (CCD) camera (Sony, Cambridge, U.K.), a PC with a frame-grabber, and the image analysis software (SIS, Olympus, Germany). Samples preparation and measurements were as described in earlier publications (Papagianni et al., 1998, 1999). A magnification of 100× was applied for measurements on mycelial particles to estimate the individual mycelia and other micro morphological features.

RESULTS

During the study we identified five species of *Aspergillus* viz. *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus japonicus* and *Aspergillus terreus* by using differential culture media like CZ, CYA and MEA for their growth. Besides, this various macroscopic (Table 1) and microscopic features (Table 2) were studied for their identification. The macroscopic characteristics shown in Table 1 revealed that the most common conidium color of the fungal colonies was green and reverse color of the colonies was white to cream. However, some more colors like yellow, brown and yellow orange were also shown by the conidia and reverse side of the isolated fungal strains. The colony diameter shown by different species ranged between a minimum of 19 mm and a maximum of 50 mm. The microscopic features of these fungal species (Table 2) revealed that conidia length, conidia width, stipe length and stipe width varied between 2.5 to 3.5 µm, 2.2 to 3 µm, 28 to 350 µm and 2 to 3.5 µm, respectively. The conidia shape shown by the strains was globose, ellipsoid, subglobose and pyriform with ornamentation of conidia and stipe as smooth, coarsely roughened and finely roughened. Moreover, the phialide shape was ampulliform and cylindrical, branching pattern was Mono-verticillate, Bi-verticillate, Ter-verticillate and Quarte-verticillate.

Table 1. Macroscopic features of different species of *Aspergillus*.

Name of fungi	Macroscopic features					
	On CYA			On MEA		
	Color of conidium	Reverse color	Diameter (mm)	Color of conidium	Reverse color	Diameter (mm)
<i>A. niger</i>	Green	White to cream, yellow,	28	Green	White to cream, yellow,	33
<i>A. flavus</i>	White to cream, yellow,	Brown	19	Green	Brown	23
<i>A. fumigatus</i>	Yellow orange	Pale brown	44	Green	Yellow	50
<i>A. japonicus</i>	Green	White to cream	31	Green	Brown	39
<i>A. terreus</i>	Green	yellow	20	White to cream	White to cream	22

Table 2. Microscopic features of different species of *Aspergillus*.

Name of fungi	Microscopic feature								
	Conidia length (µm)	Conidia width (µm)	Conidia shape	Conidia ornamentation	Stipe length (µm)	Stipe width (µm)	Stipe ornamentation	Phialide shape	Branching pattern
<i>A. niger</i>	3	2.8	Globose, ellipsoid	Coarsely roughened	295	3	Coarsely roughened Warted	Flask-shaped, (ampulliform, with constriction)	Bi-verticillate
<i>A. flavus</i>	3.5	3	Ellipsoid, Pyriform	Smooth	350	3.5	Smooth	Phialide shape	Mono-verticillate,
<i>A. fumigatus</i>	2.8	2.5	Subglobose	Smooth, finely roughened	258	3.4	Smooth	Flask-shaped, (ampulliform, with constriction)	Quarte-verticillate,
<i>A. japonicus</i>	2.5	2.2	Globose, subglobose	Coarsely roughened	35	2.7	Coarsely roughened	Cylindrical	Ter-verticillate
<i>A. terreus</i>	3.2	2.5	Ellipsoid	Smooth	28	2	Smooth, finely roughened	Flask-shaped, (ampulliform, with constriction)	Bi-verticillate

DISCUSSION

Identification of five *Aspergillus* species by using differential media like CYA and MEA

demonstrated that it was a simple and reliable technique for identification of *Aspergillus* species. A culture time of 7 days or more on differential media like CYA and MEA is generally required for

delineating the macroscopic and microscopic characteristics of fungal colonies to identify them. Some earlier studies on *Penicillium* and *Aspergillus* species with similar design were

reported by some studies (Anaissie et al., 2003; Curtis and Baker, 2005; Klich, 2002; McClenny, 2005). A recent similar study conducted in Kashmir (Bandh et al., 2011) reported the identification of five species of *Penicillium* isolated from the water samples obtained from a fresh water lake by the same approach. The follow up of the micro and macro morphological feature of these terrestrial molds is a reliable technique for their identification as was confirmed by another recent Spanish study identifying some *Aspergillus* spp. isolated from damp walls, paper and the other surfaces (Leenders and Belkum, 1999). The current study like that of our previous study (Bandh et al., 2011) on some species of genus *Penicillium* revealed that any of the general purpose media like PDA or Rose-Bengal agar is only useful for identification of the filamentous fungi upto genus level.

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