DOI: 10.21276/ijabpt.2016.7.4.21

INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com ISSN : 0976-4550

Volume-7, Issue-4, Oct-Dec-2016 Coden IJABFP-CAS-USA
Received: 4th Sept 2016 *Revised: 14th Oct 2016* *Accepted: 16th Oct 2016*

DOI: 10.21276/Ijabpt, http://dx.doi.org/10.21276/ijabpt *Research article*

PREVALENCE AND CHARACTERIZATION OF *ASPERGILLUS* SPP. FROM RICE GRAINS IN SOUTH INDIA

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ABSTRACT: Rice is one of the most important staple foods in Asian and African countries. It is prone to many pre and post-harvest biotic stresses. The rice samples are associated with a number of storage fungi like *Aspergillus*, *Rhizopus*, *Fusarium*, *Curvularia* and *Penicillium* species. Therefore, identification of fungi associated with discoloured rice grains and their characterization is needed. Hence, morphological characterization was carried out to identify toxigenic and non-toxigenic species of Aspergilli. The present study indicated that the dominant colonies included parrot green (*A. flavus*, 40%), black (*A. niger*, 30%), olive green (*A. parasiticus*, 9%) and yellow (*A. ochraceus*, 7%) coloured along with some other coloured colonies/ species. Among the four media tested, CY20S favoured luxurious growth whereas suppressed and slow growth of the fungi was observed on CZ medium, but provided additional clues on the identity of Aspergilli species. These systematic data on macroscopic and microscopic characters of the isolates were compared with each other and also with neo type cultures of *Aspergillus* species obtained from Mycological Type Culture Centre (MTCC), Chandigarh. These two sets of data were compared to arrive at and confirm the identity of the fungus isolated from discoloured rice grains. From all these data, it was concluded that the parrot green colonies were *A. flavus* (with few *A. flav*us var. *columnaris* and *A. fumigatus*), olive green colonies were *A. parasiticus*, some of the black colonies were *A. niger*, *A. aculeatus* *A. carbonarius* and yellow colonies were *A. ochraceus* species.
Key words: Rice, *Aspergillus*, morphology, neo type cultures

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INTRODUCTION

Rice, *Oryza sativa* L. (Poaceae) is a widely grown cereal crop in the world and it constitutes half of diet to the eastern and southern parts of people in India. Grain production has been steadily increasing due to advances in production technology, but improper storage results in high losses in grains. Modern scientific storage facilities are woefully scarce. More than 16 million metric tonnes of food grains are lost after the harvest each year that could feed over a hundred million people a year (Singh, 2010). Thus, the post-harvest losses impact the economy at both micro and macro levels. Deterioration caused by moulds, insects, and mites are reflected in the production of mycotoxins and entomotoxins that lead to debilitating diseases including cirrhocis of liver in human. *Aspergillus* is one of the most economically important genera of microfungi contaminated with many post-harvest and processed food products.

Identification and classification of the genus *Aspergillus* is complex and taxonomists used varied methods to delineate new species (Geiser et al 2007). Some of taxonomists used morphological characters for the identification of *Aspergillus* species (Klich 2002; McClenny 2005, Curtis et al 2005). Morphological and physiological characters may vary among species in the production of sclerotia or metabolites on different culture media (Klich et al 2001). The most important monograph on which most taxonomies are derived from is strictly based on phenotypical (micro- and macro-morphological) characters (Samson et al 2006; McClenny 2005), with the additional consideration of molecular and extrolites data used in recent years (Hong et al 2005). 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 (Klich 2002). Molecular phylogenetics has uncovered cryptic speciation in a number of taxa (Geiser et al 1998; Pringle et al 2005; Balajee et al 2005), suggesting that morphological characters provide a very broad species concept that does not reflect the true extent of evolutionary divergence and reproductive isolation, as appears to be the rule in fungi (Geiser et al 2004). As *Aspergillus* is an extremely important genus concerning pathogenicity, mycotoxins, fundamental eukaryotic genetics and biotechnological exploration, several books have been dedicated to *Aspergillus* in general (Bossche et al 1988; Bennett and Klich 1992; Powell et al 1994; Smith 1994) and to the taxonomy and phylogeny of *Aspergillus* in particular (Samson and Pitt 1990, 2000).

Traditional *Aspergillus* taxonomy, based on phenotypical characters only, gives mostly a satisfactorily delimitation of the taxa. However, in several sections of the genus much morphological variations occur, resulting in debatable taxonomic schemes. The taxonomy of *Aspergillus* and its teleomorphs has recently been re-investigated by using a polyphasic approach in order to examine the variability within the species (Frisvad et al 2004; Samson et al 2004; Geiser et al 2007; Hong et al 2005, 2006). Species belonging to the genera *Aspergillus* and *Penicillium* are commonly used or isolated and inadequate taxonomy or uncertain nomenclature of these genera can therefore lead to a tremendous confusion. Changes in the recent published International Code of Nomenclature for Algae, Fungi and Plants will lead to numerous name changes of existing *Aspergillus* and *Penicillium* species and an overview of the current names of biotechnological important species is given (Houbraken 2014). *Aspergillus* systematists had a vast diversity of opinions on this matter. Rather than dwelling on these discrepancies, it may be appropriate to accentuate on the substantial common ground among systematists, which is leading to a taxonomic system that reflects morphological, physiological, ecological and phylogenetic relationships (Geiser et al 2007). Hence, here *Aspergillus* species were identified based on morphological descriptions and growth characteristics.

MATERIAL AND METHODS

Morphological examination and identification

The colored colonies isolated from discolored rice grains (collected from different locations) were purified by single hyphal tip method on potato dextrose agar (PDA) slants and preserved at 4^{o}C in refrigerator for further studies. The molten and cooled media was poured into the sterile Petri plates and allowed for solidification under aseptic conditions. Small amount of culture of the each isolate was individually transferred to the solidified media plates and incubated at 28°C. The cultures were examined after 7 days of growth and further examined after 14 days. Colony diameter was measured and growth response of the individual isolated experimental cultures was recorded. The isolates were compared with macroscopic and microscopic characters of the (neo) type cultures of the accepted species collected from Mycological Type Culture Centre (MTCC), Chandigarh. The four cultures, *Aspergillus flavus* (MTCC- 2799), *A. niger* (MTCC -872), *A. parasiticus* (MTCC 2796) and *A. ochraceus* (MTCC-1810) were used for morphological confirmation of the species. The other species were identified by morphological features both macroscopic and microscopic noted under microscope as described by Klich (2002). Colony colour was observed by naked eye and compared with the colour charts of Methuen Handbook of Colour (Kornerup and Wanscher 1978). All species were examined under light microscope (Lica, Switzerland and Germany) using 10x, 40x and oil immersion with a compound microscope at 100x, 400x and 1000x magnifications. Digital micrographs of colonies were taken with a Nikon Coolpix 990 and 995 cameras.

Macroscopic and microscopic examination of the fungus

The macroscopic observations like colony and cultural characters were studied based on phenotypic characters visible to naked eye. The colony characters like colour, growth, size and shape; and the cultural characters like mycelia colour, structure and texture, time taken for sporulation, sclerotial production, presence of growth circles (wrinkles), and exudates or metabolites production were studied. Microscopic examinations were carried out for presence of fungal elements at 100X, 400X and 1000X magnifications. Different microscopic characters like, vesicle size, metulae, phialide size, seriation, condiophore length and breadth, conidia size, colour and texture were examined. The description and the distinguishing characters of Aspergilli were expressed as per Raper and Fennel (1965) and Klich et al (2002). Although several hundreds of isolates were obtained from discoloured grains and damaged paddy seeds, only a total of 50 representative isolates of different *Aspergillus* species are presented in the results and discussed.

Effect of different media and temperature on growth of Aspergilli

In this study, the growth abilities of all strains of *Aspergillus* sp on Czapek dox agar (CZA), Czapek yeast agar (CYA), Czapek yeast 20% sucrose (CY20S) and malt extract agar (MEA) media were tested (according to Klich et al 2002). Each strain was 3-point inoculated with a dense spore suspension onto the specific media and inspected for growth and sporulation at every 2 days interval after inoculation of the isolates up to 16 days of incubation at 25°C. Further, growth of Aspergilli at two temperatures on CYA at 25°C and 37°C were also recorded. The taxonomic systems of *Aspergillus* (Raper and Fennell 1965; Klich 2002) were followed for the identification of different species.

RESULTS

Macro and microscopic examination and identification of the *Aspergillus* species based on morphological characters

Aspergillus flavus Link: Fr. 1809

Macroscopic characters:

The colonies of green color isolates were used for identification of the species and the centre of the colony of was yellowish-green to light green on PDA and the periphery of the colony showed dull white colored mycelium, consisting of a dense felt of conidiophores. Initially, the growth was dull white and later it turned into yellowish or parrot green color in whole plate in eight days (Plate 1a). The growth radiated and the hyphae were well developed, profusely branched, septate, and hyaline. The colony growth rates ranged between 5.4-9.5 mmd^{-1} at room temperature (25 ± 2ºC). The mycelium produced an abundance of conidiophores with profuse and bulged colony appearance. The conidiophores arose singly from somatic hyphae. Some of these strains produced black coloured sclerotia (Plate 1b) upon maturation with growth rings or wrinkles, exudates and pigmentation on media plates.

Microscopic characters

The conidiophores observed were simple, long, erect, smooth, or coarsely roughened, unbranched, aseptate, hyaline hyphae each terminated in a bulbous or spherical head which was known as vesicle (Plate 1c). The length and breadth of the conidiophores were moderate and varied from 515 - 556 or from 800 µm to 1 mm in length and 11.4 to 13.0 µm in diameter. A large number of conidiogenous cells (phialides or sterigmata) are produced over entire surface of the vesicle completely covering it. Conidial heads were either uniseriate (Plate 1d) or biseriate with two layers of phialides (Plate 1e), 300 to 400 µm in diameter, typically globose to radiate (later split into loose columns in some isolates) or columnar, very light to deep yellow green, olive brown or brown. The vesicles were globose upon maturity (Plate 1f) with heads, and 25.0-33.5 µm in diameter. Conidia were hyaline, single celled, smooth and produced in chains from the tip of the second row phialides (measured 6.5 to 8.5 x 4.0 to 5.5µm in diameter) and the metulae size varied from 4.5 to 5.5 x 3.5 to 4.5µm. Conidia were typically globose to sub-globose, 3.5 to 5.0 µm in diameter, extremely roughened walls, sometimes elliptical to pyriform, and conspicuously echinulate (Plate 1g). The conidial heads were observed to radiate in the same cultures (1-2 wk-old) with the development of sclerotial bodies (100µm, **Table 1**). Based on all these characters the fungal isolates were identified as *Aspergillus flavus* Link: Fr. 1809 as per Raper and Fennell (1965).

A. flavus Link.var. *columnaris*

Some of the parrot green colonies were velvety, uniformly dense, and yellowish green color on medium. The conidial heads showed consistently columnar, typically uniseriate and the size of the head ranged from 400 to 500 µm in length and 40 µm in breadth. The shape of the vesicle was sub-globose and the length of the conidiophores was 420 to 500 µm length and 9 to 12.2 µm in breadth. The vesicle observed was globose and measured 15 to 25 µm in size. The outer walls of conidiophore were thinner and somewhat less roughened in texture and hyaline to brown color. The size of the phialides ranged from 8.4 to 11 µm x 3.2 to 4.3 µm and the conidia observed were globose to sub-globose with minutely roughened walls and were 3.1 to 4.5 µm in size. When the same cultures were examined later (1-2wk-old), single column or columnar type vesicles were observed with released conidia forming a column shape (**Table 1**). Based on the presence of uniseriate, columnar vesicle and other morphological characters, the isolated fungus was identified as *A. flavus* Link.var. *columnaris.*

A. parasiticus Speare.

Macroscopic characters:

The olive green colonies showed radiate dense growth with turmeric yellow to olive green centre and the peripheral of the colony showed dull creamy white colored mycelium. Initial growth was dull white and later it turned into dark yellowish or olive green color (Plate 2a). The growth of the colony was 60 to 78 mm at eight days after inoculation. The mycelium produced an abundance of conidiophores with profuse and bulged colony appearance. Sclerotia were absent in all isolates.

Microscopic characters

The conidiophores observed were simple, medium, erect, roughened walls with length of 200 to700 µm and breadth of 9.5 to 11 µm. Conidial heads consistently loosely radiate (400 to 500 µm) and with a vesicle size of 25 to 40 µm in diameter and they were uniseriate (Plate 2b-e). When young phialides were clearly visible and were arranged radially on vesicle. They were olive, green or brown (Plate 2f). The sterigmata size varied from 6.8 to 9.0 µm in length and 2.8 to 3.7 µm in breadth. The conidia were globose, coarsely roughened and brown to olive brown color with 3.5 to 5.5 µm size (Plate 2c). Sclerotia and cleistothecia were absent. A few isolates showed pigmentation on media (**Table 1**). Based on all these characters the fungal isolates were identified as *A. parasiticus* Speare.

***A. fumigatus* Fresenius.**

Some of the green colored colonies on PDA were white initially and then turned dark green, while its old cultures turned smoky gray. The growth was rapid and the growth rate varied from 4.72- 6.74 mmd^{-1} after three days of incubation with dense colony. The colonies showed typical columnar, uniseriate conidial heads measured 100 to 400 µm. Conidiophores are short, smooth-walled with a size 250 to 300 µm length and breadth of 6.8 to 10 µm breadth. The shape of the vesicle observed was conical shape or sub clavate, which supported a single row (uniseriate) of phialides on the upper two-thirds of the vesicle and measured 20.0-30.0 µm in size with 6.5 to 7.8 µm in length x 2.0 to 3.0 µm in breadth of phialides (Plate 3a and 3d). The conidia were sub-globose, echinulate, green in colour and 2.4 to 3.7 µm in diameter. Sclerotia absent and a few isolates showed pigmentation (**Table 1**). Considering these characters the fungus was identified as *A. fumigatus* Fresenius. The teleomorph of this fungus is *Neosartorya fumigata*.

***A. niger* van Tieghem.**

Macroscopic characters:

The black colored colonies showed fast growth and covered entire plate in 5-6 days. Initial growth of the fungus was white; later it became black giving "salt and pepper appearance" and reverse turning pale yellow in one or two days. The fungus produced abundance of conidial heads which are in shades of black (Plate 4a). The mycelium is scanty, hyaline to white or light yellow. Hyphae are septate and smooth walled. After three days, the growth rates were determined to be 4.4-9.2 mmd^{-1}. Some isolates grew rapidly and were uniformly distributed and scattered.

Microscopic characters

Conidial heads initially appeared large, globose, dark brown which become radiate, but subsequently split into a few to several irregular or well defined divergent columns of conidial chains. They were black, globose, or radiate (measured 550 to 700 µm in diameter) and biseriate (Plate 4b). The vesicle shape was globose and the size was 40 to 75µm. Conidiophores were aseptate, and are 1100 to 3000 µm long, 11-19 µm in diameter, hyaline to light brown, unbranched, long, thin, erect, brittle and terminate in an inflated apex upon which radiating phialides or sterigmata were formed (Plate 4c- e). Each primary sterigmata (8.0 to 10.0 x 4.5 to 5.0µm) bore one pair of secondary sterigmata (7.0 to 10.0 x 3.0 to 3.5µm) from which the conidia arose in chains. The conidia were single celled pale to dark brown, rough walled, ornamented with warts and ridges, more or less globose, and 3.5-5.5 µm in diameter (Plate 4f). Based on these characters the fungus was identified as *A. niger* van Tieghem.

***A. aculeatus* Iizuka.**

Macroscopic characters

Some of the black colored colonies showed slow growth with black conidiophores at centre and white mycelial threads at peripherals. The growth was radiate to sparse dense, 50 to 60 mm in 10- 12 days after inoculation and with no color to pale yellow on reverse side (Plate 5a). The conidial heads were globose initially and split into a few compact columns later.

Microscopic characters

The conidial heads measured 500 to 700 µm or more than this in size with a globose vesicle somewhat elongated when young and became globose on maturiy, commonly pigmented in brown shades and the size ranged from 60 to 80 µm in diameter. The conidiophores showed light brown color 1.0 to 1.5 or 2.0 mm in length x 9.0 to 13.0 µm in breadth. The size of the sterigmata measured 6.0 to 9.8 µm in length x 2.8 to 4.5 µm in breadth (Plate 5b-e). The conidia are elliptical to globose, echinulate, and 3.5 to 5.0 µm diameter (Plate 6f). These characters confirmed the isolated fungus as *A. aculeatus* Iizuka.

***A. carbonarius* Bainier.**

Some of the black to carbon color colonies showed slow to medium growth 40 to 50 mm in 10 days, mycelium is white and moderately compact with somewhat raised and cushion type growth. Conidial heads are globose to radiate and split into columns and 400µm in size and they arose on long conidiophores. They ranged from 1000 to 1200µm in length and 8.7 to 10µm in breadth. The shape of the vesicle was globose and measured 50 to 60µm in diameter and biseriate, primary sterigmata up to 25µm in length and 7.0 to 9.0µm in breadth and secondary sterigmata 8.5 to 11µm length x 6.0 to 7.0µm breadth. The conidia were globose, very coarsely roughened and big spores of 5.0 to 7.6µm in size. Based on these characters it was identified as *A. carbonarius* Bainier.

***A. ochraceus* Wilhelm.**

Macroscopic characters

The yellow colored colonies showed slow, sparse growth, and covered entire plate in 10 to 12 days after inoculation. The growth of mycelium was initially white to creamy white and later turned into pale yellow to dark yellow (Plate 6a). The growth rate was 5.4-7.3 mmd^{-1} from three to four days onwards. The growth of the colony was radiate to irregular and the hyphae were small, erect and less compact. Sclerotial production was absent and a very few (1 or 2 isolates) isolates showed pigmentation.

Microscopic characters

The conidial heads were initially globose and later split into dense compact columns like tri- or penta-partite columns (Plate 6b), biseriate and showed more than 200 µm in diameter. The shape of the vesicle was globose, 30 to 50 µm in diameter. The conidiophores were long and measured 500 to 700 or more than 1100 µm in length and 9.5 to 12.1 µm in breadth with hyaline to pale yellow colored with smooth to less roughened walls. The primary phialides showed 9.2 to 10.5 µm x 4.5 to 5.1 µm and secondary phialides were 5.6 to 7.2 x 2.2 to 3.0 µm breadth in size (Plate 6c-d). The conidia were small, globose to sub-globose, smooth and delicately roughened with 3.0 to 4.3 µm in size (Plate 6f). Based on these observations the fungus was identified as *A. ochraceus* Wilhelm.

From these isolates, 40% isolates were identified as *A. flavus* followed by *A. niger* (30%), *A. parasiticus* (9%), *A. flavus* var. *columnaris* (8%), *A. ochraceus* (7%), *A. aculeatus* (3%), *A. carbonarius* (2%) and *A. fumigatus* (1%) (data not shown). The results in general indicated that Aspergilli were dominant in the seed samples. However, other fungi like *Rhizopus, Helminthosporium, Curvularia, Pencillium* and *Alternaria* spp. were also found, but at insignificant levels in the seed samples.

The different species of Aspergilli are furnished and compared each with others in terms of colony and morphological characters as well as growth response and microscopic characters of individual species as well as with neo-type cultures. The neo type cultures of MTCC's 2799, 2796, 872 and 1810 showed characters similar to that of isolates of parrot green (*A. flavus)*, olive green (*A. parasiticus)*, black (*A. niger)* and yellow (*A. ochraceus*) coloured colonies respectively (data not shown). The colony color variation of the species was from green shades (*A. flavus* and *A. parasiticus*), to yellow (*A. ochraceus*) and to black shades (*A. niger* and *A. aculeatus*). Among the species, the average maximum radial mycelial growth was shown by *A. niger* (74 mm), *A. flavus* (73 mm), *A. aculeatus* (72 mm), *A. parasiticus* (66 mm) and *A. ochraceus* (59 mm) on CY20S, followed by CYA and MEA and the least growth on CZ medium (Table 2). The size of the conidiophore length ranged from 488.5 (*A. parasiticus*) to 1780 (*A. aculeatus*), 7.7 (*A. ochraceus*) or 17.8 µm (*A. aculeatus*), vesicle size varied from 26.1 (*A. parasiticus*) to 80 µm (*A. aculeatus*) and size of the conidia 2.5 (*A. ochraceus*) to 6.1 µm (*A. parasiticus*), the shape of the conidial head varied from globose or sub-globose, columnar or radiate **(Table 1**).

Effect of different media and temperature on growth of Aspergilli

a. Growth on specific media

The average growth of isolates of individual species on different media was recorded at eight days after inoculation of the fungi on to the medium. The growth of *A. flavus* on CY20S showed the maximum growth (73 mm) followed by CYA (71 mm), MEA (70 mm) and the least growth was recorded on CZ (53.4 mm). *A. parasiticus* showed maximum growth on CY20S (66 mm) followed by MEA (63 mm) and CYA (61 mm). The growth of *A. niger* similar to that found in other species with maximum growth on CY20S (74 mm) and minimum on CZ (53.4 mm). The growth of the *A. ochraceus* was less with a slow growth rate on all media, however, maximum growth was recorded on CY20S (59 mm) and the least growth was on CZ (34 mm). The growth of *A. aculeatus* was similar to all other species with the maximum growth on CY20S (72.2 mm) and minimum on CZ (51.1 mm). Irrespective of species, the growth was fast, luxurious, velvety, puffy, bulge or raised, high dense, maximum on CY20S whereas, on CZ, growth was slow, suppressed and minimum. The length of the conidiophores and the hyphal length were also suppressed on CZ medium. The CYA medium stands as the second best of the media for *A. flavus, A. niger* and *A. aculeatus*, and MEA stands as the second best for *A. parasiticus* and *A. ochraceus*. The growth on MEA was sparse and individual hyphae arose into individual conidiophores with conidial head, and the growth was sparse and looks like raised dots on media plate, and it hasn't supported any sclerotial production and growth circles or wrinkles, even if the same isolate produced sclerotial bodies on other media. The sclerotial production was highly supported or produced by CYA as well as CY20S media while CZ supported less. The pigmentation was high on CZ medium followed by CYA and MEA. Even if some isolates did not produce pigmentation on other media, they showed pigmentation on CZ medium (Table 2).

The radial mycelial growth of isolates of Aspergilli on four media at different incubation times, i.e., every alternate day after incubation, was recorded and the growth was observed to gradually increase at all intervals (Fig 1b). CY20S showed the maximum growth of all *A. flavus* isolates at all incubation times followed by CYA.

The growth on MEA and CYA was altered for different isolates of *A. parasiticus, A. aculeatus, A. niger* and *A. ochraceus* at various incubation periods (Fig 1a).

The statistical analysis of variance (ANOVA) for different media at different incubation times for *A. flavus, A. parasiticus* and *A. ochraceus* showed that all the source of variances and their interactions were significantly different at $P < 0.05\%$ level. For *A. niger*, isolate, media, time, isolate x media, media x time and isolate x time showed significant differences whereas, for isolate of *A. aculeatus*, media, time and media x time showed significant differences. The species, media, time and the interactions of species x media and species x time were significantly different for the species of Aspergilli. The coefficient of variance (CV) was below 10% for all species (Table 3).

b. Temperature effect on growth of Aspergilli on specific media

Growth rate of Aspergilli on CYA was recorded at two temperatures (25°C and 37°C) and it varied from 4.26 to 15.59 and 2.57 to 10.21mmd^{-1}, respectively. The growth was slow and comparatively suppressed at 37^{o}C than at 25^{o}C in all species. But pigmentation, growth circles and sclerotial production was more at 37^{o}C. Some isolates of *A. flavus* and *A. ochraceus* produced sclerotia that were fewer in numbers at 25^{o}C and so many dark brown to black shiny sclerotia were present at 37^{o}C including an isolate of *A. niger* (Table 4).

DISCUSSION

Identification and morphological characterization of Aspergilli

A morphological examination of species was first made with naked eye and at low magnification power of microscope. The colonies are usually fast growing, initially white to creamy white or dull white and later turn to shades of green, yellow, yellow-brown, brown to black. They mostly consist of a dense felt of erect conidiophores. Presence of conidiophores, vesicle and its shape and size; conidial head, shape, size, phialides producing conidia and in some species presence of secondary sterigmata (metulae) including all these characters led to identification as *Aspergillus* and by the difference in these characters, species were further identified. The detailed examination was made by measuring the dimensions of the microscopic structures, photographing the microscopic structures and the *Aspergillus* species were identified as *Aspergillus flavus* Link: Fr. (40%), *A. flavus* Link. var. *columnaris* (8%), *A. parasiticus* Speare (9%), *A. niger* van Tieghem (30%), *A. ochraceus* Wilhelm (7%), *A. aculeatus* Iizuka (3%), *A. fumigatus* Fresenius (1%), *A. carbonarius* Bainier (2%) using relevant literature as reference manual about the genus Aspergilli (Raper and Fennell 1965; Mc Clenny 2005; Diba et al 2007; Domsch et al 1980; Samson and Pitt 2000) and classification system (Gams et al 1985). This study revealed the importance of macroscopic characters in sub-generic classification in the genus *Aspergillus*. Conidial colours as observed on Petri plates served as a major sub-generic classification characters. Colours vary from black or white to yellow or green to blue. A color guide is used for determining these characters by using Kornerup and Wanscher (1978) color chart. Some colonies may also produce droplets of liquid (exudate) on the surface of the colony. Coloured pigment may be produced in the agar on the reverse of the colony (reverse color). Some of these may be soluble enough to extend beyond the edge of the colony (soluble pigment). Colony diameter is sometimes useful, as species in the genus vary in ability to grow at different temperatures. Colony diameter is measured by holding the plate up to the light and measuring from one edge of mycelial growth to the opposite edge. Other structures found in this genus include sclerotia that are asexually formed as firm masses of hyphae. 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 identification. Several *Aspergillus* taxonomic keys and guides are available (Klich 2002; Raper and Fennell 1965).

A detailed morphological study of ex-type and other isolates of *A. flavus*, *A. parasiticus*, *A. niger* and *A. ochraceus* was undertaken. The growth parameters and morphological characters of the isolates of the individual species were found similar to the characters of neo-type cultures of MTCC 2799 (*A. flavus*), MTCC 2796 (*A. parasiticus*), MTCC 872 (*A. niger*) and MTCC 1810 (*A. ochraceus*), respectively. Further, the microscopic features and measurements of the individual species were within the range of Klich values (data not shown). Kozakiewicz (1982) also had made similar reports on *A. flavus* and *A. parasiticus*. More worryingly, since the publication of Kozakiewicz (1982) no other than the collection at CABI, Egham, UK (formerly IMI) which has re-disbursed the particular isolates. This has confirmed that these mistakes have been rectified; implying that wrongly named material continues to be sold and distributed. Furthermore, in routine examinations of the IMI collection, 10 additional isolates have been re-identified to date (Kozakiewicz 1994). Morphologically, it resembles *A. flavus* but differs by the production of small bullet-shaped sclerotia; those in *A. flavus* being more globose.

Contemporary diagnosis of the two species was made based on the descriptions and keys of Raper and Fennell (1965). The colony color variation of the species ranged from green shades (*A. flavus* and *A. parasiticus*), yellow (*A. ochraceus*) and black shades (*A. niger* and *A. aculeatus*). The primary separation was the shape of the conidial heads presence of globose to radiate head and biseriate or uniseriate for *A. flavus* and columnar shape, typically uniseriate for *A. flavus* var. *columnaris*.

The next predominant character is the size of the conidial head, globose and measured 300 to 400µm for *A. flavus* and somewhat elongate head with 400 to 500 µm for *A. flavus* var. *columnaris* (Table 1). The conidiophore length ranged from 488.5 (*A. parasiticus*) to 1780 (*A. aculeatus*), breadth 7.7 (*A. ochraceus*) to 17.8 µm (*A. aculeatus*); vesicle size varied from 26.1 (*A. parasiticus*) to 80 µm (*A. aculeatus*) and size of conidia from 2.5 (*A. ochraceus*) to 6.1 µm (*A. parasiticus*); and shape of conidial head varied from globose or sub globose to columnar or radiate. Similar findings and observations on seriation of the individual species were also reported by Afzal et al (2013). The results showed that *A. fumigatus* and *A. flavus* were dominating species isolated and *A. fumigatus* is a rapidly growing species than *A. iccum*, *A. flavus*, *A. flavus* var. *columnaris* and *A. terricola* var. *americana* followed by *A. terreus* var. *aureus* and *Emericella rugulosa*. Out of eight species, *A. fumigatus* showed uniseriate heads, and *A. flavus* var. *columnaris* and *A. parasiticus* showed mostly uniseriate heads but few biseriate heads were also found. The remaining species showed typical biseriate conidial heads. Identifying character of *A. flavus* were yellow to green or dark green colony color and radiate heads with rough conidiophore wall. The difference between *A. flavus* and *A. flavus* var. *columnaris* was in conidial heads; on MEA *A. flavus* var. *columnaris* showed typical columnar heads whereas *A. flavus* showed radiate heads.

Table 1. Morphological characteristics of different species belonging to Aspergilli

Species	Seriation	Conidial head appearance	Conidial head size (mm)	Vesicle size (mm)	Primary sterigmata length & breadth	Secondary sterigmata length & breadth	Sclerotia size (mm)	Sclerotia Color
A. flavus	Either uni or biseriate	Globose to loose radiate/ columnar	300 to 400µm	25.0 to 33.5µm	6.5 to 8.5 x 4.0 to 5.5µm	4.5 to 5.5µm x 3.5 to 4.5µm	100µm	Dark black
A. flavus var. *columnaris*	Typical uniseriate	Colomnar and radiate	400 to 500µm	15 to 25µm	8.4 to 11 x 3.2 to 4.3µm	Absent	Absent	Absent
A. parasiticus	Uniseriate	Globose to radiate or loose columns	400 to 500µm	25 to 40µm	6.8 to 9.0 x 2.8 to 3.7µm	Absent	Absent	Absent
A. niger	Biseriate	Globose to loose columns	550 to 700µm	40 to 75µm	8.0 to 10.0 x 4.5 to 5.0 µm	7.0 to 10.0 x 3.0 to 3.5 µm	Absent	Absent
A. ochraceus	Biseriate	Initially globose later splitted into tri or penta partite columns	Morethan 200µm	30 to 50µm	9.2 to 10.5 x 4.5 to 5.1 µm	5.6 to 7.2 x 2.2 to 3.0 µm	Present in 1 or 2 isolates	Absent
A. aculeatus	Uniseriate	Globose to radiate or compact columns	500 to 700µm or more	60 to 80 µm	6.0 to 9.8 x 2.8 to 4.5 µm	Absent	Absent	Absent
A. fumigatus	Typical uniseriate	Typical columnar	100 to 400µm	20.0 to 30.0µm	6.5 to 7.8 x 2.0 to 3.0 µm	Absent	Absent	Absent
A. carbonarius	Biseriate	Globose to radiate or compact columns	400µm more than	50 to 60 µm	25 µm and 7.0 to 9.0 µm	8.5 to 11 x 6.0 to 7.0 µm	Absent	Absent

The primary separation was the presence of metulae and phialides (biseriate conidial head), parrot green color colonies for *A. flavus* and phialides only (uniseriate conidial head), olive green colonies for *A. parasiticus* (Plate 1d and 2c). In the key for *A. parasiticus*, the words "strictly uniseriate" replace the former terms of "usually" or "mostly uniseriate" (Plate 2d) as used in previous keys (Thom and Church 1926; Thom and Raper 1945).

Examination of a large number of *A. parasiticus* isolates (Kozakiewicz 1995) has shown that up to 10% of conidial heads in an *A. parasiticus* colony can have metulae and phialides (biseriate). Furthermore, not all *A. flavus* isolates consistently produce metulae (Klich and Pitt 1988). Conidia of *A. parasiticus* are more spherical and noticeably echinulate or spinulose (Plate 1e and 2e). The primary distinguishing character is the presence of pale or yellowish green shades, globose to radiate with uni- and bi-seriate heads for *A. flavus* whereas, lily green, erect, compact and strongly columnar, uniseriate heads for *A. fumigatus*. The shape of the vesicle was globose for *A. flavus* and sub-clavate shape for *A. fumigatus*. The characteristic feature was the presence of biseriate conidial head, globose vesicle for *A. niger* and uniseriate conidial head with slightly elongated vesicle when young and globose upon maturation for *A. aculeatus* (Plate 4e and 5c-d). The size of the vesicle was brown, solid and somewhat bigger than that of *A. niger*. *A. carbonarius* resembled *A. niger* in many features, and indeed the two species were very closely related. *A. carbonarius* differred from *A. niger* most notably in the production of larger spores, although other minor morphological differences also exist. All these differences were furnished by Raper and Fennell (1965) and Klich (2002).

Effect of different media and temperature on growth of Aspergilli

a. Growth on specific media

The use of differential media helps to accelerate growth rate and the production of conidia. The media components are important criteria for fungal culture and study, along with important physiological parameters that lead to maximum sporulation in fungi (Kim et al 2005; Saxena et al 2001; Saha et al 2008). Moreover, the variations in colour of spores, especially among *Aspergillus* spp. are one of the main criteria used widely for their identification and taxonomic placement (St-Germain and Summerbell 1996) which seems to be mainly attributed to the constituents of a medium. In the present investigation, type of culture media and their chemical compositions significantly affected the mycelial growth rate and conidial production in *A. flavus*, *A. parasiticus*, *A. niger*, *A. ochraceus* and *A. aculeatus*. Irrespective of species the growth was fast, luxurious, velvety, puffy, bulge or raised, high dense and maximum mycelial radial growth on CY20S shown by *A. niger* (74 mm), *A. flavus* (73 mm), *A. aculeatus* (72 mm), *A. parasiticus* (66 mm) and *A. ochraceus* (59 mm) on CY20S (Table 2), followed by CYA and MEA whereas, least, slow, suppressed, minimum growth was recorded on CZ medium. The length of the conidiophores and the hyphal length were also suppressed on CZ medium. The CYA medium stands as the second best medium for *A. flavus*, *A. niger* and *A. aculeatus* and MEA stands as the second best for *A. parasiticus* and *A. ochraceus* (**Fig 1a**). The growth on MEA was sparse and individual hyphae raised into individual conidiophores with conidial head, and the growth was sparse looked like raised dots on media plate, and did not supported any sclerotial production and growth circles/ wrinkles, even if the same isolate produced sclerotial bodies on other media. The average growth of isolates of *A. niger* was high at two and four days after incubation time and at six and eight days after inoculation *A. flavus* showed high, whereas, *A. ochraceus* showed low growth among the five species at all the intervals **(Fig 1b**). These results were on par with the results of Diba et al (2007) and Smith and Dawson (1944) and they reported, the growth rates of *A. flavus, A. parasiticus, A. niger, A. repens, A. kanagawaensis* and *A. terreus* were almost similar to that of *A. fumigatus* when colonies were observed on PDA and RBA media, after incubation for 5 days at 25°C. Growth of *A. ochraceus* was slightly slow. Several workers stated PDA to be the best media for mycelial growth (Xu et al 1984; Maheshwari et al 1999; Saha et al 2008). *A. niger* has been seen to thrive best on PDA, which is rich in nutrients, thus encouraging the mycelial growth and sporulation.

The sclerotial production was high on CYA and CY20S and less in CZ. The pigmentation was more on CZ followed by CYA and MEA. Even if some isolates did not show pigmentation on other media, they showed pigmentation on CZ medium. The results have shown that CZA and MEA media are easy, simple and reliable as also recorded by Raper and Fennell (1965). Colony diameter was more on MEA than on CZA. Various reports have been published that used morphological characters as key identifying factors (Alwakeel 2007; Morya et al 2009; Bandh et al 2012). Colony and morphological characters of *Aspergillus* species on CYA, MEA, CYSA, CZA media were also studied by Ulhan et al (2006). Diba et al (2007) identified 11 *Aspergillus* species and demonstrated that use of four differential media including CYA, CYA20S, MEA and CZ is a simple and reliable method for identification of *Aspergillus* species. Raper and Fennell (1965) and Johnston and Booth (1983) also used two differential media, Czapek solution agar and MEA, to identify *Aspergillus* species.

Table 2. Effect of media on colony characteristics of Aspergilli

Species	CYA	MEA	CY20S	CZ
Colony diameter (mm)				
A. flavus	71.0	70.0	73.0	53.4
A. parasiticus	61.0	63.0	66.0	50.0
A. niger	69.0	68.0	74.0	53.4
A. ochraceus	51.0	54.0	59.0	34.0
A. aculeatus	67.0	65.3	72.2	51.1
Colony color and shape				
A. flavus	Parrot green, circular margins	Parrot green, circular margins	Pale to dark parrot green, circular	Pale to dark parrot green, circular
A. parasiticus	Olive green, circular to wavy	Olive green, circular	Olive green, circular to wavy	Olive green, wavy margins
A. niger	Dark black, circular	Black, circular	Dark black, circular	Dark black, circular to wavy margins
A. ochraceus	Creamy white to yellow/ dark yellow, circular	Creamy white to yellow, circular	Creamy white to yellow, circular	Pale yellow, circular
A. aculeatus	Dark black, circular	Black, circular	Dark black, circular	Dark black, circular to wavy margins
Mycelium				
A. flavus	Fluffy, white to parrot green	Fluccose& white to parrot green	Fluffy parrot green	Normal parrot green
A. parasiticus	Fluffy,dull white	Dull white	Fluffy growth at centre & dull white	Dull white
A. niger	Fluffy, dull white	Fluccose, dull white	Fluffy, dull white	Fluffy, dull white
A. ochraceus	Dull white with cream mat	Fluffy white to dull wheat	Dull white to creamy white	Dull white
A. aculateus	Fluffy, dull white	Fluccose, dull white	Fluffy, dull white	Fluffy, dull white
Conidial heads				
A. flavus	Pale to parrot green	Parrot green	Parrot green	Parrot green
A. parasiticus	Pale to olive green	Olive green	Dark olive green	Olive green
A. niger	Creamy white to black	Pale black	Creamy white to black	Creamy white to black
A. ochraceus	Dark yellow buff	Dark to buff yellow	Creamy white to yellow	Creamy white to dark yellow
A. aculeatus	Creamy white to black	Pale black	Creamy white to black	Creamy white to black
Conidial head shapes				
A. flavus	Globose to columnar	Globose to columnar	Globose to columnar	Globose to columnar
A. parasiticus	Radiate	Radiate	Radiate	Radiate
A. niger	Radiate	Radiate	Radiate	Radiate
A. ochraceus	Globose to splitted into 3-5 columns	Globose to splitted into 3-5 columns	Globose to splitted into 3-5 columns	Globose to splitted into 3-5 columns
A. aculateus	Globose to radiate	Globose to radiate	Globose to radiate	Globose to radiate

Reverse side				
A. flavus	Pale yellow to orange/reddish brown	Pale yellow to orange/brown	Yellow to orange	Yellowish orange/ red tinge to light peach
A. parasiticus	Red tinge with chocolate brown	Brown	Reddish brown	Reddish tinge to dark brown
A. niger	Pale yellow to florescent yellow/ yellow	Dark orange to brown	Pale yellow to yellowish brown	Pale yellow to florescent yellow
A. ochraceus	Dark yellow to brownish yellow	Pale yellow to dark yellow	Dark yellow to brownish yellow	Yellow to brownish yellow
A. aculeatus	Pale yellow to florescent yellow/ yellow	Dark orange to brown	Pale yellow to yellowish brown	Pale yellow to florescent yellow
Presence of growth circles				
A. flavus	Present	Absent	Present	Present
A. parasiticus	Present	Absent	Present	Present
A. niger	Present	Absent	Present	Present
A. ochraceus	Present	Absent	Present	Absent
A. aculateus	Present	Absent	Present	Absent
Pigmentation				
A. flavus	Present in some isolates	Present in some isolates	Slight pigmentation in very few isolates	Present in some isolates
A. parasiticus	Present in few isolates	Absent	Absent	Present in some isolates
A. niger	Absent	Absent	Absent	Present in very few isolates
A. ochraceus	Absent	Absent	Absent	Present in few isolates
A. aculeatus	Absent	Absent	Absent	Absent
Sclerotial production				
A. flavus	Present in some isolates	Absent	Present in some isolates	Present in some isolates
A. parasiticus	Absent	Absent	Absent	Absent
A. niger	Absent	Absent	Absent	Absent
A. ochraceus	Absent	Absent	Present in one or two isolates	Absent
A. aculeatus	Absent	Absent	Absent	Absent
Sclerotialcolor and number				
A. flavus	Wheat brown & Very few	Absent	Wheat brown & few	Light brown & few
A. parasiticus	Absent	Absent	Absent	Absent
A. niger	Absent	Absent	Absent	Absent
A. ochraceus	Absent	Absent	Few	Absent
A. aculeatus	Absent	Absent	Absent	Absent

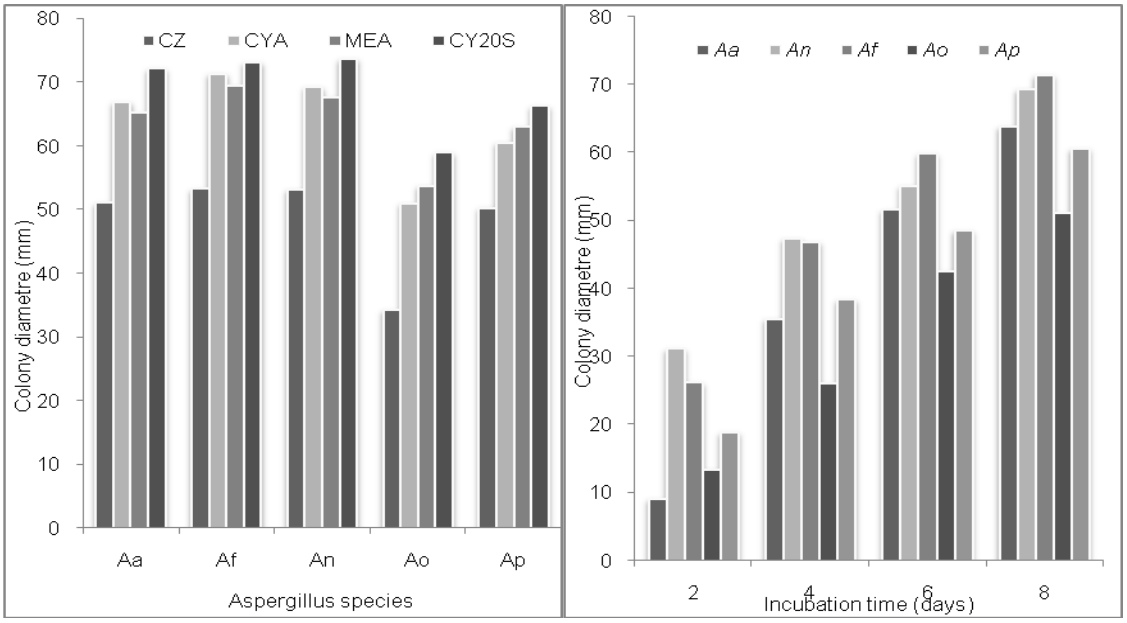
Table 3. ANOVA for Aspergilli radial growth data at different times and media

Source	Mean Squares (MS)	Factor (F)	Probability (P)
Species	6.6544	324.36	<0.0001
Isolate	0.6416	-	-
Media	8.4223	410.53	<0.0001
Time	46.7267	2277.62	<0.0001
Species x Media	0.1153	5.62	<0.0001
Species x Time	0.4523	22.05	<0.0001
Media x Time	0.0493	2.4	0.0106
Species x Media x Time	0.036	1.75	0.0039
Error	0.0205	-	-
CV	6.63		

Table 4. Effect of temperature on growth of Aspergilli on CYA medium

Characteristics*	CYA 25^{O}C					CYA 37^{O}C				
	A. flavus	***A. parasiticus***	***A. niger***	***A. ochraceus***	***A. aculeatus***	***A. flavus***	***A. parasiticus***	***A. niger***	***A. ochraceus***	***A. aculeatus***
Relative growth rate at 48h	13.09	9.45	15.59	6.74	3.81	8.92	6.55	9.45	4.91	1.87
Relative growth rate at 96h	10.36	9.77	8.13	6.27	15.81	6.1	6.34	6.21	4.79	10.21
Relative growth rate at 144h	6.5	5.09	3.88	8.28	8.02	3.37	3.98	2.1	5.87	5.96
Relative growth rate at 192h	5.77	6	7.09	4.26	5.85	4.1	3.87	4.54	2.57	2.71
Colony color	Pale to parrot green	Olive green	Dark black	Creamy white to yellow/ dark yellow		Dark parrot green	Olive green	Dark black	Turmeric yellow	Black
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular & wavy	Circular	Slight wavy	Circular
Mycelium	Fluffy white to parrot green	Fluffy, dull white	Fluffy, dull white	Dull white with cream mat	Dull white	Creamy to parrot green	Fluffy, dull white	Fluffy, dull white	Dull white with cream mat	Dull white
Growth circles	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present
Production of sclerotia	Present in some isolates & very few	Absent	Absent	Present in 1 or 2 isolates		Present in some isolates & many in number	Absent	Present	Present	Absent

*Relative growth rate measured in mm/day



Af = A. flavus; An = A. niger; Ap = A. parasiticus; Ao = A. ochraceus; Aa = A. aculeatus

Figure 1. Growth of Aspergillus species in different media (a) and at different incubation periods (b)

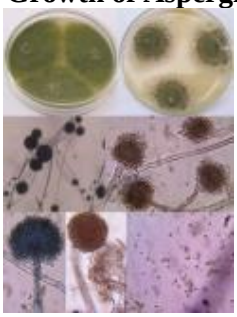


Plate 1a-g.*A. flavus*

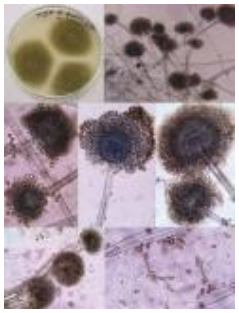


Plate 2a-g.*A. parasiticus*

Plate 3a-d.*A. fumigatus*

Plate 4a-g.*A. niger*

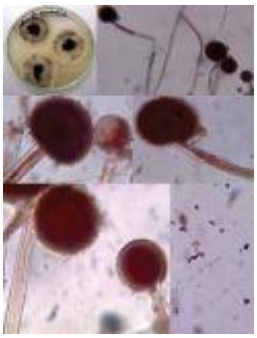


Plate 5a-g. *A. aculeatus*

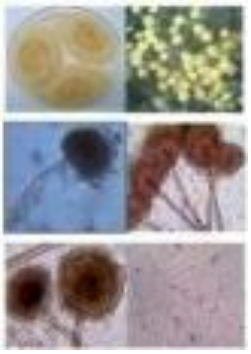


Plate 6a-f.*A. ochraceus*

b. Temperature effect on growth of Aspergilli on specific media

Growth rate of Aspergilli on CYA recorded at 25°C and 37°C varied from 4.26 - 15.59 and 2.57 - 10.21 mmd^{-1}, respectively. The growth was slow and comparatively suppressed at 37°C than at 25°C in all species. But pigmentation, growth circles and sclerotial production was more at 37°C. Sood (2011) stated that the incubation temperature on *Aspergillus umbrosus* has shown a very narrow range to tolerance. Below 20°C, growth rate was very less with minimum sporulation and light yellow colouration of the medium. Optimum growth occurred at 30°C with equally good growth at 26°C. This gives a range of 26-30°C for its best growth. Achar et al (2006) also investigated the effect of different substrates and temperatures on the growth and aflotoxin production in *A. flavus*. In contaminated peanuts in Georgia at 10°C or at 37°C neither growth nor aflotoxin was detected. Maximum growth and aflotoxin production was at 27 and 30°C in three media tested - potato dextrose agar (PDA), nutrient agar (NA) and corn meal agar (CMA) (Moss 1989). Similarly, Oladiran and Iwu (1993) reported 30°C and relative humidity of 70-90% to be optimal conditions for the growth of *A. niger* and *A. flavus*.

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ISSN : 0976-4550

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