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Mycofloral diversity of glutinous rice *Aghoni Bora* in storage condition

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Abstract

Rice grain is contaminated with various fungi depending on the storage condition. The present study was conducted to identify the possible contaminants of glutinous rice variety *Aghoni bora. Aghoni Bora* grain samples were collected from districts of Assam. Nine species of filamentous fungi from the genus *Acremonium, Aspergillus, Chaetomium, Cladosporium, Gibellula, Paecilomyces* and *Penicillium* were identified morphologically among which potential mycotoxin producers were also present.

Keywords: *Aghoni Bora,* storage fungi, morphology, mycotoxins, contaminants

Introduction

Rice (*Oryzae sativa* L.) is the most important staple food crop in India and the crop is cultivated during *kharif* or wet season. Like most of the states in the country, Assam is also a traditionally rice growing state. In Assam, rice is grown in an area of 25 lakh ha with a production of 2093 kg/ha. In India, rice is cultivated in 43.87 m ha with the production of 104.32 mt, and productivity of 2381 kg/ha 2016 (Ministry of Agriculture, GOI, 2016). Rice plays a pivotal role in the socio-cultural life of the people of the state. As a traditional practice, specific rice varieties are processed into rice products like *tilpitha, ghilapitha, sungapitha, chungachaol, chira, bhojabora, hurum, laru, bhoja chaul, sandahguri* etc. and even rice beer, which are of both ethnic and commercially important (Ahmed *et al*., 2010; Dutta *et al*., 2014). However, rice harbours many microorganisms specially fungi either at field conditions or/and during storage. Fungi such as *Alternaria* sp., *Cladosporium* sp., *Fusarium* sp., *Helminthosporium oryzae* and *Pullularia* sp., to name a few, invade seeds as they are developing on the plants in the field or after they have matured, but before they are harvested; and, for this reason, they have been designated "field fungi" (Christensen, 1957). In storage, the development of fungi, especially *Aspergillus* spp. and *Penicillium* spp., is an unsolved problem. These fungi are responsible for rice quantitative and qualitative losses and are also potential mycotoxin producers. Mycotoxin contamination in stored agricultural commodities like rice has been a serious concern for human and animal health. Mycotoxins are substances produced mostly as secondary metabolites by filamentous fungi that grow on seeds, grains, and feed in the field, or in storage. The major mycotoxin-producing fungi are species of *Aspergillus, Fusarium* and *Penicillium*. Important mycotoxins are *viz*., Aflatoxins, fumonisins, trichothecenes, ochratoxins, cyclopiazonic acid, patulin, deoxynivalenol, zearalenone, citrinin, gliotoxin, and sterigmatocystin. Several workers reported the dominance of *Aspergillus* and *Penicillium* under storage conditions (Ali and Deka, 1996; Amadi *et al*., 2009). The present study identifies and determines the presence of filamentous fungi associated with dehusked rice grains of Aghoni bora under storage.

Materials and Methods

Survey and sample collection

A roving survey was conducted in Jorhat (26°87′ N, 94°15′E), district of Assam to observe the rice storage practice and to collect dehusked rice samples. Rice grains (cultivar: *Aghoni Bora*) stored in gunny bags, plastic bags and at house hold storage bins were collected in sterilized plastic bags and were brought to laboratory for direct isolation of associated fungi.

Isolation and purification of fungi

Direct plating of stored rice grain to isolate fungi was done in potato dextrose (PDA) agar containing 200 ppm streptomycin sulphate. Any visible mycelial growth or spores were transferred to Potato Dextrose Agar (PDA) plates. The fungal cultures were purified by hyphal tip culture method in water agar media. The pure culture of the isolates was maintained on PDA slants throughout the experimental period by subsequent periodical sub-culturing on fresh medium and stored at 4°C in refrigerator.

Cultural or macromorphological studies

Apart from PDA, the isolated pathogens were also cultured on specific media, whenever needed, like Malt Extract Agar (MEA), Czapek Dox Agar(CDA), Rose Bengal Agar (RBA) and Oat Meal Agar (OMA) containing 200 ppm streptomycin sulphate for specific growth characteristics. The observations were recorded after incubation at 25±1°C for 5 days, on colony colour, diameter, elevation and type of margin. Colony colour was recorded with standard reference of Royal Horticultural Society (RHS) colour chart. The changes in pigmentation of the colonies were also recorded.

Micro-morphological studies

Colony structures of different isolates of fungal cultures were studied with different dyes *viz.,* lacto phenol; lacto phenol cotton blue; and basic fucshin. Care was taken to minimize the damage of structures during slide preparation. Structures *viz*., sporangial head, phialide, conidia etc. were measured under high power objectives using an ocular micrometer. The average size of the spores was determined by ocular and stage micrometer and shape of the spores was also recorded.

Identification

Identification and characterization of fungi were carried out with the help of relevant keys, monograph and literature (Thom and Church, 1926; Subramanium, 1971; Raper and Thom, 1984 and CBS database). For confirmation of the identity of isolated fungal cultures, pure cultures were sent to National Centre for Fungal Taxonomy (NCFT), New Delhi.

Results and Discussion

Nine filamentous fungi were isolated from stored rice samples and their macro and micromorphology were listed in **Table 1**. An isolate was identified as *Acremonium strictum* W. Gams [Synonyms: *Sarocladium strictum* (W. Gams) Summerbell (2011)] based on slow growing colony, attained 3 cm in 10 days. The mycelia of the colony showed fibrous pattern of growth. The front colour of the colony was recorded as white (155A RHS) along with the reverse colony colour of yellowish (16 B RHS) tinge on MEA. Conidia were single celled that arose from weakly branched conidiophores. The long slender phialides arose from hyphae, cylindrical to oval in shape and ranged from 4.8 to 7.0 micrometer (µm) in length and 2.0 to 3.0 µm in breadth (**Figure 1**). Perdomo *et al*., (2010); Summerbell *et al*., (2011) also described similar characteristics of *A. strictum* based on which the fungus was identified. However, the species was also confirmed as *A. strictum* by NCFT with the I.D. no. 1995.17.

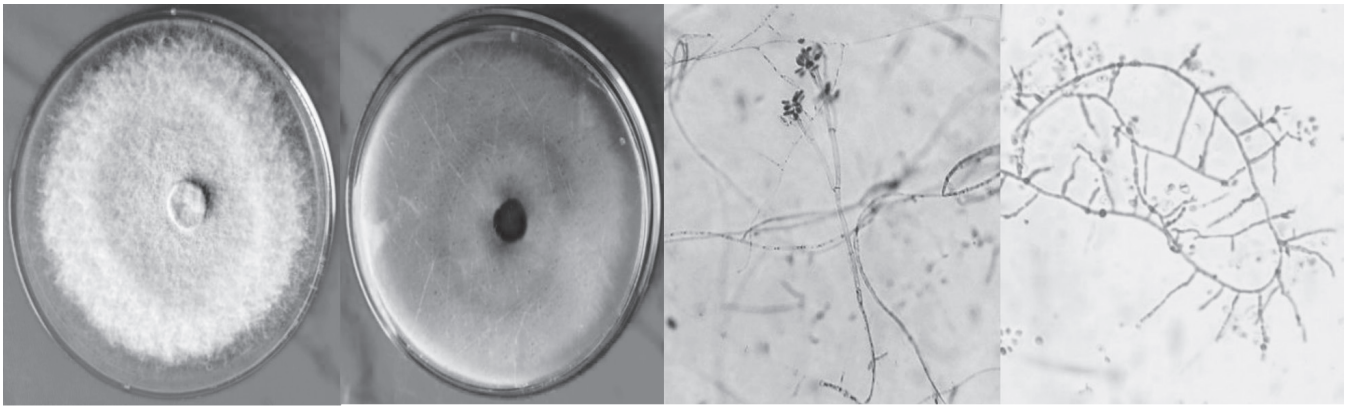

Figure 2: Macro and micromorphology of *Aspergillus niger*

(a) front view (b)reverse view on MEA (c) front view (d) reverse view on Oat Meal Agar
(e) hyaline smooth conidiophore, globose vesicle and biseriate conidial head (f) foot cell

Table 1. Macromorphology and micromorphology of filamentous fungi isolated from stored rice (*Aghoni Bora*)

Isolate	Macromorphology (Colony characters)		Micromorphology		
	Front Colour	Reverse colour	Conidia/spore shape	Conidia size (µm)	Conidium ontogeny
Acremonium strictum	White	Yellowish	cylindrical to oval, single celled	4.8 to 7.0 x 2.0 to 3.0	On Phialides
Aspergillus niger	Black	Yellow	globose to subglobose	3.5-5.0 diameter	On biseriate conidial heads with phialides
Chaetomium globosum	Grey	Grey	lemon-shaped ascospores	4.2 to 6.5 x 4.5 to 6.8	within clavate ascomata
Cladosporium sp.	Grey	Grey	Single celled or septate; oval to cylindrical	4.2 to 8.3 x 5.5 to 16.5	Budding present
Gibellula sp.	White	Whitish	cylindrical to clavate	4 to 9 x 2 to 4	On conidial heads
Paecilomyces sp.	White	Orange	spherical to ovoid	2.3 x 2.5-2.6	On awn shaped phialides
Penicillium chrysogenum	gray green	Yellowish	subglobose to elliptical	3.0 to 4 x 2.8 to 3.5	On biverticillate penicilli
Penicillium purpurogenum	Dark green	Orange red	subglobose	3.0 to 3.5 x 2.5 to 3.0	On biverticillate penicilli
Penicillium sp.	grey green	Dark green	globose to subglobose	3.5 to 4.8 x 3.2 to 4.5	On biverticillate penicilli

Another isolate was identified as *Aspergillus niger* van Tiegh. based on colonies on czapek dox agar, which consisted of a compact white or yellow (10C RHS) basal felt covered by dense layer of black conidial heads (202A RHS) **(Figure 2a-b)**. On oat meal agar the colony produced dark black spores (**Figure 2. c-d**). Conidiophores were smooth-walled, hyaline or changing dark in colour towards the vesicle. Conidial heads were 250-300 µm in diameter, with globose vesicle and dark brown in colour, radiate in nature and tend to split into several loose columns with age. Conidial heads were biseriate with the phialides borne on brown metulae. Conidia were globose to sub-globose (3.5-5.0 µm in diameter), dark brown to black color (**Figure 2.e**). These characteristics were compared with standard description of Thom and Church (1926) and the species was confirmed as *Aspergillus niger.1*

Chaetomium globosum Kunze grey coloured colony on PDA and recorded fast growth (156B RHS) (**Figure 3a-b**). Perithecial hairs were terminal, long and undulate and loosely coiled (**Figure 3c**). Sexual sporulation produced flat lemon-shaped ascospores **(Figure 3e)** , within the clavate ascomata (**Figure 3d**), ascus (up to 20-30 µm) with eight ascospores and a size varied from 4.2 to 6.5 by 4.5 to 6.8 µm. Girisham *et al*., (2016) described similar characteristics for *Chaetomium globosum* based on which isolated species was confirmed.

Cladosporium herbarum (Pers) Link produced single celled to septate small, lemon-shaped and smooth walled conidia. They formed long, fragile chains up to 10 conidia in length with distinctive darkened connective tissue between each spore. Budding was present and conidia gave rise to new conidia, ranged from 4.2 to 8.3 by 5.5 to 16.5 µm in measurement (**Figure 4**). Identification of isolated species was confirmed following the monograph of *Cladosporium* (Bensch *et al.*, 2012).

Gibellula sp. showed septate and verrucose conidiophores with reduced vesicle. Conidial structures aggregated into synnemata, conidia were more or less cylindrical to clavate, apiculate, 4-9 x 2-4 µm (**Figure 5**). Samson and Evans (1973) described similar characteristics for *Gibellula* reported from insect host, based on which the identity of the genus was confirmed.

Isolated *Paecilomyces* sp. was fast growing and white colour in nature (155A RHS) on PDA Produced orange pigment (25D RHS) on the reverse side of the colony. Conidiophore were septate, branched and bore the penicilate heads. Phialides are born in verticils on penicilate head. Phialides were awn shaped, swollen at the base with slender long neck. Conidia are spherical to ovoid and smooth in nature (**Figure 6**). Thus, the genus was identified as *Paecilomyces* sp. based on the description of Samson (1974).

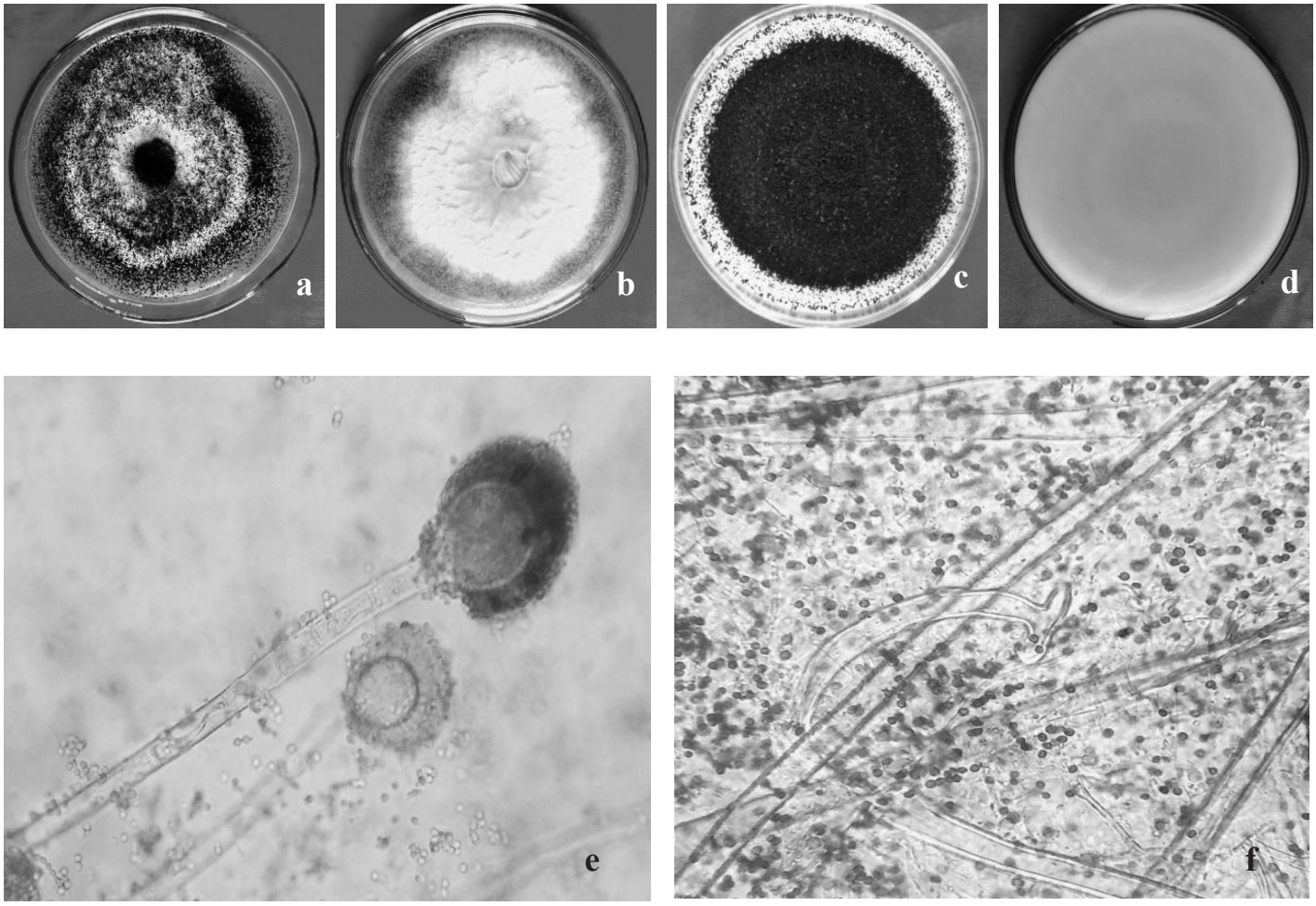

Figure 2: Macro and micromorphology of Aspergillus niger

(a) front view (b)reverse view on MEA (c) front view (d) reverse view on Oat Meal Agar

(e) hyaline smooth conidiophore, globose vesicle and biseriate conidial head (f) foot cell

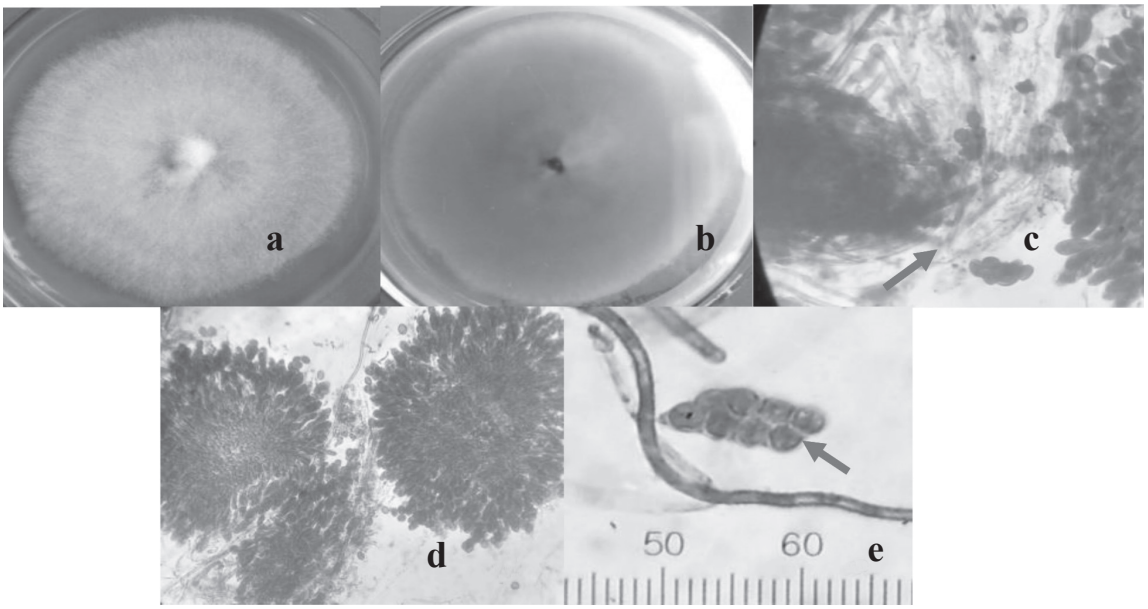

Figure 3: Macro and micromorphology of Chaetomium globosum
(a) front view (b) reverse view on PDA (c) perithecial hair (d) delinquished asci (e) ascospores

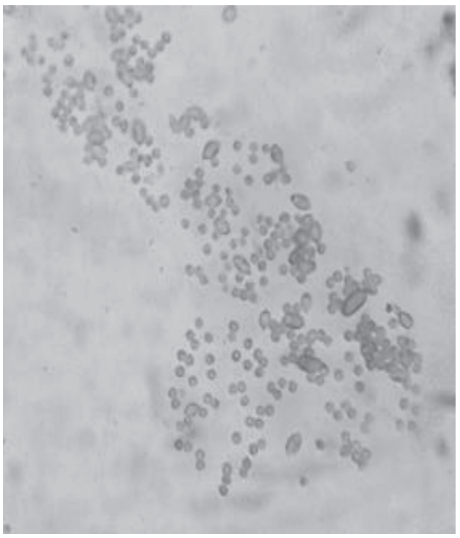

Figure 4. Conidia of *Cladosporium herbarum*

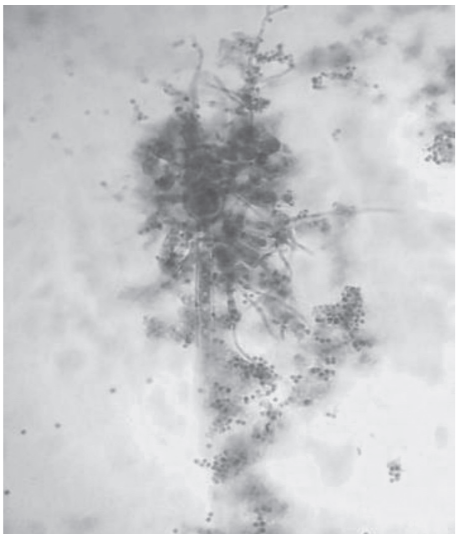

Figure 5. Conidial head of *Gibellula* sp.

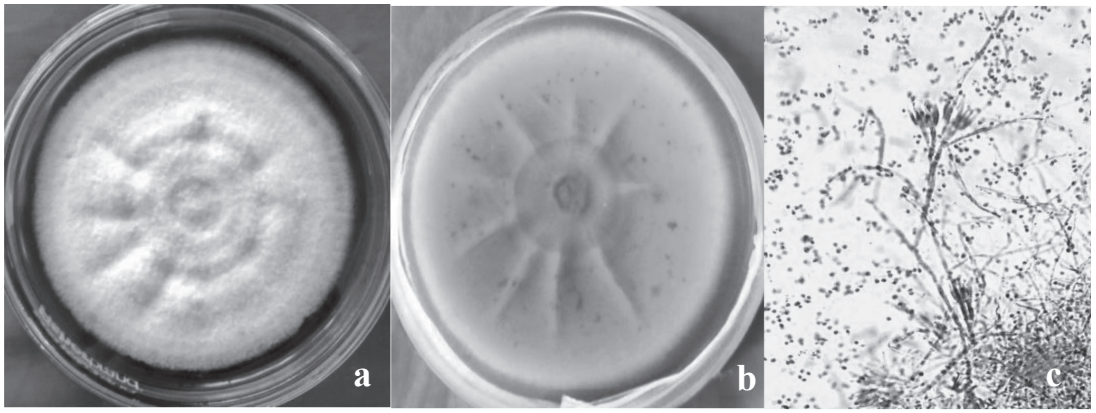

Figure 6: Macro and micromorphology of *Paecilomyces* sp.
(a) front view (b) reverse view on PDA (c) conidiophore with tenpin phialides

Three species of *Penicillium viz*., *Penicillium chrysogenum* Thom, *P. purpurogenum* Stoll and *Penicillium* sp. were identified based on their morphology.

Colonies of *P. chrysogenum* on PDA showed velvety, grey green colour, conspicuous radial furrows which lend the colony a wheel like appearance (**Figure 7a**). Colonies were yellow colour in reverse (**Figure 7b**). Penicilli were biverticillate; main axis terminated in verticils of 2 to 5 metulae, which bore sterigmata. Size of the metulae were ranged from 10 to 12 µm by 2-3 µm; sterigmata produced vertically, ranged from of 4 to 6, 8 to 10 µm by 2.0 to 2.5 µm in size. Conidial chains were well defined chains up to 200 µm in size, sub-globose to elliptical in nature and ranged from 3.0 to 4 µm by 2.8 to 3.5 µm (**Figure 7c**). Based on the reports of Raper and Thom (1984), the isolated species was identified. It was further confirmed by NCFT a with the ID. No. of 1998.17.

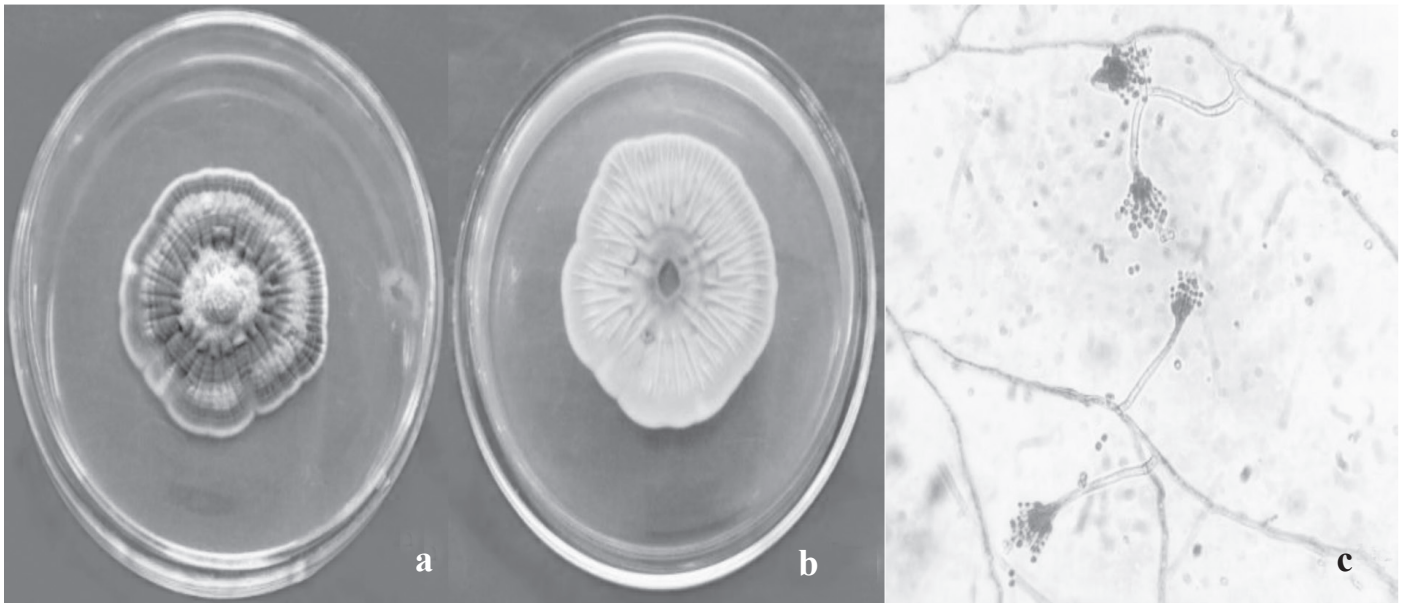

Figure 7: Macro and micromorphology of *Penicillium chrysogenum*
(a) front view (b)reverse view on PDA (c) conidiophore arising from hyphae

However, colonies of *Penicillium purpurogenum* Stoll were dark green along with (191A RHS) (**Figure 8a**), reverse colour of orange red shades (174 B RHS) (**Figure 8b**) on MEA where in the size of diameter recorded as 5 to 5.2 cm in 10 days at 25 ± 1° C. Conidiophores arose from the substratum and measured up to 100 to 120 µm in length by 2.5 to 3.0 µm in breadth. Penicilli were biverticillate symmetrical, each consisted of 5-6 metulae with successive 4 to 5 sterigmata. Metulae were 8 to 10 µm by 2.5 to 3.0 µm in size and sterigmata recorded a measurement of 10.0 to 12.0 µm by 2.0 to 2.5 µm. Conidia were arranged in short chain, smooth, sub-globose, measured 3.0-3.5 µm x 2.5-3.0 µm (**Figure 8c**). Raper and Thom (1984) described similar characteristics for *Penicillium purpurogenum* and the isolated culture identity was confirmed as *Penicillium purpurogenum*.

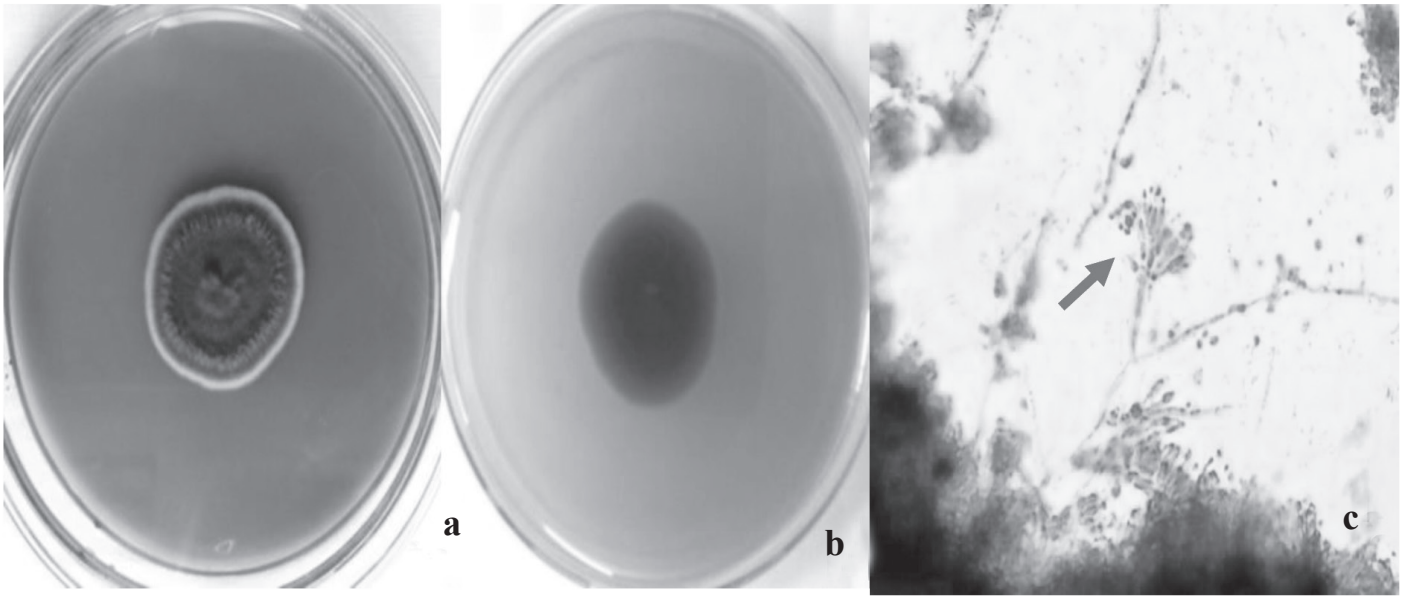

Figure 8: Macro and micromorphology *Penicillium purpurogenum*
(a) front view(b) reverse view on MEA (c) asymmetrical biverticilate penicilli

Another *Penicillium* sp. produced grey green colonies (191A RHS) (**Figure 9a**); with dark green colour (139A RHS) pigmentation on the reverse side (**Figure 9b**) on PDA. Penicilli were biverticillate and asymmetrical, with each major element bearing successive verticils of 2-5 metulae and 3 to 5 sterigmata and the size varied from9.0 to 15.0 µm by 2.5 to 3.5 µm and 9.0 to 15.0 µm by 2.5 to3.5 µm. Conidia smooth in nature and formed in chain, borne on the tip of phialides, globose to subglobose in shape and the size varied from 3.5-4.8 µm long to 3.2-4.5 µm wide (**Figure 9c**). The fungus was confirmed by following the standard descriptions of Raper and Thom (1984).

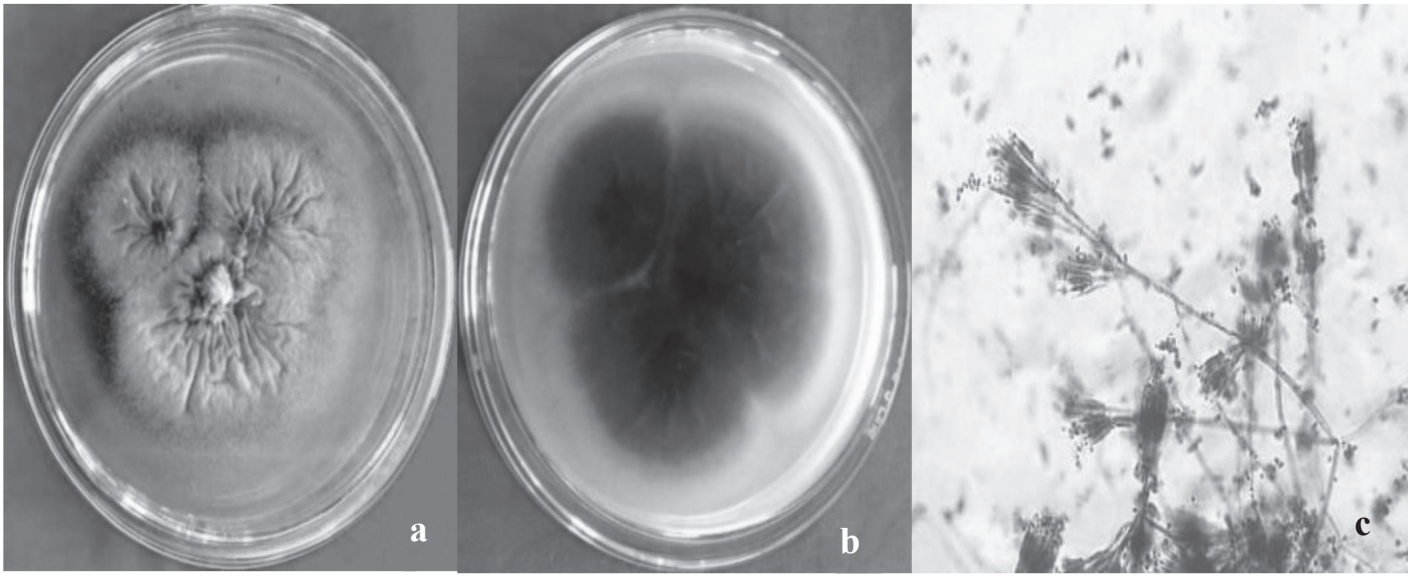

Figure 9: Macro and micromorphology *Penicillium sp.*
(a) front view (b) reverse view on PDA (c) biverticillate penicilli bearing globose conidia

Table 2. Identified fungi species from stored rice cultivar Aghoni Bora

Sl.No.	Samples	Storage type	Location/District	Fungi identified
1.	Stored rice grain (Aghoni Bora)	Storage bins	Titabar, Jorhat	*Penicillium chysogenum, Gibellula* sp., *Chaetomium globosum, Aspergillus niger, Cladosporium* sp.
		Gunny bag	Teok, Jorhat	*Penicillium* sp., *Acremonium strictum, Aspergillus niger*
		Plastic bags	Rowroiah, Jorhat	*Penicillium purpurogenum, Paecilomyces* sp.

Species wise distribution of fungi isolated from different storage structures where *Penicillium* is a common genus was found in all three types of storage *viz*., storage bins, gunny bags and plastic bags (**Table 2**). Furthermore, *Acremonium* is a potential toxin producer (Girisham *et al*., 2016) and its presence in stored rice may deteriorate the quality of the grain and may cause health hazards on consumption. *Sitophilus zeamais,* a stored grain pest, was reported to transmit *Acremonium* sp., along with other fungi including *A. niger, A. glaucus, A. candidus, Penicillium islandicum, P. citrinum, Paecilomyces, Epicoccum, F. semitectum*, yeasts and many others was reported by Mason and Mcdonough (2012). Perhaps the reason behind this was the rice sample infested by stored grain pests might have introduced the fungus to it.

Earlier scientists reported the presence of *Aspergillus niger* in the stored rice grains (Amadi *et al.,* 2009; Reddy *et al.,* 2009 and Akano 1990). Tamang (2003) and Joshi and Sandhu (2000) also reported the association of *A. niger* in stored rice. Conidia of *Aspergillus* are always present in air through which they may be introduced into the sample (Dube, 2015) and has the potential to cause allergic reaction. In addition, the fungal species was known for the production of harmful toxins like fumonisins and ochratoxins (Reddy *et al*., 2008; Frisvad *et al*., 2011). Hence, handling and consumption of rice contaminated with *A. niger* may lead to health issues.

The present study revealed that the association of *Chaetomium globosum* with stored rice grain. Similar results were reported by Ibiam *et al.,* (2008), Reddy *et al.,* (2009) and Surekha *et al*., (2011). *Chaetomium globosum* is a cellulose degrading fungus commonly present as indoor contaminants (Dube, 2015) and hence there is possibility of infection in the stored rice grains also. The only species of *Cladosporium* recorded in the present investigation was *Cladosporium herbarum*. It was reported as a common contaminant of food and food products by Reddy *et al.,* (2009) and Bensch (2012).

Gibellula sp., an entomopathogenic fungus, was also recorded in the study. The fungus reported as a habitat for spiders (Samson and Evans, 1973). It is probable that due to dual infection of both fungi and insect, the seed lot deteriorated beyond the tolerance limit and *Gibellula* was found as the predominant genus in that sample. Different insect species, such as *Sitophilus oryzae, Sitotroga cerelella, Rhizopertha dominica, Trogoderma granarium* and *Tribolium castaneum* were reported in stored samples of rice (Ali and Bhattacharya, 1991) which may harbour *Gibellula* like fungi.

One species of *Paecilomyces* was found to be associated with stored rice. Surekha *et al*., (2011) also found species of *Paecilomyces varioti* to be associated with stored rice during early storage period. However, Akano and Atanda (1990) reported *Paecilomyces varioti* from Nigeria in stored groundnut cake.

All the species of *Penicillium viz., P. chrysogenum, P. purpurogenum and Penicillium* sp. were found to be associated with stored rice. Ali and Deka (1996), Amadi *et al.,* (2009), and Ibiam *et al.,* (2008) reported species of *Penicillium* to be associated with stored rice. *Paecilomyces* sp. and *Penicillium* spp. spores were present abundantly in stored rice.

Conclusions

The study concludes that stored *Aghoni bora* rice grain samples collected from Jorhat found infected with nine fungal species *viz.*, *Acremonium strictum, Aspergillus niger, Chaetomium globosum, Cladosporium* sp., *Gibellula* sp., *Paceliomyces* sp., *Penicillium chrysogenum, Penicillium purpurogenum, Penicillium* sp. These fungi are frequently known to produce mycotoxins which may cause detrimental effect on human health.

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