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An Improved Slide Culture Technique for the Microscopic Identification of Fungal Species

Agu, Kingsley Chukwuebuka[1]; Chidozie, Chiamaka Perpetua[2]

[1]Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria
[2]Department of Medicine and Surgery, Nnamdi Azikiwe University, Teaching Hospital, Nnewi, Nigeria

ABSTRACT

Several phyto-pathogenic fungi have been discovered by numerous researchers who continue to be saddled with the problem of proper identification of these fungal agents. The conventional method requires plating out the diseased tissues of such plant materials onto culture media and observing their gross morphological features on agar plates. As soon as their colonial characteristics have been studied, the microscopic examination of fungal reproductive structures (spores) and mycelia must be done as a confirmative method of identification which must be followed by Molecular identification in order to ensure complete identification. This step often disturbs the fragile spore-hypha arrangements, thus, leading difficulties in interpretation of morphological results owing to the teasing effect in the preparation of wet mounts. The slide culture method of identification developed by Riddel in 1950 which uses an agar block of medium transferred to a glass slide and put in a moist petri dish have since been used with various modifications that have not really taken care of the slight disturbance of the mycelial arrangement of the study fungi that occurs during removal and replacement of coverslips during lactophenol cotton blue stain. We have developed a rapid slide-culture method that can diminsh this problem to the barest minimum.

KEYWORDS: *Slide Culture, Microscopy, Pathogen, Fungal Species*

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INTRODUCTION

Colonial and microscopic identification of fungi remains the most employed method for the identification of fungi even with the discovery of molecular procedures, due to its high cost (Wijedasa and Liyanapathirana, 2012). Slide culture remains the backbone of morphological identification of fungi in any mycological laboratory. This technique sustains the basic morphological characteristcs relatively intact compared with tease mounts and cello-tape mounts. However, slide cultures are also associated with problems. Taking away of the cover slip and agar block results in changes in the morphological features, such as the angle of branching and attachment of macro and micro spores to sporangiophores, which leads to problem characterizing organisms down to species level.

Also repeated viewing of same slide over a period of time to study different growth stages of the fungi and examination of structures implanted in agar is not feasible with traditional Slide culture technique (Smith, 1960). The nonexistence of a customary technique for performing submerged sporulation has precluded the development of a suitable general slide culture method based on sporulation of moulds in liquids (Madelin, 1969). The microscopic identification of many pathogenic fungi still relies heavily on morphology, location, and arrangement of individual spores or groups of spores as well as the shape of the columella for those with sporangiospores (Johnson, 1946). The teased wet-mount preparation is becoming outdated since it disrupts the hyphal and mycelia arrangements as is the case with the fungi *Blastomyces dermatitidis* or *Sporotrichum schenkii*. Slide cultures, in contrast, when suitably performed, fosters the absolute recognition of these genera, and also aid substantially with the categorization of other varieties of fungi. The available slide culture methods, however, is plagued with several intricacies

(Henrici, 1930; Brown, 1942; Lewis and Hopper, 1943).

Numerous traditional slide-culture methods exist that have been developed by various researchers. Nonetheless, these methods regularly disrupt the feeble cellular architecture thus, resulting in difficulties in elucidation of the key morphological features under the microscope (Larsen and Covey, 1979). Several fungi form very feeble propagative arrangement (spores) which are at easily disturbed by even the most meticulous operation. This famous feature has led to the formulation of assorted slide culture methods (Anthony and Walkes, 1962; Cole and Kendrick, 1968; Ellis and Ajello, 1982). Each of these was developed to solve the problems of the fragility of the fungi been evaluated and to meet the investigator's options.

This paper is aimed at explicating an improved slide culture technique which is easy to perform, inexpensive, time-saving and labour-saving in which sporulating cultures could be grown for slide mounts. It also provides important guide for microscopic recognition of fungi, as well as for pedagogic activities.

Materials and Methods

Test Fungi

The test fungi viz. *Aspergillus tamarii, Aspergillus niger, Aspergillus flavus, Mucor circinelloides, Lasiodiplodia theobromae* and *Fusarium solani* were obtained from a previous study (Ogbo and Agu, 2015) and used for this study.

Traditional Slide Culture Technique

A sterile Whatmann No. 1 filter paper was placed in a sterile petri dish. A bent sterile glass rod was placed at the bottom of the petri dish, then, 2ml of sterile distilled water was dispensed into the the petri dish. A sterile glass slide was placed on top of the bent glass rod. Using a sterile scalpel, a small square (about 1 x 1 cm) of agar block from Saboraud Dextrose Agar (SDA) was cut and placed on the centre of the slide. With the aid of a sterile inoculation needle, the agar the agar block was inoculated with a small amount of fungus under test on each of the four sides of the block. Therefter, a heat-sterilized coverslip was laid over the block and pressed down gently. The procedure was repeated for the entire seven fungi used in this study. Thereafter, the plates were incubated at room temperature for 3 to 5 days depending on the growth rate of the fungus. When desired growth was observed the coverslips were removed and few drops of Lactophenol cotton blue stain dropped on the developing cultures on the slide before the coverslips were replaced and viewed under the microscope. Referencing was done using Fungal Atlases (Barnett and Hunter, 2000; Watanabe, 2002; Ellis *et al*., 2007).

Improved Slide Culture Technique

A sterile glass slide was placed on the bottom of a sterile petri dish. With the aid of a sterile syringe 0.5 ml of molten Saboraud Dextrose Agar (SDA) maintained at 45 ^{O}C in a water bath was dispensed on the sterile glass slide. The cover of the petri dish was replaced and the molten agar allowed to gel. Upon gelling, a sterile inoculation needle was used to inoculate the agar bump with a small amount of fungus under test at the centre of the bump. Therefter, a heat-sterilized coverslip was laid just over the agar bump without pressure. The procedure was repeated for the seven fungi used in this study. The fungi used in this study were obtained from previous studies (Frank and Kingsley, 2013; Frank and Kingsley, 2013; Agu *et al.,* 2014 2015; Okigbo *et al.,* 2015; Ogbo and Agu, 2015). Thereafter, the plates were incubated at room temperature for 3 to 5 days depending on the growth rate of the fungus. When desired growth was observed, few drops of Lactophenol cotton blue stain was dropped at the interface of the developing cultures on the slide and the coverslip so as to preserve the integrity of the culture and allowed to permeate the entire culture before viewing under the microscope. Referencing was done using Fungal Atlases (Barnett and Hunter, 2000; Watanabe, 2002; Ellis *et al*., 2007).

Molecular Confirmation of Fungal Identities

The identities of the seven isolates were confirmed by the Microbial Identification Service, CABI E-UK, Bakeham Lane, Egham, Surrey, TW20 9TY, England, UK. using the partial ITS rDNA sequencing analysis and a BLAST search using the GenBank sequence database.

RESULTS

These test isolates were obtained from a previous study on the evaluation of a new method for testing the pathogenicity of molds to yam tubers (Ogbo and Agu, 2015).

Table 1: Morphological Comparisons of Test Fungi using Traditional and Improved Slide Culture Technique using Genotypic Characteristics as a Standard

Isolates	Cultural characteristics	Microscopic characteristics (Traditional slide-culture)	Microscopic characteristics (Improved slide-culture)	Partial ITS rDNA sequencing analyses	Identity
SC 1	On SDA, colonies were powdery, flat with radial grooves, yellow at first, but later turned to bright to dark yellow-green with age. Colour on the reverse side was creamy. Colonies were incubated at 30 ^{o}C for 5 days.	Conidial heads were radiate, splitting to form loose columns (200 µm in diameter). Thus, conidiophores stipes were hyaline and coarsely roughened, noticeable near the vesicle and non-septate. Conidia were globose to subglose (2 µm in diameter), pale-green and conspicuously echinulate.	Conidial heads were radiate, splitting to form loose columns (300- 400 µm in diameter). Thus, conidiophores stipes were hyaline and coarsely roughened, noticeable near the vesicle and non-septate. Conidia were globose to subglose (3-6 µm in diameter), pale-green and conspicuously echinulate.	A BLAST search of the GenBank sequence data base showed 100 % Identity to multiple ITS sequences reported from *A. oryzae* and *A. flavus*. The best matches included sequences reported in peer-reviewed literature (Yin, Y. *et al.* (2009). Molecular characterization of toxigenic and atoxigenic *A. flavus* isolates collected from peanut fields in China. *Journal of Applied Microbiology* 107 (6): 1857- 1865). This sample was distinguished from *A. oryzae* based on its smaller conidia (up to 6 µm, whereas those of *A. oryzae* are larger, up to 8 µm).	*Aspergillus flavus*
SC 2	On SDA, colonies were floccose (cottony in texture), pale greyish-brown. Growth rate was rapid, thus, colonies filled the entire petri-dish in 3 days. Colour on the reverse side was yellow. Colonies were incubated at 30 ^{o}C for 5 days.	Sporangiophores were hyaline, erect, non-septate and branched sympodially and circinate. Sporangia were terminal,dark-brown, finely echinulate to smooth and spherical (20- 80 µm in diameter). Sporangiospores were hyaline or pale-brown. Collumellae were ellipsoidal and	Sporangiophores were hyaline, erect, non-septate and branched sympodially and circinate. Sporangia were terminal,dark-brown, finely echinulate to smooth and spherical (20- 80 µm in diameter). Sporangiospores were hyaline or pale-brown. Collumellae were ellipsoidal and		*Mucor circinelloides*

		4.5-7 x3.5-5 µm in size. Chlamydospores were absent.	4.5-7 x3.5-5 µm in size. Chlamydospores were absent.		
SC 3	Colonies had rapid growth, 4.5cm in 4 days. Aerial mycelium was white to cream with orange-yellow pigmentation. Colour on the reverse side was dark-yellow. Colonies were incubated at 30 ^{o}C for 5 days.	Conidiophores were hyaline, simple, bearing spore masses at the apexes. As tall as the length of macroconidia by a few times. Conidia were hyaline and phialosporous. 1-3 macroconidia were present, which were fusiform, cylindrical, moderately curved with an indinstinctly pedicellate foot cell and a short blunt apical cell, 15-30 x 2-4 µm. Microconidia were abundant, which were cylindrical to oval, one to two-celled borne on lateral phialides, 4-8 x 1-3µm. Chlamydospores were hyaline, globose, smooth to rough-walled, borne singly and in pairs on short lateral branches, 6- 10 µm.	Conidiophores were hyaline, simple, bearing spore masses at the apexes. As tall as the length of macroconidia by a few times. Conidia were hyaline and phialosporous. 3-5 macroconidia were present, which were fusiform, cylindrical, moderately curved with an indinstinctly pedicellate foot cell and a short blunt apical cell, 28-42 x 4-6 µm. Microconidia were abundant, which were cylindrical to oval, one to two-celled borne on lateral phialides, 8-16 x 2-4.5µm. Chlamydospores were hyaline, globose, smooth to rough-walled, borne singly and in pairs on short lateral branches, 6- 10 µm.		*Fusarium solani*
SC 4	On SDA, colonies were colourless at first, then gradually turned orange-yellow to brown or light brownish-olive with age, but often presenting a suggestion of	Conidial heads varied greatly in size in the same fruiting area, from more or less columnar to nearly, but not completely, globose and up to 250m in diameter, with radiating chains	Conidial heads varied greatly in size in the same fruiting area, from more or less columnar to nearly, but not completely, globose and up to 300m in diameter, with radiating chains	A blast search using the GenBank sequence database, showed that the sequence from this sample showed 100 % identity to multiple ITS sequences reported from A. *tamari*. Best matches included sequences reported	*Aspergillus tamari*

	green that was transient and limited to areas of young heads. The reverse side was colorless. Growth rate was rapid (3 days)	and columns of conidia. Conidiophores arising from submerged hyphae, were up to 1 to 2 mm in length, colorless, with walls becoming abruptly thinner at the base of the vesicle. Vesicles were globose to subglobose, 15 to 32m in diameter with fairly thin walls which frequently crush in mounts, fertile over almost the entire surface. Sterigmata,were in one series in small heads and in two series in large heads.Conidia ranged from more or less pyriform, through sub-globose to globose, conspicuously roughened from prominent tubercles and bars of orange-yellow coloring matter deposited between the loose outer wall and the firm inner wall, commonly ranging from 5.0 to 6.5mm in diameter, occasionally up to 8mm. Sclerotia were purplish, globose with apex white.	and columns of conidia. Conidiophores arising from submerged hyphae, were up to 1 to 2 mm in length, colorless, with walls becoming abruptly thinner at the base of the vesicle. Vesicles were globose to subglobose, 25 to 50m in diameter with fairly thin walls which frequently crush in mounts, fertile over almost the entire surface. Sterigmata,were in one series in small heads and in two series in large heads.Conidia ranged from more or less pyriform, through sub-globose to globose, conspicuously roughened from prominent tubercles and bars of orange-yellow coloring matter deposited between the loose outer wall and the firm inner wall, commonly ranging from 5.0 to 6.5mm in diameter, occasionally up to 8mm. Sclerotia were purplish, globose with apex white.	in peer-reviewed literature (Rakeman, *et al*. (2005). Multilocus DNA sequence comparisons rapidly identify pathogenic molds). Journal of Clinical Microbiology **43**: 3324- 3333).	
SC 5	On SDA, colonies had rapid growth	Conidiophores were hyaline or pale-brown, erect,	Conidiophores were hyaline or pale-brown, erect,		*Aspergillus niger*

	rate. However, colonies were flat and compact with yellow basal felt covered by a dense layer of black conidial heads with powdery texture. The colour on the reverse side was pale yellow. Colonies were incubated at 30 ^{o}C for 5 days.	simple, with foot cells basally, inflated at the apex forming globose vesicles, bearing conidial heads (up to 3 mm by 15 to 20 µm in diameter), split into over 4 loose conidial columns with over 4 fragments apically composed of catenulate conidia (over 15 conidia/ chain) borne on uniseriate and biseriate phialides on pale-brown, globose vesicles and phialides acutely tapered at apex. Conidia (3.5-5 µm in diameter) are phialosporous, brown, black in mass globose and minutely echinulate	simple, with foot cells basally, inflated at the apex forming globose vesicles, bearing conidial heads (up to 3 mm by 15 to 20 µm in diameter), split into over 4 loose conidial columns with over 4 fragments apically composed of catenulate conidia (over 15 conidia/ chain) borne on uniseriate and biseriate phialides on pale-brown, globose vesicles and phialides acutely tapered at apex. Conidia (3.5-5 µm in diameter) are phialosporous, brown, black in mass globose and minutely echinulate		
SC 6	On SDA, colonies were grayish sepia to mouse grey to black, fluffy with abundant aerial mycelia; the reverse side was fuscous black to black. Colonies were incubated at 30 ^{o}C for 5 days.	Pycnidia were simple or compound, often aggregated, ostiolate, up to 5mm wide. Conidiophores were hyaline, simple, sometimes septate, rarely branched cylindrical, arising from the inner layers of cells lining the pycnidial cavity. Conidiogenous cells were hyaline, simple, cylindrical to subobpyriform to holoblastic and	Pycnidia were simple or compound, often aggregated, stromatic, ostiolate, frequently setose, up to 5mm wide. Conidiophores were hyaline, simple, sometimes septate, rarely branched cylindrical, arising from the inner layers of cells lining the pycnidial cavity. Conidiogenous cells were hyaline, simple, cylindrical to	A blast search using the GenBank sequence database, showed that the sequence obtained from sample showed 100 % identity to multiple ITS sequences described from *L. theobromae*, its perfect state *Botryosphaeria rhodina* and a single sequence described from the holotype material of *L. plurivora* (Damm et al. (2007). Botryosphaeriaceae as potential pathogens of prunus species in South Africa, with	*Lasiodiplodia theobromae*

		annelidic. Conidia were initially unicellular, hyaline, granulose, subovoid to ellipsoid-oblong,thick-walled, base truncate; mature conidia one-septate, cinnamon to fawn, longitudinally striate, 10-15 x 1-5µm. Paraphyses were hyaline, cylindrical sometimes septate up to 20 µm.	subobpyriform to holoblastic and annelidic. Conidia were initially unicellular, hyaline, granulose, subovoid to ellipsoid-oblong,thick-walled, base truncate; mature conidia one-septate, cinnamon to fawn, longitudinally striate, 20-30 x 10-15µm. Paraphyses were hyaline, cylindrical sometimes septate up to 50 µm.	descriptions of *Diplodia africana* and *L. plurivora* sp. nov. **99** (5): 664-680). *L. plurivora* was found to be a sister group to *L. theobromae* by combined ITS/Ef1 gene analysis but was differentiated morphologically by the conidia size of 26-33 x 14.5- 17 µm, whereas those of L. theobromae were shorter, about 24- 28 x 12- 15 µm.	

Fig 5: Micrograph of *Aspergillus* sp. using our Improved Method

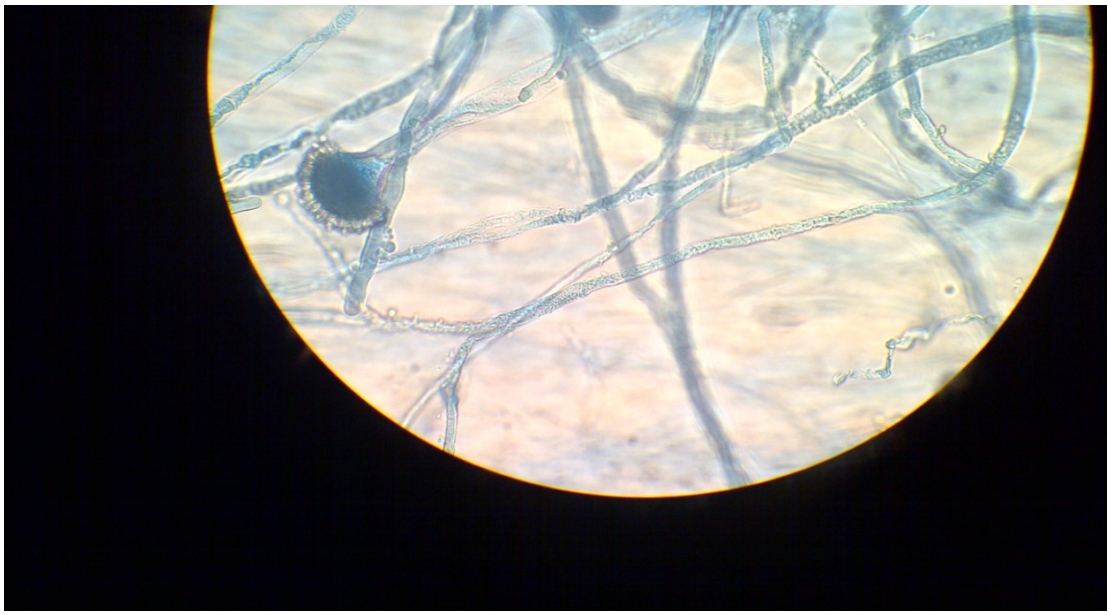


Fig 6: Micrograph of *Aspergillus* sp. using our Improved Method

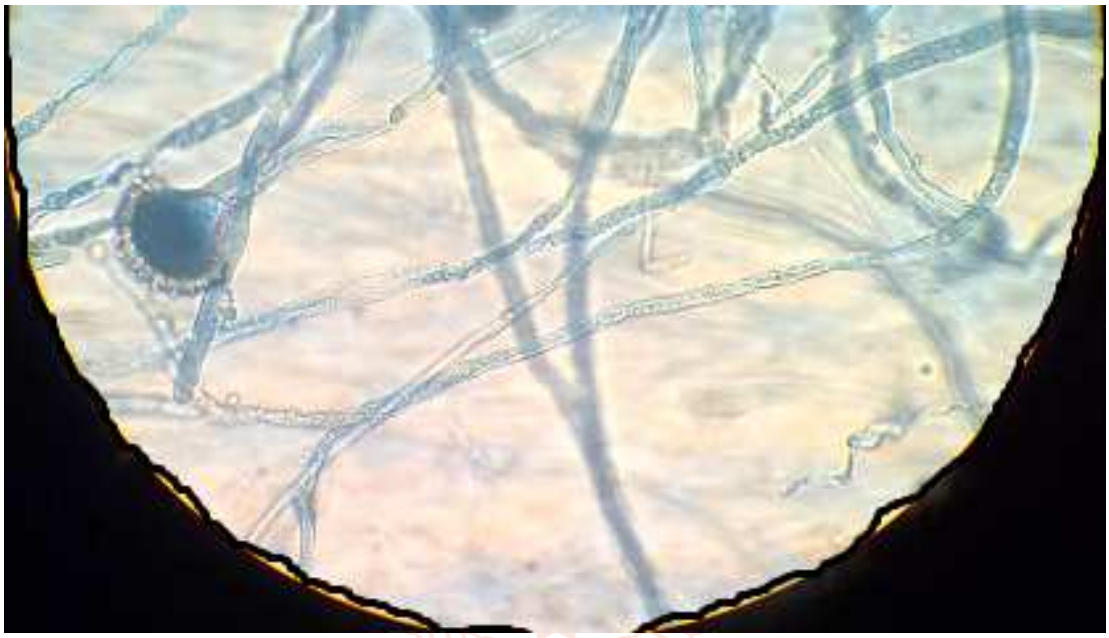


Fig 3: Micrograph of *Aspergillus flavus* using the Traditional Method

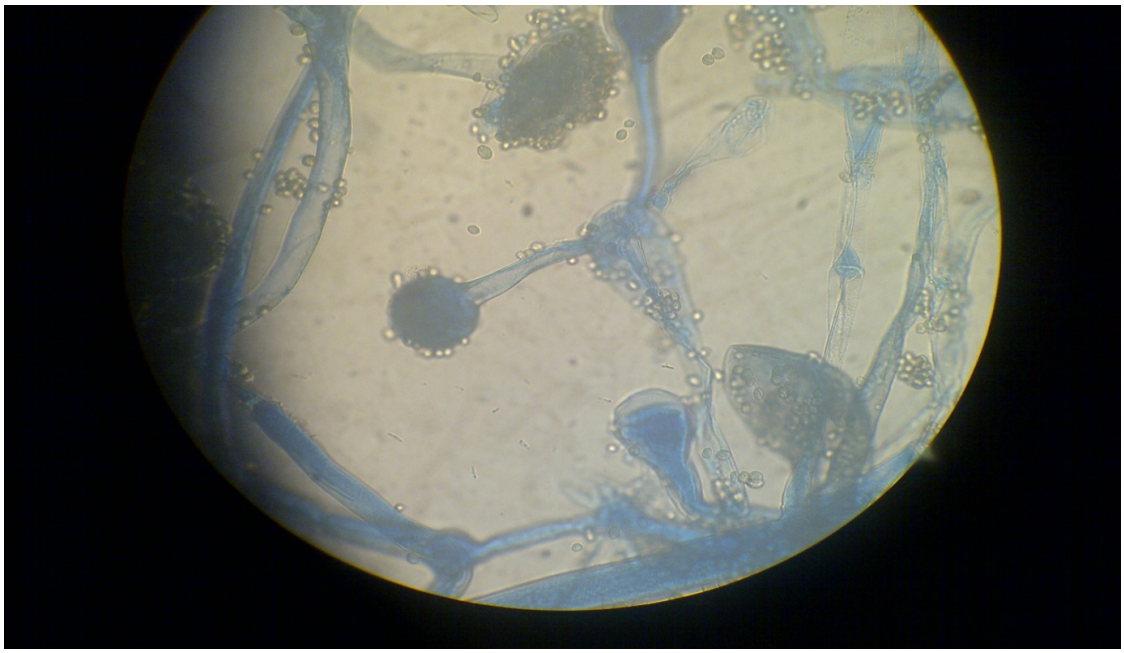


Fig 4: Micrograph of *Aspergillus flavus* using our Improved Method

Micrograph of *Mucor circinelloides*.

Fig 1: Micrograph of *Fusarium solani* using the Traditional Method

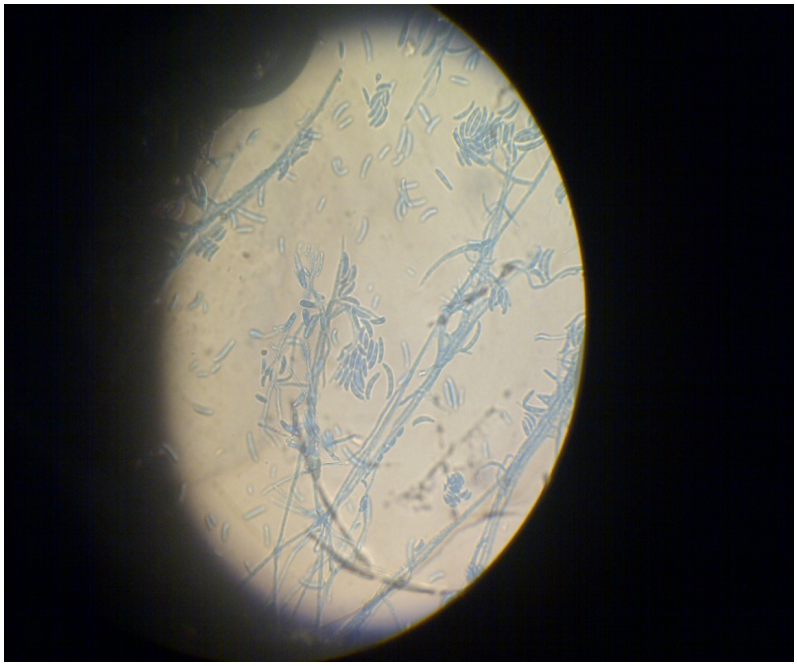
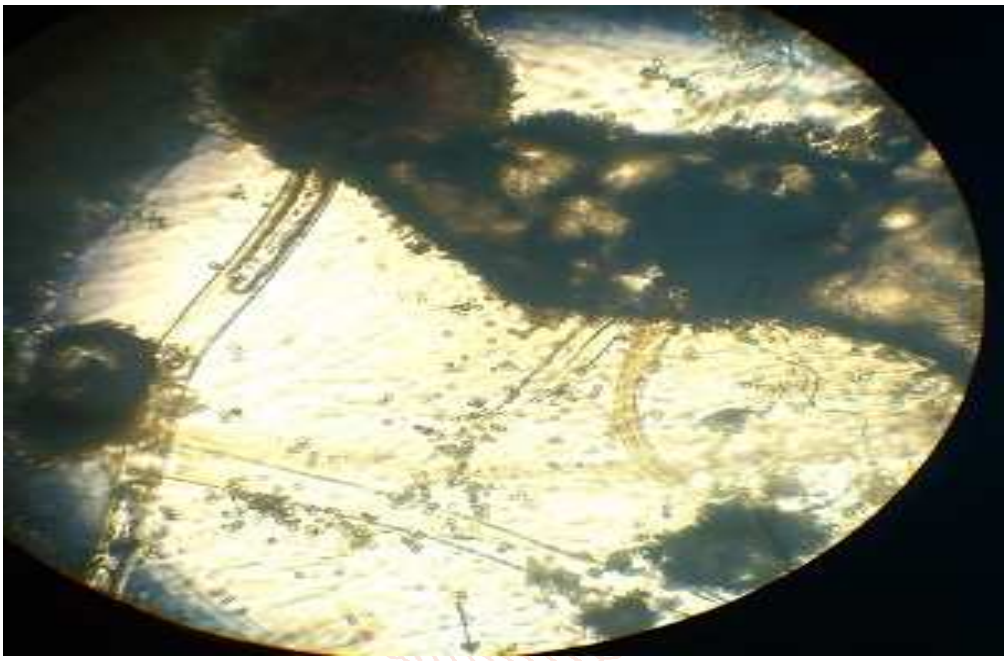
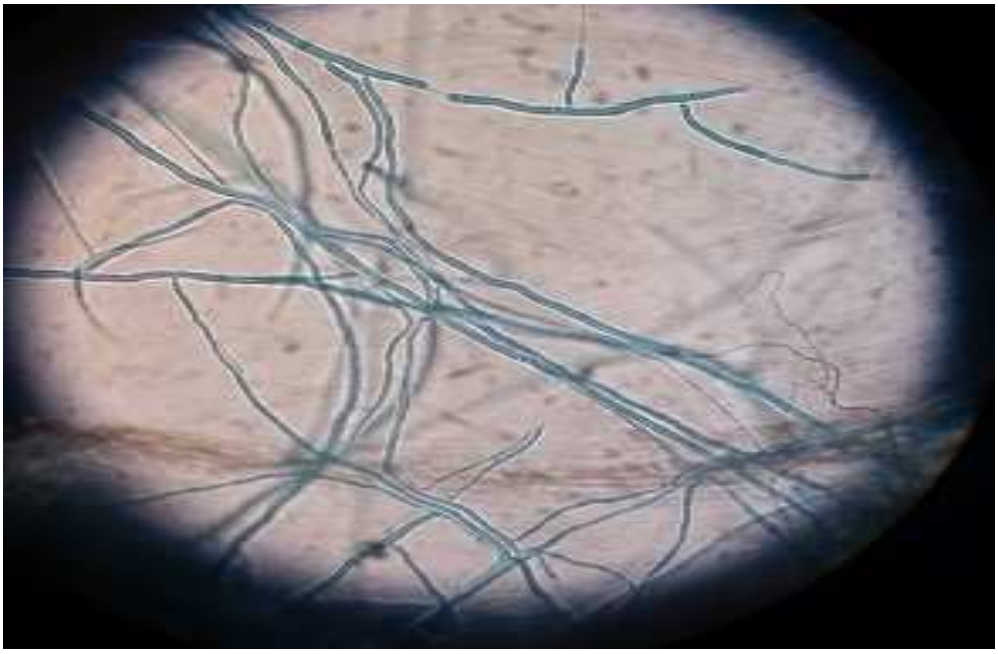


Fig 2: Micrograph of *Fusarium solani* using our Improved Method

Micrograph of *Aspergillus tamarii*



Micrograph of *Aspergillus niger*



Micrograph of *Lasiodiplodia theobromae*

DISCUSSION

The traditional teasing method does not allow for a holistic microscopic view of fungal isolates under the microscope, thereby making the study of the microscopic structures of fungi such as spore shape, spore heads and other special structures such as rhizoids, stolons, foot cells and hold fasts almost impossible. Other types of slide culture techniques are laborious, time-consuming, costly and almost impracticable to reproduce and as such the development of this simple, economical and efficient slide culture method.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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