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Variation in conidiophore complexity in *Aspergillus* v*ersicolor*

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Abstract

Aspergillus versicolor is an abundant species and habitually isolated from soil, plant debris, saline water environments, and indoor air environments. Cultural, macroscopic and microscopic features including ontogenesis process of *Aspergillus versicolor* (NRRL 238) were monitored. Fluorescence and scanning electron microscopies were used to investigate the development of conidiophore and the process of conidium formation. Ontogeny of phialides in *Aspergillus* and *Penicillium* conidiophores were studied. Although, simultaneous production of phialides are confined to *Aspergillus* conidiophores and considered unique feature to *Aspergillus* species, former studies observed fragmentary heads resembling penicillate fructifications in *Aspergills* section versicolor. Our results revealed that, *A. versicolor* has ability to produce successive phialides separately such as those in *Penicillium* species and produce proper *Penicillium* conidiophore with monoverticillate structure besides *Aspergillus* conidiophore. This study designated to defines the development of *Penicillium* and *Aspergillus* conidiophores within *A. versicolor* as a member of section Versicolor.

Key words – *Aspergillus versicolor* – conidiophore – ontogeny – scanning electron microscope

Introduction

The last complete monograph of *Aspergillus* was written in 1965 (Raper & Fennell 1965). They recognised 132 species and 18 varieties. Additional taxonomic and nomenclatural works have been carried out by Samson & Gams (1985). *Aspergillus* is one of the most economically important genera of filamentous fungi (Samson et al. 2011). Therefore, it is important to consider any taxonomic changes in order to keep the taxonomic system practical for economic and regulatory reasons. This has been accomplished through open discussion and consensus-building in meetings such as the *Aspergillus* Workshops, and in efforts such as the lists of accepted species and synonyms (Pitt et al. 2000; Samson et al. 2011). Raper & Fennell (1965) revised the genus *Aspergillus* and accepted 18 species in the *A. versicolor* group. *Aspergillus versicolor* (Vuillemin) Tiraboschi is the most broadly described species in section Versicolor (Jurjevic Z. et al. 2012). It has been isolated from soil (Domsch et al. 1980), indoor environments (Samson et al. 2001, 2010, Engelhart et al. 2002, Shelton et al. 2002, Amend et al. 2010, Anderson et al. 2011), numerous foods and feeds (Pitt & Hocking 2009), hyper-saline water (Kis-Papo et al. 2003, Mbata 2008), and related with many health problems of humans and animals Jussila 2003, Perri et al. 2005, Baddley et al. 2009, Edmondson et al. 2009, Pitt & Hocking 2009, Moreno & Arenas 2010). It is a producer of the mycotoxin sterigmatocystin that is a precursor of aflatoxin B1 (Mills & Abramson 1986, Tuomi et al. 2000, Nielsen 2003, Veršilovskis & Saeger 2010). *Aspergillus* is hyphomycetous which are characterised by, the formation of conidiophores with large,

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heavy walled stipes and swollen apices termed vesicles (Pitt & Hocking 2009). Vesicles are usually roughly spherical, but are elongated or less conspicuously swollen in a few species. Vesicles bear crowded phialides, or metulae and phialides, which are characteristically all borne simultaneously (Pitt & Hocking 2009). Conidium development in *Penicillium* resembles *Aspergillus*, but the morphological arrangement of the conidiophore structures differs (Alexopoulos et al. 1996). Phialide production in *Penicillium* and related genera is always successive, not simultaneous (Pitt & Hocking 2009). The presence of immature metulae or phialides all at the same stage of development indicates *Aspergillus*; structures with some phialides producing conidia while one or more others are still developing indicates *Penicillium* or a related genus (Pitt & Hocking 2009).To delineate *Aspergillus* from other related genera such as *Penicillim*, Smith (1969) and Pitt and Hocking (1985), considered that the simultaneous production of phialides and the presence of foot cells on conidiophore were absolute criteria for *Aspergillus* species. Some previous studies find that fragmentary heads resembling penicillate fructifications were commonly observed in *Aspergills* section versicolor (Jurjevic Z. et al. 2012). This paper aimed to study the development of penicillate-like strcture in *A. versicolor* beside *Aspergillus* conidiophore.

Materials & Methods

Organism

Organism used in the course of this study was *Aspergillus versicolor* (NRRL 238). The organism was obtained from the Culture Collection of school of Pharmacy and Bio-molecular science, Liverpool John Moores University, United Kingdom.

Culture Media

Cultures were grown on Czapek yeast extract agar (CYA), malt extract agar (MEA) and potato dextrose agar (PDA) at 28 °C for 10 days in darkness. Colony diameters and appearance were recorded and photographed.

Microscopy

Bright field microscope

Microscopic examination was performed by tearing apart a small amount of mycelium in a drop of 0.1 % lacto-phenol blue or by a modified cover slip culture method (Nugent et al. 2006) and examining the preparation under bright field microscopy fitted with camera and Differential Interference Contrast (DIC) light microscopy with an Olympus BH2 research microscope using x40 and x60. Images were captured by Camera (INFINITY 1) and were analysed by Infinity Analyse software provided with measurement functions and image enhancement options.

Fluorescent microscope

The fluorescent stain Calcofluor White (CFW) (W/V) was used to observe the young conidia, scars and septa (Romero & Minter 1988) using a solution of calcofluor 17352 (Polysciences, Washington, PA) with fluorescent brightener 28.F6259 (Sigma, St. Louis, MO). Samples were examined using an Olympus BX51 fluorescence microscope with appropriate filters. The stain CFW was exited at 412 nm and emitted at 347 nm. Images were captured by digital camera (HAMAMATSU) ORCA-ER C742-95 and were analysed by OPENLAB 4.0.2. soft-ware provided with measurement function and image enhancement option.

Scanning electron microscope

For examination by scanning Electron Microscopy, small sections from the fresh culture of *A. versicolor* were fixed in 2.5% Glutaraldehyde for 20 minutes to provide a rapid inter and intra-cellular penetration. The fixed specimens were dehydrated through a series of increasing concentrations of ethanol, ending in a 100% dehydrating liquid of the highest possible purity. Typically these are steps of

10, 20, 30, 50, 70, 90, 95, 100% at 10 minutes for each, with 3 changes at 100%. Acetone was used as the intermediate fluid because it is miscible with carbon dioxide. Critical point drying (CPD) was used to prevent collapse in ESEM. This is achieved by replacing acetone with liquid CO2 and then the liquid CO2 is taken to a critical temperature and pressure (34. 5°C and 1200 psi (pounds per square inch)). At these parameters, the shearing forces and surface tension on the samples are minimal and the CO2 is in equilibrium between liquid and gas. This stops the samples collapsing whilst they are dried. The samples were sputter-coated with gold using an Emitech K550X coating unit. The coated specimens were then loaded into FEI (Quanta 200) ESEM (Environmental Scanning Electron Microscopy, 2008) and observed over a range of magnifications. Images were obtained using an image capture system (Oxford Instruments, INCA system, Oxford, UK). Another method used for examination of living cells was a low-temperature environmental scanning electron microscopy (ESEM). Specimens were rapidly frozen in liquid nitrogen slush (at -180 ˚C) under vacuum and transferred to the Cryo-preparation chamber, where the frozen samples were sublimated at -80 ˚C to remove surface ice crystals. Samples were then re-cooled to -130 ˚C and sputter coated with gold in the preparation chamber using a voltage of 1.2 kV Specimens were viewed at an accelerating voltage of 5 kV at -130 ˚C with a FEI Quanta 200 ESEM fitted with a Quorum Technology Cryo-preservation system. Images for all methods were obtained using an image capture system (Oxford Instruments, INCA system, Oxford, UK).

Results

Description of *Aspergillus versicolor* (NRRL 238) on different media

Colonies grown 10 days on CYA at 28 ℃ attained 21–28 mm diam., sulcate, centrally raised 4–5 mm, sporulating well, conidial heads pale greyish green near tea green, central area mealy from aggregated aerial hyphae, exudate present in mostly clear to pale pink shades (brownish red in one isolate), faint to very obvious pinkish soluble pigment, reverse vinaceous or brown or scarlet. Colonies grown 10 days on MEA at 28 °C attained 25–32 mm diam., low, with funicular hyphal aggregates, sometimes dominating colony appearance, sporulating in pale to dark bluish green to grey green colour, no exudate seen, soluble pigment yellow in some isolates, not present in others, reverse pale yellow, yellow orange or orange. Colonies grown 10 days on PDA at 28 °C attaining 18–28 mm diam., centrally raised, often good sporulating, either consisting of a dense felt of conidiophores or of aerial and closely interwoven mycelium bearing the conidiophores. Colour at first white, then changing to yellow, orange-yellow to yellow-green, often intermixed with flesh to pink colours, reverse pale yellow, yellow orange or orange. In most culture media conidiophores including stipes hyaline or slightly pigmented, smooth-walled, (45–) 200–750(–1050) × (4–) 5–8(–12) µm. Vesicles subglobose to ellipsoidal, or pyriform to spatulate, 9-17µm in diam. Conidial heads biseriate, metulae covering half to entire vesicle (no single image shows metulae covering just half of the vesicle), 3–6(–9) × 2.5–4.5 µm, Phialides borne on metulae, 5.5–8.0 × 2.5–3.0 µm. Metulae 5.0–7.5 × 2.0–2.5 µm. Conidia spherical to sub spherical, occasionally ellipsoidal, (2–) 2.5–3.5(–6.5)µm, finely roughened wall (Fig.1).

Aspergillus versicolor conidiophores

A penicillus like structure is easy to recognize by microscopic examination. After 2–3 days of vegetative growth, vegetative hyphae of *A. versicolor* start to produce very short stalks from non-specialised cell and at irregular intervals along vegetative hyphae. These so-called stalks can differentiate into conidiophores or stipes. Conidiophore stalk extends about 5–10 µm into the air. During conidiophore extension, the tip swells and forms the phialides one by one. These phialides give rise of mainly uninucleate conidia. A penicillus conidiophores or stipes is characterized by very short, solitary, borne at irregular intervals along vegetative hyphae and no foot cell noticed by microscopy (Figs. 2 & 3).

The attachment of phialides to a stipe directly as a single whorl or verticillus called monoverticillate. Sometimes phialiedes bear a short chain of conidia, but more commonly a single developing conidium, shed at maturity and succeeded by another blown out terminally from the conidiophore (Fig. 4). Scanning electron microscope showed that the phialide production in *Penicillium*

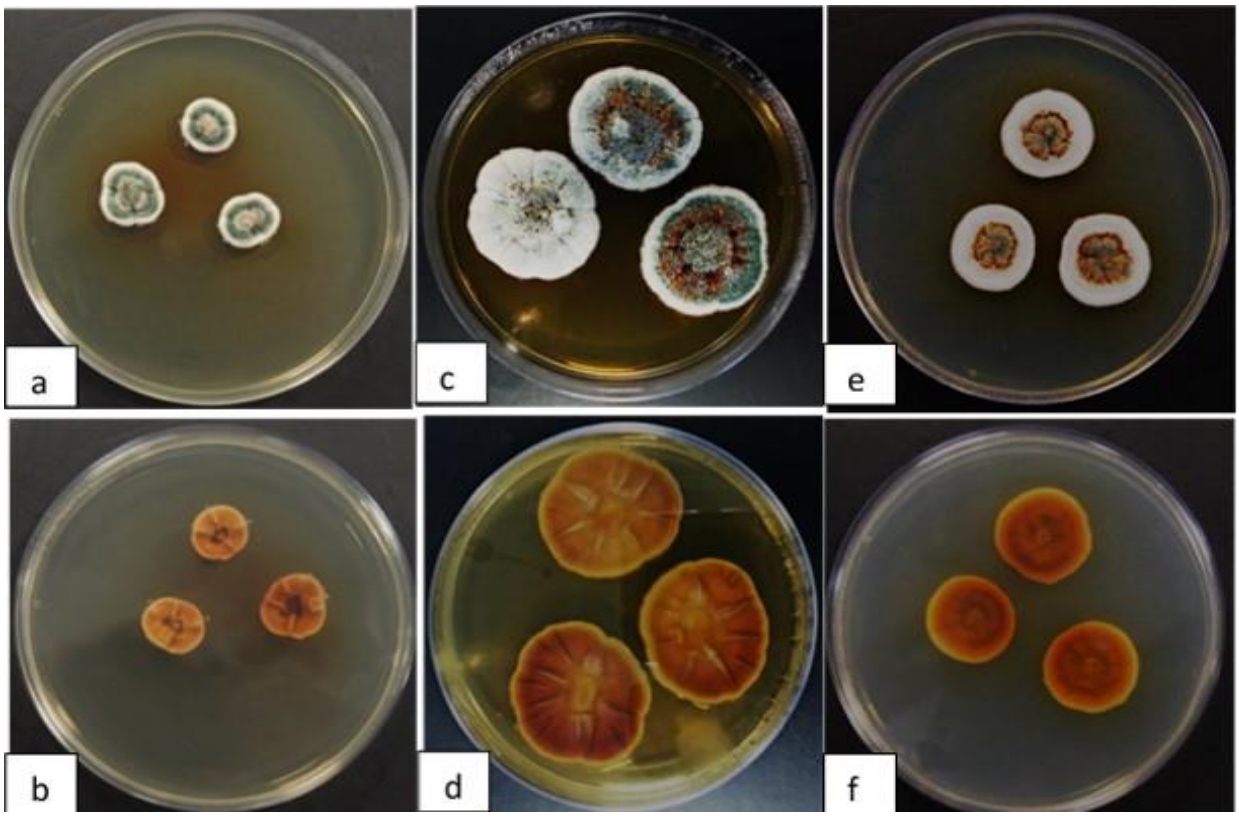

Fig. 1 – Petri dishes are 9 cm diam., colonies grown at 28 °C for 10 days. a. CYA colonies. b. CYA colony reverse. c. MEA colonies. d. MEA colony reverse. e. PDA colonies f. PDA colony reverse

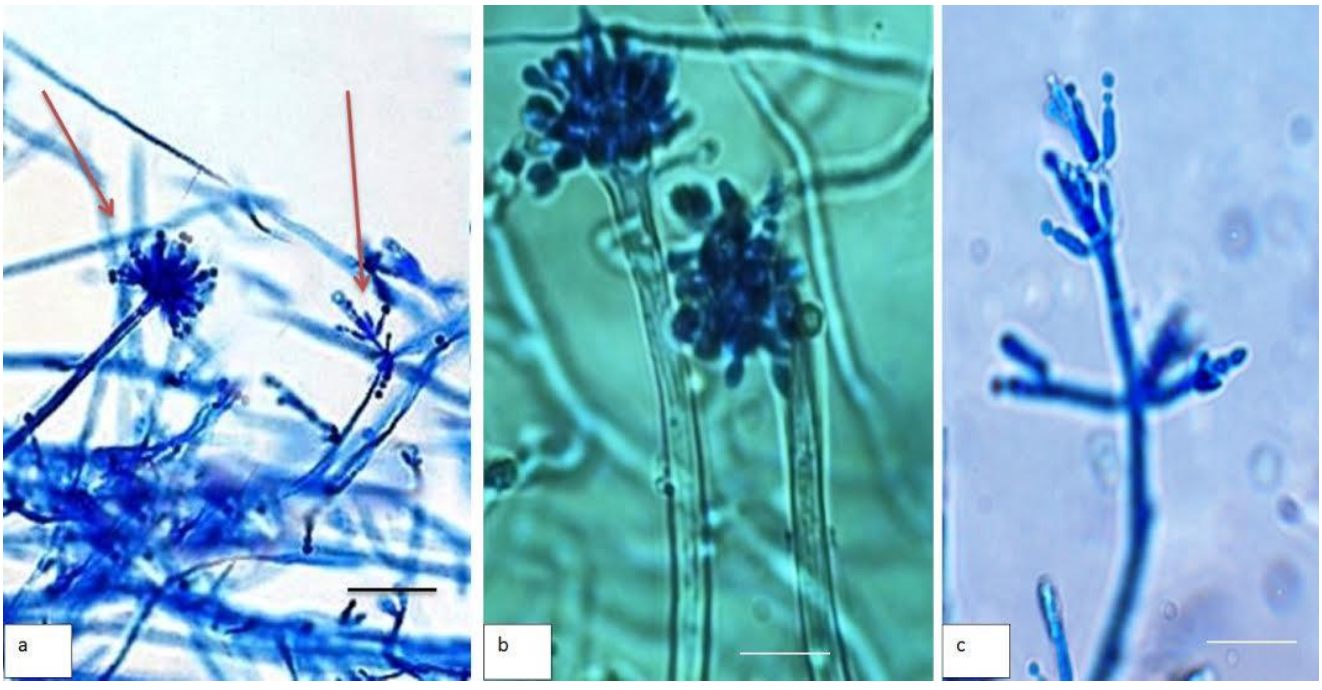

Fig. 2 – Bright field micrographs of *Aspergillus versicolor* showing. a. Both *Aspergillus* and *Penicillium* conidiophores in one spot b *Aspergillus* conidiophores, subglobose vesicle, smooth stipe and conidia c *Penicillium* conidiophores from aerial hyphae, phialids and conidia. Bar = a. 500 µm, b. 50 µm, c. 100 µm

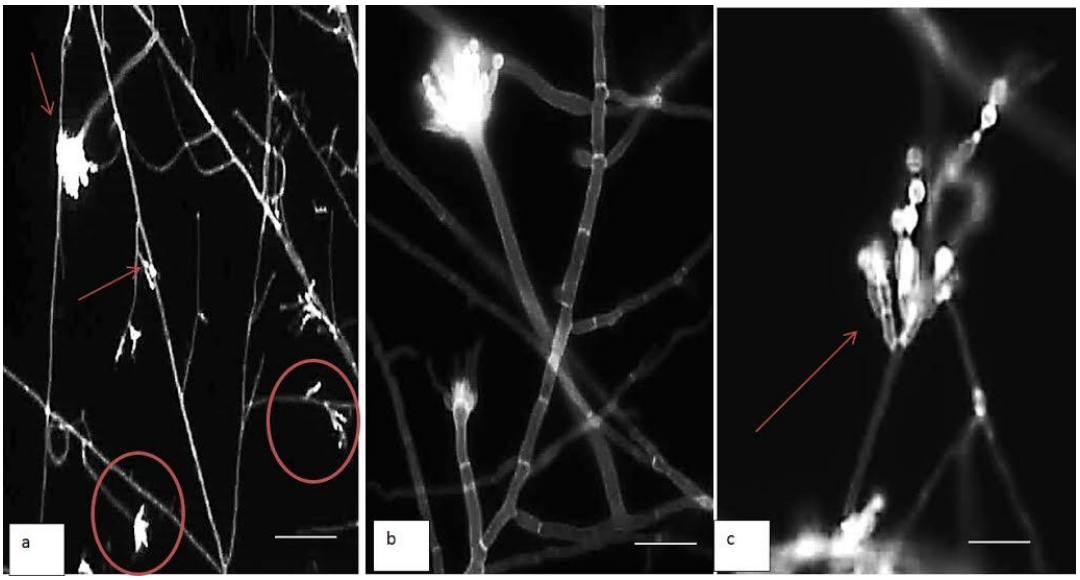

Fig. 3 – Fluorescence micrographs of *Aspergillus versicolor* showing a. Both *Pinicillium* and *Aspergillus* conidiophores (arrowed) in one spot b. *Aspergillus* conidiophores, non- septate stipe, subglobose vesicle, smooth stipe, phialid, foot cell and conidia. c. *Penicillium* conidiophores from aerial hyphae, phialids (arrowed) and conidia. Bar = a. 500µm, b. 100µm, c. 40µm

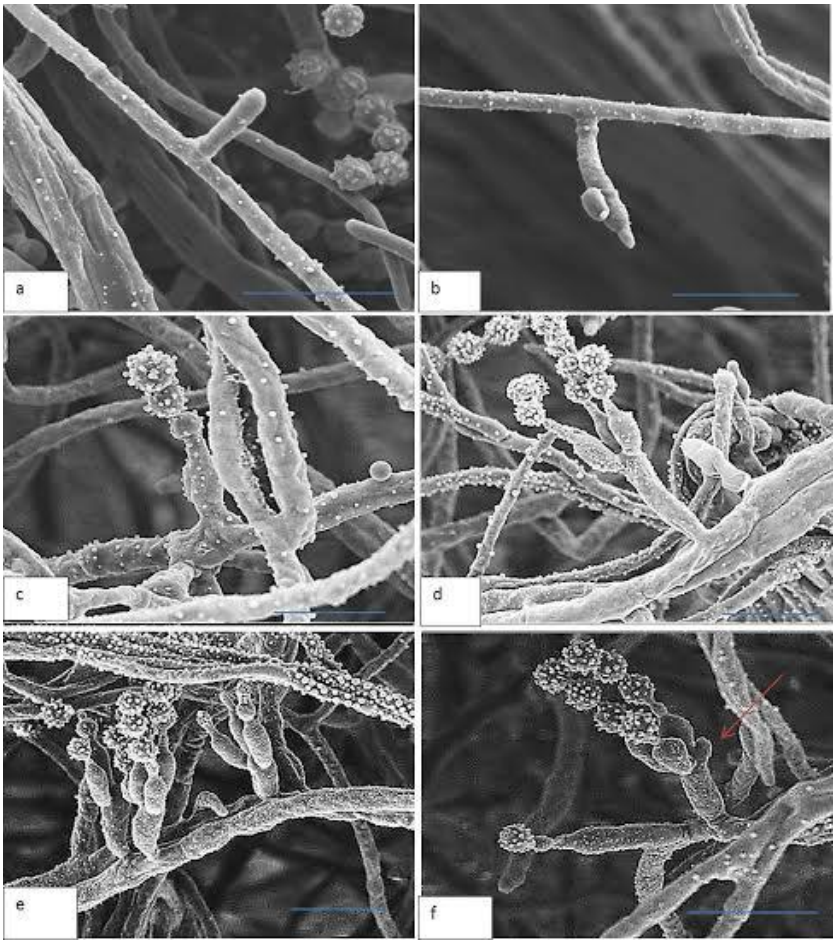

Fig. 4 – Scanning electron micrographs of *Aspergillus versicolor* showing ontogeny of Penicillium-like conidiophore and conidia a – b Pinicillium-like conidiophores raised from vegetative hyphae, very short stipe, and size of stipe is very close to vegetative hyphae. c – d phialides attached directly to a stipe e. Penicillium-like structure rose from vegetative hyphae on interval areaes. f. Penicillium-like structure with some phialides and conidia while other phialids are still developing. Bar = 10 µm

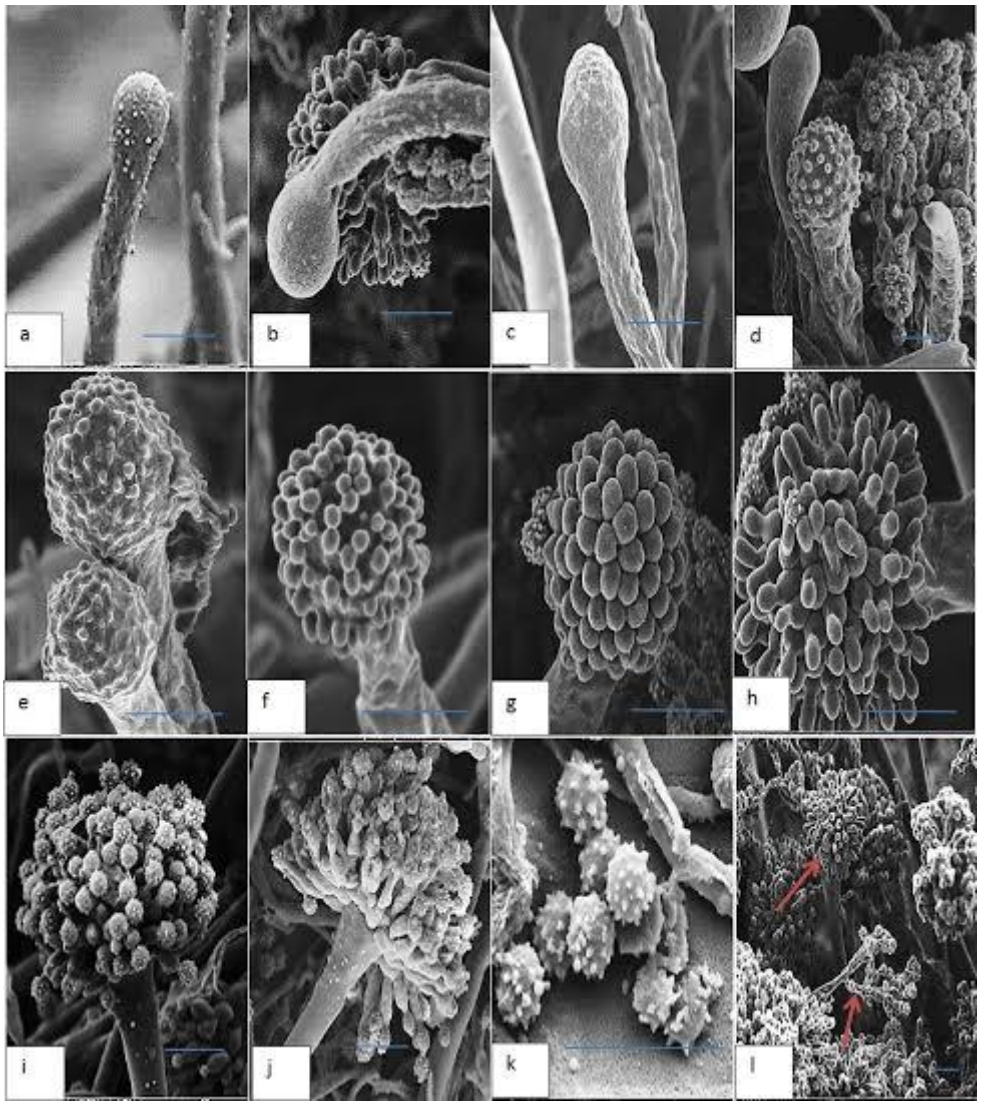

Fig. 5 – Scanning electron micrographs of *Aspergillus versicolor* showing ontogeny of *Aspergillus* conidiophore and conidia a – j formation of conidiophore and conidial head k. Rough walled conidia l. Both *Aspergillus* and *Penicillium* conidiophores in one spot (arrowed) while the above arrow refer to *Aspergillus* while the below refer to *penicillium* structure. Bar =10µm

like structure is successive, not simultaneous. The presence of immature phialides all at the same stage of development as in *Aspergillus* structure not found. Conidiophore with some phialides producing conidia while one or more other phialids are still developing is very obvious (Fig. 4). Scanning electron microscope showed no difference between conidia produced from both *Aspergillus* and *Penicillium* conidiophores. After a period of vegetative growth, air-exposed colonies of *A. versicolor* form two types of aerial hyphae (Figs. 2 & 3). One type is quite similar to vegetative hyphae and has a diameter of about 2–3 µm. The second type of aerial hyphae has a diameter of about 5–6 µm. These so-called stalks can differentiate into conidiophores (Fig. 2 & 3). The conidiophore stalk extends about 200 – 750 µm into the air and is formed from a specialised foot-cell within the substrate mycelium. When the stalk has reached its maximum height, the tip swells and forms a vesicle with a diameter of 9-–17 µm. Vesicles are usually roughly subglobose to pyriform or spatulate. In biseriate *A. versicolor*, the vesicle surface buds resulting in a layer of primary sterigmata termed metulae. The metulae in turn bud twice. These results in a second layer of sterigmata called phialides. Metulae and phialides are typically borne simultaneously i.e. immature metulae or phialides are all present at the same stage of development. The phialides give rise to chains of mainly uninucleate basipetal conidia. Conidia are spherical to subspherical, occasionally ellipsoidal and finely rough walled (Fig. 5).

Discussion

Aspergillus versicolor is the most reported fungal species in section *Versicolores* from damp indoor environments (Jussila 2003, Rydjord et al. 2005) and its presence is used as an indicator of Sick Building Syndrome (SBS) (Schwab & Straus 2004). The media components are important criteria for fungal growth, along with important physiological parameters that lead to maximum sporulation in fungi (Kim et al. 2005). In the present investigation, type of culture media and their chemical compositions considerably affected the mycelial growth rate and conidial production in *A. versicolor*. However, types of culture media had no noticeable affected on conidiophores formation in *A. versicolor*. *Aspergillus* and *Penicillium* are clearly revealed in some species such as *Aspergillus versicolor* group that produce in addition to the typical *Aspergillus* head, fragmentary heads resembling those of *Penicillium* (Vanden Bossche et al. 1988). This study showed that, *Penicillium* heads are produced only during the first days of incubation period. From our point of view, *Aspergillus versicolor* has a tendency to produce penicillium-like structure at the beginning of incubation period due to spreading purposes. Correspondingly, the production of conidia by monoverticillate *Penicillium* heads is much faster than its production by *Aspergillus* heads. In *A. versicolor* the monoverticillate *Penicillium* heads produced phialides in successive way while only *Aspergillus* heads produced simultaneous phialids. Therefore, *A. versicolor* group cannot be used as an evidence for producing simultaneous phialides by monoverticillate penicillia in contrasting with Vanden-Bossche et al. (1988). In conclusion, *A. versicolor* has ability to produce a true penicillium-like structure with monoverticillte conidiophores rather than fragmentary heads resembling penicillate fructifications. Moreover, There is no doubt of that *Aspergillus versicolor* belongs to *Aspergillus* species but the production of *Penicillium* like structure should be considered and used as a unique morphological character for this species.

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