Nigerian Journal of Pharmaceutical and Applied Science Research, 2012, 1(2):37-45

Photomicrographic report on filamentation and sporulation in *Byssochlamys nivea* westling, a fungal contaminant of bottled Raphia Palmwine

Esiegbuya Daniel Oforitse [1,2] and [1]*Okungbowa Francisca Iziegbe

[1] Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin
[2]Plant Pathology Division, Nigerian Institute for Oil Palm Research, Benin City, Nigeria.

*Corresponding author. Dr Francisca I. Okungbowa
Email: fiokun2002@yahoo.com. Phone 08055376204.

ABSTRACT

Growth and sporulation studies were investigated in *B. nivea* obtained from the Plant Pathology Division of the Nigerian Institute for Oil Palm Research (NIFOR) where it was originally isolated from pasteurized raphia palm wine. Potato Dextrose Agar and Broth cultures were incubated at room temperature (26 ±2^{o}C) for 15 days during which morphological changes in the solid cultures were observed daily under a microscope fitted with a Motic Digital Camera, using the cover-slip method. Photomicrographs were taken. Liquid cultures were autoclaved at various temperatures. Streaked plates of the autoclaved cultures were observed for fungal growth. For the solid cultures, single filaments of *B. nivea* were seen after two days, while septation began on the third day. Profuse filamentation on the eighth day was followed by formation of conidia in chains (pseudohyphae). Chlamydospores were formed terminally on the profusely branched filaments on the tenth day while ascospores appeared from day 12-15. In addition, clusters of asci were present. Growth of autoclaved cultures decreased with increase in temperature. There was no growth at 80^{o}C. Although a simple study, this is the first documented report on sequential growth and sporulation studies in *B. nivea*, a major contaminant of most processed drinks and juices.

Keywords: *Byssochlamys*,contamination, filaments, growth,spores, palmwine.

INTRODUCTION

The genus *Byssochlamys* (Family Trichocomaceae) is an ascomycete characterized by the absence of a cleistothecium or any other structure that envelops the ascus in Ascomycetes during development. *Byssochlamys* contains two important species, *Byssochlamys nivea* and *B. fulva*. Both species cause spoilage of processed fruit products and are among the most commonly encountered fungi associated with spoilage of heat-processed fruits, world-wide (Tournas, 1994). The ascospores of *Byssochlamys* species are heat-resistant and survive temperatures of 85^{o}C (Beuchat and Rice, 1979; Splittstoesser, 1987). Also, *Byssochlamys* species can grow under very low oxygen tension (Taniwaki, 1995) and can form pectinolytic enzymes (Samson *et al*.,2009). The combination of these three physiological properties makes *Byssochlamys nivea* a very important fungus in pasteurized and canned fruits. Some potentially toxic secondary metabolites such as patulin, are produced by *Byssochlamys nivea* (Rice *et al.*, 1997; Dombrink-Kurtzman and Engberg, 2006).The anamorphic form of *Byssochlamys* is *Paecilomyces* (Samson *et al*., 2009).

Raphia hookeri is the most economically important plant among the eight raphia species indigenous to Nigeria (Okolo, 2008). The *Raphia* palm (*R. hookeri*) is a monocarpic crop with a terminal inflorescence. It flowers once and dies after fruit maturity (Bassir, 1968). The plant grows abundantly in southern Nigeria where it is cultivated as a multipurpose crop (Obahiagbon, 2009). *Raphia* palm wine is obtained from tapping the inflorescence of the raphia palm (*Raphia hookeri, R. vinifera* or *R. sudanica*) sap which should have been used for the development of the inflorescence (Ndon, 2003). The *Raphia* palm sap is the exudate or liquid which flows when the base of the inflorescence of the palm is tapped (Obahiagbon and Oviasogie, 2007) and it is colourless and becomes whitish after some time due to impartation of some organoleptic properties. The fermented sap (caused mainly by *Saccharomyces cerevisiae***)** is called palm wine, ''emu'' in Nigeria and ''nsafufuo'' in Ghana, and is very nutritious (Obahiagbon and Oviasogie, 2007). Local equipment such as ladder, chisel, machete (for creating tapping channels), collection pot, tapping rope, collection funnel and leaf cover are required for tapping. The nutritional composition of palm wine as reported by Esechie (1978) shows that it contains sugar, protein, titrable organic acids and alcohol. The raphia palm wine is also rich in vitamins, carbohydrates, yeast, mineral elements and water (Ukhun *et al*., 2005; Obahiagbon *et al.*, 2007). The health implication of the sap has been reported by Bassir (1968) who stated that it could be used for the cure of malaria, measles, and jaundice, and aids the flow of milk in nursing mothers. Eleven elements that the body cannot synthesize which were recommended by the NAS/NRC (2001) for the maintenance of good health have been detected in raphia palmwine (Obahiagbon *et al.*, 2007).

The sap of *R. hookeri* palm, drunk by millions of people as beverage in Africa has a shelf life of 24 months (Obahiagbon and Oviasogie, 2007). Eziashi *et al*., (2010) studied the thermal destruction temperatures of *B. nivea* in a bid to contribute to the proper preservation of raphia palmwine. *Byssochlamys nivea* was observed to produce white bubbles in pasteurized and un-pasteurized bottled raphia palmwine (Eziashi *et al*., 2010) which these workers said might have been due to inadequate pasteurization, contaminants from the host palm, sub-standard condition of the palm wine tapping panel and the bottling unit. They also found that *B. nivea* ascospores grew at pasteurization temperature of 80-85^{o}C.

The objective of the current investigation was to build on the initial report above and to document information on the time-based growth and sporulation patterns of *B. nivea* which would contribute to the understanding of the biology of this fungus.

MATERIAL AND METHODS

Fungal culture

A pure culture of *B. nivea* (IMI Number 396923) was obtained from the Plant Pathology Division of the Nigerian Institute for Oil Palm Research (NIFOR) where it was originally isolated from raphia palm wine (RPW) and identified by CABI Identification services, UK where a voucher specimen was deposited. It was then subcultured on Potato Dextrose Agar (PDA) supplemented with 1ml of 40g/ml gentamycin.

Morphological study

From the revived culture, a spore suspension (containing 1x10^{6} spores/ml distilled water) was made. Then 100ml conical flasks containing 50ml Potato Dextrose Broth (PDB) were inoculated, each with 1ml spore suspension. Also 1ml spore suspension was spread on fresh PDA plates. The culture flasks and plates were incubated at room temperature (26± 2^{o}C) for 15 days. The fungal morphological changes in the solid cultures were observed daily under a microscope fitted with a camera (Motic B1 Digital camera) using the cover-slip method in which a little quantity of each culture was transferred onto the base of cover slips buried in PDA (Williams and Cross, 1971). Day of inoculation = Day 0. Each observation was repeated by viewing three separate slides of each culture. The photomicrographs of observed morphological forms at each time point were taken.

Determination of thermal destruction temperature

At the end of incubation (after 15days) each culture flask was autoclaved at various predetermined temperatures (40, 50, 60, 70, 80^{o}C) for 20 min. Thereafter, the cultures were spread on fresh PDA plates and examined daily

microscopically during incubation for presence of mycelia. A growth rating scale of + to +++ and - was used, in which:

+ ++ = mycelial growth observed two days after inoculation

++ = mycelial growth observed after 3 days

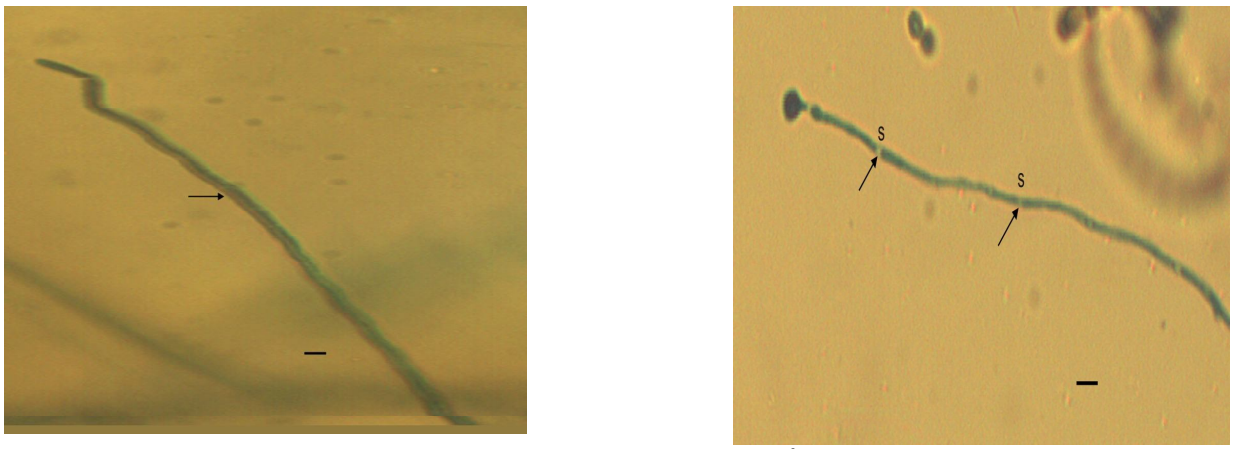
+ = mycelial growth observed after four days

- = No growth.

RESULTS

Morphology and sporulation

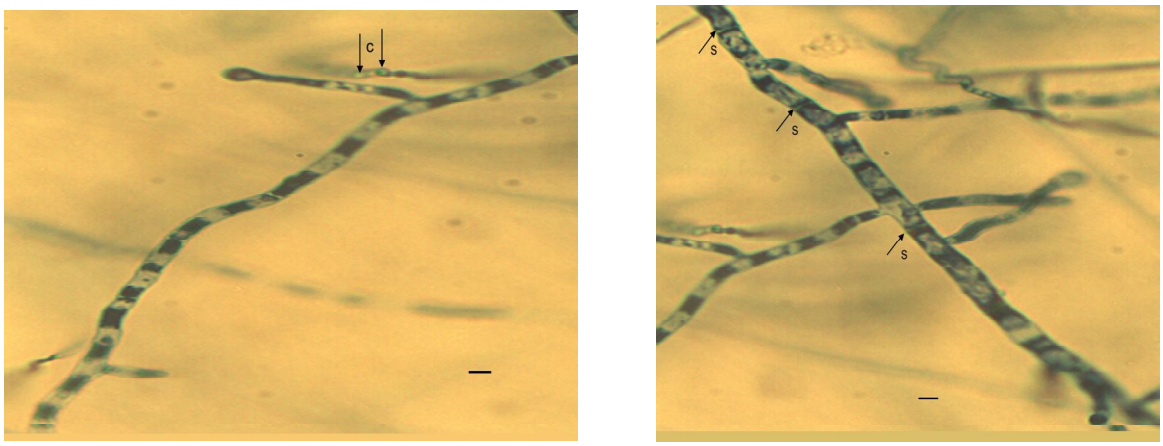
Results of morphological study showed that two days after incubation, single filaments of *B. nivea* (Fig. 1a) were seen while on days 3 and 4, points for septation were evident (Fig.1b). Septation became very clear with cells in compartments on day 5 (Fig. 2a). By day 8 water droplets were observed in cultures. There was profuse filamentation with side branches which was followed by production of conidia (Fig. 2b). Pseudohyphae were observed also (Fig. 3a). Single ascospores and clusters of ascospores were noticed (Fig. 3b and 3c). Figures 4a and b show ascocarps (fruiting bodies) and terminally borne chlamydospores respectively,which were seen from day 12-15. Conidia were the first spores observed while ascospores (sexual reproductive structures) appeared much later.



a b

Figure 1: Filament formation in *B. nivea* during incubation on PDA at 26±2°C.

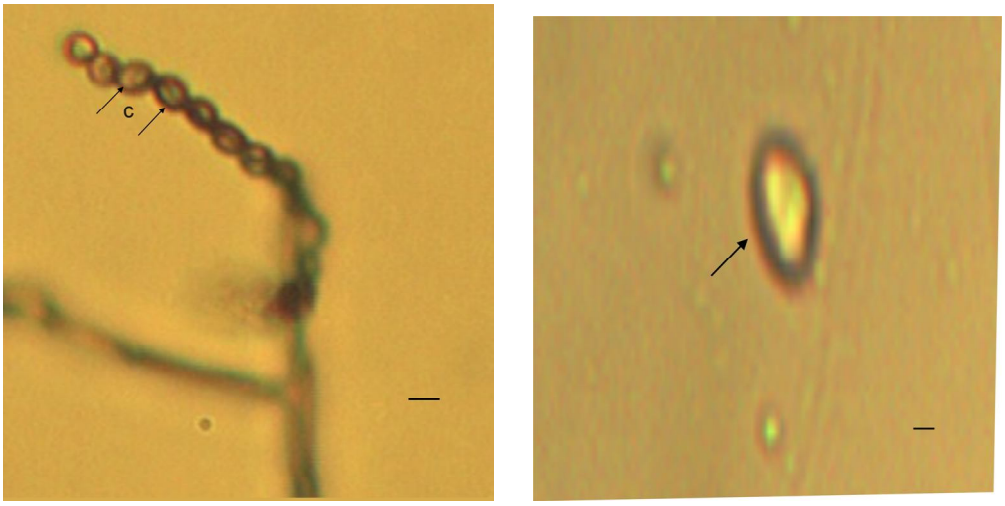
a = single filament of *B. nivea* seen after 2 days of incubation;
b = single filament showing signs
of septation (arrows show septa, S) after 3 days of incubation. Bar =10µm.



a b

Figure 2: Advanced stage of filamentation in *B. nivea* during incubation on PDA at 26±2°C for 5 days. a =filamentation with prominent septation (arrows show septa, S) and compartmentization of cells. b = profuse filamentation and conidia (C) formation. Bar =10µm.

a b



c

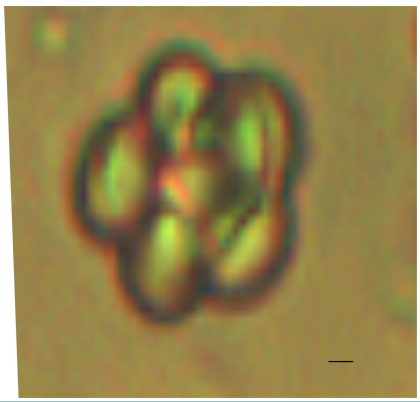


Figure 3: Sporulation in *B. nivea* after 8 days of incubation on PDA at 26±2°C; a = conidia, C in chains, b = single ascospore, c = cluster of ascospores. Bar =10µm.

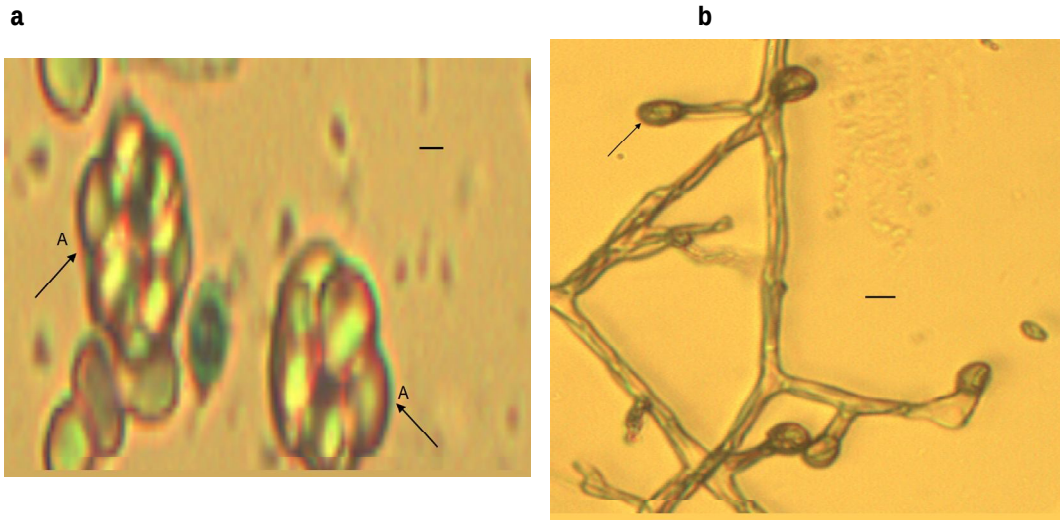


Figure 4: Production of fruiting bodies (ascocarps, A) and chlamydospores in *B. nivea* after incubation on PDA at 26±2°C for 12-15 days. a = ascocarps; b = terminally borne chlamydospores (arrow). Bar =10µm.

Table 1: Time taken for hyphae to grow out of autoclaved *Byssochlamys nivea* cultures during incubation on PDA at 26°C.

Temperature (°C)	Emergence of hyphae*
40	+++
50	+++
60	++
70	+
80	-

*Duration of incubation (days) before hyphae could be seen. +++ = 2 days; ++ = 3 days; + = 4 days; - = no growth.

Effect of Temperature Growth

Growth was least affected at 40^{o}C as mycelia were evident after two days of incubation, growth became slower with increased temperatures (Table 1). At 80^{o}C, no growth occurred (spores and mycelia were not seen).

DISCUSSION Filamentous fungi are morphologically complex microorganisms exhibiting different structural forms throughout their life cycles (Adrio and Demain, 2003). In submerged cultures, these fungi have different morphological forms ranging from dispersal of mycelia filaments to dense mycelial masses (Xu andYank, 2007). The life cycle of filamentous fungi starts and ends in the form of spores (Xu and Yang, 2007). The conidia are asexual reproductive spores produced for rapid proliferation of the fungus. They are usually the first to be produced as a fungus colonizes a very rich growth medium. Okungbowa and Usifo (2010) in their study, transferred the conidia of *Aspergillus and Penicillium* species onto growth media and these grew and produced mycelia (mass of filaments). Ascospores of *B. nivea* are heat-resistant (Samson *et al*., 2009; Eziashi *et al*., 2010). The chlamydospores are thick-walled spores produced during unfavourable conditions. They usually remain dormant until favourable conditions of growth (for example availability of nutrients, adequate moisture content and right pH) return. The conidia are not heat resistant (King *et al*., 1969). All spores observed in this study were similar to those described by Samson *et al*. (2009) and also reported by Eziashi *et al*. (2010). Each conidium was 3.0 -3.9 × 2.8- 3.1 µm. The genus *Byssochlamys* is morphologically well defined and characterized by ascomata (ascocarps) in which crosiers and globose asci are formed with ellipsoidal ascospores (Eziashi *et al*., 2010). Chlamydospores and characteristic clusters of ascospores (absent in the work of Eziashi *et al*., 2010) are reported here. The inhibition of growth in *B. nivea* at 80^{o}C corroborates earlier reports that the fungus can withstand high temperatures (Samson *et al.,* 2009; Eziashi *et al*., 2010) and this property is conferred on it by its ascospores.

CONCLUSION Although simple, this is the first documented report on sequential growth and sporulation studies in B. nivea, a mycotoxigenic contaminant of bottled Raphia palmwine. Pasteurization temperatures of 85-90^{o}C should be used for sterilization of RPW. Further research would have to determine other environmental factors affecting sporulation since spores are the major source of heat resistance. Also, a study of the molecular basis of heat resistance in *B. nivea* is timely.

REFERENCES

Adrio JL, Demain AL (2003). Fungal biotechnology. Intern Microbiol 6:191-199.

Bassir O (1968). Some Nigerian Wines. West Afric J Biol Chem 10: 42-45.

Beuchat LR, Rice SL (1979). *Byssochlamys* spp. and their importance in processed fruits. Adv Food Res 25:237-288.

Dombrink-Kurtzman MA, Engberg AE (2006). *Byssochlamys nivea* with patulin-producing capability has an isoepoxydon dehydrogenase gene (idh) with sequence homology to *Penicillium expansum and P. griseofulvum*. Mycol R e s 1 1 0 :1 1 11 – 1 1 1 8.

Esechie HA (1978). Effects of Different Preservative on the Major Chemical Constituents of Bottled Palm Wine During Storage. Nigerian Agricultural Journal, 15: 158-167.

Eziashi EI, Omamor IB, Airede CE, Udozen CV, Chidi N (2010). Heat resistance of genus *Byssochlamys* isolated from bottled raphia palm wine. J Yeast Fungal Res 1(8):142 – 145.

King ADJR, Michener HD, Ito KA (1969). Control of Byssochlamys and related heat-resistant fungi in grape products. Appl Microbiol 18 (2): 166-173.

NAS/NRC (2001). National Academy of Sciences/National Research Council Dietary References Intakes for Vitamins and Mineral Elements, National Academy Press, Washington, D.C. p.773.

Ndon BA (2003). The Raphia Palm. Concept Publications, Lagos. 156pp.

Obahiagbon FI (2009). A review of the origin, morphology, cultivation, economic products, health and physiological implications of raphia palm. Afric J Food Sci 3(13):447-453.

Obahiagbon FI, Oviasogie P (2007). Changes in the Physico-Chemical Characteristics of Processed and Stored *Raphia hookeri* Palm sap (Shelf Life Studies). Americ J Food Technol 2 (4): 323-326.

Obahiagbon FI, Ukhun ME, Eke CR (2007). *Raphia* hookeri Sap: recommended dietary allowances and roles of its dietary constituents in human nutrition. Intern J Bios 2(1): 62-65.

Okungbowa FI, Usifo AO (2010). Radial growth of *Aspergillus* and *Penicillium* spores exposed to laboratory disinfectants. Micol Aplicada Intern 22 (2): 59-61.

Okolo EC (2008). Evaluation of raphia hookeri progenies. NIFOR in - House Research Review pp 147-148.

Rice SL, Beuchat LR, Worthington RE (1997). Patulin production by *Byssochlamys* spp. in fruit juices. Appl Environ Microbiol 34:791-796.

Samson RA, Houbraken J, Varga J, Frisvad JC (2009). Polyphasic taxonomy of heat resistant ascomycete genus *Byssochlamys* and its *Paecilomyces* anamorphs. Persoonia 22: 14-27.

Splittstoesser DF (1987). Fruits and fruit products In: Beuchat LR (ed) Food and Beverage Mycology. 2nd edn. Van Nostrand Reinhold, New York. 101-128.

Taniwaki MH (1995). Growth and mycotoxin production by fungi under modified atmospheres. PhD Thesis, University of New South Wales, Australia.

Tournas V (1994). Heat-resistant fungi of importance to the food and beverage industry. Critic Rev Microbiol 20 (4): 243-263.

Ukhun ME, Okolie NP, Oyerinde AO (2005). Some mineral profiles of fresh and bottled palm wine: A comparative study. Afric J Biotechnol 4: 829-832.

Williams ST, Cross T (1971). Actinomycetes Isolation from Soil. In: Booth C (ed). Methods in Microbiology Vol. 4. Academic Press, London, pp 295-334.

Xu Z, Yang S (2007). Production of mycopherolic and *Penicillium brevicompactum* immobilized in a rotating fibrous-bed bioreactor enzyme. Microbiol Technol 40: 623-628.