INFLUENCE OF PHYSIOLOGICAL FACTORS ON MYCELIAL GROWTH OF BOTRYODIPLODIA THEOBROMAE PAT., ISOLATED FROM ANNONA MURICATA

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ABSTRACT
Influence of physiological factors on the mycelial growth of Botryodiplodia theobromae Pat., isolated from Annona muricata was investigated in the Forestry and Environment Laboratory (Pathology unit). The experiment was laid in Completely Randomized Design (CRD) with three replicates. Botryodiplodia theobromae, is the major causal organism of soursop fruit. This fungus grew and sporulated at a room temperature 28 ± 2°C using Potato Dextrose Agar (PDA) culture medium. Maximum pigment formation occurred on PDA (white 95%, black 25%). However, addition of 15g of glucose to Potato Agar Medium (PA) showed minimum pigment formation (white 25%, black 75%). The addition of 10g of glucose into potato agar significantly increased the mycelial growth of B. theobromae (28.6 ± 0.7mm – 32.0 ± 0.4mm). The highest number of pcyndia formed on Potato Agar (PA) without glucose was (10) and 15g of glucose recorded 4 pcyndia. Results revealed that there was no significant difference (P ≥ 0.05) on the growth of test fungus in both continuous light and darkness. These results will be useful for the study of fungi physiology and soursop consumer’s awareness on the dangers of infected fruits. It is observed that low population and poor yield in soursop may be due to the influence of deforestation, oil and gas activities within the region. It is recommended that post harvest losses could be reduced through proper handling and storing of fruits to avoid wounds and it consequent infections by B. theobromae.

Keywords: Mycelial growth, sporulation, Culture medium, Botryodiplodia theobromae.

INTRODUCTION
Botryodiplodia theobromae Pathogen
Fungi grow on diverse habitats in nature and are cosmopolitan in distribution requiring several specific elements for growth and reproduction. In laboratory, these are isolated on specific culture media for cultivation, preservation, microscopic examination biochemical and physiological characterization (Kuhn and Ghannoum, 2003).

A wide range of media are used for isolation of different groups of fungi that influence the vegetative growth and colony morphology, pigmentation and sporulation depending upon the composition of specific culture medium, pH, temperature, light, water availability and surrounding atmospheric gas mixture (Northolt and Bullerman, 1982; Kumara and Rawal, 2008).

However, the requirements for fungal growth are generally less stringent than for the sporulation. Physical and chemical factors have a pronounced effect on diagnostic characters of fungi. Hence, it is often necessary to use several media to identify fungus in culture since mycelial growth and sporulation on artificial media are important biological characteristics (St-Germain and Summerbell, 1996).
Botryothplodia theobromae Pat., is a known parasite causing both field and storage diseases of different crops, fruits and plantation trees (Khurana and Sing, 1972., Talukdar, 1974., Singh et al., 1977, Liag and Marfil, 1977). Botryodiplodia theobromae is an important pathogen of mango and other tropical fruits (Alam and Nahar, 1990) and causes die-back disease of jute, crown rot diseases of banana fruit, rot of coconut fruit, stem-end rot of mango fruit, soft rot of papaw, guava, and back in lemon plants.

Fungi exhibit varying responses to light, depending on the light intensity, quality, and duration of exposure and temperature. Exposure to light is needed by some fungi for sporulation (Marsh et al., 1959), whereas other fungi sporulate better in dark). Decrease in germination of conidia as the period of darkness increased (Rewal and Grewal, 1989). Fungi are spore-forming, non-chlorophytic, eukaryotic (cells having true nuclei) organisms and most of the true fungi are filamentous and branched. Over 100,000 species of fungi are saprophytes (Erinle, 1982).

However, over 20,000 species of fungi are parasites and cause diseases in crops and plants (USEPA, 2005). Fungal parasites are by far the most prevalent plant pathogenic organism. All plants are attacked by one species or another of phytopathogenic fungi. However, individual species of fungi can parasitize one or many different kinds of plants. Micro-organisms especially fungi have been reported to cause extensive deterioration of fruits and vegetables (Fajola, 1979; Amadioha and Uchendu, 2003). Some of these micro-organisms cause rotting, discoloration or fermentation of the fruits which affect their preservation. Several studies have shown that the fruits of A. muricata are attacked by various fungi which cause their deterioration.

Olunloyo (1986) found that fungal pathogens can impact negatively on pre-harvest of soursop leading to its deterioration. Olunloyo stated that this impact on the fruits of soursop might have probably arisen by the presence of pathogens which resided in dead stems and then dispersed by rain splash into the growing fruits to initiate infection spore, and that it was also possible that insect vectors dispersed the pathogens. Morton (1987) and Escobar and Sanchez (1993) reported that Colletotrichum gloeosporoides and Rhizopus stolonifer were associated with the pre-harvesting deterioration of soursop fruit in Colombian plantation. Botryodiplodia theobromae, Fusarium spp, Rhizopus stolonifer and Aspergillus niger fungal isolates were associated with the pre-harvest deterioration of soursop, (Morton, 2000). The pre-harvest rot of immature fruits was associated with B. theobromae Fajola, 1979).

The negative impact of pathogenic activities might also be responsible for the relative reduction in the protein, fat, fibre and carbohydrate contents of the infected ones. The protein, fat, fibre and carbohydrate might have been broken down by the fungi into smaller molecules which they absorbed. Bonner (1997) reported that deficiency of fibre in our diet leads to artherosclerosis, and intestinal cancer. The relative reduction in the vitamins (A and C) content of the infected fruits may be attributed to the activities of these pathogens. Nogata et al (2006) observed that vitamins act as co-enzymes in carboxylation, fatty acid metabolism, pyruvic carboxylases and also as important growth factors in all fungi. This means that the consumption of the fungal-infected fruits by man could lead to such vitamin-deficiency diseases as scurvy, dry skin and
dermatitis since the pathogen has utilized the vitamin content of the fruits for their growth, this phenomenon will lead to increase in the mineral compositions of the infected fruits relative to the healthy ones (Bonner, 1997). Nogata et al., (2006) reported that the deterioration of tropical fruits by pathogens may lead to the release of minerals from organic or biochemical complexes leading to increase in mineral content. This might be responsible for the increase in the ash content of the infected fruits. According to Bonner (1997), minerals contribute to the structures of essential enzymes and the regulation of the activities of some enzymes. Stainer et al., (1980) reported that fungi require minerals for satisfactory growth. The relative increase in the mineral contents of the fungal-infected fruits could also constitute health hazards to the consumer. According to Wayne (2004), excess of sodium in the diet causes oedema and hypertension. Mercer (2001) reported that copper in excess of metabolic requirements can lead to copper toxicity.

Underwood (2007) stated that lead results in a resultant general depression of energy metabolism and protein synthesis which causes a reduction in cell division and effects on growth. Robinson (2003) in his investigation reported that lead causes inhibitory effects on various stages of haembiosynthesis and that the continued inhibition of haembiosynthesis and protein synthesis could result to anaemia. Hence, consumption of these heavy metals could create health problems for man.

The pathogen (B. theobromae) first appears as whitish cottony colonies on potato-dextrose agar at 25% becoming heavily speckled by the presence of sporangia and then brownish black in age, spreading rapidly by means of stolons fixed at various points to the substrate by rhizoids (Lunn, 2004). Bilai (1988) stated that once a spore lands on a wounded tissue, it germinates and starts growing on the surface, producing a thick mycelium which at the same time procures cell degrading enzymes (pectinases, amylases) that denature the tissues in advance, thus, leading to infection. Lunn (2004) observed that the infection process takes place in a wide temperature range of 20 to 30°C.

This research is aimed at investigating the influence of physiological factors on mycelial growth of Botryodiplodia theobromae isolated from Annona muricata.

Specific objectives of this research were to:

i. isolate and identify fungal pathogens associated with soursop fruits;

ii. determine effect of different glucose concentrations on mycelial growth, pigmentation, and pcyndia formation;

iii. access the effect of light and darkness on mycelial growth of test fungus

MATERIALS AND METHODS

Sources of the fruits

Samples of Annona muricata fruits were collected from Choba and Mile Three Markets in Port Harcourt, Rivers State. The fruits were transferred into sterile black polyethene bags to the laboratory for investigation and kept in the refrigerator for use.
Microbiological Analysis
Isolation and identification of fungi

The detection of seed-borne and fruit borne mycoflora was carried by surface sterilizing the fruits and seeds in diluted sodium hypochlorite for 5 minutes. 5 seeds and fruit mesocarp respectively were placed on moistened filter paper and incubated for 5-7 days at 28 ± 2 °C room temperature (Chukunda and Stephen, 2015; Ukoima et al., 2009). The pure cultures of fungal isolates were obtained from potato dextrose agar (PDA). The fungi were identified and characterized based on their macroscopic appearance on culture medium C.M.I., 2010). Percentage Frequency occurrence of the fruit/seed-borne fungi was obtained using the formula: % Frequency occurrence = Number of fruits/seeds containing fungi x 100

Total number of fruits/seeds isolated

Preparation of Culture Medium
The culture medium used for this study was potato dextrose agar (PDA). PDA was mixed with 1 litre of sterile distilled water for 20 minutes in a conical flask plugged with sterile cotton wool. The content was sterilized in an autoclave at 1.03 kg cm\(^{-2}\) for 10 minutes and later dispensed into sterile Petri dishes which were allowed to solidify. All the isolated fungi sub-cultured on potato dextrose agar (PDA) medium were allowed to sporulate and later identified under binocular microscope for fungal growth according to (Ukoima et al., 2013).

Effect of light and darkness on mycelial growth of *B. theobromae*
To study the effect of light and darkness on mycelial growth of isolated fungi, 5 mm culture discs were cut with the sterilized Cork borer from advancing margin of the colonies of *B. theobromae* and inoculated on PDA plates at 5 days interval for 15 days. Carbon paper was used to wrap the Petri dishes for darkness, while unwrapped Petri dishes were exposed to light. All the Petri dishes were incubated at 28 ± 2 °C in six replicates under continuous light and darkness, (Kausar et al., 2009).

Effect of different glucose concentration on mycelial growth of *B. theobromae*
Effect of different concentrations of glucose (0, 5, 10, 15g) on fungi mycelial growth, sporulation and pigmentation of *B. theobromae*. Each Petri dish was inoculated with different glucose concentrations for five times with 4mm mycelial disc and kept at 28 ± 2°C. Control Petri dishes did not contain any glucose. Sporulation, Pigmentation and mycelial growth were observed at 5 days interval until 15 days in line with Saha et al., (2008).

Experimental Design and Statistical Analysis
The experiment was carried out in a Completely Randomized Design (CRD). Data collected were analyzed used Analysis of variance (ANOVA). The Least Significant Difference (LSD) at 5% probability and standard deviation were used to test for the level of significant between the treatments.
RESULTS AND DISCUSSION

The results on the frequency of occurrence of fungal pathogens of Soursop fruit (*Annona muricata*) from two markets; Choba and Mile 3 (Table 1) indicated that *Botryodiplodia theobromae* and *Rhizophus stolonifer* were found to be responsible for the rot of soursop fruits collected from the two markets. **The fruit/seed-borne pathogens of soursop have earlier been implicated with fruits rot by some researchers.** Chukunda (2014) reported that some of these fungi, *Aspergillus niger*, *Rhizopus* spp and *Fusarium* spp caused serious rotting in tomato fruits. Similarly, Ukoima *et al.*, (2013) isolated *Aspergillus niger* from seeds of *Jatropha*, while Baraka *et al.*, (2010) implicated *Colletotrichum gloeosporoides*, *Penicillium* spp and *Aspergillus niger* to be responsible for the rotting of groundnut seeds.

Chukunda (2014) found *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus stolonifer*, *Fusarium pallidoroseum*, *Botryodiplodia theobromae*, *Colletotrichum gloeosporoides*, *Penicillium expansum* and *Botrytis cinera* to be responsible for the serious decay of avocado pears obtained in the Niger Delta ecosystem. These finding agreed with those of George and Agrios (2005) who observed that symptoms of post-harvest disease of *Annona muricata* develop during storage, but infection of fruit by decay-causing pathogens could occur prior to harvest or during the post-harvest handling process and storage. Everett and Thomas (2001) observed that infection after harvest might be due to contamination of wounds by decay-causing pathogens during the drenching and packing process, and by fruit-to-fruit spread during storage.

*B. theobromae* has been reported to be one of the most important fruit rot pathogens in Southwestern Nigeria (Adisa and Fajola, 1982). *R. stolonifer* and *A. niger* are air borne fungi, and probably secondary invaders as well as opportunistic pathogens (Raper and Fennell, 2008). They reported that the post-harvest fungal rot pathogens of *Annona muricata* may include the following: *Botryodiplodia theobromae*, *Colletotrichum gloeosporoides*, *Rhizopus stolonifer*, *Rhizopus nigrican*, *Aspergillus flavus* and *Aspergillus niger*. *Rhizopus* spp is known to be omnipresent in the air as a contaminant. Robert, (2008) stated that usually saprophytic, fungi infect fleshy fruits in the field through wounds or bruises (made by harvesting implements) where the tissue is in a pre-necrotic process.

The results of the effects of light and darkness on fungal growth (Table 2) revealed that there was an increase in growth of *Botryodiplodia theobromae* in both light and darkness. The findings from research totally agreed with the report of Rewal and Grewal (1989) who studied the effect of light on conidial germination of three strains of *Botrytis cinerea* infecting chickpea, and found that conidia of *B. theobromae* germinated more under continuous light and strain B2 of *B. theobromae* germinated well under light and darkness treatment. From the study, it implied that light and darkness are necessary for growth and sporulation of test fungi. This is in agreement with Ahmed (1985) who observed that light promoted the growth and sporulation of *Collectotrichum gloeosporides*. Similarly, Marshi *et al.*, (1959) reported that fungi exhibited varying response to light depending on the light intensity, quality and duration of exposure.
Oladiran and Iwu (1993) and Pihet et al., (2009) reported that ultra violet (UV) radiation or sunlight affected the survival of fungal spores, sclerotia and pycndia. Some fungi need light to sporulate whereas other fungi sporulate better in darkness. In their investigation, *Aspergillus ornatus* produced abundant conidia when grown in continuous light and virtually none when grown in dark while cleistothecia and ascospores are produced in the dark whereas neither is produced in continuous light. Schwemmin, (1960) and Hill, (1976) further explained that light inhibits glucose uptake and phosphorylation which caused starvation and retards fungi growth and conidia formation. Conversely the growth of *Mycospharella pinodes, Aspergillus niger* increased when exposed to darkness.

On the contrary, Alam et al., (2001) reported that light is not necessary for growth and sporulation of *B. theobromae*, but it enhanced the growth and the number of conidia formed which agreed with the observation of Rewal and Grewal (1989) According to Cochrane (1958), temperature range permitting reproduction is usually narrower than that permitting growth. Alam et al., (2001) obtained the more growth of *B. theobromae* under conditions of continuous light and less in continuous darkness.

However, Teygaga and Clerk (1972) earlier demonstrated the relationship between *Cercospora canescens* conidia longevity and storage humidity, and observed that in the dark there was longest survival of conidia at low humidities than those under light. Generally the spores stored in the darkness appeared to be more viable than those in light. This may be due to metabolic disruption by light or that light inhibited the spores of test fungi thus reducing their percentage conidial germination. The effect of different glucose concentration (Table 4) revealed that 10g of glucose supported the best growth of *B. theobromae*.

Alam et al., (2001) reported that highest mycelial growth and sporulation of *B. theobromae* was recorded on PDA, which was in agreement to the present work. Several other researchers also stated that PDA was the best media for mycelial growth (Maheswari et al., 1999). Kumar and Singh, (2000) also stated that *L. theobromae* grew well in potato dextrose medium. Result of this study agrees with Alam et al (2001), who observed high mycelial growth of *Altermaria zinniae* on potato dextrose agar medium.

Light had no significant influence on mycelial growth, which was found to be equally good under complete light, complete dark and alternate 12 hour light and dark conditions. Sporulation was excellent and noticed after 10 days when the fungus was grown under complete light condition. Ray, (2004) showed that lactose and glucose had similar effect on growth of *Botryodiplodia theobromae*. Jash at al., (2003) also observed that sucrose was the best carbon source for growth of *Altermaria zinniae* followed by starch and maltose and mannitol produced least growth.
### Table 1: Fungi isolated from soursop fruits and seeds

<table>
<thead>
<tr>
<th>Market</th>
<th>Botryodiplodia theobromae</th>
<th>Rhizopus stolonifer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choba</td>
<td>84 ± 0.03</td>
<td>68 ± 0.83</td>
</tr>
<tr>
<td>Mile three</td>
<td>98 ± 0.12</td>
<td>57 ± 0.55</td>
</tr>
</tbody>
</table>

LSD (P ≤ 0.05) 8.00 5.25

### Table 2: Effect of light and darkness on mycelial growth of Botryodiplodia theobromae on PDA medium incubated at 28 ± 2°C

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Mycelial growth (mm) / days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Continuous light</td>
<td>16.0 ± 0.7</td>
</tr>
<tr>
<td>Continuous darkness</td>
<td>17.0 ± 0.6</td>
</tr>
</tbody>
</table>

### Table 3: Effect of different concentration of glucose in potato agar medium for pycndia formation and pigmentation of B.theobromae after 5, days interval of incubation at 28 ± 2°C

<table>
<thead>
<tr>
<th>Glucose Concentrations on Potato Agar (g)</th>
<th>% Pigmentation</th>
<th>Number of Pycndia/Petri dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>White 95, Black 5</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>White 85, black 15</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>White 70, black 30</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>White 25, black 75</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 4: Effect of Different Glucose Concentration On Mycelial Growth of B.theobroma

<table>
<thead>
<tr>
<th>Glucose Concentration (g)</th>
<th>Incubation period (days)/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>0 (control)</td>
<td>8.3 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>20.2 ± 0.6</td>
</tr>
<tr>
<td>10</td>
<td>22.0 ± 0.8</td>
</tr>
<tr>
<td>15</td>
<td>20.0 ± 0.5</td>
</tr>
</tbody>
</table>

Conclusion and Recommendations
The deterioration of the fruits of Annona muricata by botryodiploida theobromae has posed a serious post-harvest loses of the fruit. The effect of increasing glucose concentrations in medium (Potato Agar) we observed that B. theobromae utilize it in a certain level and grow properly and after certain level the fungal physiology does not permit the utilization of glucose for growth which most probable the fungus might have utilize the glucose for pigment formation. These micro-organisms cause rotting, discolouration fermentation of the fruits which affect their preservation.

Recommendations
Based on the present findings the following recommendations are made;
1. To reduce much post harvest losses, careful measures should be taken in cause of handing and storing the fruits to avoid wounds and its consequent infections.
2. It is revealed from the study Botryodiplodia theobromae that light enhanced sporulation while continous darkness promoted maximum mycelial growth of the fungus.
3. It was also proven that glucose was a good carbon. Source for growth of Botryodiplodia theobromae
4. From the study potato dextrose agar (PDA) was found to be a good medium of growth that supported the growth of Botryodiplodia theobromae.

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