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Fungi associated with black rot disease of pineapple (*Ananas comosus* L.) fruits and the effects of the disease on nutritional value of the fruits

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Pineapple (*Ananas comosus* L.) is one of the most important crops grown in Africa. The fruit is consumed fresh by many people and hence, source of essential mineral elements, nutrients and vitamins. This study was conducted to investigate the fungi associated with black rot disease of pineapple fruits and the effect of the disease on nutritional value of the fruits. The fungi isolated from black rot pineapple fruits got from Uselu, Ikpoba Hill, Oba, Oluku and New-Benin Markets in Benin City, were grown on Potato Dextrose Agar (PDA) at room temperature (28±2°C) and observed morphologically and microscopically for cultural and spore characteristics. Assimilative property of yeasts was determined with carbon substrates and peptone. Freshly harvested pineapple fruits (six wounded and six unwounded) were inoculated with the fungus responsible for black rot and incubated for six days (pathogenicity test). Healthy fruits as well as infected ones were analysed for carbohydrate, fibre, moisture, protein, and minerals. Two isolates of *Ceratocystis paradoxa* (black rot fungus), four isolates of yeasts, *Aspergillus niger* and *Penicillium* species were identified. Black rot was prominent in wounded pineapple fruits after six days of inoculation. The four isolates of yeast assimilated the carbon substrates and peptone. Furthermore, the infected fruits showed relative decrease in nutrient composition as compared to healthy fruits. With this basic knowledge of growth of *C. paradoxa,* the cause of black rot of pineapple fruit, better handling and storage can be planned in order to reduce crop losses.

Key words: Black rot, *Ananas comosus* fruit, *Ceratocystis paradoxa*, nutritional value.

INTRODUCTION

History and origin of pineapple

Pineapple (*Ananas comosus* L.) is a tropical plant with edible multiple fruit consisting of coalesced berries named for resemblance to the pine cone. The crop is propagated vegetatively. The cultivar of the crop is

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derived from spontaneous mutation and natural selection without controlled breeding (Oseik-kofi et al., 1997). It is the most economically important plant with a cylindrical false fruit in the Bromeliaceae family. It consists of thickened, fleshy, very juicy axis core and inedible, scaly warty skin. Pineapples may be cultivated from a crown cutting of the fruit, possibly flowering in 20 to 24 months and fruiting the following six months (Onuorah et al., 2013). Pineapple may be consumed fresh, canned, juiced and are found in a wide array of food stuffs, dessert, fruit salad, jam, yogurt, ice cream, candy and as a complement to meat dishes (Kochhar, 2006; Lobo and Paull, 2016).

Health benefits of pineapple

Anti-inflammatory and digestive benefits

Bromelain is a complex mixture of substances that can be extracted from the stem and core fruit of the pineapple. Among dozens of components known to exist in this crude extract, the best studied components are a group of protein-digesting enzymes (called cysteine proteinases) (Debnath et al., 2012). Originally, researchers believed that these enzymes provided the key health benefits found in bromelain, a popular dietary supplement containing these pineapple extracts. In addition, researchers believed that these benefits were primarily limited to help with digestion in the intestinal tract (Nwachukwu et al., 2008). One healthy ripe pineapple fruit can supply about 16.2% of daily requirement for vitamin C (Hemalatha and Anbuselvi, 2013).

However, pineapple contains high level of sugars and other nutrients and their low pH values make them desirable to fungi decay. Fungi can survive on pineapple because of the nutrients present such as proteins, fat and carbohydrates that support the growth of pathogens. The ripening of pineapple fruits make it susceptible to fungi infection. This disease subsequently developed and damaged the fruit during nutrition and thereby reducing the quality of the fruit. In Nigeria, due to poor transport and storage facility, postharvest losses are severe (Droby, 1998).

Pineapple being a cheap, nutritious and easily accessible commodity in Nigeria, the consumption of the fruits is at a high rate (Nwachukwu et al., 2008). However, increase consumption rate has also be known with a parallel increase in food borne diseases. The study therefore called for isolation and identifying the fungi associated with the disease.

Ceratocystis paradoxa

Pineapple disease caused by the ascomycetous fungus *Ceratocysis paradoxa* (Dade) Moreau belonging to the order Microascales, is an important rot (Dede and Okungbowa, 2007). The disease affects sugarcane and pineapple setts in the first week after planting leading to poor germination of bud and emergence of young shoots. As a result, gabby stands are evident and young crops have a patchy and uneven appearance (Agoris, 1997).

C. *paradoxa* is parasitic on a variety of economic and food crops among which are *Ananas sativa, Cocos nucifera, Elaeis guineensis, Ipomoea batatas, Musa sapientum, Phoenix africanas, Sandoricum indicum, Sorghum vulgare, Theobroma cacao* and *Zea mays* (Agrios, 1997). In the oil palm, *C. paradoxa* is associated with dry basal rot in which it attacks the stems, leaves and fruit causing premature fruit drop (Agrois, 1997). *C. paradoxa* also causes disease in a variety of other tropical plants such as banana, coconut, and sugarcane making it a somewhat dangerous pathogen. Pineapple black rot is the most common and well-known post-harvest disease of the pineapple fruit and is responsible for serious losses in the fresh pineapple fruit world industry. The pathogen is a polyphagous wound parasite and gains entry into the fruit via wounds sustained during and after harvest (Dede and Okungbowa, 2007). The disease only shows up in fresh fruit because the time from harvest to processing it too short for infection to occur. Infection can also occur out in the field, but it is not nearly as common as post-harvest infection.

Yeasts

Yeasts are eukaryotic microorganisms classified in the kingdom fungi, with 1,500 species currently described (Serge et al., 2015). Yeasts are unicellular although some species with yeast forms may become multicellular through the formation of a string of connected budding cells known as pseudo-hyphae, or false hyphae, as seen in most molds (Lima et al., 1999).

Nutrition and growth of yeasts

Yeasts are Chemoorganotrophs, as they use organic compounds as a source of energy and do not require sunlight to grow (Kurtzman, 1990). Carbon is obtained mostly from hexose sugars, such as glucose and fructose, or disaccharides such as sucrose and maltose. Some species can metabolize pentose sugar like ribose, alcohols and organic acids. Yeast species either require oxygen for aerobic cellular respiration (obligate aerobes) or are anaerobic, but also have aerobic methods of energy production (facultative anaerobes) (Kutzman, 1990), yeast grows best in a neutral or slightly acidic pH environment. In general, yeast is grown in the laboratory on solid growth media or in liquid broths. Common media

used for cultivation of yeast include potato dextrose agar (PDA), Wallerstain laboratories nutrient (WLN), and yeast mould agar (Teramoto et al., 2005).

Reproduction of yeast

Yeasts like all fungi, may have asexual and sexual reproductive cycles. The most common mode of vegetation growth in yeast is asexual reproduction by budding (Ogawa et al., 2000). Here, a small bud or daughter cell is formed on the parent cell. The nucleus of the parent cell splits into a daughter nucleus and migrates into the daughter cell. The bud continues to grow until it separates from the parent cell, forming a new cell. The daughter cell produced during the budding process is generally smaller than the mother cell (Ogawa et al., 2000). The objectives of this study were to isolate and identify fungi associated with black rot disease of pineapple; to carry out pathogenicity test and the impact of the organism (causal agent of black rot disease of pineapple) on nutritional composition of pineapple fruit; and determine how to control the growth of *C. paradoxa* in pineapple fruits.

MATERIALS AND METHODS

Source of pineapple

Pineapple fruits used for this investigation were purchased from 5 markets in Benin City in Edo State. The species examined was *A. comosus.* The fruit rot was collected from Uselu, Oba Market, Ikpoba Hill, Oluku and New-Benin markets.

Sterilization

All glass wares and slides were washed in 'Omo' detergent solution, rinsed in several change of tap water and finally with distilled water and allowed to dry. They were sterilized in an electric oven at a temperature of 60°C for 24 h. Cover slips were flame sterilized just before use. The droppers, pipettes, cotton wool and plugs were covered with aluminium foil to prevent entry of condensed water vapour into the media.

Detection of fruit rot

Pineapple fruits were examined usually by disserting the fruit with a clean knife after which the symptoms were described.

Isolation of fungi associated with fruit rot

Small portion (including rotted and healthy portions) of 5 mm diameter were cut with a flamed scalpel blade from mesocarp and scale, these were sterilized in 0.1% mercuric chloride solution for 2 min and rinsed in 3 changes of distilled water, dried with sterile tissue paper and crushed before plating in petri dishes were incubated on a laboratory bench at laboratory temperature of 28 to 2°C for 3 to 7 days. After the period of incubation different colonies of fungi associated with the fruit rot was aseptically sub-cultured using a flamed inoculating loop, into a sterile plate containing PDA.

Fifteen McCartney bottles divided into two groups label 10^{-1} to 10^{-4} and the stock bottle labeled S, S1, S2 and S3 prepared in triplicate were sterilized while for yeast isolates the stock bottle labeled S1, S3, S3, S4, S5, S6, S7, and S8 were sterilized. The sterilization is done in autoclave at 121°C for 15 min. After sterilization, the black rot diseased samples were transferred into each individual stock bottle labeled 10^{1} to 10^{-4} and yeast 10^{-1} to 10^{-8} containing 9 ml of sterilized distilled water for serial dilution preparation.

Pathogenicity test

Healthy pineapple fruit sixteen were purchased from market and sterilized with ethanol and rinsed with distilled water. Six sterilized pineapples were punctured with sterilized pin and inoculated with 2 ml solution of two isolates of *C. paradoxa,* respectively isolated from naturally infected fruits and 6 unwounded sterilized pineapples were also inoculated with 2 ml solution of two isolates of *C. paradoxa,* respectively. These infected central were healthy incubated in sterilized bucket for 6 days at low temperature. The organisms were isolates from injected fruit to confirm the causative agent.

Morphological identification of isolates

Fungal cultures were examined for characteristic macroscopic and microscope features based on descriptions of Chukwura et al. (2010). Cultured samples were also confirmed by Nigeria Institute of Oil Palm Research Benin City. The yeast colonies were described on morphological characteristic based on the surface, margin, colour, and elevation. The yeast cells examination was identified on the microscope.

Physiological investigation of yeast

A loop of yeast culture was inoculated in thirteen test tubes containing 9 ml of carbon substrate and nitrogen substrate media containing glucose, lactose, saccharose, dextrose, xylose, starch, mannitol, maltose, Raffinose, fructose, citric acid, Galactose and peptone. Each substrate was represented in triplicates and used for testing assimilation properties of yeast by visualizing the turbidity of the substrates in the test tubes and comparing the inoculated carbon and nitrogen substrates with the substrates without yeast strains (control).

Determination of Vitamin C

The reagents are as follows: 0.2829 g of dried $K_2Cr_2O_7$ in 100 ml of distilled water (1000 ppm); 10 ppm $K_2Cr_2O_7$ from stock: 1 ml in 100 ml flask and made to mark; 1 M H_2SO_4: 5.45 ml in 100 ml flask; 0.25% diphenylcarbazide (DPC): 0.25 g in 100 ml of acetone.

Crude protein determination

The crude protein in the sample was determined by the routine semi-micro Kjeldahl, procedure/technique. This consists of three techniques of analysis, namely digestion, distillation and titration.

Digestion

Each finely ground dried sample (0.5 g) was weighed carefully into the Kjeldahl digestion tubes to ensure that all sample materials got to the bottom of the tubes. To this were added 1 Kjeldahl catalyst tablet and 10 ml of Conc. H_2SO_4. These were set in the appropriate hole of the Digestion Block Heaters in a fume cupboard. The digestion was left on for 4 h, after which a clear colourless solution was left in the tube. The digest was cooled and carefully transferred into 100 ml volumetric flask, thoroughly rinsing the digestion tube with distilled water and the flask was made up to mark with distilled water.

Distillation

Distillation was done with Markham Distillation Apparatus which allows volatile substances such as ammonia to be steam distilled with complete collection of the distillate. The apparatus was steamed out for about 10 min. The steam generator is then removed from the heat source to the entire developing vacuum to remove condensed water. The steam generator is then placed on the heat source (that is, heating mantle) and each component of the apparatus was fixed up appropriately.

Determination

Portion of the digest (5 ml) was pipette into the body of the apparatus via the small funnel aperture. To this was added 5 ml of 40% (W/V) NaOH through the same opening with the 5 ml pipette.

The mixture was steam-distilled for 2 min into a 50 ml conical flask containing 10 ml of 2% boric acid plus mixed indicator solution placed at the receiving tip of the condenser. The boric acid plus indicator solution changes colour from red to green showing that all the ammonia liberated have been trapped.

Titration

The green colour solution obtained was then titrated against 0.01 N HCL contained in a 50 ml Burette. At the end point or equivalent point, the green colour turns to wine colour which indicates that all the nitrogen trapped as ammonium borate [$(NH_4)_2BO_3$] have been removed as ammonium chloride (NH_4CL). The percentage nitrogen in this analysis was calculated using the formula:

%N = Titre value × Atomic mass of Nitrogen × Normality of HCL used × 4
or
%N = Titre value × Normality/Molarity of HCL used × Atomic mass of N × Volume of flask containing the digest × 100/1

Weight of sample digested in milligram × volume of digest for steam distillation. The crude protein content is determined by multiplying percentage nitrogen by a constant factor of 6.25, that is, %CP = %N × 6.25.

Dry matter and moisture determination

Sample (2 g) was weighed into a previously weighed crucible. The crucible plus sample taken was then transferred into the oven set at 10°C to dry to a constant weight for 24 h overnight. At the end of the 24 h, the crucible plus sample was removed from the oven and transferred to desiccator, cooled for 10 min and weighed.

If the weight of empty crucible is Wo, the weight of crucible plus sample is W_1, and the weight of crucible plus oven-dried sample is W_3.

%Dry Matter = W_3 - Wo/W_1 - Wo × 100
% Moisture = W_1 - W_3/W_1 - Wo × 100
% Moisture = 100 – %DM.

Determination of ash

Sample (2.0 g) was weighed into a porcelain crucible. This was transferred into the muffle furnace set at 550°C and left for about 4 h. About this time, it had turned to white ash. The crucible and its content were cooled to about 100°C in air, then room temperature in a desiccator and weighed. This was done in duplicate. The percentage of ash was calculated from the formula:

Ash content = Weight of ash / Original weight of the sample × 100

Fibre determination

Sample (2.0 g) was accurately weighed into the fibre flask and 100 ml of 0.255 N H_2SO_4 was added. The mixture was heated under reflux for 1 h with the heating mantle. The hot mixture was filtered through a fibre sieve cloth. The filtrate obtained was thrown off and the residue was returned to the fibre flask to which 100 ml of 0.313 N NaOH was added and heated under reflux for another 1 h. The mixture was filtered through a fibre sieve cloth and 10 ml of acetone added to dissolve any organic constituent. The residue was washed with about 50 ml hot water on the sieve cloth before it was finally transferred into the crucible. The crucible and the residue were oven-dried at 105°C overnight to drive off moisture. The oven-dried crucible containing the residue was cooled in a desiccator and later weighed to obtain the weight W_1. The crucible with weight W_1 was transferred to the muffle furnace for Ashing at 550°C for 4 h.

The crucible containing white or grey ash (free of carbonaceous material) was cooled in the desiccator and weight to obtain W_2. The difference W_1 - W_2 gives the weight of fibre. The percentage fibre was obtained by the formula:

%Fibre = W_1 - W_2 / Weight of sample × 100

Determination of mineral element (Calcium, Potassium and Sodium)

The ash of each sample obtained was digested by adding 10 ml Aquaregar and filtered through a Whatmann No. 1 filter paper into a 25 ml volumetric flask. The filtrate was made up to mark with distilled water stoppered and made ready for reading of concentration of Calcium, Potassium, and Sodium on the Jenway Digital Flame Photometer (PFP7 Model) using the filter corresponding to each mineral element. While the Iron, Manganese and Copper were read in a Perkinhelmer AAS model 2300AA.

The concentration of each of the element was calculated using the formula:

%Ca or %K or %Na = Meter Reading (MR) x Slope x Dilution factor / 1000

where MR × slope × dilution factor will give you the concentration in part per million (ppm or mg/kg). You get concentration in % when you divide by 10000.

Table 1. Morphological characteristics of yeast isolated from Pineapple fruit rot from five markets in Benin City.

S/N	Morphological characteristic	*Candida* spp.	*Kodamaeae ohmeri*
1	Surface	Smooth	Rough
2	Margin	Entire	Undulating
3	Colour	Cream	yellow
4	Elevation	Convex	Convex
5	Cell shape	Spherical ellipsoidal	Ellipsoid filamentous pseudo mycelium
6	Pseudo mycelium	Present	Absent
7	Mycelium	Absent	Absent
8	Growth at 37°C	+	+
9	Locations	1.Uselu, 2.New Benin, 3.Ikpoba hill markets	New Benin and Oluku market

Table 2. Physiological characteristics of yeast strains isolated from pineapple fruit rot.

Assimilation test	*Candida* spp.	*Kodamaea ohmeri*
Glucose	++	++
Lactose	++	+++
Saccharose	++	+
Dextrose	++	+
Xylose	+++	++
Starch	+++	++
Mamnitol	++	+
Masltose	++	+
Raffinose	+++	+++
Fructose	+++	-
Citric acid	+	-
Galatose	++	++
Peptone	+++	+++

+Low turbidity, -No turbidity, ++High turbidity.

Determination of carbohydrate

Procedure for extraction

Dried wood (1 g) was weighed. 5 ml of 72% H_2SO_4 was added. The mixture was later shaked and filtered. 1 ml of the filtrate was thereafter pipetted into a conical flask and 5 ml of anthrone reagent was added to it and shake. 30 ml of the anthrone reagent was added and then heated for 10 min. The absorbent was allowed to cool to room temperature and measured at 620 nM.

Standard

D glucose (0.01 g) was weighed into a 100 ml of volumetric flask and the 100 ml mark was made up with distilled water. 0, 0.1, 0.2, 0.3, and 0.4 ml of stock (100 ppm) was pipetted into a conical flask which was later made up to 1 ml with water. 5 ml of the anthrone reagent was added and heated for 10 min thereafter allowed to cool to room temperature. The absorbent was measured at 620n.

RESULTS

Description of fungal isolates

Cultures from mesocarp of rotted fruit of pineapple showed the presence of black fungus identified and confirmed by NIFOR as *C. paradoxa*. Mycelium of the fungus was whitish and later turned black due to the presences of mature chlamydospores measuring 5 mm in diameter. The micro-conidia consisted of a basal and an apical cell, tapering towards the free end (Table 1).

The physiological test result shown in Table 2 shows that isolates 1 and 2 utilized the carbon substrates and nitrate substrate (peptone) for their growth. Isolate 3 utilized some of the carbon substrate and peptone (nitrate substrate) except citric acid, fructose, reffinose, maltose, mannitol and xylose. Isolate 4 assimilated most of the carbon substrates except fructose and citric acid

Table 3. Morphological characteristics of contaminated fungi isolates from fruit rot of pineapple from five markets in Benin City.

S/N	Morphological description	Suspected organisms
1	Black mycelia growth	*Aspergillus niger*
2	Grey colour mycelia growth	*Pencillium* spp.

Table 4. Proximate analysis result.

Parameter	Infected with isolate 1 of *C. paradoxa*	Infected with isolate 2 of the *C. paradoxa*	Uninfected (control)
Calcium (mg/kg)	$455^{c} \pm 14.64$	$422^{b} \pm 11.02$	$417.5^{a} \pm 1.32$
Manganese (mg/kg)	$2.84^{a} \pm 0.44$	$2.40^{a} \pm 0.31$	$4.85^{b} \pm 0.45$
Potassium (mg/kg)	$1202.5^{a} \pm 1.89$	$1210^{a} \pm 7.52$	$1477.5^{b} \pm 10.41$
Ash (%)	$0.13^{a} \pm 0.50$	$0.15^{a} \pm 0.15$	$0.10^{a} \pm 0.01$
Moisture (%)	$10.05^{b} \pm 0.01$	$8.24^{a} \pm 0.15$	$20.95^{c} \pm 1.03$
Fibre (%)	$35.11^{c} \pm 0.68$	$31.72^{b} \pm 0.89$	$21.32^{a} \pm 0.35$
Vitamin C (mg/kg)	$2.78^{a} \pm 0.23$	$2.44^{a} \pm 0.33$	$5.22^{c} \pm 0.05$
Carbohydarate (mg/kg)	$22.42^{b} \pm 0.30$	$20.63^{a} \pm 0.19$	$27.22^{b} \pm 0.49$
Protein (mg/kg)	$12.06^{a} \pm 0.21$	$12.11^{a} \pm 0.02$	$15.43^{b} \pm 0.30$
Energy (mg/kg)	$14.36^{a} \pm 0.32$	$14.15^{a} \pm 0.74$	$17.65^{b} \pm 0.18$

Means followed by the same letter in the same row are not significantly different with Duncan Multiple Range test (DMRT) at $p<0.05$.

(Table 3).

The result shown in Table 4 shows relative decrease in mineral composition of vitamin C, protein, energy, carbohydrates, potassium and manganese and increase in calcium, ash, and fibres in the infected pineapple fruit. This shows that *C. paradoxa* utilized the macronutrient in the pineapple fruits growth.

Pathogenicity test

Six days after inoculation, symptoms of the disease manifested on the healthy pineapples but more prominent on wounded samples.

DISCUSSION

The most common and well known post-harvest disease of the pineapple fruit which is responsible for serious losses in fresh pineapple fruit world industry is caused by black rot fungus *C. paradoxa.* The disease occurs in field through wounds and crevices between individual fruits and normally remains quiescent or especially appears during harvest and marketing. The symptoms are characterized by a soft watery rot that starts at the point of detachment of the fruit. Tissues affected by the fungus mycelium and chlamydospores darken during the course of the disease.

The physiological study showed that the yeast strains assimilated most of the carbon and nitrate substrates, which indicates that the yeasts utilized most of mineral composition of pineapple fruit for growth thereby degrading the macro nutrients and this led to fermentation and off-flavour of pineapple fruit.

The other isolated fungi, especially Penicillium spp. have the potential to induce rot on fresh fruits which might have a remarkable effect on the value of the fruit especially in food industry as well as on human health. This agrees with the report of Amusa et al. (2003) that stated that the presence of fungi in pineapple fruits is a health risk to the consumer. *Aspergillus niger* produces mycotoxins which causes food intoxication in man and other animal (Vargea et al., 2009).

Symptoms observed and organism re-isolated in the pathogenicity test were similar to the pineapple fruit rot samples found in the two markets in Benin City which confirmed that *C. paradoxa* is responsible for the fruit rot of the pineapple fruits. However, twelve days after inoculation, it was observed that the symptoms of black rot of pineapple caused by *C. paradoxa* were no longer visible. This may be as a result of fermentation caused by mycoparasitic reactions of antagonistic yeasts cells present in the fruits, which competed for nutrients. This

finding is similar to that of Droby et al. (1989) that reported that fermentation by microoganisms on food stuff often affect the sypmtoms expression of the causal agent. Moreso, if yeast cells were responsible for disease inhibition it means that yeasts cells have biocontrol efficacy against infection of *C. paradoxa*, which is related to the findings of Arras et al. (1999) who reported that interaction between plants and certain fungi such as yeast was associated with beneficial effects such as biocontrol of soil borne fungal pathogens.

The increased calcium level in the infected pineapple fruit showed that the mycoparasitic reaction of *C. paradoxa* induced calcium hydroxide on the fruit thus leading to bioaccumulation of calcium in fruits. This is related to the study of Awuku et al. (1991). The conversion of organic or biochemical compounds of the fruits into inorganic complexes lead to relative increase in ash content of the infected pineapple fruits. This agrees with the report of Onuorah et al. (2013) that stated that biochemical processes within fruits often affected its ash composition.

The utilization of macronutrients by the fungus for growth caused nutrient deficiency of the infected pineapple fruit, which can lead to health problem such as inability to decrease severity of respiratory tract infection. Perifollicular hemorrhage which can lead to scurvy, retarded growth, muscle wasting, low metabolic activities, psychomotor disturbances, and reduction in energy (Diribe and Elom, 2002).

Conclusion

Black rot disease is a universal fresh fruit problem which occurs in field when fruit is injured in poorly drained soil and in low acidic soil. The knowledge of survival and growth of *C. paradoxa* gotten from this study shows that black rot disease can be properly managed by careful handling of fruits so as to avoid bruises.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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