Incidence and Aetiology of Stem End Rot and Anthracnose Diseases Affecting Soursop Fruits at Pre-Harvest and Post-Harvest Stages in Ghana

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Abstract
Soursop is increasingly becoming an important fruit crop in Ghana especially within the urban areas. A research work was carried out to identify the major diseases and their associated pathogens affecting the crop at both the pre-harvest and post-harvest stages of fruits in the country. Diseased soursop fruits of the local varieties were collected from the coastal savannah zone of Ghana, disease symptoms expression on fruits studied and the causal agents identified using morphological and molecular approaches. Two major diseases namely, stem end rot and anthracnose were identified on the fruits. Cultural characteristics of isolates and the amplification of expected PCR products using species specific primers confirmed that Lasiodiplodia theobromae and Colletotrichum gloeosporioides were responsible for stem end rot and anthracnose diseases respectively. Mean disease incidences of stem end rot and anthracnose at the pre harvesting stage for a period of 3 years was 25.5% and 21.4% while at the post-harvest stage were 33.4% and 65.5% respectively. Though stem end rot was found to be more damaging as the fruits infected by the causal agent could not be utilized in any way, both diseases destroy the aesthetic and marketing values of the fruits and hence deserve attention. The information obtained in this study will be very useful for any future studies of the soursop crop in Ghana.

Keywords: Soursop, Stem end rot, Anthracnose, Post-harvest

Incidence et Étiologie de la Pourriture Terminale des Tiges et des Maladies de L'anthracnose Affectant les Fruits Soursop aux Stades Pré-Récolte et Post-Récolte au Ghana.

Résumé
Soursop est de plus en plus une culture fruitière importante au Ghana, en particulier dans les zones urbaines. Un travail de recherche a été effectué pour identifier les principales maladies et leurs pathogènes associés affectant la culture aux stades pré-récolte et post-récolte des fruits dans le pays. On a prélevé des fruits malades des variétés locales dans la savane côtière du Ghana, on a exprimé les symptômes de la maladie sur les fruits étudiés et on a identifié les agents causals à l'aide d'approches morphologiques et moléculaires. Deux grandes maladies, à savoir

la pourriture des tiges et l'anthracnose, ont été identifiées sur les fruits. Les caractéristiques culturelles des isolats et l'amplification des produits de PCR attendus à l'aide d'amorces spécifiques à l'espèce ont confirmé que Lasiodiplodia theobromae et Colletotrichum gloeosporioides étaient responsables respectivement de la pourriture des tiges et de l'anthracnose. L'incidence moyenne de la pourriture terminale et de l'anthracnose avant la récolte pendant une période de trois ans était de 25,5 % et de 21,4 %, alors qu'elle était de 33,4 % et de 65,5 % respectivement après la récolte. Bien que la pourriture terminale de la tige se soit avérée plus dommageable que les fruits infectés par l'agent causal ne pouvaient être utilisés en aucune façon, les deux maladies détruisent les valeurs esthétiques et de commercialisation des fruits et méritent donc l'attention. Les renseignements obtenus dans le cadre de cette étude seront très utiles pour toute étude future de la culture de l'acide sulfurique.

Mots clés: Soursop, pourriture des tiges, anthracnose, post-récolte

Introduction

Soursop (*Annona muricata* L.) also called guanabana, graviola, or Brazilian pawpaw, belongs to the Annonaceae family. Although the crop is a native of the American tropics, it is widely introduced in the tropics (Morton, 1987). The 'fruits fibrous white flesh, which combines the flavours of mango and pineapple, can be eaten fresh and is strained to make custards, ice creams, and drinks (Grant, 2018). A study by Alates *et al*. (2019) showed that soursop supplementation can lower blood pressure and serum uric acid levels. Gyesi *et al*. (2019) also found that two essential oils in soursop leaf and fruit pulp could serve as antioxidants in food processing and preservation as well as cosmetic and pharmaceutical industries. The crop has long been labelled as a promising tropical fruit for the European market since 1964 (Morton, 1987). Reports indicated that international demand for soursop has been growing at 3.8% per year (Alvarez et al., 2005). Therefore, cultivation of soursop fruits has a potential of improving the incomes of people who will be involved in its production and marketing (Alberto and Otanes, 2016).

Soursop fruits are not considered an important export commodity in Ghana, hence the trees are treated same as local varieties of pawpaw, mango and avocado. The production of the crop is restricted to backyard gardens with very little attention. However, few fruits harvested from such trees are found in several grocery shops dotted in several urban areas all over the country. The potential of the crop to serve as a supplementary source of livelihood to the women engaged in its marketing locally is therefore huge. However, this great potential of the crop is under threat by several diseases which are found on the fruits both in the field and on grocery stalls in the country (Personal observation).

In most areas where soursop fruits are produced, two major diseases have been reported affecting the fruits both in the field and after harvest. These are fruit rot and anthracnose. Pathogens such as *Colletotrichum gloeosporioides*, *Lasiodiplodia theobromae, Fusarium* sp., *Rhizopus stolonifer*, *Colletotrichum acutatum*, *Fusarium chlamydosporum* and *Aspergillus* species have been associated with rots of fruits in the field and after harvest (Amusa *et al*., 2003; Alberto and Otanes, 2016). Generally, *L. theobromae* is known to cause stem end rot in several tropical crops while *C. gloeosporioides* is associated with anthracnose on tropical fruit crops (Honger *et al*., 2015; Honger *et al*., 2014; Agrios, 2005). These are distinct diseases both of which eventually reduce quality of soursop fruits

even before they are harvested (Alberto and Otanes, 2016). In Venezuela, anthracnose disease attributed to *C. gloeosporioides* has been reported as causing losses of up to 90% in the production of soursop by attacking leaves, branches, flowers and fruits, producing black fruit rot, especially during rainy season (Alvarez *et al*., 2005).

In Ghana, information concerning the types of diseases affecting soursop fruits and their causal agents is scanty. However, field observations and market surveys of soursop fruits show evidence of fruit deterioration by microbial agents in Ghana (Asare, 2014). For an effective mitigation of postharvest rot of soursop fruits, research studies on microbial agents associated with these rots is necessary. Therefore, this research work was carried out to determine the major types of disease symptoms and their associated pathogens that are causing deterioration of soursop fruits in Ghana at both the pre and postharvest stages of production.

Materials and Methods

Experimental sites

Sampling of soursop fruits for the experiment was carried out at several locations in the Lower Manya Krobo and Yilo Krobo municipalities of the Eastern region. There were no soursop farms in the survey area, however, a total of 25 soursop trees scattered at random in the area were identified and used in the study. In addition, regular visits were made to markets and roadside grocery shops in the Greater Accra, Eastern and Volta regions to collect diseased soursop fruits from the vendors. Markets visited included Kantamanto and Makola in Accra, Kasoa New market in Central region, Agomanya and Somanya markets in Eastern region and Juapong market in the Volta region. The roadside grocery shops were found at Ashyiyie in the Greater Accra region, Akuse and Trom in the Eastern region. These locations were selected since fruits from all over the country are sent there for marketing.

Pre-harvest determination of disease incidence

A total of 25 fruit-bearing soursop trees were inspected once during their fruiting season in each of the three years of the experiment. Fruits were inspected and the number of fruits showing disease symptoms was recorded. The disease symptom for each year was calculated using the formula:

$$\text{Disease incidence} = \frac{\text{Number of fruits showing particular disease}}{\text{Total number of fruits}} \times 100$$

Post-harvest determination of disease incidence

A total of 293 healthy fruits, made up of 135 harvested from soursop trees (those used in the assessment of disease incidence at the pre-harvesting stage) and 158 obtained from vendors of approximately equal sizes were washed with soapy water, rinsed 3 times with clean water and air dried. They were then packed in cardboard boxes at not more than 10 fruits per box, ensuring that fruits do not sit on each other and incubated in the laboratory at 22 ± 2º C and 65% RH. Fruits were observed on a daily basis until they began to ripen. Disease symptoms observed on fruits were documented and the number of fruits showing symptoms of the different diseases observed was recorded. The incidence of each recorded disease was determined as stated above.

Isolation of causal agents

Microbial agents associated with observed disease symptoms on fruits were isolated on artificial water agar (Agar powder; Oxoid) and potato dextrose agar (PDA; Oxoid) prepared at rates of 20 g/l and 39 g/l respectively. A total of 1005 diseased soursop fruits, made up of 120 diseased fruits harvested from trees in the study area, 592 diseased fruits

collected from grocery sellers and 293 fruits that were initially healthy after harvesting but later developed the disease symptoms, were used in the study. Pieces of the fruit tissues (5 mm x 3 mm) taken from the advancing edge of the lesion at all parts of the fruits (stem end, middle and bottom portions) were surface sterilised with 1% sodium hypochlorite for 15 seconds, washed in 3 changes of sterile distilled water and blotted dry using a paper towel. These were plated singly on water agar in Petri dishes and incubated for 5 days under at a temperature of 22 ± 2º C and relative humidity of 65%. The fungal colonies that grew were then sub-cultured onto PDA until pure cultures were obtained.

Cultural and morphological identification of isolates

Mycelial plugs of each isolate were taken from the periphery of each mycelium on PDA, using an 8 mm cork borer and placed singly in the middle of plates and incubated for 8 days. The nature of growth and colour of the mycelium were recorded to aid in the identification of isolates. Diameter of colony growth was measured daily until the mycelium filled the entire plate and average of mean daily growth was calculated to represent the mycelial growth rate (millimetres per day). Conidial dimensions of isolates were measured under the microscope and means for 20 individual conidia per isolate was calculated (Prihastuti *et al*., 2009). The cultural and morphological characteristics of isolates were used to identify them to the genus level. Ten isolates each belonging to the *Lasiodiplodia* and *Colletotrichum* genera, which were consistently obtained from the two major disease symptoms on the fruits were selected for further studies.

Molecular identification of isolates

DNA extraction and polymerase chain reaction

DNA extraction and polymerase chain reactions were carried out in the Biotechnology Laboratory of the School of Agriculture, University of Ghana. The DNA was extracted from the isolates using the Sigma's GenFlute Plant Genomic DNA Miniprep Kit (St. Louis, MO, USA), following the manufacturer's instructions. The DNA obtained was used as templates in a polymerase chain reaction (PCR). Two sets of PCRs were carried out using the primer pair, CgInt/ITS4, specific to *C. gloeosporioides* (Mills *et al*., 1992) and *L. theobromae* species specific primer Lt347-F (AACGTACCTCTGTTGCTTTGGC) and Lt347-R (TACTACGCTTGAGGGCTGAACA) (Xu *et al*., 2015). The PCR reaction mixture was made up of 2 µl target DNA, 5 µl of 10X PCR buffer (Invitrogen, Carlsbad, CA), 2.5 µl of deoxynucleoside-triphosphate mix (2.5 mM each), 0.25 µl bovine serum albumin (20 mg/ml), 2 µl each of the forward and reverse primer, 1.8 µl of magnesium chloride (50 mM) and 0.2 µl of taq polymerase (Invitrogen, Carlsbad, CA) added to 34.25 µl of double distilled water. Each PCR was performed in a total reaction volume of 50 µl. The reaction was carried out in a Thermo Hybaid PXE Thermal Cycler (Thermo Electron Corporation, USA). The reaction cycles were denatured for 2 min at 94° C followed by 35 cycles of 1min at 94° C, 1 min at 55° C, 2 min at 72° C and a final extension of 10 min at 72° C. Amplification products were separated by 1.5% w/v agarose gel (Invitrogen, Carlsbad, CA), stained with Ethidium bromide or gel red alongside 1.0 kb marker at 100 V for about 1.5 hours. Bands were observed under UV light and Polaroid photographs taken. The presence of the expected PCR bands was a confirmation of the identity of the isolates at the species level.

Pathogenicity test

Pathogenicity of the isolates was tested on physiologically mature but unripe fruits of soursop. Physiologically mature but unripe fruits were washed, surface sterilised with 5%

dilution of household bleach and rinsed 3 times with sterile distilled water and air dried. Two 5 mm deep holes were aseptically punched on one side of each fruit with a 4 mm diameter stainless steel cork borer. On each fruit, one hole was only filled with PDA plug while the other hole was filled with a plug of mycelia of each of the isolated fungi. The 10 isolates each of *L. theobromae* and *C. gloeosporioides* that were selected in the study were used to inoculate the fruits separately. Each isolate was used to inoculate three fruits. The plugs of the fruit removed were replaced and the wounds were sealed with parafilm tape. Inoculated fruits were kept on laboratory benches at 22±2ºC and 65% RH and observed daily for the development of disease symptoms. A fungus was considered the causal agent of the disease when it was able to cause the same symptoms on the inoculated fruits and subsequently being re-isolated from the rotten tissues to complete Koch's postulates.

Results

Description of disease symptoms observed on soursop fruits

Two very distinct disease symptoms were observed on the soursop fruits hanging on trees in the study area. The same types of symptoms were observed after incubation of healthy fruits that were harvested from the same trees. Also, fruits collected from markets and roadside grocery shops exhibited the same symptoms. These disease symptoms were identified as stem end rot and anthracnose.

Stem end rot was characterized by a soft watery rot shown as dark brown patches on the fruit surface (Fig. 1A). These were found to be originating from the stem end portion of the fruit hanging on trees. This type of rot was common on very young fruits, which in most cases were covered entirely by the disease lesion. This disease symptom was also found on fruits collected from vendors and also from the healthy ones that were incubated. In most cases, extensive areas of the fruits were covered and the mycelia of the causative organism accompanied some of the spots. In most cases, it was found that the necrosis remains beneath the cuticle and had penetrated all fruit flesh without showing on the entire fruit pericarp. However, with a slight pressure using the finger, the pericarp breaks opened to reveal extensive rotten innermost parts.

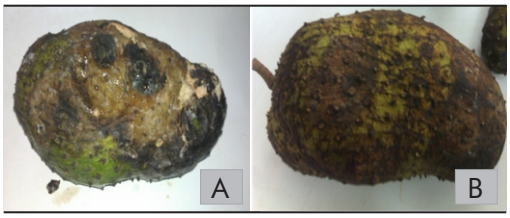

Fig. 1 Nature of disease symptoms observed on soursop fruits collected in the study. A=Stem end rot, B=Anthracnose.

Anthracnose was characterised by dark sunken lesions, the diameter of which ranged from pin-point spots to about 5 mm diameter round spots (Fig. 1B). On the few occasions that the symptom was encountered on the trees, it was found to be developing around wounds created on the infected fruit surface. The symptom was also found on the fruits collected from vendors and after incubation of healthy fruits. In these cases, the lesions were found to be accompanied by numerous acervuli of the causal agent.

Disease incidence of stem end rot and anthracnose at the pre-harvesting stage

The incidence of stem end rot disease ranged from 21.2% in 2018 to 27.6% in 2017. The mean incidence during the period of the experiment was 25.5% (Table 1). Also, during the period, incidence of anthracnose ranged from 10.8% in 2019 to 28.2% in 2018 with a

mean incidence of 21.4% (Table 1).

Incidence of stem end rot and anthracnose of soursop fruits at the postharvest stage.
The disease incidence of stem end rot ranged from 31.6% in 2017 to 34.4 % in 2019 with mean incidence of 33.4% within the period of the experiment (Table 2). On the other hand, anthracnose disease incidence ranged from 65.7% in 2018 to 68.4% in 2017 with mean incidence of 66.5% (Table 2).

Cultural and morphological characteristics of isolates
Isolates of the fungus consistently isolated from the stem end rot symptoms, produced a mycelium which was initially white and turned dark as the culture aged (Fig. 2A). Mycelia grew and filled the entire 9 mm plate in four days, with a mean growth rate of 2.25 mm/day. The hyphae were initially hyaline and turned dark, and were septated. Two types of conidia; mature and immature were observed. The immature conidia were hyaline, aseptate, granular, ovoid and thick-walled (Fig. 2B). The matured ones were uniseptate, brown walled and had longitudinal striations (Fig. 2B). The average size of mature conidium of isolates ranged from 15.5-16.5 x 9.9-10.1 µm. These conidia were produced in dark coloured pycnidia. The cultural and morphological features of the isolates showed they were *Lasiodiplodia theobromae.*

Table 1. Incidence of stem end rot and anthracnose diseases on soursop fruits in the Lower Manya Krobo District in the indicated years

Year	Disease incidence (%)	
	Stem end rot	Anthracnose
2017	27.6	25.3
2018	21.2	28.2
2019	22.7	10.8
Mean	25.5	21.4

Table 2: Incidence of stem end rot and anthracnose diseases on soursop fruits after harvest in the indicated years

Year	Disease incidence (%)	
	Stem end rot	Anthracnose
2017	31.6	68.4
2018	34.3	65.7
2019	34.4	65.6
Mean	33.4	66.5

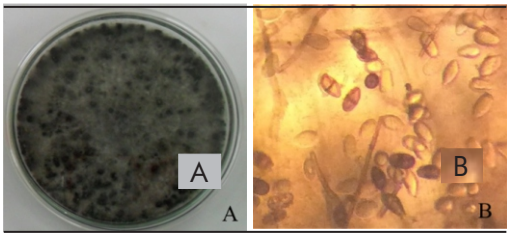

Fig. 2. Cultural and morphological characteristics of *Lasiodiplodia theobromae* consistently isolated from stem end rot lesions on soursop fruits. A=cultural growth on PDA. Note the presence of numerous pycnidia which are the black structures in the culture, B=immature (hyaline and unicellular) and mature (darker and double celled) conidia (Mg X400).

Isolates of the fungus which were consistently isolated from the anthracnose disease symptoms produced mycelia which were initially white but turned to grey is as the culture aged (Fig. 3A). Mycelium grew and filled the entire 9 mm Petri dish in 9 days, with a mean growth rate of 1.1 mm/day. Hyphae were hyaline and septated. Most isolates

produced bright orange coloured acervuli which grew in an annular ring manner in culture. The acervuli contained numerous conidia which were short, with dimensions ranging from 14.8-16 x 5.0-5.5 conical and rounded at the edges (Fig. 3B).

Molecular identification of isolates

The species-specific primer pair CgInt/ITS4 resulted in the amplification of an approximately 480 bp product when DNA from the isolates identified as *C. gloeosporioides* were used as templates in the PCR. The product was absent in the water control (Fig. 4). Also, the use of the species-specific primer pair, Lt347-F/Lt347-R resulted in the amplification of an approximately 347 bp product when DNA from *L. theobromae* isolates were used in the PCR (Fig 5).

Pathogenicity test

All isolates of *L. theobromae* used for the inoculation studies were able to induce the stem end rot disease symptoms on the inoculated fruits. Three days after inoculation, large portions of the fruit around the point of inoculation were found to show light coloured watery portions. By the 5th day after inoculation, the lesion has darkened and the fruit was completely rotten. The disease symptoms were not observed on the fruits inoculated with PDA only. The pathogen was re-isolated from the symptoms to complete Koch's postulates.

Also, isolates of *C. gloeosporioides* were able to induce anthracnose disease symptoms on the artificially inoculated fruits. The symptoms were first seen as dark areas around the points where the wounds were created. This was also seen on the wounds inoculated with the PDA only. However, by the 5th day after inoculation, the lesion around the wounds inoculated with the mycelia of the fungus expanded and was accompanied by the bright orange coloured acervuli of the causal agent. This expansion was not observed in the wounds inoculated with the PDA only. The pathogen was re-isolated from the symptoms to complete Koch's postulates.

Discussion

Prior to this work, crown gall caused by

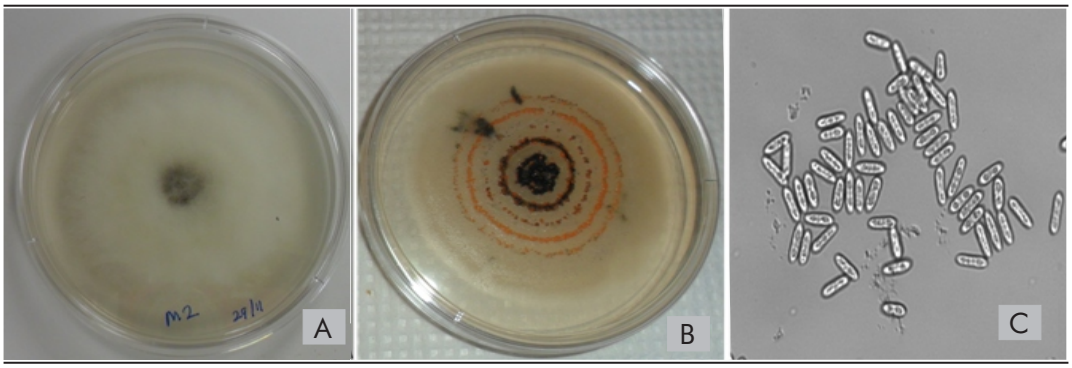

Fig. 3. Cultural and morphological characteristics of *Colletotrichum gloeosporioides* consistently isolated from anthracnose lesions on soursop fruits. A=white mycelium growth on PDA, B=white mycelium accompanied by orange coloured acervuli growing in an annular ring form, B=short conical spores of the fungus (Mg X400).

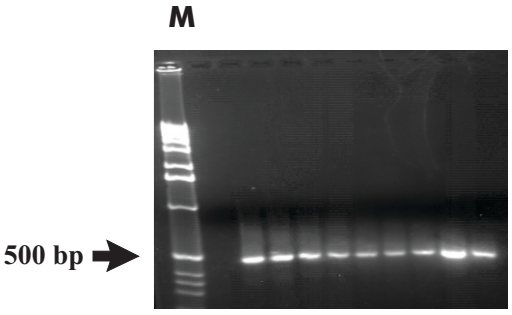

Fig. 4. A gel showing an approximately 480 bp PCR product amplified from DNA from some of the *C. gloeosporioides* obtained from soursop fruits. Lane 1=water control, M=100 bp marker, Lane 2-9= DNA from isolates.

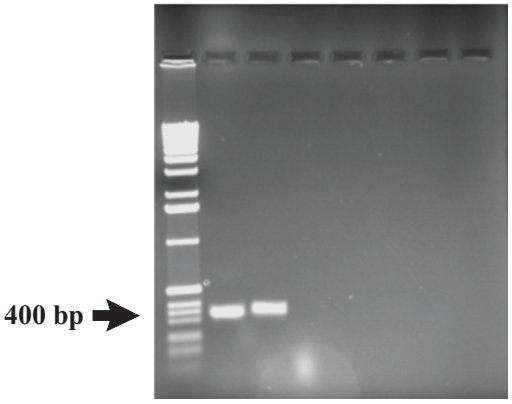

Fig. 5. A gel showing an approximately 347 bp PCR product amplified from DNA of isolates of *L. theobromae* obtained from soursop fruits. Lane 1=100 bp marker, Lane 2 and 3, DNA from two isolates and lane 4=water control.

Calonectria rigidiuscula was the only disease reported on soursop in Ghana (Oduro, 2000). However, few diseases such as tip rot and fruit rot have been reported in other countries (Morton, 1984; Amusa *et al*., 2003). Findings of this work have shown that two major diseases namely, stem end rot caused by *L. theobromae* and anthracnose caused by *C. gloeosporioides* were prevalent on fruits of soursop both at the pre and postharvest stages in Ghana.

Stem end rot is a common disease of tropical fruit crops (Johnson, 1998). In most of these crops, the pathogen has been identified as *L. theobromae*. In this study, the pathogen was consistently isolated from the stem end rot symptoms on the soursop fruits. The characteristic mature and immature conidia of isolates and the amplification of the expected 347 bp PCR product from their DNA conclusively identified them as isolates of *L. theobromae* (Honger *et al.,* 2014; Xu *et al.*, 2015; Phillips *et al.*, 2013). *L. theobromae* is a ubiquitous pathogen and has a wide host range. It has been reported as causing diseases on cocoa, kola and mango in Ghana and stem end rot of papaya in Hawaii (Saeed, 2012; Offei *et al*., 2008; Nishijima, 1993). It has also been reported to be a major cause of soursop fruit deterioration in Nigeria (Amusa *et al.*, 2003). Its ability to induce the disease symptoms on healthy fruits confirms its pathogenicity and hence the type of disease on the fruits. Stem end rot disease on soursop fruits renders infected fruits unsuitable for both marketing and utilization, due to the rapid and massive rots caused to the internal parts of the fruit. In this study, the disease was found to be destructive at the pre-harvesting stage causing about 25.5% losses to fruits on trees while at the postharvest stage, damages due to the disease was more than 30%. Several samples of soursop fruits collected from grocery shops in the study area were infected by the disease. This translates into losses in revenue to the retailers due to the fact that fruits infected by the disease are discarded.

Anthracnose is a common disease of most sub-tropical to tropical fruits. In most of the tropical areas, the disease on fruit crops such as mango, pawpaw and avocado have been

ascribed to *C. gloeosporioides* (Honger *et al*., 2016; Agrios, 2005). The pathogen was isolated from the anthracnose disease lesions on soursop fruits in this study and was identified based on its characteristic short rod spores that are rounded at the edges. Its identification was further confirmed by the amplification of the expected 480 bp PCR product from the DNA of the isolates (Honger *et al*., 2014; Agrios, 2005). The fungus was able to induce the anthracnose disease symptoms on inoculated fruits in this study and has been associated with anthracnose disease on soursop fruit in major producing countries (Alvarez *et al*., 2005; Amusa *et al.,* 2003).

Anthracnose was found to be an important threat to both the production and marketing of soursop fruits. In the field, the disease incidence of more than 21% means a significant proportion of the fruits produced would not be sold because there are not many soursop trees currently under cultivation in the study area. Also, about 66.5% of the healthy fruits incubated showed the disease symptoms. This means a large amount of the fruits which will be sent to the markets will have their aesthetic value and hence their marketability and price, reduced, if not sold on time. This finding corroborates reports that the disease could cause as much as 90% loss in the production of the crop (Alvarez *et al*., 2005). Therefore, anthracnose disease on soursop is likely to generate the same problems it causes in the production and marketing of mango fruits worldwide (Arauz, 2000) and therefore, deserves much pathological attention.

Out of a total of 1,005 fruits collected in this study, none was free from the two diseases identified. The high disease incidence could be attributed to the absence of control measures against the diseases both on the field and post harvest in Ghana. Soursop fruits found in grocery shops are harvested from few neglected trees found in people's backyards. Interactions with some farmers in the study area revealed that most of them consider the production of the crop for local markets as a non-profitable venture, since markets for the exports of the crop have not been developed. They also considered fruit crops such as local varieties of pawpaw, mango and avocado and soursop as tree crops that do not need any special treatment to thrive. However, judging from the large quantities of the soursop fruits flooding the grocery markets, lately, the local marketing could be very profitable, not excepting the future export potential, if the trees are given the much-needed attention.

Conclusion

Two major diseases namely; stem end rot and anthracnose caused by *L. theobromae* and *C. gloeosporioides* respectively were found causing destruction of soursop fruits collected in the Coastal savannah zone of Ghana. Both diseases cause reduction in the aesthetic value of the fruits and hence their marketability. The disease incidence was found to be high especially at the postharvest stage, which could be attributed to the absence of disease control measures at both the pre and post harvesting stages of the crop which has predisposed the fruits to rapid microbial degradation. The information obtained in this study would be very useful for future studies aimed at improving the shelf life of the soursop fruits and improving the profitability of any future soursop enterprise in Ghana.

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