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Biomolecular characterization, identification, enzyme activities of molds and physiological changes in sweet potatoes (*Ipomea batatas*) stored under controlled atmospheric conditions

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Abstract: Microbial attacks during storage are one of the primary causes of product deterioration, and can limit the process of prolonging the shelf-life of harvested food. In this study, sweet potatoes were stored at temperatures of 13, 21, and 29 °C for 4 weeks. Samples were collected during storage and plated on potato dextrose agar, from which axenic mold cultures were obtained and identified using 26S rRNA gene sequences. Physiological changes of potato tubers were assessed with respect to pathogenicity, enzyme activity, and atmospheric storage conditions. Six fungal species were identified, namely *Penicillium chrysogenum* (*P. rubens*), *P. brevicompactum*, *Mucor circinelloides*, *Cladosporium cladosporiodes*, *P. expansum*, and *P. crustosum.* The following fungal isolates, namely *P. expansum*, *P. brevicompactum*, and *Rhizopus oryzae*, were recovered from the re-infected samples and selected according to their levels of enzyme activity. This study revealed high levels of activity for cellulase and pectinase, which were most notable during the initial three days of testing, and were followed by a steady decrease (P<0.05). Polygalacturonase activity was prominent with values ranging from 12.64 to 56.79 U/mg (*P. expansum*) and 18.36 to 79.01 U/mg (*P. brevicompactum*). Spoilage was obvious in the control group, which had a 100% decay at the end of the experimental period compared with samples treated with iprodione and sodium hypochlorite, in which the decay rates were 5% and 55%, respectively. The data for the iprodione- and sodium hypochlorite-treated samples at the end of the 3-month storage period showed that they were significantly different (P=0.041), with the sodium hypochlorite-treated samples producing twice the rate of infection compared to the iprodione-treated samples. The comparative rate of the progression of decay in the treated samples can be expressed as iprodione<sodium hypochlorite<control. This study demonstrates that sweet potato tissue damage is due to the activities of microbial enzymes and, in particular, the pectinases of the organisms isolated from the infected potato tissues, and suggests the advantages of utilizing iprodione as a curing agent for potato tubers before storage.

Key words: Controlled atmosphere, Enzyme activity, Molds characterization, Potato spoilage, Sodium hypochlorite, Iprodione

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1 Introduction

Sweet potatoes (*Ipomea batatas* Lam.) are grown in over one hundred different countries in tropical and sub-tropical regions. The production of sweet potatoes across the world is estimated to be

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approximately 140 903 000 t/a and 92% of these are reportedly produced in Asia and the Pacific Islands (FAO, 2002). According to Horton *et al.* (1989) and Dayal *et al.* (1991), sweet potatoes are the world's seventh most cultivated food crop. The tubers vary greatly in size, shape, color, and quality of taste, depending on the variety. When the roots are stored they are sensitive to changes in soil temperature, which is largely dependent on their stage of root development (Williams *et al*., 1980). Storage of sweet potato tubers after harvest is imperative, as this practice may prevent a surfeit of potatoes entering the food markets at any given time and prolong the period of fresh tuber availability, especially when the crop is not in season or when the economic circumstances and/or regional climates in a particular area of production dictate its production during the year. Ali *et al*. (1991) have emphasized the effect of seasonal production, especially during the winter period, in countries such as India and Bangladesh on the reduction of sweet potato prices. The authors attributed price reduction to the enormous supply of different varieties of edible vegetables, which periodically deprives farmers of an adequate monetary return on their potato crops. Onwueme and Charles (1994) and Hall (1993) postulated divergent cropping methods and increased crop cultivation as a necessity to facilitate the required long-term storage of roots, thereby meeting world food requirements during intervening seasons. Rapid deterioration in the condition of the produce is a result of exposure to environmental conditions and is exacerbated by mechanical damage during handling and transport (Rees *et al.*, 2003). Sweet potatoes can lose up to 1–2 months of storage time because of wilting and root rot, which can affect 15% to 65% of the crop (Ray and Balagopalan, 1997). Sweet potatoes are affected by a number of diseases, both fungal and bacterial (Clark, 1992). Fungal diseases which can afflict sweet potatoes include: surface rot and root rot caused by various species of the genus *Fusarium*; soft rot caused by the fungus *Rhizopus stolonifer*; Java black rot caused by the fungus *Diplodia gossypina*; scurf disease caused by the fungus *Monilochaetes infuscans*; black rot of the tuber which is caused by the species *Ceratocystis fimbriata*, among others. The activities of the spoilage-causing organisms are usually supported by microbial enzymes, which are secreted into the sweet potato tubers. These enzymes constitute the main agents of deterioration, and it is also worth noting that enzymes of microbial origin have been exploited in food and medical research, paper and textile industries, as well as in waste utilization and biotechnologies (Lim *et al.*, 1985). The ability of many plant pathogens to produce these enzymes in a culture is not a sufficient reason for ascribing to them a role in pathogenicity (Byrde, 1979). Nevertheless, the study of enzyme secretion in vitro can be an indication of the types of enzymes that a pathogen is capable of producing in relation to infection and disease. The progress of any plant infection depends upon the growth potential and enzymatic production capabilities of the associated microorganism, in addition to the physiological status of the tissues that are infected. The shelf-life of sweet potato roots varies from a few days to a few weeks according to the cultivar, as well as to the conditions prevailing at the time of harvest and during storage (Kurup and Balagopalan, 1991; Acedo *et al*., 1996; Cabanilla, 1996; Rees *et al*., 1998; Mtunda *et al*., 2001). The short shelf-life of sweet potato tubers has led to many countries across the world to avoid storage of the crop, including Ethiopia (Doku, 1989), Indonesia (Jusuf *et al*., 1997), Kenya (Doku, 1989), Tanzania (Mtunda *et al*., 2001; Rees *et al*., 2001), Uganda (Rees *et al*., 1998), and Papua New Guinea (Bourke, 1982) where as an alternative larger potato roots are harvested from individual plants, leaving smaller tubers to increase in size before being subsequently harvested when needed (Karuri and Ojijo, 1994). Consequently, improved storage methods which decrease losses without resulting in undesirable raw product quality changes are of considerable interest. When potato tubers are not properly cured and well packed or when the storage conditions are inadequate, it may result in tuber injury, skinning, dehydration, and microbial attack, all factors that can lead to substantial post-harvest losses (Aidoo, 1993; Jusuf *et al*., 1997; Mtunda *et al*., 2001; Rees *et al*., 2001). Ray and Ravi (2005) reported that the loss of weight in sweet potatoes after 113 d of storage was 17% in the cured samples, in which the tubers were kept in storage at 29–30 °C and relative humidity of 92%, compared with 42% for uncured tubers. By controlling the atmosphere, where a steady regulated environment consisting of a special blend of oxygen, nitrogen, and carbon dioxide is monitored and maintained, the shelf-life of certain products can be extended in storage environments. When the oxygen or carbon

dioxide concentration of the storage atmosphere around a food material is reduced or increased, respectively, this could precipitate an increase in the rate of respiration of fresh crops such as fruits and vegetables, and will ideally inhibit the growth of spoilage microorganisms (Fellows, 2000). Many methods have been used in the post-harvest preservation of sweet potatoes with the singular aim of preventing deterioration and thereby prolonging shelf-life. These methods include, but are not restricted to, curing under controlled temperature and relative humidity, the use of disinfectants and cold storage. These methods are often used in isolation or in limited combinations in order to impede the progress of the spoilage agents. However, it is evident that these approaches have not achieved the anticipated or desired results in terms of preventing post-harvest spoilage of sweet potatoes or prolonging their shelf-life. In an attempt to suggest an alternative approach, this study investigates the activities of enzymes produced by filamentous fungi that have been isolated from infected sweet potato tubers. This study also examines the combination of curing, disinfection, low temperature (8 °C), relative humidity (92%) with the use of a modified atmosphere containing low oxygen (1%), high carbon dioxide (20%), and assesses the performance of this regime on the storage of sweet potatoes over a period of 3 months. The results obtained from these studies were encouraging, showing a low percentage of spoilage of the crop. In particular, samples disinfected with 0.1% (1 g/L) iprodione were better maintained when compared with those disinfected with 20% sodium hypochlorite solution or left untreated.

2 Materials and methods

Four kilogrammes of fresh healthy sweet potato tubers were purchased from the Oja-ale Market in Offa Town, Oyun Local Government Area, Kwara State, Nigeria. The potato samples were packaged in sterile plastic containers for further analysis.

2.1 Sample storage and isolation of fungi

The potato samples were divided into 4-L capacity sterile plastic sample containers and then stored at three different temperatures, 13, 21, and 29 °C, for a 4-week duration. After each of the sample groups had been stored for 7 d, samples were taken from the incubators for microbiological analysis. Two sets of serial dilutions (10^{-1}–10^{-9}) were prepared using both the surface peel and the deep tissue cored from the samples. Potato dextrose agar (PDA; Oxoid, UK) was used to isolate the filamentous fungi. The medium was prepared according to the manufacturer's specifications.

2.2 Estimation of total microbial counts

The microbial loads of surface peels and deep tissue samples were estimated using tryptone soy agar (TSA) for bacteria and PDA for fungi. Two sets of serial dilutions (10^{-1}–10^{-9}) were prepared using both the surface peel and the deep tissue cored from the samples and then plated using the spread plate method on TSA and PDA in three replicates. The TSA plates were incubated at 37 °C for 24 h while the PDA plates were incubated at 25 °C for 1–3 d. At the end of the incubation periods, total viable aerobic counts were estimated using a colony counter and expressed in colony forming units (cfu/g). From the mixed cultures, sub-culturing was repeated until distinct axenic mold cultures were obtained.

2.3 Extraction of filamentous fungal genomic DNA

Filamentous fungal genomic DNA extraction was carried out as described by Hautala *et al.* (1977). Fungal mycelial mats were grown in Sabouraud dextrose broth supplemented with 2% (0.02 g/ml) glucose using a shaking incubator at 25 °C for a period of 5 d. Afterwards, they were harvested using vacuum filtration. Harvested mycelial mats were washed in 0.9% (9 g/L) sodium chloride solution three times, frozen in liquid nitrogen, and freeze-dried overnight in a freeze dryer. The freeze-dried mats were pulverized with a glass rod until a fine powder was obtained. A 2 ml volume of extraction buffer (250 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 8.0, 50 mmol/L NaCl, 0.5% (5 g/L) of Triton X-100, 0.25 μg/ml Pronase) was added to 50 mg of the powder. This was incubated in a shaking incubator at 37 °C for a period of 3 d. After incubation, the solution was centrifuged at 8000 r/min for 15 min in a microfuge and thereafter the supernatant was decanted into a tube while the pellet was discarded. The supernatant was precipitated with two volumes of absolute ethanol and left at room temperature for 15 min before subsequently being centrifuged at

8000 r/min for another 15 min. The pellet obtained from the sample was re-suspended in 500 μl of salt buffer (1 mmol/L NaCl, 1 mmol/L Tris-HCl pH 7.4, 0.1 mmol/L Na_2EDTA, 3 ml ethanolic perchlorate reagent: 140 g $NaClO_4{\cdot}H_2O$, 381 ml absolute ethanol, 36 ml distilled water), which was then mixed through inversion. It was then centrifuged again at 13 000 r/min for 3 min to pellet the DNA. The DNA was drained and re-suspended in 500 μl of low salt buffer plus 250 μl of high salt buffer (0.5 mol/L NaCl, 25 mmol/L Tris-HCl pH 7.4, 2 mmol/L EDTA) and 300 μg/ml pre-boiled RNaseA. This was incubated at 37 °C for 3 h. After incubation, 750 μl of chloroform and isoamyl alcohol in the ratio of 24:1 (v/v) was added and mixed on a windmill rotator for 2–24 h. It was then centrifuged at 13 000 r/min for 5 min. The upper phase was carefully removed and transferred to an Eppendorf containing 450 μl of isopropanol. These were mixed by inversion and left at −70 °C for 30 min in order to precipitate. Centrifugation was carried out again at 13 000 r/min for 5 min, after which the sample was re-suspended in 200 μl of salt buffer solution. To this 600 μl of 100% ethanol was added to re-precipitate at ambient temperature for 30 min. Any fibrous DNA which appeared was removed and re-suspended in a salt buffer solution. The remaining DNA was pelleted by centrifugation at 13 000 r/min for 6 min. This was then re-suspended in a low salt buffer solution.

2.4 Amplification of fungal genomic DNA by PCR

Polymerase chain reactions (PCR) were carried out according to the methods described by Borneman and Hartin (2000) and Cocolin *et al.* (2002). A master mix of 50 μl (final volume) was prepared as follows: 4 μl 2.5 mmol/L $MgCl_2$, 2 μl 0.25 mmol/L dNTPs, 5 μl 10× PCR buffer, 4 μl forward primer NL1 (100 pmol/μl or 0.1 mmol/L stock: 5'-GGCCATAT CAATAAGCGGAGGAAAAG-3') and 4 μl reverse primer LS2 (100 pmol/μl or 0.1 mmol/L stock: 5'-ATT CCCAAACAACTCGACTC-3') (Cocolin *et al*., 2002), 10 μl genomic DNA template, 2 μl 5 U Taq polymerase (Sigma, UK), and 4 μl sterile reverse osmosis (RO) water. The reagents were combined and heated in a thermal cycler at 94 °C for 2 min. Thirty-five cycles of PCR were then performed using a regime of 94 °C over a period of 10 s, 56 °C for 10 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 2 min. From this solution, 5 μl of the PCR products were electrophoresed in a 1% (0.01 g/ml) agarose gel for 3 h. The primers amplify the D1 region of the 26S ribosomal DNA (rDNA).

2.5 Sequencing and identification of purified filamentous fungal DNA-PCR products

Aliquots of 20 μl for each of the purified PCR products amplified from fungal DNA were transferred into PCR tubes labeled with the appropriate bar codes. These were then sent to MWG Biotech in Germany for DNA sequencing using the amplification primers. Subsequently, the nucleotide sequence of each isolate was matched with existing sequence databases using the basic local alignment search tool (BLAST) to identify the isolates based on query coverage and maximum identity in the databases. Identification was further confirmed using DISTANCE TREE matching.

2.6 Infection of sweet potato tubers

The ability of the fungal isolates to establish infection (pathogenicity) and cause spoilage was determined according to the methods of Ekundayo and Daniels (1973) and Ikediagwu and Ejale (1980) inclusive of modifications outlined below. Fresh healthy sweet potato tubers were surface-sterilized in 10% sodium hypochlorite solution for 1 h. They were then rinsed with six changes of sterile RO water to remove the residual effects of sodium hypochlorite. A sterile 5-mm cork borer was used to remove cylindrical cores (1 cm) from the middle portion of the tuber. Each well was inoculated with 100 μl of each of the inoculums (10^{-4}–10^{-9}). The cores were immediately replaced and sealed off with sterile petroleum jelly. Some cores were inoculated with 100 μl maximum recovery diluent (MRD) to serve as control samples. Alternatively, tubers were cut into slices and either surface-sterilized, rinsed with six changes of sterile RO water, and inoculated with each of the isolates or inoculated with the isolates without surface sterilization. After inoculation, the tubers and the slices were distributed into sterile plastic containers and sterile Petri dishes, respectively, and incubated at 13, 21, and 29 °C. The sweet potato tubers and slices were inspected weekly and photographic evidence was recorded. Two of the filamentous fungi, *Penicillium expansum* (FSP11) and *P. brevicompatum* (FDC3),

were selected for further investigations based on their clear ability to spoil the tubers.

2.7 Recovery and confirmation of pathogenic fungal isolates

To confirm that the damage observed was caused by the action of the pathogenic fungal isolates, samples were collected from the sites of infection. Inoculums were prepared and plated on PDA plates. The plates were then incubated at 25 °C for 3 d. Sub-culturing was carried out until pure isolates were obtained. Using the molecular methods previously outlined (Hautala *et al*., 1977), DNA was extracted from these isolates and amplified with 26S primers. PCR products were purified and sequenced using the MWG Biotech service. The nucleotide sequences of the isolates exhibited 100% similarity to pre-existing DNA sequences and their relationships to database sequences were identified using the BLAST and DISTANCE TREE matching.

2.8 Measurement of enzyme activity

To establish if the selected spoilage fungal isolates have the capacity to secret plant cell wall degrading enzymes on sweet potatoes, the isolates were grown in a Sabouraud broth medium supplemented with 2% (0.02 g/ml) mashed sweet potato over a period of 9 d. Using culture supernatants from each of the organisms, the activities of nine different enzymes were studied. The enzymes analyzed included cellulose, amylase, xylanase, polygalacturonase, xylosidase, arabinofuranosidase, and ferulic acid esterase using the modified method of Bernfeld (1955). The substrates used for the respective enzymes were carboxy-methyl cellulose (Acros Organics, UK), starch (Fisher Scientific, UK), birchwood xylan (Sigma Adrich, UK), *p*-nitrophenol xylopyranosidase (Fisher Scientific, UK), and methyl ferulate (Sigma Aldrich, UK). Potassium hydrogen phosphate (1 mol/L, pH 6.5) was used as a buffer. Each of the selected filamentous fungal isolates was first grown in a Sabouraud dextrose medium supplemented with 2% (0.02 g/ml) glucose for 3–5 d in a shaking incubator at 25 °C. The mycelia were then harvested using a sterile vacuum filtration unit. The harvested mycelia were then transferred into a fresh Sabouraud medium supplemented with 2% (0.02 g/ml) mashed sweet potato and grown in a shaking incubator for 9 d at 25 °C. On a daily basis, aliquots of the culture medium were collected and centrifuged at 13 000 r/min for 5 min. The supernatant was carefully withdrawn while the sediment was discarded. The supernatant served as a crude secreted enzyme extract. A similar volume of the supernatant was boiled in a water bath for 10–20 min before being used as a control sample. Two millilitres of the crude extract was mixed with 1 ml of 1% (0.01 g/ml) substrate solution and incubated at 40 °C for 1 h. The reaction was halted by the addition of 3 ml dinitrosalicylic acid (DNSA) reagent. The mixture was cooled in cold water and then diluted with 18 ml water and the absorbance of the resultant solution was measured at 550 nm. The control was subjected to similar treatment. The quantity of the reducing sugars formed was calculated from a standard curve prepared with known concentrations of substrates. Enzyme units (U) are recorded as the release of 1 μmol of product per minute.

2.9 Measurement of proteins

Protein standard curves were prepared according to the method of Bradford (1976). A 20-μl aliquot of each of the bovine serum albumin (BSA) protein standards (BIO-RAD, USA) was transferred into a clean Eppendorf tube. One milliliter of the 1× dye reagent was added to each tube and mixed by inversion. The mixture was incubated at room temperature for 5 min. After incubation, the absorbance of each of the standards was measured with a spectrophotometer at 595 nm and a standard BSA calibration curve was plotted.

2.10 Pre-storage treatments of sweet potato samples

Organic sweet potato samples were purchased from the TESCO stores in Loughborough, Leicestershire in the United Kingdom and brought to the laboratory before being transferred into storage containers under aseptic conditions. They were partitioned into groups A, B, and control. Sixty potato tubers were divided into three replicates with each replicate containing 20 tubers. The group labeled sample A was treated according to the methods of Afek *et al*. (1998) and Afek and Orenstein (2003) with the following modifications: the sample was cured in a storage incubator (29 °C and relative humidity 92%) for 1 week. This procedure was followed by surface sterilization in 0.1% (1 g/L) iprodione for

5 s and then stored for 3 months at 8 °C under a controlled atmosphere (oxygen 1%, carbon dioxide 20%, and relative humidity 92%).

Another 60 potato tubers were divided into three replicates with each replicate containing 20 tubers and labeled sample B. This sample was cured in a storage incubator (29 °C and relative humidity 92%) for 1 week. The curing process was followed by surface sterilization in 20% sodium hypochlorite solution for 5 min and then stored for 3 months in a controlled atmosphere (8 °C, oxygen 1%, carbon dioxide 20%, relative humidity 92%).

Sixty uncured and non-disinfected potato tubers were, again, divided into three replicates with each replicate containing 20 potato tubers and used as controls for the treated samples A and B. The treated samples and the control were put in sterile boxes before being placed in the incubator. At the end of each month, samples were photographed to record evidence of spoilage or lack thereof. At the end of the 3-month storage period, the percentages of spoilage in both samples A and B were calculated by comparing the number of spoiled samples with the number of unspoiled samples in the treatments and the control. Microbiological analyses were also carried out on the spoiled samples to enumerate the spoilage organisms.

2.11 Storage of treated sweet potato samples under a controlled atmosphere

The aim of this study was to determine which of the two disinfectants (0.1% (1 g/L) iprodione (Fisher Scientific, UK) or 20% sodium hypochlorite solution) in conjunction with curing would best prevent the decay of stored sweet potato samples. The first experiment (sample A) involved curing and dipping the samples in the iprodione solution before storage in the KBF240 Binder incubator set at 8 °C and 92% relative humidity for a period of 3 months under a controlled atmosphere. A gas mixture of 1% oxygen and 20% carbon dioxide balanced with nitrogen flowed continuously throughout the storage area during this period. The second experiment (sample B) was similar to the first, except that a 20% sodium hypochlorite solution was used instead of iprodione. Uncured and untreated samples (sample C) were used as the control. In each case, the samples were put in perforated plastic boxes before being put in the storage incubator.

2.12 Estimation of percentage decay

At the end of each month of storage, the number of spoiled samples in each batch was determined. This was done by careful and aseptic examination of each batch and by identifying spoiled samples through color change and microbial growth. Spoiled samples were removed and the number recorded. Percentage decay in each treatment was calculated in relation to the original number of potato tubers used.

2.13 Statistical analysis

The data collected were subjected to a two-factor analysis of variance (ANOVA) as described in the Design Expert, Version 6.0.11 from Stat-Ease Minneapolis software. A significant difference was accepted at 5% probability by SAS V8.3 (SAS Institute Inc., Cary, NC, USA).

3 Results

The total fungal counts of the surface peels and deep tissue cuts from the sweet potatoes stored at different temperatures are presented in Fig. 1. The results show the presence of more countable fungal colonies on the surface of the tubers than in the deep tissue. The surface and deep tissue counts increased over the first two-month period at all temperatures, except that the deep tissue cut at 13 °C decreased after 2 weeks, before becoming static towards the end of the storage period. Similarly, after 2 weeks of storage, the total counts (9.6 $\log_{10}$ cfu/g) of deep tissue cuts at 21 °C indicated a decrease when compared with the values of the 5.8 $\log_{10}$ cfu/g at 4 weeks. Conversely, there were consistent increases in the total viable counts of deep tissue cuts at 29 °C (5.5–7.2 $\log_{10}$ cfu/g) and surface peels at 29 °C (8.7–11.0 $\log_{10}$ cfu/g) from the onset of the experiment until its termination after 4-week storage.

An exception to the general trend was a significant increase noted in Week 2 for the sweet potatoes stored at 21 °C. Fungal counts on the PDA at 13 °C ranged between 10.4 and 8.9 $\log_{10}$ cfu/g on the surface peels compared with the 6.5 to 7.4 $\log_{10}$ cfu/g in the deep tissues. The total fungal counts at 21 °C ranged from 8.9 to 9.9 $\log_{10}$ cfu/g on the surface peels compared with 5.6 to 9.6 $\log_{10}$ cfu/g in the deep tissues.

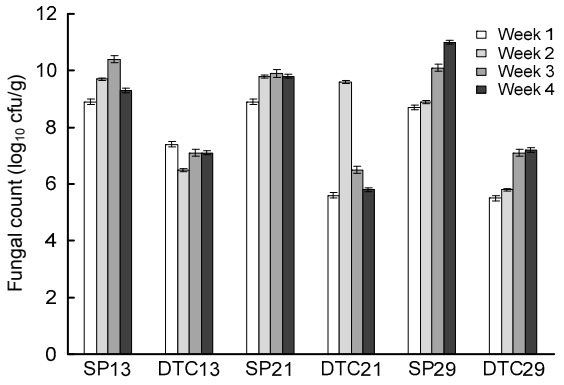

Fig. 1 Fungal counts of surface peel and deep tissue cuts of sweet potato tubers on potato dextrose agar after storage at different temperatures

SP13: surface peel at 13 °C; DTC13: deep tissue cut at 13 °C; SP21: surface peel at 21 °C; DTC21: deep tissue cut at 21 °C; SP29: surface peel at 29 °C; DTC29: deep tissue cut at 29 °C

At 29 °C the fungal counts were 8.7–11.0 $\log_{10}$ cfu/g on the surface peels and 5.5–7.2 $\log_{10}$ cfu/g in the deep tissues. The surface peel counts at 29 °C increased throughout the period with a maximum count of 11.0 $\log_{10}$ cfu/g by the end of Week 4. Ray and Byju (2003) reported substantial numbers of isolated organisms associated with the surface peels of sweet potato tubers.

A total number of 13 fungal isolates were obtained and sequenced. All of the isolates belong to seven species of fungi based on the query coverage and maximum identity (98%–100%) in the databases as presented in Table 1, and include *P. chrysogenum* (FDC22A) recently renamed *P. rubens*, *Mucor circinelloides* (FDC2), *P. expansum* (FSP11), *Cladosporium cladosporioides* (FDC1A), *P. brevicompactum* (FDC3), *P. crustosum* (FSP14A), and *R. oryzae* (FDC1B). The pathogenicity capacity of the isolated molds on sweet potato tubers is illustrated in Figs. 2 and 3. The majority of the fungal isolates showed clear evidence of infection in the sliced and whole potato tubers when the site of the inoculation was compared with the control site. Three of the isolated molds, namely *P. expansum*, *P. brevicompactum*, and *R. oryzae*, were recovered with maximum identities of between 99% and 100% from the re-infected sweet potato samples. The enzyme activity of *P. expansum* (FSP11) is presented in Fig. 4. Polygalacturonase activity was predominant in the cultures of *P. expansum* (FSP11) with values ranging from 56.79 U/mg (Day 1) to 12.64 U/mg (Day 9). The

Table 1 Molds identified from the sequences obtained and from the subsequent BLAST and DISTANCE TREE matching

Isolate	Maximum identity (%)	Organism identified
FDC22A	99	*Penicillium chrysogenum*
FDC2	99	*Mucor circinelloides*
FSP11	100	*P. expansum*
FS5	99	*P. expansum*
FDC1A	100	*Cladosporium cladosporioides*
FDC3	99	*P. brevicompactum*
FSP14A	99	*P. crustosum*
FDC1B	98	*Rhizopus oryzae*

F: filamentous fungi; SP: surface peel; DC: deep tissue cut

ranges of values obtained for the activities of other enzymes were: cellulase 0.78–22.68 U/mg, amylase 0.30–8.59 U/mg, xylanase 1.42–27.79 U/mg, glucanase 0.59–10.54 U/mg, xylosidase 8.81–16.97 U/mg, arabinofuranosidase 10.53–24.39 U/mg and ferulic acid esterase 1.37–4.01 U/mg. The organisms demonstrated optimum activity for most of the enzymes assayed within the first three days of incubation. Thereafter, there was a steady decrease ($P<0.05$) in activity up to the last day (Day 9) when the experiment was terminated. The enzyme activity of *P. brevicompactum* (FDC3) on the Sabouraud dextrose medium is indicated in Fig. 5. The fungi demonstrated the highest activity for secreted polygalacturonase (18.36–79.01 U/mg) and the lowest activity for secreted glucanase (0.91–9.73 U/mg). The ranges of activities obtained for the other enzymes were: cellulase 0.83–36.69 U/mg, amylase 0.50–12.93 U/mg, xylanase 2.21–54.57 U/mg, xylosidase 12.05–25.79 U/mg, arabinofuranosidase 15.87–37.39 U/mg, and ferulic acid esterase 1.71–4.44 U/mg. Optimum activities of the enzymes produced by the fungal species were mostly demonstrated within the earlier part of the experiment.

The results of pre-storage treatments of the sweet potato samples at the end of the three-month storage period are presented in Table 2. There was an increase in the percentage of infected tubers in the three samples including the control with time ($P=0.0088$) as storage progressed.

Fig. 6 shows the appearance of the sweet potato samples after storage in a controlled atmosphere for 1 month (Fig. 6a), 2 months (Fig. 6b), and 3 months (Fig. 6c), all at 8 °C, relative humidity 92%, 1% oxygen,

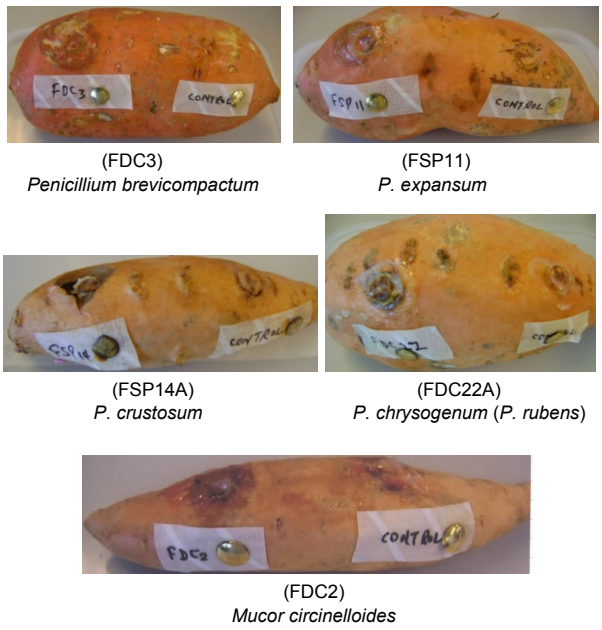

Fig. 2 Typical pathogenicity test with fungal isolates (molds) on sweet potato tubers
F: filamentous fungi; SP: surface peel; DC: deep tissue cut

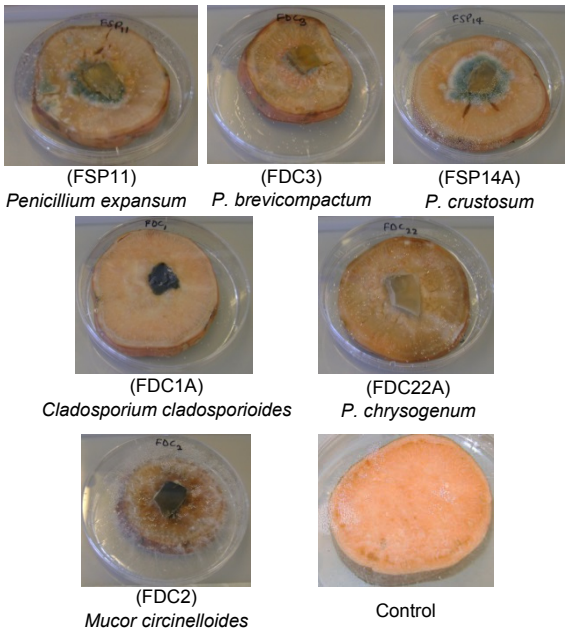

Fig. 3 Typical pathogenicity test with fungal isolates on sweet potato slices
F: filamentous fungi; SP: surface peel; DC: deep tissue cut

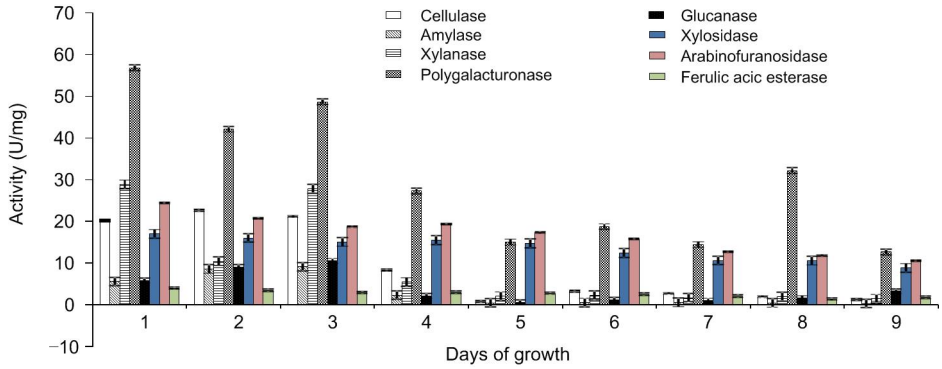

Fig. 4 Enzyme activities detected in culture supernatants of *Penicillium expansum* (FSP11) grown in Sabouraud dextrose medium supplemented with 2% (0.02 g/ml) mashed sweet potato as a function of the incubation time (1–9 d)

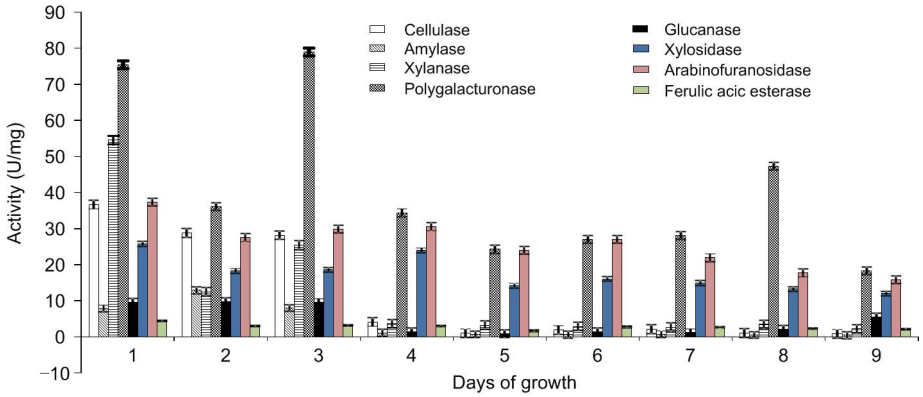

Fig. 5 Enzyme activities detected in culture supernatants of *Penicillium brevicompactum* (FDC3) grown in Sabouraud dextrose medium containing 2% (0.02 g/ml) mashed sweet potato as a function of the incubation time (1–9 d)

and 20% carbon dioxide, which is balanced with nitrogen. Spoilage of stored samples was prominent in the control, which recorded 100% decay at the end of 3 months when the experiment was terminated. The spoilage values obtained from other treatments after storage were 5% (iprodione) and 55% (sodium hypochlorite), respectively. Statistical analysis of the infection data presented in Table 2 confirmed that the various treatments affected the level of infection (P=0.041) with the control showing the highest level of infection and the iprodione samples the least. Two-factor ANOVA of the data for the iprodione- and sodium hypochlorite-treated samples showed that these were significantly different with respect to treatment (P=0.031) and time of incubation (P=0.015). The sodium hypochlorite-treated samples yielded twice the rate of infection in comparison to the iprodione-treated samples by the end of the 3-month storage period. The total viable aerobic bacterial and fungal counts in the stored samples from both the surface peels and the deep tissue cuts of the samples are presented in Table 3. Estimates of the total viable counts carried out at the end of the 3-month storage period revealed higher fungal counts than bacterial counts on average, particularly in the deep tissue cuts as indicated in Table 3. Iprodione was very effective at reducing the number of viable bacteria but not the number of viable fungi. Sodium hypochlorite was ineffective in reducing the number of viable bacteria or the number of viable fungi. At the end of the storage period, visual fungal growth was evident in sample B and the control but was negligible in sample A, where little or no evidence of bacterial growth was observed. Analysis of the viable aerobic

Table 2 Percentage decay of sweet potato tubers after different treatments during 3-month storage (8 °C, 92% relative humidity, 1% oxygen and 20% carbon dioxide balanced with nitrogen)

Treatment	Quantity in storage	Cumulative quantity spoiled per month			Cumulative percentage spoiled per month (%)		
		1st	2nd	3rd	1st	2nd	3rd
A	60	0	3	15	0	5	25
B	60	6	18	33	10	30	55
C	60	12	24	60	20	40	100

A: cured+iprodione; B: cured+sodium hypochlorite; C: uncured and untreated (control)

Fig. 6 Sweet potato samples after storage under a controlled atmosphere for 1 (a), 2 (b), and 3 (c) months at 8 °C, 92% relative humidity, 1% oxygen and 20% carbon dioxide balanced with N_2

counts showed statistically significant differences for bacteria and fungi (P<0.001). The progression can be expressed as: iprodione-treated samples<sodium hypochlorite-treated samples<control in both the surface peels and the deep tissue cuts.

Table 3 Total viable aerobic counts from surface peel (SP) and deep tissue cut (DTC) of stored sweet potato samples

Organism	Sample	Total viable counts ($\log_{10}$ cfu/g)	
		SP	DTC
Bacteria	A	7.8 (±0.044)*	0.0 (No growth)
	B	9.0 (±0.020)	8.7 (±0.043)
	C	9.2 (±0.005)	9.3 (±0.061)
Fungi	A	7.7 (±0.036)	7.8 (±0.043)
	B	9.1 (±0.007)	9.2 (±0.009)
	C	10.2 (±0.032)	10.3 (±0.030)

A: cured+iprodione; B: cured+sodium hypochlorite; C: uncured and untreated (control). * ±SD (n=3)

4 Discussion

The presence of fungi was recorded both on the surface peels and in the deep tissues of the stored sweet potato tubers. This observation supports the findings of Clark (1992), Clark *et al*. (1992), and Ray and Balagopalan (1997) who similarly reported diverse groups of fungi as the primary spoilage agents of food products in storage. Some fungal genera, which are often linked with post-harvest deterioration of sweet potato tubers, include *Penicillium*, *Aspergillus* and *Rhizopus*. Some authors have reported that *R. oryzae*, one of the fungi isolated and confirmed as an agent of spoilage in this study, causes soft rot disease in sweet potatoes (Ray and Misra, 1995; Ray *et al*., 1997). It was further established that the fungus could infect potato roots through in juries to the tubers due to deficiencies in the handling or packaging processes (Ray and Misra, 1995). The molds, causing rot during the storage of sweet potato tubers among other food crops, do so through their pectinolytic enzyme activities which often lead to cell separation, maceration, and tissue disintegration (Chesson, 1980). Additionally, according to Ray and Balagopalan (1997), mechanical injury may predispose the roots of sweet potatoes to be attacked by microorganisms, where the proximal end is especially susceptible to invasion because of the natural presence of dead tissues at the site of the wound, which can occur at the time of harvest or packaging. Wounds arising at harvest can be advantageous for opportunistic *R. oryzae* infection. In this study, fungi were isolated from both the surface peels and the deep tissue cuts of stored sweet potatoes. This suggested that the spoilage fungi, once they successfully colonize the surface of the tuber, and that these infections can easily proceed into the deep tissue to cause tissue spoilage. Phytopathogenic microorganisms are assisted by the enzymes they secrete. The production of extracellular hydrolytic enzymes and, in particular, cellulolytic and pectinolytic enzymes capable of breaking down storage tubers have been previously reported (Amadioha, 1993; Ray, 2004). In this study, isolates of *P. expansum* (FSP11) and *P. brevicompactum* (FDC3) demonstrated the production of pectinases in Sabouraud dextrose broth supplemented with sweet potato mash. These observations support earlier reports on the production of cellulolytic and pectinolytic enzymes by several species of fungi associated with soft rots in tuber crops (Chesson, 1980; Obi and Moneke, 1986). However, it must be pointed out that the evidence of pectinolytic activities in vitro is not sufficient to confirm their ability to cause soft rot. This is because groups of pectic enzymes are not able to cause rot by themselves (Chesson, 1980). The ability to cause rot is restricted to those pectic depolymerases having an endo-mode of attack and being able to cause extensive solubilization of pectic substances by the internal cleavage of relatively few linkages (Chesson and Codner, 1978). Hence, the ability of the fungal isolates used in this study to soften sweet potato tissue suggests that they probably have an endo-mode of action in causing spoilage rots in sweet potatoes. The storage temperatures at which these organisms grew and caused spoilage in the tubers (13–29 °C) also reveal that many of these organisms are capable of growing and causing spoilage at a range of temperatures. The results of the investigation of these activities of enzymes in the selected filamentous fungal isolates revealed high activities for secreted cellulases and pectinases, which were notably maximal within the first three days of the experiment. Glucanase activities in *P. expansum* and *P. brevicompactum* were observed to climb during the first three days of measurement but decreased from Days 4 to 8 before picking up again on Day 9.

Xylanase activity appeared in the culture supernatants of all the cultures after 24 h of growth but declined after Day 3. Xylosidase activities which contribute to the utilization of hemicelluloses by acting as either post endo-xylanase activities or directly upon the side chains of the arabinoxylan remained low throughout the period of measurement in *P. expansum* and *P. brevicompactum*. Arabinofuranosidase activities in *P. expansum* and *P. brevicompactum* remained at medium levels (38–10 U/mg) throughout the period of measurement. Chávez *et al.* (2006) studied the production of xylanases from the genus *Penicillium* and noted that for insoluble sources of xylan the initial release of oligosaccharides necessary for full expression of the enzyme family may require mechanical disruption or the action of other enzymes, otherwise the xylanase activities would remain at basal levels. Ferulic acid esterases maintained a relatively low level of activity throughout the investigation period in all the organisms tested. Sweet potatoes may not contain the necessary substrates to induce this enzyme class. Indeed Donaghy and McKay (1995) reported that complex carbon sources such as wheat bran and sugar beet pulp were required for the production of extracellular esterases by *P. expansum* and *P. brevicompactum* in both liquid- and solid-state cultures when using methyl ferulate as the enzyme substrate. Sweet potato tubers are, however, known to be rich in starch and therefore are a good potential substrate for amylase production. Nonetheless, although amylase levels were evident on Days 1–3 (2–12 U/mg) in all the organisms tested, they did not reach the activities of the cellulases and had declined to low levels by Day 4. It is generally believed that different enzymes work together in synergy by complementing the efforts of one another in the degradation of plant tissues. Obi and Moneke (1986) demonstrated cellulolytic and pectinase enzyme production by soft rot causal organisms. The fact that the activities of some of these enzymes appeared low may not necessarily be an indication of their non-participation in the softening or tissue degradation process. de Vries *et al.* (2002) concluded that the efficient degradation of polysaccharides requires the cooperation or synergistic interactions between the enzymes responsible for splitting the different linkages. For example, the release of ferulic acid from xylan by a feruloyl esterase from *Aspergillus niger* was strongly enhanced by the addition of endoxylanases. According to their research, synergy is a general phenomenon between polysaccharide-degrading enzymes. The extent of tissue damage has been shown to parallel levels of pectolytic enzymes from organisms isolated from infected tissues (Ragaert *et al.*, 2007). The results of this experiment indicate that it is possible to extend the shelf-life of sweet potatoes in storage. In one of our experiments, we were able to achieve a 0% decay level in the first month of storage, a 5% decay level in the second month of storage, and a 25% decay level in the third month of storage by using iprodione in conjunction with curing and a controlled atmosphere storage, as indicated in Table 2. These results are in agreement with those of Afek *et al.* (1998), in which they reported a 14% decay rate in the Georgia Jet variety of sweet potatoes when cured, treated with iprodione, and stored for 5 months. However, it is not clear whether they stored the sweet potatoes, after treatment, under a controlled or non-controlled atmosphere. Until now, the necessary skills and adequate knowledge of the methods for sweet potato storage have been lacking among the farmers in Nigeria. In most places, once harvested, sweet potatoes are spread on the floor in a store room as they are ostensibly destined for domestic consumption. If the stock is not exhausted within 2 weeks, it will decay completely and will not be fit for human consumption. The report of Olorunda (1979) highlighted these shortcomings, and noted that even when traditional methods of storage were coupled with fires being lit on a weekly basis to fumigate the tubers, losses of up to 95% were common. Statistical analysis conducted on the data presented here showed that sweet potato samples treated with iprodione (0.1% (1 g/L)) prior to storage fared better than those treated with sodium hypochlorite solution (20%). On the basis of the results obtained in this study, the shelf-life of sweet potatoes in Nigeria and other tropical countries could be prolonged or extended to 3 months with minimal losses when cured, and disinfected with iprodione, and stored under a controlled atmosphere. However, the provision for controlled atmosphere storage facilities may be limited to urban centers.

In most cases, the first step toward the control of post-harvest spoilage of vegetable crops is curing. The use of curing has for a long time been considered

a standard procedure for treating sweet potatoes prior to storage (Hall, 1993). In this study, sweet potato samples were cured for 7 d at 29 °C and 92% relative humidity in the storage incubator. According to some authors, for example Kays *et al*. (1992) and Wang *et al*. (1998), the environmental conditions for adequate curing of sweet potato tubers have been reported to be at temperatures ranging from 29 to 33 °C and with a relative humidity 80%–95% for 4–7 d, which could be considered as ambient conditions in the hot and humid climates of tropical countries (Kays *et al*., 1992; Ray and Balagopalan, 1997). According to Ray and Balagopalan (1997) and Sowley and Oduro (2002), the curing of potato tubers may facilitate the toughening of the skin and could encourage the healing of injuries sustained during the harvest, resulting in the reduction of post-harvest infection and deterioration. In addition, curing induces suberization; that is to say the development of wound periderm and lignification of exposed parenchyma cells which often protects the potato tubers against microbial invasion (Ray and Ravi, 2005).

In addition to curing, 0.1% (1 g/L) iprodione and 20% sodium hypochlorite solutions were applied to samples A and B, respectively. Ekundayo and Daniel (1973) have reported on the use of sodium hypochlorite solutions in the root surface sterilization of cassava tubers prior to storage. Iprodione has been found to be an efficient fungicide that can be used for the control of post-harvest deterioration of sweet potato tubers (Afek *et al*., 1998; 1999). According to Katan (1982) and Ritchie (1982), iprodione has been a common fungicide that has been used for the control of the post-harvest spoilage of vegetable crops, applied as both a pre- and post-harvest spray. When iprodione is combined with wax and oil, the decay control potential is increased, and in such applications it can be used to control post-harvest spoilage fungi such as *Alternaria* and *Rhizopus* species (Ogawa *et al*., 1992). According to Eckert and Ogawa (1988), the physiological storage life of many fruits and vegetables can be realized only by treating them with an antifungal agent before they are stored in an environment that is optimum for retention of the desired crop qualities. The diversity of fungi isolated from the decayed samples is consistent with this view. According to Ray and Misra (1995), filamentous fungi have been implicated in the soft rot disease which affects sweet potatoes. The fungal counts are estimated to be higher than the bacterial counts, especially in the deep tissue cuts, which is consistent with previous reports that fungi are more likely than bacteria to be implicated in soft rot diseases found in sweet potatoes in the temperate and tropical regions (Clark *et al*., 1992; Thankappan, 1994; Afek *et al*., 1999; Holmes and Stange, 2002; Sowley and Oduro, 2002). However, it is possible that the bacterial flora may have colonized the tubers before succession of the fungi.

Apart from curing and disinfection, one other factor which has contributed to the success of this study was the use of a controlled atmosphere consisting of oxygen and carbon dioxide balanced with nitrogen. According to Fellows (2000), an increase in the proportion of carbon dioxide and/or a reduction in the proportion of oxygen within specified limits will maintain product quality and extend the product shelf-life. This is achieved by inhibiting bacterial and mold growth, reducing moisture loss and oxidative changes, and establishing control of biochemical and enzymatic activities. Furthermore, carbon dioxide inhibits microbial activity by dissolving in water within the food to form mild carbonic acid and thus lowering the pH of the product, which has negative effects on enzymatic and biochemical activities in the cells comprising the food and the colonizing microorganisms. Bennik *et al.* (1998) stated that the inhibitory effect of CO_2 on the growth of bacteria is believed to predominantly result from the diffusion of hydrogen carbonate across the bacterial membrane, causing intracellular changes in pH. Such changes may affect enzymes involved in metabolic pathways within the cells. The direct inhibition of enzymatic processes in bacteria using CO_2 has been previously demonstrated. These effects are stronger at lower temperatures as a result of the greater solubility of CO_2 at decreased temperatures (Bennik *et al*., 1998).

In this study, 1% O_2 was used in conjunction with 20% CO_2 in addition to curing and disinfection. Sholberg *et al.* (2001) stated that low oxygen concentration does not appreciably suppress the growth of fungi until the concentration is below 2%, where important growth reductions result if the oxygen concentration is lowered to 1% or below. According to Yoshihiro and Kazuo (2007), cured sweet potatoes can tolerate controlled atmosphere treatments, which

have potential as quarantine procedures. However, they also noted that it is critical to optimize atmospheric conditions, temperature, and the duration of treatments during crop exposure to low oxygen.

In this study, sweet potato tubers were cured at 29 °C and 92% relative humidity for 1 week before being stored at 8 °C and a relative humidity of 92%. Low temperatures and high relative humidity in the post-harvest environment have been reported to affect the activity of decay-causing organisms. This was established during the course of this study, particularly in relation to sample A during the first 2 months of storage as indicated in Table 2.

Improved storage methods which decrease produce loss without resulting in undesirable raw product quality changes are of interest for the development of flexible sweet potato storage applications. It was recently reported that biological post-harvest control methods can be used to preserve and extend the shelf-life of sweet potatoes. According to Wisniewski and Wilson (1992) and Tian (2006), the primary control of microorganisms, especially with antagonistic yeasts, has indicated positive effects in the control of several post-harvest diseases in harvested vegetables and fruits. Sholberg *et al.* (2001) have advocated an integrated post-harvest decay control method. These authors conclusively accepted this concept as the most promising for the future and encouraged society not to rely on only one or two control strategies but to enlist an entire spectrum of strategies to help reduce post-harvest losses of this important tuber crop.

5 Conclusions

Based on the query coverage and maximum identity in the nucleotide sequence databases, seven species of filamentous fungi were identified from the spoiled sweet potatoes. Most of the fungi expressed clear evidence of infection of the sliced and whole potato tubers when the site of inoculation was compared with the control site. *P. expansum*, *P. brevicompactum*, and *R. oryzae* were recovered from the re-infected sweet potato samples. The enzyme activities of *P. expansum* and *P. brevicompactum* were optimum at the earlier time points of infection. Furthermore, the extent of tissue damage was observed to parallel to the secretion of the enzymes used by the spoilage organisms.

Due to the considerable economic importance of sweet potato tubers, their storage after harvest is of great concern. This concern is heightened when the harvest exceeds initial demand in the period of production but with the prospect of shortages later in the year. Storage provides an opportunity to market sweet potatoes out of season since there are specific seasons of the year when the crop can be grown, particularly in semi-tropical and temperate regions. Besides good handling and transportation practices, sweet potato tubers should be stored in an environment which discourages the growth of spoilage organisms, particularly molds. Rapid deterioration of the product results from exposure to environmental conditions, and is exacerbated by mechanical damage during handling and transportation (Rees *et al.*, 2003). Sweet potatoes remain a significant crop for many countries, and it is hereby suggested that fresh efforts should be made to see that the techniques suggested in this study are fully utilized.

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Compliance with ethics guidelines

C. O. OLADOYE, I. F. CONNERTON, R. M. O. KAYODE, P. F. OMOJASOLA, and I. B. KAYODE declare that they have no conflicts of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：甘薯贮藏环境中霉菌的生物分子特征、鉴定和酶活性及甘薯生理变化的研究

目　的：鉴定甘薯贮藏环境中霉菌的生物分子特征和酶活性，并比较不同试剂处理后甘薯的生理变化。

方　法：从不同温度下保存的甘薯得到霉菌菌株，提取其细菌基因组 DNA，进行聚合酶链反应（PCR）及测序鉴定。将甘薯分成扑海因处理组、次氯酸钠处理组和对照组，在为期三个月的贮藏时间内，对霉菌致病性、酶活性和空气条件对甘薯的生理变化的影响进行评估。

结　论：实验结束后，扑海因处理组的变质率为 5%，次氯酸钠组为 55%，对照组为 100%。研究发现，甘薯组织变质主要由于不同的微生物酶的活动，尤其是受感染组织的果胶酶活性。因此，建议将扑海因作为甘薯贮藏之前的保鲜剂。

关键词：气调贮藏；酶活性；扑海因；霉菌特征；腐败；次氯酸钠