ISOLATION, IDENTIFICATION AND PATHOGENICITY OF POST-HARVEST DECAY-INDUCING PATHOGEN(S) IN *CUCUMIS AFRICANUS* AND *CUCUMIS MYRIOCARPUS* FRUITS

BY

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DECLARATION

I declare that the mini-dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Agriculture (Plant Protection) has not previously been submitted by me for a degree at this or any other University; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

Mphahlela R.R (Miss) Date

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ABSTRACT

Crude extracts of wild watermelon (*Cucumis africanus*) and wild cucumber (*C. myriocarpus*) fruits are widely used for both medicinal and ritual purposes in South Africa. Fruits are collected fresh from the wild, but have high incidence of post-harvest decay. A study was conducted to isolate and identify the pathogen responsible for post-harvest fruit decay, followed by the pathogenicity tests. Decayed fruits were individually surface-sterilised using 0.5% NaOCl, incubated at 25ºC to allow for decay, small rotten pieces were severed and placed on solidified plates of potato dextrose agar and incubated. At harvest, seven days after incubation, isolated fungus was repeatedly cultured for 21 days for verification of diagnostic characteristics. Based on the morphological characteristics, the pathogen associated with fruit rot of both *Cucumis* species was identified through the assistance of an expert as *Penicillium simplicissimum* (Oudem) Thom. Pathogenicity results suggested that *P. simplicissimum* was responsible for the observed fruit decay in both species, with the higher incidence being in *C. africanus,* probably due to its low pH. Due to the antibiotics that *P. simplicissimum* releases and its reduction of medium pH, the culture retained its purity, without any contamination. In conclusion, the pathogen that induces post-harvest fruit decay in *C. africanus* and *C. myriocarpus* is *P. simplicissimum*, which has the ability to reduce the pH of the growing medium and also produce antibiotics.

CHAPTER 1
RESEARCH PROBLEM

1.1 Background

Post-harvest rots from pathogens cause considerable losses in fruits, vegetables and cut flowers in many countries during storage or transportation (Abdel-Rahim, 1988; Adikaram, 1988; Agbor-Egbe and Richard, 1991; Bernhardt *et al*., 1988; Ceponis *et al.*, 1987; Hide, 1981; Shaul *et al.,* 1992; Snowdown, 1990; Sommer, 1982). Worldwide, there is little work concerning post-harvest diseases of indigenous fruits due to their uneconomic status. In South Africa, indigenous fruits are widely used by the locals, particularly in rural areas, where more than 60% of the citizens live. The fruits are used for their nutrition, medicinal, pesticidal and/or alcoholic roles.

Losses in fruit crops due to post-harvest diseases have become a major focus for fresh crop producers (Kelman, 1989). Worldwide, the single most important driving factor in determining the marketability of the fruits is quality. In developing countries, post-harvest fruit losses range between 15% and 30% of the harvested crops (Buys and Nortjie, 1997). However, when excluding South African census, post-harvest fruit losses in African countries are estimated at 50% (Eckert and Ogawa, 1985). Pre-harvest treatment, harvest handling and the type of storage have great influence on fruit shelf-lives.

The most important effect of post-harvest fruit decays is the induction of diseases of animals and humans caused by consumption of food invaded by certain common fungi (Agrios, 2005). Fungi produce toxic substances called mycotoxins which pose threat to human and animal health when present in relatively high concentrations (Agrios, 2005). Post-harvest losses in crops result in financial losses that exceed those incurred during pre-harvest (Agrios, 2005).

Condition of high relative humidity, mild temperature and tissue susceptibility are important factors that affect natural infections in fruits (Eckert and Ratnayake, 1983; Parker and Sutton, 1993; Reuveni *et al*.,

2002). Generally, fungal spore germination is inhibited at low relative humidity and small differences in relative humidity can have significant effects in relation to the degree of post-harvest decay (Spotts and Peters, 1981). Fungi causing fruit rot generally grow at 20 to 25ºC and can be conveniently divided into those with growth minimum temperature of between 5ºC and 10ºC or negative 6ºC and 0ºC. However, at low temperatures fungal growth is significantly slowed and thus, reduces decay (Agrios, 2005). The level of moisture content when crops are growing may also contribute to unacceptable high level incidences of post-harvest decays. Favourable conditions for infection during the growing season result in heavy pre-harvest infections, which are invariably carried to the post-harvest storage facilities (Conway, 1984).

For instance, when fruits remain wet for a day or more prior to harvest, fungal infection is eminent. Therefore, the condition of fruits at harvest determines how long the crop can be safely stored. For example, bananas are picked mature but unripe to ensure that they can be stored safely for several months, whereas peaches, have to ripen on the tree (Hartmann *et al*., 2002). The onset of ripening and senescence in various fruits and vegetables render them more susceptible to infection by pathogens (Kader, 1985). Generally, soon after harvest, most fruits enter climacteric process, which is characterised by a burst of respiration and other metabolic changes such as fruit ripening that is stimulated by ethylene leading to the eventual death of fruits (Hartmann *et al*., 2002).

Fruits of wild watermelon (*Cucumis africanus)* and wild cucumber (*C. myriocarpus)* are collected from the wild. Fruits of these *Cucumis* species are widely used for medicinal purposes (Van Wyk *et al*., 1997) and may serve as an alternative to synthetic nematicides in plant protection against nematodes (Mashela, 2002). Fruits of both plant species rot soon after harvest. The identification of the pathogen involved will have positive effects on overall fruit utilization of these species. The aim of this study is to

isolate, identify and to investigate the efficacy of decay-inducing pathogen(s) in *C. africanus* and *C. myriocarpus* fruits.

1.2 Problem statement

The causal agent that causes decay of *C. africanus* and *C. myriocarpus* fruits is not documented. The researcher proposes to isolate, identify and to determine the pathogenicity of the pathogen involved in post-harvest rotting of *C. africanus* and *C. myriocarpus* fruits.

1.3 Motivation

Isolation of pathogens that cause fruit-rot in *C. africanus* and *C. myriocarpus* will facilitate the development of appropriate mass storage of fresh fruits prior to drying in the development of commercial products.

1.4 Aim and objectives

1.4.1 Aim

The aim of the study was to isolate, identify and determine the pathogenicity of fungi, which are responsible for the post-harvest rotting of *C. africanus* and *C. myriocarpus* fruits.

1.4.2 Objectives

Objective 1: To determine whether post-harvest decay-inducing pathogen(s) in *C. africanus* and *C. myriocarpus* fruits can be isolated using potato-dextrose agar.

Objective 2: To determine whether isolated and identified pathogen(s) can induce post-harvest fruit-decay of *C. africanus* and *C. myriocarpus* under aseptic conditions.

1.5 Null hypotheses

Hypothesis 1: Post-harvest decay-inducing pathogens in *C. africanus* and *C. myriocarpus* fruits cannot be isolated using potato-dextrose agar.

Hypothesis 2: Isolated and identified pathogen(s) cannot induce post-harvest fruit-decay of *C. africanus* and *C. myriocarpus* under aseptic conditions.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

The Cucurbitaceae Family has 115 genera, with wild *Cucumis* species being of great interest as alternative crops (Mashela, 2002). South Africa is considered as the centre of diversity for wild *Cucumis* species (Kristkova *et al*., 2003). The genus contains domesticated species which are highly susceptible to the root-knot nematodes (*Meloidogyne species)*, with emerging evidence suggesting the presence of resistance in wild types. The root-knot nematode species infecting exotic cucurbitaceous plants induce root-galling and considerable pre- and post-harvest yield losses (Di Vito *et al*., 1983; Ferris, 1985; Ploeg and Phillips, 2001). Due to the withdrawal of synthetic nematicides, alternatives are underway to test various strategies of suppressing nematode numbers.

2.2 Uses of *Cucumis* species

Extracts from roots and fruits of wild *Cucumis* species have a wide range of uses. In this study, only medicinal and crop protection uses were reviewed.

2.2.1 Medicinal uses

Fruits and roots of wild watermelon (*Cucumis africanus*) and wild cucumber (*C. myriocarpus*) contain pharmacological properties used in treatments of numerous diseases. The often cited diseases cured using materials from these plants include liver damage, weakening of body immunity, lumps, jaundice, acute and chronic viral hepatitis, hepatocirrhosis, persistent dyspepsia, epilepsy due to wind-phlegm, gonorrhoea, boils and intestinal roundworm infection. Materials are used either in fresh, cooked, powder or infusion forms**.**

2.2.2 Crop protection uses

Various studies demonstrated that crude extracts of C. *myriocarpus* and *C. africanus* fruits have potent nematicidal properties (Mashela, 2002; Mashela and Mphosi, 2001; Mofokeng, 2005; Mphosi, 2004). Mashela (2002) demonstrated that ground fruits of C. *myriocarpus* have the potential for suppressing *Meloidogyne* species. Ground *C. myriocarpus* fruit applied at quantities ranging from 0.2 to 0.7 mt/ha consistently suppressed root and soil stages of *M. incognita* race 2. The material increased the productivity of tomato and improved soil electrical conductivity, without affecting soil pH. The efficacy of ground *C. myriocarpus* fruit on nematode suppression and improving of tomato productivity was comparable to that of synthetic nematicides, namely, aldicarb and fenamiphos (Mashela and Khoza, 2005; Mashela *et al*., 2008). Also, biotest solutions from *C. myriocarpus* fruit extracts demonstrated that *C. myriocarpus* has antibacterial properties (Muedi, 2005). Bioactivity tests are used to assess the impact of chemicals on living organisms (Hench and Wilson, 1993).

2.3 Chemical constituents

Fruits of these plant species contain the most toxic compounds in the history of man, which have been identified as cucurbitacins (Jeffrey, 1978; Rimington, 1938; Van Wyk *et al*., 1997). Toxic chemicals in *Cucumis* species are cucumins ($C_{27}H_{40}O_9$) and leptodermins ($C_{27}H_{38}O_8$), collectively referred to as cucurbitacins (Jeffrey, 1978; Rimington, 1998; Van Wyk *et al*., 1997). The water-soluble cucurbitacin A, mostly occurring in *C. myriocarpus*, has strong cutaneous adsorptive properties and amongst the bitterest substances known to man. In *C. myriocarpus* the toxic compounds occur in fruits (especially in seeds) and in roots (Haynes and Jones, 1976), whereas in *C. africanus* these potent chemicals (Curcubitacin B) occur in all organs of the plant (Rimington, 1938). Chen *et al*. (2005) reviewed the structural and chemical properties of 12 cucurbitacins and emphasised that cucurbitacin A is the only cucurbitacin that is soluble in water.

2.4 Shelf-life of *Cucumis* fruits

Fruits of *C. africanus* and *C. myriocarpus* have a short shelf-life. Rotting of this fruit is a major concern as it limits the time during which the fruits are stored, thus, forcing the user to use them over a short period of time. Rotting of *C. africanus* and *C. myriocarpus* fruits become a major concern in the cited studies since fruits were collected from the wild. *Cucumis africanus* and *C. myriocarpus* fruits are not commercially produced, they are usually considered as weeds. Other wild species originating mostly from arid land or semi-arid regions of Africa are cultivated as ornamental plants (Rubatzky and Yamaguchi, 1997).

Figure 1.1 A. Photograph of Cucumis myriocarpus plant with fruit. B Photograph of Cucumis africanus plant with fruit.

In horticultural produce, post-harvest diseases caused by fungi usually begin as either latent infections established in the field or from infection through wounds during post-harvest handling (Terry and Joyce, 2004). Post-harvest decays are caused by a wide variety of microorganisms, including fungal species (Sommer, 1985). Most fruits are infected with one or more fungi that produce highly toxic compounds known as mycotoxins and the related aflatoxins (Agrios, 2005). Studies have since shown aflatoxins to be potent carcinogens to people (Ellis *et al.,* 1991).

Fruits of *C. africanus* and *C. myriocarpus* are attacked by white mould during storage. However, no previous report exists to verify that rotting of fresh fruit is induced by fungi. According to Zitter *et al.* (1996), the causal agents that cause most fruit rots are fungi. Rots incited by fungi have been reported

to cause heavy losses of many fruits both in the field and during storage (Oludemokum, 1976). Many fungal species can cause fruit decay in different crops and they usually flourish under warm and moist conditions. Fungi are generally more difficult to eradicate than bacteria, since they are much larger and produce spores that are highly resistant to drying and other environmental stresses (Agrios, 2005). Approximately, 1.5 million fungal species exists, although most of them are not pathogenetic (Hawksworth, 2006).

2.5 Pre-harvest diseases of cucurbits

Cucurbits are a large, diverse group that are susceptible to over 200 diseases (Zitter, 1996). Anthracnose is a disease of foliage, stems or fruit that typically appear as dark-coloured spots and in some fruit, the spots are raised and have corky surfaces (Sitterly and Keinath, 1996). Additionally, circular, watery, dark and sunken lesions appear on the surface of the fruit. Anthracnose, caused by the fungus *Collectotrichum lagenarium*, is a destructive disease of cucurbits and it occurs during warm and moist seasons (Agrios, 2005; Zitter, 1996).

Many pathogens such as fungi and bacteria cause fruit rot, fruit spotting and other fruit abnormalities prior to storage, majority of fruit rotting organism being fungal although several bacteria can also cause soft rot (Zitter *et al*., 1996). Diseases such as anthracnose and scab occur in various parts of the world and can cause severe losses when they infect the fruit grown for fresh market (Agrios, 2005). Both anthracnose and scab diseases attack cucurbits in the field before the fruits can be harvested.

The fruit becomes susceptible to infection at about the time of ripening (Agrios, 2005). Significant damage can occur to cucumber, muskmelon and watermelon (Zitter, 1996). Frequent rains, warm temperatures (23ºC – 25ºC) and high humidity favour the development of anthracnose (Zitter, 1996). The fungus does not require a wound for infection to occur (Howard *et al*., 1994). Anthracnose fungus

overwinters on diseased residues and may also be carried on cucurbits seed (Agrios, 2005; Zitter *et al*., 1996).

Scab is primarily a disease of cucumber, cantaloupe and muskmelon caused by fungus *Cladosporium cucumerinum* (Zitter *et al*., 1996). The scab fungus can attack any above ground portion of the plant including fruits (Zitter *et al*., 1996). Scab can produce the greatest damage especially if fruits are infected when young. Spots on the fruits first appear as small sunken areas similar to insect sting, later enlarge, and finally become distinct sunken cavities (Babadoost, 2000; Zitter *et al*., 1996). When moist, the cavity is lined with a dark, olive green, velvety layer of spores of the scab fungus (Babadoost, 2000; Zitter *et al*., 1996).

Scab is a common causal agent of storage decay in muskmelon and occasionally of late-planted squash (Babadoost, 2000). Zitter *et al*. (1996) established that fungus causing scab overwinters mainly in squash and pumpkin vines, but may also be seed-borne. Cucumber scab appears as small and sunken spots on the fruit and such spots sometimes ooze out rather clear fluid (Sherf and MacNab, 1986). The most favourable weather conditions for disease development are wet weather and temperatures near or below 21ºC (Zitter *et al*., 1996).

Plant pathogenic bacteria have been known to cause a variety of diseases in plants (Agrios, 2005). Bacterial fruit blotch caused by *Acidovorax avenae* subsp. *Citrulli,* has great potential to cause significant economic losses in cucurbits production in the field (Latin and Hopkins, 1995). The bacterium has been responsible for up to 90% losses of marketable yield in some watermelon fields (Somodi *et al*., 1991). The bacterial pathogen is transmitted through seeds and affects all stages of cucurbit crops, eventually causing destructive fruit rot (Webb and Goth, 1965). Warm temperature and high humidity favour the development of bacterial diseases (Latin *et al*., 1995). Symptoms develop as

small irregularly shaped lesions that rapidly expand into large blotches that can cover most of the fruit (Isakeit *et al*., 1997). Blotches are shallow infections that usually do not penetrate into the flesh of fruit which may appear water soaked, dull-green, and gray-green or dark green in colour (Frankle *et al.,* 1993). In time, the older area of lesions can turn brown to red-brown and necrotic, with the epidermis cracking and an amber-coloured exudates oozing out of the central blotch (Walcott *et al*., 2003).

2.6 Post-harvest pathogens of cucurbits

Fungal pathogens that cause post-harvest decay of cucurbits include *Sclerotinia sclerotium, Penicillium* species, *Rhizoctonia solani*, *Fusarium* species, *Phytophthora capsici*, *Alternaria* species and *Rhizopus* species. Among these genera*, Fusarium* and *Sclerotinia species* cause both pre-harvest and post-harvest rotting (Arvayo-Ortiz *et al*., 1994; Rath *et al*., 1990; Vigliola, 1993). Species of damping-off namely, *Pythium aphonidermatum*, *Rhizoctonia solani* and *Botrytis cinerea,* attack fleshy organs in plants, which rot in the field or in storage (Agrios, 2005). Such infection results in a cottony growth on the surface of the fleshy organs, whereas the interior turns into a soft, watery and rotten mass called leak, especially during the extended wet periods (Thomas and Straub*,* 1992).

Pythium species occur in surface waters and soils throughout the world (Agrios, 2005). The fungus may penetrate the fruit directly under moist or wet conditions and also wounds facilitate fungal entry. Infections of fruit occur in the region that is in contact with the soil. In *C. africanus* and *C. myriocarpus* fruits are directly in contact with the soil, which accords them potential candidacy for fungal infection.

Alternaria species attack fruit closer to maturity which cause spots in crops such as cucurbits and at other point through wounds and many may produce toxins (Agrios, 2005). The spots may be small and sunken or may enlarge to cover most of the fruit, and they may be leathery and have a black, velvety surface layer of fungus growth and spores (Zitter *et al*., 1996). *Alternaria* spores are present in the air

and are one of the most common fungal causes of hay fever (Agrios, 2005). Many species of *Alternaria* are saprophytic, that is, they can infect living plant tissue but only grow on dead or decaying plant tissue (Agrios, 2005).

Alternaria cucumerina affects the leaves of melon and watermelon which causes the leaves to shrivel exposing the fruit to the sun, which may lead to sunscald (Babadoost, 2000; Zitter *et al*., 1996). Fruit rot may develop on summer squash, which gets a brown rot at the blossom end that progresses until the fruit is black and shrivelled. On muskmelon, watermelon and cucumber, rot may begin on overripe fruit or fruit that has been damaged by sunscald (Zitter *et al*., 1996).

Babadoost (2000) observed that sunken spots develop on muskmelon, summer squash, watermelon and cucumber fruits, often the spots later become covered with a dark olive green to black, the mycelium and spores of *Alternaria* fungus. *Alternaria* fungus over-winter as dormant mycelium in diseased and partly decayed crop refuse in weeds of the cucurbits family and possibly in the soil (Babadoost, 2000).

Didymella bryoniae (*Phoma cucurbitacaerum*) is known to cause black rot in all cucurbits (Zitter *et al.,* 1996). Initially, a brown to pink, water-soaked area develops in which numerous, conspicuous black fruiting bodies are embedded. *Didymella bryoniae* attacks the leaves and stems of watermelon, cucumber, cantaloupe and the fruit of squash and pumpkin (Agrios, 2005).

According to Zhang and Bruton (1999), black discouloration associated with the common name of the disease on fruit is normally observed until fruit is near maturity. The disease has many characteristics similar to those of *fusarium* fruit rot (Bruton and Duthie, 1996). They differ in that the brown tissue/tan tissue surrounding *fusarium* fruit rot lesions is often raised, whereas the brown/tan tissue surrounding black rot lesions is not (Zhang and Bruton, 1999). The pathogen is soil-and seed-borne and can over-winter in infected crop debris as dormant mycelium or chlamydospore (Zitter *et al*., 1996). Temperature

and moisture are conducive to disease development, but high relative humidity, rainfall and wetness duration are the most critical (Adams *et al*., 1992).

Erwin and Ribeiro (1996) reported that 49 species of various plants could be infected by *Phytophthora capsici*. Among the major hosts are red and green peppers (*Capsicum annuum*), watermelon (*Citrullus lanatus*), cantaloupe (*Cucumis melo*), honeydew melon (*Cucumis melo*), cucumber (*Cucumis sativus*), blue Hubbard squash (*Cucurbita maxima*), gourd (*Cucurbita moschata*), processing pumpkin (*C. moschata*), yellow squash (*Cucurbita pepo*), tomato (*Lycopersicon esculentum*), black pepper (*Piper nigrum*) and eggplant (*Solanum melongena).* Fruit rot can occur from time of fruit set until harvest (Babadoost, 2000). Fruit rot begins as a water-soaked lesion, which expands, eventually covering the fruit with white mould and resulting in complete collapse of the fruit (Babadoost, 2000; Erwin *et al*., 1995; Farr *et al*., 1989). P*hytophthora capsici* is a soil-borne pathogen that can survive in the field for several years (Babadoost, 2000). *Phytophthora capsici* survives between crops as oospores in soil or mycelium in plant debris (Erwin and Ribiero, 1996; Hausbeck, 2004; Papavizas *et al*., 1981; Zitter *et al*., 1996).

Oospores are resistant to desiccation, cold temperatures and other extreme environmental conditions, and can survive in the absence of host plant, for many years (Hausbeck, 2004; Zitter *et al*., 1996). Fruit rot can occur from the time of fruit set until harvest especially on the fruits that are in contact with the soil (Babadoost, 2000; Latin and Rane, 1999; Zitter *et al*., 1996). Zitter (1992) demonstrated that *Sclerotinia* white mould caused by *S. sclerotiorum* could develop in the field on melons (*Cucurbita melo*) and pumpkins (*C. maxima*) or in storage on winter squash (*C. maschata*). White cottony mould and the presence of black, pea-sized resting bodies (*sclerotia)* are important signs of the fungal infection and affects all stages of fruit development and post-harvested materials (Agrios, 2005). The common storage diseases of squash are *Alternaria* rot (*Alternaria cucumerina*), black rot *(Didymella*

bryoniae) and pink rot *(Fusarium equiseti),* which can occur in the field during the growing season or during post-harvest handling and storage (Snowdon, 1992).

Fusarium fruit rot (*Fusarium solani* f.sp *cucurbitae*) is one of the most common pre-harvest and post-harvest diseases of cucurbit fruits (Babadoost, 2000). Fruits of all cucurbits are susceptible to one or more species of *Fusarium* (Zitter, 1992). Occasionally, the fungus can reach the fruit and contaminate the seed, more especially when the soil moisture is high and temperatures are relatively low (Zitter *et al*., 1996). When harvested fruits are contaminated with fungus, post-harvest diseases develop, especially, in crops with edible underground parts and prostrate plants such as cucurbits (Agrios, 2005). Pumpkin fruits are attacked by *Fusarium* at the soil line and the severity of infection varies with soil moisture and the age of the ring when infection occurs (Zitter *et al*., 1996). *Fusarium* is also one of the fungal genera that produce mycotoxins. Since 1984, at least 20 toxins had been described from about 30 species of *Fusarium* (Moss and Smith, 1984).

Rhizopus stolonifer is a post-harvest disease that can be economically important without proper harvest and post-harvest practices. Cucurbits are among the crops most affected by *Rhizopus* soft rot, when conditions are favourable, the disease spreads rapidly and losses can be great in a short period of time (Agrios, 2005). *Rhizopus* species are omnipresent in the air as a contaminant, are usually saprophytic, and enter fruits through injuries or bruises (Lunn, 1977). Infected fleshy organs appear water soaked at first and are very soft, if the skin remains intact, the tissue losses moisture gradually until the fruits shrivels into a mummy (Agrios, 2005). High humidity and temperature of about 25ºC during storage are favourable for *Rhizopus stolonifer* (Alvarez and Nishijima, 1987).

Several *Penicillium* species are among the most important pathogenic fungi of harvested fruit, and are usually the most destructive of all post-harvest diseases affecting fruits and vegetables. According to

Agrios (2005), *Penicillium* species have a blue, bluish-green, or olive green colours usually surrounded by white mycelium and a band of water soaked tissue, and are known to produce several mycotoxins. Some infections may take place in the field, but blue moulds or green mould are essentially post-harvest diseases and often account for 90% of decay in transit and in storage (Sugar and Spotts, 1999). *Penicillium* first appear as soft rot , watery, slightly discoloured spots of varying size and on any part of the fruit (Agrios, 2005). Moist and warm air is conducive to fungal development, under dry conditions fruit may shrink and become mummified (Amari and Bompeix, 2005).

2.7 Mechanism in fruit decay

In this literature review, emphasis was placed on those enzymes which are responsible for rotting of fruits in absorption nutrition in the Fungi Kingdom, which require extracellular breakdown of food molecules (Campbell, 1992).

In order for the pathogen to induce a disease, the pathogen has to overcome several host barriers like cell wall, pectin layer and protein matrix (Williams and Heitefuss, 1976). Most phytopathogenic microorganisms produce enzymes involve in degrading the different cell wall polysaccharides, such as pectins and hemicelluloses (Annis and Goodwin, 1997; Collmer and Keen, 1986; Walton, 1994) and permit the fungus to attack the cells. Pectinases are the only cell wall degrading enzymes capable of macerating plant tissue and killing plant cells on their own (Cooper, 1983). In addition to the degradation of pectic polymers, degradation-associated changes in hemicellulosic polysaccharides are likely to be important in the breakdown of plant cell walls and the colonisation of plant tissue (Carpita and Gibeaut, 1993).

Ripening of *C. africanus* and *C. myriocarpus* is characterised by softening of the flesh. Fruit softening is associated with cell wall disassembly (Seymour and Gross, 1996) and changes to the pectin fraction

are some of the most apparent changes that take place in the cell wall during ripening (Marin-Rodriquez *et al*., 2002). As such, the cell wall enzymes might be responsible for increased disease susceptibility of ripening of fruits in *C. africanus* and *C. myriocarpus.* Softening of the fruit is accompanied by solubilisation of pectin, involving the action of enzymes pectinesterase, polygalacturonase and pectate lyases (White, 2002). Polygalacturonase splits the long pectin chains into smaller units of galacturonic acid (Deacon, 1997). However, data on cell wall degrading enzyme in *C. africanus* and *C.myriocarpus* is not yet known.

Prusky (1996) suggested several hypotheses that explain resistance mechanism of unripe fruits:

(i) Nutrients available to the pathogen may be limited in unripe hosts,

(ii) Preformed antifungal compounds present in unripe fruits decline during ripening,

(iii) Inducible antifungal compounds in unripe fruits decline during ripening and

(iv) Fungal pathogenicity factors may be activated mainly in ripening fruits.

Endogenous enzymes play a major role in determining the quality of fruits. Adejuwon *et al*. (2009) demonstrated that extract from tomato fruit infected with *Penicillium funiculosum* exhibited cellulase activity which might be responsible enzyme in pathogenicity of the fungus. Hence, the cellulolytic components of the fruits were degraded (Adejuwon *et al*., 2009). Cellulose is major structural constituent of cell walls. Cellulases have been reported to be associated with the pathogenicity of a number of microorganisms (Jan and Chen, 2003; Kalogeris *et al*., 2003). Curren (1969) reported that polygalacturanase seemed to be an important enzyme involved in the breakdown of squash fruit by *Didymella bryoniae.*

Miedes and Lorences (2004) observed in apple fruit infected with *Pencillium expansum* a significant increase in polygalacturonase and pectin methylesterase, whereas in tomato fruit the only increase in enzymatic activity significantly related to infection was in polygalacturonase. Difference between apple

and tomato fruit during fungus infection may be related to differences in cell wall structure and composition and also the specificity of *P. expansum* infection spectrum (Miedes and Lorences, 2004). Pectin depolymerisation might increase the porosity of the wall and allow increased access to fungus colonization and facilitate the progress of the fungal infection (Miedes and Lorences, 2004).

Secretion of cell-wall degrading enzymes has also been reported in many *Pythium* diseases (Cherif *et al*., 1991; Dube and Prabakaran, 1989). According to Zamski and Peretz (1996), softening of cucumber fruit in cottony leak disease, induced by *Pythium aphanidermatum*, has been established to be related to the activities of endo-polygalacturanase and cellulase. In other study xylanase activities were also found in avocado (*Persea americana* Mill.) which may be involved also in fruit softening (Ronen *et al*., 1991).

2.8 Industrial uses of pathogens

Fungi are omnipresent in natural environment and are important in industrial processes. Their most important role is as decomposers of organic materials, as pathogens and symbionts of animals and plants. Fungi are utilized as producers of economically important substances such as ethanol, citric acids, antibiotics, polysaccharides, enzymes and vitamins (Gadd, 1993). *Penicillium simplicissimum* is known to excrete citric acid under pH range between 4 and 7 (Franz *et al*., 1993) which is mainly used as flavouring and preservatives in food and beverages. In another study, *P. simplicissimum* excreted large amount of citric acid in the presence of insoluble metal oxides such industrial filter dust, zinc oxide and synthetic mixture of metal oxides (Franz *et al.,* 1991). The accumulation of filter dust onto *P. simplicissimum* mycelium was not only responsible for citric acid production but also leaching of zinc (Franz *et al.,* 1991). Franz *et al.* (1991) suggested that induction of citric acid excretion by *P. simplicissimum* involved the plasma membrane H+-ATPase in the presence of industrial filter dust. The importance of metallic ions to fungal metabolism had been known for long time (Gadd, 1993).

Enzyme xylanases excreted by *P. simplicissimum* is responsible for degradation of xylan in plant cells in low pH and optimum temperature (Schmidt *et al*., 1998). Xylan is the major hemicellulose component of the plant cell wall (Biely, 1985). Also, *P. simplicissimum* was isolated from plants such as black pine, oak forest and grapes (Pitt, 1999). Xylanases have been widely detected in bacteria and filamentous fungi (Beg *et al*., 2001; Sunna and Antranikian, 1997). Schimdt *et al*. (1998) indicated that this enzyme has found several biotechnological applications where they can be used as bleaching agents or boosters despite its relatively low pH and temperature optimum.

Recently, several xylanases from different families and with different molecular structures were compared with respect to their performance in the bleaching process. Much research has been focused on isolation of powerful thermostable xylanases because of their potential application in various industrial processes, such as bioconversion of lignocellulosic materials into fermentative product and improvement of digestibility of animal feedstock and clarification of juices (Beg *et al*., 2001; Sunna and Antranikian, 1997). Thermostable xylanases have been reported in a wide range of microorganisms including fungi (Lee *et al*., 2006; Mata and Savoie, 1998). Fungal xylanases are secreted extracellularly and their activity is much higher than xylanases from bacteria and yeasts (Krisana *et al*., 2005; Twomey *et al*., 2003).

2.9 Influence of pre-harvest handling in fruit decay

The quality and condition of fresh produce cannot be changed after harvest. Pre-harvest conditions may have a greater impact on post-harvest quality than post-harvest handling systems (Ippolito and Nigro, 2000). Environmental conditions during growth such as unfavourable high and low temperature, wind, rain or hail, can all affect the crop by reducing both yield and quality of stored fruits (Barkai-Golan, 2001). As the fruit ripens, tissues soften due to the solubility of cell wall compounds such as pectin or

hemicelluloses (Eckert, 1978; Paull *et al*., 1999). The ability of tissues to produce antimicrobial compounds which inhibit the pathogenic infections and their growth diminishes (Verhoeff and Liem, 1975). After harvest, seeds, fruits and vegetables continue to have physiological functions such as respiration and enzymatic activities and as senescence sets in their natural resistance to post-harvest diseases is reduced (Narayarasamy, 2000).

Various cultivars may vary greatly in their susceptibility to diseases. Melons with thick skin texture, for instance, withstand the rigours of harvesting and handling better tha thin skin ones, and therefore, have longer storage lives (Barkai-Golan, 2001). Pathogens may contaminate the planting material and cause diseases in the field or in the storage, hence the importance of obtaining clean seeds and establishment of certification schemes (Barkai-Golan, 2001). Generally, pre-harvest conditions may even have a greater impact on post-harvest handling systems (Ippolito and Nigro, 2000).

2.10 Influence of post-harvest handling in fruit decay

The degree of maturity when fruits are harvested is crucial to its subsequent storage and shelf-life before processing. Crop losses after harvest remain unacceptably high. Hartmann *et al.* (2002) indicated that specific measures must be taken to maintain crop quality from time of harvest onward in order to reduce physiological deterioration as this can become conducive to spoilage pathogens. Generally, mishandling during harvest and beyond result in high incidences of fruit rot.

2.11 Isolation of fruit-rot pathogens

Most fungi are difficult to isolate from infected tissues, infested soil or decaying organic materials due to the rapid and often antagonistic development of associated fungi, bacteria and actinomycetes with faster growth rates. Successful isolation of many of these fungi can be achieved by the used of selective media that either slow down the growth or inhibit the growth of these antagonistic organisms.

Fungi isolation is an ongoing process that was introduced for identification purposes due to major losses of stored fruits. Development and introduction of techniques for growing various fungi in pure cultures dated back to Brefeld, Koch, Petri and others 1875-1912 (Agrios, 2005). Koch (Cited by Agrios, 2005) also proposed a set of four rules, known as Koch's postulates, which should be satisfied before it can be accepted that a particular microorganism causes a disease or not.

2.12 Pathogenicity tests

According to Koch's rule (Agrios, 2005), the following steps should be taken before a microorganism isolated from diseased fruits can be considered as the cause of the diseases:

a) The pathogen must be found associated with the disease in all diseased plants examined.
b) The pathogen must be isolated and grown in a pure culture on nutrient media and its characteristics described (non-obligate) or it must be grown on a susceptible host plant (obligate parasites) and its appearance and effects recorded.
c) The pathogen from the pure culture must be inoculated on healthy plant of the same species or variety on which disease appears and it must produce the same disease on inoculated plants.
d) The pathogen must be isolated in pure culture again and its characteristics must be exactly like those observed in step (b).

The aim of the study was to isolate, identify and assess the pathogenicity of rot-inducing pathogens in *C. myriocarpus* and *C. africanus* fruits. These will help identify pathogens that are responsible for storage decay of *C. myriocarpus* and *C. africanus* fruits, thus prolonging their shelf-life.

CHAPTER 3
ISOLATION, IDENTIFICATION AND PATHOGENICITY OF POST-HARVEST DECAY PATHOGEN(S) IN WILD *CUCUMIS* SPECIES

3.1 Introduction

The shelf-life of the two *Cucumis* species, *viz.* wild watermelon (*Cucumis africanus*) and wild cucumber (*C. myriocarpus*), is limited. Rotting fruits of both species showed signs of mycelia when stored fresh for a short period, suggesting the presence of fungal pathogens. Generally, association does not necessarily imply causal. According to Koch's rule (Agrios, 2005), causality is tested through the four steps; *viz*. (i) the pathogen must be found associated with the disease in diseased plants examined; (ii) the suspected causal agent must be isolated from the diseased plant and grown in pure culture; (iii) the pathogen from the pure must be inoculated on healthy plant of the same species or variety on which disease appears and (iv) it must produce the same disease on inoculated plants and the pathogen must be isolated in pure culture again and its characteristics must be exactly like those observed in step 2 above. The objective of this study was two-fold: (i) To determine whether post-harvest decay-inducing pathogen(s) in *C. africanus* and *C. myriocarpus* fruits can be isolated using potato-dextrose agar, and (ii) To determine whether isolated and identified pathogen(s) can induce post-harvest fruit decay in *C. africanus* and *C. myriocarpus* fruits.

3.2 Materials and methods

The experiment was conducted in the Nematology Laboratory, University of Limpopo, South Africa (23º53'10"S, 29º44'15"E). Ripe fruits of *C. africanus* and *C. myriocarpus* were collected locally and separately washed with distilled water and stored in batches of two at 25°C and 80% RH to allow fruit decay to occur.

Isolation and identification: The growing medium was prepared by mixing 10 g potato dextrose agar (PDA) in 250 ml distilled water and sterilised at 121°C and 15-psi pressure for 20 minutes, with pH

adjusted within 6.5 and 7.0 after autoclaving (Ali *et al*., 2006). After cooling, the medium was poured into sterile Petri dishes. Fruits of *C. africanus* and *C. myriocarpus* were surface-sterilised using 0.5 % NaOCl (bleach) with small pieces being cut off using sterile scalpels that were intermittently dipped in 10% bleach, placed separately on solidified plates of PDA and incubated at 25°C and 80% RH in the growth chamber (Model LTIM 70). Repeated culturing on PDA was done from the first to the fifth generations after inoculation in order to verify the purity of the observed fungal accessions. Isolates from *C. africanus* and *C. myriocarpus* were code-named Cuit and Cont, respectively, stored at 5°C and delivered to the Biosystematics Division of the Plant Protection Research Institute of the Agricultural Research Council (ARC-PPRI) for identification to the species level. Repeated culturing on PDA at ARC was done to confirm whether the morphological characteristics of the delivered isolates would not change over three generations.

Pathogenicity test: Separate studies were conducted for the two *Cucumis* species. A 2 × 4 factorial experiment was conducted in the growth chamber. The first main factor comprised two inoculation sites and the second was treatments, *viz.* (i) untreated control, (ii) bleach alone, (iii) isolate alone and (iv) bleach + isolate. The factors were arranged in a randomised complete block design, with five replications. Bleach alone and bleach + isolate treatments were achieved by first placing healthy fruits in 500 ml bottles, covering them with 1% bleach solution and mechanically shaking for 30 minutes, with the untreated control and isolate alone being shaken in distilled water. Fruits were then rinsed with distilled water to remove excess solution and wiped off by wrapping individual fruits in tissue paper and squeezing slightly.

A 5-mm-diameter cork borer was used to drill holes, with either one or two inoculation sites, which were inoculated with and without spores from the isolate. Approximately 10 ml tap-water was poured into 22 mm x 12 mm transparent plastic bags and twisted prior to placing in individual fruit to ensure that fruits

did not come into contact with water meant to provide constant humidity. Bags were placed in rails of growth chamber for 16 days. An arbitrary decay scale was developed: 0 = not rotten, 1 = rotten around inoculation site, 3 = half of the fruit is rotten and 6 = the whole fruit is rotten.

Fruit pulp from three fruits per treatment that were not rotten was removed and centrifuged at 1500 rpm to separate seeds from juice. The sugar content of the juice was determined using a hand-held refractometer. The pH of juice was measured using a pH meter.

3.3 Data analysis

Fruit decay data were transformed using LN(x + 1) prior to analysis in order to homogenize the variances (Gomez and Gomez, 1984), but untransformed data are reported. Analysis of variance was performed on treatments using Statistix 8 software (Statistix, Analytic Software, Statistix; Tallahassee, FL, USA, 1985-2003). Fisher's least significant difference (LSD) test at 0.05 probability level was used to determine treatment differences among the means.

3.4 Results

Isolation and identification: Seven days after incubation of infected tissues from decayed fruits, green to dark green mycelia with greyish coloured spores were observed on the PDA (Appendix 3.1). Repeated culturing indicated that the fungus retained its morphological characteristics from the first generation to the fifth generation. Repeated culturing of the two isolates at the ARC-PPRI also confirmed the morphological characteristics observed in the Nematology Laboratory. The pathogen associated with post-harvest fruit decay of *C. africanus* and *C. myriocarpus* was identified at ARC-PPRI as *Penicillium simplicissimum* (Oudem.) Thom (Table 3.1).

Table 3.1 Pathogens associated with decay in *Cucumis africanus* and *Cucumis myriocarpus* fruits as identified at the Biosystematics Division of the Plant Protection Research Institute of the Agricultural Research Council.

Cucumis	**Code A[x]**	**Code B[y]**	**Identification**
C. africanus	Cuit	M-48/377	*Penicillium simplicissimum* (Oudem.) Thom.
C. myriocarpus	Cont	M-48/378	*Penicillium simplicissimum* (Oudem.) Thom.

[x] = code for the sender, University of Limpopo.
y = code determiner (Ms M. Truter), at the Biosystematics Division of the Plant Protection Research Institute of Agricultural Research Council.

Pathogenicity test: Treatment effects for the pathogenicity test were highly significant at the probability level of 0.05% for both *Cucumis* species, accounting for 22% and 18% total treatment variation in fruit rotting in *C. africanus* and *C. myriocarpus* fruits, respectively (Table 3.2). However, the number of infection sites had no effect on fruit decay. In *C. africanus*, inoculation alone and bleach + inoculation had the highest incidence on fruit decay, which was significantly different ($P \leq 0.05$) from the untreated control (Table 3.3). Bleach alone resulted in the least incidence of fruit decay. In *C. myriocarpus*, the treatment bleach alone and inoculation alone had the lowest incidence of fruit decay, whereas bleach + inoculation had the highest incidence of fruit decay when compared with the untreated control and the other two treatments. Compared with *C. myriocarpus* fruits, fruits of *C. africanus* had the highest incidence of fruit decay (Table 3.4). Also, juice of *C. africanus* had significantly lower pH than that of *C. myriocarpus*, whereas the sugar content did not differ.

Table 3.2 Analysis of variance for responses of *Cucumis africanus* and *Cucumis myriocarpus* fruit sterilized with and without bleach when inoculated with *Penicillium simplicissimum* in one and two holes.

		Cucumis africanus		*Cucumis myriocarpus*	
Source of variation	DF	SS	Percentage	SS	Percentage
Replication (A)	4	11.15	4	4.60	4
Treatment (B)	3	71.08	22**	22.90	18**
Hole (C)	1	0.03	0ns	3.60	3ns
B × C	3	20.68	7	2.60	2
Error	28	214.85	67	95.40	73
TOTAL	39	317.775		129.100	

** = Significant at P ≤ 0.05; ns = Not significant at P ≤ 0.05.

Table 3.3 Responses of *Cucumis africanus* and *Cucumis myriocarpus* fruits sterilized with and without bleach (NaOCl) when inoculated with *Penicillium simplicissimum.*

Treatment	*Cucumis africanus*	*Cucumis myriocarpus*
Untreated control	1.30b	0.60b
Bleach alone	0.90c	0.03c
Inoculation alone	3.80a	0.10c
Bleach + inoculation	3.70a	1.90a

Column means followed by the same letter are not different (P ≤ 0.05) according to the least significant difference test.

Table 3.4 Incidence of fruit decay, juice pH and sugar content of *Cucumis africanus* and *Cucumis myriocarpus* fruits when inoculated with *Penicillium simplicissimum*.

Species	Decayed fruit	Juice pH	Sugar content (Brix %)
Cucumis africanus	3.43a	4.90b	4.84a
Cucumis myriocarpus	1.65b	5.92a	5.17a
Standard error	0.514	0.246	0.335

Column means followed by the same letter are not different (P ≤ 0.05) according to the least significant difference test.

3.5 Discussion

The pathogenicity test confirmed that the fungus *P. simplicissimum* was associated with post-harvest fruit decay in both *C. africanus* and *C. myriocarpus*. Previously, *P. simplicissimum* was isolated and identified from rotten fruits of apples (*Malus domestica*), grapes (*Vitis vinifera*) and certain fruiting vegetables (Ali *et al*., 2006; Domsch *et al*., 1980; Sage *et al*., 2004). Approximately, 150 identified species had been identified in the genus *Penicillium*, with at least 50 species being common (Pitt, 1979; 1988). *Penicillium* species resemble each other in colour, mycelium growth characteristics and decay symptoms (Tournas, 2005). Consequently, expert advice is almost always necessary for the identification of this genus to the species level.

In *C. africanus* and *C. myriocarpus*, the *P. simplicissimum*-infected fruits were hardly contaminated as observed in field-collected decaying fruits. The Kingdom Fungi is known to excrete substances which increase pH of the growth medium (Gadd, 1993). Mild temperature, sugar content and pH level of fruit juices are important variables in natural infections of pathogens (Vadhyasekaran, 2008). Generally, most fungal pathogens prefer fruits with juice that contains high sugar content and pH values (De Roever, 1999).

In *P. simplicissimum,* the hydrolytic enzymes had been isolated and identified as an intracellular hydroperoxidase, which is the first dimeric catalase-peroxidase of eukaryotic origin (Fraaije *et al*., (1996). Additionally, Fraaije *et al*. (1996) isolated and partially purified an atypical catalase which was located in the periplasm and contains a chlorin-type heme as prosthetic group and had the ability to operate over a broad pH range. These atypical catalases are active in the pH range 5-10 (Fraaije *et al.,* 1996). Since fungal degradation occurs over alkaline conditions (Agrios, 2005), the presence of this unnatural catalase in *P. simplicissimum* suggest that this fungus may also induce fruit rotting under acidic conditions.

Penicillium simplicissimum is known for degradation of natural lignin in making aromatic dyes and is also important for decouloration which suggests that the species has ligninolytic abilities (Hong-yan *et al*., 2005). Lignin is one of the most widely distributed natural organic polymers, and decomposes slowly in the environment due to its complex structure.

Ligninolytic enzymes have a potential in several industrial and biotechnological processes within a wide variety of organic and inorganic substrate specificities (Couto and Herrera, 2006). According to Agrios (2005), lignin polymer is perhaps more resistance to enzymatic degradation than any other plant substances. However, it is accepted that only a small group of microorganisms is capable of degrading lignin (Agrios, 2005). Hong-yan *et al*. (2005) indicated that enzymes such as lignin peroxidase, laccase and hemicellulase are believed to be the most important catalyses in the biodegradable process. The physiological roles of fungal laccase include pigment production and degradation of lignocellulosic materials (Thurston, 1994).

Penicillium simplicissimum excretes citric acids, which reduce pH of the growth media to exclude the growth and development of other fungal species (Franz *et al*., 1993; Schinner and Burgstaller, 1989). In addition to the reduction of pH, the identified isolate of *P. simplicissimum* also excrete an antibiotic, *viz*. 4-allyl-2-azetidinone (B-143), which suppresses all forms of microbial activity except for *P. simplicissimum* (Kobayashi *et al*., 1997). In the same study, Kobayashi *et al*. (1997), demonstrated that B-143 exerted reduction in proliferation of *Fusarium* species in commercial cucumber (*C. sativus*) cultivars. The observation is in line with the definition of antibiotics, *viz.* chemical compounds that kill or inhibit the growth of microorganisms, including both bacteria and fungi (Hardman *et al*., 1996). The association of B-143 and the reduction in pH by *P. simplicissimum* is unique to this fungus. However, when used as a bionematicide for tomato cultivation, crude extracts of the two *Cucumis* species had no effect on soil pH (Mashela, 2002; Mashela *et al*., 2008), which suggests that *P. simplicissimum* plays a

negligent role in ground leaching technology, as demonstrated in other studies (Mashela and Ntangeni, 2002, Mphosi, 2004).

The higher incidence of post-harvest decay in *C. africanus* fruits when compared with *C. myriocarpus* fruits is probably due to the lower pH of juice in the former, which improves the growth of *P. simplicissimum* as an acid-loving fungus. Lack of effect of number of holes on fruits may suggest that fruit bruising is less of a factor in the infection of *P. simplicissimum* in the two *Cucumis* species.

In conclusion, post-harvest fruit decay of *C. africanus* and *C. myriocarpus* fruits are induced by *P. simplicissimum*, which reduces the pH of the affected organ which suppresses the growth of other pathogens, but also suppresses growth of other pathogens through the release of antibiotics. Further studies are necessary to establish whether *P. simplicissimum* and the highly sensitive *C. africanus* may not offer opportunities to produce innovative antibiotic products for soil health in an attempt to suppress alkaline-loving economic soil pathogens such, fungi and plant-parasitic nematodes.

CHAPTER 4
SUMMARY AND CONCLUSION

Fruits of *Cucumis africanus* and *C. myriocarpus* play a major role in traditional medicine and crop protection in South Africa. Fruits are used either fresh or in dried forms. However, fruits of the two *Cucumis* species are sensitive to post-harvest decay, thus, reducing the utility of these fruits in fresh form.

The pathogen that induces post-harvest fruit decay in *C. africanus* and *C. myriocarpus* fruits was identified as *P. simplicissimum,* which is a cosmopolitan pathogen in the Cucurbitaceae family. This fungus produces antibiotics and therefore suppresses other potential pathogens. The fungus is acid-philic pathogen and it proliferated in *C. africanus* which has low juice pH.

In conclusion, the first empirical work of post-harvest fruit decay of *C. africanus* and *C. myriocarpus* fruits suggested that *P. simplicissimum* is the causal agent. Since this pathogen is acid–philic, storage conditions that increase pH may attempt to eliminate the pathogen.

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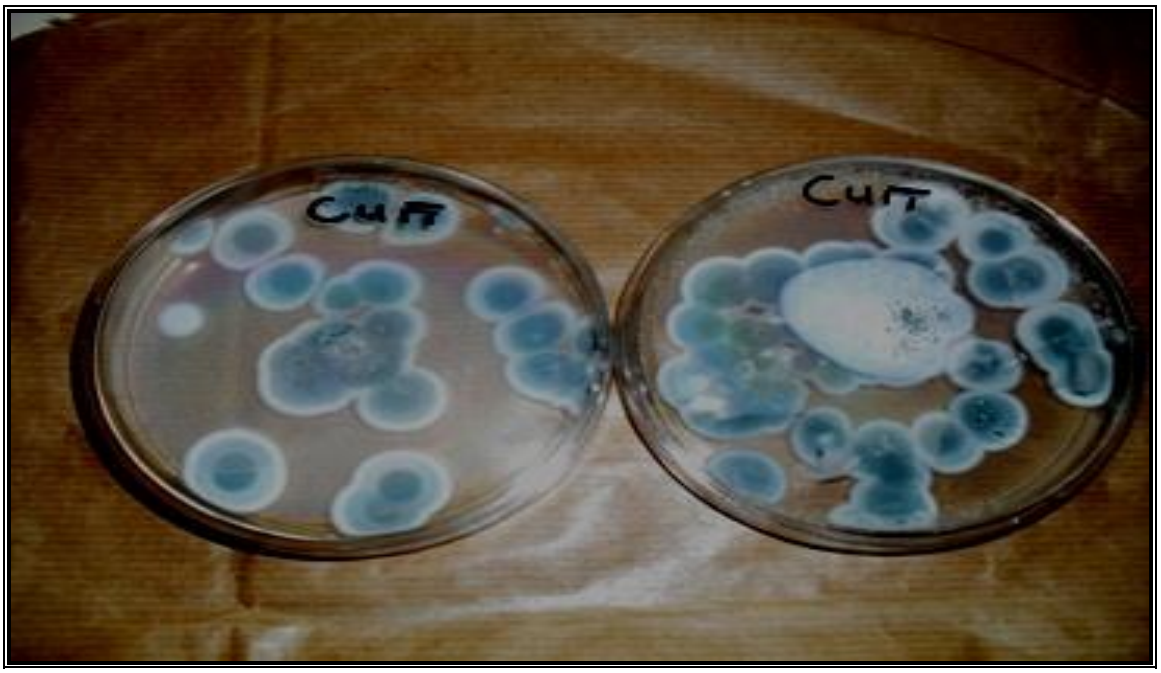
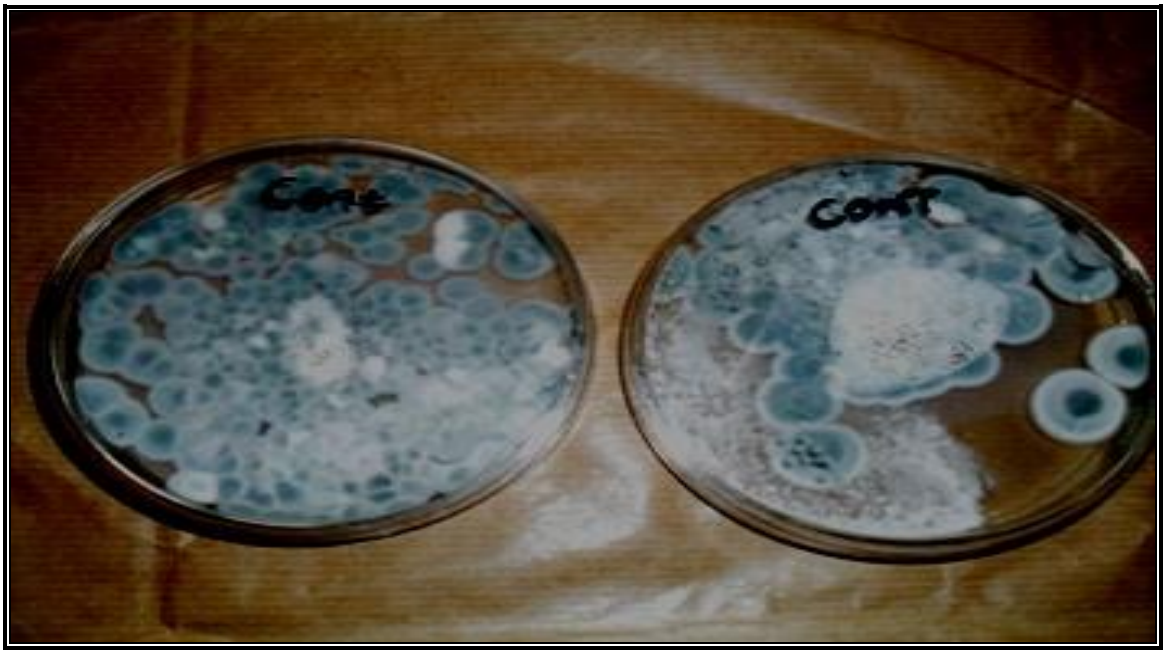
APPENDICES

Appendix 3.1 *Penicillium simplicissimum* isolates.



(a) Isolated from *Cucumis africanus* and the growth pattern of *Penicillium simplicissimum* colonies at the end of 5 days of incubation at 25ºC.



(b) Isolated from *Cucumis myriocarpus* and the growth pattern of *Penicillium simplicissimum* colonies at the end of 5 days of incubation at 25ºC.

Appendix 3.2 Analysis of variance for pH in *Cucumis africanus* and *Cucumis myriocarpus* fruit juice.

Source of variation	DF	SS	MS	F-value	P-value
Treatment	1	7.77243	7.77243	17.19	0.0003**
Error	28	12.6621	0.45222		
Total	29	20.4345			

** = Significant at P ≤ 0.05

Appendix 2.2 Analysis of variance for sugar *Cucumis africanus* and *Cucumis myriocarpus* fresh fruits.

Source of variation	DF	SS	MS	F-value	P-value
Treatment	1	0.83333	0.83333	0.99	0.3284^{ns}
Error	28	23.5853	0.84233		
Total	29	24.4187			

ns = Significant at P ≤ 0.05