MYCOTOXIN PRODUCTION AND MOLECULAR CHARACTERISATION OF *Penicillium* SPECIES ISOLATED FROM MILLET GRAINS (*Pennisetum glaucum*) (*L.*) R. Br.) IN SOUTHWESTERN NIGERIA

By

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ABSTRACT

Millet, a widely consumed food crop, is subject to fungal contamination during storage. These fungi produce mycotoxins in stored food products. Mycotoxins such as citrinin and ochratoxin produced by *Penicillium* species have been reported to be injurious to consumers. Knowledge of the fungi involved in the production of these mycotoxins will help in the control of its spread in food products. However, there is dearth of information on the mycotoxins produced by *Penicillium* on millet grain and the species characterisation with molecular technique in Nigeria. Hence, this study was designed to investigate the distribution of *Penicillium* species and determine the presence of mycotoxins in the millet grains with molecular techniques.

Samples of millet grains were purchased from three randomly selected markets each in Lagos, Ogun, Ondo, Oyo and Ekiti states all in southwestern Nigeria. One hundred grains from each of the millet samples were employed for isolation of fungi using Dichloran rose bengal chloramphenicol agar. Morphological identification of the fungal isolates was done using Pitt`s manual. The DNA of the *Penicillium* species were isolated using standard procedures. Similarities between species were determined by amplifying their internal transcribed spacer (*ITS1, ITS4*) region and partial beta tubulin gene (*Bt2a, Bt2b*). Physiological assessment of the *Penicillium* isolates which includes optimum pH, carbon and nitrogen sources, temperature and incubation time for mycelial growth were carried out using standard methods. Toxigenic screenings of the isolates were carried out by amplifying the genes *pksCT, ctnA, orf3* and *otanps* responsible for citrinin and ochratoxin production. The effects of pH and temperature on mycotoxin production were determined and quantified using Enzyme linked immunosorbent assay. Data were analysed using descriptive statistics.

Thirty-four isolates were obtained, out of which twenty-three were identified as *Penicillium citrinum* (17), *P. capsulatum* (1), *P. simplicissimum* (1), *P. oxalicum* (1), *P. steckii* (2) and *P. chrysogenum* (1). Distributions among the states were Ondo: *Penicillium citrinum*, *P. simplicissimum* and *P. oxalicum*, Ogun: *Penicillium citrinum* and *P. chrysogenum* while only *Penicillium citrinum* was obtained from Lagos, Oyo and Ekiti states. Twenty-one isolates showed amplification to the beta tubulin gene with a uniform amplicon size of 500 base pair, while *P. simplicissimum* and *P. oxalicum* exhibited variation in amplicon fragement sizes (450 and 550 base pair), respectively. Physiological assessment of the isolates showed optimum growth at pH 8 having 2.51 g Mycelia Weight (MW) and starch as carbon source having (2.14 g MW), $(NH_4)_2SO_4$ as nitrogen source having (0.86 g MW), optimum temperature was at 30 °C having (0.96 g MW) and 14 days as best incubation time with 0.94 g as dry MW. Toxigenic genes *ctnA* (678-679 base pair), *orf3* (428-447 base pair) and *otanps* (788 base pair) was detected in six *Penicillium* (26%) isolates. Ochratoxin production was highest at pH 8 with 7.0 ppb, and at 25 °C with 5.0 ppb.

Distribution of *Penicillium* on millet grain across the study area was determined, the isolates were characterised and mycotoxin from *Penicillium citrinum* was found to be above the standard permissible limit of ochratoxin.

Keywords: Millet grain, Mycotoxigenic *Penicillium* species, Ochratoxin and citrinin genes

Word count: 497

CERTIFICATION

I certify that this research work is original and was carried out by Bunmi Comfort Kotun in the Department of Botany, Faculty of Science, University of Ibadan, Ibadan Nigeria, under my supervision.

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DEDICATION

This research work is wholly dedication to The Alpha and Omega,

Omnipresent, Omnipotent and Omniscience who IS, WAS

and IS TO COME.

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I ascribe All Glory, Honour and Adorations to the Almighty one, who has seen me through this research work. He alone is worthy of my praises.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER ONE

1.0 INTRODUCTION

Millet belongs to the Family *Poaceae*, Subfamily *Panicoideae*, Genus *Pennisetum* and Species *P.glaucum*. Most widely cultivated species are Pearl Millet (*Pennisetum glaucum*), Foxtail Millet (*Setaria italica*), Common Millet or Proso Millet (*Panicum miliaceum*) and Finger Millet (*Eleusine coracana*).pearl millet comprises of 40% of world production (Oelke *et al*., 1990). Pearl and Finger Millet are African varieties that had gained world acceptance (Josep, 2001). Millet is widely grown around the world as cereal crops or grains for both human and animal consumption, they are important in the semi-arid tropics of Asia and Africa especially in India, Nigeria and Niger, 97% of these are produced in developing countries (McDonough *et al*., 2000). Millet is ranked the sixth most important grain in the world, which has been reported to sustain one third of the world's population (Karen, 2011), they are mainly produced by China, India and Nigeria as the largest 41% producers in West Africa.Millet grain can be eaten whole after roasting, cooking or boiling like rice, it is becoming commonly used as baby foods due to its nutritional value, it is a rich source of calcium and has a pleasant flavor. The flour can be used for making wine and beer. It is also used in making thick dough called Fura (a popular drink in the north), a custard like food called Kwoko and Kunnun zaki a millet juice.

Pearl millet is one of the most extensively cultivated cereals in the world after rice, wheat and sorghum particularly in the arid to semi-arid regions, it is the principal food cultivated in drought prone regions in Africa, in USA, Australia and South America. It is extensively grown as a forage crop mainly for animal feed (FAO, 2005). Its residue provides building materials, fuel for cooking in dry areas and also used as fodder.

Makun *et al*. (2009) reported the presence of *Aspergillus, Fusarium, Penicillium, Rhizopus, Mucor, Syncephalastrum and Helminthosporium* in pearl millet. Susceptibility of millet to fungal growth and mycotoxin contamination has been documented (Okoye, 1992: Amadi and Adeniyi, 2009). Most species of *Aspergillus* and *Penicillium* are able to grow on a wide range of organic substrate. They are essentially saprophytic and are particularly associated with stored moldy plant product they are metabolically very versatile and a number of species have been found to produce toxic metabolites (Lacey, 1991). Many *Aspergillus, Penicillium* and *Cladosporium* species are known to produce mycotoxins. At present Aflatoxin is considered to be one of the most toxic, carcinogenic compounds produced by several members of the genus *Aspergillus* in foods and seeds (Gourama and Bullerman, 1995: Cotty, 1997; Ezekial *et al*., 2013). The ubiquity of potentially aflatoxigenic moulds and the large number of agricultural produce in which the natural occurrence of aflatoxin has been reported has led many countries to introduce legislation aimed at limiting and controlling exposure to these toxic substances (Lacey, 1990; Munimbazi and Bullerman, 1996).

Penicillium species are commonly occurring worldwide, and have been located from various substrates including air, soil, various food and feed products and indoor environments (Pitt and Hockings, 1997; Pitt and Hockings, 2009; Samson *et al*., 2010; Houbraken *et al*., 2010).

Penicillium fungi are versatile and opportunistic. They are post-harvest pathogens. The most important toxigenic *Penicillium* species in foods are *Penicillium citreonigrum,* which produces citreoviridin, *Penicillium citrinin* produces citrinin, *Penicillium crustosum*

produces Penitrem A, *Penicillium islandicum* produces cyclochlorotine, islanditoxin, luteoskyrin and erythroskyrin, *Penicillium verrucosum* produces Ochratoxin A. They are one of the most common causes of fungal spoilage in fruits and vegetables.

Penicillium italicum and *Penicillium digitatum* are the most common attackers of citrus fruits, while *Penicillium expansum* is known to attack apples. *Penicillium digitatum* works by producing ethylene to accelerate ripening, and covers the citrus fruit with green conidia, causing the fruit to shrivel and dry out. *Penicillium italicum* causes slimy rot and produces blue-green conidia. These species like cooler temperatures, which explains why they are usually found on foods left too long in the refrigerator. Most of these species resemble each other in color characteristics, style of decay, and infection symptoms; they fall under a general category called blue mold. Sometimes *Penicillium italicum* and *Penicillium expansum* will adhere to each other to create synnemata. *Penicillium expansum* is one of the most aggressive species. These fungi live a long time and are quite durable, even under adverse conditions. *Penicillium purpurogenum* is a common indoor contaminant associated with water intrusion. It produces the chemotherapeutic agent known as Rubratoxin**.** *Penicillium* species attack more than just fruit. For example, *Penicillium verrucosum* grows on cereal products. *Penicillium rubrum* produces **rubratoxin** which causes abdominal pain, jaundice and convulsion. *Penicillium Citrinum* produce **Citrinin** which is a renal toxin to monogastric domestic animals, **Citrinin** causes watery diarrhoea, increased food consumption and reduced weight gain due to kidney degeneration in chickens (Mehdi *et al*., 1981). *Penicillium citrinum* has been isolated from nearly every kind of food surveyed for fungi, the most common sources are milled grains and flour, whole cereals especially rice, wheat and corn (Pitt and Hocking, 1985a). Instances of spoilage are rare but growth and toxin production are likely to be a common occurrence.

Penicillium infections are most commonly exhibited in immunosuppressed individuals. For example, *Penicillium marneffei* is a fungus abundant in Southeast Asia that typically infects patients with AIDS in this area. Infection with *Penicillium marneffei* is acquired via inhalation and initially results in a pulmonary infection and then spreads to other areas of the body (lymphatic system, liver, spleen, and bones), and is often fatal. An indication of infection is the appearance of papules that resemble acne on the face, trunk and extremities

(Lo *et al*., 2000.). One of the things *Penicillium* is most famous for is the drug penicillin. It was used to produce the first antibiotic. The original strain, *Penicillium notatum*, was discovered in 1920 by Sir Alexander Fleming. However, it was replaced with *Penicillium chrysogenum*, a more productive species, which is now the species used in manufacturing penicillin.

However, *Penicillium* is not merely a harmful fungus, it also has many useful species. An example is *Penicillium roqueforti* used to make blue cheese. The color of the cheese comes from the spores (conidia) of the fungus. The spores are injected into the cheese curd during fermentation. *Penicillium camemberti* is another species used to produce cheese. *Penicillium chrysogenum* produces glucose oxidase, which is used as a preservative in fruit juices. This fungus has been isolated from patients with keratitis, ear infections, pneumonia, endocarditis, peritonitis, and urinary tract infections.

1.2 Statement of problem and Justification

Penicillium species are important grain storage fungi and several species have been implicated as Mycotoxin producers. It is necessary to assay the *Penicillium* isolates for production of these secondary metabolites which are considered as highly toxic to animals including humans. Species identification of *Penicillium* is also still a problematic issue leading to many *Penicillium* isolates been misidentified in literature, *Penicillium citrinum*, *Penicillium frequentans*, *Penicillium puberulum* and *Penicillium variabile* have been misidentified as aflatoxin producers. Till date, the Genus *Penicillium* has been misidentified morphologically leading to incorrect classification due to the large diversity present in the Genus. There is also very little information on *Penicillium* species isolated from Millet grains in Nigeria, Major focus has been on *Aspergillus* and *Fusarium.* One of the main challenges is the need for a rapid and reliable identification system which has led to outdated list of species and lack of verified complete database sequence. Molecular marker is a useful tool for assessing genetic similarity and resolving species identities. Hence Molecular detection of toxigenic *Penicillium* species by targeting the toxin producing gene is therefore important to boost database of information especially in Nigeria.

1.3 Objectives of the Study

Aim

This study is to determine the distribution of molds on millet grains and the *Penicillium* toxins present in the *Penicillium* species isolated from Millet grain from five southwestern regions of Nigeria.

Objectives of the Study

1. To isolate fungi present on the millet grains obtained from southwestern Nigeria.
2. To isolate and morphologically identify *Penicillium* species found as contaminants in the millet grains.
3. To determine the Phenotypic, genotypic and physiological characterization of the *Penicillium* isolates.
4. To study Mycotoxin production by the isolates under different incubation temperature and pH.
5. To molecularly detect the genes producing ochratoxin and citrinin.

CHAPTER TWO
LITERATURE REVIEW

2.0 The genus *Penicillium* (Link, 1809)

The genus was first described in the scientific literature by Johann Heinrich Friedrich Link in his 1809 work Observationes in ordines plantarum naturales (Link *et al*., 1809). Link included three species *Penicillium candidum, Penicillium expansum, and Penicillium glaucum* all of which produced brush-like conidiophores (asexual fruiting structure). The common apple rot fungus *Penicillium expansum* was selected as the type species (Samson and Pitt, 1985).

In a 1979 monograph, John I. Pitt divided *Penicillium* into four subgenera based on conidiophores morphology and branching pattern: *Aspergilloides, Biverticillium, Furcatum,* and *Penicillium* (Pitt *et.al*., 1979).

Penicillium is classified as a genus of anamorphic fungi (Kirk *et al.,* 2008) in the division Ascomycotina (Class Eurotiomycetes, Order Eurotiales, Family Trichocomaceae). The genus name is derived from the Latin root *Penicillium* meaning "painter's brush", and refers to the chains of conidia that resemble a broom (Densed brush-like spores bearing structures known as penicillia) (Haubrich, 2003).

The colonies of *Penicillium* other than *Penicillium marneffei* are rapid growing, flat, filamentous, and velvety, woolly, or cottony in texture. The colonies are initially white and become blue green, gray green, olive gray, yellow or pinkish with time. The plate reverse is usually pale to yellowish (de Larone, 1995), the thallus (mycelium) mostly consists of a branched network of multinucleate, septate, usually colorless hyphae. Many-branched conidiophores sprout on the mycelia, bearing individually constricted conidiospores. The conidiospores are the main dispersal route of the fungi, and often are green in color (Sutton *et al*., 1998).

Penicillium is a large and difficult genus found almost everywhere and mostly abundant in soil; this genus is famous for secondary metabolite production which is of commercial importance (Guillamon *et al*., 1998).

They are commonly found as agents in food spoilage (Carlile *et al*., 2001). Several members of *Penicillium* genus are good assimilators of hydrocarbon; reports have proven their ability to breakdown xenobiotic compounds into less mutagenic products. Examples are *Penicillium simplicissimum* SK9117 on phenol also *Penicillium chrysogenum* (Erika *et al*., 2012).

2.1 Important toxigenic *Penicillium* species

2.1.1 *Penicillium citreonigrum*

Produces the toxin citreoviridin which was isolated from moldy "yellow rice" implicated to be the causative agent of an acute cardiac beriberi dieases in japan. Ueno and Ueno (1972) proved that the disease was due to the presence of *Penicillium citreonigrum* in rice. This species was first discovered in 1901; in 1979 Pitt revived the name. *Penicillium citreonigrum* has a distinctive slow growth on Czapek yeast autolysate agar (CYA), pale grey green conidia and yellow mycelium. Citreoviridin is a neurotoxin which is acutely toxic to mice. (Ueno and Ueno, 1972). This toxin causes vomiting, convulsions, ascending paralysis and respiratory arrest in many animal species, in higher mammals' neurological symptoms or depressed sensory responses could occur (Uraguchi, 1969).

2.1.2 *Penicillium islandicum*

Raper and Thom (1949), were the first to recognize this species while Scopp was first to describe this species, it produces many toxic compounds, though its toxigenic status is still in doubt. It is also a slow growing species with dense colonies of brilliant orange to brown colours in both mycelium and reverse colonies. It produces four Mycotoxins which includes cyclochlorotine, islanditoxin, luteoskyrin and erythroskyrin and cyclochlorotine

and islanditoxin are very toxic causing liver cirrhosis , fibrosis and tumors in mice (Uraguchi *et al*.,1972), the other two are both liver and kidney toxins less toxic compared to the first two but are also known to be carcinogenic. *Penicillium islandicum* also causes yellowing of rice, but significance of toxin production is still unclear (Saito *et al*., 1971).

2.1.3 *Penicillium verrucosum*

Has been associated with moldy grains and *Penicillium viridicatum.* Krogh (1978) first isolated oxalic acid, Citrinin and Ochratoxin A from *Penicillium verrucosum* present in barley. Pitt (1987) later proved that Ochratoxin A was a product of this species in the temperate regions. Ochratoxin A is fat soluble and not readily excreted, thereby accumulates in the fat tissues posing a serious health risk to humans. *Penicillium verrucosum* is a slow grower on CYA agar producing bright green conidia, clear to pale yellow exudates and rough stipes (Pitt,1987) Ochratoxin A is a nephrotoxin and appears to be responsible for kidney degeneration in humans leading to death in extreme cases. A few strains produce Citrinin.

2.1.4 *Penicillium crustosum*

Raper and Thom (1949) regarded *P. crustosum* as uncommon. Recently serious outbreaks of tremogenic and neurotoxicity in domestic animals were linked to *Penicillium cyclopium* (NOW Penicillium aurantiogriseum); the toxin responsible is now known as Penitrem A which is now linked to *Penicillium crustosum.*

Penicillium crustosum is now a very common species in foods and feeds, it is the fastest growing species in genus *Penicillium* producing dull green colonies on CYA, and it produces large rough walled spherical conidia, with a distinct production of enormous numbers of conidia on MEA. It produces the toxin Penitrem A, cyclopiazonic acid and roquefortine (Pitt and Leistner, 1988). Penitrem A is a potent neurotoxin (Hockings *et al*., 1988) symptoms include trembling. Potential hazard to man is yet unknown, only symptom attributed to man was instances of dizziness and vomiting after consumption of contaminated beverages, yet recovery of patient was complete in all cases.

2.1.5 *Penicillium citrinum*

Is a well-recognized species since 1910, it is an important species in the fact that the production of toxins are of human significance due to its widespread nature, its mycotoxin can be expected to be found widely distributed in food and feed. It is the major producer of Citrinin discovered in the 1940s. It was considered antibiotics but proved to be too toxic for therapeutic use and now considered a potentially hazardous mycotoxin. *Penicillium citrinum* produces benicillus consisting of 3 to 5 divergent metulae under the stereomicroscope. They grow moderately on Czapek yeast autolysate agar (CYA). Growth seldom exceeds 10 mm after 7 days. Citrinin is a renal toxin to monogastric domestic animals (Cariton *et al*., 1974), effects on humans remains undocumented, though kidney damage appears after prolonged ingestion. Citrinin is a nephrotoxin produced by several species of the genera *Aspergillus*, *Penicillium* and *Monascus*. It is mostly formed after harvest and occurs mainly in stored grains. *Penicillium citrinum* is a main citrinin producer. This Mycotoxin has ability to cause nervous and carcinogenic effects (Benneth and klich, 2003, Singh *et al.*, 2007).

2.1.6 *Penicillium simplicissimum*

Penicillium simplicissimum is an anamorphic species which promotes plant growth, it's found on food and decaying vegetations (Pitt and Hockings, 2012). *P. simplicissimum* produces verruculogene.

2.2 Plant Pathogenic *Penicillium* species

2.2.1 *Penicillium ulaiense*

Authors familiar with green and blue molds of citrus had mistaken the fungus for *Penicillium italicum* and dismissed its unique features as variations due to particular environmental conditions. *Penicillium ulaiense* causes whisker mold in citrus fruits (Hsieh *et al*., 1987), it is a post-harvest pathogenic fungus, and it has been reported to be a post-harvest pathogen of orange in Egypt (Youssef *et al*., 2010).

2.2.2 *Penicillium expansum*

Penicillium expansum is one of the most prevalent post-harvest soft rots that infect apples. Although it is a major economic problem in apples this plant pathogen can be isolated from a wide host range, including pears, strawberries, tomatoes, corn, and rice (Domsch *et al*., 1980). This mold is a post-harvest pathogen of apples causing apple rots. This species also produces patulin a carcinogenic metabolite. Its neurotoxin nature makes it harmful if present in apple juice and products (Morales *et al*., 2007). This toxin is produced after rotting of host, since the presence of wounds on host brings about infection, careful handling can limit amount of infections, chemical treatment using chlorine bath has been shown to reduce amount of spores and decay, biofungicides have also proven effective to prevent infection but not to cure existing ones. Citrinin is also another secondary metabolite produced by *Penicillium expansum* (Janisiewicz and Wojciech, 2012).

Penicillium expansum infection acidifies the host through secretion of organic acids, also the acidification of potential hosts was found to increase *Penicillium_expansum* development, meaning an acidic environment could enhance *Penicillium_expansum* development Andersen *et al*. (2004) also reported the toxicoses of domestic animals by *Penicillium expansum.*

2.2.3 *Penicillium digitatum*

Penicillium digitatum is also a common postharvest pathogen of citrus commonly called green mold. *P. digitatum* is a necrotrophic wound pathogen requiring preexisting injured fruit peel to penetrate the host tissue and colonizes through deployment of maceration enzymes (Marcet-Houben, 2012). It acts by producing ethylene which accelerates the ripening process and covers fruit with green conidia causing it to shrivel and dry out.

2.3 Animal pathogenic *Penicillium* species

2.3.1. *Penicillium marneffei*

Penicillium marneffei (Anamorphic name) is also known as *Talaromyces marneffei.* (Telemorphic name) (Min Chen *et al.*, 2013). Discovered in 1956, is the only known thermally dimorphic *Penicillium* causing lethal systemic infection called penicilliosis, fever and anemia.

Penicillium marneffei is a unicellular member of the genus *Penicillium* with macrophages that forms extracellular elongated cells giving a special feature which differentiates it from other species, a thermal dimorphic feature, which can grow as a mycelium at 25°C (and produces filamentous, flat, radially sulcate colonies at 25°C which are bluish-gray-green at center and white at the periphery. The red, rapidly diffusing, soluble pigment observed from the reverse is very typical) and as a yeast at 37°C (*Penicillium marneffei* colonies are cream to slightly pink in color and glabrous to convolute in texture) which is its virulent form.

Penicillium marneffei is microscopically similar to other *Penicillium* species, but in its yeast phase, *Penicillium marneffei* is visualized as globose to elongated sausage-shaped cells (3 to 5 µm) that multiply by fission (Larone, 1995; de Hoog *et al*., 2000 and Sutton *et al*., 1998). It is a highly emerging pathogenic member of the genus *Penicillium* causing fatal systemic mycosis in HIV patients. Mode of transmission is not yet clear and not documented (Yeun *et al*., 2003), but have been found in bamboo rats faeces, liver, lungs and spleen, this animal was suggested to be a reservoir for this fungus since it is still unclear whether the rats are affected or are merely asymptomatic carriers of the diseases, spores of the fungus could have also been airborne and inhaled by susceptible individuals (Lo *et al.*, 2000).

Some other animal pathogens include *Penicillium corylophilum, Penicillium fellutanum, Penicillium implicatum* and *Penicillium janthinellum* while *Penicillium viridicatum* and *Penicillium waksmanii* are mosquito pathogens (de Costa *et al*., 1998).

2.4 Industrially Important *Penicillium* species

2.4.1 *Penicillium chrysogenum*

Ikram and Hina (2006) reported this species as a potent producer of protease by shake flask fermentation. It has also being implicated in penicillin production at laboratory and commercial levels in various countries (Eriksen *et al*., 1994).

Penicillium chrysogenum is a fungus, common in temperate and subtropical regions and can be found on salted food products, but it is mostly found in indoor environments, especially in damp or water damaged buildings. It was previously known as *Penicillium notatum*. It is the source of several β-lactam antibiotics most significantly penicillin, other secondary metabolites are roquefortine C, meleagrin, chrysogine, xanthocillins, secalonic acids, sorrentanone, sorbicillin and PR-toxins (de-Hoog *et al*., 2000).

Penicillium chrysogenum conidia are blue to blue-green, and the mold sometimes exudes a yellow pigment. However, *Penicillium chrysogenum* cannot be identified based on color alone. Observations of morphology and microscopic features are needed to confirm its identity and DNA sequencing is essential to distinguish it from closely related species such as *Penicillium rubens*. The sexual stage of *Penicillium chrysogenum* was discovered in 2013 by mating cultures in the dark on oatmeal agar supplemented with biotin, after the mating types (MAT1-1 or MAT1-2) of the strains had been determined using PCR amplification (Bohm *et al*., 2013). The airborne asexual spores of *Penicillium chrysogenum* are important human allergens. Vacuolar and alkaline serine proteases have been implicated as the major allergenic proteins (Shen *et al*., 2003). Industrially Penicillium chrysogenum has been used to produce penicillin and xanthocillin x used to treat pulp mill waste and for production of polyamine oxidase, phosophogluconate and glucose oxidase (de-Hoog *et al*., 2000). Penicillin is produced in liquid culture by *Penicillium chrysogenum*, and the antibiotic is used primarily against Gram positive bacteria. Today, most penicillin is manufactured by synthetic processes. Penicillin was discovered in 1928 when Alexander Fleming's lab assistant left a window open overnight and had mold spores cover his *Staphylococcus* bacteria specimens in a Petri dish (Ligon, 2004).

2.4.2 *Penicillium glabrum*

Penicillium glabrum is frequently involved in food contamination, due to its large number of spore dispersal (Pitt and Hockings, 1997). *Penicillium glabrum* produces the enzyme

xylanase which can be used for clarification and maceration of juices and wines (Knob and Carmona, 2010).

2.4.3 *Penicillium camemberti*

Penicillium camemberti is an industrially important species used in the production of Camembert, Brie, Coulommiers and Cambozola cheeses, on which colonies of *Penicillium camemberti* form a hard, white crust. It is responsible for giving these cheeses their distinctive taste. It is a close relative of *Penicillium chrysogenum* (Wolke and Robert, 2014).

When making soft cheese that involves *Penicillium camemberti*, the mold may be mixed into the ingredients before being placed in the molds, or it may be added to the outside of the cheese after it is removed from the cheese molds. *Penicillium camemberti* is responsible for the soft, buttery texture of brie and Camembert, but a too high concentration may lead to an undesirable bitter taste (Michelson and Patricia, 2010), desirable flavor could be done by maintaining the mycelia growth of the fungus using PCR techniques (Drean *et al*., 2010). Also produces the toxin cyclopiazonic acid, hence weaker strains are used to prevent production of toxin in cheese production which is unhealthy for consumers.

2.4.4 *Penicillium glaucum*

Penicillium glaucum is a mold that is used in the making of some types of blue cheese, including Bleu de Gex, Rochebaron, and some varieties of Bleu d'Auvergne and Gorgonzola (Other blue cheeses, including Bleu de Bresse, Bleu du Vercors-Sassenage, Brebiblu, Cambozola, Cashel Blue, Danish blue, Fourmed' Ambert, Fourme de Montbrison, Lanark Blue, Roquefort, Shropshire Blue, and Stilton use *Penicillium roqueforti*).

2.4.5 *Penicillium roqueforti*

Penicillium roqueforti is a common saprotrophic fungus widespread in nature; it can be isolated from soil, decaying organic matter and plants. It is known to be a common spoilage mold of silage (Skaar, 1996), and can also be found on bread as a spoilage agent (Nielsen *et*

al., 2006; Boysen *et al.*, 2000). *Penicillium roqueforti* can be used industrially for the production of blue cheeses, flavoring agents, antifungal, polysaccharides, proteases and other enzymes, it has also been used to prepare Roquefort cheese, Stilton cheese, Danish blue cheese, Cabrales cheese and other blue cheeses edible to man (Judy, 2004).

Most strain are producers of harmful secondary metabolites under certain growth conditions (Finoli *et al*., 2001) such as Aristolochene a precursor of PR toxin (Proctor and Hohn, 1993) this toxin is not stable in cheese ,it breaks down into a less toxic PR imine (Siemens and Zawitowski, 1993). Andrastins A-D on the other hand is found in blue cheese, it acts on the cheese by inhibiting proteins in the efflux of anticancer drugs from multidrug resistant cancer cells (Nielsen *et al*., 2005), neurotoxin and roquefortine C produced in cheese is at a very low level which inhibits their toxic effects. *Penicillium roqueforti* is also used to produce proteases.

Other *Penicillium* species include

Penicillium olsonii, Penicillium melanoconidium, Penicillium italicum, Penicillium griseofulvum, Penicillium commune, Penicillium nordicum, Penicillium palitans, Penicillium scleotigenum, Penicillium crustosum, Penicillium bialowiezense, Penicillium brevicompactum and *Penicillium carneum.* (Svendsen and Frisvad, 1994).

2.5 Isolation and ecology of *Penicillium* species

Penicillium can be isolated on Czapek's agar and malt-extract agar, incubated for seven days in the dark at 25^{o}C. *Penicillium* can grow in the temperature range of 22^{o} to 27^{o} C, in some laboratories; they are grown at 37^{o}C correlating to the human body temperature, an optimum temperature to determine species differentiation. *Penicillium* grows optimally at neutral to slightly acidic pH. Yeast extract is added to media for rapid and uniform fungal growth due to the presence of trace minerals and organic nitrogen needed by *Penicillium.*

Penicillium species are versatile and opportunistic, due to their osmotolerant nature they are commonly found in temperate soils since they can survive and even grow in low water activity environments.

It also found in soils that contain a high amount of organic matter, especially in forest soils that have abundant amounts of leaf litter on the surface. *Penicillium* is a very common mold that is found in most households and is known to regularly infect such foods as citrus, other fruits, and cheeses. Species of *Penicillium* are ubiquitous soil fungi preferring cool and moderate climates, present where organic material is available. Saprophytic species of *Penicillium* are among the best-known representatives of the Eurotiales which live mainly on organic biodegradable substances. They are among the main causes of food spoilage, especially species of subgenus *Penicillium*. (Samson *et al*., 2004).

Some species have a blue color, found growing on old bread and giving it a blue fuzzy texture. Some species affect the fruits and bulbs of plants, apples, pears, citrus fruits (Balgrie, 2003) and garlic (Valdez *et al*., 2006). *Penicillium viridicatum* and *Penicillium waksmanii* are potential pathogens of mosquitoes (da Coasta *et al*., 1998), *Penicillium marneffei*, which causes mortality in the Vietnamese bamboo rats (Ustianowski *et al*., 2008). *Penicillium* species are also present in the air and dust of indoor environments, such as homes and public buildings. The fungus can be readily transported from the outdoors, and grow indoors using building material or accumulated soil to obtain nutrients for growth. Growth still occur indoors even at low relative humidity, once there is sufficient moisture available on a given surface.

A British study determined that *Aspergillus* and *Penicillium* type spores were the most prevalent in the indoor air of residential properties, and exceeded outdoor levels (Fairs *et al*., 2010). Even ceiling tiles can support the growth of *Penicillium* as one study demonstrated if the relative humidity is 85% and the moisture content of the tiles is greater than 2.2% (Chang *et al*., 1995). Some *Penicillium* species cause damage to machinery and the combustible materials and lubricants used to run and maintain them. For example, *Penicillium chrysogenum, Penicillium steckii, Penicillium notatum, Penicillium cyclopium,* and *Penicillium nalgiovensis* affect fuels; *Penicillium chrysogenum, Penicillium rubrum,* and *Penicillium verrucosum* cause damage to oils and lubricants; *Penicillium regulosum* damages optical and protective glass (Semenov *et al*., 2003).

2.6 History of mycotoxins

In the year 1962, mycotoxin was coined as a result of an unusual veterinary crisis near London, England, during which approximately 100,000 turkey poults died. This mysterious turkey X disease was linked to a peanut (groundnut) meal contaminated with secondary metabolites from *Aspergillus flavus* known as aflatoxins, it sensitized scientists to the possibility that other occult (toxigenic) fungi metabolites might be deadly (Bennett and Klich, 2003). About 10,000 years ago, when mankind first began to cultivate crops and to store them from one season to the next, these toxigenic fungi and mycotoxins entered human food supplies (storage of cereals) and probably could have initiated the transition by mankind from hunter-gatherer to cultivator, at the same time providing a vast new ecological niche for fungi pathogens on grain crops or saprophytes on harvested grain, many of which produced mycotoxins.

Grains have been the major source of mycotoxins in the diet of man and his domestic animals. In the historical context, ergotism from *Claviceps purpurea* in rye has been known probably for more than 2000 years and caused the deaths of many thousands of people in Europe in the last millennium. Known in Japan since the 17th century, acute cardiac beriberi associated with the consumption of moldy rice was found to be due to citreoviridin produced by *Penicillium citreonigrum*, this toxin citreovitidin was believed to be only of historic importance until its reemergence in Brazil a few years ago. Ochratoxin A, which was once considered to be a possible cause of Balkan endemic nephropathy, is treated in a historical context (Pitt and Miller, 2016).

Currently, more than 300 mycotoxins are known, scientific attention is focused mainly on those that have proven to be carcinogenic and/or toxic. Human exposure to mycotoxins may result from consumption of plant-derived foods that are contaminated with toxins, the carry-over of mycotoxins and their metabolites in animal products such as meat and eggs (CAST, 2003) or from exposure to air and dust containing toxins (Jarvis, 2002).

Mycotoxins of greatest public health and agro-economic significance include aflatoxins (AF), ochratoxins (OT), trichothecenes, zearalenone (ZEN), fumonisins (F), tremorgenic toxins, and ergot alkaloids. These toxins account for millions of dollars annually in losses

Worldwide in human health, animal health, and condemned agricultural products. Factors contributing to the presence or production of mycotoxins in foods or feeds include storage, environmental, and ecological conditions. Often times most factors are beyond human control (Hussein and Brasel, 2001). The most important genera of mycotoxigenic fungi are *Aspergillus, Alternaria, Claviceps, Fusarium, Penicillium* and *Stachybotrys.*

The principal classes of mycotoxins include a metabolite of *A. flavus* and *Aspergillus parasiticus,* aflatoxin B_1 (AFB_1) which is the most potent hepatocarcinogenic substance known, and has been recently proven to also be genotoxic. In dairy cattle, another problem arises from the transformation of AFB_1 and AFB_2 into hydroxylated metabolites, aflatoxin M_1 and M_2 (AFM_1 and AFM_2), which are found in milk and milk products obtained from livestock that have ingested contaminated feed (Boudra *et al*., 2007). In 1993, the WHO-International Agency for Research on Cancer (World Health Organization International Agency for Research on Cancer (WHO-IARC), 1993a and World Health Organization International Agency for Research on Cancer (WHO-IARC), 1993b) evaluated the carcinogenic potential of aflatoxin (AF), ochratoxin (OT), trichothecenes, zearalenone (ZEN), and fumonisins (F). Naturally occurring AF were classified as carcinogenic to humans (Group 1) while OT and F were classified as possible carcinogens (Group 2B). Trichothecenes and ZEN, however, were not classified as human carcinogens (Group 3). The health hazards of mycotoxins to humans or animals have been reviewed extensively in recent years (Averkieva, 2009 and Yaling *et al*., 2008).

Due to the diverse chemical structures and biosynthetic origins of mycotoxins, their various biological effects, and their production by a wide number of different fungal species, their classification is based on the training of the personel categorizing the mycotoxin. Classification based on organs they affect places mycotoxins into hepatotoxins (affect the liver), nephrotoxins (affects the kidneys), neurotoxins (destroys nerve cells), immunotoxins (affects the immune system). Classification based on knowledge of cell biology groups mycotoxins into teratogens (affects the embryo), mutagens (causes genetic mutation), carcinogens (cancer causing), and allergens (causes allergic reactions). Classification based on chemical structures; they are grouped as lactones (cyclic esters of hydroxycarboxylic acids) and coumarins(a vanilla-scented compound found in many plants), biochemists

according to their biosynthetic origins groups them as (polyketides, amino acid-derived, etc.); physicians by the illnesses they cause (e.g., St. Anthony's fire, stachybotryotoxicosis), and mycologists by the fungi that produce them (e.g., *Aspergillus* toxins, *Penicillium* toxins) (Bennett and Klich, 2003).

2.7 Mycotoxin Production

Fungi are ubiquitous plant pathogens that are major spoilage agents of foods and feedstuffs. The infection of plants by various fungi not only results in reduction in crop yield and quality with significant economic losses but also contamination of grains with poisonous fungal secondary metabolites called Mycotoxins (Hussaini, 2013).

Mycotoxins are metabolites from molds that are toxic to humans and domestic animals feed including wild birds and raw materials (Moss *et al*., 2002). Mycotoxins are secondary metabolites produced by fungi which can cause diseases and death in humans and animals. All mycotoxins are from fungal origin but not all toxic compounds produced by fungi are mycotoxins. Mycotoxins could be classified based on their diverse chemical structures, biosynthetic origin, biological effects, species that produces the toxin. Classification using their biosynthetic pathway, groups Mycotoxins into Polyketides. Hajjaj *et al.* (2000) confirmed that red pigments and citrinin belong to oxygenated polyketides metabolites and are derived from the polyketides biosynthetic pathway. Mycotoxin exposure is more common where poor handling and storage methods are observed. The major groups of Mycotoxins are AFLATOXIN B1, B2,G1 and G2, OCHRATOXIN A(OTA), OCHRATOXIN B (OTB) non chlorinated form of (OTA), OCHRATOXIN C (OTC) is an ethyl ester form of (OTA), CITRININ, ERGOT, PATULIN and FUSARIUM TOXINS (Desjardins and Procter ,2007;Moss, 2008; Bennett and Klich,2003;Bayman and Baker,2006; Yin *et al*., 2008). *Penicillium* species produce a much diversified array of active secondary metabolites, including antibacterial (Lucas *et al*., 2007), antifungal

substances (Nicoletti *et al*., 2007), immunosuppressant, cholesterol-lowering agents (Kwon *et al*., 2002), and also potent mycotoxins (Frisvad and Samson, 2004).

Penicillium Mycotoxins of importance includes Ochratoxin A, Citreoviridin, Penicillic acid, Verricarum toxin, Xanthomegnin, Patulin, Cyclopiazonic acid, Citrinin, Fumitremorgin B, Verruculogen, Luteoskyrin, Verrucosidin, Roquefortine C and PR toxin (Thomas and Alistair, 2002).

2.7.1 *Aspergillus* Toxins

- **Aflatoxins**

Aflatoxins are potent carcinogens affecting man and all tested animal species. Four compounds are commonly produced in foods: aflatoxins B1, B2, G1 and G2, named for the colour of their fluorescence under ultra violet light, and their relative position on TLC plates. Sources of aflatoxin include *Aspergillus flavus* species; this species has a special affinity with three crops, maize, peanuts and cotton seed, and usually produces only B aflatoxins. Only about 40% this species produce aflatoxin. *Aspergillus parasiticus* occurs commonly in peanuts, but is quite rare in other foods. It is also restricted geographically, (Pitt *et al*., 1993). *A. parasiticus* produces both aflatoxin B and G and virtually all known isolates are toxigenic. Other species known to be capable of producing aflatoxin in culture include *A. nomius*, *A. toxicarius* and *A. parvisclerotigenus* , *but* it is very difficult to distinguish between these species and isolates may easily have been identified as *A. flavus* or *A. parasiticus*.

- **Cyclopiazonic acid**

Cyclopiazonic acid (CPA) also produced by *Penicillium* species (Holzapfel, 1968) is a potent mycotoxin that produces focal necrosis in most vertebrate inner organs. It was originally believed that aflatoxins were responsible for all the toxic effects of *Aspergillus flavus* contaminated peanuts to turkeys in the Turkey X disease syndrome, but it was later shown that cyclopiazonic acid had an additional severe effect on the muscles and bones of the turkeys (Jand *et al*., 2005).

Aspergillus flavus and *A. oryzae* often produce large amounts of CPA. *A. flavus* is common on oil seeds, nuts, peanuts and cereals, but may also produce aflatoxin on dried fruits (Pitt

and Hocking, 1997). Other producers of CPA in *Aspergillus* include *A. tamarii*, *A. pseudotamarii*, *A. parvisclerotigenus*, but the role of these fungi concerning CPA production in foods or feeds is not clear.

- **Nitropropionic Acid (BNP)**

Nitropropionic acid has been reported to be involved in sugar cane poisoning of children, but may potentially also causes other intoxications, as producers are widespread (Burdock *et al*., 2001). It can be produced by *A. oryzae* when artificially inoculated on cheese and peanuts. Unfortunately *A. flavus* has not been tested for the production of BNP, but BNP production by *A. oryzae* on peanuts indicates that *A. flavus* may be able to produce this mycotoxin in combination with aflatoxin B1, cyclopiazonic acid and kojic acid. The possible synergistic effect of these mycotoxins on mammals is unknown.

A. flavus may be an important producer of this mycotoxin in foods, but there are no surveys that include analytical determination of BNP alongside cyclopiazonic acid and aflatoxin B1. *A. oryzae* and *A. sojae* can produce BNP in miso and shoyu, but it is probably more important that their wild-type forms, *A. flavus* and *A. parasiticus* respectively, may produce BNP in foods. More research is needed in this area.

Other fungi producing BNP from sugar cane are *Arthrinium phaeospermum* and *Art. sacchari*, but other species such as *Art. terminalis*, *Art. saccharicola*, *Art. aureum* and *Art. sereanis* also produce BNP (Burdock *et al*., 2001) *Penicillium atrovenetum* is another authenticated producer of BNP, but this fungus is only found in soil.

- **Ochratoxin A**

Ochratoxin A (OA) is a nephrotoxin is also produced by *Penicillium* species, affecting all tested animal, though it's effects in man have been difficult to establish. It has been listed as a probable human carcinogen (Class 2B) , Links between OA and Balkan Endemic Nephropathy have long been sought, but not established (JECFA, 2001). *Aspergillus ochraceus* a major producer of OA (van der Merwe *et al*., 1965), and occurs in stored cereals (Pitt and Hocking, 1997) and coffee (Taniwaki *et al*., 2003). *A. ochraceus* has been shown to consist of two strains (Varga *et al*., 2000a, b; Frisvad *et al*., 2004b). The second and new strain produces large amounts of ochratoxin A consistently and has been described as *A.westerdijkiae*. The original producer of ochratoxin A was from *Andropogon sorghum*

in South Africa, has been designated as the type culture of *A. westerdijkiae* NRRL, 3174 (Frisvad *et al*., 2004b). *A. westerdijkiae* is both a better and more consistent ochratoxin producer than *A. ochraceus*, and it may also be more prevalent in coffee than *A. ochraceus*. The ex type culture of *A. ochraceus* CBS 108.08 only produces trace amounts of ochratoxin A. *Aspergillus carbonarius* (Horie, 1995) is also is a major OA producer, occurs in grapes and produces OA in grape products, including grape juice, wines and dried vine fruits (IARC, 2002; Leong *et al*., 2004) and sometimes on coffee beans (Abarca *et al*., 2004; Taniwaki *et al*., 2003). *Aspergillus niger* is an extremely common species, but only few strains appear to be producers of OA, so this species may be of much less importance than *A. carbonarius* in grapes, wine and green coffee beans (Abarca *et al*., 1994; Taniwaki *et al*., 2003; Leong *et al*., 2004). However, *A. niger* NRRL 337, referred to as the "food fungus", produces large amounts of OA in pure culture and is used for fermentation of potato peel waste and for animal feed (Schuster *et al*., 2002).

Aspergillus steynii, from the *Aspergillus* section *Circumdati*, is also a very efficient producer of OA, and has been found in green coffee beans, mouldy soy beans and rice (Frisvad *et al*., 2004b). Several Aspergilli can produce ochratoxin A in large amounts, but they appear to be relatively rare. In *Aspergillus* section *Circumdati* (formerly the *Aspergillus ochraceus* group), the following species can produce ochratoxin A: *Aspergillus cretensis*, *A. flocculosus*, *A. pseudoelegans*, *A. roseoglobulosus*, *A. sclerotiorum*, *A. sulphureus* and *Neopetromyces muricatus* (Frisvad *et al*., 2004b).

- **Sterigmatocystin**

Sterigmatocystin is a possible carcinogen. However, its low solubility in water or gastric juices limits its potential to cause human illness. The major source of sterigmatocystin in foods is *Aspergillus versicolor* which is common on cheese, but may also occur on other substrates (Pitt and Hocking, 1997). A large number of species are able to produce sterigmatocystin, including *Chaetomium* spp., *Emericella* spp., *Monocillium nordinii* and *Humicola fuscoatra (*Joshi *et al*., 2002). These species are unlikely to contaminate foods

2.7.2 *Fusarium* Toxins

- **Culmorin**

Culmorin has been found to have a low toxicity in several biological assays (Pedersen and Miller, 1999) but a synergistic effect with deoxynivalenol towards caterpillars has been demonstrated (Dowd *et al*., 1989). Culmorin and hydroxyculmorins which also contained deoxynivalenol and acetyl-deoxynivalenol have been detected in cereals (Ghebremeskel and Langseth, 2001). *F. culmorum* and *F. graminearum* are major producers of culmorin and are found in cereals, the less widely distributed species of *F. poae* and *F. langsethiae* are also consistent producers of culmorin and their derivatives (Thrane *et al*., 2004). Other species producing culmorin are *F. crookwellense* and *F. sporotrichioides* and are also found in cereals.

- **Fumonisins**

Since the discovery of fumonisins in the late 1980s much attention has been paid to this highly toxic compounds. Major sources of fumonisins include *F. verticillioides* (formerly known as *F. moniliforme*; Seifert *et al*., 2003) and *F. proliferatum* which are found in maize. These species produce fumonisins in maize and to a lesser extent other cereal crops. Other fumonisin producing species are *Fusarium nygamai, F. napiforme, F. thapsinum, F. anthophilum* and *F. dlamini* from millet, sorghum and rice. Some strains of these species have also been isolated from soil debris.

- **Fusaproliferin**

Fusaproliferin is a recently discovered mycotoxin which shows teratogenic and pathological effects in cell assays. *Fusarium proliferatum* and *F. subglutinans* are the major sources in maize and other cereal grains. The fungi and fusaproliferin have been detected in Europe, North America and South Africa (Wu *et al*., 2003). A few strains of *F. globosum, F. guttiforme, F. pseudocircinatum, F. pseudonygamai* and *F. verticillioides* have been found to produce fusaproliferin.

- **Moniliformin**

Moniliformin is cytotoxic in nature, it acts by inhibiting protein synthesis and enzymes, it causes chromosome damages and induces heart failure in mammals and poultry. Moniliformin has been found worldwide in cereal samples. In maize *F. proliferatum* and *F. subglutinans* are the main producers of moniliformin, whereas *F. avenaceum* and *F. tricinctum* are the key sources in cereals grown in temperate climates. In sorghum, millet and rice *F. napiforme*, *F. nygamai*, *F. verticillioides* and *F. thapsinum* may be responsible for moniliformin production. Some strains of *F. oxysporum* produce a significant amount of moniliformin under laboratory condition (Schütt *et al*., 1998).

- **Trichothecenes**

Trichothecenes are haematotoxic and immunosuppressive in nature. In animals, vomiting, feed refusal and diarrhoea are typical symptoms of the infections, Skin oedema in humans has also been observed. An Europian union working group has reported on trichothecenes in food (Schothorst and van Egmond, 2004).

- **Deoxynivalenol (DON) and Acetylated Derivatives (3ADON, 15ADON)**

Deoxynivalenol (DON) and its acetylated derivatives (3ADON, 15ADON) are the most important trichothecenes. Numerous reports on world-wide occurrence have been published and several international symposia and workshops have focussed on DON (Larsen *et al*., 2004). *Fusarium graminearum* and *F. culmorum* are consistent producers of DON, especially in cereals (Nielsen and Thrane, 2001). Recently, *F. graminearum* has been divided into nine phylogenetic species based on DON production (O'Donnell *et al*., 2004). Production of DON by *F. pseudograminearum* has been reported, but this species is restricted to warmer climates.

- **Nivalenol (NIV) and Fusarenon X (FX, 4ANIV)**

Nivalenol (NIV) and fusarenon X (FX, 4ANIV) occur in the same commodities as DON due to the high degree of similiarity between them. NIV is often detected in much lower concentrations than DON, but is considered to be more toxic.

Fusarium graminearum is a well known producer of NIV and FX in cereals. In temperate climates *F. poae*, which is a consistent producer of NIV (Thrane *et al*., 2004), may be responsible for NIV in cereals. Strains of *F. culmorum* that produce NIV are less commonly isolated than those that produce DON producers. *F. equiseti* and *F. crookwellense* found in some cereal samples and in vegetables may also produce NIV. In potatoes *F. venenatum* strains that produce NIV have been detected (Nielsen and Thrane, 2001).

- **T-2 toxin**

T-2 toxin is one of the most toxic trichothecenes, whereas the derivative HT-2 toxin is less toxic. Due to structural similarity these toxins are often included in the same analytical method. *Fusarium sporotrichioides* and *F. langsethiae*, which are frequently isolated from cereals in Europe are consistent producers of T-2 and HT-2, only a few T-2 and HT-2 producing strains of *F. poae* and *F. sambucinum* have been found (Nielsen and Thrane,2001; Thrane *et al.*, 2004).

- **Diacetoxyscirpenol (DAS)**

Diacetoxyscirpenol (DAS) and monoacetylated derivatives (MAS) are a fourth group of important trichothecenes in food. *Fusarium venenatum* isolates, often produce high levels of DAS and this species is frequently isolated from cereals and potatoes (Nielsen and Thrane, 2001). *F. poae* isolates also often produces high levels of DAS. *Fusarium equiseti* isolates can produce DAS and MAS in high amounts, but this species is not frequently isolated from cereals and vegetables. *F. sporotrichioides* and *F. langsethiae* also produce DAS and MAS; however at lower levels (Thrane *et al.*, 2004). *F. sambucinum* isolates which also produce DAS and MAS are possibly the cause of DAS in potatoes (Ellner, 2002).

- **Zearalenone**

Zearalenone causes hyperoestrogenism in swine and its possible effects in humans have also been reported. Derivatives of zearalenone have been used as growth promoters in livestock; however, this is now banned in European Union (Launay *et al*., 2004). *Fusarium graminearum* and *F. culmorum* are the most pronounced producers of zearalenone and its several derivatives. They occur frequently in cereals all over the world. Recently, *F. graminearum* has been divided into nine phylogenetic species based on the production of Zearalenone (O'Donnell *et al*., 2004). Under laboratory conditions *Fusarium equiseti* produces a number of zearalenone derivatives in high amounts, but little is known about production under natural conditions. *F. crookwellense* also produces zearalenone.

2.7.3 *Penicillium* Toxins

- **Chaetoglobosins**

The chaetoglobosins are toxic compounds that may be involved in mycotoxicosis. They are produced by common food-borne Penicillia and have been found to occur naturally (Andersen *et al*.,2004). *Penicillium expansum* and *P. discolor* are major sources of the chaetoglobosins. Both species cause spoilage in fruits and vegetables, and the latter species also occurs on cheese (Frisvad and Samson, 2004b).

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- **Citreoviridin**

Citreoviridin was reported as a cause of acute cardiac beriberi (Ueno, 1974), but a more in depth toxicological evaluation of this metabolite is needed. It has been associated with yellow rice disease, but this disease has also been associated with *P. islandicum* and its toxic metabolites cyclic peptides cyclochlorotine and islanditoxin, and anthraquinones luteoskyrin and rugulosin (Enomoto and Ueno, 1974).

- **Citrinin**

Citrinin is a nephrotoxin, but probably of less importance than ochratoxin A. However, producers of citrinin are widespread and common in foods. Citrinin has been found in cereals, peanuts and meat products (Reddy and Berndt, 1991). *P. citrinum* is an efficient and consistent producer of citrinin and has been found in foods world-wide (Pitt and Hocking, 1997). *P. verrucosum* is predominantly cereal-borne in Europe and often produces citrinin as well as ochratoxin A (Frisvad *et al*., 2005b). *P. expansum* which is common in fruits and other foods sometimes produces citrinin. *P. radicicola* is commonly found in onions, carrots and potatoes (Overy and Frisvad, 2003). *Aspergillus terreus, A. carneus, P. odoratum* and *P. westlingii* have been reported as producers of citrinin, but are not likely to occur often in foods.

- **Cyclopiazonic acid**

Penicillium commune and its domesticated form *P. camemberti*, and the closely related species *P. palitans*, are common on cheese and meat products and may produce cyclopiazonic acid in these products (Frisvad *et al*., 2004c). *P. griseofulvum* is also a major producer of cyclopiazonic acid, and may occur in long stored cereals and cereal products such as pasta (Pitt and Hocking, 1997). *P. dipodomyicola* occurs in the environs of the kangaroo rat in the USA, but has also been reported from rice in Australia and in a chicken feed mixture in Slovakia (Frisvad and Samson, 2004b).

- **Mycophenolic acid**

Despite having a low acute toxicity, mycophenolic acid may be a very important indirect mycotoxin as it is highly immunosuppressive, perhaps influencing the course of bacterial and fungal infections (Bentley, 2000). *Penicillium brevicompactum* is a ubiquitous species and may produce mycophenolic acid in foods such as ginger (Overy and Frisvad, 2005). Two other major species producing mycophenolic acid are *P. roqueforti* and *P. carneum*. Another important producer is *Byssochlamys nivea* (Puel *et al*., 2005). Mycophenolic acid has been found to occur naturally in blue cheeses (Lafont *et al*., 1979). The soil-borne species *Penicillium fagi* also produces mycophenolic acid (Frisvad and Filtenborg, 1990)

- **Ochratoxin A**

Penicillium verrucosum (Frisvad, 1985; Pitt, 1987) is the major producer of ochratoxin A in cool climate stored cereals (Lund and Frisvad, 2003). *Penicillium nordicum* (Larsen *et al*., 2001) is the main OA producer found in manufactured meat products such as salami and ham. Both OA producing *Penicillium* species have been found on cheese also, but have only been reported to be of high occurrence on Swiss hard cheeses (as *P. casei.* Staub, 1911). The ex type culture of *P. casei* is a *P. verrucosum (*Larsen *et al*., 2001).

- **Patulin**

Patulin is generally very toxic for both prokaryotes and eukaryotes, but the toxicity for humans has not been conclusively demonstrated. Several countries in Europe and the USA have now set limits on the level of patulin in apple juice. *Penicillium expansum* is the most important source of patulin. *P. expansum* is the major species causing spoilage of apples and pears, and is the major source of patulin in apple juice and other apple and pear products. *Byssochlamys nivea* may be present in pasteurised fruit juices and may produce patulin and mycophenolic acid (Puel *et al.*, 2005). *Penicillium griseofulvum* is a very efficient producer of high levels of patulin in pure culture, and it may potentially produce patulin in cereals, pasta and similar products. *P. carneum* may produce patulin in beer, wine, meat products and rye-bread as it has been found in those substrates (Frisvad and Samson, 2004b), but there are no reports yet on patulin production by this species in those foods. *P. carneum* also produces mycophenolic acid, roquefortine C and penitrem A (Frisvad *et al*., 2004c). *P. paneum* occurs in rye-bread (Frisvad and Samson, 2004b), but actual production of patulin in this product has not been reported. *P. sclerotigenum* is common in yams and has the ability to produce patulin in laboratory cultures. The coprophilous fungi *P. concentricum, P. clavigerum, P. coprobium, P. formosanum, P. glandicola, P. vulpinum, Aspergillus clavatus, A. longivesica* and *A. giganteus* are very efficient producers of patulin in the laboratory, but only *A. clavatus* may play any role in human health, as it may be present in beer malt (Lopez-Diaz and Flannigan, 1997). *Aspergillus terreus, Penicillium novae-zeelandiae, P. marinum, P. melinii* and other soil-borne fungi may produce patulin in pure culture, but are less likely to occur in any foods.

- **Penicillic acid**

Penicillic acid (Alsberg and Black, 1913) and dehydropenicillic acid (Obana *et al*., 1995) are small toxic polyketides, but their major role in mycotoxicology may be in their possible synergistic toxic effect with OA (Stoev *et al*., 2001) and possible additive or synergistic effect with the naphtoquinones hepatotoxins xanthomegnin, viomellein and vioxanthin. Penicillic acid is likely to co-occur with OA, xanthomegnin, viomellein and vioxanthin produced by members of Aspergillus section *Circumdati* and *Penicillium* series *Viridicata* (which often co-occur with *P. verrucosum*). The *Aspergillus* species often occur in coffee and the Penicillia are common in cereals. The major sources of penicillic acid are *P. aurantiogriseum*, *P. cyclopium*, *P. melanoconidium* and *P. polonicum* (Frisvad and Samson, 2004b) and all members of *Aspergillus* section *Circumdati* (Frisvad and Samson, 2000). Penicillic acid is produced by *P. tulipae* and *P. radicicola*, which are occasionally found on onions, carrots and potatoes (Overy and Frisvad, 2003). Penicillic acid has been found in one strain of *P. carneum* (Frisvad and Samson, 2004b).

- **Penitrem A**

Penitrem A is a highly toxic tremorgenic indol-terpene. It has primarily been implicated in animal mycotoxicoses (Rundberget and Wilkins, 2002), but has also been suspected to cause tremors in humans (Cole *et al*., 1983; Lewis *et al*., 2005). *Penicillium crustosum* is the most important producer of penitrem A (Pitt, 1979). This species is of world-wide distribution and often found in foods. This mycotoxin is produced by all isolates of *P. crustosum* examined by Pitt (1979) and Sonjak *et al*. (2005). *P. melanoconidium* is common in cereals (Frisvad and Samson, 2004b), but it is not known whether this species can produce penitrem A in infected cereals. *P. glandicola*, *P. clavigerum*, and *P. janczewskii* are further producers of penitrem A (Frisvad and Samson, 2004b; Frisvad and Filtenborg, 1990), but have been recovered from foods only sporadically.

- **Rubratoxin**

Rubratoxin is a potent hepatotoxin (Engelhardt and Carlton, 1991) and is of particular interest as it has been implicated in severe liver damage in three Canadian boys, who drank rhubarb wine contaminated with *Penicillium crateriforme*. One of the boys needed to have his liver transplanted (Richer *et al*., 1997). *P. crateriforme* is the only known major producer of rubratoxin A and B (Frisvad, 1989).

2.8. Effects of mycotoxins on Humans

Human's exposure to mycotoxins has led to a condition known as Mycotoxicoses, a toxicological syndrome (mycotoxicoses) which can be categorized as acute or chronic. Acute syndrome generally has a rapid onset and an obvious toxic response, while chronic syndrome is characterized by low-dose exposure over a long time period, resulting in cancers and other generally irreversible effects (James, 2005), before the discovery and implementation of modern milling practices, *Fusarium* species have been implicated in several human outbreaks of mycotoxicoses. Cereal grains contaminated with *F. sporitrichoides* and *F. poae* were implicated in alimentary toxic aleukia in Russia from 1932 to 1947. Symptoms included mucous membrane hyperaemia, oesophageal pain, laryngitis, asphyxiation, gastroenteritis, and vertigo (Lewis *et al*., 2005). Aflatoxicosis is a toxic hepatitis leading to jaundice and, in severe cases causes death. Repetitive incidents of this nature have occurred in Kenya (during 1981, 2001, 2004 and 2005), India, and Malaysia (Shephard, 2004 and Lewis *et al*., 2005). AFB_1 has been extensively linked to human primary liver cancer in which it acts synergistically with Hepatitis B virus (HBV) infection and was classified by the International Agency for Research on Cancer (IARC) as a human carcinogen (Group 1 carcinogen) (IARC, 1993). This combination represents a heavy cancer burden in developing countries. A recent comparison of the estimated population risk between Kenya and France highlighted the greater burden that can be placed on developing countries (Shephard, 2006).

The largest risk of AF to humans is usually the result of chronic dietary exposure. Such dietary AF exposures have been associated with human hepatocellular carcinomas, which may be compounded by hepatitis B virus. Approximately 250,000 deaths are caused by hepatocellular carcinomas in China and Sub-Saharan Africa annually and are attributed to

risk factors such as high daily intake (1.4 μg) of AF and high incidence of hepatitis B (Wild *et al*., 1992). Aflatoxins have been found in tissues of children suffering from Kwashiorkor and Reye's syndrome and were thought to be a contributing factor to these diseases. Reye's syndrome, which is characterized by encephalopathy and visceral deterioration, results in liver and kidney enlargement and cerebral edema (Blunden *et al*., 1991). Aflatoxin has long been linked to Kwashiorkor, a disease usually considered a form of protein energy malnutrition, although some characteristics of the disease are known to be among the pathological effects caused by aflatoxins in animals. Aflatoxin exposure was associated with reduced levels of secretory immunoglobulin A (IgA) in Gambian children (Turner *et al*., 2003). Changes in differential subset distributions and functional alterations of specific lymphocyte subsets have been correlated with aflatoxin exposure in Ghanaian adults and indicate that aflatoxins could cause impairment of human cellular immunity that could decrease resistance to infections (Jiang *et al*., 2005), of the other health risk factors, the morbidity and mortality associated with unsafe sex, unsafe water and indoor smoke, arises from infectious diseases, such as HIV/AIDS, infectious diarrhoea and lower respiratory tract infection, respectively. The immunological suppression associated with aflatoxin and possibly DON could adversely affect all these outcomes. The modulating effect of aflatoxins in cases of zinc, iron and vitamin A deficiency in human health is less clear, but evidence from animal nutrition would suggest it could be significant (Williams *et al*., 2004). Fumonisins have been implicated in one incident of acute food-borne disease in India in which the occurrence of borborygmy, abdominal pain, and diarrhoea was associated with the consumption of maize and sorghum contaminated with high levels of fumonisins. Fumonisin B_1, the most abundant of the numerous fumonisin analogues, was classified by the IARC as a Group 2B carcinogen (possibly carcinogenic in humans) (IARC, 2002). Fumonisins, which inhibit the uptake of folic acid via the folate receptor, have also been implicated in the high incidence of neural tube defects in rural populations known to consume contaminated maize, such as the former Transkei region of South Africa and areas of Northern China (Marasas *et al*., 2004). Both DON and ZEN from toxic *Fusaria* have been linked to scabby grain toxicoses in the USA, China, Japan, and Australia, symptoms include nausea, vomiting, and diarrhea. Fumonisin B_1 was associated with an illness outbreak in India with symptoms of acute onset of abdominal pain and

diarrhea. Fumonisins also have been implicated in oesophageal cancer in China (Yoshizawa *et al*., 1994). However, with limited causal relationships and the presence of several confounding factors, data compiled by the International Agency for Research on Cancer were not conclusive for F carcinogenicity in humans (Casegnaro and Wild, 1995). Trichothecenes have been suggested as potential biological warfare agents. For example, T-2 toxin was implicated as the chemical agent of 'yellow rain' used against the Lao Peoples Democratic Republic from 1975 through 1981 (Peraica *et al.*, 1999). In an investigation of similar biological warfare agents in Cambodia from 1978 to 1981, T-2 toxin, DON, ZEN, nivalenol, and DAS were isolated from water and leaf samples collected from the affected areas (Peraica *et al*., 1999). Clinical symptoms preceding death included vomiting, diarrhea, hemorrhage, breathing difficulty, chest pain, blisters, headache, fatigue, and dizziness. In addition to nephritic congestion, autopsy findings included necrosis of the lining of the stomach and upper small intestine, lungs, and liver. It should be noted, however, that the origin of the samples of yellow rain is still a subject of debate. For instance, one theory attributed the source of illnesses to unidentified endemic factors because the yellow rain was found to be a native bee fecal material devoid of mycotoxins (Seeley *et al*., 1985).

2.9 Effects of mycotoxin on animals

As with other species, the kidney is the primary target organ of OTA in dogs and cats. In a study with dogs, pacing and vomiting were observed at an OTA dose of 0.2 mg/kg. At doses between 0.2 and 3.0 mg/kg symptoms of intoxication in dogs included anorexia, polydipsea, polyuria, anxiety, prostration, and death. The necropsy findings included epithelial degeneration (proximal tubules), mucohemorrhagic enteritis (cecum, colon, and rectum) and necrosis of the lymphoid tissues (spleen, tonsil, thymus, and peripheral lymph nodes) (Bird, 2000).

Ruminants such as cattle, sheep, goats, and deer are less known for their sensitivity to the negative effects of mycotoxins than are non-ruminants. However, production (milk, beef, or wool), reproduction, and growth can be altered when ruminants consume mycotoxin-contaminated feed for extended periods of time (Hussein and Brasel, 2001).

2.10 Molecular Identification of *Penicillium*

Classification of fungi still relies mainly on morphological and cultural characters, the same holds true for the monographs of aspergilli and penicillia (Raper and Fennell, 1965). Additional tools for the classification of fungi are substrate utilization patterns. Identification of *Penicillium* is quite difficult due to the large size of the genus, and so many common species look alike to the inexperienced. There is also a great deal of variability within the species; therefore, unambiguous identification of the species requires molecular identification (Guerche *et al*., 2004). Seifert *et al.* (2000); Kiil and Sasa (2000) described the use of the BIOLOG system for classifying and identifying *Penicillium* species. Today, comparison of DNA sequences for taxonomic studies is the approach most widely used. Berbee and Taylor (2001) give an overview on fungal molecular evolution and useful genes. However, DNA sequencing is still rather expensive, alternative methods for DNA base comparison have been developed (Scott and Strauss, 2000). DNA fingerprinting is comparably cheap and allows discrimination of fungal strains from the genus down to clone level (Chulze *et al*., 2000, Louws *et al*., 1999). RFLP was the first DNA fingerprinting technique used in microbial taxonomy. This technique has the drawback that it requires vast quantities of high quality DNA. RFLP was applied to examine the taxonomic position of the species in the *Aspergillus* section *Nigri* (Varga *et al*., 2000b, Parenicova *et al*., 2000). RAPD or AP-PCR is a PCR-based fingerprinting technique relying on the random amplification of DNA fragments. This technique is straightforward and has been applied for the characterization of *Penicillium roqueforti* strains (Geisen *et al*., 2001).

The polymerase chain reaction first described by Saki *et al*. (1985) requires the presence of specific target sequences. Polymerase chain reaction (PCR) is simply used to amplify or replicate a single region of DNA (Deoxyribonucleic acid) i.e. the sequence can be altered in predetermined ways (Kennedy and Oswald, 2011).

Sequencing of PCR amplicon (a piece DNA or RNA fragment) of conserved regions of DNA (i.e. marker genes) is done to identify and described taxonomic group distributions, many research has focused on sequencing functional gene amplicon (Vetrovsky and Baldrian, 2013).

The locus mostly used for the description of fungal taxons is the ITS (internal transcribed spacer) region of the ribosomal RNA genes, which identifies fungi to its genus or species level and has highly been represented in sequence database (Seifert, 2009). The ITS region is amplified from the target fungus and sequenced to identify regions of DNA unique to the fungus of interest. The ITS region refers to the spacer DNA (non coding DNA) found between the small subunit ribosomal RNA (rRNA) and the large subunit rRNA genes in the chromosomes, it has been recommended as the universal fungal barcode sequence (Peay *et al*, 2008;Schoch *et al*., 2012). due to limitations associated with ITS as species marker in *Penicillium*, secondary barcodes(identification markers) are employed for species level identification such as β-tubulin(BenA),Calmodulin(CAM) or RNA polymerase II second largest subunit(RPB2) genes (Frisvad and Samson, 2004). Among the molecular tools available, β-tubulin gene has proven useful for identification of closely related *Penicillium* species (Seifert and Louis-Seize, 2000; Samson *et al*., 2004; Kim *et al*., 2006).

2.11 Amplification of *Penicillium* species

Amplification is the production of copies of a target sequence (the amplicon). Amplicons are direct repeat of head to tail or inverted repeat (head to head or tail to tail) gene sequence (Cohn and Moldave, 1996).

The standard thermal cycle for the amplification process for ITS, BenA and CaM genes involves an initial denaturing temperature of 94^{o}C for 5 minutes in 35 cycles, denaturing temperature of 94^{o}C for 45 minutes, annealing temperature of 55^{o}C for 45seconds, elongation temperature of 72^{o}C for 60 seconds and a final elongation temperature of 72^{o}C for 7 minutes and a rest period of 10^{o}C (Visagie *et al*., 2014).

Table 1: Primers used for amplification of ITS, BenA, CaM and RPB2 regions

Locus	Primer name	Direction	Sequence	Reference
Internal Transcribed Spacer (ITS).	ITS1	Forward	TCCGTAGGTGAACCTGCGG	White *et al*., 1990.
	ITS4	Reverse	TCCTCCGCTTATTGATATGC	
B-Tubulin (BenA)	Bt2a	Forward	GGTAACCAAATCGGTGCTGCTTTC	Glass & Donaldson, 1995.
	Bt2b	Reverse	ACCCTCAGTGTAGTGACCCTTGGC	
Calmodulin (CaM)	CMD5	Forward	CCGAGTACAAGGARGCCTTC	Hong *et al.*, 2006.
	CMD6	Reverse	CCGATRGAGGTCATRACGTGG	
	CF1	Forward	GCCGACTCTTTGACYGARGAR	Peterson *et al*., 2005
	CF4	Reverse	TTTYTGCATCATRAGYTGGAC	
RNA polymerase II second largest subunit(RPB2)	5F	Forward	GAYGAYMGWGATCAYTTYGG	Liu *et al.*,1999
	7CR	Reverse	CCCATRGCTTGYTTRCCCAT	
	5Feur	Forward	GAYGAYCGKGAYCAYTTCGG	Houbraken *et al.*, 2012b
	7CReur	Reverse	CCCATRGCYTGYTTRCCCAT	

Source: Studies in Mycology. Visagie *et al*. (2014).

2.12 Detection of Mycotoxigenic species by multiplex PCR

To detect Mycotoxigenic fungi unique DNA sequences of the organisms are chosen as primer binding sites, these genes which are involved in the Mycotoxin biosynthetic pathway forms a basis for specific detection of these toxigenic strains. A number of approaches have been taken to develop assays with the desired level of specificity, whether to detect a single species or to detect strains from different species with the potential to produce a common Mycotoxin (Brown *et al*., 2001). Most researchers have used more specific and sensitive molecular tests like Enzyme Linked Immuno assays and Polymerase Chain reaction (PCR). Both the approaches provide fast analysis of results but the PCR technology is more specific and sensitive for the early detection of toxigenic fungi.

Multiplex PCR refers to the use of PCR to amplify several different DNA sequences at the same time (performing many separate PCR reactions together in one reaction). It involves amplifying the DNA of target samples using multiple primers; a temperature mediated DNA polymerase in a thermal cycler to produce amplicons of varying sizes that are specific to different DNA sequences. Annealing temperature for each primer sets must be optimized to work within a single reaction, the base pair lengths (amplicons sizes) must be distinct enough to form different bands when visualized by gel electrophoresis. If overlap of sizes occurs, then the different amplicons can be visualized using primers that have been dyed with different color fluorescent dyes (Hayden, *et al*., 2008). Studies by Shimizu *et al.* (2007) was the first report on the presence of an activator of citrinin (CT) biosynthesis in CT producing fungi, this Mycotoxin (CT) is a secondary metabolite from *Penicillium citrinum* and has been found in fungal such as *Penicillium*, *Aspergillus* and *Monascus*. CT belongs to the group of polyketides synthesized by the type I polyketides synthase (PKS). In a previous study a polyketides synthase (PKS) gene for CT (pksCT) was cloned from *M.purpureus* (Shimizu *et al*., 2005).

CHAPTER THREE
MATERIALS AND METHODS

3.1.1. Geographical focus: Southwestern Nigeria

3.1.2. Field survey, study area and selection of site: Five states were chosen as study areas in southwestern Nigeria. In each study area three markets were randomly selected for sampling.

3.1.3. Collection of samples

Millet samples (500g) were collected within the Southwestern region of Nigeria namely Lagos, Ogun, Oyo, Osun and Ondo state (study area), were obtained from three major markets in each study area and were randomly collected from three different market women from each market, to make a total of nine samples from each study area, were placed in sterile polyethylene bags labeled properly and taken to the laboratory for further studies.

3.1.4. Sterilization of glassware and other equipment

All glass wares such as McCartney bottles, Measuring cylinders, Beakers, Conical Flasks were all made of Pyrex and were washed with liquid detergent rinsed in several changes of tap water, air dried and arranged inside an oven(Model Gallenkamp Hotbox Oven, Gallenkamp, UK), Oven was heated for 160^0C for 1hour for sterilization of glass wares which were used immediately.

3.2. Preparation and sterilization of culture media

Dichloran Rose Bengal Chloramphenicol Agar (DRBC), Potato Dextrose Agar (LAB M), Potato dextrose broth, Czapek yeast auto lysate agar (LAB), Yeast extract sucrose agar were prepared according to manufacturers' specifications and autoclaved at 121^0C for 15minutes at 1.1 kg/cm^2. Each medium was prepared in 500ml of distilled water in 500ml conical flasks. Conical flasks with agar suspensions were cotton-plugged with non-absorbent cotton wool wrapped with aluminum foil paper. Distilled water was poured into the chamber of (a market forge sterilmatic, Everett) the autoclave and all the air was removed before closing the exhaust valve. Media were autoclaved and allowed to cool to 45^0C inside the laminar flow hood (model EACI ENVIRCO) approximately 12-15ml quantity was poured into sterile disposable petri dishes. The plates were left in the hood to solidify before inoculation.

3.3. Determination of moisture content of millet grain

Fifty grams of each sample were milled and dried in an oven at 80^0C to obtain a constant weight.

The moisture content was calculated as percentage of the dry weight. i.e.

$$\frac{\text{Wet weight – dry weight x 100\%}}{\text{Wet weight}}$$

3.4. Isolation of Fungi.

One hundred grains from each millet samples per location was assayed for the presence of mold using direct plating technique for internal infestation (Pitt and Hocking, 1997). Grains were surface sterilized for l min in sodium hypochlorite (NaOCl), washed in three changes of sterile distilled water and plated (10 grains per plate) directly on the surface of Dichloran Rose-Bengal Chloramphenicol Agar (DRBC), (King *et al*., 1979). Containing 16.527g/L DRBC and 15g/L agar amended with 0.1g/L of chloramphenicol to suppress bacterial contamination. Five plates of each sample for surface sterilized grains and five plates of each sample for non-surface sterilized. Plates were incubated at 28^0C +/-2^0C for 7days.

All isolates were also inoculated at three points onto Czapek Yeast Agar (CYA, for colony characters). The pH was adjusted to 6.3 for CYA, measurements and observations were performed in duplicate from cultures inoculated. Plates were incubated at 28^0C+/-2^0C for 7 days.

3.5 Identification of Fungi

The number of grains on which fungal growth occurred revealed morphologies consistent with *Aspergillus, Rhizopus and Penicillium.* Morphological features such as colony pigmentation, colony texture and shape were observed on culture plates.

3.6 Identification of *Penicillium* species

Penicillium isolates were identified with the help of keys developed by Pitt (1979 & 2000) and Frisvad and Samson (2004). Macromorphological identification was done using media (Czapek Yeast Autolysate agar (CYA) and Yeast extract sucrose agar (YES) which were modified by adding copper sulphate and zink sulphate ensure proper development of the green pigmentation of the conidial color of the *Penicillium* is isolates as proposed by Filtenborg *et al*., 1990. Micromorphology characters slides were prepared from 5-7day old Malt extract agar (MEA) colonies, lactophenol blue was used as mounting agar as described by Frisvad and Samson (2004). Micrographs of these slides were taken to observe features such as conidiophores branching pattern, metulae, phialides and conidia which are used in classification of *Penicillium* as described by Pitt (1979).

3.6.1 Ehrlich test

All Isolates were examined for production of cyclopiazonic acid and other alkaloids reacting with Ehrlich reagent using a filter paper method. Ehrlich reagent consists of 2g of 4-dimethylaminobenzaldehyde in 96% ethanol (85ml) added 15ml 10N HCL.A *ca*. four mm agar plug was cut out of the center of a colony grown on CYA and incubated at 25°C for 7 days. A round piece (1cm diameter) of wetted filter paper (What man No. 1) was placed on the mycelia side of the plug. The appearance of a violet ring after 2-6 minutes shows a positive test that culture contains cyclopiazonic acid or related alkaloids. Reaction

after 7-10minutes was regarded as weak. After 10minutes violet ring fades away. Some fungi produce alkaloids that react with Ehrlich reagent to give pink to red or yellow rings. Lund (1995a). *Penicillium* isolated were authenticated by Odebode A.C. (2014).

3.7 Maintenance of cultures

Only suspected *Penicillium* cultures were transferred to PDA plates and slants, which were stored for molecular identification and Mycotoxigenic gene detection.

3.8 Inoculum preparation

The *Penicillium* isolates were grown on PDA broth in Petri dishes at 28^0C (+/-2^0C) for 7 days. Each flasks of the growth medium was inoculated with one disc of agar plus mycelia 5mm of each isolate obtained by using a sterile cork borer for first inoculation, subsequent inoculations were done by transferring seven (7) day old culture of 5mm diameter mycelia disc into fresh broth as inoculums.

After incubation period the mycelium produced was harvested with preweighed filter paper (What man no.1, 15cm diameter). The filter paper with mycelia were dried in an oven at 80°C and weighed. The difference in weight of filter paper and the mycelium gave the dry weight.

3.9 Physiological studies

The *Penicillium* isolates were used for physiological experiment. Growth medium used was Potato dextrose Broth, two hundred (200) grams of Irish potato (*Solanum tuberosum*) peeled, washed and cut into pieces boiled with 1 liter of water till potato pieces were soft. The potato preparation was mashed and squeezed through a sieve to obtain a pulp which was placed in a liter flask. Twenty (20) grams of dextrose was added and the medium was made up to 1 liter with distilled water. This was dispensed into conical flask, plugged with cotton wool and foil, autoclaved at 121^0C for 15minutes at 15-Pa pressure and allowed to cool. Three (3) drops of chloramphenicol added (Ataga *et al*., 2010).

3.9.1 Determination of incubation time

Incubation time studied in this work was 7days, 14days and 21days. The growth medium was sterilized and dispensed in 25ml portions into each of several 250ml conical flasks and inoculated with fungal isolates. All flasks were incubated at 30°C for an incubation period of 7, 14 and 21 days each after which the mycelia produced was harvested and the dried weight was determined (Ataga *et al*., 2010).

3.9.2 Determination of growth temperature

The growth temperature used in this research work are 20°C, 25°C, 30°C , 35°C,40°C and 45°C respectively. The growth medium was sterilized and dispensed in 25ml portions into each of several 250ml conical flasks and inoculated with fungal isolates. All flasks were incubated for 7days at each of the 6 different growth temperatures after which the mycelia produced was harvested and the dried weight was determined (Ataga *et al*., 2010).

3.9.3 Determination of pH for growth

The pH of the growth medium was adjusted to 2, 4, 5, 6 and 8 with the electronic pH meter (pHo p®)-pH Tester CE from Hanna instruments, Italy.), drops of 0.1ml HCL was used to reduce the pH to an acidic value while drops of 0.1ml of NaOH was used to adjust the pH to a basic value and dispensed in 25ml portions into each of several 250ml conical flasks sterilized allowed to cool and then inoculated with fungal isolates. All flasks were incubated for 7days at 30°C after which the mycelia produced was harvested and dried weight was determined (Ataga *et al*., 2010).

3.9.4 Determination of carbon-source

The carbon sources used were glucose, sucrose, fructose, lactose, maltose, galactose and starch and CYA. Twenty (20) grams of each sugar per liter was incorporated into the growth medium as sole carbon source. The simple sugar was autoclaved at 115°C for 10 minutes due to the thermo labile nature. The flask was inoculated with *Penicillium* isolates at 30°C for 7days. A control flask was without carbon source. The dried weight of each mycelium was determined (Ataga *et al*., 2010).

3.9.5 Determination of Nitrogen source

Nitrogen sources used in this study include Peptone, sodium nitrite (NaNO3), ammonium sulphate $(NH_4)_2SO_4$ and Yeast using the method of Prnay and Ram (2010), 1% of each of the nitrogen sources were inoculated into the growth medium, which was sterilized and dispensed in 25ml portions into each of several 250ml conical flasks and inoculated with fungal isolates. All flasks were incubated for 7days in each of the 4 different nitrogen sources after which the mycelia produced was harvested and the dried weight was determined.

3.10 DNA extraction

Extraction was carried out by following the manufacturer's instructions from the DNA extraction kit (Solis BioDyne, Estonia). Isolates were sub cultured on potato dextrose agar for 3 days at 25℃. Fungal surface were scoop using a wire loop into a mortar with 1ml of phosphate buffer solution (PBS) and pipette into tubes. These tubes were centrifuge for 2 minutes, supernatant was decanted while lysis buffer added before vortex of tubes, incubation was at 37℃ for 1hour. Cells were centrifuge for 10minutes at 3000 x g. supernatant decanted. 180µl of digestion solution and 20 µl of proteinase k was added and vortex. Incubation was done at 56℃ for 45 minutes with occasional vortexing. 20 µl of RNase A Solution was added, vortexed and incubated at room temperature for 10minutes. 200 µl of lysis solution was added and vortex for 15 s. 400 µl of 50 % ethanol was added and vortex. Lysate was transferred to a gene jet genomic DNA purification column inserted in a collection tube. Column was centrifuge for 1minute at 600 x g. collection tube containing the flow through solution was discarded. The gene jet genomic DNA purification column was placed into a new 2ml collection tube.500 µl of wash buffer I (ethanol added) was added and centrifuge for 1minute at 800 x g. flow through was discarded while purification column was placed back into collection tubes.500 µl of wash buffer II (ethanol added) was added and centrifuged for 3 minutes at > 1200 x g. 200 µl of elution buffer was added, incubated at room temperature for 2 minutes, centrifuged for 1minute at 800 x g. purification column was discarded while purified DNA was stored at -20℃ for polymerase chain reaction.

3.11 PCR Amplification

Amplification of ITS Gene using primer ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC)(White *et al*.,1990) and β-tubulin gene, using primers Bt2a (5'- GTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') (Glass and Donaldson, 1995) were used. PCR mixture contained 0.5 pmol of each primer, 0.2 mM of dNTP's, 10 mM Tris-HCl, 50 mM KCl, 1.5 $MgCl_2$, 2.5U *Taq* polymerase and 15 ng of template DNA. PCR amplification was carried out in a PCR machine (Eppendorf Vapo Protect , Nexus Series) for 30 cycles of 94℃ for 40 s denaturing, 55℃ for 40 s annealing and 72℃ for 1 min extension. Initial denaturing at 94℃ was extended to 3 min and the final extension was at 72℃ for 15 min. PCR products were sent to Germany for sequencing. Identification of homologous sequences were carried out with Basic Local Alignment Search tool (BLAST) search. (Altschul *et al*., 1997) using the beta tubulin gene as a query tool. The phylogenetic tree was constructed using the maximum likelihood from Mega 6 software at 1000 bootstrap.

3.12 Mycotoxin detection using multiplex pcr

Multiplex PCR was carried out to detect Mycotoxin producing genes (majorly citrinin and ochratoxin), using a combined method of Reddy *et al.* (2013) and Chen *et al.*(2008), briefly in an Eppendorf Vapo Protect (Nexus Series) 50µl reaction mixture containing 100ng of fungal DNA as template, 0.2 mM of primers otanps and primer sets of citrinin biosynthesis genes were *pksCT*-F8, AACTGGTCTCTTCCCCAAGC; *pksCT*-R12, ATACTGCATCGCAAACAGCG; *ctnA*-F12, TCGTTATCTAGGCTGGGCCA; *ctnA*-R1,CGTCTGGTGCAGTTAATGCG; *orf3*-F5, CGTGCACCTCTACAGGGTTC; and *orf3*-R13, GCCGCCCCATTGAAGAATAC.PCR cycling conditions were carried out with an initial denaturation at 94°C for 4min, followed by 30cycles of 94°C for 1min,58°C for 1min and 72°C for 1.5 min, with a final extension of 72 °C for 8min.

3.13 Ochratoxin quantification

Ochratoxin quantification was done using ELISA Kit Method (Agra Quant Ochratoxin (2-40ppb) Test Kit) according to the manufactures instruction. Samples were weighed and

grinded and extracted with methanol in the ratio 1:5(w:v) sample to extraction solution respectively, after sample settles, top layer of extract is filtered off with a What man filter paper to collect the filterate, 200ul of conjugate solution is pipetted into dilution wells of test kit. 100ul of each sample extract is added to the dilution wells, mixed together and 100ul was transferred from dilution wells into antibody coated wells and was incubated at room temperature for 10minutes then washed five times with distilled water. Wells were tap dried. 100ul of substrate solution was then pipetted into the antibody coated wells and incubated at room temperature for 5minutes.100ul of stop solution was added into the antibody coated wells. Strips were then read with ELISA Reader using 450 nm and 650 nm differential filters.

CHAPTER FOUR
RESULTS

4.0 Field Survey Information

The samples were collected randomly from three markets in each state studied in this work as shown on Plate A. The field survey information revealed the percentage (%) moisture content of the millet grains obtained from each of the study area (Figure 4.1- Figure 4.5), the percentage moisture content value ranges from 12 %(highest values found in grains obtained from Ogun and Osun markets) to 1.1% (lowest value found from grains obtained from Lagos markets.

The result of fungal isolations and frequency of occurrence (Table 4.0, figure 4.6, 4.7and table 4.1) of fungal contamination was found both on surface disinfected and non-surface disinfected grains the highest percentage occurrence of isolation was found to be 64.2 % for surface disinfected grains from Ondo market, followed by a 57.5% percentage frequency of occurrence from non-surface disinfected grains from Osun market.

The fungal isolation per state ranges from 939 isolates (lowest value were obtained from Lagos market) to 1832 isolates (highest value obtained from Osun market), the percentage fungal isolated had the highest value at 26.7% from Osun market and lowest value was 13.7% obtained from Lagos market.

Results from Studies on periodic isolation from stored millet grains gave the highest value (500 fungal isolates) at 6^{th} month and the lowest value was (5 fungal isolates) at the 9^{th} month of storage.

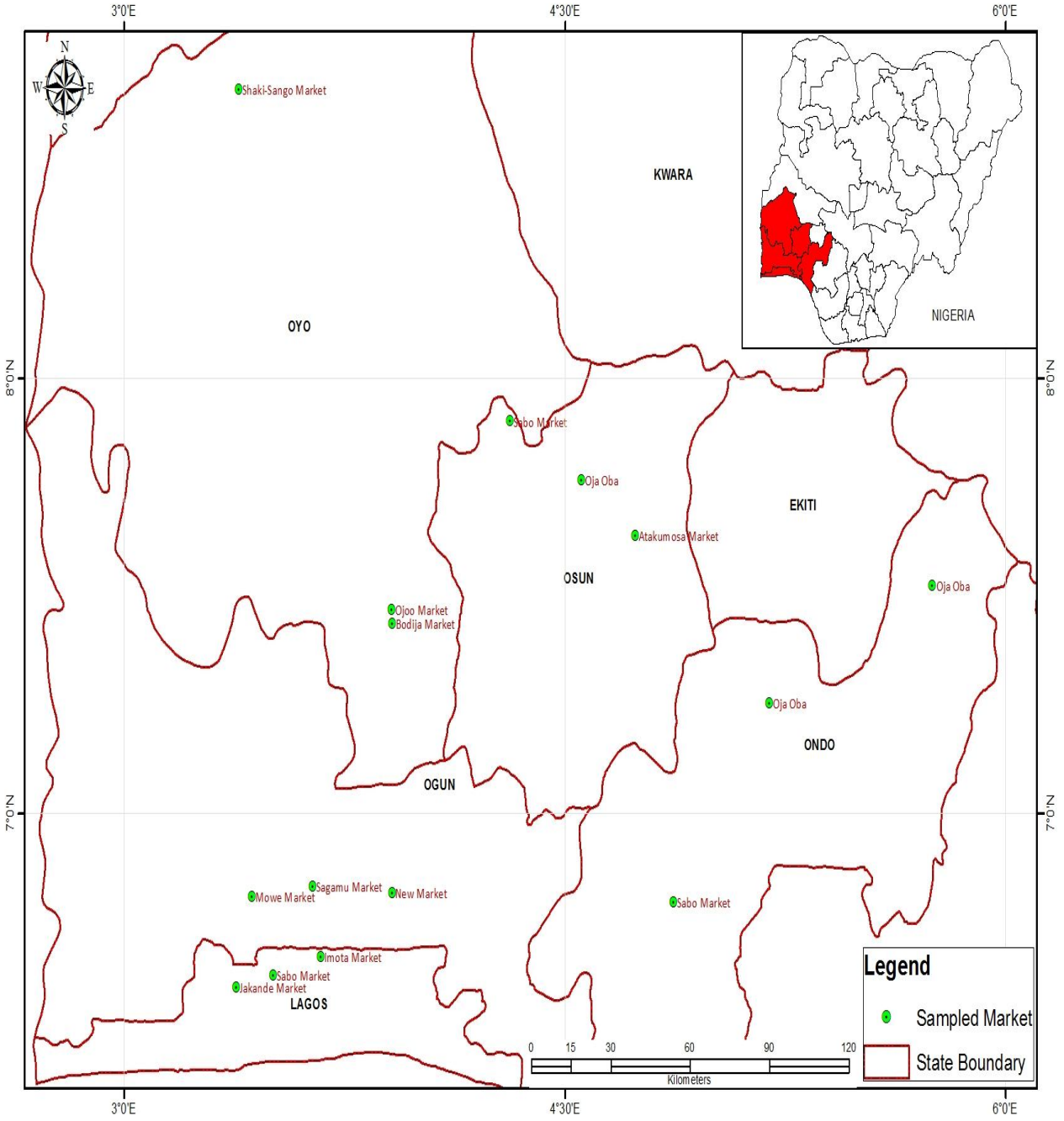


Plate A: Study Areas from which Millet samples were collected.

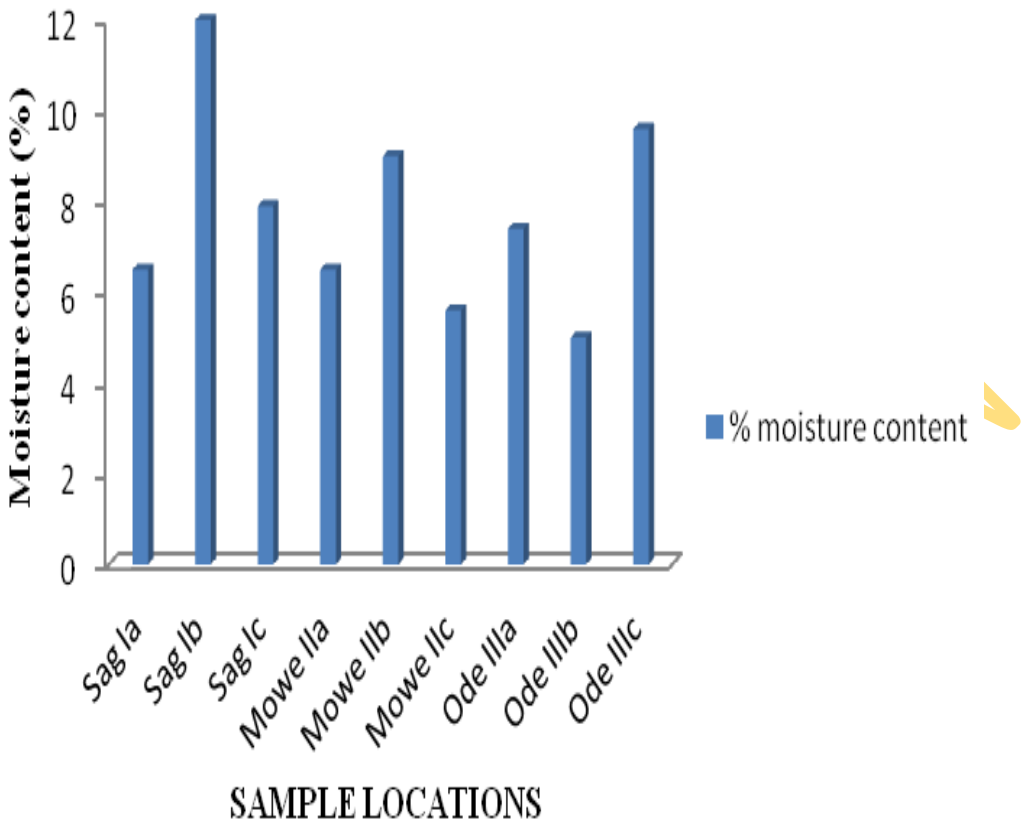


Fig 4.1: Percentage (%) moisture content of the millet grains obtained from Ogun state

Legend

SAG IA, IB and IC = Sagamu market (seller 1, 2 and 3)

Mowe IIA, IIB and IIC = Mowe market (seller 1, 2 and 3)

ODE IIIA, IIIB and IIIC= Ijebu ode market (seller 1,2 and 3)

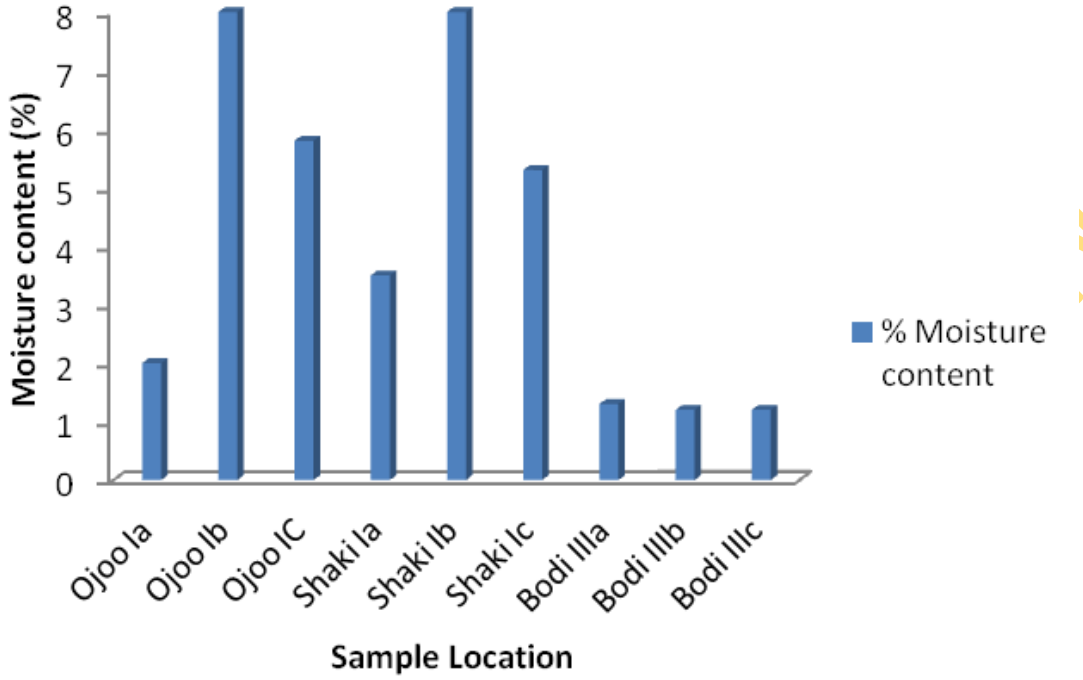


Fig 4.2: Percentage (%) moisture content of millet grains obtained from Oyo State

Legend

Ojoo IA,IB and IC = Ojo market (seller 1, 2 and 3)

Shaki IIA, IIB and IIC= Shaki market (seller 1, 2 and 3)

Bodija IIIA, IIIB and IIIC= bodija maerket (seller 1, 2 and 3)

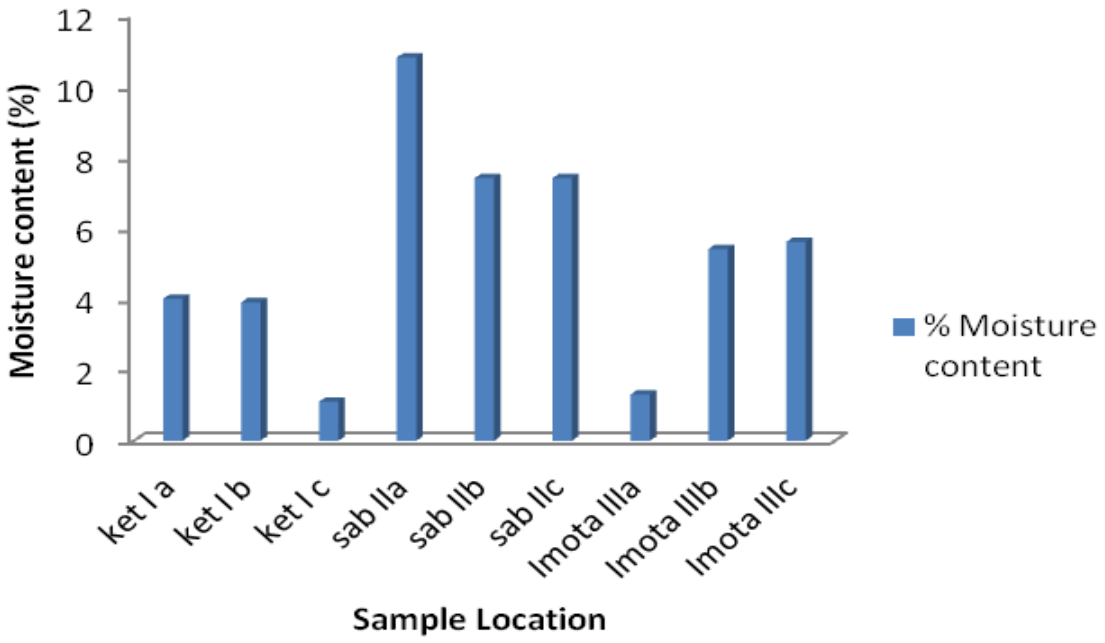


Fig 4.3: Percentage (%) moisture content of millet grains from Lagos State

Legend

KET IA, IB and IC= jakande market (seller 1, 2 and 3)

SAB IIA, IIB and IIC= Sabo market (seller 1, 2 and 3)

IMO IIIA, IIIB and IIIC= Imota market (seller 1, 2 and 3)

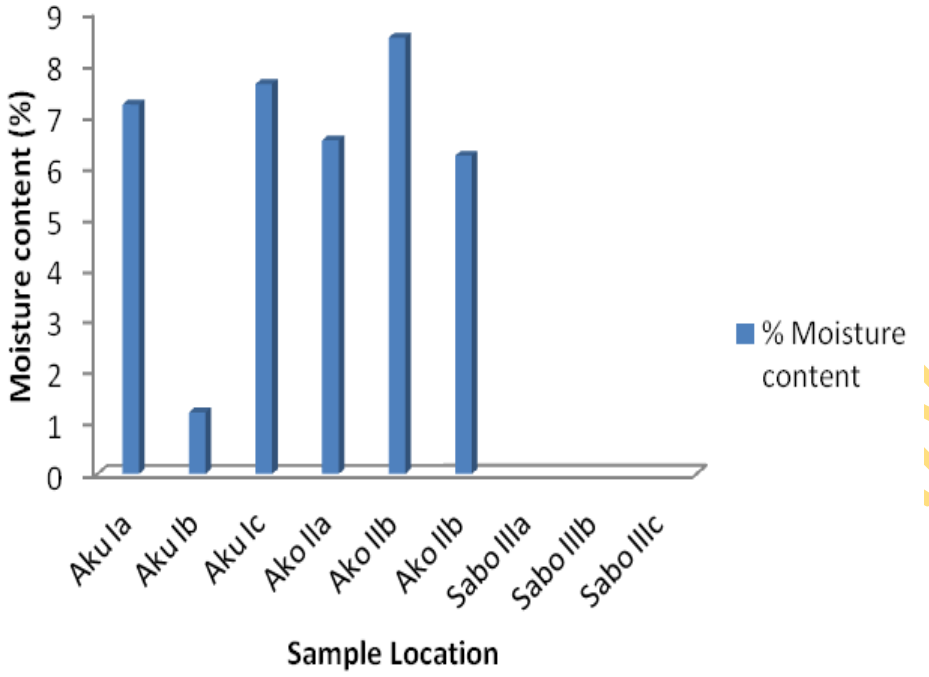


Fig 4.4: Percentage (%) moisture content of millet grains obtained from Ondo State

Legend

AKU IA, IB and IC= oja oba market (seller 1, 2 and 3)

AKO IIA, IIB and IIC= oa oba market (seller 1, 2 and 3)

Sabo IIIA, IIIB and IIIC= Sabo main market (seller 1, 2 and 3)

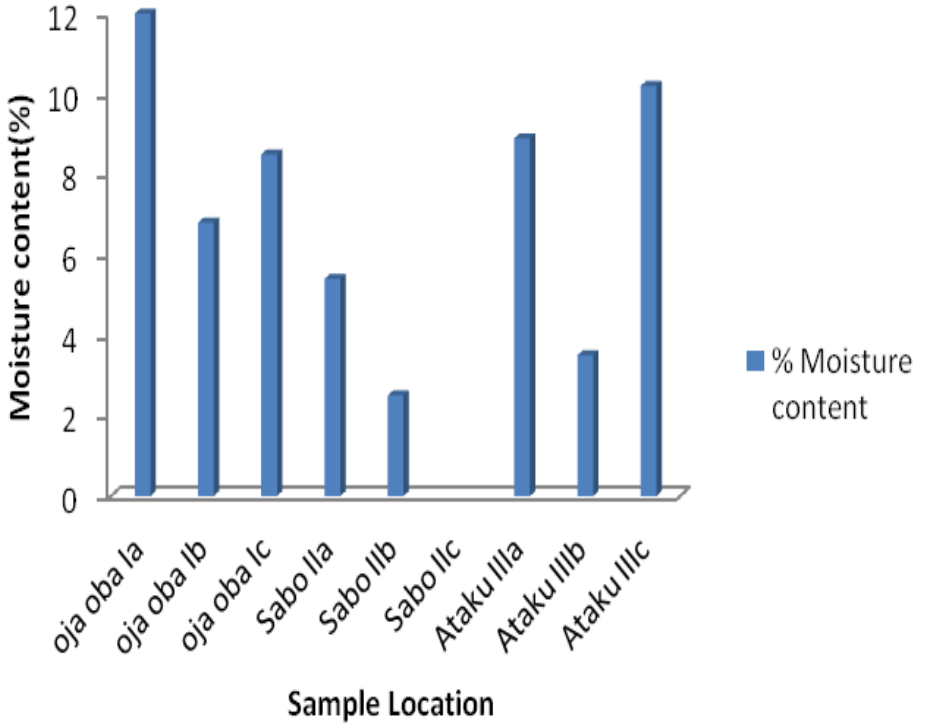


Fig 4.5: Percentage (%) moisture content of millet grains obtained from Osun State

Legend

Oja oba market IA, IB and IC= Oshogbo (seller 1, 2 and 3)

Sabo market IIA, IIB and IIC= Ede (seller 1, 2 and 3)

Atakumosa market IIIA, IIIB and IIIC= Ilesha (seller 1, 2 and 3)

Table 4.0: Frequency of occurrence of fungal isolated from Surface Disinfected Grains (SDG) and Non-surface Disinfected Grains (NSDG)

State	SDG Surface Disinfected Grains	NSG Non-Surface Disinfected Grains	Total Fungal Occurrence	Frequency of Isolation of SDG %	Frequency of Isolation of NSG %
Ogun	643	784	1427	45	54.9
Oyo	901	683	1584	56.9	43.1
Lagos	492	447	939	52.4	47.6
Ondo	690	385	1075	64.2	35.8
Osun	779	1053	1832	42.5	57.5

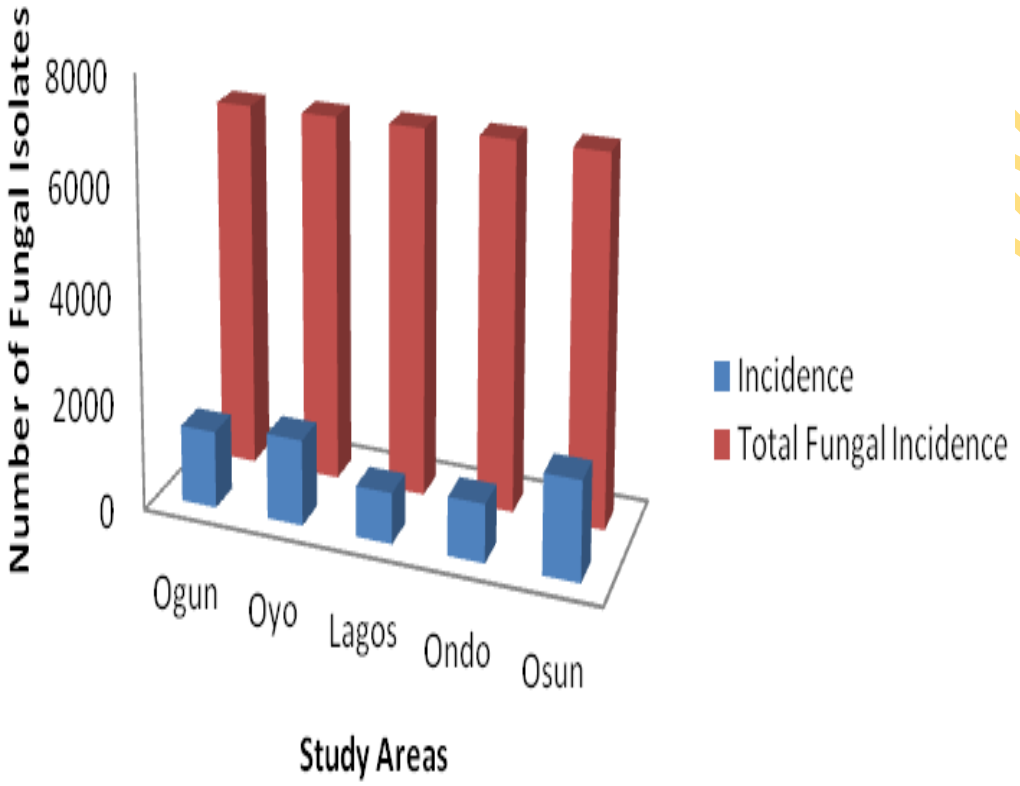


Fig 4.6: Fungal isolation per state (study area)

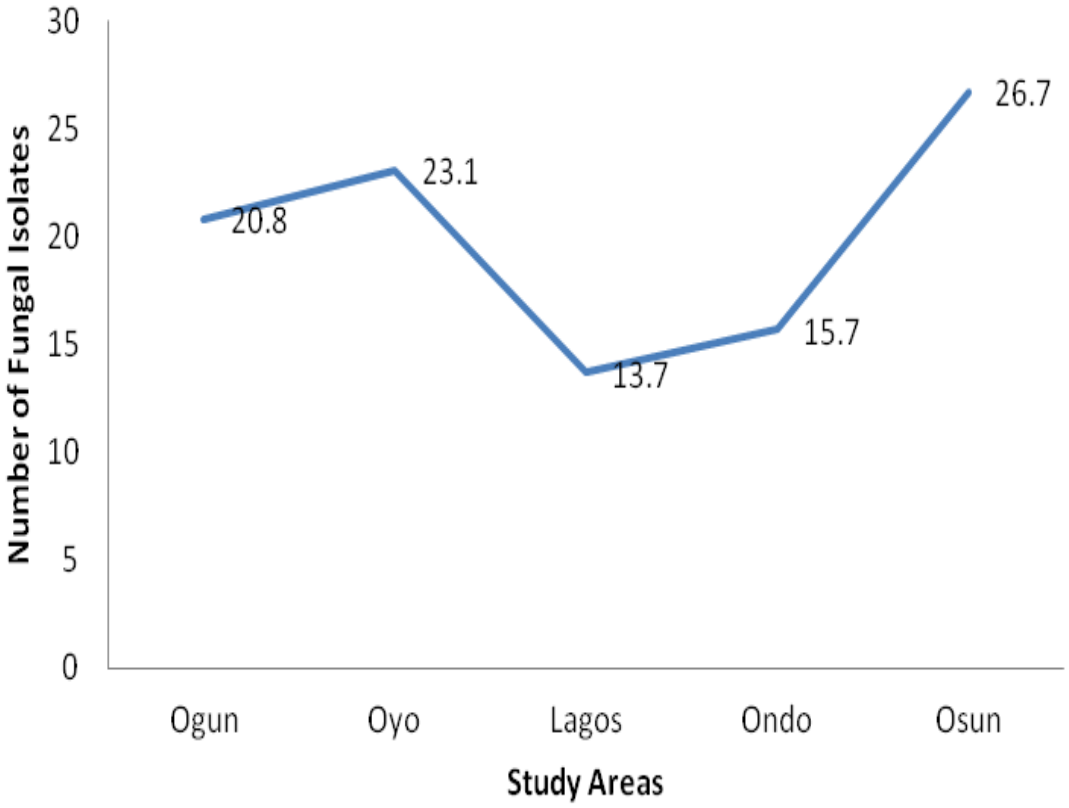


Fig 4.7: Percentage fungal isolation per state (study area)

Table 4.1: Periodic fungal isolation from stored Millet grains

State	Location of samples	1st month	3rd month	6th month	9th month
OGUN	I Sagamu	90	96	125	48
	II Mowe	112	50	168	56
	II Ode	359	109	149	65
OYO	I Ojoo	6	58	500	0
	II Sha	7	122	157	9
	II Bodi	213	156	269	87
LAGOS	I Ket	19	143	186	5
	II SAB	0	109	0	131
	III IMO	40	135	151	20
ONDO	I Aku	300	92	41	80
	II Ako	0	55	106	0
	III Sabo	31	211	0	159
OSUN	I Oso	454	244	283	49
	II Ede	13	190	132	26
	III Ile	224	0	188	29

4.1: Cultural characteristics of *Penicillium* cultures

Results of cultural characteristics of the *Penicillium* cultures (Table 4.2) revealed a range of values from 1.3cm to 4.4cm on Czapex yeast autolysate agar (CYA) and a range of 1.3cm to 4cm on Potato dextrose agar (PDA).

All isolates gave a negative test to Erlich reaction. Plates 1, 3, 5, 7, 9 and 11 Shows the morphological characteristics of isolates of the genus *Penicillium.*

Penicillium capsulatum: Colonial mophology showed rapid growth on CYA with a diameter of 3.37cm, color of colony was grayish green and the back side of colony was cream in color on MEA media. Colony texture was sulcate, centrally raised and velvety. Exudates were present and seen as clear droplets on surface of colony. Microscopic features observed the branching pattern of the conidiophores as biverticillate, phialides were long and ampuliform, metulae were short and in whorls of three and conidia were elliptical in nature.

Penicillium citrinum: These isolate showed moderate to rapid growth on CYA with a colony diameter ranging from 1.3- 4.4cm, colony pigmentation was pale green center and the back side of colony was cream on MEA. Texture of the colony was radially sulcate and centrally raised. Exudates were present in some isolates and absent in others. The microscopic features observed branching patterns of the coniophore as biverticillate (one stage branching), phialides which were lanceolate in structure (narrow basal part tapering to a pointed apex) were found to be shorter than the metulae when compared to other species of *Penicillium* isolated. The metulae were in whorls of three to five (3-5) divergent structures. Conidia were spherical and produced in chains.

Penicillium chrysogenum: Showed rapid growth on CYA with colony diameter of 3.37cm, a dark green color of colony with white at the centre and edges was observed on MEA with a creamy color back side. Powdery lose colony texture. No exudates observed. Microscopic features observed the branching pattern of the conidiophores as biverticillate, phialides were long and ampliform in structure, metulae in verticils of three and conidia were ellipsoidal.

Penicillium steckii: Showed moderate growth with colony diameter of 1.73cm on CYA. A dark green colony with white edges and creamy back view was observed on MEA. Colony texture was observed to be sulcate with a floccose center. Microscopic features observed the branching pattern of the conidiophores as biverticillate, phialides were long and ampuliform, metulae were in whorls of 3-5 while conidia were ellipsoidal.

Penicillium simplicissium: Showed moderate growth on CYA having a 2cm colony diameter. Dark green color with white edges and cream rare on PDA media was observed. Colony texture was slightly sulcate with a floccose centre. Microscopic features observed the branching pattern of the conidiophores as biverticillate, phialides were long and lanceolate, metulae in verticils of three and conidia are ellipsoidal.

Penicillium oxalicum: Showed moderate growth, colony diameter on CYA was 2.1cm. dark green color of colony with white edges and off- white on the back side of colony on PDA was observed. Colony texture was compact and powdry. No exudate was observed on colony. Microscopic features observed the branching pattern of the conidiophores as biverticillate, phialides were long, metulae in verticils of three and conidia are ellipsoidal and in chains.

4.2: Microscopic characteristics

Representative isolates from the 23 isolates obtained as examined under the microscope revealed the structures shown in Plates 2, 4, 6, 8, 10 and 12 below, showing branched metaluae and brush like appearances called penicillus with stripes. All isolates showing a one stage branched *Penicillium* species.

4.3: Distribution of the *Penicillium* isolates and preliminary mycotoxin detection

Table 4.3, reveals the distribution of the *Penicillium* isolates from the study areas, Five of the isolated species were obtained on grains from Ogun , four from and Ondo market, eleven from grains on Osun market, one on Oyo and two from Lagos markets.

Also from table 4.3, results of the preliminary mycotoxin detection revealed 10 isolates as positive to citrinin, while all isolates were negative to aflatoxin detection

Table 4.2: Cultural characteristics of *Penicillium* isolates

Isolate number	Location	Erlich test	Colony diameter on PDA	Colony diameter on CYA
2	Ogun	No rxn	NG	3.37cm
3	Osun	No rxn	1.9cm	2.76cm
5	Osun	No rxn	2cm	4.4cm
6	Ondo	No rxn	2.1cm	1.37cm
7	Osun	No rxn	2.1cm	1.65cm
10	Ondo	No rxn	2.1cm	
11	Ogun	Light yellow	4cm	3.73cm
12	Osun	No rxn	1.8cm	1.9cm
13	Osun	No rxn	2.1cm	2.16cm
14	Ogun	Very weak	2cm	1.63cm
15	Ondo	No rxn	2.1cm	1.63cm
17	Ogun	No rxn	2.1cm	1.46cm
18	Lagos	No rxn	1.9cm	1.96cm
20	Osun	No rxn	1.7cm	1.73cm
22	Ogun	No rxn	1.6cm	1.3cm
24	Oyo	No rxn	1.9cm	2.2cm
25	Lagos	No rxn		2.06cm

28	Ondo	No rxn	1.6cm	1.96cm
29	Osun	No rxn	2.4cm	1.83cm
31	Osun	No rxn	2.6cm	2cm
32	Osun	No rxn		1.4cm
34	Osun	No rxn	3.6cm	2.1cm

No rxn= No reaction

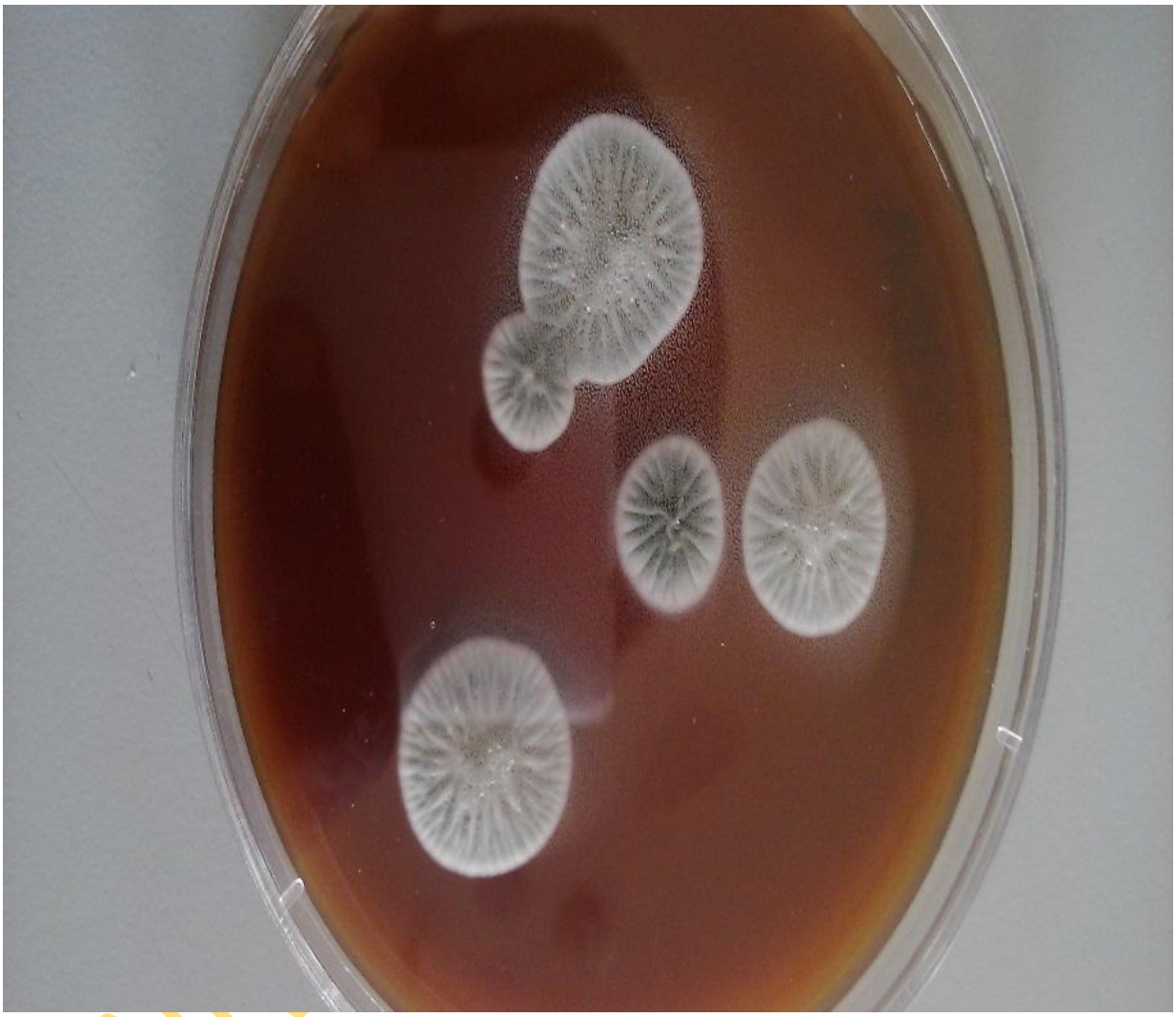


PLATE 1: *Penicillium capsulatum* on MEA

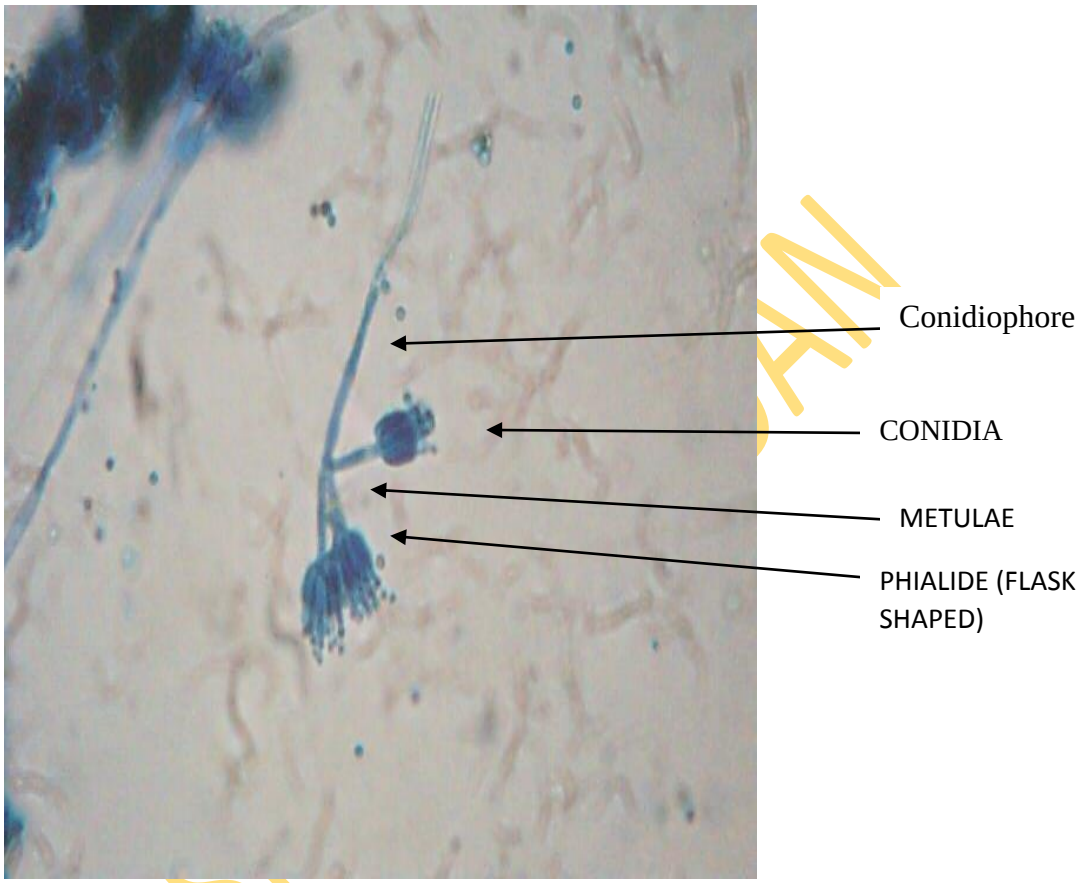


Plate 2: Photomicrograph of *Penicillium capsulatum* x 100

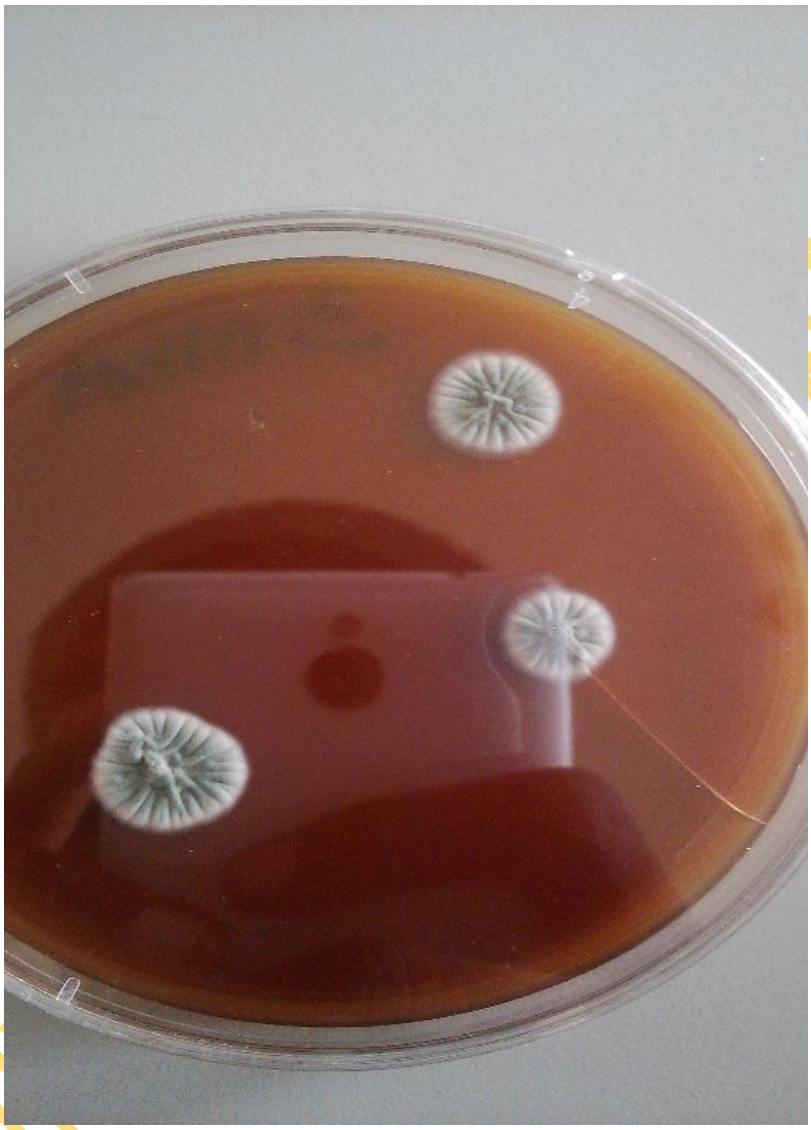


PLATE 3: *Penicillium citrinum* on MEA

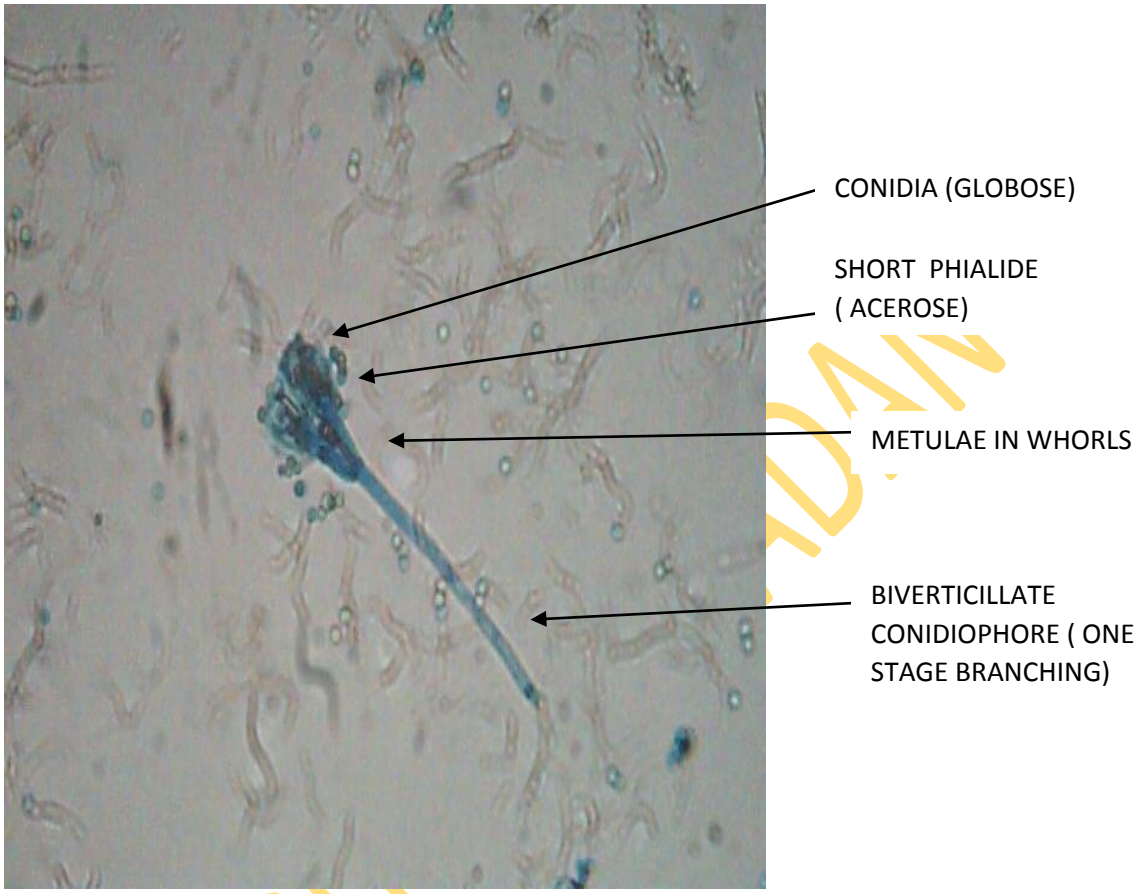


Plate 4: Photo micrograph of *Penicillium citrinum* x400

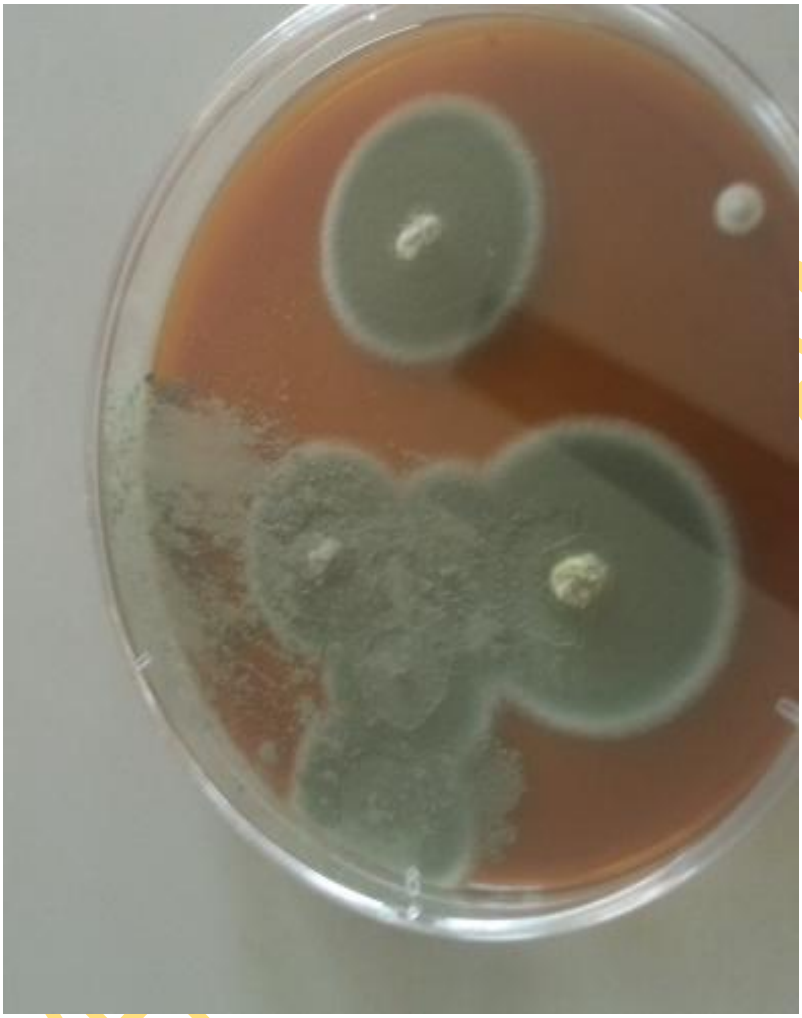


Plate 5: *Penicillium chrysogenum* on MEA

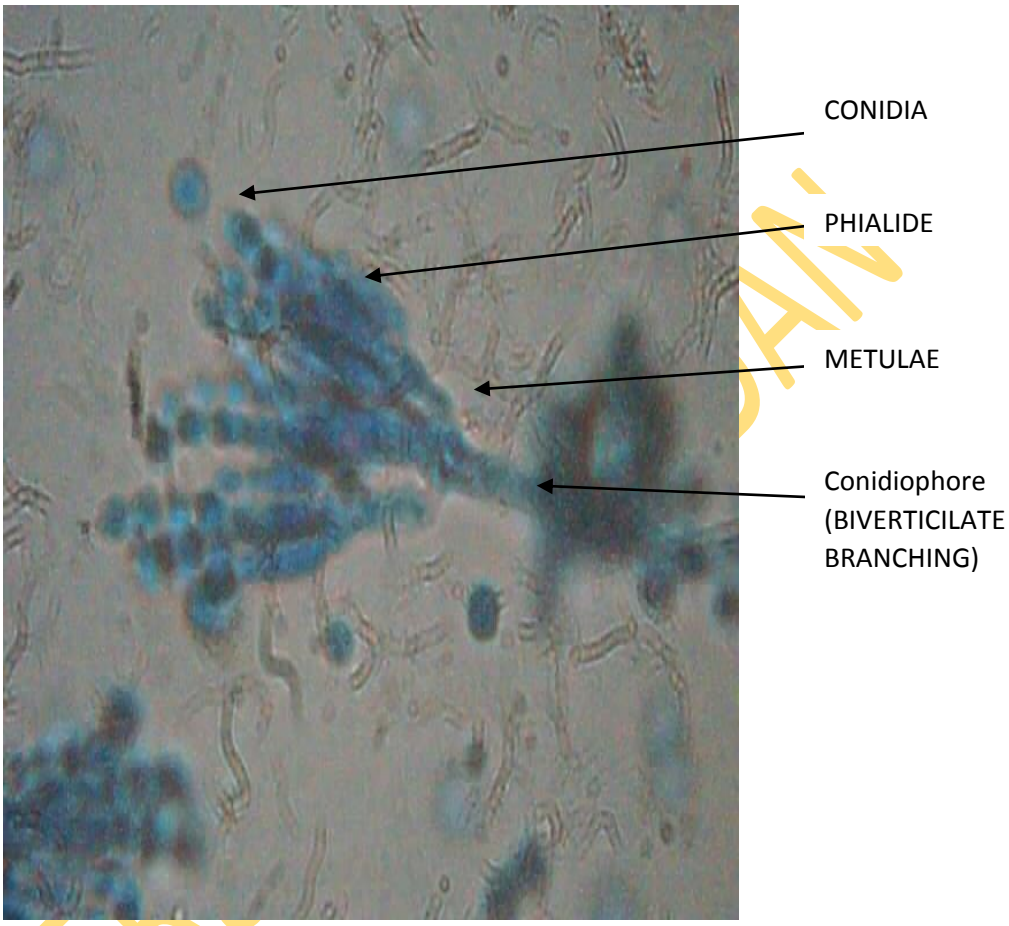


Plate 6 : Photomicrograph of *Penicillium crysogenum* x 1000

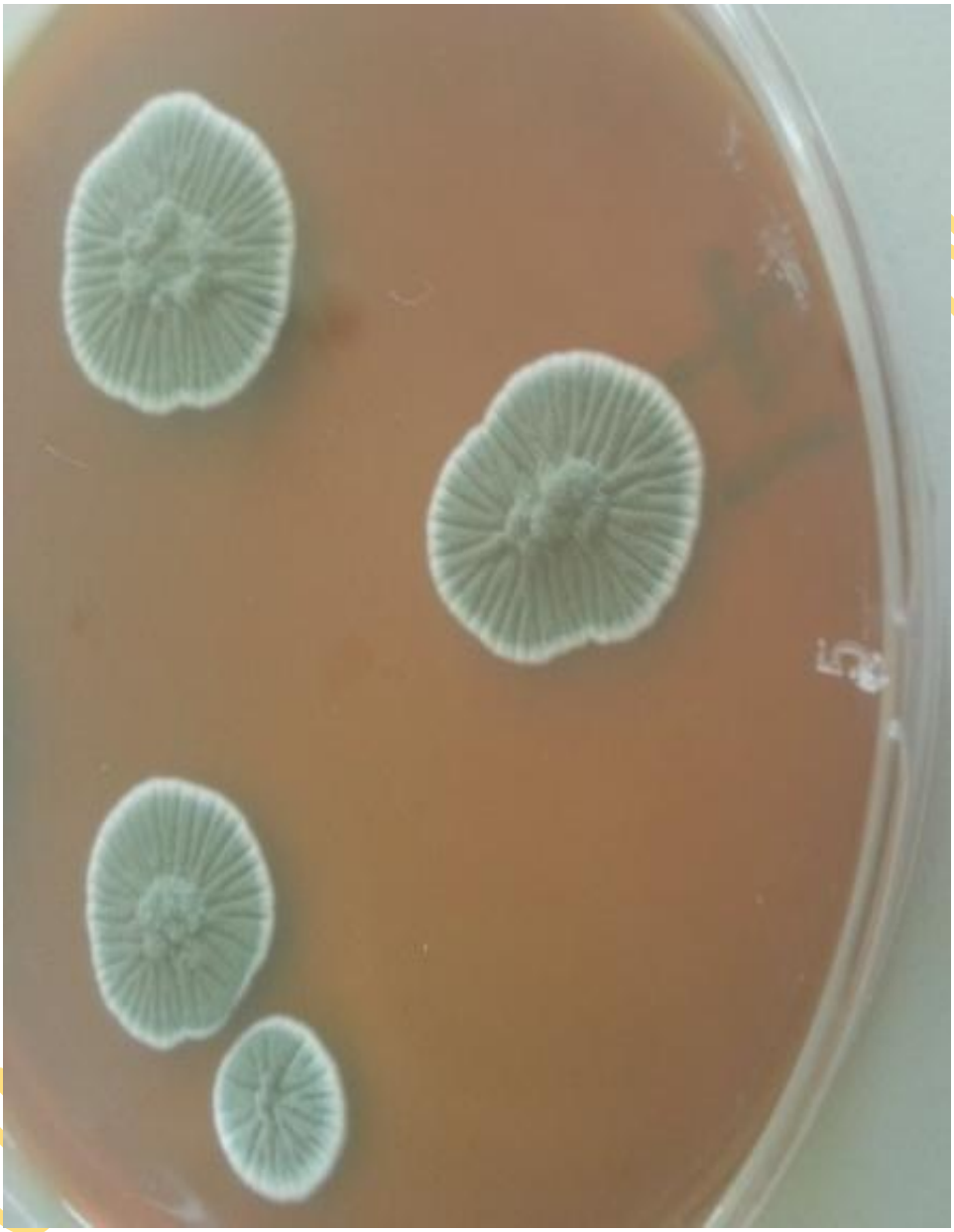


Plate 7: *Penicillium steckii* on MEA

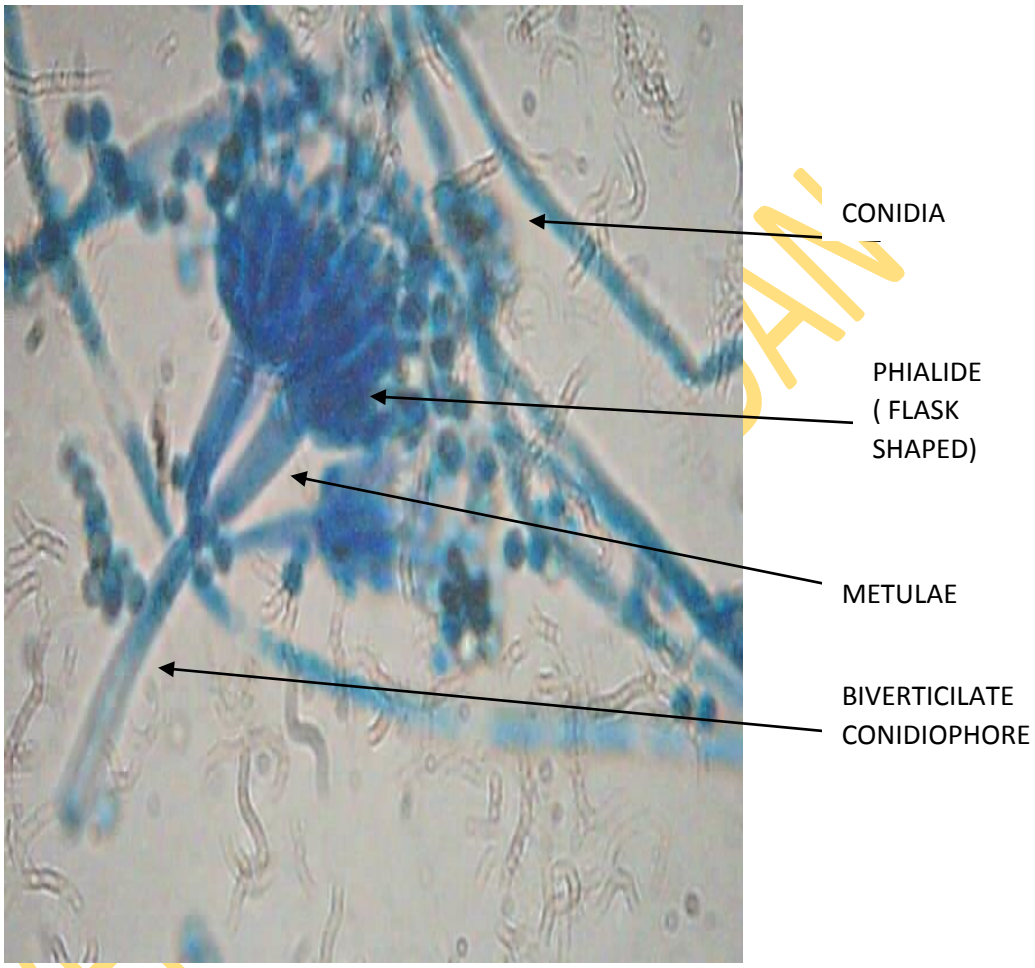


Plate 8: Photomicrograph of *Penicillium steckii* x1000

Plate 9: *Penicillium oxalicum* on PDA

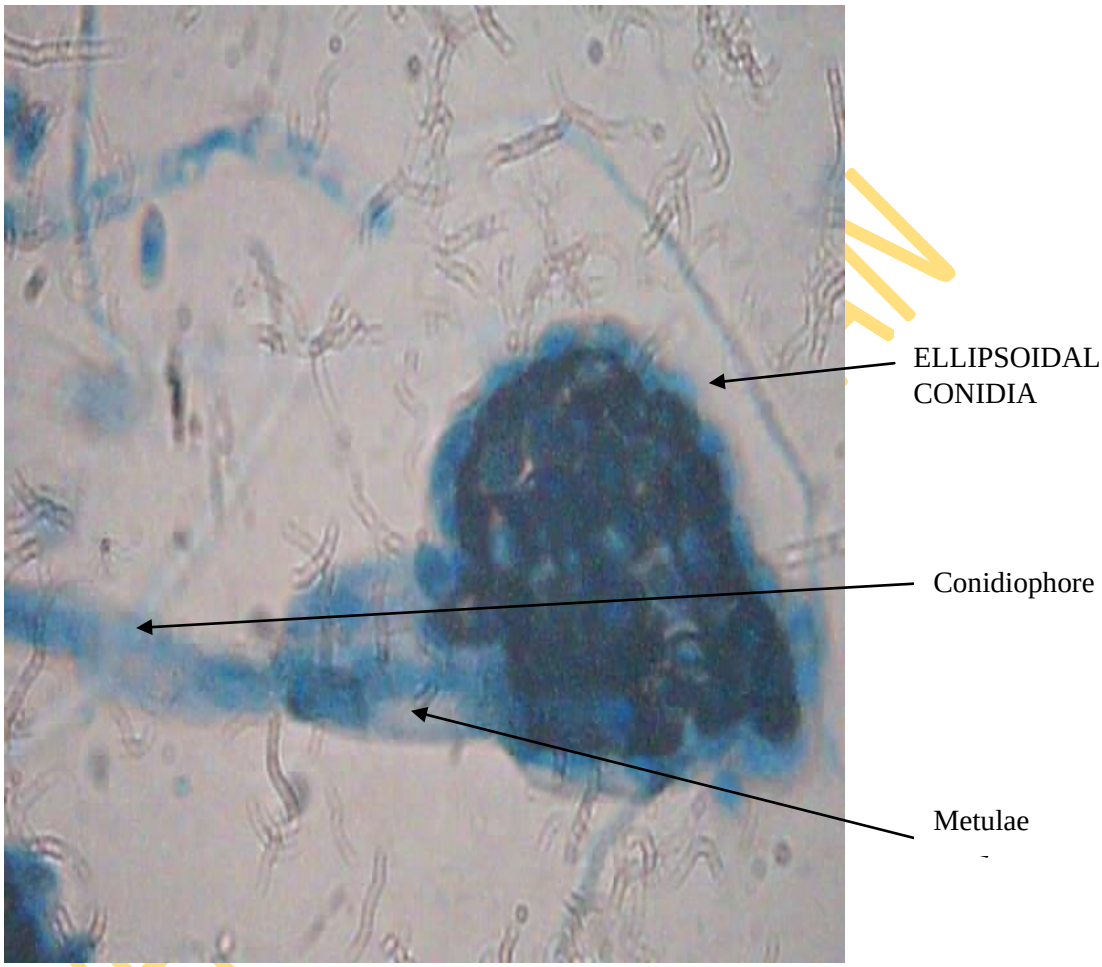


Plate 10: Photomicrograph of *Penicillium oxalicum* x1000

Plate 11: *Penicillium simplicissimum* on PDA

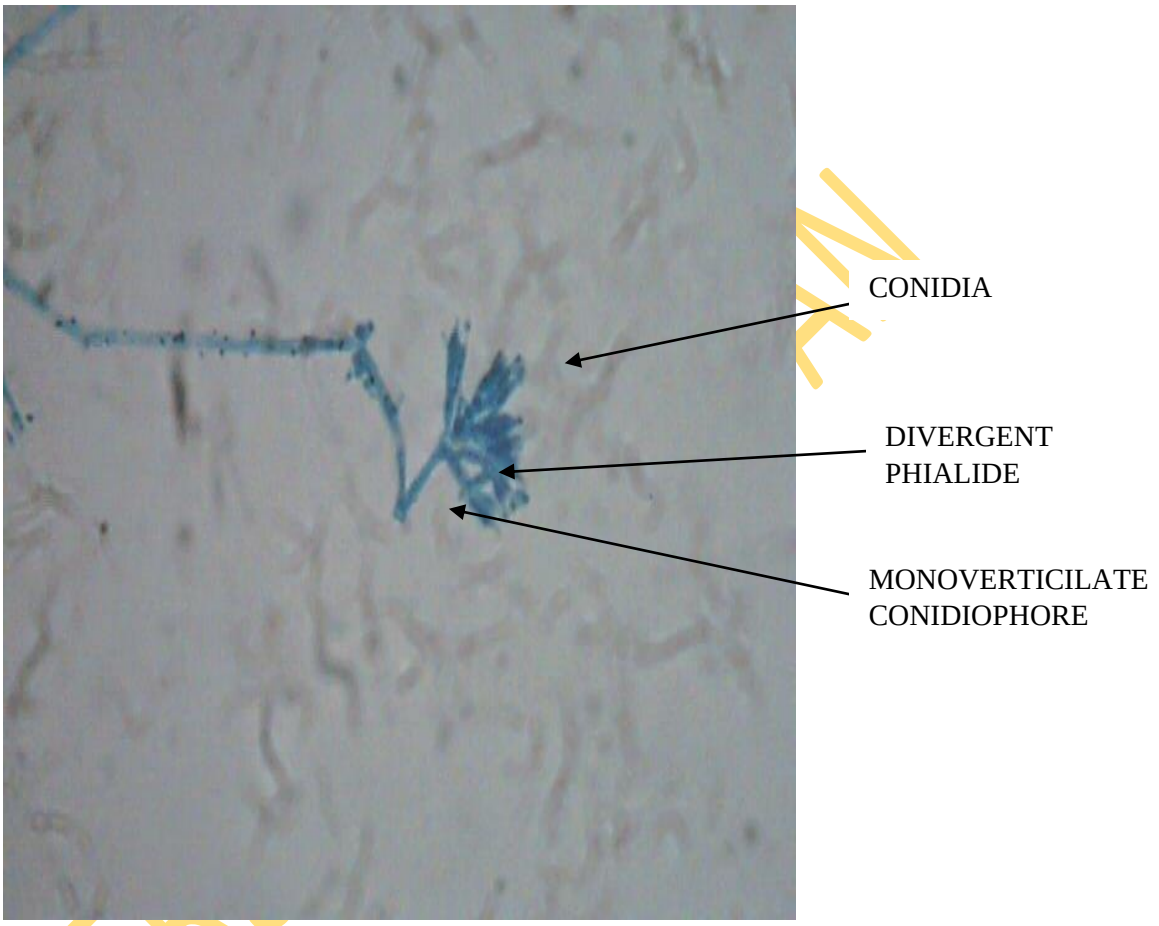


Plate 12: Photomicrograph of *Penicillium simplicissium* x400

Table 4.3: Prelminary mycotoxin test on Coconut cream agar

Isolate number	Location	Result on CCA for citrinin	Result on CCA for aflatoxin
2	Ogun	Negative	Negative
3	Osun	Positive	Negative
5	Osun	Positive	Negative
6	Ondo	Positive	Negative
7	Osun	Positive	Negative
10	Ondo	Positive	Negative
11	Ogun	Negative	Negtive
12	Osun	Negative	Negative
13	Osun	Negative	Negative
14	Ogun	Negative	Negative
15	Ondo	Positive	Negative
17	Ogun	Positive	Negative
18	Lagos	Positve	Negative
20	Osun	Positive	Negative
22	Ogun	Negative	Negative
24	Oyo	Negative	Negative
25	Lagos	Negative	Negative

28	Ondo	Positive	Negative
29	Osun	Negative	Negative
31	Osun	Negative	Negative
32	Osun	Negative	Negative
34	Osun	Negative	Negative

4.4: Physiological studies of the *Penicillium* isolates

4.4.4 Utilization of carbon sources

Results obtained from the ultilization of different carbon sources from this study are shown on Table 8 and Figure 4.4.4. All isolates in this study utilized all the eight carbon sources tested, it was observed that highest mycelia growth was recorded on starch with a value of 2.14g,while least growth was seen in czapek yeast autolysate agar (CYA). Generally, growth media without carbon source resulted in poor mycelia growth,while sucrose, fructose and starch gave better mycelia growth.

4.4.3 Determination of best pH for growth

All the isolates grew on all the pH ranges tested as shown on fig 4.4.3. Results revealed that the highest growth occurred at pH8 (2.51g),while lowest mycelia growth was at pH2 (0.00g).

4.4.1 Determination of best incubation time on growth

Best incubation time for mycelia growth was observed from this report as shown on fig:4.4.1, it was observed that mycelia growth improved with increase in incubation time with 14 days as the optimium incubation time for mycelia growth. Highest mycelia growth was also found at day 14 with a value at 0.76g,while lowest mycelia growth was found as 0.02g at day 7 .

4.4.2 Determination of best growth temperature

Results obtained from the assay for the best temperature at which mycelia growth is best favoured is given on fig 4.4.2, which shows growth at all temperatures tested in this study,the best growth was observed at 30°C with a value 0.96g,while the lowest value was 0.03g at 25°C.

4.4.5 Determination of best nitrogen source for growth

Mycelia growth was supported by all nitrogen sources used in this study fig 4.4.5,, however the best nitrogen source as observed from this study was$(NH_4)_2SO_4$ with a value of 0.86g and lowest value was observed in $NaNO_3$ (0.56g).

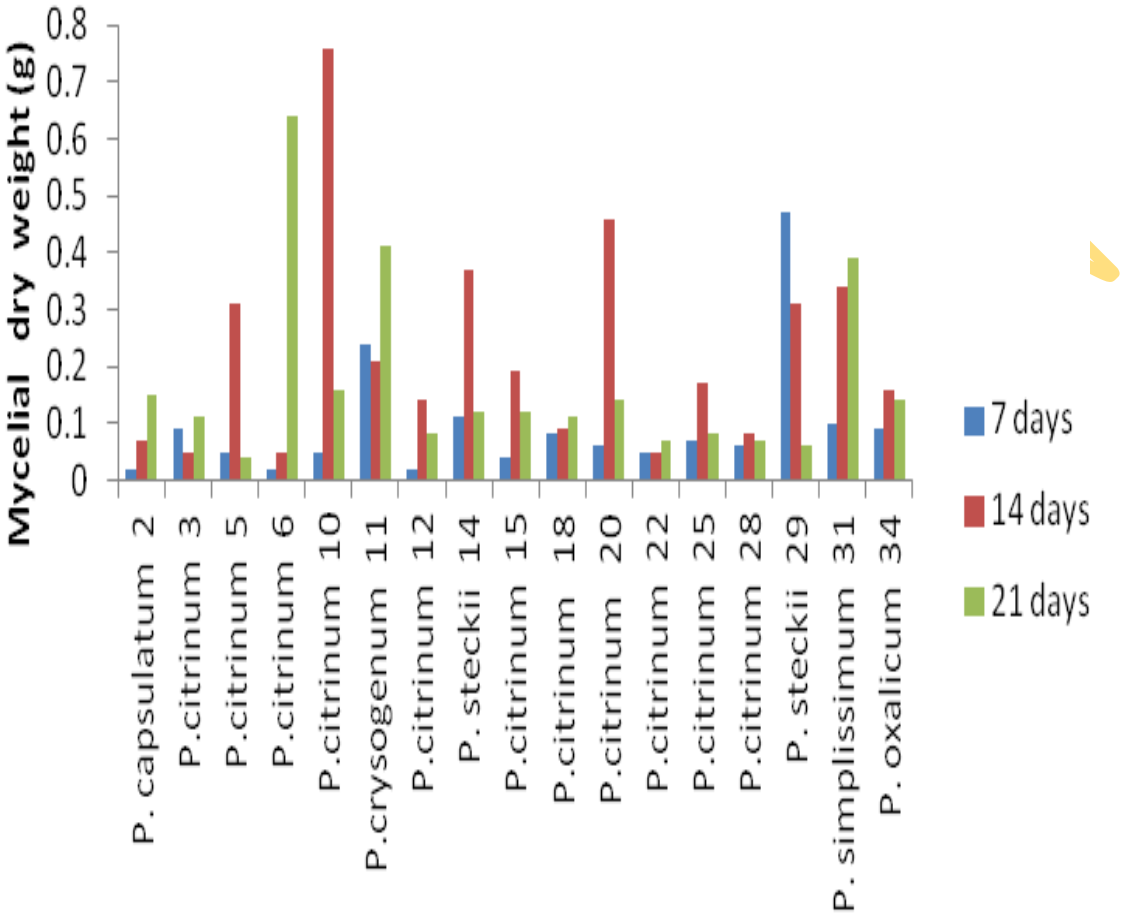


Fig 4.4.1: Mycelial dry weight of the *Penicillium* isolates at different incubation time

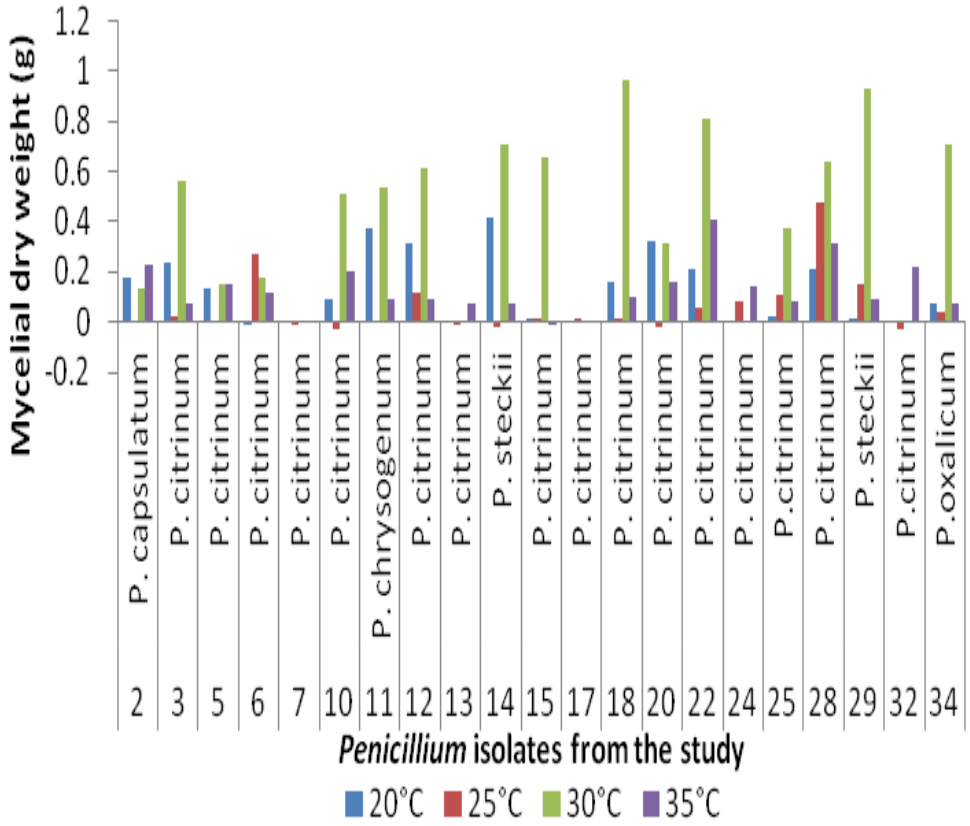


Fig 4.4.2: Mycelial dry weight of the *Penicillium* isolates at different temperatures

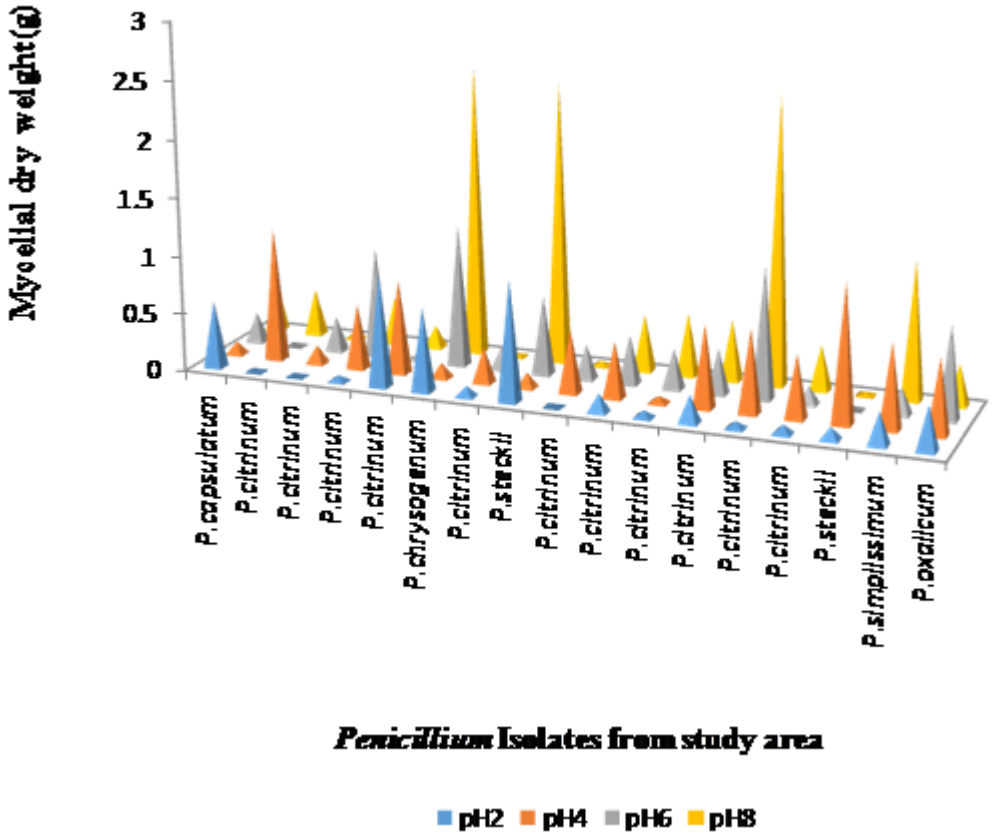


Fig 4.4.3: Mycelial dry weight of the *Penicillium* isolates at different pH

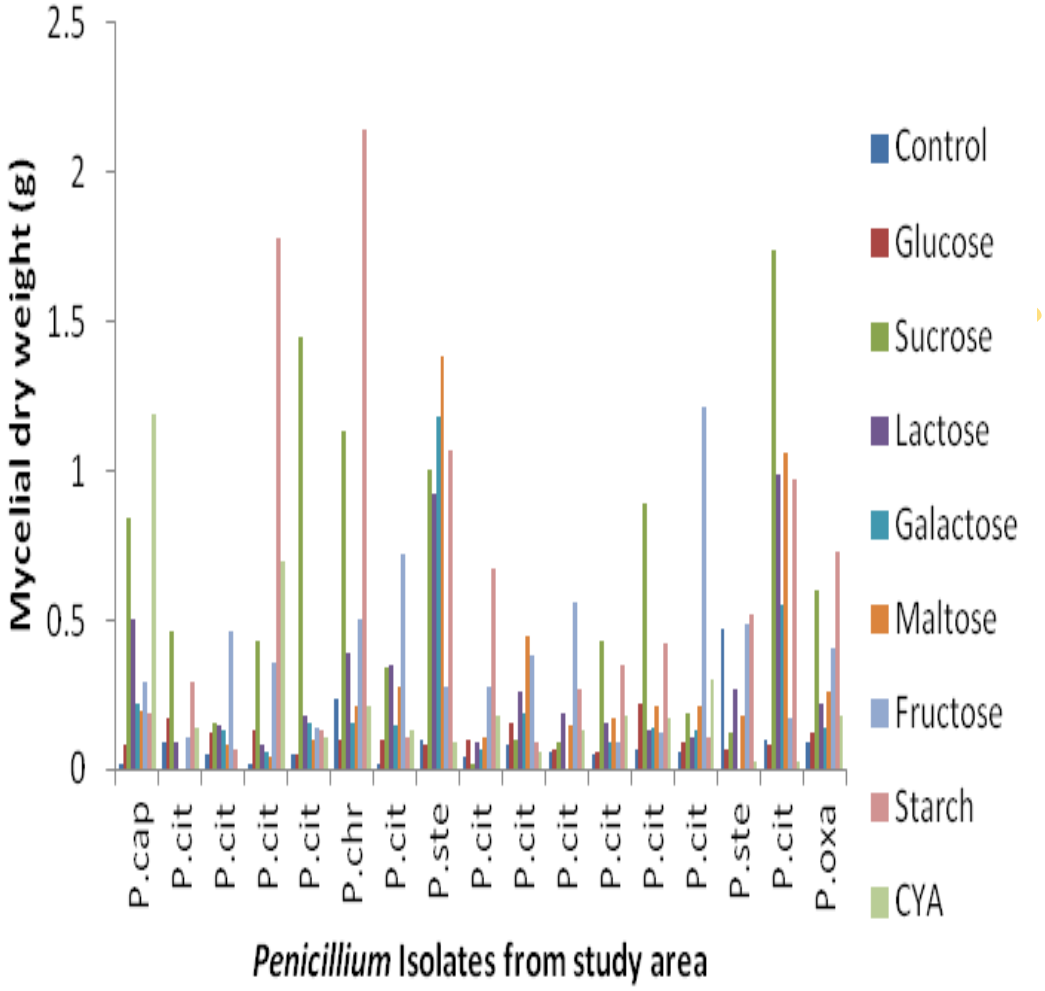


Fig 4.4.4 Mycelial dry weight of the *Penicillium* isolates on different carbon sources

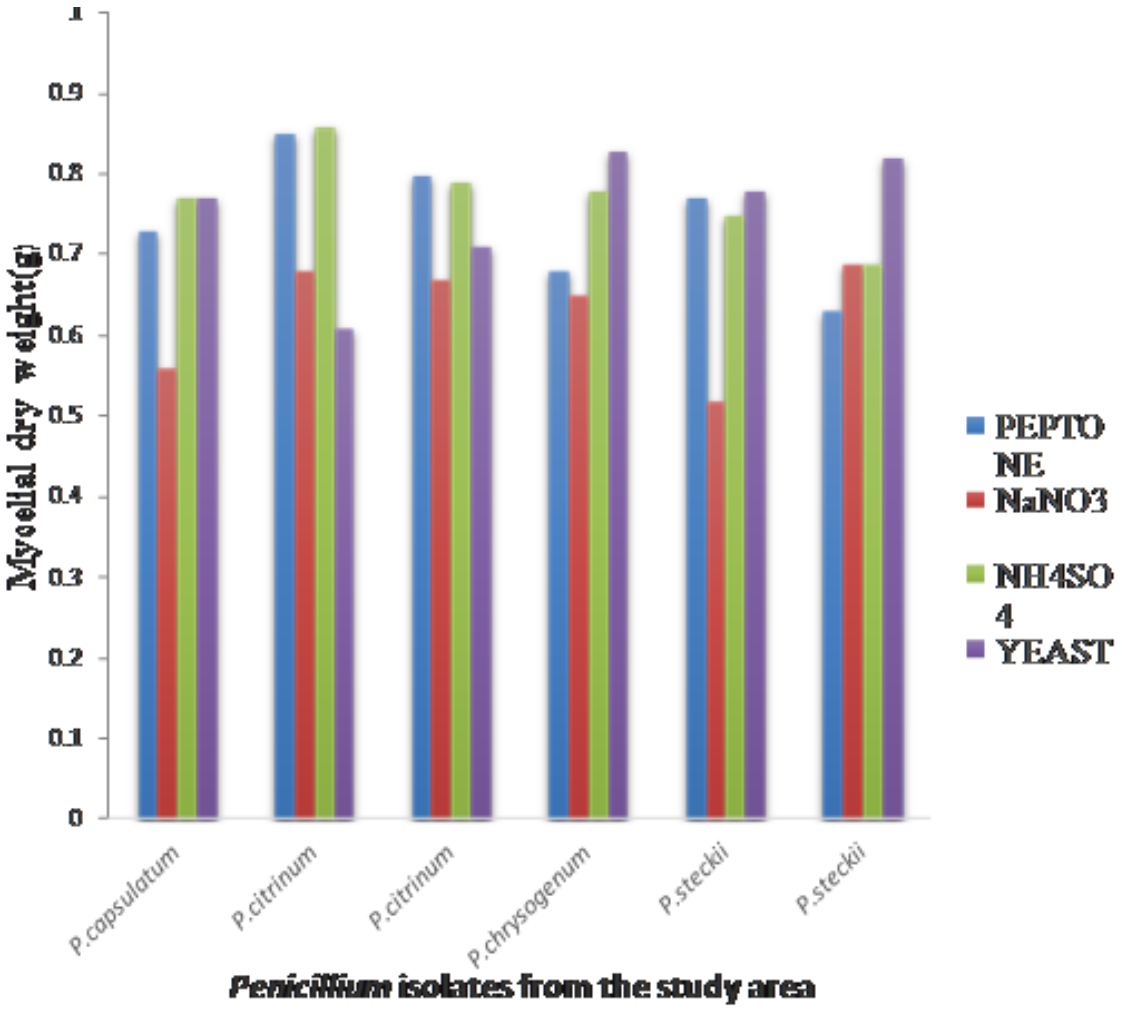


Fig 4.4.5: Mycelial dry weight of the *Penicillium* isolates on different Nitrogen sources

4.5: Molecular Identification of isolates obtained

4.5.0 Amplification of Internal Transcribed Spacer

The ITS regions of all the isolates from this study was amplified as shown in plate 13and 14, the ITS regions were mostly negative to the amplification, with only isolates 18,25 and 31 showing positive amplification with an amplicon size between 500-600 base pair.

4.5.1 Amplification of Beta tubulin gene

Results of the amplification of the beta tubulin gene is shown in plate 15 and 16, the amplification of the beta tubulin genes gave a better result compared to the results of the ITS region, all isolates were positive to the expected amplicon size of 500base pair, with a little variation in the amplicon size of isolate 31 which was around 490base pair and isolate 34 was a little above 500 base pair.

4.6 Gene sequence of *Penicillium* species

The gene sequence results (Appendix) provides an indication of possible relationships and similarities with reference sequences. This is shown on Table 4.4, which revealed the sequence of isolate 2 with ascension number FJ004390.1 when compared with the sequences available within the gene bank was found to be 97% identical to that of *Penicillium capsulatum* strain NRRL2056 Beta tubulin (Ben A) Gene, partial cds from the gene bank sequence. (3,4,5,6,7,10,12,13,17,18,20,22,24,25 and 32) with ascension number KC345003.1 were compared with sequences available in the Gene Bank and was found to be 98-100% identical to that of *Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds from the gene bank sequence.

Sequence of isolate 15 with ascension number KM491315.1 was 99% identical with that of *Penicillium citrinum* strainCICC2478 Beta Tubulin(tub)gene, partial cds, while sequence of isolate 28 with ascension number KJ413333.1 was 99% identical to that of *Penicillium citrinum* strain AC1101 III beta tubulin(tub)gene partial cds. Sequences of isolates (14 and 29) with ascension numbers JF521539.1 were identical to that of *Penicillium steckii* strain KACC45933 Beta tubulin gene, partial cds. Sequence of isolate 31 with ascension number KJ767048.1 were identical to that of *Penicillium simplicissimum* isolateA1S5-D20 beta-tubulin gene, partial cds. Sequence of isolate 34 with ascension number JX091528.1 was identical to that of *Penicillium oxalicum* strain CV822 Beta-tubulin gene, partial cds from the gene bank. From the result of beta tubulin sequence, the *Penicillium* species were identification in this study as 17 *Penicillium citrinum*, 2 *Penicillium steckii*, 1 *Penicillium capsulatum*, 1 *Penicillium chrysogenum*, 1 *Penicillium simplicissimum and 1 Penicillium oxalicum*. Sequencing revealed the length of isolates was about 460 base pair. Sequences were deposited at gene bank and accession numbers were given in Table 4.3.

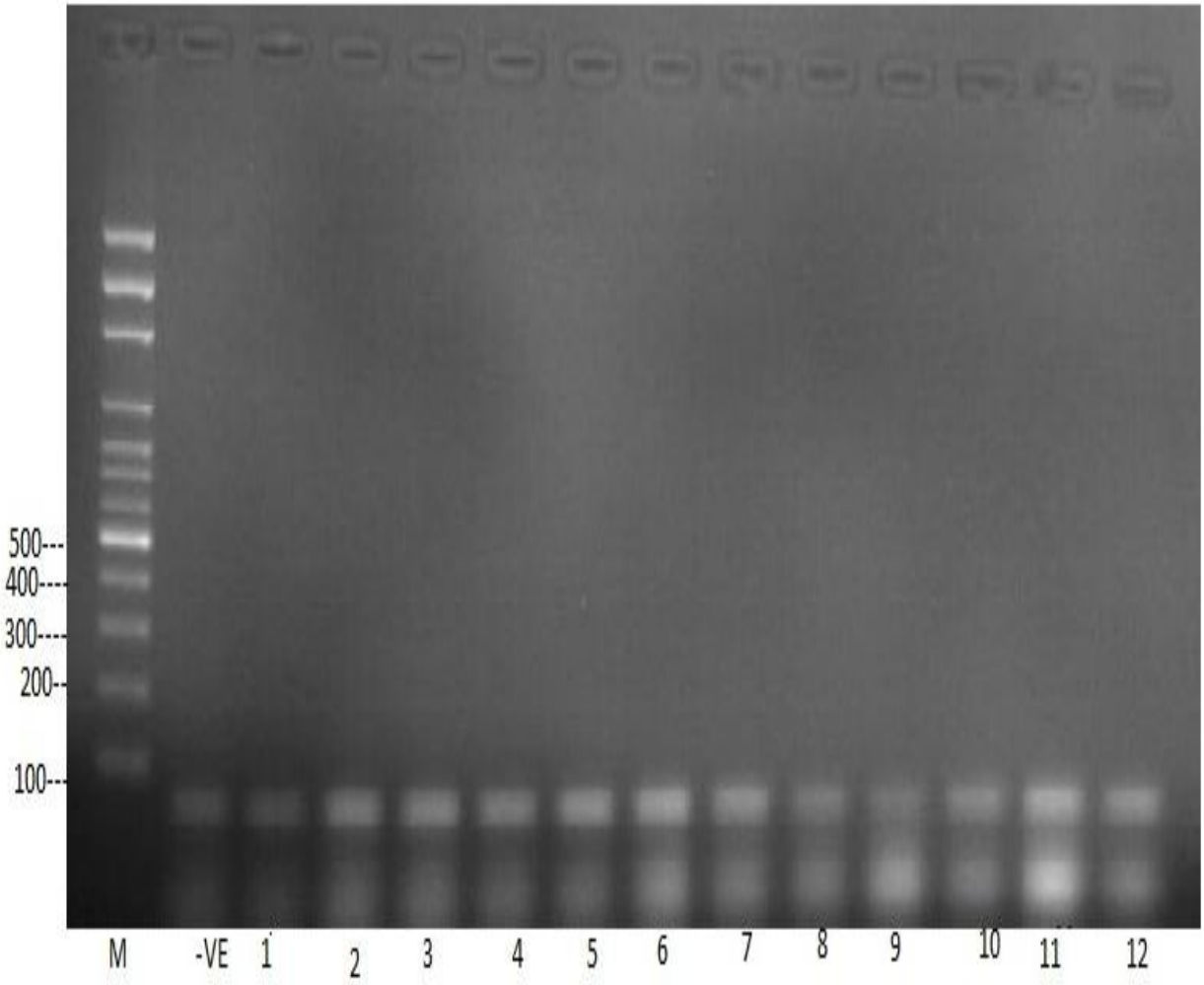


Plate 13: ITS gene amplification of the Penicillium isolates on Agarose gel electrophoresis. No amplification obtained from gel picture

Keys:

M=Marker, -VE= negative, 1= unknown, 2= *Penicillium capsulatum*

3,4,5,6,7,8,9 and 12= *Penicillium citrinum*, 11= *Penicillium chrysogenum*

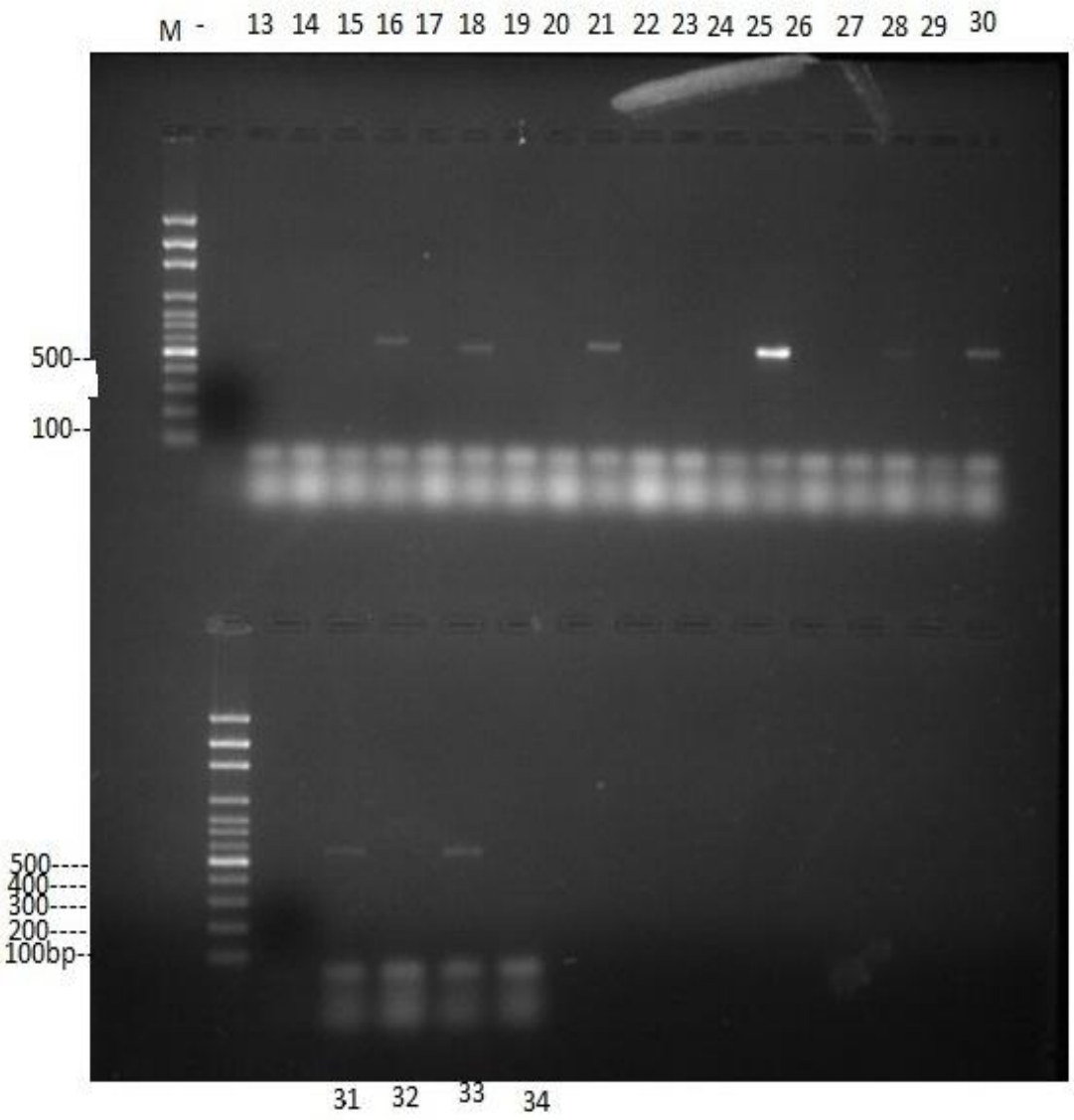


Plate 14: ITS gene amplification of the *Penicillium* isolates on Agarose gel electrophoresis, amplification observed in isolates 13,16,18,20 25, 28, 30, 31 and 33 with amplicon sizes between 500-600 base pair.

Keys:

M=Marker, -VE= negative, 1= unknown, 14 and 29= *Penicillium steckii*, 31= *Penicillium simplissimum*, 34= *Penicillium oxalicum*, 13, 15, 17,18, 20,22, 24, 25, 28 and 32 = *Penicillium citrinum.*

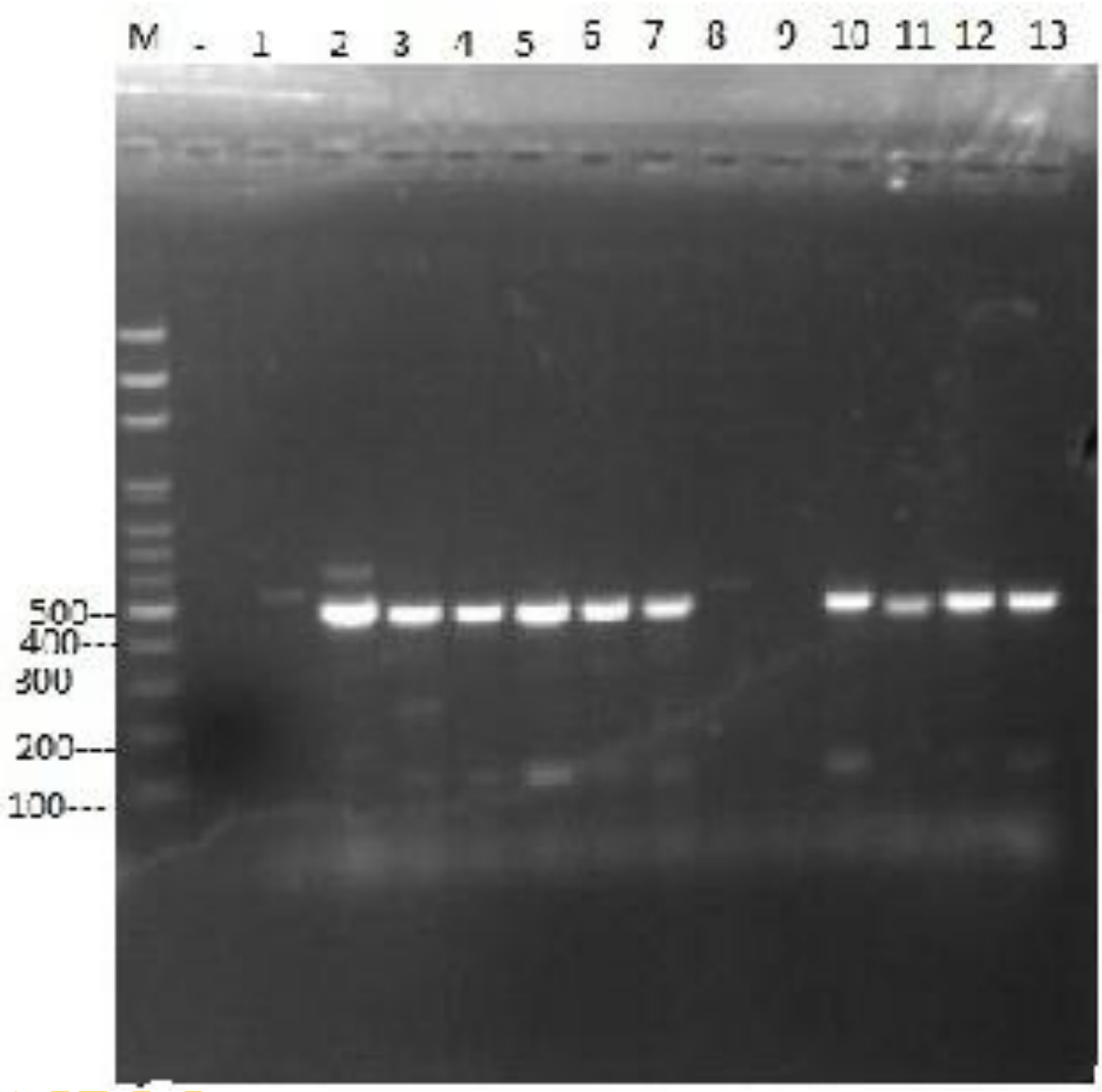


Plate 15: Beta-tubulin gene amplification of the *Penicillium* isolates on Agarose gel. All isolates obtained an expected amplicons size of 500bp expect for isolates 8 and 9.

Keys:

M=Marker, -VE= negative, 1= unknown, 2= *Penicillium capsulatum*

3, 4, 5, 6, 7, 8, 9 ,10, 12 and 13= *Penicillium citrinum*, 11= *Penicillium chrysogenum*.

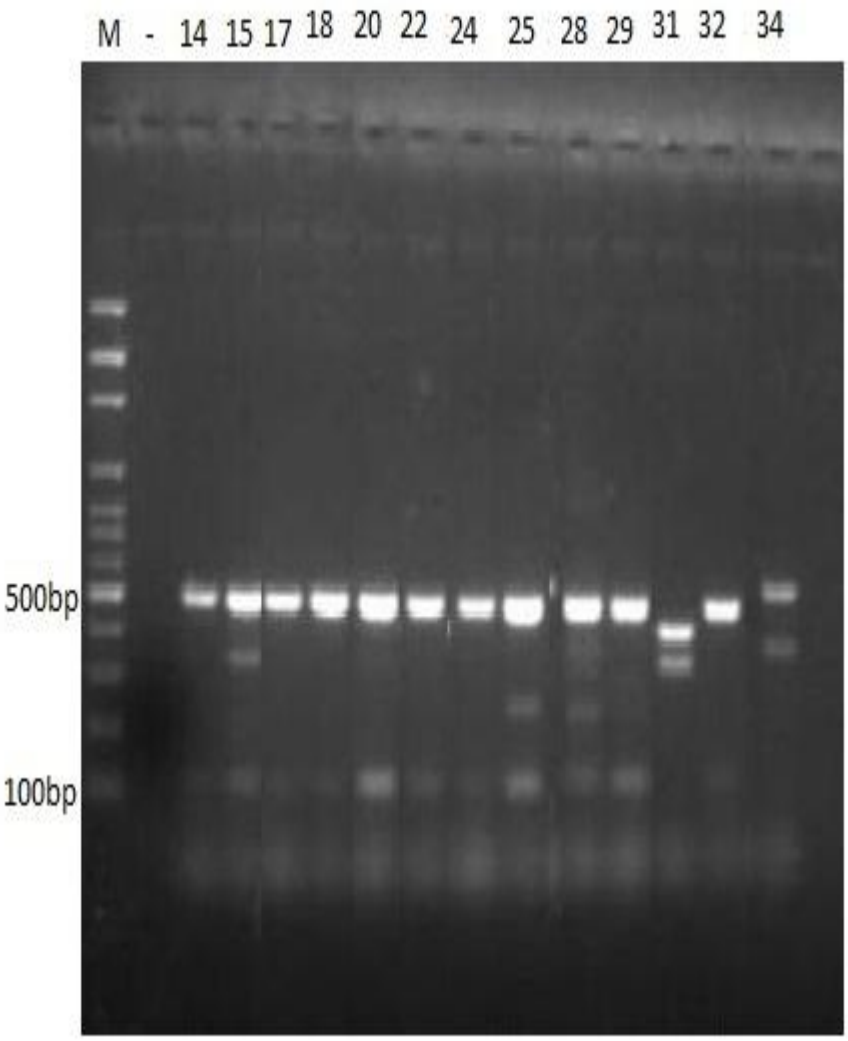


Plate 16: Beta-tubulin gene amplification of the *Penicillium* isolates on Agarose gel. Isolate 31 at below 500bp and isolate 34 a little above 500bp.

Keys: M=Marker, -VE= negative,14 and 29= *Penicillium steckii*, 31= *Penicillium simplissimum*, 34= *Penicillium oxalicum*, 15,17,18, 20, 22, 24, 25, 28 and 32 = *Penicillium citrinum.*

Table 4.4: *Penicillium* strains isolated from Millet grains, identified by partial beta tubulin gene sequence.

Isolate	Description	Max Score	Query Cover	E.Value	Identification	Ascension Number
1	No identifiable sequence					
2	*Penicillium capsulatum* NRRL2056 Beta tubulin (Ben A) gene, partial cds	649	90%	0.0	97%	FJ004390.1
3	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	780	99%	0.0	98%	KC345003.1
4	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	695	98%	0.0	99%	KC345003.1
5	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	695	98%	0.0	99%	KC345003.1

6	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	713	100%	0.0	99%	KC345003.1
7	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	697	97%	0.0	99%	KC345003.1
10	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	699	100%	0.0	99%	KC345003.1
11	*Penicillium chrysogenum* strain Gr170 beta-tubulin(Ben A)gene , partial sequence	636	99%	8e-179	98%	FJ904907.1
12	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	795	100%	0.0	99%	KC345003.1
13	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	771	100%	0.0	99%	KC345003.1

14	*Penicillium steckii* strain KACC45933 beta-tubulin gene, Partial cds	719	94%	0.0	99%	JF521539.1
15	*Penicillium citrinum* strain CICC2478beta tubulin (tub)gene, partial cds	667	100%	0.0	99%	KM491315.1
17	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	806	100%	0.0	99%	KC345003.1
18	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	798	100%	0.0	99%	KC345003.1
20	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	695	97%	0.0	99%	KC345003.1
22	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	701	100%	0.0	100%	KC345003.1
24	*Penicillium citrinum* strain a2s6 6 beta	704	100%	0.0	99%	KC345003.1

	tubulin gene, partial cds					
25	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	704	100%	0.0	99%	KC345003.1
26	No identifiable sequence					
28	*Penicillium citrinum* isolate AC1101 IIIbeta tubulin(tub) gene, partial cds	767	98%	0.0	99%	KJ413333.1
29	*Penicillium steckii* strain KACC 45933 beta-tubulin gene, partial cds	664	93%	0.0	100%	JF521539.1
31	*Penicillium simplicissimum* isolate A1S5-D20beta-tubulin gene, partial cds	148	100%	7e-33	100%	KJ767048.1
32	*Penicillium citrinum* strain a2s6 6 beta tubulin gene, partial cds	715	99%	0.0	99%	KC345003.1
33	No identifiable sequence					

34	*Penicillium oxalicum* strain CV822 beta-tubulin gene, partial cds	252	98%	1e-63	98%	JX091528.1

4.7: Occurrence of *Penicillium* species isolated

Fig 4.6 reveals the percentage occurrence of the isolated *Penicillium* species from this study, *Penicillium citrinum* had the highest prevalence of occurrence at a value of 73.91%, followed by *Penicillium steckii* with its prevalence value at 8.69% while other isolates had the lowest prevalence at 4.35% each.

4.8: Diversity of isolates

The *Penicillium* isolates obtained were grouped into five clades (clusters). Isolates 28,25,22,15,12,10,3,5,20,4,6,7,13,17,18,24,32,14 and 29 formed the largest cluster(Cluster I).isolates 14 and 29(*P.steckii*)formed cluster II,, Isolates 31(*Penicillium simplicissimum*) cluster III, Isolate 11(*Penicillium chrysogenum*) cluster IV, while isolates 2 (*P. capsulatum*) and isolates 34 (*P. oxalicum*) were grouped into cluster V, on Table 4.5, 4.6 and fig 4.7.

The result of alignment of nucleotide sequences obtained is seen on appendix page:, isolates were mostly related to beta tubulin of *P.citrinum* strain a2s6_6, strain FJAT-31016, strain: T-2 and strain KAS 2602. Isolates had a minimum of 99%similiarity to each other with a major polymorphism of 1% at position 113 and also at position 112.isolate 3 differed most from this group with a C rather than A at position 54 and a CA deletion at position 487 and 488, single organism mutation was also found in isolate 15 having a G indel rather than A in position 63, and isolate 20 had a G rather than T in position 109. Isolates 14 and 29 were clustered to *P.steckii* strain KACC 45933 and only differed with an A indel at position 46, alignment with other closely related isolates of *P.steckii* indicated that this indel was not consistent as it was only in isolate 14 that the nucleotide A was deleted. Other species isolated and did not form cluster of major groupings.

Isolates 31was 100% identical to *P. simplicissimum*, Isolates 11was 98% identical to *P. chrysogenum* strain Gr170, isolate 2 was 97% identical to *P. capsulatum* strain NRRL 2056 and isolates 34 was 99% identical to *P. oxalicum* strain CV822.

Also found on the appendix page is the translation of the gene to amino acids revealed that all point mutation were silenced except for the AC transversion mutation at the 113 position resulting in the substitution of Glutamic acid (isolates with allele A) to Alanine (isolates with allele C). *P.citrinum* isolates gave rise to an Allele C gene frequency of 44% and Allele A frequency of 55%.

Polymorphic content of the *Penicillium* isolates as shown on Table 4: reveals the occurrence of *P. citrinum* at 18, the Allelic frequency was at a ratio of 5:4, while the number of polymorphism was recorded as 9, 1.80% was obtained as percentage polymorphism.

4.9 Mycotoxin detection

The result obtained from the detection of Mycotoxin from the *Penicillium* isolates using multiplex PCR is shown on the Agarose gel picture in plate 17 and 18,the citrinin biosynthetic gene cluster from *Monascus purpureus* with primer sets R12 (pksCT gene),R1 andF12 (ctnA gene),R13and F5 (orf3 gene),also primer Otanps(ochratoxin detection). All isolates were negative to the amplification of pksCT gene, isolates 2 (*Penicillium capsulatum*), 3 (*Penicillium citrinum*), 6 (*Penicillium citrinum*), 11 (*Penicillium chrysogenum*) and 14 (*Penicillium steckii*) were positive to the amplicon size obtained between 678-697 base pair for amplification on ctnA Gene. Isolates 2 (*Penicillium capsulatum*), 3 *(Penicillium citrinum)*, 4 (*Penicillium citrinum*), 6 (*Penicillium citrinum*), 7 (*Penicillium citrinum*), 11 (*Penicillium chrysogenum*), 14 (*Penicillium steckii*) and 17 (*Penicillium citrinum*) were positive to the amplification of orf3 gene at amplicon size 428-447base pair. Isolates 2 (*Penicillium capsulatum*), 3 (*Penicillium citrinum*), 6 (*Penicillium*

citrinum), 11(*Penicillium chrysogenum*), 14 (*Penicillium steckii*) and 29 (*Penicillium steckii*) were positive to amplification of Otanps genes at amplicon size at 788base pair.

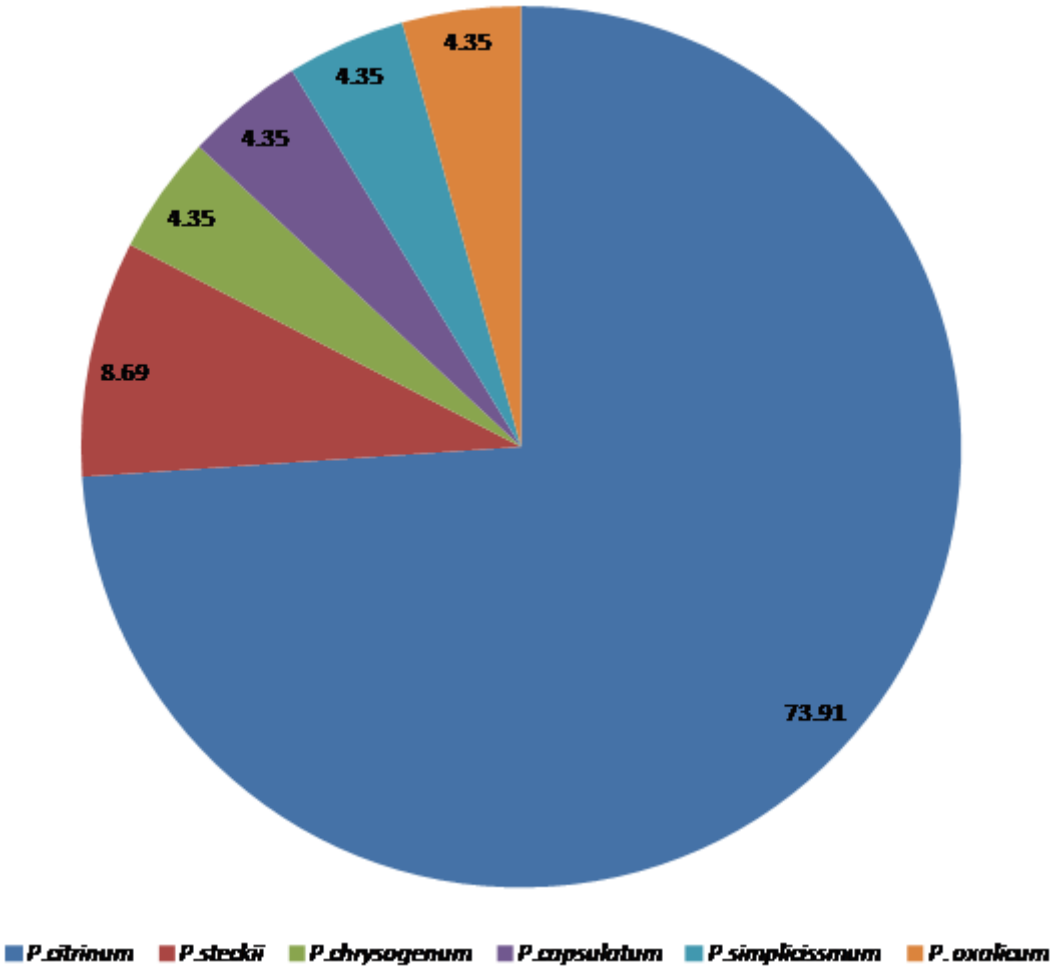


Fig: 4.6: Percentage (%) occurrence of *Penicillium* isolated from Millet grains obtained from the study area

Table 4.5: Cluster groups of the *Penicillium* isolates

Cluster Group	Total Number of isolates	Isolates Codes
I	17	28,25,22,15,12,10,3 5,20,4,6,7,13,17,18,24,32
II	2	14,29
III	1	31
IV	1	11
V	2	2,34

Table 4.6: Polymorphic Information content (PIC) of the *Penicillium* isolates

Sample type	Occurance	Allelic frequency	No. of neucleotide polymorphism	% polymorphism	polymorphic type	no. of occurance
Penicillium citrinum	**18**	**5:4**	**9**	**1.80%**	**C A transversion**	**2**
					G T transversion	**2**
					G A transistion	**1**
					T indel	**2**
					C indel	**1**
					A indel	**1**
Penicillium steckii	**2**		**1**	**0.02%**	**G indel**	**1**

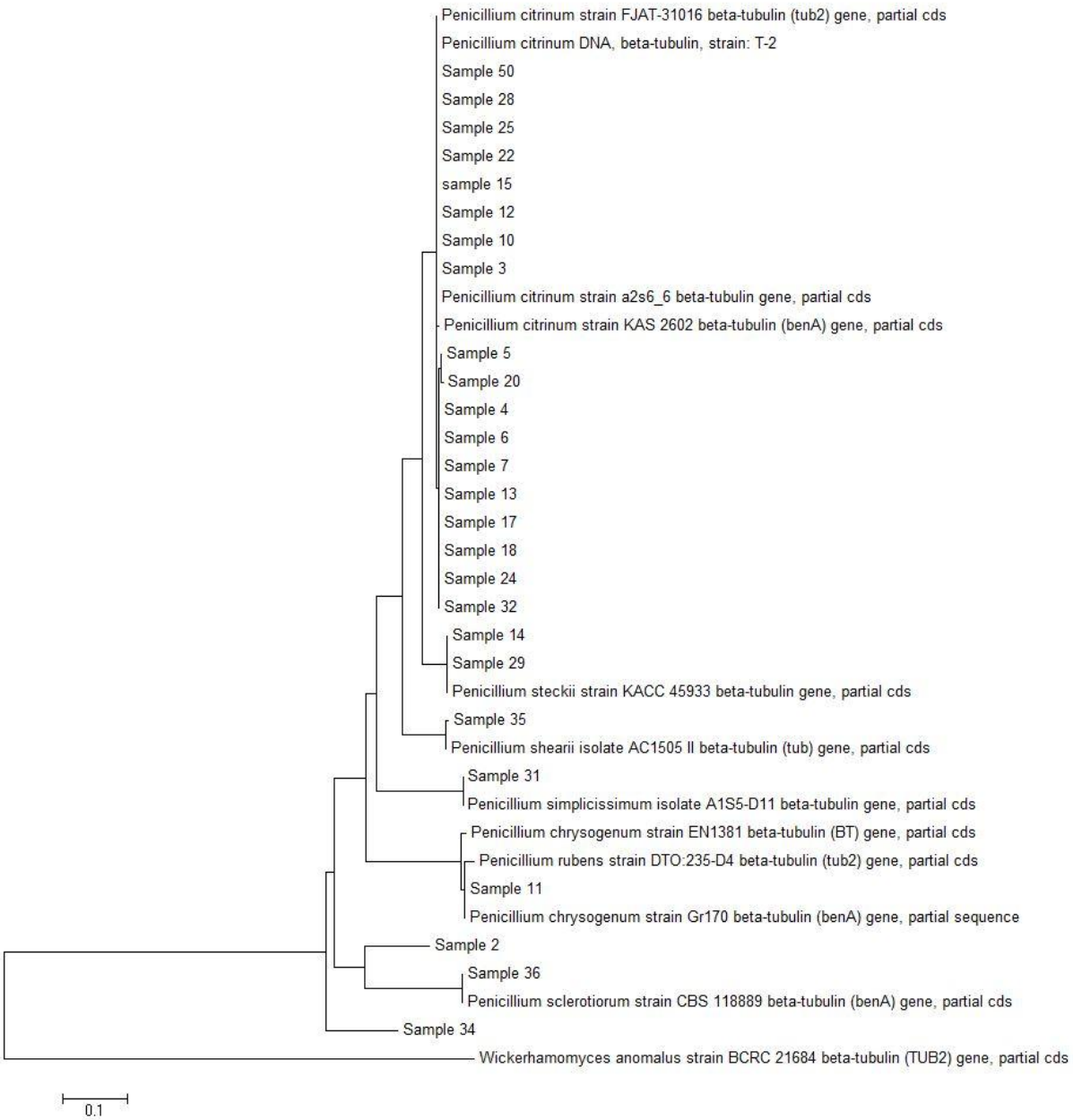


Fig 4.7: Dendogram showing the clusters of the *Penicillium* isolate

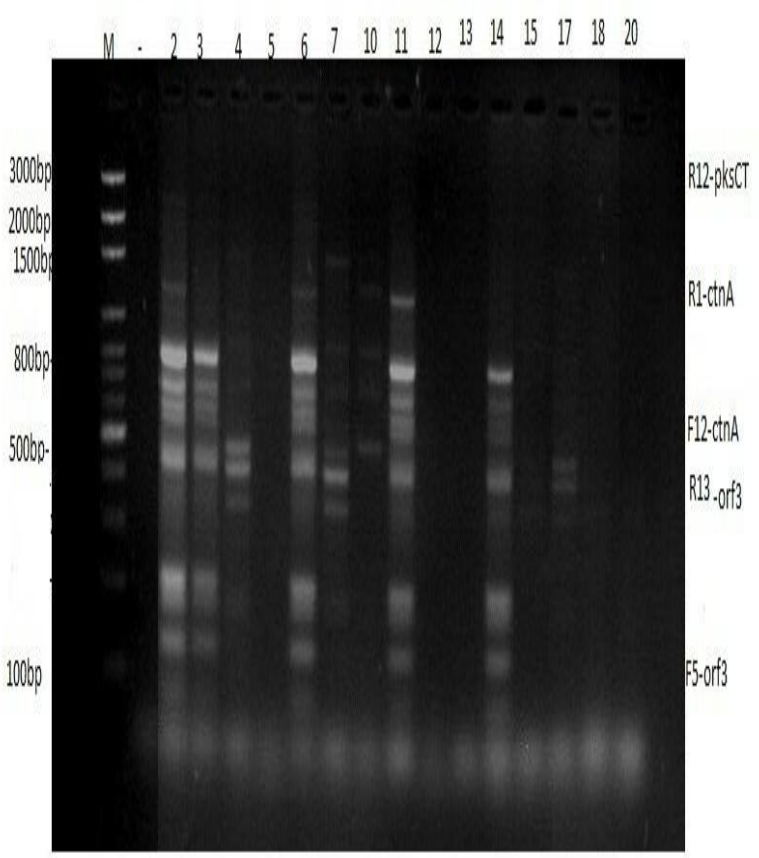


Plate 17: Multiplex Agarose gel: Amplification of mycotoxigenic gene from the *Penicillium* isolates

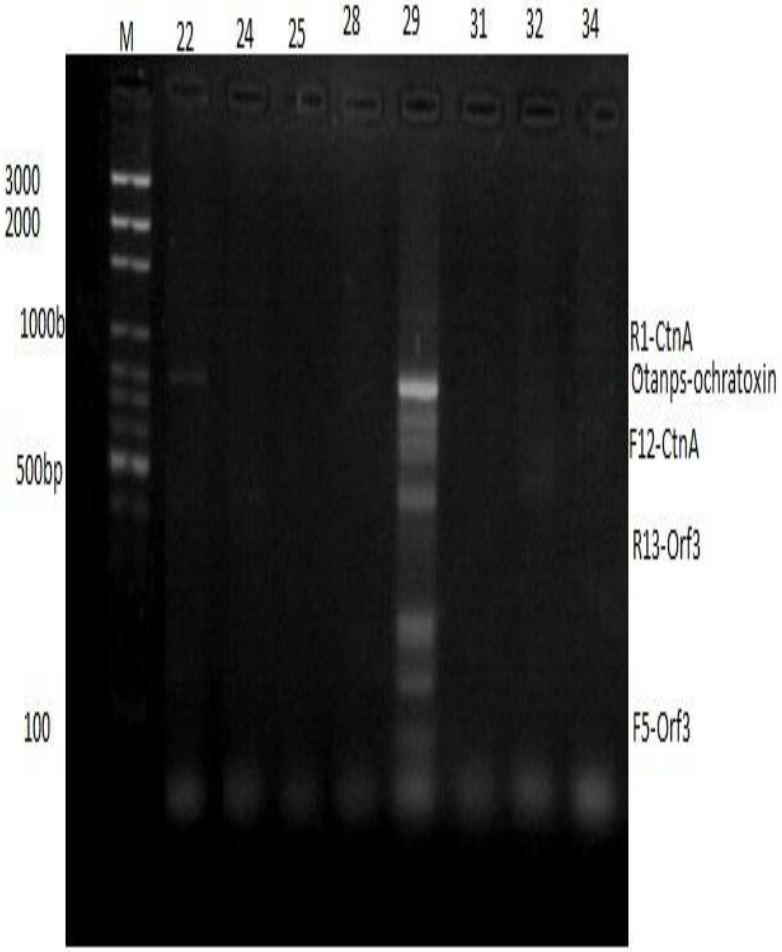


Plate 18: Multiplex Agarose gel: Amplification of mycotoxigenic gene from the *Penicillium* isolates with isolate 29 as the only amplified strain.

4.10 Ochratoxin quantification

The result of ochratoxin quantification revealed a high ochratoxin value of 7.0ppb at a pH value of 8 with lowest value of 0.1ppb at a pH value of 4 while effect of temperature on production resulted in a high yield of 5.0ppb at 25^0C, as observed on Fig 4.10a -4.10b.

Ochratoxin production obtained from each pH is shown on appendix page, at pH2 isolate 11 (*P.chrysogenum*) had the highest production at a value of 5.0ppb with lowest yield of 0ppb from isolate 14(*P. steckii*), at pH3 highest yield was at 1.5ppb from isolate 2 (*P. capsulatum*), lowest was produced by isolate 11 (*P. chrysogenum*),at pH4 isolate 11 (*P. chrysogenum*) gave highest value of 3.0ppb while lowest value 0.1ppb was obtained from isolate 29 (*P.steckii*).

Ochratoxin production at pH5, isolate11 (*P.chrysogenum*) gave a high yield at 5.0ppb and lowest was at 2.0ppb from isolate 6 (*P.citrinum*), at pH6 isolate 11 (*P. chrysogenum*) produced the highest ochratoxin value of 4.0ppb with lowest value of 0ppb by isolate 2 (*P. capsulatum*), at pH7 isolate 14 (*P.steckii*) with highest production at 4.0ppb,while lowest value of 0ppb was obtained from isolate 29 (*P.steckii*).

Highest value at pH8 was 7.0ppb produced by isolate 3 (*P.citrinum*), while isolates 6 (*P.citrinum*) and isolate 14 (*P.steckii*) both produced at lowest value of 2.0ppb, at pH9 isolate 2 (*P. capsulatum*) produced highest value at 4.5ppb with 0.5ppb as lowest value produced by isolate 14 (*P.steckii*), at pH10 isolate 29 gave highest ochratoxin yield at 5.0ppb while lowest yield was obtained from isolate 14 (*P.steckii*) at 0ppb.

Ochratoxin production from each temperature can be observed on appendix page, at 20^{o}C isolate 6 (*P.citrinum*) produced ochratoxin at value of 4.0ppb with 0ppb production from isolate 29 (*P.steckii*), at 25 ^{o}C ochratoxin production was highest at a value of 5.0ppb from isolate 6 (*P.citrinum*) and lowest at 0ppb from isolate 3 (*P. citrinum*).

highest ochratoxin value of 4.0ppb was at 30 ^{o}C from isolate 6 (*P.citrinum*) and lowest value from isolates 29 (*P.steckii*) at value of 0ppb,at 35 ^{o}C ochratoxin production was highest from isolate 6 (*P.citrinum*) with the value 1.0ppb and lowest from isolate 29 (*P. steckii*) with a value of 0ppb,at 40 ^{o}C ochratoxin production was highest with a value of 3.1ppb from isolate 6 (*P.citrinum*) while isolates 2 (*P. capsulatum*),29 (*P.steckii*),11 (*P. chrysogenum*) and 14 (*P.steckii*) produced the lowest value at 0ppb.

At room temperature highest production was obtained from isolate 2 (*P. capsulatum*) with a value of 2.4ppb while lowest value of 0.5ppb was observed from isolate 3 (*P.citrinum*).

Ochratoxin production from individual isolates using different temperature and pH can also be observed on the appendix page.

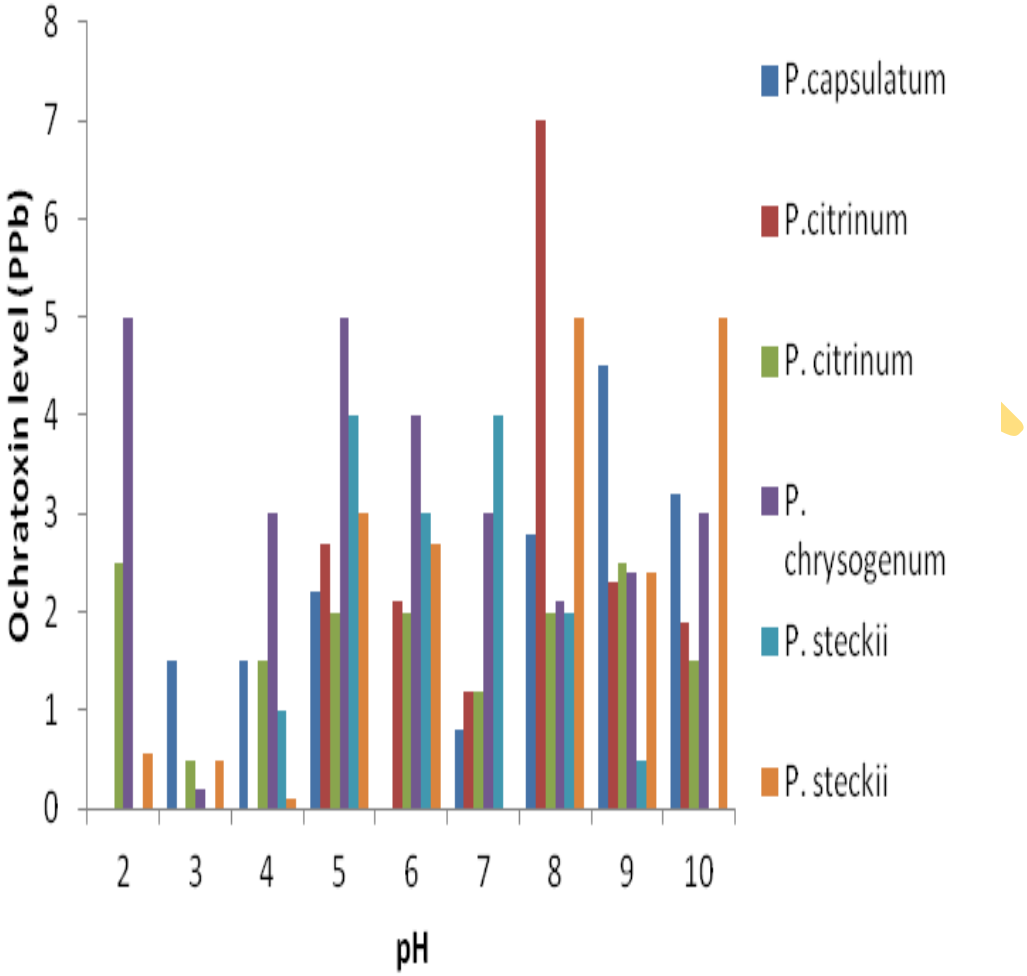
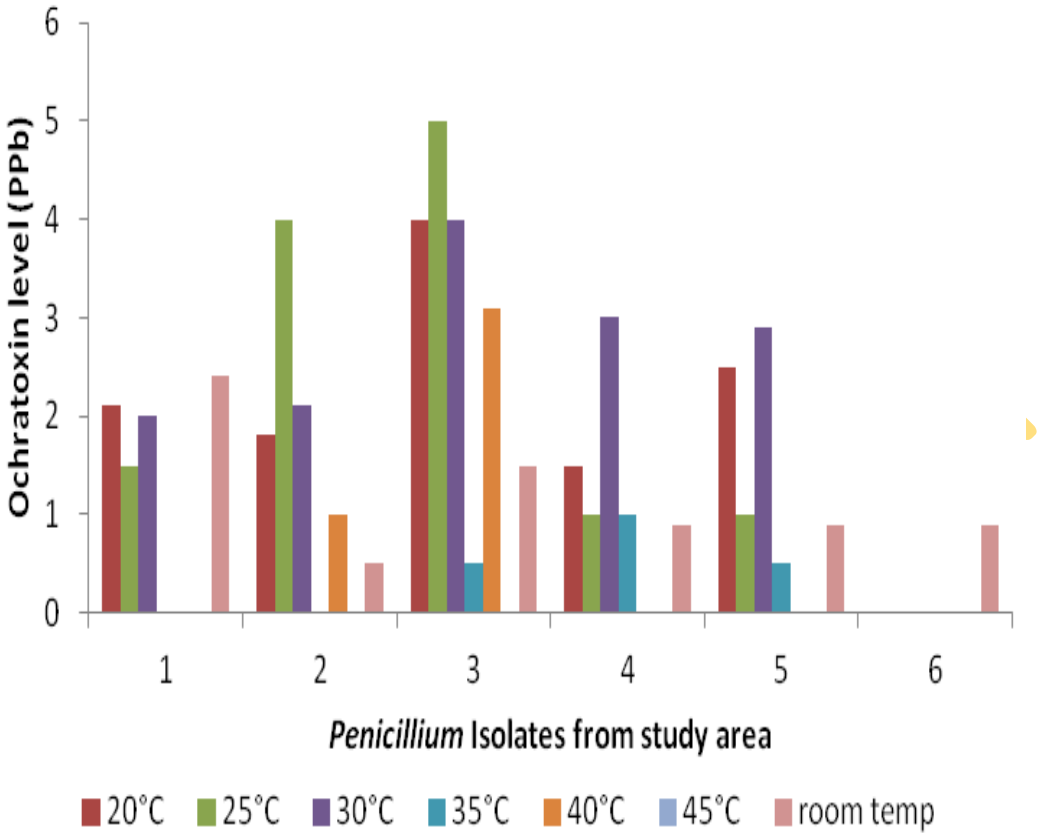


Fig 4.10a: Ochratoxin production by the *Penicillium* isolates at different pH.



Legend

1= *Penicillium capsulatum*, 2= *Penicillium citrinum*, 3= *Penicillium citrinum*,

4= *Penicillium chrysogenum*, 5= *Penicillium steckii*, 6= *Penicillium steckii*

Fig 4.10b: Ochratoxin production by the Penicillium isolates at different temperatures

CHAPTER FIVE

DISCUSSION

(*Pennisetum glaucum* (L) R.Br.) Millet grain, is the sixth most cultivated grain in the world, has been reported to sustain one third of the world's population especially in Africa (Karen, 2011). Nigeria produces 40% (nearly half) of the millets grown in Africa (Oelke *et al.*, 1990), therefore the presence of deteriorative fungi with Mycotoxin producing potentials in these grains represent a serious hazard to both human health and animals (Gassen, 1999).

The presence and isolation of fungi species from the millet grains in this study confirms the presence of fungi as contaminants of grains in storage as reported by Suleiman *et al*. (2013) and Syed *et al*. (2013).

In this research work, the results obtained show the presence of three major Fungal genus frequently isolated namely *Rhizopus, Aspergillus* and *Penicillium*, which were in accordance with the work of (Makun *et al*., 2009:Amadi and Adeniyi, 2009) which identified *Aspergillus, Fusarium, Helminthosporium, Mucor, Penicillium* and *Rhizopus* from millet. Miller (1995) documented the fact that of the large numbers of filamentous fungi found as contaminants in food only a fraction of them produce secondary metabolites such as mycotoxins which are toxic to vertebrates (humans and animals) these Fungi includes deteriorating storage fungi, especially *Fusarium, Aspergillus* and *Penicillium* species, this is also supported by (Erdogan, 2004; Zain, 2011). Pitt (2000) listed aflatoxins, fumonisins, trichothecenes, ochratoxins and zearalenone as some important mycotoxins occurring in grains.

Microbial activity has been reported to occur when moisture is present, therefore moisture content of the grain and that of the surrounding should be reduced and monitored (Jayas and White, 2003). Moisture content of all samples was determined, result obtained is close to the report of Laura (2013) who stated that as moisture content increases above 13.5% in sorghum, invasion by fungi also increases with temperature and time. Two states were observed to have the highest fungal counts from non-surface disinfected grains, these result could be linked to the environmental conditions for growth, insect damage, late harvesting, improper storage conditions, poor processing such as use of contaminated equipment, unhygienic personnel handling, milling and transportation processes, variable climatic conditions of the area which could have also favored the chances of fungal contamination of grains (Essono *et al*., 2007). Moisture content is closely related to temperature, under certain conditions such as premature storage of inadequately dried grain, re-absorption of water while in store from leaking containers or uptake from floors or store walls could bring about re-distribution of moisture leading to fungal growth. The Low fungal incidence observed in Lagos from this study could have been as a result of the quick disposal of grains, which are not kept for long before they are consumed, reprocessed into various food products and re-distribution to other parts of the state, giving little or no time for fungal growth.

The direct plating methods may be used to detect fungi in foods. The dilution plating method is the traditional method used in examining foods. The direct plating method have been found to be more efficient than the dilution plating method for detecting individual mould species, including most of the toxin producers. It can also be used to determine whether the presence of mould is due to external contamination or internal invasion. Dichloran rose Bengal chloramphenicol (DRBC) agar is recommended as a general-purpose medium for direct plating of grain kernels and for plate counts of flours, meals, and processed products for total counts, Bullerman and Bianchini (2011). The simplest way to evaluate the internal microflora of seeds and kernels is the direct plating method, supported by (Lund and Frisvad 2003, Park *et al.,* 2005) which involves surface sanitizing seeds or kernels in full strength or 50% household bleach for one minute to kill surface micro flora.

The amount of internal infection of the grain is an indicator of quality and storability of the grain.

The application of direct plating method in this study suggested that the contamination of these samples were both external and internal invasion, therefore supports the use of direct plating method for comparison of contamination of food sample as described by Mislivec (2000). The result obtained from this work implies that contamination was mostly external from Ogun and Osun states (field fungi were found to dominate the millet grains) which was observed in the non-surface disinfected grain.

Storage fungi are usually not present in large quantities before harvest but are widely distributed and almost always present. Contamination occurs through small quantities of spores contaminating the grain as it goes into storage from the harvest, from handling and storage equipment or from spores already present in storage structures (IRRI, 2006). From the work on effect of storage period on the fungal isolation, showed that fungal isolation showed little or no difference at the 1st and 3rd month of storage, at the 6th month isolation was high which could be as a result of the presence and redistribution of fungal inoculum in the grain during a short break in electricity which made the conditions favorable for their growth and multiplication, this could be supported by the work done by Owolade *et al*. (2011), who reported that sorghum seeds can be stored in freezer without loss of viability of seed provided electricity was available for at least 8 hours each day. Similar work by Dejene (2004) recorded that low temperature conditions associated with lower moisture content are most suitable for storage of sorghum grains. Molds development during storage can be controlled or prevented by ensuring that grain is adequately dry at intake, further protection can be provided by preventing the development of temperature and moisture gradients by cooling and/or aeration of the grain (Hockings, 2003). Fungal isolation from the storage of Millet grains in this study revealed a variation in fungal population which could have resulted from fluctuation in moisture content and temperature of storage as also observed by Marin *et al.* (1998) and Rees (2004). The findings of shahin *et al*. (2003) and Jonathan *et al*. (2011) which reported that the total fungal populations of wheat grains were increased significantly by increasing the storage period, they observed that the longer the

storage time, the higher the fungal population could also be used to explain the results obtained from this study.

This study focused on the *Penicillium* species present in the millet grains, nine isolates were obtained from Osun state, four from Ondo, three from Ogun, one each from Oyo and Lagos. Lund (1995a), developed a simple test to distinguish among several species of *Penicillium* present in cheese and bread factories in Demark which involved using Erlich reagent to visualize the production of indole compounds as secondary metabolites from culture plates. Species for which test is positive (which is indicated by the production of a violet ring on the filter paper after 2-6 minutes) includes *P. camembati*, *P. commune*, *P. expansum* and *P. roqueforti*. A few species give produce yellow colors while others are negative. The cultural characteristics of the isolates obtained from this study gave a Negative reaction to Erlich tests, these results are supported by the findings of Frisvad and Samson (2004) who also carried out Ehrlich test to detect specific extrolites formation by some species in series Viridicata (*Penicillium* species), the use of these simple tests was proposed to aid identification, since chromatographic and spectrometric equipment is not available in most mycological laboratories. Colony diameter of the *Penicillium citrinum* isolated from this study is close to the descriptions of Kim *et al*. (2007). Results obtained from the presumptive Mycotoxin test on coconut cream agar (CCA), is related to the findings of Mohamed *et al*. (2013) who obtained yellow green fluorescence on CCA as positive test for citrinin detection and also confirmed findings with Thin Layer Chromatography.

Apart from the work of some foreign authors among which are (Javadi *et al*., 2012; Abastabar *et al*., 2016), there has been no extensive attempt to the identification of the genus *Penicillium* using the beta tubulin gene marker approach until now in Nigeria, focus has been more on macroscopic and microscopic features, RAPD, AFLP for *Penicillium* identification while extensive identification in Nigeria has been on other molds such as *Aspergillus* and *Fusarium* species.

Suspected *Penicillium* isolates from this study were therefore further subjected to molecular identification, by amplifying the internal transcribed spacer (ITS) and beta tubulin gene (Ben A) regions. In this study the ITS primer amplified the rDNA regions of the *Penicillium*, three isolates were in accordance with results obtained by Tiwari *et al. (*2011) who amplified *Penicillium* ITS 1 and 4 regions with resulting amplicons ranging from 550,565and 540 base pair, other studies by Sunnucks (2000) and Erika *et al*. (2012) also reported a fragment of around 600 base pair.

Other isolates in this research were not amplified by the ITS 1 and 4 regions; this is corroborated by the findings of Peterson. (2000); and Samson *et al*. (2007) who stated that the slow evolution of the ITS has hence made it difficult to resolve the Phylogenetic history of some closely related economically important species like *Penicillium* and *Aspergillus*. The ITS region is not variable enough for distinguishing all closely related species of *Penicillium* species (Seifert *et al*., 2007; Skouboe *et al*., 1999). Houbraken *et al*. (2011), also reported similar values. Visagie *et al*. (2014), therefore suggested another region which is the β-tubulin gene region which can be successfully used for accurate identification of *Penicillium* species.

The results for Amplification of the partial beta-tubulin gene with primers Bt2a and Bt2b was successful in all isolates tested, this agrees with the preliminary findings of Seifert and Loius-Seize (2000) who demonstrated the utility of beta tubulin sequences for revealing Phylogenetic relationship and molecular support for phenotypically based species, the beta tubulin analysis had more resolution compared to the ITS analysis. The amount of variation is suitable for phylogenetic relationship among closely related species of *Penicillium* (Samson *et al*., 2004). Amplicon sizes obtained were similar to those obtained by Won *et al*. (2007). Lutzoni *et al.* (2000), their work revealed amplification of the β-tubulin gene region resulting in amplicons ca. 450 base pair long and aligned dataset containing sequences of the new species discovered, as well as its closely related sister species was 432 base pair long, results obtained by Kim *et al*. (2007) also yielded fragments approximately 500bp. Erika *et al*. (2012) obtained a fragment of 455 bp in size.

In this study Identification of *Penicillium* species was acquired by reliance on sequencing of this high resolution gene marker (beta tubulin) for quick accurate and reliable database

identification of all isolates (Frisvad and Samson, 2004 and Abastabar *et al*., 2014). Sequence results obtained revealed similarities between 15 out of the 17 *Penicillium citrinin* isolates. *Penicillium citrinum* was the predominant specie among the isolates studied which is in accordance with Shen *et al*. (1999). This study also indicates that the isolates obtained are genetically diverse with little correlation to sample they were isolated from and geographical location where they were obtained this is also confirmed by the findings of Roslan *et al*. (2010). Other factors which could bring about genetic diversity include mutation, chromosome deletions and environmental variations where isolates were collected (Nath *et al*., 2012).

Physiological studies of these *Penicillium* species is very crucial in the aspect of preventing their occurrence as food spoilage agents, their ability to grow at low temperatures and water activity has given them an edge at being universally present in cereals, refrigerated foods and many other environment, therefore adequate knowledge of their physiology could help in their possible control (Pitt and Hockings, 1997).

The results obtained from the physiological assessment of the *Penicillium* isolates showed growth over the range of incubation time used in this study, mycelial growth increased as incubation time increased, this indicates that the mycelial growth improved with length of time, growth was optimal at the 14th day and was reduced at 21day, which could have been as a result of depletion of nutrients in growth media and possible production of secondary metabolites. The *Penicillium* isolates also grew on the range of growth temperature of 20°C -35°C, mycelial growth was best at 30°C and was drastically low at 25°C this result is supported by the findings of Gunasekaran (2008) who discovered, the optimal temperature for both mycelia growth and pigment production to be 30°C, Gonzalez *et al*. (1988) and Montani *et al*. (1988) also reported *P. citrinum* to grow at 30°C. Consequently, the optimal temperature for both mycelial growth and pigment production was found to be 30°C, this optimal temperature is regarded as a favourable for *Penicillium* sp. This is similar to anthroquinone production by *P. oxalicum* (Sardaryan *et al*., 2004). This result however, differs from that of Larous *et al*. (2007), Kanan (2008) and Thiyam and Sharma (2014) who reported that 25°C was the optimum growth temperature for *Penicillium* species, this was also reported in studies relating to *P. expansum* (Lahlali *et al*., 2005), *P.*

digitatum and *P. italicum* (Plaza *et al.*, 2003). From pH evaluation, *Penicillium chrysogenum* had the highest mycelial growth at pH 8, similar results were observed by Thompson *et al.* (1993) who studied the growth of *Penicillium* species on pH ranging from 4.0-10.0, and Wheeler *et al*. (1991).

The *Penicillium* isolates were further examined for their growth in various carbon sources, which were relatively favorable to the mycelia growth of *Penicillium* species, is in corroboration with the studies by Gunasekaran and Poorniammal (2008) whose results showed glucose, fructose, mannose and sucrose as favorable for mycelia growth of *Penicillium* species. From Nitrogen source studied, best growth was observed on ammonimun sulphate this complies with the findings of Zahoor and Zafar (2014), who obtained better mycelia growth in medium containing ammonium as nitrogen source, and Cheng *et al*. (2013), also had higher mycelia growth on calcium nitrate. Both finding had lower mycelia growth on ammonium nitrate. However, the findings of Kim *et al*. (1997) observed that higher mycelia production was found in organic nitrogen sources.

Chromatographic methods have been used from the beginning of mycotoxin research and are still used for detecting, quantifying, and confirming the presence of mycotoxins. Various detection methods, such as fluorescence, ultraviolet absorption, and others have been combined with chromatographic methods. Polymerase chain reaction methods have also come to limelight. Selma *et al*. (2008) carried out a real-time PCR assay for ochratoxigenic *Aspergillus* detection using primers pertaining to the b-ketosynthase domain of a polyketide synthase from *A. carbonarius*. PCR methods for the detection other toxigenic fungi are documented by Niessen (2007).

The *Penicillium* isolates from this study were subjected to multiplex PCR to detect their toxigenic status, the citrinin biosynthetic gene cluster in *Monascus purpureus*BCRC33325 (IFO30873) was proposed in Shimizu's studies (2005, 2007), polyketides synthase (*pksCT*) and transcriptional activator (*ctnA*) were proven to be involved in citrinin biosynthesis, similar work was done by Shimuzu *et al*. (2005) and Chen *et al*.(2008), the amplicons fragment size used in this study were obtained from Chen *et al*.(2008), related work by

Sukthan *et al.* (2010), using RT-PCR revealed 879 bp of the partial citrinin biosynthesis gene products and a 454 bp actin gene products from *M. purpureus* TISTR3541 wild type (G0) and mutants (G1 to G4).

In this work the two primers employed to detect pksCT genes were not amplified. This result could be explained from the work of Shimizu *et al*. (2005) since PKS gene for citrinin (CT) (pksCT) was cloned from *Monascus purpureus* from the studies of Hajjaj *et al*.(1999b) revealed that the biosynthesis of citrinin was from a tetraketide pathway in *Monascus ruber* which differs from that of *Penicillium* and *Aspergillus* species which is a pentaketide, also confirmed by Abou-Zeid (2012) stating that the citrinin spectra label in *M.ruber* was strikingly different from those obtained from *P.citrinum* and *A.terrus*. Abou-Zeid (2012) further deduces that the tetraketide precursor for citrinin from *M. ruber* could have accounted for the difference in production of the other two Polyketides. This could have led to the negative result obtained for amplification of the pksCT gene from this study. Eleven (11) isolates showed amplification for the primers used for the multiplex PCR, this supported the results obtained by Ramana *et al*. (2011), who discovered that toxin metabolic pathway genes may be present under field conditions thus influencing toxin production.

This study proposes to be the first in Nigeria to have detected citrinin from *Penicillium citrinum* using citrinin biosynthetic genes obtained from *Monasus purpureus*, other related studies are those of Khosravi *et al*., (2012) using randomly amplified polymorphic DNA (RAPD) to type toxigenic *P.citrinum* strains, showing amplifications ranging from 376bp to 3.5kb.

Ochratoxin A (OTA) is a mycotoxin produced by secondary metabolism of many filamentous species belonging to the genera *Aspergillus* and *Penicillium* (Miller and Trenholm, 1994). In the genus *Penicillium*, *P*. *verrucosum* has been regarded to be the only species of the genus *Penicillium* to synthesize OTA (Geisen *et al*., 2004). However, it has been shown that two species of the genus *Penicillium* have this capacity, namely *P*. *verrucosum* and *P*. *nordicum* (Frisvad *et al*., 2006). *Penicillium verrucosum* is the major producer of OTA in stored cereals (Geisen *et al*., 2004), *Penicillium nordicum*

(Lund and Frisvad, 2003) is the main OTA producer found in meat products. With respect to liquorice, Chen *et al*. (2011) found that *P. polonicum* and *P. chrysogenum* were the primary OTA contributors in moldy liquorice in China. From this study, *Penicillium capsulatum, P. chrysogenum, P.citrinum* and *P.steckii* have been implicated in ochratoxin production.

Mycotoxin production has been found to be influenced by environment in which the toxin is being produced; they have been reported to resist being destroyed by heat. Ochratoxin has been reported to persist in food (Lanyasunya *et al*., 2005). Pardo *et al*., 2004; Kapetanakou *et al*., 2011 reported the effects of Ecological factors such as water activity, temperature and pH on OTA production by A. ochraceus and A. carbonarius. According to Leong *et al*. (2006), OTA production by A. carbonarius and A. niger was not related to the growth length, colony size was strongly controlled by temperature.

Detection methods based on the production of antibodies specific for individual mycotoxins have been developed which include enzyme-linked immunosorbent assays and immunoaffinity columns. These methods allow for specific and precise detection and quantification of specific mycotoxins, using test kits for mycotoxins which are rapid and simple to use.

Quantification of ochratoxin from this study revealed that ochratoxin production was best favored at pH 8 and this agrees with the reports of Keller *et al*. (1997) while the optimum temperature range of 20-30°C for ochratoxin production was also observed by Pardo *et al* . (2004). This study has shown that the production of ochratoxin is temperature dependent and 25ºC was the most favorable temperature to produce significant quantity of OTA as compared to other temperatures. Most of the times the temperature for storage conditions for commodities/grains, falls within this range and could encourage OTA production through air born fungal infestations. In this study ochratoxin production by some isolates fell within while an isolate exceeded the maximum tolerance limit for OTA in cereals (5ng/g) as fixed by the European Community Regulation no. 472/2002 (Commission Regulation EC, 2005). A sufficient number of literature is available regarding OTA production by different fungal strains, temperatures and substrates, however there are fewer studies available from Nigeria on this issue. The presence of these toxigenic *Penicillium* on millet grains from this study therefore is of great concern since production of the mycotoxin

is also favored at temperatures available within storage conditions.storage conditions should therefore be modified by adopting some suitable preventive measures, and prolonged storage conditions should be avoided, to prevent the production of OTA in stored grains.

The presence of OTA in food and feed is a health hazard, storage should be done under situations which can restrict fungal growth and the consumption of contaminated foods should be avoided.

CONCLUSION

The species characterization gave a better result by amplifying the Partial beta tubulin gene, band variations which were observed in only two species, isolate 31 (*P. simplicissimum*) and 34 (*P. oxalicum*). The partial beta tubulin gene therefore should be suggested as a molecular marker for species level identification of the genus *Penicillium.*

The Dendogram of isolates formed five clusters, the similarity coefficient ranged from 0.539-0.814 indicating that no two isolates were 100% similar.

Mycotoxin detection on the Multiplex Agarose gel showed no amplification of the Polyketide synthesis (pksCT) gene cloned from *Monascus purpureus* using primer *F12* and *F8.*

This study has been able to use the apparent CT (Citrinin) biosynthetic gene cluster (pksCT and orf3) in *Monascus purpureus* to identify corresponding gene clusters in the genus *Penicillium* which appears to be the first to be reported in Nigeria.

This research has also proven that the use of the polyketides synthase (*pksCT*) from *Monascus purpureus* is from a different origin and therefore could not be used to amplify Polyketide gene in *Penicillium.* It could be concluded that the pksCT gene is therefore species specific gene.

More rigorous studies are required on this critical issue of ochratoxin A to create awareness to consumers and the concerned administration.

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APPENDIX

Appendix 1: DRBC (Dichloran Rose-Bengal Chloramphenicol agar. King *et al*., 1979)

Peptone	5g
Glucose	10g
KH_2PO_4	1g
$MgSO_4.7H_2O$	0.5g
Dichloran	0.002g
Rose Bengal	0.025g
Chloramphenicol	0.1g
Agar	15g\l
pH	5.6+/-0.2

Appendix 2: CYA Broth (Czapek Yeast auto lysate agar. Pitt,1979)

$NaNo_3$	3g
Yeast extracts	5g
Sucrose	30g
$K_2HPO_4.3H_2O$	1.3g
$MgSO_4.7H_2O$	0.5g
KCL	0.5g
$FeSO_4.7H_2O$	0.01g
$CuSO_4.5H_2O$	0.005g
$ZnSO_4.7H_2O$	0.01g
Agar	15g
pH	6.3+\-0.2

Appendix 3: YES (Yeast Extract Sucrose Agar. Frisvad, 1981; Filtenborg*et al*., 1990)

Yeast extracts	20g
Sucrose	150g
$MgSO_4.7H_2O$	0.5g
$CuSO_4.5H_2O$	0.005g
$ZnSO_4.7H_2O$	0.01g
Agar	20g

Appendix 4: Ehrlich test

Test for production of cyclopiazonic acid and other alkaloids using filter paper method.

4-dimethylaminobenzaldehyde	2g
Ethanol	85ml
10N Hcl	15ml

CCA (Coconut cream agar)

Coconut milk	600ml
Agar	16g
Distilled water	make up to 1000ml

APPENDIX 5: Gene sequences of *Penicillium* isolates obtained

>Sample 2 *Penicillium capsulatum*

TGCTTTCTGGTACGTGCCCGACGCGGAACCTCCCCCCCAAaaAcaAATACaGGGA
GatTCAATTGTTCATCTTCTGGGAcGACATGCTGACGGTATTGCAGGCAAAACAT
CTCCGCTGAGCACGGCCTCGATGGCGATGGCCAGTAAGTTTAAAAAACGATCG
ATTTCAAAGTAGAATGGCGGTCTAATGGTCTGGTTAGCTACAATGGTTCCTCCG
ACCTCCAGCTGGAGCGCATGAACGTCTACTTCAACCACGTAAGTAGCACTAAC
AAACAACATTCAATGTGAACAACATCTTTACTTATATTTACTGATAGGCTACCG
GTGACCGTTACGTTCCCCGTGCTGTTCTGGTCGACCTGGAGCCCGGTACCATGG
ATGCTGTCCGTGCCGGTCCCTTCGGCAAGCTCTTCCGTCCCGACAACTTTGTCTT
CGGTCAGTCTGGTGCTGGTAACAACTGGGCCAAGGgTCACT

>Sample 3 *Penicillium citrinum*

GGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCcaT
TgtTGGataCaaaGAGCaaTATACTGaccaaTTCtaTagGCaaACCATTGctggcgaGCACGGC
CTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGTTTACGTTTTTTC
GAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACCTCCGATCTCCAG
CTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACTGACCCCAATGCC
ATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATAGGCTTCCGGTGA
CAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGGTACCATGGACGC
TGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAACTTCGTTTTCGGT
CAATCCGGTGCTGGTAACAACTGGGCCAAGGGtCACTAcCTGaGGGTA

>Sample 4 *Penicillium citrinum*

TGGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCA
ATTGTTGGATACAAAGAGCAATATACTGACCAATTCTATAGGCAAACCATTGCT
TTGgAgAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGT
GTTTACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAA
CCTCCGATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATA
CTGACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAA
TAGGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCC
GGTACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGAC
AACTTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTac
ACCTGAGGGTA

>Sample 5 *Penicillium citrinum*

TGGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCA
ATTGTTGGATACAAAGAGCAATATACTGACCAATTCTATAGGCAAACCATTGCT
TGTAgAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTG
TTTACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAAC
CTCCGATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATAC
TGACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAAT
AGGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCG
GTACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACA
ACTTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTacAC
CTGAGGGTA

>Sample 6 *Penicillium citrinum*

TGGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCA
ATTGTTGGATACAAAGAGCAATATACTGACCAATTCTATAGGCAACCATtGcTG
GAGAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGT
TTACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACC
TCCGATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACT
GACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATA
GGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGG
TACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAA
CTTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTACAC
-TGAG

>Sample 7 *Penicillium citrinum*

TTGctGGAGAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGA
AGTGTTTACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACG
GAACCTCCGATCTCCAGCTGgAGCGCATGAACGTCTACTTCACCCACGTAAGTG
ATACTGACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTAT
CAATAGGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAG
CCCGGTACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCC
GACAACTTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCAC
TAcAcCTGAGGGTA

>Sample 10 *Penicillium citrinum*

TGGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCA
ATTGTTGGATACAAAGAGCAATATACTGACCAATTCTATAGGCAAACCATTGCT
GGCGAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTG
TTTACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAAC
CTCCGATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATAC
TGACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAAT
AGGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCG
GTACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACA
ACTTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTAcA
CCTGAGGGTA

>Sample 11 *Penicillium chrysogenum*

CTCTGGCGAGCACGGTCTCGatGgcGATGgacagtaAGTTTAACAGtGATGgggattctGgt
GgatcaCACGtCTGAtatCtTGCtAGGtAcaatGgtaCCTCCgAcCTCCAGcTCGaGCGtATGA
ACGtCTACtTCAACCaTGtgAGTACAAtGacTGggAAtctTaattGtGCATCAtCTGATCGG
gCGTTTTTCTTTGACAATCTAGGCCAGCGgtGACAAGTACGTTCCCCGtGCCGTTC
TGgtCgATTTGgAGCCCGGtACCATGGAcGCTGTCCGCTCCGGTCCCTTCGGCAAG
CTTTTCCGCCCCGACAACTTCGTCTTCGGTCAGTCCGGtGCTGGTAACAAcTGggC
CAaGGgtCAcTACAcTGAgggta

>Sample 12 *Penicillium citrinum*

TGGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCA
ATTGTTGGaTACAAAGAGCAATATACTGACCAATTCTATAGGCaAACCATTGcTG
gcGAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGTT
TACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACCT
CCGATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACTG
ACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATAG
GCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGGT
ACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAAC
TTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTACAC-
TGAGGGTA

>Sample 13 *Penicillium citrinum*

GAGCAATATACTGACCAATTCTATAGGCAAACCATTGcTGgAgAGCACGGCCTT
GATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGTTTACGTTTTTTCGAG
AATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACCTCCGATCTCCAGCTG
GAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACTGACCCCAATGCCATT
GGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATAGGCTTCCGGTGACA
AGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGGTACCATGGACGCTG
TCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAACTTCGTTTTCGGTCA
ATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTACAC-TGAGGGTA

>Sample 14 *Penicillium steckii*

TACGTGCTGCAAATCCTGAATGATCAATTGTTGGATATAAAAGGCATAtgcTGAC
CaATTctAtaggCaAaCcattgctggcgAGCACGGcCttgaTGGCGATGgCCAGtAAGTTcTttcg
ACAAAGAAGCGTTTGTGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGC
TACaACGGAACCTCTGACCTCCAGCTGGAGCGCATGAACGTTTACTTCACCCAC
GTAAGTGATACTGACCTCAATGGGATTGAAAACATTATCTAACGATTGTTTGTT
TGTTTTTTTGAATAGGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTTCTGGTCG
ACTTGGAGCCCGGTACCATGGACGCTGTCCGTGCCGGTCCCTTCGGCAAGCTTT
TCCGCCCCGACAACTTCGTTTTCGGTCAGTCTGGTGCTGGTAACAACTGGGCCA
AGGGTCACTacAccTGAG

>sample 15 *Penicillium citrinum*

GCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCAATTGTTGGGTACAAAGAGC
AATATACTGACCAATTCTATAGGCAAACCATTGCTGGCGAGCACGGCCTTGATG
GCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGTTTACGTTTTTTCGAGAATG
GCGGTCTGATATTTTTGGGCAGCTACAACGGAACCTCCGATCTCCAGCTGGAGC
GCATGAACGTCTACTTCACCCACGTAAGTGATACTGACCCCAATGCCATTGGAA
TCATTATCTAACCAACTATTGTTTGTTTTATCAATAGGCTTCCGGTGACAAGTAT
GTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGGTACCATGGACGCTGTCCGT
GCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAACTTCGTTTTCGGTCAATCCG
GTGCTGGTAACAACTGGGCCAAGGGTC

>Sample 17 *Penicillium citrinum*

TGGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCA
ATTGTTGGATACAAAGAGCAATATACTGACCAATTCTATAGGCAAACCATTGcT
GGaGAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTG
TTTACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAAC
CTCCGATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATAC
TGACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAAT
AGGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCG
GTACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACA
ACTTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTACA
C-TGAGGGTA

>Sample 18 *Penicillium citrinum*

TGGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCA
ATTGTTGGATACAAAGAGCAATATACTGACCAATTCTATAGGCAAACCATTGct
GgaGAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGT
TTACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACC
TCCGATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACT
GACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATA
GGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGG
TACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAA
CTTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTACAC
TGAGGGTA

>Sample 20 *Penicillium citrinum*

TGGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCA
ATTGTTGGATACAAAGAGCAATATACTGACCAATTCTATAGGCAAACCATTGcG
GTAgAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGT
TTACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACC
TCCGATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACT
GACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATA
GGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGG
TACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAA
CTTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTacACC
TGAGGGTA

>Sample 22 *Penicillium citrinum*

TGGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCA
ATTGTTGGATACAAAGAGCAATATACTGACCAATTCTATAGGCAAACCATTGCT
GGCAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGT
TTACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACC
TCCGATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACT
GACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATA
GGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGG
TACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAA
CTTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTACAC

>Sample 24 *Penicillium citrinum*

TGGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCA
ATTGTTGGATACAAGAGCAATATACTGACCAATTCTATAGGCaAACCATTGcTG
GAGAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGT
TTACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACC
TCCGATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACT
GACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATA
GGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGG
TACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAA
CTTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTACAC
TGAG

>Sample 25 *Penicillium citrinum*

ACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCAATTGT
TGGATACAAAGAGCAATATACTGACCAATTCTATAGGCAAACCATTGCTGGCG
AGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGTTTA
CGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACCTCC
GATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACTGAC
CCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATAGGC
TTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGGTAC
CATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAACTT
CGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTACACTGA
GGGTA>

>Sample 28 *Penicillium citrinum*

GTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCAAT
TGTTGGATACAAAGAGCAATATACTGACCAATTCTATAGGCAAACCATTGctGgc
gAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGTTTA
CGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACCTCC
GATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACTGAC
CCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATAGGC
TTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGGTAC
CATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAACTT
CGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTAcACCTG
AG

>Sample 29 *Penicillium steckii*

TACGTGCTGCAAATCCTGAATGATCAATTGTTGGATATAAAAGGCAATATGCTG
ACCAATTCTATAGGCAAACCATTGCTGGCGAGCACGGCCTTGATGGCGATGGC
CAGTAAGTTCTTTCGACAAAGAAGCGTTTGTGTTTTTTCGAGAATGGCGGTCTG
ATATTTTTGGGCAGCTACAACGGAACCTCTGACCTCCAGCTGGAGCGCATGAAC
GTTTACTTCACCCACGTAAGTGATACTGACCTCAATGGGATTGAAAACATTATC
TAACGATTGTTTGTTTGTTTTTTTGAATAGGCTTCCGGTGACAAGTATGTTCCCC
GTGCCGTTCTGGTCGACTTGGAGCCCGGTACCATGGACGCTGTCCGTGCCGGTC
CCTTCGGCAAGCTTTTCCGCCCCGACAACTTCGTTTTCGGTCAGTCTGGTGCTGG
TAACAACTGGGCCAAGGGTCACTAcACtGAGGGTA

>Sample 31 *Penicillium simplicissimum*

ACCAAATCGGTGCTGCTTTCTGGTACGTGCCACGCCCACCCCAAAAACCTCCTG
GCCACAACAGAACGACGTTTCAATTGCGAGTCAAATCGGCGGAGTTGTACTGA
CTCAATTTACAGGCAGACCATTGCTGGTGAGCACGGCCTCGATGGCGATGGCC
AGTAAGTTATTTCGACTTCCATCGATAATTCCCCCGCGACACGGAATGGCGGTC
TGATATTTTTTTTGGCTAGCTACTCTGGCACTTCCGACCTCCAGCTGGAGCGTTT
GAACGTCTACTTCACCCACGTAAGTTCGCGCGTATGGTGGATGCTGGTACGCTG
GAATTGAATCTAACCCATGAATTTCGTGATTTTCTTAGGCCGGTGGTGACAAGT
ACGTTCCCCGTGCCGTCCTCGTCGATTTGGAACCCGGTACCATGGACGCCGTCC
GTGCCGGTCCCTTTGGCAAGCTCTTCCGTCCCGACAACTTCGTCTTTGGCCAGTC
CGGTGCTGGTAACAACTGGGCCAAGGGTCACTACACTGAGGGTAG

>Sample 32 *Penicillium citrinum*

GGTAACCAAATCGGTGCTGCTTTCTGGTACGTGCTGCAAATCCTGAAAGATCAA
TTGTTGGATACAAAGAGCAATATACTGACCAATTCTATAGGCaAACCATTGcTG
GAGAGCACGGCCTTGATGGCGATGGACAGTAAGTTCTTTCGACAAAGAAGTGT
TTACGTTTTTTCGAGAATGGCGGTCTGATATTTTTGGGCAGCTACAACGGAACC
TCCGATCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTGATACT
GACCCCAATGCCATTGGAATCATTATCTAACCAACTATTGTTTGTTTTATCAATA
GGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGACTTGGAGCCCGG
TACCATGGACGCTGTCCGTGCTGGTCCCTTCGGCAAGCTTTTCCGTCCCGACAA
CTTCGTTTTCGGTCAATCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTAcAC
CTGAGGGTA

>Sample 34 *Penicillium oxalicum*

cGAGcAcaacACGAcaATTCTTTGTGTCGTGAtTCGATCTAATATGAGAaccAAACTG
ACTCGATATTCAGGCAAACCATTGcTGGTGAGCACGGcCTTGacGGCGATGGCCA
GtAAGtCAACAGCTTGCCCCCCCCCCCCCCCcCCCACGATGGGATCCTGACGAC
AGAGAATGGCGGTCTGATATTTTTCGGTTTAGCTACAATGGTTCCTCCGACCTC
CAGCTGGAGCGCATGAACGTCTACTTCACCCACGTAAGTCGTGAAAAACGTCTT
CCCAATCAAAGAAATCTCCCAACCCTGATCATTGAAACGTTCGATTTTCCAGGC
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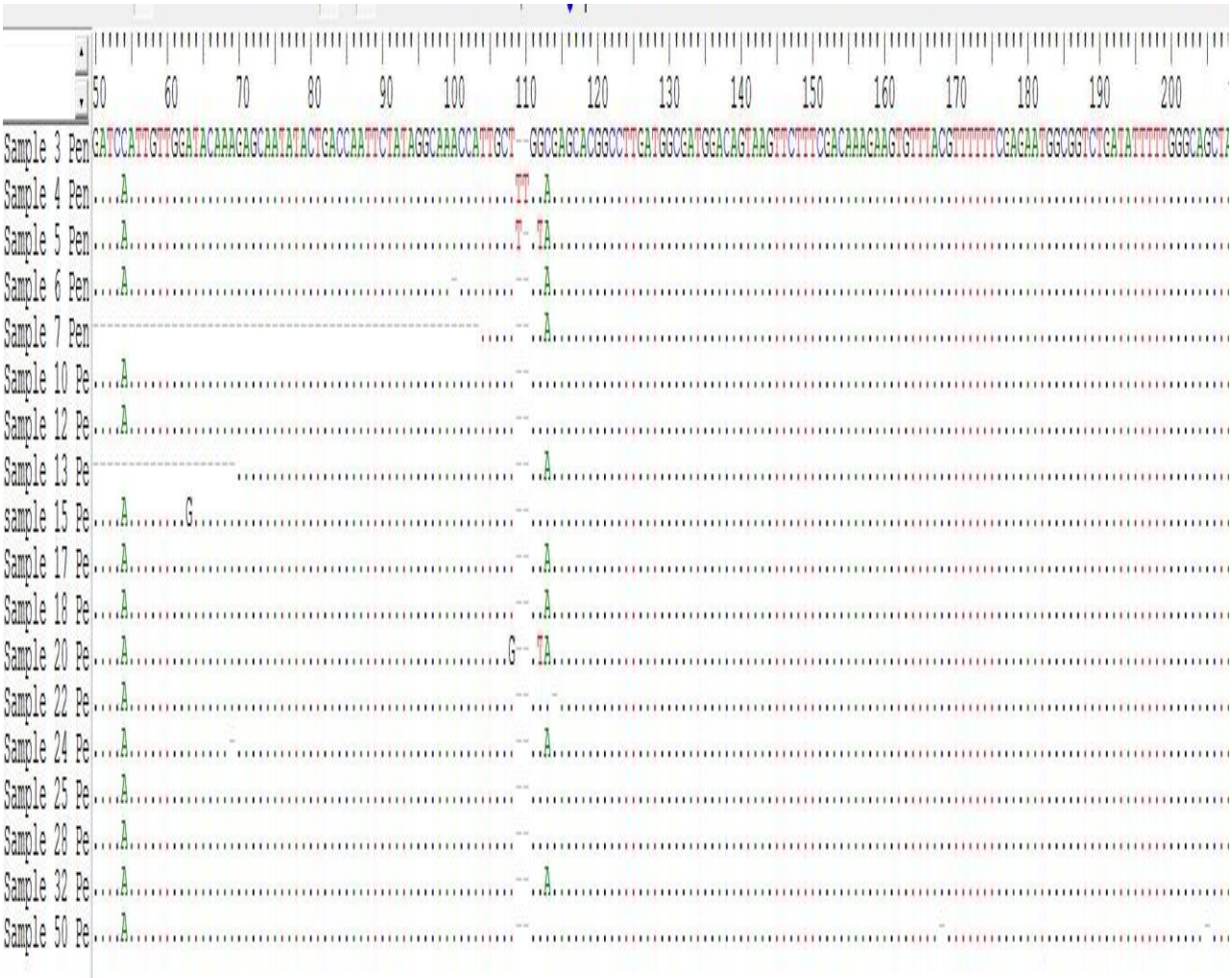
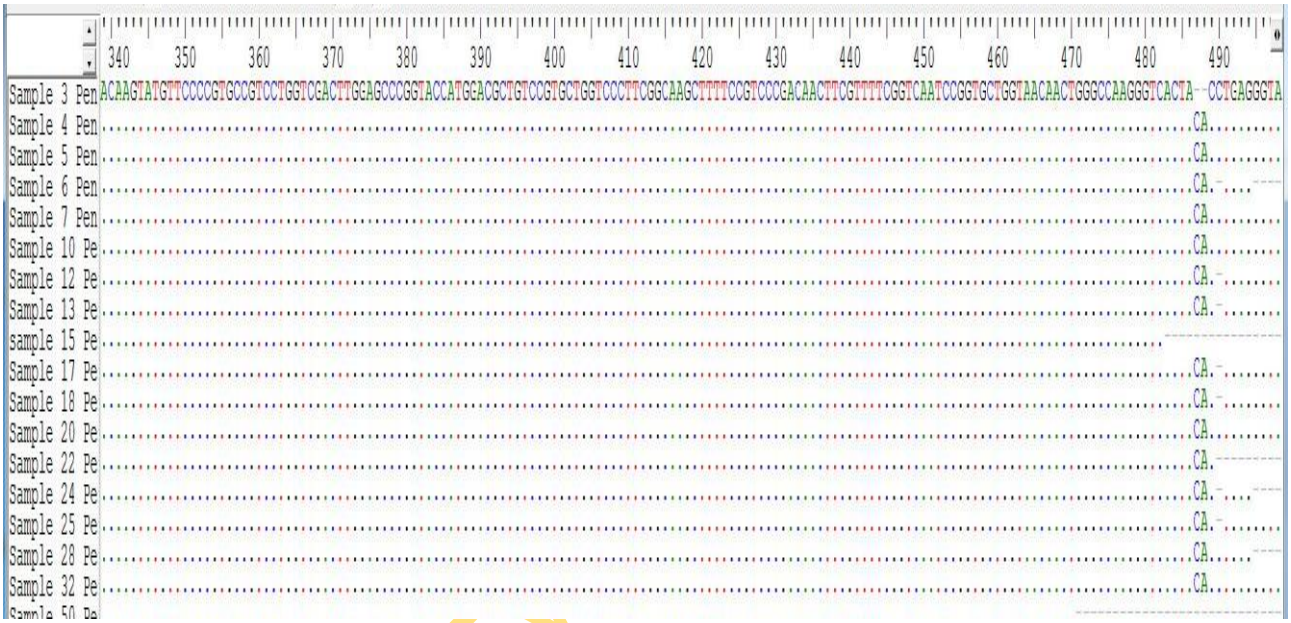
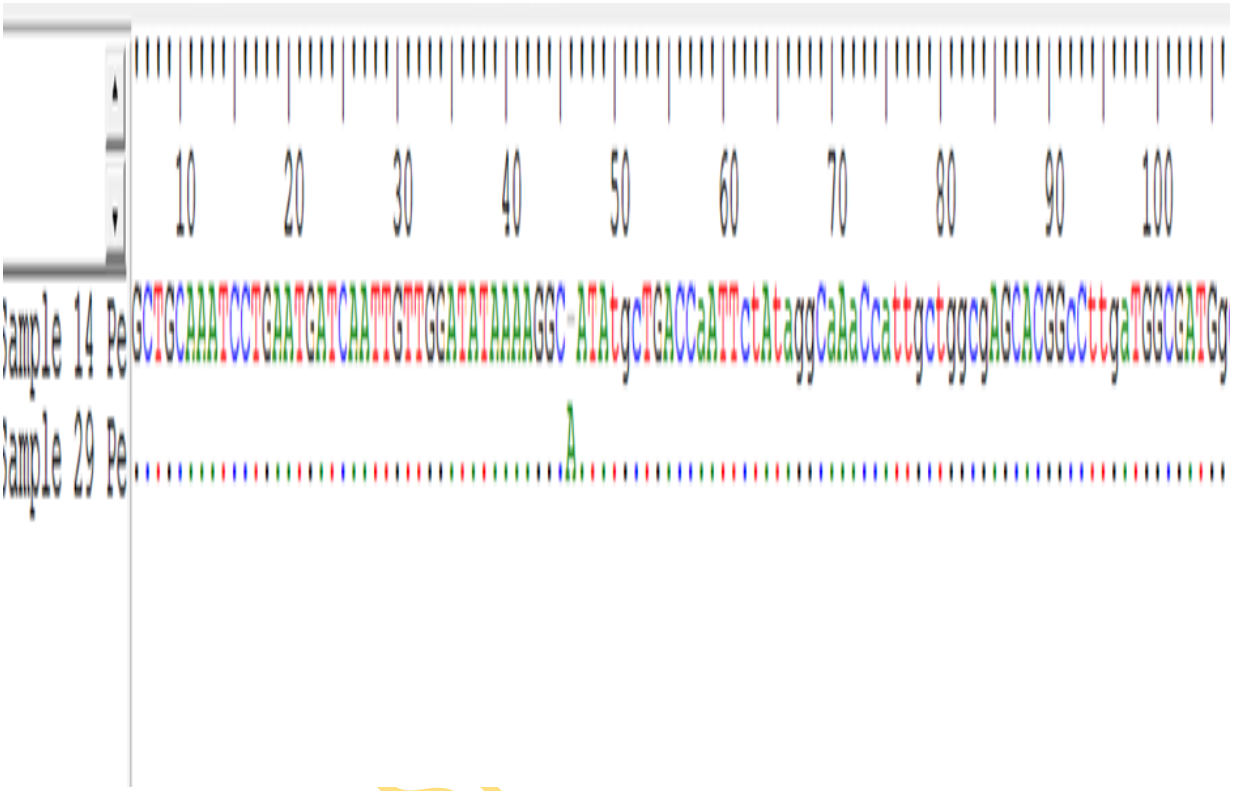
CGTC



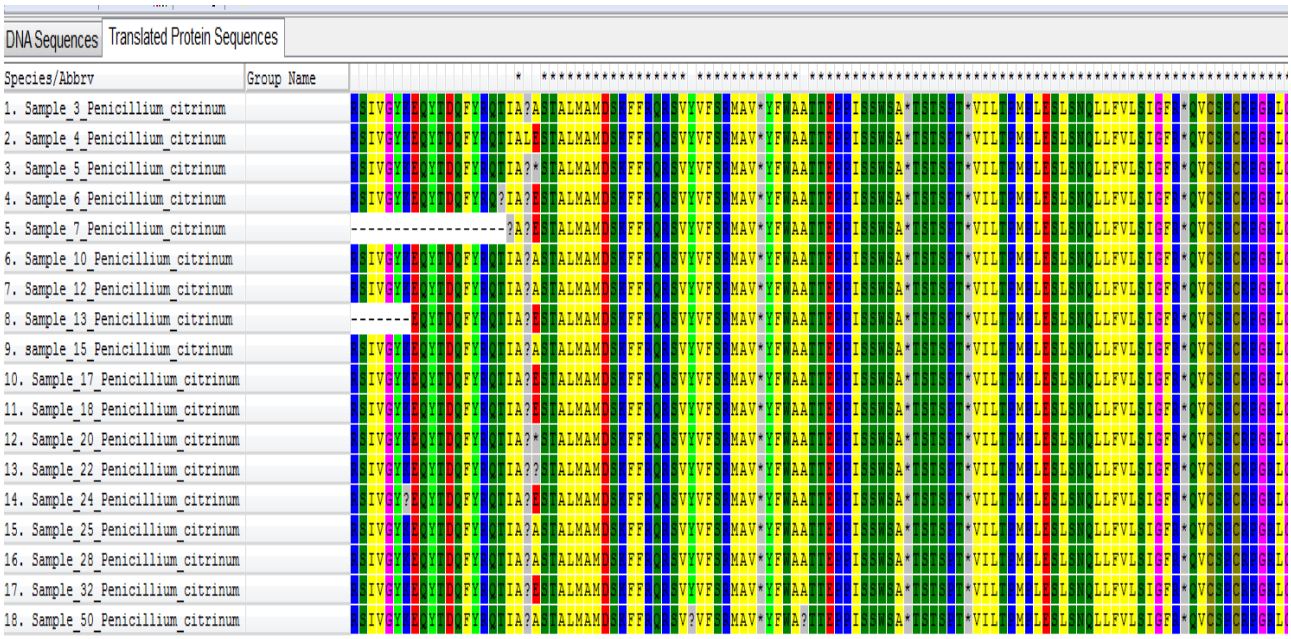
Alignment of the *Penicillium citrinum* nucleotide sequence obtained from amplified beta tubulin gene.



Alignment of the *Penicillium citrinum* nucleotide sequence obtained from amplified beta tubulin gene.

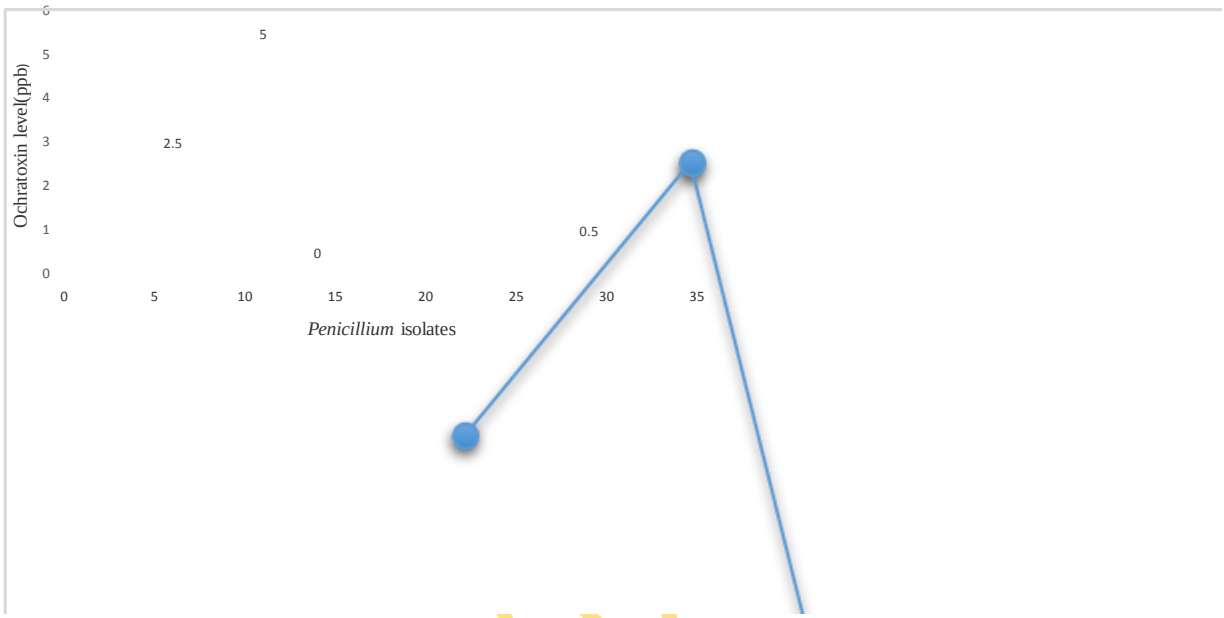


Alignment of the *Penicillium steckii* nucleotide sequence obtained from amplified beta tubulin gene.

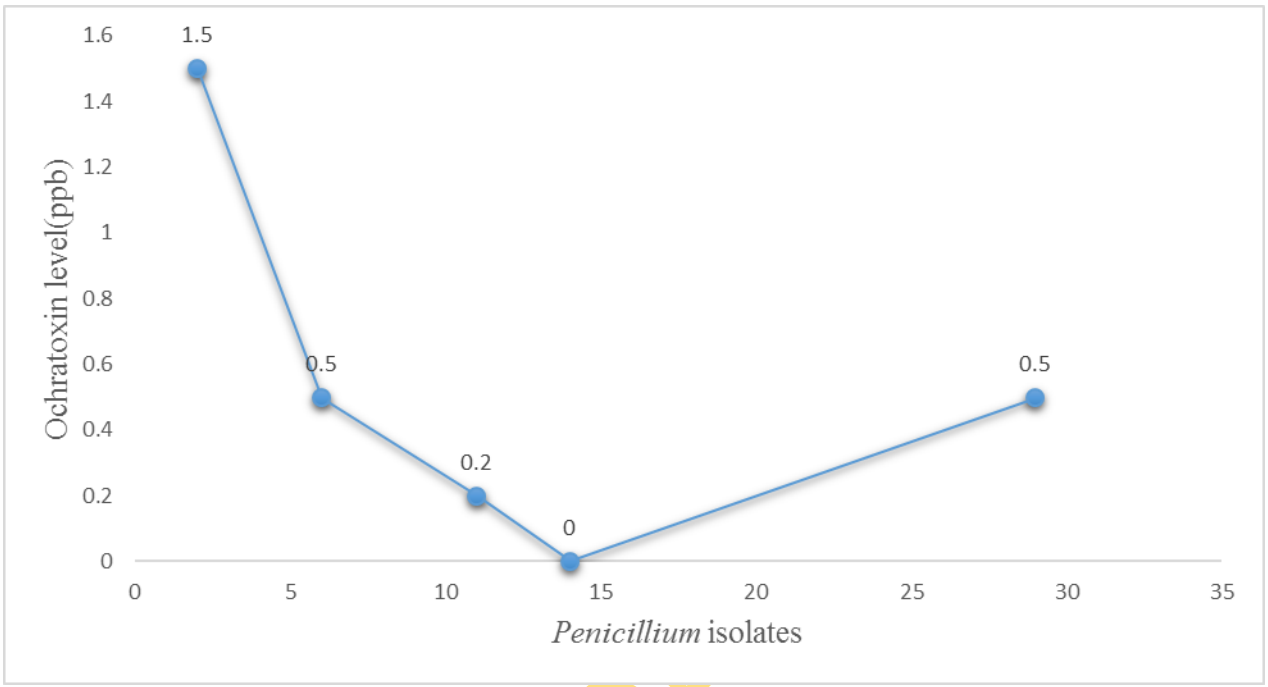


Translated protein sequences from the *P. citrinum* isolates

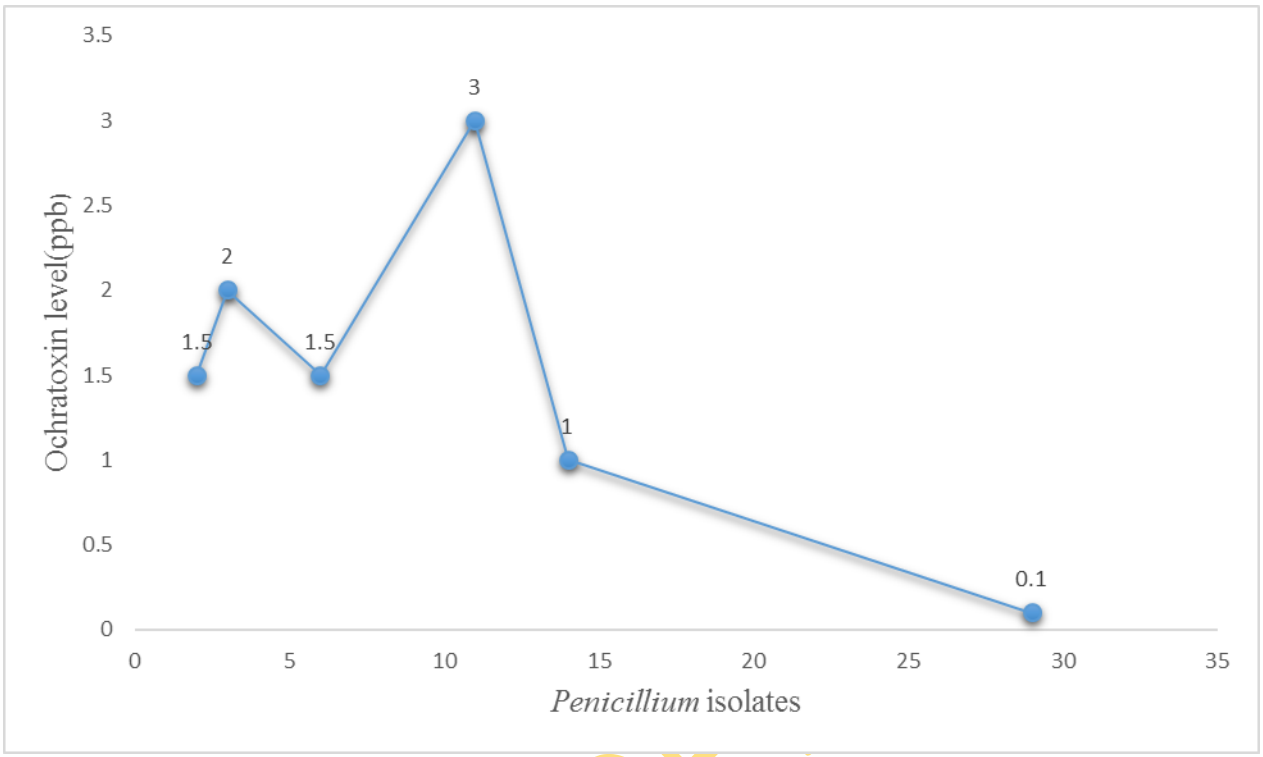
APPENDIX 6



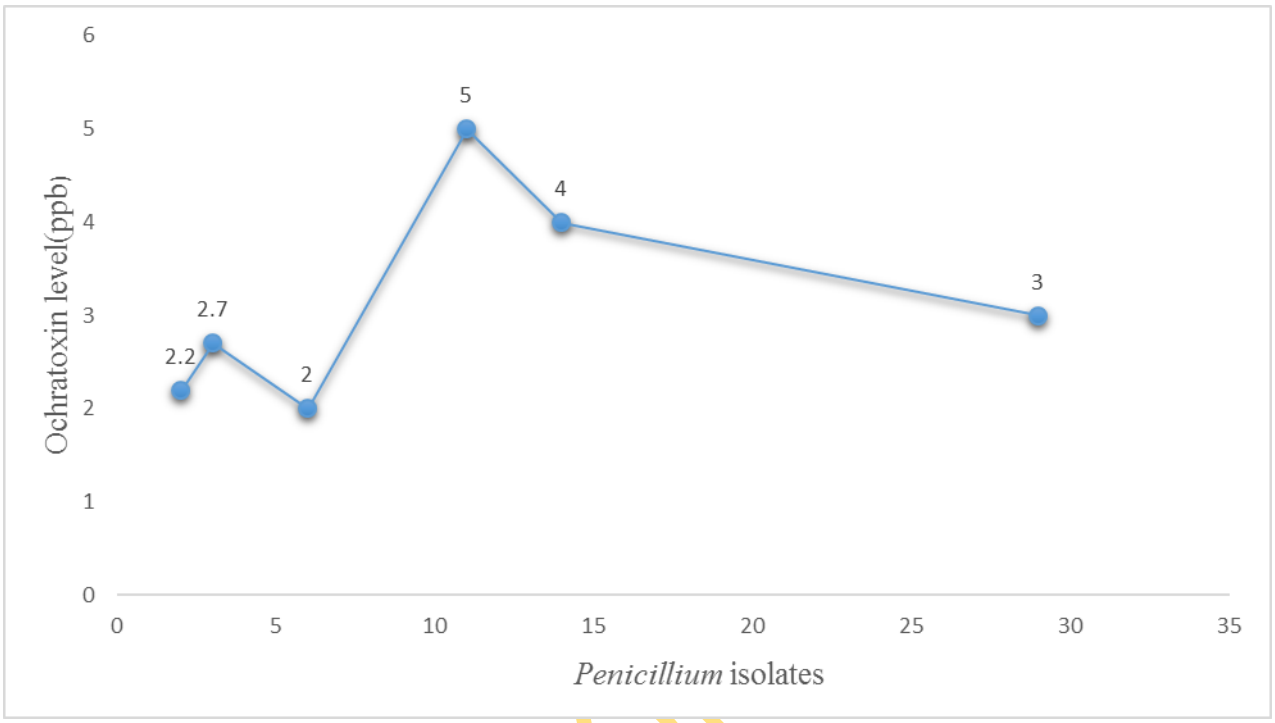
Ochratoxin production from the *Penicillium* isolates at pH 2



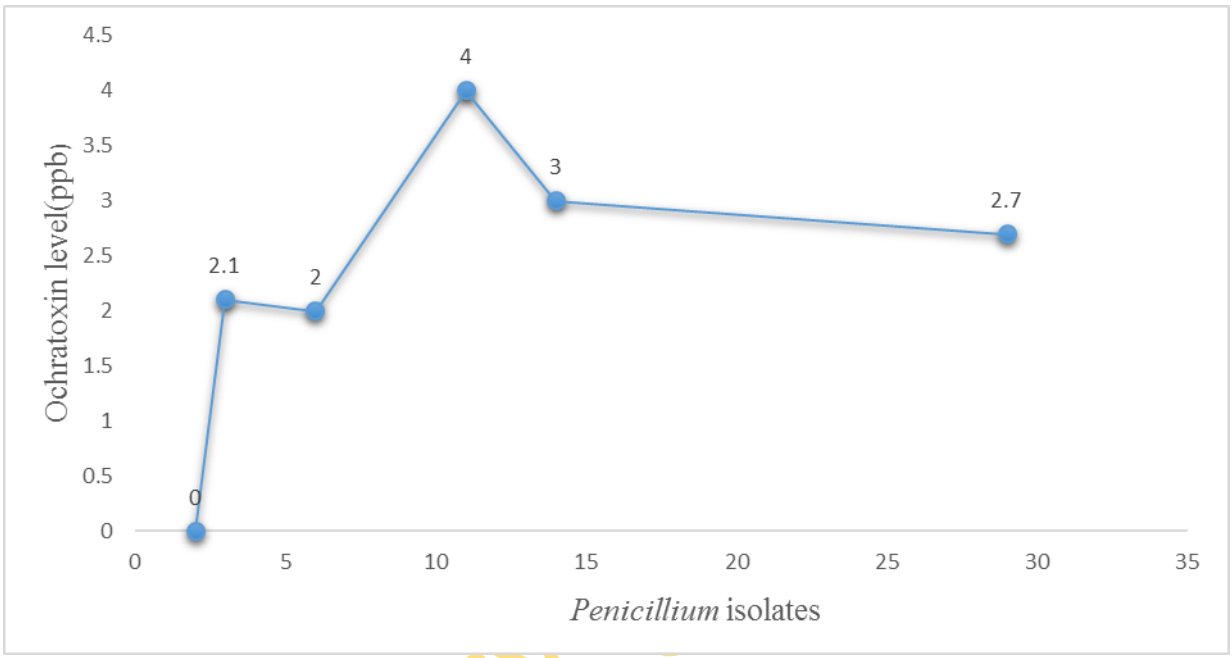
Ochratoxin production from the *Penicillium* isolates at pH 3



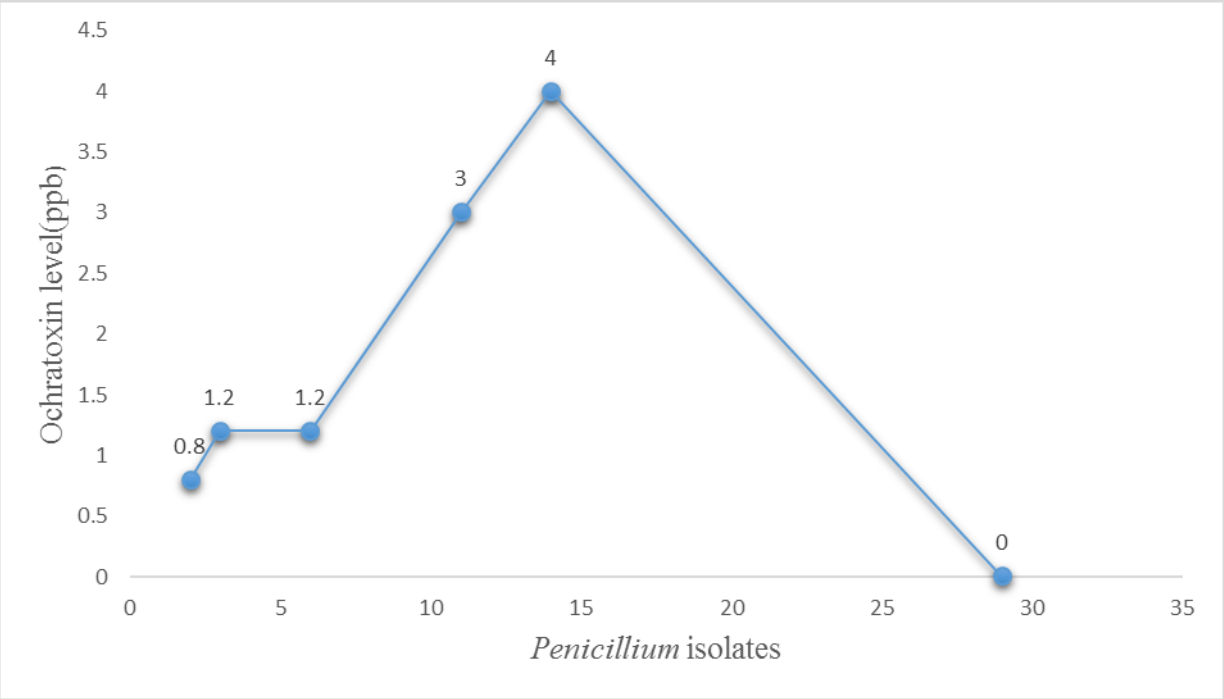
Ochratoxin production from the *Penicillium* isolates at pH 4



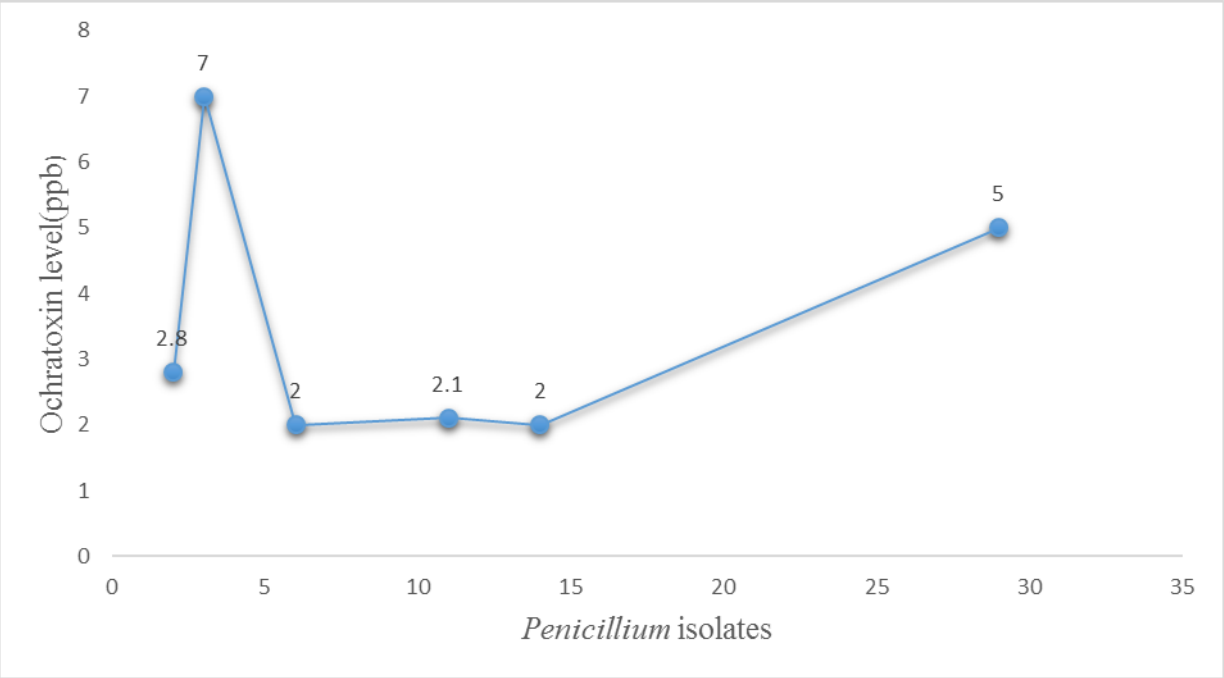
Ochratoxin production from the *Penicillium* isolates at pH 5



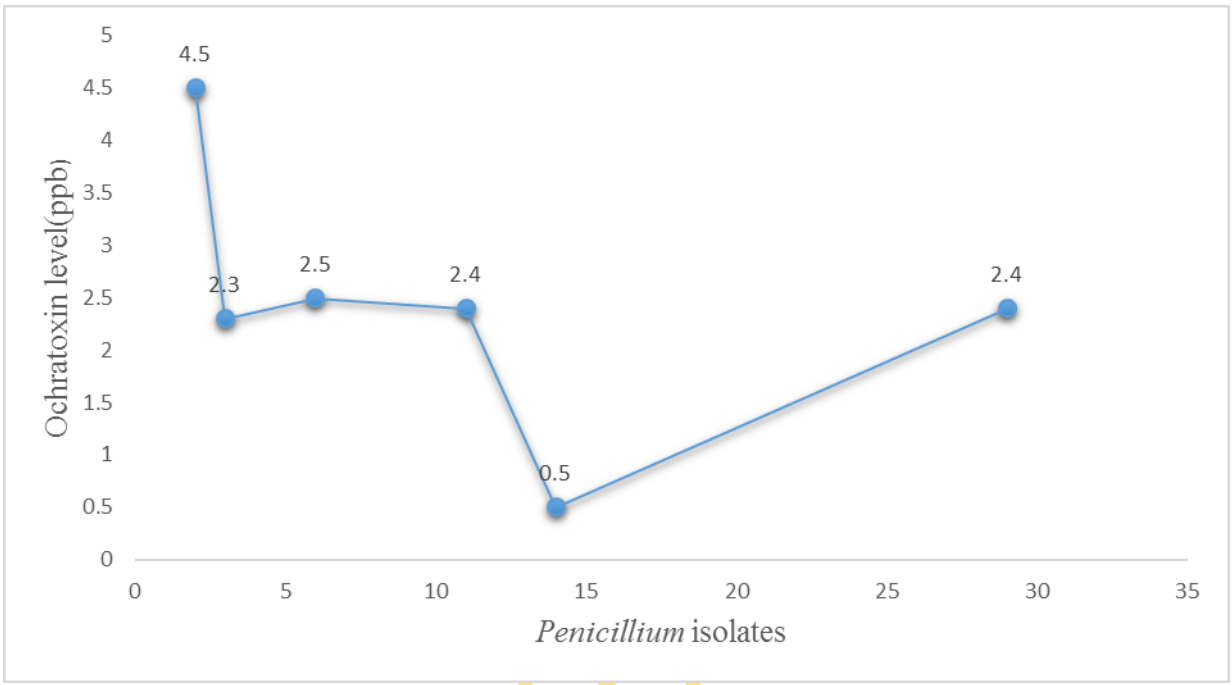
Ochratoxin production from the *Penicillium* isolates at pH 6



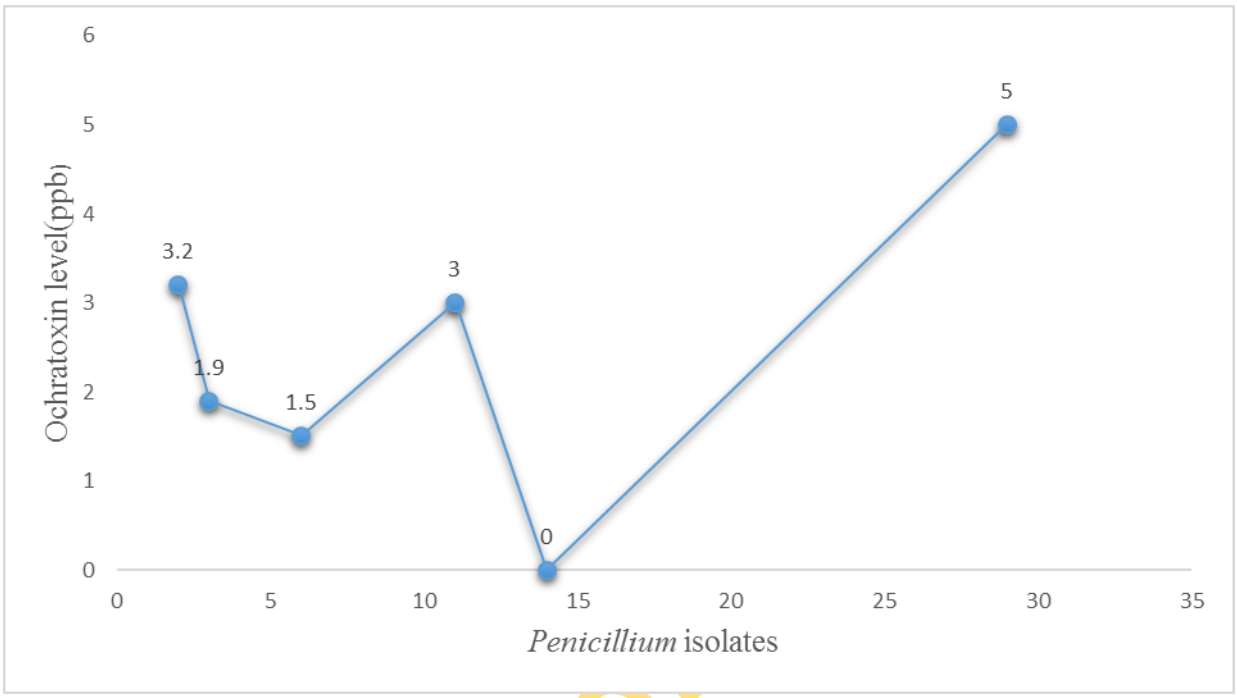
Ochratoxin production from the *Penicillium* isolates at pH 7



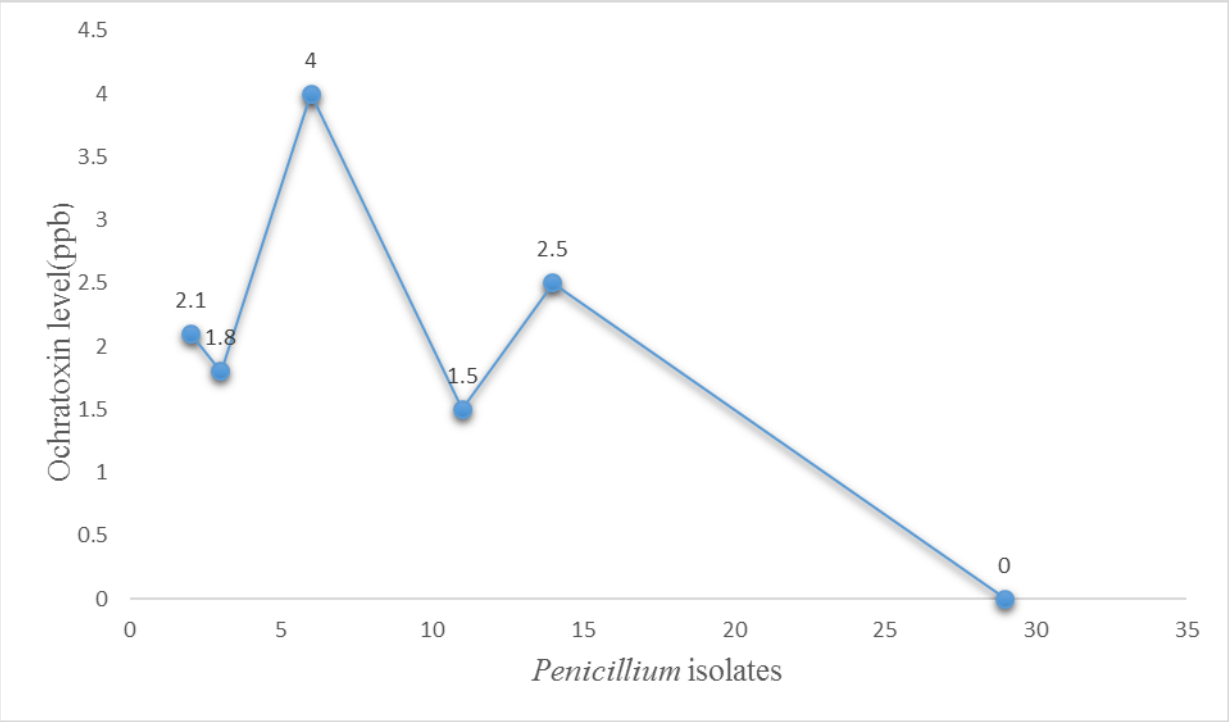
Ochratoxin production from the *Penicillium* isolates at pH 8



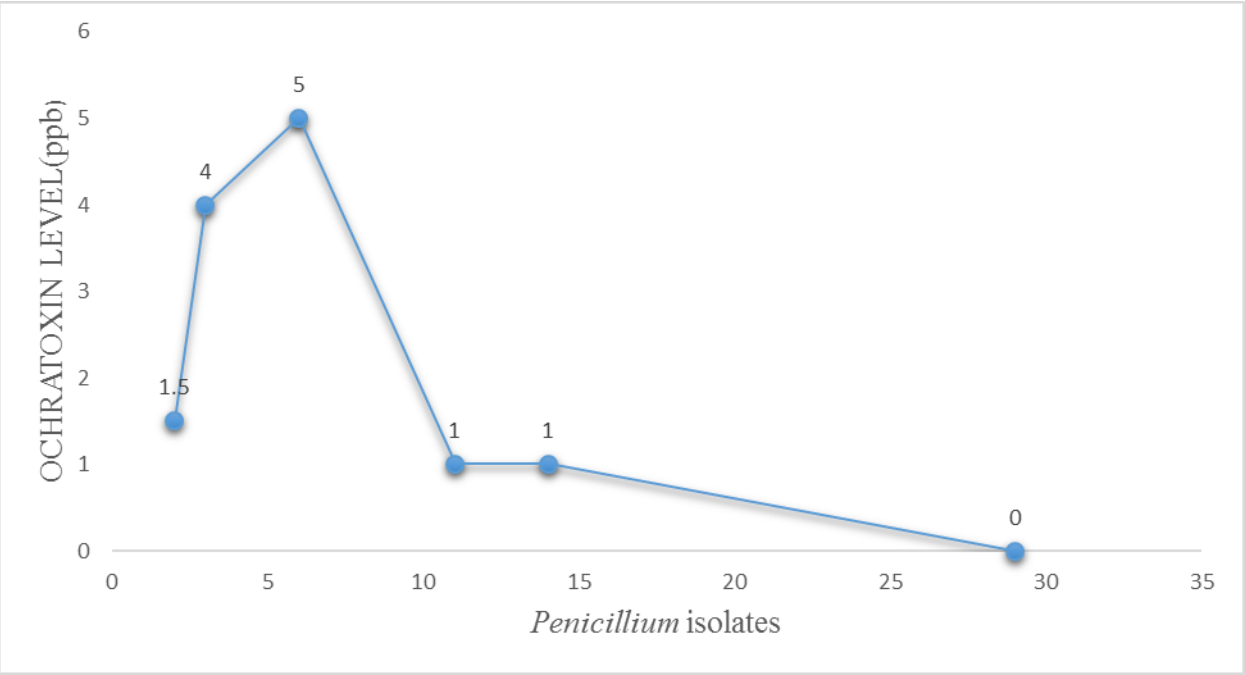
Ochratoxin production from the *Penicillium* isolates at pH 9



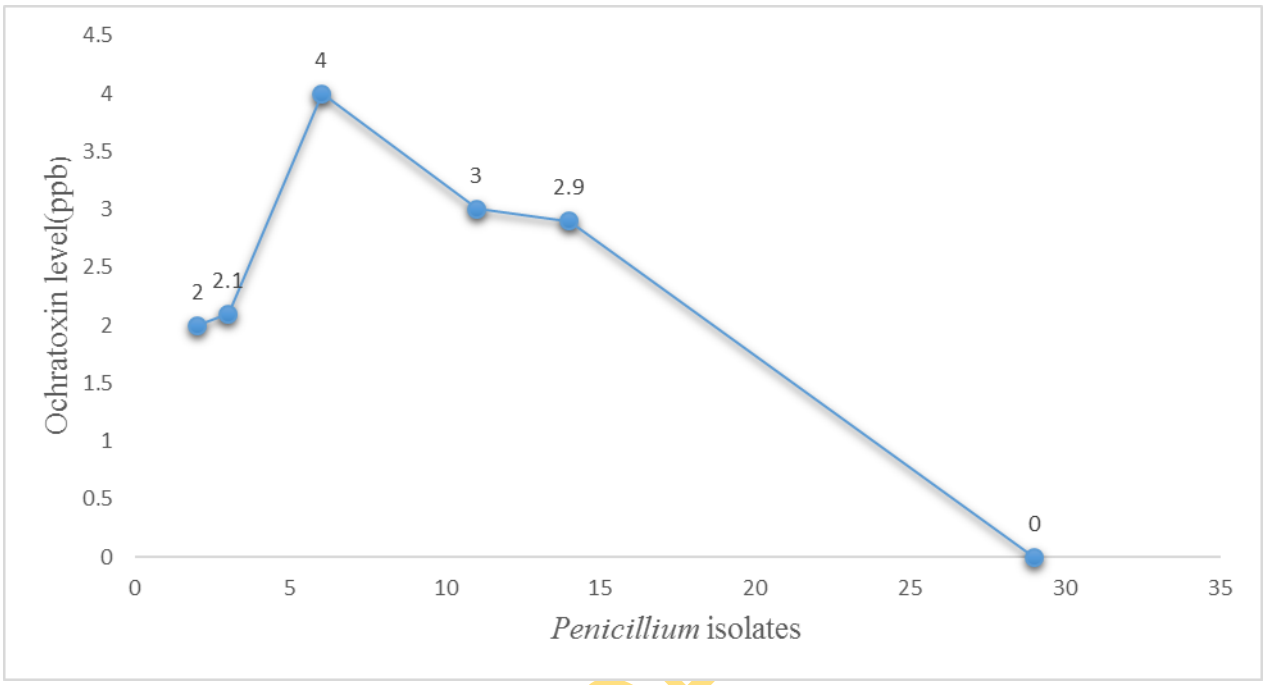
Ochratoxin production from the *Penicillium* isolates at pH 10



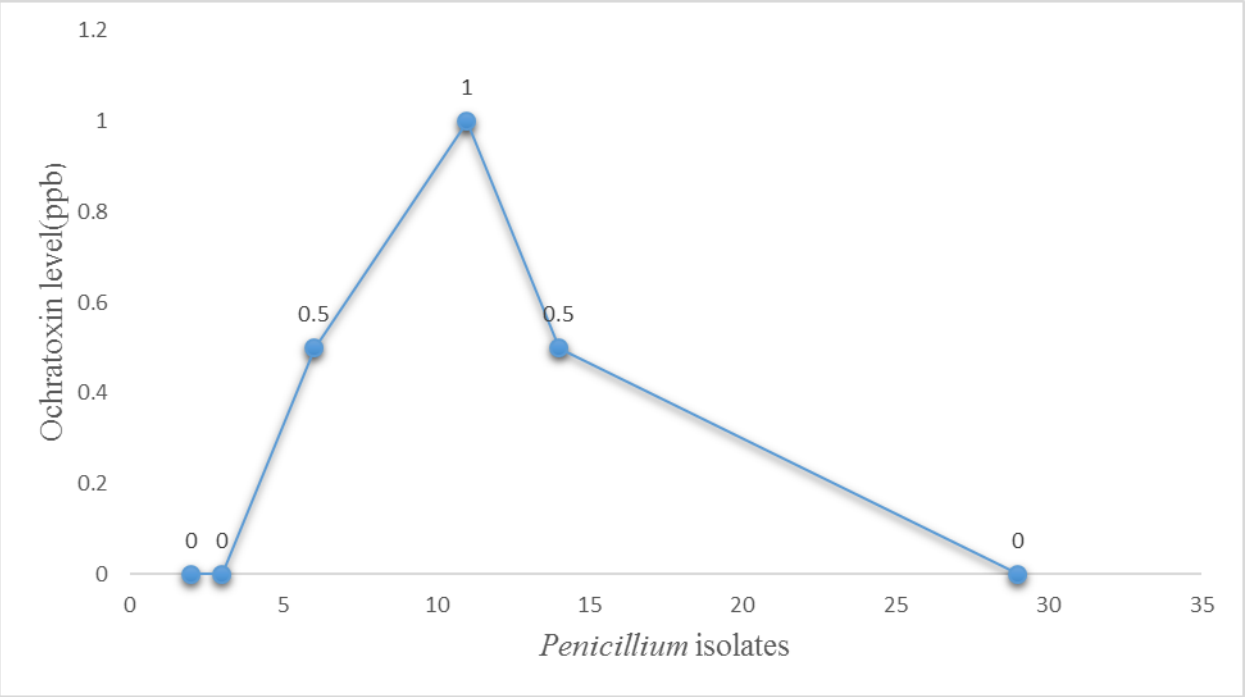
Ochratoxin production from the *Penicillium* isolates at 20^{o}C



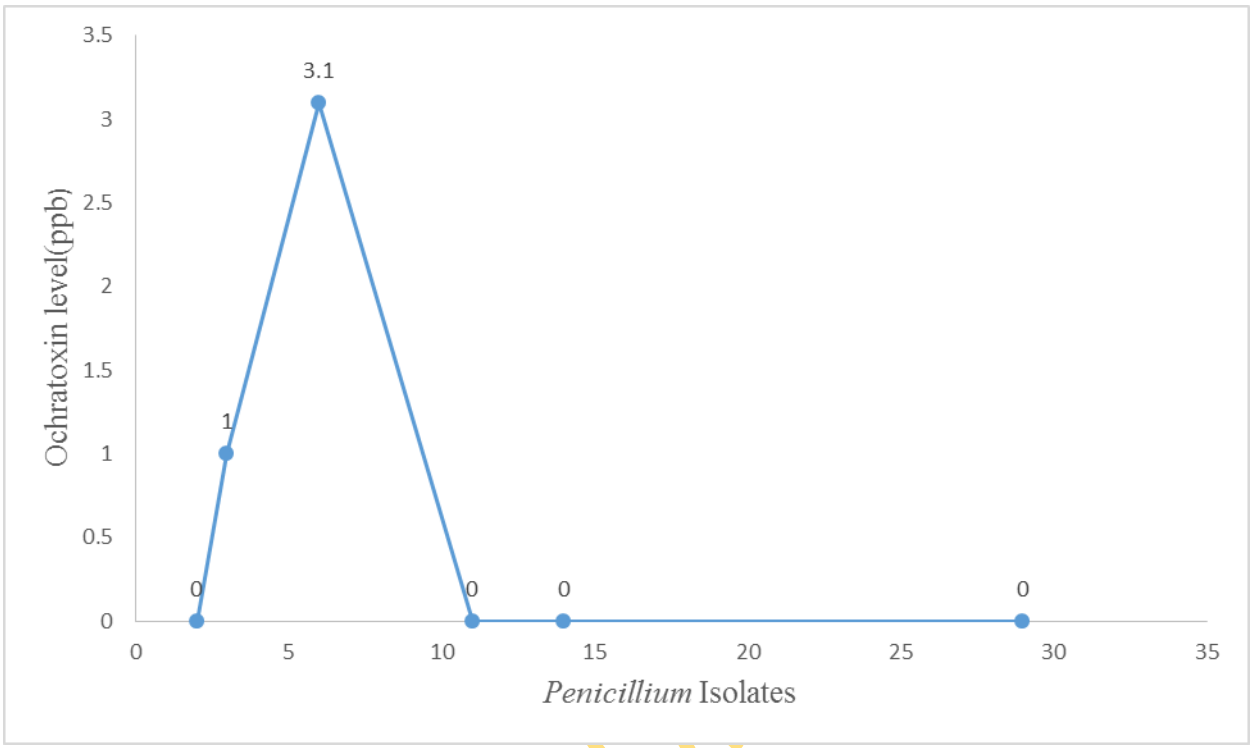
Ochratoxin production from the *Penicillium* isolates at 25°C



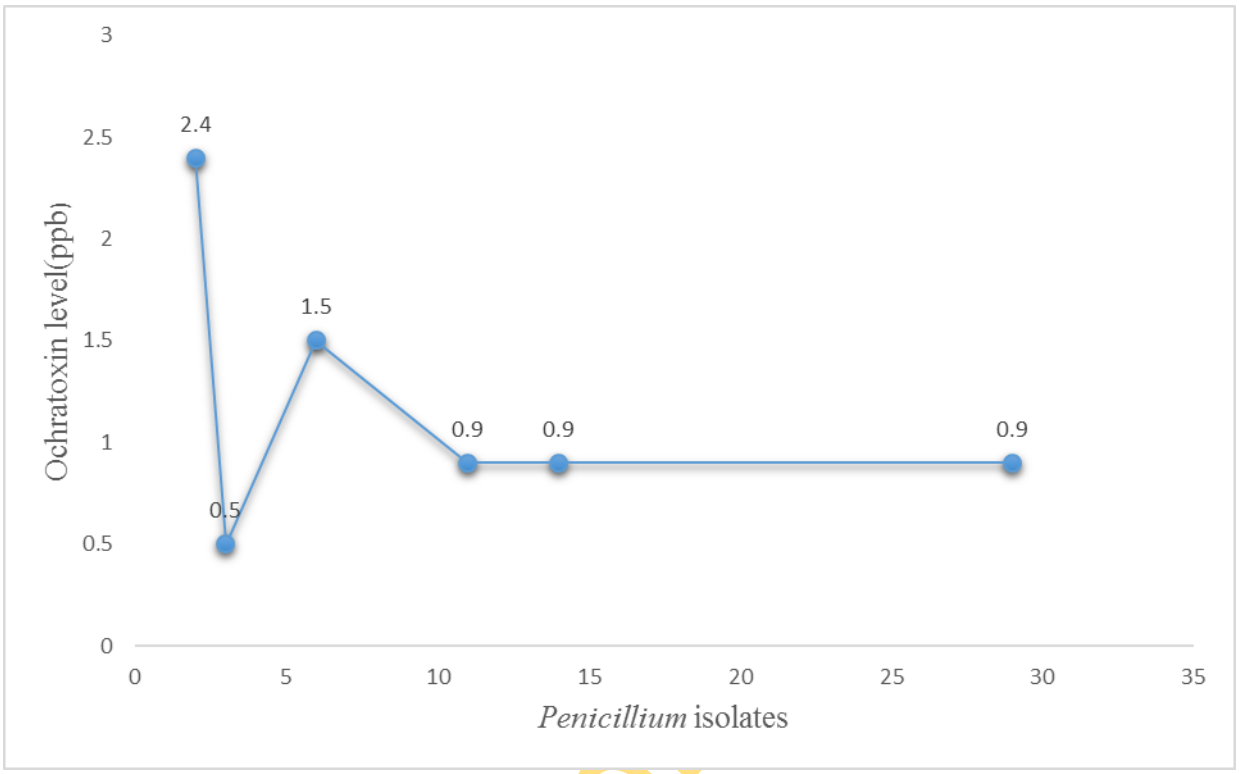
Ochratoxin production from the *Penicillium* isolates at 30°C



Ochratoxin production from the *Penicillium* isolates at 35°C



Ochratoxin production from the *Penicillium* isolates at 40°C



Ochratoxin production from the *Penicillium* isolates at room temp