Molecular Characterization of Fungi Associated with *Senecio biafrae* (Oliv. & Hiern) in Ibadan Metropolis

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Chapter One

Introduction

1.1 Background of the study

Senecio biafrae is one of the Indigenous green leafy vegetables, notable for its promising nutritional, nutraceutical and economic potentials[1]. *Senecio* Hiern, *Crassocephalum biafrae* (Oliv. & Hiern) S. Moore) belongs to Kingdom (Plantae), family (Asteraceae)[2]. The vegetable is called worowo, Gbologi/Bologi, Ako amunimuye, Boludo (Ijebu-Ibefun), Molepo/Malepo (Ode-Aye), worowo (Ibadan) in Yoruba; ota ekein Ibo (Owerri), all in Nigeria; Bologi in Sierra Leone; worowo in Tanzania[3].

It grows in large number as undercover in tree crop plantation, majorly in cocoa and kola nut plantations, especially in the Southwestern part (Osun, Ekiti, and Oyo States) of Nigeria[3].It can be found in West Central Africa's rainforest region, where annual rainfall can reach 1500 mm and elevations of up to 1800 meters[4].

About 8.9% of farmers in South-western part of Nigeria grow *Senecio biafrae* as their only crop, while 57.7% of farmers grow it in combination with other crops. As a conservation measure, 49.6% of farmers use continuous cultivation, and about 75% of farmers use organic fertilizers[1]. *Senecio biafrae* is mostly cultivated by vegetative propagation. To grow the plant, farmers frequently utilize stem cuttings, which are with two and three nodes that were 2–12 cm long. These cuttings need regular hydration and light shade to flourish, and they are planted in soil that drains properly[4].

Senecio biafrae is rich in micronutrients such as iron, magnesium, potassium, copper, zinc, calcium, vitamins, fibre and very low in fat and anti-nutrients[1]. Senecio biafrae contains Zinc

(Zn), which is an essential trace element for living organisms. It also plays an important role in the DNA replication, transcription, and protein synthesis, influencing cell division and differentiation (5). Zn has a relationship with many enzymes in the body and can prevent cell damage through activation of the antioxidant system[5].

The vitamins, minerals, fibres, proteins and non-nutritive bioactive compounds (flavonoids, phenolic compounds and bioactive peptides) in vegetables have proven health-promoting effect. Foods that are rich in vegetables are essential for sustainable and healthy diets and are excellent and promising strategies for reducing the risks of non-communicable diseases such as cancers, cardiovascular diseases, diabetes and micronutrient malnutrition[1.]

Green leafy vegetables e.g. *Senecio biafrae* are medicinal and about 80% of the population in developing countries such as Nigeria use medicinal plants and plant products in handling some of their primary medical problems, due to their accessibility, availability and affordability [5].

Ethnobotanical investigation established that the leaves are employed in treating diabetes, infertility, cough, rheumatism, hypertension, dysentery, piles, and sore eyes and the leaf extract is also used to stop bleeding from injury[1]. Several bioactive compounds in *S. biafrae* include gallic acid, chlorogenic acid, caffeic acid, rutin, quercetin, and kaempferol. These compounds exhibit significant antioxidant activities and inhibitory effects on key enzymes associated with type II diabetes mellitus and Alzheimer's disease[6]. The multipurpose value of this vegetable was revealed in the various uses of *Senecio biafrae* in the preparation of delicacies or soups. This leafy vegetable is also considered for its high medicinal value as the juice extracted from the leaves are wholly applied to fresh wounds or cuts as styptic in the rural community for man and animal use[5]. *Senecio biafrae* has both hypoglycemic and anti-anaemic properties. The aqueous leaf extract was found to reduce glucose levels. It is used to treat oedema, cough, heart troubles and as a rheumatic pain reliever [7].

In Nigeria, especially Southwest, approximately 50% of farmers harvest *S. biafrae* for household use, while the other half sell their produce in local markets. This indicates that the vegetable contributes to the livelihoods of farmers by providing a source of income [1].

In many parts of Nigeria, fungus is common as the organism that causes some edible vegetables to deteriorate. Direct contact with dirt, dust, and water, as well as handling during pre-harvest, harvest or postharvest processing, expose vegetables to microbial infection. Fungi produce an abundance of extracellular pectinases and hemicellulases that are important factors for fungal spoilage. Some spoilage microbes are capable of colonizing and creating lesions on healthy, undamaged plant tissue[8].

Fungal species found associated with the deterioration of the various vegetables include *Aspergillus niger*, *Aspergillus flavus, Aspergillus fumigatus, Penicillium maniffei, Trichoderma harzianum, Microsporum specie, Fusarium oxysporum, Trichophyton terrestre, Bipolaris hawaiiensis, Geotrichum candidum, Rhizopus specie* and *Rhizopus stolonifer*[9].

Alternaria alternata fungus is a fungus that cause leaf spot disease on *Senecio* genus plants. The fungus could infect the leaves of this species, leading to characteristic symptoms [10]. *Puccinia lagenophorae*, a rust fungus has been identified on *Senecio madagascariensis* and *Senecio vulgaris* in Colombia. The disease manifests as powdery orange spores on leaves, leading to necrosis and potential plant death. The presence of this pathogen in *Senecio* genus species suggests a possible threat to *Senecio biafrae* cultivation[11].

In regions like Southwestern Nigeria (Ibadan, Osogbo, Ekiti) where *Senecio biafrae* is cultivated, ambient temperatures typically range from 25°C to 35°C, especially during the rainy season. This climate is conducive to the growth of mesophilic fungi such as *Botrytis cinerea,* which is commonly associated with post-harvest spoilage of vegetables[12]. *Senecio biafrae* leaves are tender and contain a high percentage of water. This makes them highly perishable, as moisture accelerates enzymatic activity and microbial growth, both of which lead to rot. When exposed to environmental fungi or handling surfaces, leafy vegetables with low structural rigidity and high moisture content, like *Senecio biafrae*, deteriorate more quickly after harvest. Post-harvest contamination with fungi like *Aspergillus niger*, *Aspergillus parasiticus*, *Cladosporium sp.*, and *Rhizopus sp.* have been observed. Therefore, management strategies for fungal diseases in *S. biafrae* are essential to ensure sustainable production and consumer safety[13].

Mycotoxins are toxic secondary metabolites produced by various molds, including *Aspergillus*, *Penicillium*, and *Fusarium species*. These toxins contaminate various agricultural products, including vegetables. The presence of mycotoxins is a significant concern due to the potential health risks they pose to consumers. These toxins not only affect the quality and marketability of vegetables but have also been related to a range of severe health consequences, including hepatotoxicity, nephrotoxicity, carcinogenicity, and immunosuppression[14].

The identification of fungal pathogens affecting *Senecio biafrae* (locally known as *worowo*) is an area that requires more focused research. Fungal species on rotten *S. biafrae* leave have been done macroscopically and microscopically to identify the species based on morphological characteristics[8] but Molecular tools like Molecular markers, ITS sequencing and PCR are the gold standard for identifying and characterizing fungi associated with vegetables, including *Senecio biafrae* because the methods are sensitive, specific, and reliable[15].

1.2 Statement of the Problem

Fungi associated with the *Senecio biafrae*, a significant medicinal vegetable, represent a crucial area of study, particularly in the context of agricultural and ecological sustainability. The lack of comprehensive data on the specific fungal species contributing to the decay of *Senecio biafrae* hampers effective management and conservation strategies. Identifying the fungi involved and understanding their molecular characteristics is essential for developing integrated disease management practices, promoting the health of *Senecio biafrae* populations, and ensuring the availability of this plant for both ecological and medicinal purposes.

1.3 Justification of the Study

Fungi play a significant role in decomposition and nutrient cycling; however, pathogenic fungi can disrupt these processes, leading to declines in plant populations. S*enecio biafrae* is utilized in traditional medicine to cure primary medical problems, dietary and nutrition security but despite the usefulness of these vegetables, they are under-exploited due to phytopathogenic fungi species that are attributed to enormous loss of its yield, and also one of the most important factors affecting the postharvest quality and shelf life. Identifying the fungi causing rot will help farmers implement preventive measures, thus enhancing the plant's viability and increase yield. Additionally, due to conventional methods of identifying the fungi species, the selection of appropriate biocontrol measure and developing resistant strains of *Senecio biafrae* have not been detected.

Some fungi associated with *Senecio genus* can be opportunistic pathogens in humans and animal but isolation and identification of these fungi at molecular level can contribute to development of disease-resistant varieties, improve public health knowledge, particularly in rural and urban areas where human-plant interactions are common. Also, targeted food safety measures and actions to lower mycotoxin levels can be informed by identification of the fungal species that cause contamination.

1.4 Aim and Objective of the Study

The aim of this study is to identify and characterize fungi associated with *Senecio biafrae* in Ibadan metropolis using molecular techniques.

Specific Objectives

The specific objectives to:

i. Isolate the fungi associated with *Senecio biafrae* sold in Ibadan metropolis.
ii. Determine the pathogenicity of the isolates.
iii. Identify and characterize the isolates using molecular techniques.
iv. Determine Aflatoxin concentration of *Senecio biafrae* sold in Ibadan metropolis using ELISA method.

1.5 Significance of the Study

The identification of fungal species offers essential insights into the fungal diseases linked to *Senecio biafrae*, a significant green vegetable in West Africa. Morphological identification and characterization may occasionally result in misdiagnosis, as various fungal species might exhibit analogous physical characteristics. Moreover, it lacks genetic information, which is crucial for accurate identification and comprehension of fungal evolution. The utilization of molecular techniques, including DNA sequencing, molecular markers, and PCR, is essential since they facilitate accurate identification of fungal diseases, hence minimizing misdiagnosis.

These approaches detect fungus at an early stage, even before obvious symptoms occur, allowing for timely action and help discriminate between fungal species that may seem identical but have different harmful effects. Identifying and characterizing fungal species molecularly enable targeted treatment plans, increase production, control postharvest losses, and evaluate possible food safety hazards related to eating contaminated vegetables. Additionally, it advances our knowledge of fungal biodiversity and their ecological involvement in the decomposition of vegetable crops.

1.6 Scope of the study

The scope focuses on the assessment of fungi associated with *Senecio biafrae* sold in selected markets in Ibadan metropolis. The research involves the collection of *Senecio biafrae* from Orita merin, Mapo, Shasha and Oje markets. Mycological technique was used for the isolation and identification of the fungal isolates. Molecular characterization of the fungal isolates was done and Aflatoxin content was further determined using ELISA method.

1.7 Limitations of the study

This research solely focuses on *Senecio biafrae*, excluding other leafy vegetables. The markets were restricted to four in Ibadan metropolis because of how scarce the vegetable was in the markets. Fungal identification and molecular characterization were based only on isolates that could be successfully cultured in the laboratory. Aflatoxin detection was restricted to *Aspergillus* species, and other potential mycotoxin-producing fungi were not examined in this research because *Aspergillus spp* are associated with production of Aflatoxins which has been implicated in hepatotoxicity and hepatocellular carcinoma.

1.8 Operational Definition of Terms

DNA- Deoxyribonucleic acid, organic chemical of complex molecular structure that is found in all living organisms. DNA codes genetic information for the transmission of inherited traits**.**

ITS Sequence- The internal transcribed spacer is defined as a region located between the small and large subunits of ribosomal RNA (rRNA) gene sequences, characterized by rapid evolutionary change and valuable for analyzing intraspecific variation in various taxa.

PCR- Polymerase chain reaction (PCR), a molecular technique used to make numerous copies of a specific segment of DNA quickly and accurately.

ELISA- Enzyme-Linked Immunosorbent Assay is a laboratory technique that detects and quantifies specific antigens, antibodies, proteins, or hormones in a sample.

Mycotoxin- A toxic, poisonous chemical compound produced by certain types of molds that contaminate vegetables, fruits etc.

Molecular Characterization- This term refers to a method used to determine the genetic characteristics of the fungi.

Morphological Identification- Visually examination of fungal colony on an agar plate to observe its characteristics.

Aflatoxin- This is cancer-causing toxins produced by Aspergillus molds that can contaminate crops, fruits and vegetables during growth and storage.

Mycotoxin- A toxic chemical substance produced by certain fungi, such as molds, which can contaminate crops like nuts, cereals, fruits and vegetables.

Mesophilic- Organism that grows best in moderate temperature, neither too hot nor too cold, with an optimum growth range from 20 to 45 °C (68 to 113 °F).

Part Per Billion (ppb)- A unit of concentration representing one part of a substance per one billion parts of a total mixture.

Mycology- The branch of biology focused on the scientific study of fungi, encompassing their taxonomy, genetics, biochemical properties, and various uses.

Endnotes

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Chapter Two

Literature Review

2.1 Origin and History of *Senecio biafrae*

It was originally published as *Solanecio biafrae* in Floral of Tropical Africa. Later, it was renamed as *S. biafrae* and *Crassocephalum biafrae*[1]. Natural habitats for *S. biafrae* include Nigeria, Uganda, Liberia, Ghana, Zaire, Sierra Leone, Upper guinea, Ivory Coast, Congo, and the Cameroon Mountains. Its widespread presence in cocoa plots in southwest Nigeria (Osun, Ekiti, and Oyo States) was attributed to its intentional protection and lack of unplanned planting because of its culinary value as a potherb [1]. The native vegetable *Senecio biafrae*, also referred to as worowo, is underrated and grows natively in semi-wild or wild settings with little cultivation. It's called Worowo in Yoruba and Bologi in Sierra Leone and Nigeria, respectively. Worowo's soft, fresh leaves are frequently prepared as a vegetable and seasoned with onions, tomatoes, and pepper. Worowo leaves are typically boiled or steam-cooked and served with fish and okra in Sierra Leone [2]. In South-Western Nigeria, worowo is a weed that is protected during weeding because the cocoa trees offer shade, support, and stakes. When given appropriate moisture, shade, and wooden pole stakes, worowo can be grown in home gardens as well as in fields. The vegetable's typical growth is restricted when it is exposed to direct sunlight. The plant grows on soils that are rich in nutrients, especially organic matter, and that are moist, well-drained and fertile. It is commonly cultivated beneath cocoa trees because the wet conditions guarantee that development continues during the dry season. In humid conditions, the crop stays fresh for three days. Due to the lack of documentation regarding interregional trade, *Senecio biafrae* is marketed locally for home consumption[3].

The juice extracted from the vegetable is put on to injured eyes and has therapeutic qualities. Additionally, it can be substituted for spinach while making soups and stews [3]. It is comparatively more expensive than the rest of the leafy greens in the area because there is a higher demand for this kind of vegetable in South-Western Nigeria than there are supply levels in the markets. In addition to being gathered from the wild, *Senecio biafrae* continues to be raised in plantation crops like cocoa and coffee [4]. Native to southern Nigeria, *Senecio biafrae* is mostly farmed there, but market supply typically falls well short of customer demand. They believed that because this vegetable has not yet been introduced into regular cultivation in the conventional cropping methods, farmers and vendors are unable to satisfy consumer demand. Ghana, Uganda, Liberia, Nigeria, Sierra Leone, the Democratic Republic of the Congo, and Upper Guinea are among the African nations where *Senecio biafrae* grows natively in the forest zone [2].

It is referred to as Ota eke (Igbo; Nigeria), worowo (Yoruba; Nigeria), Bologi (Sierra Leon), Yankonteh (Ghana), Balo dede (Ivory Coast), and Gnanvule (Eastern Cote d'Ivore) [4]. The leaves, stem, and rich edible mucilaginous fiber are used as a laxative, purgative, and to relieve indigestion. Additionally, Sierra Leone, Ghana, Benin, Nigeria, Cameroon, and Gabon all use *Senecio biafrae* fresh and succulent leaves as a leafy vegetable. They are particularly well-liked in southwest Nigeria. Typically, they are prepared with onions, tomatoes, and pepper. The Yoruba saying "vegetable soup cooked with *Senecio biafrae* doesn't require meat" reflects the exceptional qualities of the vegetable being used in these recipes, which eliminate the necessity for meat or fish. But you can add meat or fish to the soup. The leaves are consumed cooked alongside okra and fish in Sierra Leone, where they are referred to as "bologi." To extract the sticky substance from the leaves, they are first steam-cooked in hot water and then squeezed. To get rid of the mucilage as thoroughly as possible, two or three washes with cold water are performed after the squeezing. The leaves are infused and consumed as a beverage [2].

The vegetable is considered seasonal because it grows most abundantly during the rainy season[5]. *Senecio biafrae* has not been completely tamed and incorporated into the standard agricultural systems like the widely consumed crops like spinach and amaranth therefore, it is in danger of going into extinction. Many species of plants have experienced genetic loss due to rapid urbanization brought on by population growth and a lack of deliberate attempts to safeguard them[6]. However, methods of preservation that can efficiently increase the duration of storage and make the season vegetable available during the off-season are required [5]. *Senecio biafrae* domestication for periodic cultivation is being prompted by growing knowledge of its potential in terms of yield, nutritional contents, and nutritional health roles that are on par with or better than those of commonly grown leaf vegetables [6].

Table 2.1 *Senecio biafrae* Common/Vernacular Names and Countries[4].

Common names	**Country/region**
Gnanvule	Eastern Cote d'Ivoire (Anyi- Ndenye)
Yankonfeh	Ghana (Akan- Fante)
Kokolé titi	Ivory Coast (Kru- Guere)
Balo dédé	Ivory Coast (Nekedie)
Doua	Cameroon: Baham (Ghomala'a)
Ota eke	Nigeria: Igbo (Owerri)

Akọ amùnimúyè; g- bologi; worowo	Nigeria: Yoruba
Boludo	Nigeria: Yoruba (Ijebu- Ibefun)
Molepo/Malepo	Nigeria: Yoruba (Ode- Aye)
Rọrọwọ	Nigeria: Yoruba (Ilorin)
Lambe pundo (English spinach)	Sierra Leone (Kissi, Krio)
Bologi	Sierra Leone (Krio)
Worowo	Tanzania

2.2 Taxonomical Classification of *Senecio Biafrae*

S. biafrae belong to

Kingdom - Plantae

Division - Tracheophyta

Class - Magnoliopsida

Subclass - Magnoliidae

Superorder - Asteranae

Order - Asterales

Family - Asteraceae

Subfamily - Asteroideae

Tribe - Senecioneae

Subtribe - Senecioninae

Genus - Solanecio

Species - *S. biafrae* (Olive and Hierne) (C. Jeffrey) [1].

2.3 Description of *Senecio biafrae*

In the absence of fertilizer, *Senecio biafrae* (Fig 2.1) grows very slowly. With a stem up to 3 meters tall and numerous branches, *S. biafrae* is a perennial scandent shrubby herb that has glabrous, succulent stems and branches. The petiole is slender and glabrous, 0.8–5.5 cm long and ex auriculate; the blade is triangular sub hastate to hastate or sagittate, 2.2–9 cm × 1.3–6.2 cm, with a lower margin that is remotely sinuate and paucidentate; the base is weakly cordate or subtruncate and quickly decurrent onto the petiole; the apex is more or less attenuate, acute, apiculate, and glabrous. The leaves are alternate, simple or thoroughly pinnately lobed, more or less succulent and petiolate, and stipules are absent [1].

Within the terminal loose thyrses of stalked congested sub umbelliform corymbs, there are several discoid capitula. The individual capitula's stalk is short and sparsely pubescent, with a cylindrical involucre measuring 7–10 mm × 2–4 mm and bracts of calyculus 2–6 that are lax and glabrous, measuring 6.5–10 mm in length. Bisexual, tubular, pentamerous flowers with pale yellow disc florets, a corolla that is 6–8.5 mm long, a tube that is glabrous and slightly enlarged from above in the middle, lobes that are 1.2–1.5 mm long, and glabrous ovaries. When ripe, the achene is 3 mm long, ribbed, glabrous, and has pappus that is 6–8 mm long [1].

At elevations ranging from sea level to around 1,300 meters, *S. biafrae* can be found. It is found in regions with a pronounced dry season and an average annual rainfall of roughly 1,500 mm. It is unable to withstand dry conditions and reacts strongly to water pressure by shriveling its stems and turning its leaves yellow. It grows best in soil that is well-drained and rich in organic matter;

it prefers a spot in the light shade; crops start to yield about 70 days after planting and continue to do so for at least a year; plants can be harvested at any time of year if the blooming stems are removed; it yields 15 kg per plant annually; even with careful handling, the stems are very fragile and brittle; flowering branches are removed to increase leaf output; many varieties are of excellent quality [7].

Fig 2.1: *Senecio biafrae* before staking [6].

2.4 Nutritional and Economic Importance of *Senecio biafrae*

Several nutritional problems in Nigeria can be addressed by eating vegetables, especially at this time when the high poverty rate in the nation makes it difficult for many Nigerians to purchase milk, eggs, and meat. Phytochemicals are substances found in vegetables that help prevent cancer and other illnesses. A small number of compounds have been identified, including indole-3 carbinol (which can be discovered in caulifers and cabbage), capsiacin (which was discovered in pepper), and allylic (discovered in onions and garlic). In addition to being low in fat and containing no cholesterol, vegetables are also recognized for being excellent sources of dietary fiber, or roughages, which help to avoid illness and constipation [8].

According to reports, the 100 g dry matter of the leaves of the purple-stemmed and green-stemmed varieties of *S. biafrae* contain 12.3 g and 11.6 g of crude protein, 11.8 g and 10.5 g of crude fiber, 342 mg and 320 mg of calcium, 39 mg and 46 mg of phosphorus, and 52 mg and 53 mg of iron, respectively[9]. Protein (14.26±2.01%) and fiber (15.78±0.13%) were found in *S. biafrae* leaves, according to the results of proximate analysis. The minerals that were found using a technique called atomic absorption spectrophotometer were sodium, iron, potassium, calcium, selenium, magnesium, aluminum, cobalt and iron[9].

Flavonoids, phenolics, ascorbic acid, vitamin A, folic acid, niacin, riboflavin, thiamine, and vitamins E, C, K, and B, crude protein, linoleic, threonine, linolenic, and arachidonic acids, isoleucine, valine, tyrosine, leucne, phenylalanine, tryptophan, and histidine, crude fiber, sodium, iron, potassium, phosphorous, iron, aluminum, calcium, zinc, selenium, magnesium, and cobalt, as well as terpenoids, primarily sesquiterpene D, are among the phytochemicals and phytonutrients found in *Senecio biafrae*. According to reports, its delicate leaves are low in fat and anti-nutrients and high in vitamins and minerals. *Senecio biafrae* was demonstrated to have a number of bioactivities, such as anti-trypanosomal and hepatoprotective properties. It has hypoglycemic and anti-anemic effects. In ethnopharmacology, its leafy components are utilized to treat pulmonary disorders, cough, wounds, and heart issues [10].

Senecio biafrae is rich in proteins (29%), dietary fibers, and minerals like manganese, salt, potassium, magnesium, and calcium are all abundant in it [11]. *S. biafrae* is well-known for its medicinal properties, particularly among the Yoruba-speaking inhabitants of South Western Nigeria, who use its leaf extract to treat pulmonary defects, diabetes, and cuts. In Sierra Leone and Cameroon, it is also used to treat eye soreness [2, 12]. In Benin, Côte d'Ivoire, and Cameroon, it has been utilized in traditional medicines to cure a wide range of various conditions, including rheumatism, coughing, oedema, infertility, and painful eyes [12].

Pulped leaves are used as a galactagogue on the breasts in Côte d'Ivoire. *Senecio biafrae* is utilized as a tonic, to cure cough and heart problems, and to reduce localized oedemas, prurient allergies, and rheumatic discomfort in the Congo. It is also used in burial and initiation ceremonies in the Congo, and it is linked to smallpox prevention ceremonies in Yoruba culture [2]. Ethnobotanical research in Cameroon's western and northwest regions has shown that it is used to treat infertility in women [1,13]. Traditional healers employ both the stems and the leaves of *Senecio biafrae* either marinated in water or via palm wine to treat infertility in women [13].

2.4.1 Phytochemicals in *Senecio biafrae*

Phytochemicals are the medicinal chemical components of vegetables that have been linked to their healing properties. Alkaloids, flavonoids, saponins, and cardiac glycosides are examples of phytochemicals that actively contribute to the improvement of illnesses. They have been shown to have antimalarial, anti-cancer, antibacterial, antioxidant, and antidiabetic properties. However, species and variety may have different quantities of these plant compounds [12]. Vitamins, terpenoids, polyphenols, lignins, tannins, stilbenes, amines, flavonoids, quinones, betalains, alkaloids, coumarins, and other metabolites are among the many phytochemical compounds found in plants that are abundant in scavengers of free radicals [14]. They are antioxidant chemicals with antiviral, antibacterial, anticancer, antimutagenic, anticarcinogenic, anti-inflammatory, and antiatherosclerosis properties. Additionally, eating fresh antioxidants has been linked to lower chances of diabetes, heart disease, cancer, and other aging-related illnesses. The usage of natural phytochemicals found in berry crops, oilseeds, teas, herbs, beans, fruits, and vegetables has been popular worldwide in recent years [14]. Different plant components, including leaves, fruits, flowers, roots, stems, and seeds, collect phytochemicals. Phytochemicals found in a wide variety of foods including vegetables, fruit, legumes, whole grains, seeds, fungus, herbs, and spices. They possess significant qualities that help prevent or combat a number of prevalent illnesses [15]. Numerous secondary metabolites of various polarity, including amino acids, terpenoids, sesquiterpenes, and dihydroisocoumarins, are found in *S. biafrae* and have a variety of medicinal uses [9, 13].

The leaves include vitamins E, C, K, A, and B complex, as well as important fatty acids (linoleic, linolenic, and arachidonic acids) and essential amino acids (threonine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, and histidine). Alkaloids, steroids, tannins, phlobatanins, flavonoids, phenols, saponins, glycosides, terpenes, and chalcones are among the qualitative phytochemical elements of *S. biafrae* leaves [9]. By blocking the activity of free radicals like hydroxyl (OH-), superoxide (O-), nitric oxide (NO), nitrogen dioxide (NO-2), and peroxyl (ROO), as well as non-free radicals like hydrogen peroxide and singlet oxygen, antioxidants stop oxidative damage. This has been linked to the etiology of various clinical illnesses, including diabetes, cancer, inflammatory diseases, cardiovascular diseases, the aging process, and maybe dementia [9].

2.5 Varieties of Senecio Plants

In the Asteraceae (daisy) family, the genus Senecio is one of the largest, with more than 1,000 species of succulents, shrubs, vines, and herbs. These plants are used for a number of purposes, including ornamental (*S. rowleyanu),* medicinal(*S.vulgaris*), edible ones(*S.biafrae*) and there are some that are for taxonomic and conservation purposes *(S. namibensis, S.* sisparensis). They can be found all over the world, from temperate to tropical climates.

2.5.1 *Senecio namibensis*

In the northern Namib Desert, *Senecio namibensis* (Fig 2.2) is a small shrub that grows on rocky outcrops. It can persist for several years or finish its life cycle in a single year because it possesses both annual and perennial traits. The plant is characterized by its radiate capitula, which are flower heads with yellow ray florets (usually 3-6 in number), and its succulent leaves. The species is additionally distinguished by its yellow-floretted, disciform or faintly radiating capitula. Senecio namibensis is grown mainly for its distinctive appearance and ability to withstand drought. It is suited for certain growing circumstances because it flourishes in arid, rocky, and severe areas.

Fig 2.2 *Senecio namibensis* [16].

2.5.2 *Senecio sisparensis*

From Silent Valley National Park in Palakkad, Kerala, India, a new species of *Senecio* (Asteraceae), S. sisparensis, (Fig 2.3) has been discovered. Although its morphology is similar to that of S. griffithii, its habit, leaves, capitula, bracts, ray florets, and achene characteristics are different[17].

As with other Senecio species, cultivation of *Senecio sisparensis* benefits from bright light, well-draining soil, and sparing watering. It grows best outside in pots with drainage or in soil that drains well and contains grit or perlite. Low humidity and moderate temperatures are preferred by the majority of Senecio species. Stem cuttings are an easy way to reproduce Senecio plants. Before planting the cuttings in soil that drains properly, let them callous over for a few days[17].

Fig 2.3 *Senecio sisparensis*[17].

2.5.3 *Senecio umbrosus*

Senecio umbrosus (Fig 2.4) is a genus of flowering plants in the daisy family (Asteraceae) that includes ragworts and groundsels. The flower heads are typically rayed and carried in branched clusters; they are typically entirely yellow; however, they can also be green, purple, white, or blue. Leaves of *Senecio umbrosus* usually show a rosette growth habit. The leaves' slightly hairy texture and typical dark green color help to retain moisture. Depending on the surroundings, the plant can reach a height of roughly 30 to 100 cm. Stem cuttings and leaf cuttings are simple methods of propagation[18].

Fungal communities are extremely variable in the phyllo sphere in temperate regions and exhibit even greater diversity than in the phyllo sphere of tropical trees. Phyllo spheric microorganisms infiltrate the leaf surface, causing damage by first breaking down the cuticle and wax layer. The physical structure of the plant community influences several environmental elements and other biotic and abiotic parameters that can impact pathogen populations. Temperature, humidity, and light levels are all influenced by the vegetation cover [18].

Fig 2.4 *Senecio umbrosus*[18].

2.5.4 *Senecio vulgaris*

Although common groundsel is a common winter annual broadleaf, it can grow year-round in California's coastal regions. It lives in disturbed areas and on agricultural land. Common groundsel grows up to 4900 feet (1500 m) in elevation throughout California, with the exception of deserts. Infestations are particularly troublesome when it's cool and moist. Long stretches of heat and dryness kill plants. Toxic substances known as pyrrolizidine alkaloids are found in common groundsel. They are poisonous to people and animals when consumed in large quantities or even in tiny amounts over a period of weeks or months[19].

Common groundsel develops a single stem or branches from the base, and its mature stems are roughly erect. Its maximum height is two feet (60 cm). The leaves vary greatly; the upper leaves are smaller than the lower leaves and adhere directly to the stem (without stalks); the lower leaves have short stalks; and the upper leaves are either hairless or lightly covered in long, wavy, or cotton-like hairs. Generally speaking, leaves are uniformly distributed on the stems and have serrated edges and deep lobed surfaces[19].

Almost all year long, flowers blossom. At the top of the blooming stalk, single, tiny yellow blooms form unshowy flower heads. Groundsel is distinguished from other members of the thistle family by the green reduced leaflike structures (bracts) that hug the base of the flower head and have noticeable black tips. Eventually, the flower head develops into a white puffball that resembles a dandelion seed head[19,20].

Fig 2.5 *Senecio vulgaris* [19, 20].

2.6 Fungi Associated with Vegetable Rots

One crucial component of horticultural crops is their look, which is impacted by pests and diseases that damage vegetables and fruits in the field and shorten their shelf lives. Bacterial, viral, fugal, and nutritional disorders can affect fruits and vegetables [21]. Different parasitic bacteria, yeasts, and fungi typically penetrate aboveground plant sections in order to get host resources necessary for growth, survival, and reproduction. Since a parasite uses its host's nutrition, parasitism is usually classified as a trophic relationship. Diseases affecting the leaf, stem, root, vascular system, and fruit are brought on by plant pathogens. It can cause moderate to severe damage, sometimes destroying all plants, and in other circumstances, leading to the extinction of whole plant species[18].

With estimates ranging from 1.5 to 5.1 million species, fungi are the second largest category of eukaryotic creatures on Earth. Fungal species can play significant roles in human life and can live in a variety of natural and artificial environments. Identification of fungi at the species level is essential for both fundamental (taxonomy, ecology) and applied (genomics, bioprospecting) scientific research applications. Fungi create a multitude of natural products; they have substantial industrial applications and are well-known for their potential to produce secondary metabolism products with biological activity that can be utilized for drug discovery. Secondary metabolites from fungus provide a considerable fraction of our modern medications, which includes often-cited penicillin, and also those employed as cholesterol-lowering, antibacterial, or immunomodulatory substances[22].

Many crops and vegetables are spoiled by fungi. They cause the quality of plants to be destroyed and lower crop yields. Fungal infections frequently cause our veggies to deteriorate, resulting in a reduction in their yield [23]. Fungi and plants can have a variety of relationships, from parasitism to mutualism. They play a crucial role in the nutrient cycle, which releases important nutrients for plants into the soil. They are plant diseases that have the potential to seriously harm forestry and agriculture [24]. Fungal illnesses frequently spread passively; the spores' path is determined by their mode of transportation, which can be wind, water, or animals. Individual plant distances within the population determine how effective this "disease escape in space" is. Therefore, the primary spread can occur between plants that are physically adjacent to one another, i.e., in populations that are crowded or occasionally overcrowded. The majority of pathogenic fungus also have inactive resting stages. They can produce spores that wait for better circumstances and endure for many years, such as in soil, a phenomenon known as "disease escape in time." Additionally, some of them might use a wide variety of species of plants as hosts. The term "host jumping" describes the process by which typical (susceptible) hosts become "non-host" plants by infecting tissue asymptomatically and then exploring other susceptible hosts. For these reasons, even in consecutive seasons, a pathogen might not be present on the host plant species [18].

Numerous fungal species can cause plant diseases, affecting a variety of hosts with varying tissue specificities and a wide range of symptoms. Several hosts are susceptible to anthracnose, foliar disease, rot, seedling blights, and post-bloom fruit drop caused by *Colletotrichum* species. Diaporthe species are linked to a variety of host diseases, including dieback, leaf spots, stem cankers, root and fruit rots, and seed decay. Several commercially significant crops are susceptible to post-harvest illnesses, fruit rots, severe chlorosis, needle blight, tip blight, grey blight, canker lesions, shoot dieback, and leaf spots caused by *Pestalotiopsis* species. In addition to affecting ornamental crops and the agricultural industry, fungal infections can cause debilitating diseases which can have a significant financial impact [24].

Since the early 2000s, fungal infections have been found on the White Mountain population of the ragwort species *Senecio umbrosus* [18]. Former names for *Senecio umbrosus* were *S. doria* subsp. *umbrosus* and *S. macrophyllus*. The ragwort occupies a rather limited ecological niche, occurring in thermophilous shrubs, loose woodlands, calcareous meadows, fens, and xerothermic grasslands. Regardless of the substrate's composition and humidity, the neutral or alkaline reaction and high CaCO3 level are consistent characteristics. The ideal environments for *S. umbrosus* growth and development are well-lit areas with occasional or transient shadow. Necrosis, distortions, obvious discoloration or deformations, and etiological evidence, such as piles of spores and/or sclerotia that are blackish, brown, and rusty in color, with a greyish-white tinge, are all indications of microfungal infections on the leaves of *S. umbrosus* plants [18]. Originating in Australia and known by a variety of names, *Puccinia lagenophorae* Cooke is a rust that has spread throughout Europe. *Senecio madagascariensis* and *S. vulgaris* plants have been observed to be parasitized by *Puccinia lagenophorae* disease in Colombia[25].

Numerous plant species, including *S. vulgaris*, that are members of the Compositae family have been identified as hosts for this fungus. It has been discovered in several parts of Africa, including South Africa on *Calendula* sp., Tunisia on *S. crassifolius*, and Tanzania on *Cineraria* sp. [26]. Both the native rust fungus *Coleosporium tussilaginis* and the invasive rust *Puccinia lagenophorae*, which was first identified in the UK in the 1960s, attack *S. vulgaris* in Europe. These two diseases are fungi that cause no systemic foliar rust [25].

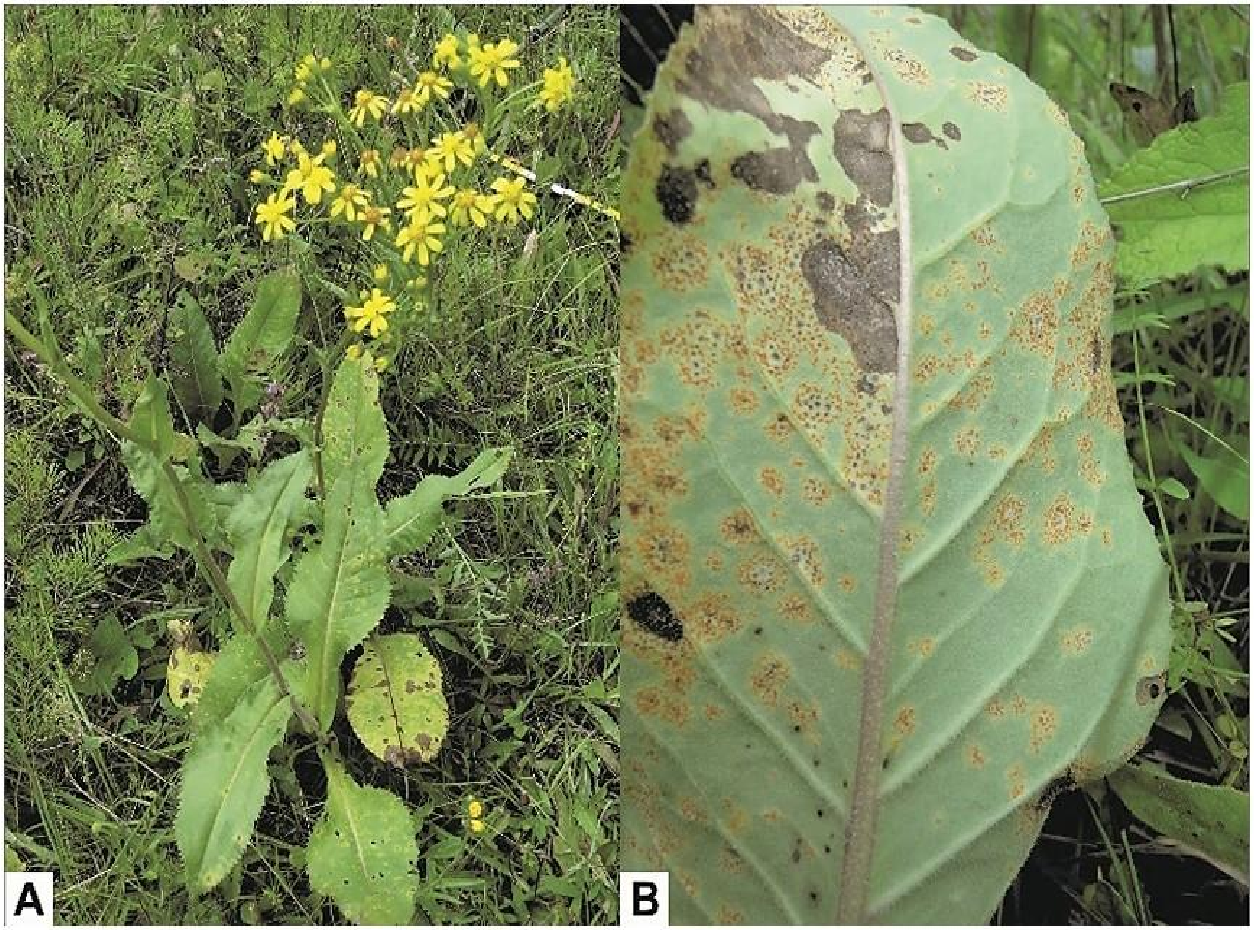

Figure 2.6: *Coleosporium tussilaginis* on *Senecio umbrosus* leaves: (A) general view of infected plant; (B) abaxial (lower) side of leaf [18].

2.7 Common Fungal Diseases in *Senecio* Plants

The diversity of fungal diseases is astounding, encompassing both enormous multicellular creatures and single-celled organisms. For plants, powdery mildew, rusts, blights, wilts, and downy mildew are the most prevalent fungal diseases. Numerous plant species, including ornamental plants, fruits, and vegetables[27].

i. **Powdery Mildew:** Fungal disease known as powdery mildew is identified by the white, powder-like layer that forms on senecio plant leaves. Insufficient air circulation and excessive moisture are the main causes of the disease.

ii. **Downy Mildew:** Poor drainage and excessive water can lead to the fungal disease known as downy mildew. The grayish-white covering on the underside of the leaves is what makes it identifiable.

iii. **Leaf Spot:** Leaf spot is a common disease in senecio plants caused by too much water and poor drainage. It is recognizable by its small, dark spots on the leaves.

iv. **Root Rot:** Excessive water and inadequate drainage can lead to the fungal disease known as root rot. It can be identified by its yellowing, withering leaves and foul odor.

v. Wilts caused by Fusarium and Verticillium fungi infect and block the plant's water-conducting tissues, leading to wilting and death of the vegetables[27].

2.8 Prevention and Management of Diseases Caused by Fungi in *Senecio* Plants

Fungal diseases strike plants when conditions such as moisture and temperature encourage their development and spread. Those conditions vary according to the disease, but these simple cultural practices can help limit their effect.

i. Choosing high-quality plants is the first step in preventing disease in Senecio plants. Seek choose robust, healthy plants that show no symptoms of withering or browning and have bright green foliage. Plants that are weak or unhealthy should not be purchased because they are more prone to get diseases.

ii. It is also crucial to ensure that the soil is well drained and does not contain an excessive amount of nitrogen, as this may make the plant excessively lush and prone to diseases.

iii. Practicing appropriate watering practices is the very important. Watering Senecio plants deeply but sparingly is recommended. While insufficient irrigation might result in dehydration and other problems, excessive watering can cause the roots to rot.

iv. Regular pruning of the Senecio plant lowers the incidence of fungal diseases like powdery mildew by keeping the form open and airy. Furthermore, disease risk can be decreased by trimming off any dead or dying branches.

v. Preventive and routine use of well-established fungicides, and removal of any infected leaves, play a significant role in safeguarding gardens and farm from fungal infections. It also increases yields by mitigating disease establishment and severity [28].

2.9 Molecular Techniques Used in Identification of Fungi

New technologies, like molecular biology technology for detection, offer more reliable, accurate, and precise methods for studying and diagnosing vegetable diseases caused by pathogens and physiological races[29]. Since molecular-based technologies focus on an organism's genetic makeup, they are the most trustworthy instruments for characterizing microorganisms. In-depth information on assessments of systems impacted by climate change, food, agriculture, industrial settings, and other environmental sciences has become possible thanks to molecular technologies.

Highly conserved oligonucleotide primers have been created, such as those used to amplify the fungal internal transcribed spacer (ITS) region [30].

High throughput molecular detection techniques for plant-infecting fungus are used in recent advancements. These consist of nucleic acid sequence-based amplification (NASBA), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), RPA/CRISPR-Cas12a, genomics, standard polymerase chain reaction (PCR), real-time PCR, nested PCR, and loop-mediated isothermal amplification (LAMP). The techniques that work best for genotyping rather than species identification are PCR restriction fragment length polymorphism (PCR-RFLP) and PCR denaturing-gradient gel electrophoresis (PCR-DGGE). Additionally, molecular techniques include DNA macro and micro arrays, next generation sequencing (particularly RNA-Seq based), multiplex PCR, in situ PCR, cooperative PCR, magnetic capture hybridization PCR (MCH-PCR), etc. [31, 32].

Even though phytopathogens are present at lower DNA concentrations, their identification in the primary stages of infection is made possible by the increased confidence, precision, sensitivity, and sensitivity of DNA-based molecular techniques. Furthermore, mycological nomenclatural novelties can be documented using bioinformatics databases like the GenBank at the National Centre for Biotechnology Information (NCBI), Nucleotide Sequence Database Collaboration at the European Bioinformatics Institute (EBI), MycoBank, etc. These databases also store and retrieve nucleotide sequences of plant-infecting fungi, which speeds up the molecular tools of diagnosing and performing species delimitation among existing and evolving fungal species [31,32].

2.9.1 PCR Based Detection Technology

One of the greatest diagnostic procedures is Polymerase Chain Reaction (PCR), which, in contrast to conventional chemical and biological diagnostic approaches, allows for precise, quick, and specific differentiation between fungal species and subspecies. When fungal colonies are dead, in the early phases of growth, or lack diagnostic traits, it is especially helpful for diagnosing fungus. Through the enzymatic replication of a particular type of organism, PCR amplifies huge quantities of targeted DNA fragments from one or a few mold spores, enabling DNA sequencing[33]. There have been numerous reports of the application of PCR-based detection method for vegetable soil-borne diseases, such as *Fusarium oxysporum, R. solani, Verticillium dahliae, Phytophthora capsici,* etc[29].

2.9.2 Nested-PCR Based Detection Technology

When amplifying a specific member of a polymorphic gene family or amplifying a cDNA copy of an mRNA present at very low abundance in a specimen containing a heterogeneous population of cell types, nested polymerase chain reaction (PCR) is utilized when it is necessary to increase the sensitivity and/or specificity of PCR. Two consecutive amplification processes using distinct primer pairs are often used in nested PCR.The product of the first amplification reaction is used as the template for the second PCR, which is primed by oligonucleotides that are placed internal to the first primer pair. The use of two pairs of oligonucleotides allows a higher number of cycles to be performed, thereby increasing the sensitivity of the PCR. Two different sets of primers bind to the same target template, increasing the reaction's specificity. Although it needs knowledge of the target's sequence, nested PCR is an effective technique for amplifying large template segments[37]. Nested PCR detection method has been used in detecting *Fusarium culmorum.* A nested PCR approach was developed using specific primers based on the DNA

(RAPD) marker sequence with a band width of 1958 bp. Nevertheless, nested PCR-based detection method still has several drawbacks. First of all, this method takes a lot of time because it calls for two PCRs and agarose gel electrophoresis to confirm the positive result. Second, nested PCR is more vulnerable to contamination in open settings where several samples are processed [29].

2.9.3 Multiplex PCR Based Detection Technology

By combining the benefits of nested PCR and conventional PCR, multiplex PCR (M-PCR) is a PCR variation in which two or more sequences of interest are amplified simultaneously in the same process. Since many pathogens frequently infect the same produce, M-PCR is more useful for diagnosis and research on vegetable crops, saving time and money. Notably, testing the compatibility of particular primers from various pathogens is necessary for the M-PCR system's creation. Tomato scab (Xav) and tomato canker (Cmm) have been identified using dual PCR. For the pathogenic bacteria *Cladosporium cucumerinum, F. oxysporum*, and *Mycosphaerella melonis* in diseased plant tissues, an M-PCR detection method was created. *Pseudomonas syringae, C. michiganensis* subsp. *michiganensis, R. solanacearum*, and *Xanthomonas campestris* can all be detected using this quick and reliable method [29].

The drawback is that non-specific amplification of several primer pairs, templates, etc. in the same reaction can result in false-positive detection results. Other evident restrictions include the possibility that the detection sensitivity may be impacted if the level of resolution of agarose gel electrophoresis limits the length differences of amplified fragments. The virulence and infectivity of the bacterium identified by M-PCR technology could not be determined. Additionally, it could not infer information on the integrity of microbial cells, which affects epidemiological evaluation. Therefore, selecting a reliable PCR amplification system, particularly the annealing temperature, is essential when building primers. This is the only technique to increase the detection effectiveness of soil-borne pathogens [29].

2.9.4 RT–qPCR-Based Detection Technology

Quantitative PCR, another name for real-time polymerase chain reaction (real-time PCR), is a PCR method modification that enables real-time PCR process monitoring. The enzymatic procedure known as PCR is used in vitro to produce thousands to millions of copies of a certain DNA segment by amplifying a chosen DNA region through several orders of magnitude. Template DNA, primers, nucleotides (dNTPs), and thermostable DNA are required polymerase ingredients. One of the main benefits of real-time PCR over basic PCR is that it offers a trustworthy quantification relationship between the number of starting target sequences (prior to amplification by PCR) and the amount of amplicon accumulated in a specific PCR cycle. This is in addition to enhanced accuracy, sensitivity, and rapidity. This is crucial for accurately quantifying the target nucleic acids, which is necessary for determining the viral load of a clinical specimen and for quantifying mRNA in gene expression analysis[34]. Additionally, post-PCR procedures are not required, which reduces the possibility of cross-contamination from earlier amplicons. This real-time PCR technique, therefore, has revolutionized the detection and quantification of target nucleic acids and gained a wide range of applications[34].For *Rhizoctonia solani*, particular primers have been created for the RT-qPCR method, which is a hundredfold more accurate than the traditional PCR method[24].

Fusarium-specific primers for the TEF gene have been developed, and an RT-qPCR detection technique with a sensitivity 10,000 times greater than traditional PCR [29]. Vegetable pathogen levels can be objectively measured using PCR by comparing them to a standard curve. The RT-qPCR technology can detect the presence of infections but cannot distinguish between their living and dead cells. The quantitative determination of soil-borne pathogens is limited by this method, despite its effectiveness and applicability. Research in the area of managing soil-borne diseases is its primary use. The need for commercial identification is anticipated to rise as farmers become more aware of RT-qPCR detection techniques [29].

2.9.5 PMA-qPCR Based Detection Technology

Propidium monoazide, or PMA, is a unique membrane-impermeable dye that may pass through damaged cell membranes and produce fluorescent signals without affecting healthy cells directly. After entering dead or damaged cells, PMA mixes with their DNA after a predetermined amount of light reaction time. It disregards the number of dead cells and results in a lack of fluorescence signals during bacterial DNA amplification. The PMA concentration and illumination time are screened as part of the PMA-qPCR detection system construction procedure for vegetable diseases. PMA reduces the overestimation of cell count brought on by dead-cell DNA in qPCR detection by blocking the PCR amplification of dead-cell DNA [29].

The translation elongation factor (TEF) gene, the screening PMA concentration (50 mmol·L−1), and the illumination time (15 min) have all been taken into consideration while designing specific primers F8-1/F8-2. As a result, they developed a PMA-qPCR method to detect and measure living *Fusarium* cells in soil. This technology is very useful in medication screening and field disease control since it can distinguish between infections' living and dead cells. Nevertheless, PMA is costly and only appropriate for skilled laboratory workers; it is not appropriate for field use [29].

2.9.6 Bio-PCR

After increasing the fungal pathogen's biomass on the seeds through a pre-assay incubation stage, DNA is extracted and amplified using PCR in a process known as Bio-PCR. This method was first used mostly for phytopathogenic bacteria since they can be quickly and easily cultivated in a growth medium for two to three days. Later, it was also demonstrated to work well for fungi. A number of incubation techniques, such as soaking seeds in liquid fungal growth medium, have been tried to promote consistent and quick fungal development. The PCR assay's reliability is reduced because the presence of bacteria may limit or suppress fungal growth, and adding antibiotics to the substrates may not always stop bacterial growth. To boost the low levels of target *Fusarium oxysporum* f. sp. lactucae on lettuce seeds and *Alternaria radicina* on carrot seeds, other pre-assay techniques include incubating on moistened filter paper or simply on the surface of a plastic Petri dish in an environment with high humidity [35].

Compared to conventional PCR, BIO-PCR offers a number of benefits, including higher sensitivity, the removal of PCR inhibitors, and the ability to detect only living cells, preventing false positives from detecting DNA from dead cells. The drawbacks of BIO PCR include its higher cost compared to traditional PCR, particularly when selective medium is utilized, and the fact that fungal growth typically takes 5–7 days, which greatly lengthens the time needed to finish the experiments [35]. Using certain ITS primers in rDNA and a deep-freeze blotter technique, the seed-borne fungal diseases *Alternaria alternata, Alternaria radicina*, and *Alternaria dauci* were identified during the BIO-PCR assay. The benefits above end point PCR techniques include high sensitivity, PCR inhibitor removal, and live cell detection to prevent false positives. This technique's drawbacks are its time commitment and expenses associated with using selective medium for the assay [31].

2.9.7 LAMP Based Detection Technology

An isothermal nucleic acid amplification platform called loop-mediated isothermal amplification, or LAMP, has become a widely used method for phytoplasma molecular identification. Designing four distinct primers for six distinct places on the target sequence, two internal primers (FIP and BIP), two exterior primers (F3 and B3), and two ring primers (LF and LB), is the fundamental idea behind this strategy. BstDNA polymerase exhibiting elevated displacement activity makes it easy to perform the LAMP reaction under isothermal conditions. Because LAMP technique is applicable in open field operations, it has been widely employed in the detection of soil-borne pathogens in vegetables, including bacteria, viruses, and oomycetes. Furthermore, the application of LAMP technology to identify soil-borne pathogenic fungus in vegetables has been widely reported [29].

The sensitivity of LAMP method, which was created to detect *R. solanacearum* in vegetables, was ten times more than that of traditional PCR [24]. LAMP detection shows high sensitivity and accuracy for detecting pathogen DNA at 100 pg·µL−1 for *Colletotrichum* species. The following are LAMP's benefits: (1) strong specificity, as the four specific primers for LAMP correspond to six conserved sites in the target gene's sequence, and DNA amplification cannot be carried out if any site does not match; (2) high speed, the entire reaction can be finished in 30 to 60 minutes; (3) high amplification efficiency, 10–1,000 times larger than that of conventional PCR; (4) easy to use and (5) inexpensive. LAMP's inability to be employed for quantitative detection and solely for qualitative analysis is a drawback. Furthermore, the results are impacted by LAMP's open operation, which makes it susceptible to false positives [29].

2.9.8 Genomics-Based Detection Technology

The identification of soil-borne infections in vegetables has significantly improved since the development of high-throughput sequencing technologies. Bacterial identification accuracy was increased using single-molecule long-read third-generation sequencing and high-throughput second-generation sequencing. Additionally, this method may detect a wide range of distinct microbial populations, including unidentified bacteria, viruses, and viroid. This method eliminates the need for plate culture and primer design for particular microbial sequences, which is crucial because only about 10% of bacteria can be cultured. Research on soil-borne vegetable diseases has made extensive use of metagenomics, pyrosequencing, and next-generation sequencing (NGS), for which there are several platforms, including Solexa, AB SOLiD technology, 454 Roche, Illumina, and Ion Torrent [29, 35]. Several fungal genera have been identified through the use of 454 pyrosequencing in the investigation of the mycobiome of wheat seed [35]. Using high-throughput sequencing technology, it has been possible to identify high concentrations of *Streptomyces, Bradyrhizobiaceae, Comamonadaceae,* and *Mortierella* in healthy soil and *F. oxysporum, Gibberella, Bacillaceae*, and *Xanthomonadaceae* in unhealthy soil. The oomycetes and fungi *P. ultimum, P. irregulare, P. aphanidermatum, P. nicotianae, P. capsici, P. cinnamomi, R. solani,* and *F. oxysporum* have been identified in soil using genomics technology in earlier investigations. The computational resources needed for further data analysis are likewise relatively significant, as is the expense of genome sequencing [29].

2.9.9 Biospeckle

The "biospeckle" laser technology is a newly used tool that may detect the presence of harmful fungi on vegetables. The optical phenomena of interference produced by a laser light interacting with the seed coat is the basis for this procedure. Vegetable seeds examined under laser light make it possible to identify regions with various activity. The color, texture, and chemical makeup of vegetable seed surfaces can be ascertained using a multispectral vision system, such as the VideometerLab device, which is another tool that can differentiate between healthy and sick vegetable seeds. The ability to distinguish between healthy spinach seeds and seeds infected with *Stemphylium botryosum, Cladosporium* spp., *Fusarium* spp., *Verticillium* spp., or *A. alternata* was made possible by the combination of features from images taken with visible light wavelengths and near-infrared wave lengths. To find *Fusarium* spp., a similar method was previously employed in a study on malting barley (S. Bodevin, Carlsberg Research Centre, Copenhagen, Denmark, personal communication) [35].

A biotin-labeled PCR product and an oligonucleotide probe attached to different beads that specifically detect variations in nucleotide sequences hybridize to form the basis of the Luminex PCR technology. This technology has a lot of potential and could be helpful in the area of seed diagnosis because it eliminates the need for time-consuming and arduous procedures. An alternative to ELISA is the Luminex xMAP method, which uses paramagnetic microspheres (immunobeads) coated with antibodies and internally stained with fluorochromes. These beads function as tiny ELISA wells. These methods were first used in clinical settings and are being employed in plant pathology applications, such as multiplex detection of bacteria and viruses that are transmitted through seeds. Lettuce mosaic virus and pepino mosaic virus are two viral diseases that have been detected in seeds using the Luminex xMAP technology. Although this technology is still being evaluated on fungal pathogens in seeds, it is an appealing technology for laboratory-based sample testing because to its rapid analysis and massive multiplexing capability, which allows up to 100 pathogens to be examined simultaneously in a single run [35].

2.9.10 Sequencing Technology

High-throughput sequencing technologies have revolutionized the study of microbial population dynamics and plant pathogen diagnostics by making it easier to use large datasets, allowing for deeper analysis, and evaluating a wider range of relevant data. The introduction of Next-Generation Sequencing (NGS) platforms like 454 pyrosequencing, Illumina, and Ion Torrent first made it easier to use metagenomics and metabarcoding techniques. Third-generation sequencing technologies created by Oxford Nanopore Technologies and Pacific Biosciences (PacBio) came next[36].

Next-generation sequencing (NGS), which has improved our knowledge of the dynamics, structure, and function of the genome, has revolutionized genomic research. In previously unheard-of methods, scientists can now explore the complexities of genetic information. NGS's high-throughput capability and affordability have made it an essential tool for researchers in a variety of domains. NGS has facilitated the study of transcriptomics, epigenomics, metagenomics, and other omics and enabled comprehensive genome sequencing[37].

Chain termination-based sequencing method by Fredrick Sanger uses dideoxynucleotides to stop the chain elongation of DNA strands during replication. This method enables the creation of sequence reads up to a few hundred nucleotides long. Since it made it possible to quickly sequence DNA and RNA, Sanger's technique was widely used and transformed the area of molecular biology[37].

Since second-generation sequencing techniques allow for the simultaneous sequencing of thousands to millions of DNA fragments, DNA sequencing has undergone a revolutionary change. These techniques can do parallel sequencing, which sets them apart from conventional Sanger sequencing. The Roche 454 sequencing technology, which uses pyrosequencing to identify the sequence by detecting the release of pyrophosphate when nucleotides are added to the DNA template, is one of several popular second-generation sequencing technologies that have surfaced. Another technology is Ion Torrent sequencing, which determines the sequence by detecting the emission of hydrogen ions during DNA synthesis. The popular Illumina sequencing platform uses a reversible dye terminator-based sequencing-by-synthesis technique[37].

2.9.11 Electrophoresis

A potent analytical method for separating charged molecules according to their size and charge in an electric field is electrophoresis. It has grown to be a pillar since its founding in forensic, chemical, and biological sciences[38]. The technique has revolutionized fields like genomics, proteomics, and molecular biology since it allows for the accurate separation and analysis of DNA, RNA, and proteins[38].

Agarose is a heteropolysaccharide that, when dissolved in a hot buffered solution (between 50 and 55 °C), creates a viscous solution; but when cooled, it solidifies into a gel[39]. The polymerase chain reaction (PCR) products, hemoglobin, serum proteins, and nucleic acids are all separated by this support medium[39]. Using ultrapure agarose gel with a low sulfate concentration can prevent the fixed sulfate groups in agarose from decreasing the resolution of bands because they increase electroendosmosis[39].

2.9.12 Nanodrop spectrophotometer

DNA analysis and quantification are now routine everyday processes that serve as the foundation for the various molecular biology laboratory operations[40]. The measurement of sample absorbance at 260 nm is one of the most often used techniques to quantify nucleic acid concentration. During the DNA extraction procedure, the 260/280, 260/230, and 260/325 absorbance ratios are used to assess the purity of the DNA and the existence of impurities in the biological samples. At the moment, the Nanodrop spectrophotometer's absorbance measurements of samples' microvolumes provide the most practical means of estimating DNA content and purity[40].

2.10 Phylogenetic Tree

A phylogenetic tree, sometimes referred to as a cladogram, tree of life, or evolutionary tree, is a tree-like graphic representation that shows the evolutionary and phylogenetic links between biological taxa according to their genetic or physical traits. Nodes in a phylogenetic tree represent taxonomic units, whereas branches show the estimated temporal relationships between these units[41]. The phylogenetic tree visually presents the evolutionary history and phylogenetic relationships between different taxonomic units, facilitating people's understanding of the causes of species' morphological diversity and evolutionary patterns[41].

Methods for constructing phylogenetic trees fall into two major types[42]. The first method was distance-based techniques, like the UPGMA and NJ approaches[43]. These techniques use clustering algorithms to assess the fungi species and infer the phylogenetic tree after first converting the feature matrix into a distance matrix to represent the evolutionary distances between species pairs[44]. Character-based techniques, such the probability and parsimony methods were the second method[45]. These techniques, which include the MP, ML, and BI methods, usually produce a huge number of hypothetical trees based on an algorithm before inducing an optimal tree based on specific criteria[41].

2.11 Aflatoxin

The secondary metabolites known as mycotoxins, which are produced by different fungal strains, have a major negative impact on food products' quality and can seriously harm both humans and animals. Mycotoxigenic fungal strains in impacted crops were traditionally divided into two classes: "field fungi," which typically infiltrate vegetables prior to harvest (like *Fusarium spp*.), and "storage fungi," also known as saprophytic fungi, which contaminate vegetables after harvest (like *Aspergillus* and *Penicillium* genera) and cause serious issues. The most significant and economically relevant mycotoxigenic fungi and their mycotoxins of stored fruits and vegetables (Fig 2.11) are; *Aspergillus* spp. [aflatoxins (AF)], *Fusarium* spp. [fumonisins (FUM), trichothecenes (TCT), zearalenone (ZEA), deoxynivalenol (DON)], *Penicillium* spp. [ochratoxin A (OTA), patulin (PAT), and citrinin (CIT)], and *Alternaria* spp[46].

Aflatoxins are a group of structurally related toxic, mutagenic, and carcinogenic mycotoxins that contaminate large numbers of food and agricultural products such as vegetables[47]. From a food safety perspective, the B-series (aflatoxin B1 and B2), G-series (aflatoxin G1 and G2), and M-series (aflatoxin M1) are the most significant of the numerous aflatoxins' analogues and derivatives that have been discovered[47].

Fungal growth and consequent mycotoxin excretion may occur at any point in the agricultural production chain, depending on environmental variables (temperature, humidity, and rainfall) and farm management techniques (cropping, harvesting, and storage conditions). Aflatoxin synthesis in agricultural products is also known to be facilitated by plant immunocompromising variables as drought stress, damage, pest infestation, and inadequate fertilizer[47].

Aflatoxigenic fungi can effectively colonize the host through wounds or natural orifices on the surface of fruits and vegetables prior to harvest, which is the primary way that they infect them. The host is highly resistant to external, biological, or abiotic stress during the development phase[47]. However, fruits and vegetables' resistance impact is greatly diminished because of the high energy consumption at the postharvest stage. The infection can spread quickly once the right humidity and temperature are reached. Then, in the intermediate and late phases of growth, the secondary metabolite harmful mycotoxins are generated[48].

Aflatoxins are produced by certain *Aspergillus species* (*Aspergillus flavus and Aspergillus parasiticus*, Aspergillus niger). *Aspergillus spp* are widespread microscopic molds that grow and produce toxins in variety of substrates, with the highest concentrations of aflatoxins being accumulated in products[48]. The entry of fungus in crops not only compromises the self-defense of crop plants by the fungal attack but also contaminates the agricultural products which lead the aflatoxin production. The fungal infections affect the crop's growth, yield, and also result in loss of market value[49].

2.11.1 Health Risk Associated with Aflatoxin

The high risk associated with aflatoxins is due to their extremely toxic effect on liver cells. They disrupt bile secretion, induce necrosis, and paralyze their metabolic processes. They are the most potent known hepatocarcinogens, meaning they cause liver cancer in addition to being hepatotoxic. Immunogenicity, vitamin D metabolism, iron metabolism, and other mineral metabolism are linked to other lesions. The spread and growth of hazardous molds are particularly severe in areas with hot, humid climates[50].

2.11.1.1 Hepatotoxicity and Liver Cancer

One of the most prevalent and fatal malignancies in the world, hepatocellular carcinoma (HCC), is significantly increased by aflatoxin exposure (Figure 6). Aflatoxin B1 has been categorized as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC). Liver cancer can develop as a result of cumulative DNA damage brought on by prolonged exposure to even low amounts of aflatoxins [51].

2.11.1.2 Immunosuppression

The body's capacity to combat infections is diminished by aflatoxins, which compromise both innate and adaptive immunity. This immunosuppressive impact is especially problematic in areas where infectious illnesses are prevalent[51].

2.11.1.3 Aspergillosis

In immunocompromised people, Aspergillus species can cause aspergillosis, a lung infection. Although there are twenty species of Aspergillus that can cause it, the two most common ones that cause aspergillosis in humans and animals are Aspergillus fumigatus and Aspergillus flavus. The majority of human aspergillosis infections worldwide are brought on by excessive Aspergillus spore inhalation, with spore transmission through infected wounds ranking as the second most common route of infection[52]. Allergic bronchopulmonary aspergillosis (ABPA) accumulates in 1–15% of the world’s population already infected with cystic fibrosis and also in 2.5% of asthma patients, which in total comprises 4.8 million people globally. Out of the 4.8

million of the world's population affected with ABPA, 400,000 people are also affected with chronic pulmonary aspergillosis[52].

2.11.1.4 Cancer

Long-term exposure to aflatoxins has been linked to kidney, liver, lung, or colon cancer in both humans and animals. They are classified as a Group 1 carcinogen. Hepatocellular carcinoma, the predominant liver cancer seen in Africa and Asia, is linked to aflatoxin B1, and aflatoxin ingestion is thought to be the cause of 4.6% to 28.2% of hepatocellular carcinoma worldwide. Aflatoxin B1, a Group 1 carcinogen, is also harmful if ingested daily for 1–3 weeks at a dose of 20–120 µg/kg. Aflatoxin toxicity, however, is mostly dependent on the host's immunity. 75–85% of liver cancer cases globally are caused by hepatocellular carcinoma, the primary consequence of aflatoxin exposure[52].

2.11.1.5 Aflatoxicosis

Acute aflatoxin exposure can cause aflatoxicosis (Fig 2.7), a potentially fatal condition characterized by vomiting, abdominal pain, and liver failure. Outbreaks of aflatoxicosis have been reported in several countries, particularly in regions with high levels of aflatoxin contamination in staple foods[51]. The majority of aflatoxin contamination occurs in tropical and subtropical areas, where Aspergillus species thrive due to the favorable climate. Bad storage habits and high temperatures and humidity provide the perfect environment for the growth of fungi and the creation of aflatoxin. However, aflatoxin contamination is now a worldwide problem due to the expansion of its geographic spread brought about by global trade and climate change. It is anticipated that formerly unaffected areas may be more vulnerable to aflatoxin contamination due to rising temperatures and shifting precipitation patterns [51].

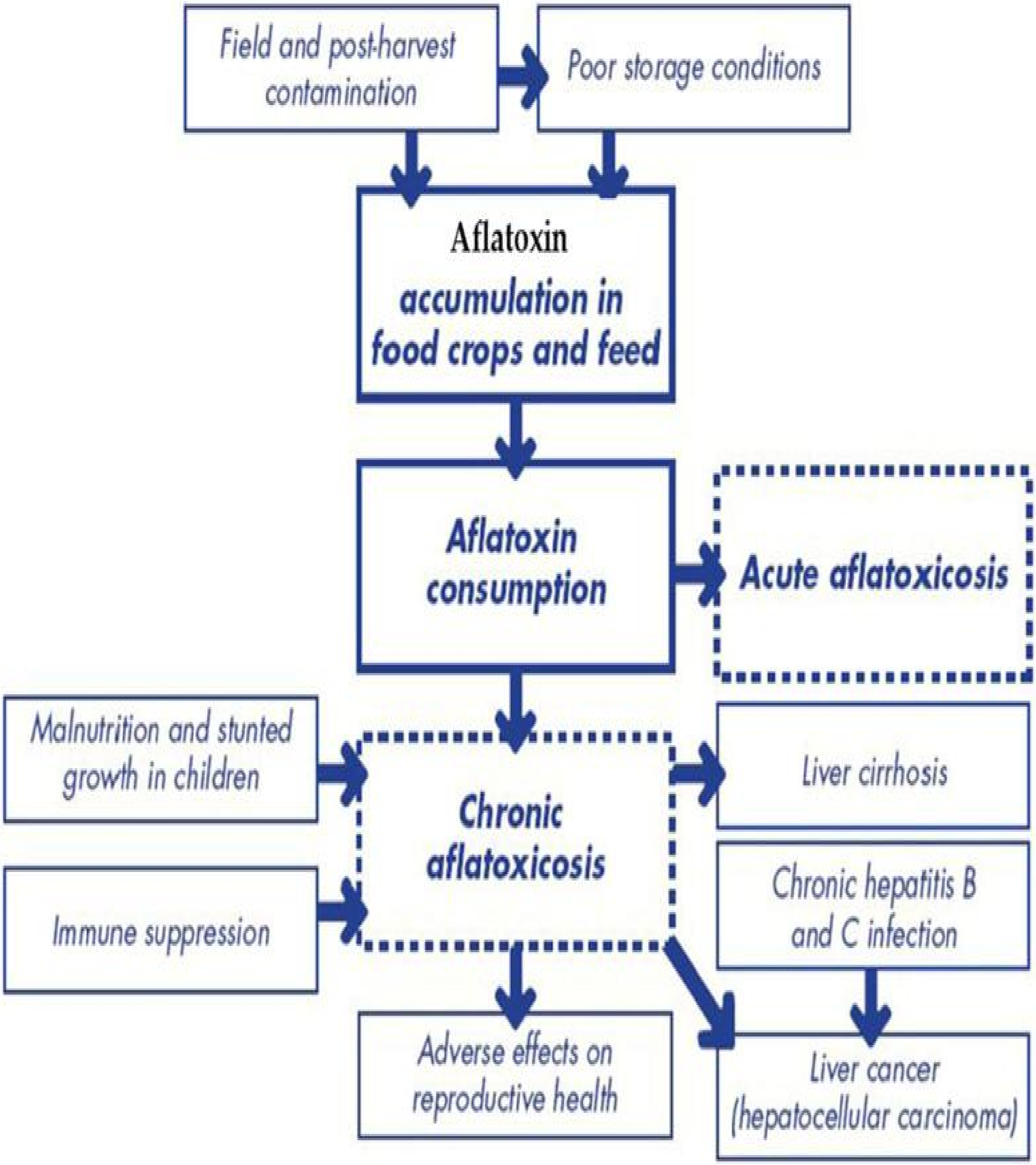

Figure 2.7: Acute aflatoxin exposure to cause aflatoxicosis[51].

2.11.2 Factors affecting Aflatoxin productions

The two most significant factors affecting the development of mycotoxin-producing fungus and mycotoxin production are temperature and water activity[53]. Furthermore, at their ideal growing temperature, the majority of fungal species produce their highest concentration of mycotoxins. There may be some outliers, but generally speaking, mycotoxins are best formed between 24 and 28°C. The most common mycotoxin-producing fungus species have different minimum and ideal temperatures and water activities for growth and mycotoxin formation[53]. Aflatoxin production thrives in warm climates, which is why mycotoxin levels are higher in tropical and subtropical regions. The ideal water activity and temperature for aflatoxin production were 0.99 and 33◦C, respectively. Even if toxin generation is still visible at 32°C, temperatures above 33°C clearly disrupt aflatoxin production, even if there is a purported increase in fungal growth[53].

2.11.3 Measures to control Aflatoxin contamination

Mycotoxin-producing molds can contaminate fruits and vegetables in the field, during and after harvesting, and during raw material storage and transit[50]. In order to minimize aflatoxin generation and fungal growth in the field, pre-harvest practices are essential. One of the best strategies is to adopt resistant crop varieties, which are less prone to infection by Aspergillus[51]. Good agricultural practices also play a crucial role in minimizing aflatoxin contamination. Practices such as crop rotation, timely harvesting, and proper irrigation The proper drying of crops is one of the most effective ways to reduce mycotoxin-producing fungi, especially when the vegetables are not being used fresh. can reduce fungal growth and aflatoxin production[51]. Post-harvest measures are critical for preventing aflatoxin contamination during storage and processing. Moisture levels should be maintained below 13% to inhibit Aspergillus growth and aflatoxin production[51].

Physical methods such as steam under pressure, dry roasting, and other cooking methods are found to be effective in the control or to reduce the aflatoxin contamination in many crops[49]. However, preventive method such as, storing the product in clean, mold-free areas, maintaining suitable low temperatures, good aeration at low humidity, regular monitoring and cleaning of the agricultural produce are equally important[50].

2.11.4 Determination Techniques of Aflatoxin in vegetables

2.11.4.1 Enzyme-Linked Immunosorbent Assay (ELISA)

There are numerous approved techniques for identifying crop plants contaminated with aflatoxin. The most widely used technique is the Enzyme-Linked Immunosorbent Assay (ELISA)[49]. The immunological reaction between an antigen and a particular antibody in the presence of a catalytic enzyme is known as ELISA. ELISA is frequently employed because is a rapid, easy, and reasonably priced technology. To identify common mycotoxins such Aflatoxins, OTA, trichothecenes, FBs, and CIT, the ELISA approach uses commercial kits. The majority of these kits were used to identify mycotoxins in food such as vegetables[53].

2.11.4.2 Biosensor- Based Techniques

Biosensors identify various biological components by using antigens or antibodies. As a signal transducer, the graphite, carbon, or gold that is affixed detects their binding to complimentary species. QCMs, or piezoelectric quartz crystal microbalances, are extremely sensitive, unlabeled instruments that have direct antigen detection capabilities. An antigen and an antibody are contained to the quartz crystal's surface when they come into contact, changing the electrode surface's mass, which is what causes the phenomenon of piezoelectric quartz crystals[52]. This method enables the detection and quantification of the immunological complex because the concentration of the antigen and antibody complex on the surface of a quartz crystal is exactly proportional to the change in mass of the electrode surface. There has been success using QCMs to identify aflatoxin B1[52].

2.11.4.3 Molecular-Based Techniques

The genes involved in the aflatoxins' production pathway are amplified by the use of several markers. Afflatoxin production pathways involve several genes, including nor-1, apa-2, omtA, ver-1, aflRS, aflJ, and omtB, which can be amplified using multiplex and real-time PCR tests. There are three distinct systems used to amplify these genes. First, the genes nor-1, omtA (omt-1), and apa-2 are amplified; second, nor-1, omtA (omt-1), and ver-1 are targeted; and third, omtB, aflRS, and aflJ are amplified by PCR[52].

2.12 Other Mycotoxins

2.12.1 Ochratoxins

A. carbonarius, A. niger, A. tubingensis, and other strains of microscopic fungi belonging to the genera *Aspergillus* and *Penicillium*—including the widely dispersed A. ochraceus and, in some cases, P. viridicatum—are producers. We know about ochratoxins A, B, and C. Ochratoxin A is the most powerful member of this class of mycotoxins, but it also has the highest toxicity[50]. The risk of ochratoxin A transition to products of fruit and vegetable processing is quite real[50].

Ochratoxin A has been shown to have harmful effects on immunogenesis, erythrocytes, liver, and kidneys. It possesses genotoxic, teratogenic, and carcinogenic qualities[50].

2.12.2 Patulin

Patulin mycotoxin is associated with fruits. Fungal species such as *Penicillium expansum*, *Aspergillus clavatus*, and *Byssochlamys nivea* are producers[54]. Patulin mycotoxin (a polyketide lactone 4-hydroxy-4H-furo (3,2c) pyran-2 (6H)-one ;(Fig 2.8) belongs to a class of toxic compound with low molecular weight (154.121 g/mol). The molecular formula of patulin (PAT) is $C_7H_6O_4$; it is stable in aqueous media at 105–125 °C with melting point of 110 °C [54]. During growth, harvest, storage, or food processing, these fungi that produce patulin target vulnerable items[54]. The primary source of PAT among several fungal species is *Penicillium expansum*, which is frequently found in a wide range of fruit kinds. Nevertheless, the toxin can also contaminate rotting vegetables, moldy feed, other fruits, and leftover wheat straw. It has been proposed that climate change may make colder climates more susceptible to mild issues with patulin in food[54].

When contaminated fruits and their derivatives are consumed, PAT mycotoxin poses health risks. PAT toxicity causes both acute and long-term toxicity issues in humans and animals by forming harmful adducts with sulfhydryl groups. Immunological, neurological, and gastrointestinal effects such distension, ulceration, and bleeding are linked to exposure to this mycotoxin[52]. The kidney, liver, gut, spleen, and stomach are among the body organs that are impacted by PAT. PAT toxicity in animals and mammalian cells encompasses immunotoxicity, genotoxicity, teratogenicity, and embryotoxicity[54].

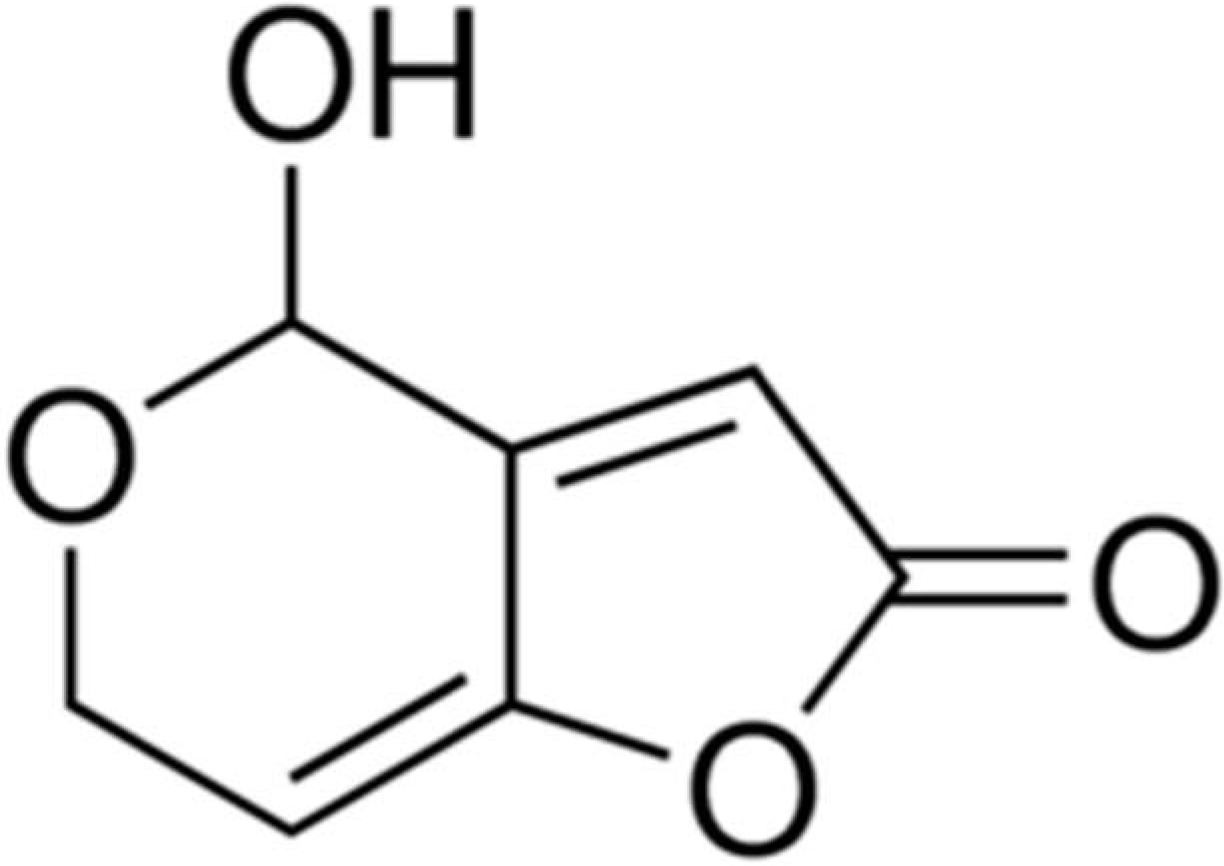

Fig 2.8: Molecular Structure of Patulin[54]

2.12.3 Fumonisin

The two main genera that produce fumonisin are *Aspergillus* and *Fusarium*. Notably, they both create distinct toxin profiles and colonize various substrates. Above-ground and below-ground plants are impacted by *Fusarium*, particularly *Fusarium verticilliode*, *Fusarium proliferatum*, and related *Fusarium spp*., which produce FB1, FB2, and FB3. Of these, FB1 is the most prevalent and toxic. They live in the soil as saprophytes and colonize the rhizospheres[55]. In contrast, the only fumonisin producing *Aspergillus* is *A. niger*, which produces FB2, FB4, and FB6 [54]. The most frequent symptoms linked to fumonisins include pulmonary edema, hydrothorax in pigs, and leukoencephalomalacia in horses. and apoptosis, carcinogenicity, and hepatotoxicity in rats' livers. The risk of esophageal cancer in people is also possible[55].

2.12.4 Trichothecenes

Trichothecenes are the largest group of mycotoxins produced as secondary metabolites, particularly by *Fusarium* species. TCNs are also produced by a number of other fungal genera, such as Stachybotrys, Cephalosporium, Cyclindrocarpon, Myrothecium, Trichoderma, Phomopsis, and Verticimonosporium species. These trichothecenes-producing fungi thrive in a variety of environmental conditions, including temperatures between 0 and 50 °C, humidity levels above 70%, moisture contents between 10 and 20%, water activity above 0.88, the growth medium's oxygen content, and nutritional compositions[56]. The TCT common structure consists of a three-ring molecule known as 12,13-epoxytrichothec-9-ene (Fig 2.9). The cyclohexene (A-ring) is fused to the tetrahydropyran (B-ring), which is bridged by a two-carbon chain at C-2 and C-5, thus forming a cyclopentyl moiety (C-ring). TCT are divided based on the substitution pattern of EPT into four types (A–D). Type A TCT is distinguished by a hydroxyl (OH) group at C-8, an ester function at C-8, or no oxygen substitution at C-8[57].

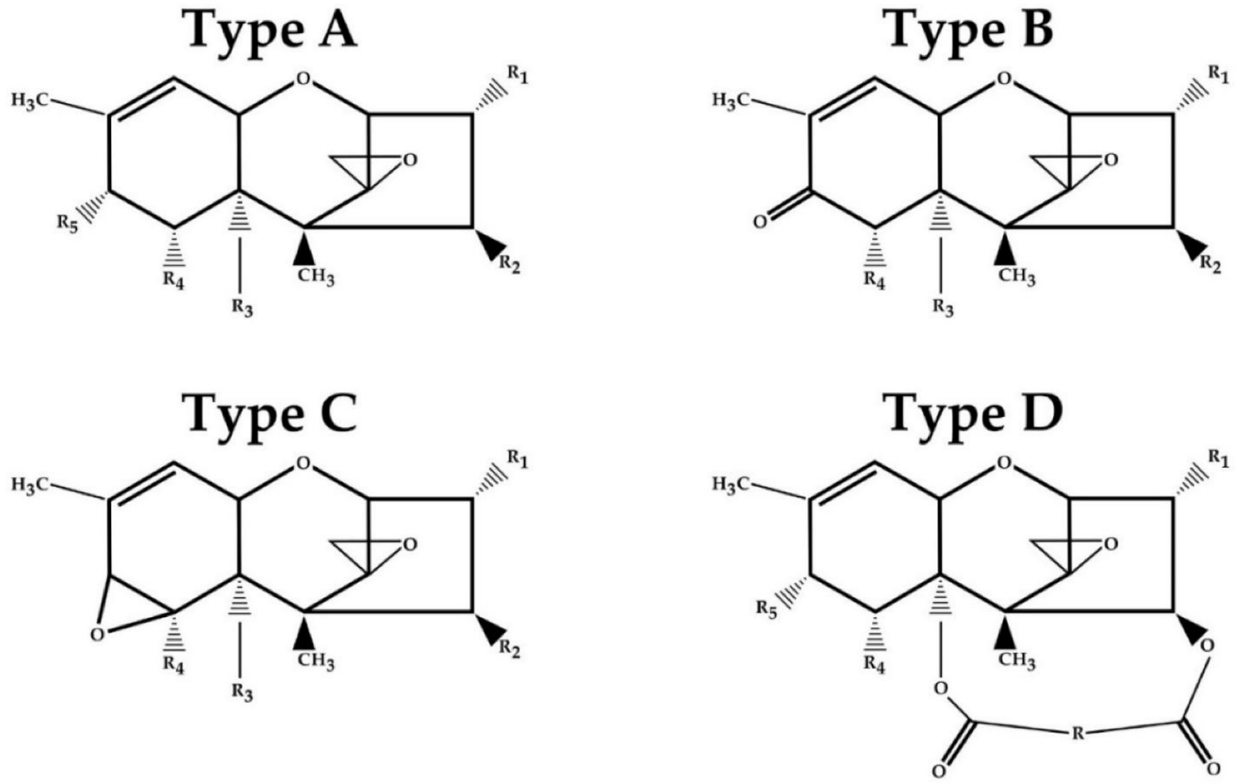

Fig 2.9: The core structures for A, B, C, and D trichothecenes (TCT) types [57]

2.12.5 Zearalenone

Zearalenone is a mycotoxin produced by fungi of the genus Fusarium, mainly *F. graminearum, F. culmorum, F. cerealis, F. equiseti, F. crookwellense, F. semitectum, F. verticillioides, F. acuminatum F. sporotrichioides,* and *F. oxysporum.* Fungi produce ZEN in temperate and warmer climates. Zearalenone has the general formula C18H22O5(Fig 2.10) and is a 6-(10-hydroxy-6-oxy-trans-1-undecenyl-beta-resorcylic acid lactone)[58]. Zearalenone is a mycotoxin that has xenogenic, hepatotoxic, and immunotoxic properties[59]. Both vegetation and prolonged storage, if uncontrolled, might result in ZEN production. Animal feed's grain and vegetable protein content are vital sources of nutrients for fungus growth, endangering the feed's safety. Typically, pre-harvest and improper storage conditions are when mycotoxin production in feed takes place[58].

It causes various changes and disorders related to the reproductive system, generating considerable economic losses. Mammals may be at risk from zearalenone and its metabolites, particularly if exposed to high concentrations over extended periods of time[58].

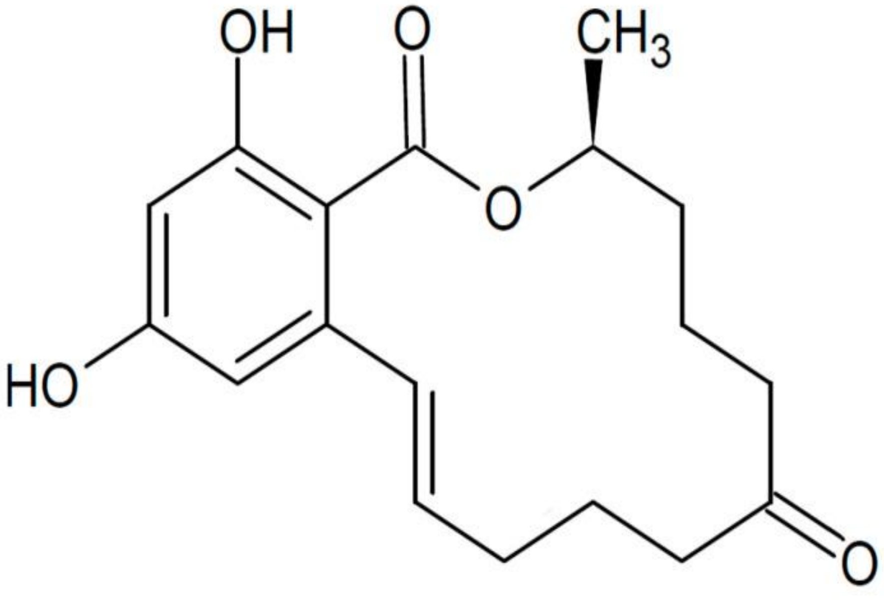

Fig 2.10: Structural formula of Zearalenone[58]

2.12.6 Alternaria Toxins

Toxins produced by *Alternaria spp.* are secondary metabolites that cause mutagenicity and cancer in both humans and animals. It can also turn into an opportunistic pathogen that occasionally causes a variety of diseases in fruits, vegetables, ornamentals, and cereal crops[60.] In plants linked to both host-specific and non-host-specific toxins, Alternaria pathogenic species produce blight, leaf rot, and leaf spot diseases that result in black spots on a variety of fruits and vegetables during the post-harvest storage and marketing phase[60]. Numerous fruits and their byproducts can get contaminated by the diverse mycotoxins produced by Alternaria fungus. Cancer, allergies, and other toxicities are among the negative health outcomes associated with exposure to these mycotoxins[60]. It has been consistently documented that ATs naturally occur in a variety of foods, such as cereal grains, legumes, oil seeds, edible oils, vegetables, fruits, and their processed products[61,62].

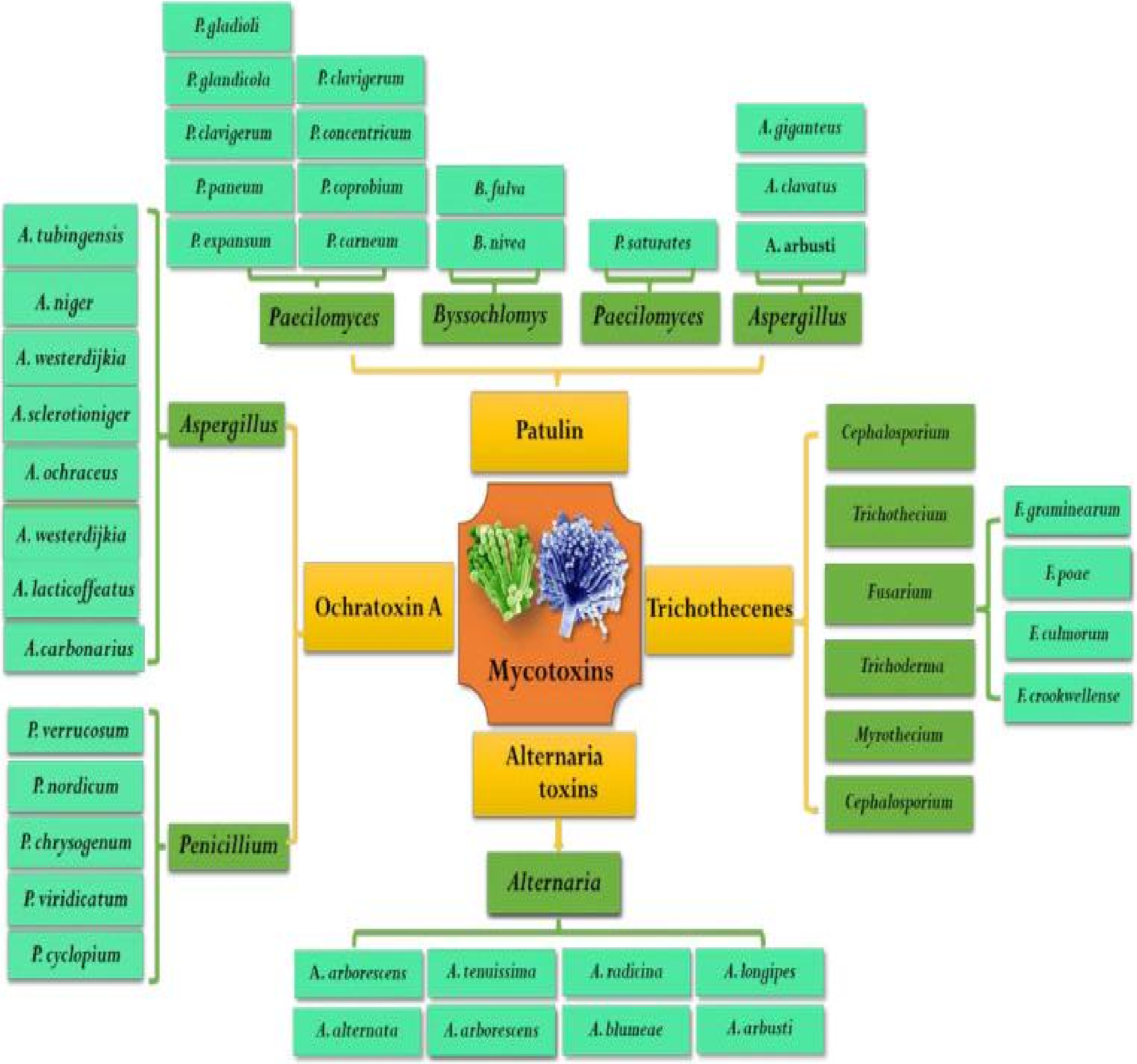
P. gladioli
P. glandicola
P. clavigerum
P. paneum
P. expansum
P. clavigerum
P. concentricum
P. coprobium
P. carneum
B. fulva
B. nivea
P. saturates
A. giganteus
A. clavatus
A. arbusti
Paecilomyces
Byssochlomys
Paecilomyces
Aspergillus
Patulin
A. tubingensis
A. niger
A. westerdijkia
A. sclerotioniger
A. ochraceus
A. westerdijkia
A. lacticoffeatus
A. carbonarius
Aspergillus
P. verrucosum
P. nordicum
P. chrysogenum
P. viridicatum
P. cyclopium
Penicillium
Ochratoxin A
Mycotoxins
Trichothecenes
Cephalosporium
Trichothecium
Fusarium
Trichoderma
Myrothecium
Cephalosporium
F. graminearum
F. poae
F. culmorum
F. crookwellense
Alternaria toxins
Alternaria
A. arborescens
A. tenuissima
A. radicina
A. longipes
A. alternata
A. arborescens
A. blumeae
A. arbusti

Figure 2.11: Common mycotoxigenic fungal species in fruits and vegetables[48]

Endnotes

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Chapter Three

Methodology

3.1 Collection of Samples

A total of 48 *Senecio biafrae* samples were collected from four markets known to have high concentration of vegetable marketers. The four markets were across four Local Government area in Ibadan Metropolis. The Local Governments were: Ibadan North-West Local Government (Orita merin market is denoted as Market A); Ibadan North-East Local Government (Mapo market is denoted as Market B); Ibadan North Local Government (Shasha market is denoted as Market C) and Ido Local Government (Oje market is denoted as Market D). The samples were identified and verified in the Department of Botany, University of Ibadan, Nigeria by Mr. Ezimekhuai before depositing it in herbarium under voucher number UIH-23681. Then, Samples were arranged and labelled appropriately in polythene bags, and transported to Lead City University, Microbiology laboratory Ibadan, Oyo State for analysis.

3.2 Preparation of Vegetable Samples

The leaves of the vegetables were washed under tap water to get rid of dust and then washed with distilled water three times and air dried on sterile filter paper[1].

3.3 Sterilization of Materials

The glass wares were thoroughly washed in detergent, rinsed in distilled water and then wrapped in aluminum foil. Additionally, the utilized media were also sterilized by autoclaving at 121°C at presence of 1.02kgcm^{-2} for 15 minutes. Scalpels and inoculating needles were sterilized in 70% ethanol and heating them in flames until red hot[2].

3.4 Preparation of Culture Media

Thirty-nine grams (39g) of Potato Dextrose Agar (PDA) was dissolved in one litre of water according to manufacturer's instruction, and the mixture was homogenized by boiling for 15 minutes. The solution was sterilized using an autoclave at 121ºC at presence of 1.02kgcm^{-2} for 15 minutes. Futhermore,1% of streptomycin sulphate was added to prevent the growth of bacteria and allowed to cool[2]. Then, 20ml of the mixture was poured into petri-dishes and allowed to solidified at room temperature.

3.5 Isolation of Fungi

Leaves from apparently unhealthy area were cut into pieces (1-2mm) with a sterilized scalpel and surface sterilized with 1% sodium hypochlorite for 1 min, then rinsed with sterilized distilled water three times and dried on a sterile filter paper. The sterilized samples were directly placed on PDA (Potato Dextrose Agar) plate which was supplemented with 1% Streptomycin antibiotic and incubated at 28±2°C for 72 hours [3]. Plates were observed daily and emerging fungal mycelia growing at the edge of leaf tissues were transferred to fresh PDA plates and was repeated to obtain pure cultures. These isolates were maintained and preserved in slant position of McCartney bottles, incubated in the refrigerator till used[4].

3.6 Morphological Identification of Fungi

The morphology of the fungal isolates was examined and described based on color, texture and growth rate. Small portion of the isolates were placed on a clean microscope glass slide, using sterilized needle. A drop of lactophenol in cotton blue was added to stain the culture on the slide and the mixture were gently covered with a coverslip to avoid air bubbles. The covered slide was then viewed under the x10 and x40 and x60 objectives of the microscope. The features of the isolates obtained (arrangement of hyphae, spore, presence of reproductive structures like conidiophores, sporangia) were compared with those described in fungal identification guide[5]. Then, the micrographs of the observed spores and conidiophores were obtained.

3.6.1 Determination of Percentage of Fungal Occurrence

Frequency of occurrence for each genus of fungi was defined as the number of isolates from each genus as a percentage of the total number of isolates[3,6].

$$\text{Frequency} = \frac{\text{Number of times of occurrence of the individual isolate over the period}}{\text{Total number of microorganisms isolated in the study over the period}} \times \frac{100}{1}$$

3.7 Pathogenicity Test

Pathogenicity tests on all fungal isolates were carried out on healthy leaves of *Senecio biafrae*. Leaves were soaked in 1% sodium hypochlorite for 1 min, washed three times with sterile distilled water, and dried on a sterile filter paper [7]. The leaves were placed in sterilized transparent Petri dishes, which were lined with autoclaved cotton wool wetted with sterile distilled water to keep a relatively high humidity[3].Two inoculation sites for each leaf were gently wounded with a sterilized needle and then fungal hyphae of pure isolated culture were introduced on the marked portions and incubated at room temperature for 7 days. Healthy leaves inoculated with distilled water were kept as control [7]. Sterilized forceps were used to remove portions from the diseased areas on the 7th day and placed on freshly prepared PDA plates and incubated at 28 ±2 ºC for 3 days[7]. The plates were monitored each day for fungal growth resembling the inoculated fungal species. The identifications of the fungi re-isolated from the inoculated *Senecio biafrae* leaves were based on morphological identification. Pure cultures were identified and the fungal growth that appeared were similar to the isolated fungi.

3.8 Molecular Identification

3.8.1 DNA Extraction

Genomic DNA was extracted from the samples received using the Quick-DNA™ Fungal/Bacterial kit (Zymo Research, Catalogue No. D6005). DNA Miniprep Kit. Nanodrop Spectrophotometer revealed the quality and quantity of the extracted DNA. Purity is measured as a ratio of absorbance at 260 nm to that of 280 nm[2].

Wet weight (50-100) mg fungal cells that have been resuspended in up to 200 µl of isotonic buffer (e.g., PBS) was added to a ZR BashingBead™ Lysis Tube (0.1 mm & 0.5 mm) and 750 ul Bashing Bead TM Buffer to the tube. Secured in a bead beater fitted with a 2 ml tube holder assembly and was process at maximum speed for ≥ 5 minutes followed by Centrifuging the ZR Bashing Bead TM Lysis Tube (0.1 & 0.5 mm) in a microcentrifuge at 10,000 x g for 1 minute. 400 µl supernatant was transferred to a Zymo-SpinTM III-F Filter in a Collection Tube and centrifuge at 8,000 × g for 1 minute. 1,200 µl of Genomic Lysis Buffer was added to the filtrate in the Collection Tube. 800 µl of the mixture from 1,200 µl previously prepared was transferred to a Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10,000 × g for 1 minute. The flow through was discarded from the Collection Tube followed by repeating the transfer 800 µl of the mixture to a Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10,000 × g for 1 minute .200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin TM IICR Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute. 500 µl g-DNA Wash Buffer was added to the Zymo-Spin TM IICR Column andcentrifuge at 10,000 × g for 1 minute. Zymo-Spin IICR Column was transferred to a clean 1.5 mil microcentrifuge tube and 100 µl (35 ul minimum) DNA Elution Buffer was added directly to the column matrix and was Centrifuge at 10,000 × g for 30 seconds to elute the DNA[2].

3.8.2 Polymerase Chain Reaction

The ITS target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers (ITS-1 ITS rDNA sequence TCCGTAGGTGAACCTGCGG) and (ITS-4 ITS rDNA sequence TCCTCCGCTTATTGATATGC). The PCR condition included an initial denaturation at 95ºC for 5 min, followed by denaturation at 95º C for 1min, annealing at 52ºC for 1min and a final extension at 72ºC for 10 min[8]. The PCR products were run on a gel and cleaned up enzymatically using the EXOSAP method. The amplicons, 5 µL aliquots of the PCR products were analyzed by electrophoresis in 2% (w/v) standard agarose gel with Ethidium bromide. A 100 bp DNA Marker ladder was used as the standard. Gels were electrophoresed at 100 V for 30min, then viewed under UV transilluminator and photographed[2].

3.8.3 Sequencing

The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050)[8].

3.8.4 Nucleotide Blast

The sequence data obtained were compared by aligning with the sequences in GenBank using the Basic Local Alignment Search Tool (BLASTN) of the National Centre for Biotechnology Information (NCBI)[9].

3.8.5 Phylogenetic Analysis

The evolutionary history was inferred using the UPGMA method. The optimal tree is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA11[10].

3.9 Determination of Aflatoxin Concentration

Aflatoxin content of the *Senecio biafrae* was measured using AgraQuant® TotalAflatoxin Assay 4/40.100 mL of 70/30 (v/v) methanol/water extraction solution was added to the fungi isolate,100µL each of the sample filtrate was transferred accordingly using a single pipette into corresponding Dilution Well containing 200 µL of Conjugate. The content of the dilution well was mixed using an 8-channel pipettor with fresh tips for each 8-well strip, by carefully pipetting it up and down 3 times, after which 100 µL of the contents from each Dilution Well was immediately transfer into a corresponding Antibody Coated Microwell stripe. This was incubated at room temperature for 15 minutes by allowing it to stand. The contents of the Micro well strips were then emptied into a waste container. The Micro well was washed filling each well with distilled water, and then pouring the water from the micro well strips. This step was done repeatedly for three times and tap dried. 100 µL of Substrate was pipetted into each Micro well strip using an 8-channel pipettor and then incubated at room temperature for 5 minutes. The stock solution (100µl) was dispensed into the antibody coated wells and the absorbance of each well was observed at 450 nm with a differential filter of 630 nm[11].

3.10 Statistical Analysis

The frequency of occurrence of the isolated fungi obtained from the four markets was calculated using SPSS for Windows version 24.0. The purified fragments were analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample. DNASTAR was used to analyze sequences generated by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI)[2].

Endnotes

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Chapter Four

Results and Discussion of Findings

4.1 Results of the Findings

4.1.1 Occurrence of Fungal Isolates from *Senecio biafrae* Samples from Four Different Markets in Ibadan Metropolis

Molecular characterization of fungi associated with *Senecio biafrae* were investigated by visiting four different retailers in four different markets. The markets are Orita merin, Mapo, Shasha, and Oje. A total of fourty-one fungal isolates consisting of thirty *Aspergillus spp,* eight *Penicillium spp* and three *Mucor spp* were isolated from the market using Potato Dextrose Agar (PDA) culture medium. Isolated fungi were subjected to macroscopic and microscopic investigation before being subjected to molecular characterization and aflatoxin analysis.

From the investigation, the six different fungal isolates obtained from the four vegetable retailers across the four markets (Orita merin, Mapo, Shasha and Oje) in Ibadan Metropolis are*: Aspergillus niger, Aspergillus brunneoviolaceus, Penicillium oxalicum, Aspergillus fumigatus, Aspergillus tamariis, Mucor irregularris.*

The results of the fungi isolated from the samples across the four markets are presented in (Table 4.1). *Aspergillus niger, Aspergillus brunneoviolaceus, Penicillium oxalicum* were isolated from the samples purchased from all the four markets. *Aspergillus fumigatus* was isolated from samples obtained from Orita merin, Shasha and Oje. *Aspergillus tamariis* was obtained from Orita merin, and Oje. *Mucor irregularris* was obtained from Shasha and Oje.

4.1 Occurrence of Fungal Isolates from *Senecio biafrae* Samples in Four different Markets in Ibadan

	Orita merin				Mapo				Shasha				Oje			
Fungal Isolates	AR1	AR2	AR3	AR4	BR1	BR2	BR3	BR4	CR1	CR2	CR3	CR4	DR1	DR2	DR3	DR4
Aspergillus. niger	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
A. brunneoviolaceus	+	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+
Penicillium oxalicum	-	-	-	+	-	-	+	+	+	+	-	-	-	+	+	-
Aspergillus fumigatus	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-
Aspergillus tamarii	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
Mucor irregularis	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-

Key note: + = present; - = absent

A (R1, R2, R3, R4) = Samples collected from Orita-merin market were replicated four times.

B (R1, R2, R3, R4) = Samples collected from Mapo market were replicated four times.

C (R1, R2, R3, R4) = Samples collected from Shasha market were replicated four times.

D (R1, R2, R3, R4) = Samples collected from Oje market were replicated four times.

Source: Laboratory Analysis, 2025.

4.1.2: Frequency and Percentage Occurrence of Fungal Isolates on *Senecio biafrae* from Four Markets in Ibadan Metropolis

The results of the percentage frequency of occurrence of the fungi isolates are presented in (Table 4.2). The samples collected from Oje market has the highest fungi isolates, (14 out of 41) of the total isolates, followed by Orita merin with (11 out of 41) of fungal isolates. Mapo and Shasha markets have the lowest isolates, 8 each out of 41 fungal isolates.

In all the four markets, *Aspergillus niger* was the most prevalent with an occurrence of (31.71%). It was followed by *Aspergillus brunneviolaceus* (26.83%). *Penicillium oxalicum* has (19.51%). *Aspergillus fumigatus, Aspergillus tamariis* and *Mucor irregularris* occurred with same percentage (7.32%). Based on location, (34.15%) of all the fungal isolates were isolated from Oje market, (26.83%) were obtained from Orita merin while the least occurrence of isolates was obtained from Mapo and Shasha, each with (19.51%) of the isolates.

4.2 Frequency (%) of Occurrence of Fungal Isolates on Senecio biafrae Samples in Four Different Markets in Ibadan Metropolis

	Percentage Frequency of Occurrence of Isolated Fungi by Location					
Fungal Isolates	Orita Merin	Mapo	Shasha	Oje	Total	
Aspergillus. niger	3	3	3	4	13	31.71%
A. brunneoviolaceus	5	2	1	3	11	26.83%
Penicillium oxalicum	1	3	2	2	8	19.51%
Aspergillus fumigatus	1	0	1	1	3	7.32%
Aspergillus tamarii	1	0	0	2	3	7.32%
Mucor irregularis	0	0	1	2	3	7.32%
Total	11(26.83)	8(19.51)	8(19.51)	14(34.15)	41(100)	100

Key note: n = 4

Source: Laboratory Analysis, 2025.

Table 4.3 Macroscopic and Microscopic Morphologies of Fungal Isolates Obtained on *Senecio biafrae* from Four Different Markets in Ibadan Metropolis

Fungal Isolates	Macroscopic view on PDA	Microscopic view
Aspergillus niger	Colonies color is black, velvety and it matured within 3 to 5 days.	The spores are spherical and the conidiophore was long and smooth.
Aspergillus brunneoviolaceus	Colonies are brown; wooly and it matured within 3 to 5 days.	The conidia are spherical and the conidiophore showed mop-like head.
Penicillium oxalicum	Colonies appeared blue-green.	The spores are ellipsoidal in shape. Repeatedly branched conidiophores of long chains.
Aspergillus fumigatus	Colonies appeared gray-green, powdery and it matured within 3 to 5 days.	The spores are spherical. Conidiophores are smooth and unbranched.
Aspergillus tamarii	Colonies are yellowish-green	The conidia are globose. Conidiophores are hyaline.
Mucor irregularis	Colonies are whitish and hairy.	The sporangia are spherical. The sporangiophores erect are branched.

Source: Laboratory Analysis, 2025

Fig 4.1: Photomicrograph of *Aspergillus niger* at 200x Mag

Source: Author’s Laboratory Work, 2025.

Fig 4.2: Photomicrograph of *Aspergillus brunneoviolaceus* at 100x Mag

Source: Author’s Laboratory Work, 2025.

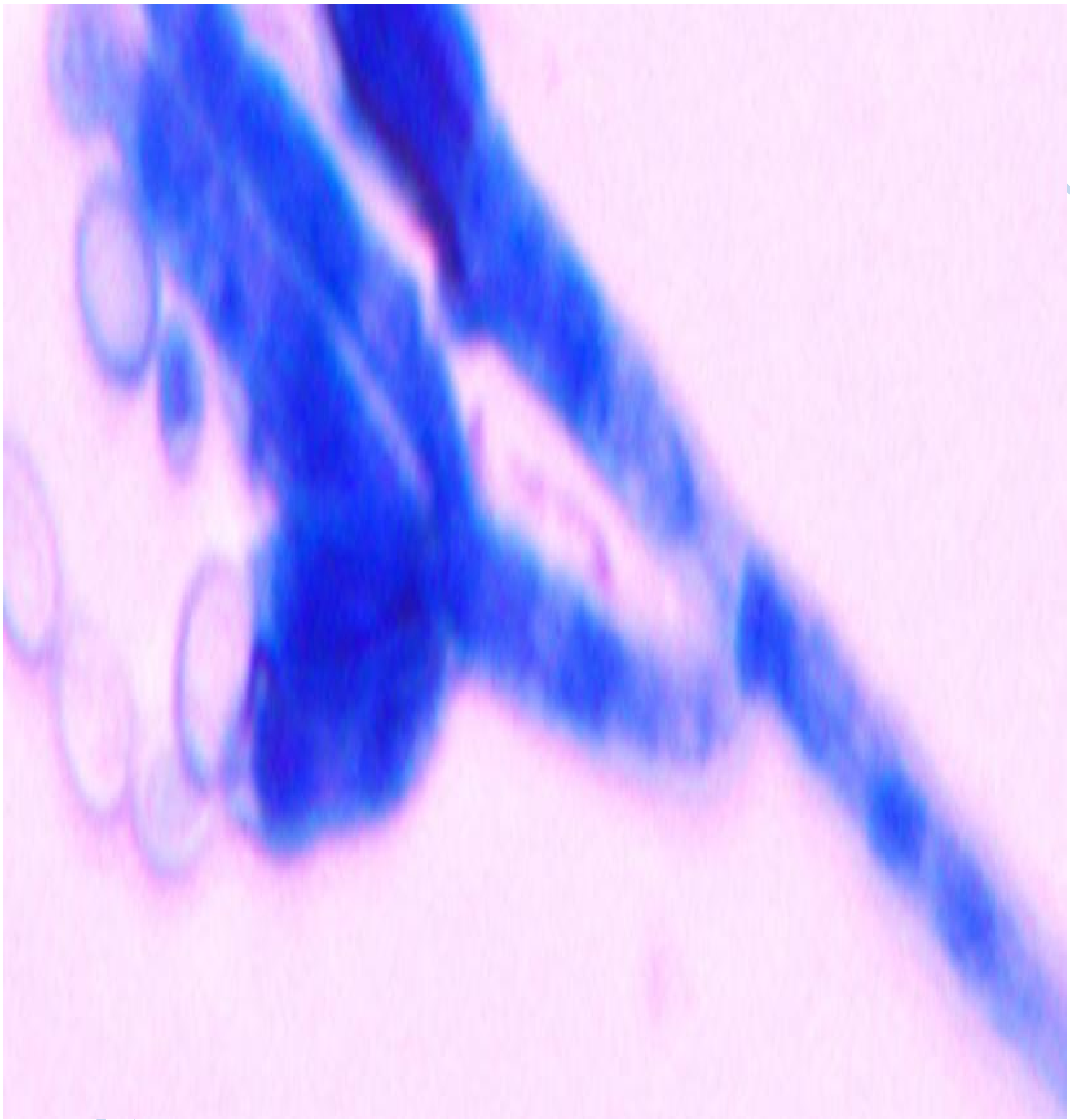

Fig 4.3: Photomicrograph of *Penicillium oxalicum* at 200x Mag

Source: Author’s Laboratory Work, 2025.

Fig 4.4: Photomicrograph of *Aspergillus fumigatus* at 400x Mag

Source: Author's Laboratory Work, 2025.

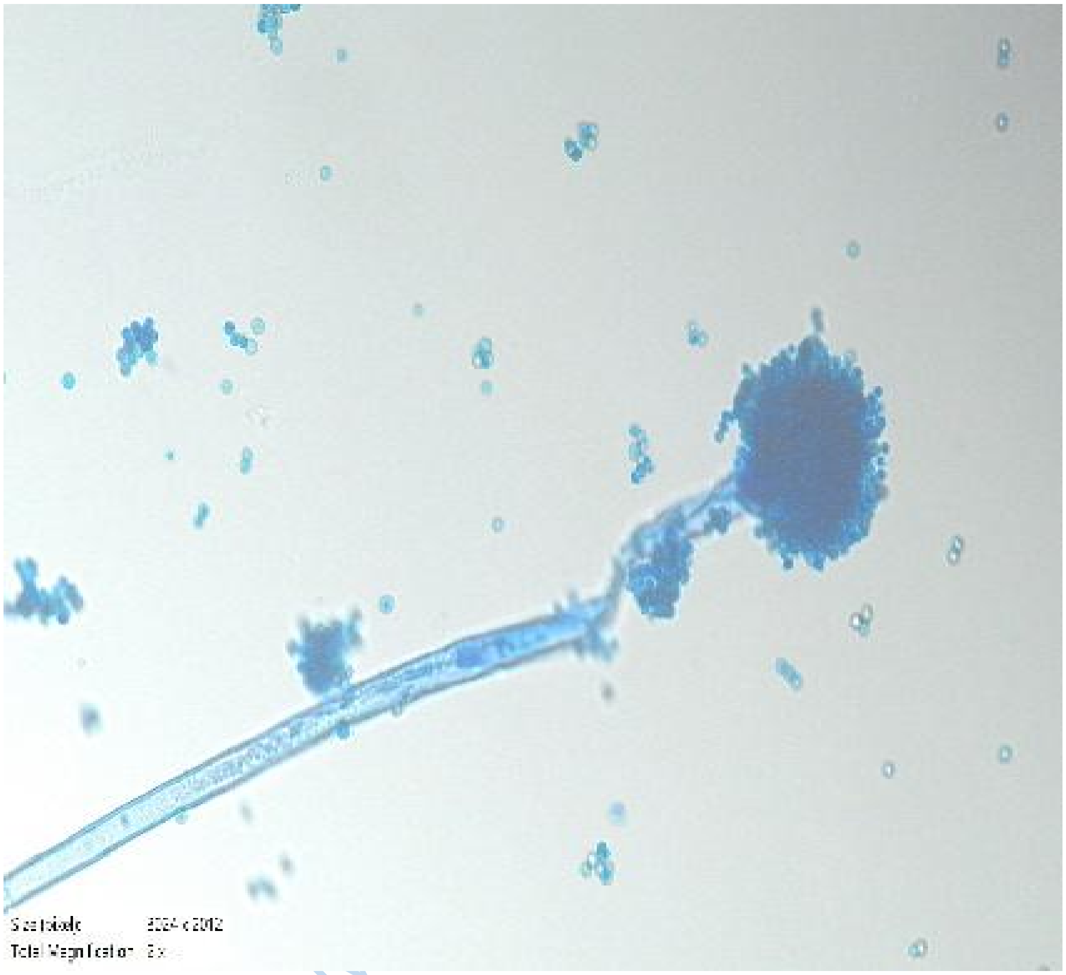

Fig 4.5: Photomicrograph of *Aspergillus tamarii* at 400x Mag

Source: Author’s Laboratory Work, 2025.

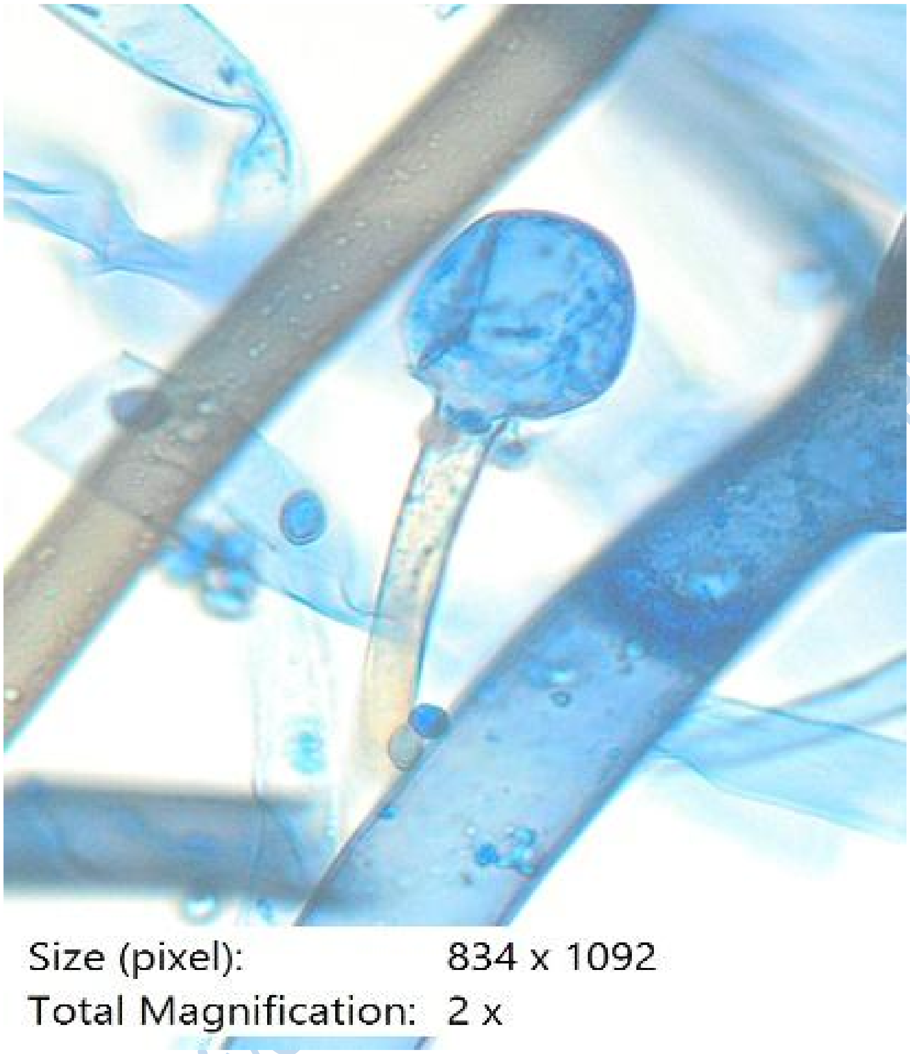

Fig 4.6: Photomicrograph of *Mucor irregularis* at 400x Mag

Source: Author’s Laboratory Work, 2025.

4.1.3 Molecular Characterization of the Fungi Isolated on *Senecio biafrae* Obtained from Four Markets in Ibadan Metropolis

From the molecular analysis results obtained, (Fig 4.7) showed the DNA extraction and PCR amplification of the internal transcribed spacer region of the fungal isolates. The DNA molecular weight is relatively high. No contamination was confirmed and the PCR amplification was successful. (Table 4.4) showed fungal species identified from each isolate obtained from the four different market using sequence analysis; *Aspergillus niger, Aspergillus brunneoviolaceus, Penicillium oxalicum, Aspergillus fumigatus, Aspergillus tamariis, Mucor irregularris.* Sequence length of *Mucor irregularris* was the longest (636%), followed by *Penicillium oxalicum* (592%) and the least was *Aspergillus brunneoviolaceus* with (534%) in (Table 4.4). The six (6) fungi were identified, designated, and assigned accession numbers as *Aspergillus fumigatus* (PP556163.1)*, Aspergillus tamariis* (PV926753.1)*, Aspergillus brunneoviolaceus* (PP967376.1)*, Aspergillus niger* (MG659662.1)*, Penicillium oxalicum* (OQ550968.1) and *Mucor irregularris* (OP237503.1) respectively in Table 4.5. Also, Identity value of the isolates ranged from (99.69%) to (100%) and the all isolates had 100% query coverage (Table 4.5).

Figure 4.7: Photographic Image of an Agarose Gel Indicating the Amplification of the ITS Target Region.

Source: Laboratory Analysis, 2025.

Table 4.4 Sequence Prediction Analysis for the Fungi Isolated from the four Markets in Ibadan Metropolis

S/no	Isolates ID	Identified organisms	Sequence length (%)
1	CR33 B	*Aspergillus fumigatus*	562
2	DR31 B	*Aspergillus tamarii*	573
3	AR11 B	*A. brunneoviolaceus*	534
4	AR12 A	*Aspergillus niger*	561
5	BR33 A	*Penicillium oxalicum*	592
6	CR32 B	*Mucor irregularis*	636

Source: Laboratory Analysis, 2025.

Plate 4.1: Pure culture of predicted organism (*Aspergillus fumigatus)* on PDA obtained from group C (Shasha market)

Sequence of *Aspergillus fumigatus*

CGAGGTAGGCTCTGGGTCACCTCCCACCCGTGTCTATCGTACCTTGTTGCTTCGGCG

GGCCCGCCGTTTCGACGGCCGCCGGGGAGGCCTTGCGCCCCCGGGCCCGCGCCCGC

CGAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTATCGTA

ATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAG

CGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAAC

GCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCC

CTCAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGA

AAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTC

TGTAGGCCCGGCCGGCGCCAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCG

GATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

Source: Laboratory Analysis,2025.

Plate 4.2: Pure culture of predicted organism (*Aspergillus tamarii)* on PDA obtained from group D (Oje market).

Sequence of *Aspergillus tamarii*

GATCATTAGAATTTAGGGTTCTAGCGAGCCCACCTCCCACCCGTGTTTACTGTAACC
TTAGTTGCTTCGGCGGGCCCGCCTTTAAGGCCGCCGGGGGGCATCAGCCCCCGGGCC
CGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTG
TATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAG
AACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGT
CTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCA
TTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTTCGGGGGGA
CGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTG
TCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAAACAACCATTCTTTCC
AGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCG
GAGGAA

Source: Laboratory Analysis,2025.

Table 4.3: Pure culture of predicted organism (*A. brunneoviolaceus)* on PDA obtained from group A (Orita merin market).

Sequence of *A. brunneoviolaceus*

GCCGGGTCTTCGGGGCCAACCTCCCACCCGTGCTTACCGTCCTGTTGTTCGGGGGCC

CGCCTTCGGGCGGCCCGGGGCCTGCCCCCGGGACCGCGCCCGCCGGAGACCCCAAT

GGAACACTGTCTGAAAGCGTGCAGTCTGAGTCGATTGATACCAATCAGTCAAAACTT

TCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAA

CTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCC

CCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCTCCCCTCCAGCCCCGCT

GGTTGTTGGGCCGCGCCCCCCCGGGGGCGGGCCTCGAGAGAAACGGCGGCACCGTC

CGGTCCTCGAGCGTATGGGGCTCTGTCACCCGCTCTATGGGCCCGGCCGGGGCTTGC

CTCGACCCCCAATCTTCTCAGATTGACCTCGGATCAGGTAGGGATACCCGCTGAACT

TAAGCATATCAATAAGCGGAGGAA

Source: Laboratory Analysis,2025.

Plate 4.4: Pure culture of predicted organism (*Aspergillus niger)* on PDA obtained from group A (Orita merin).

Sequence of *Aspergillus niger*

CGGGGTCTTTGGGCAACCTCCCATCCGTGTCTATTGTACCCTGTTGCTTCGGCGGGCC
CGCCGCTTGTCGGCCGCCGGGGGGGCGCCTCTGCCCCCCGGGCCCGTGCCCGCCGG
AGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAAT
CAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCG
AAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGC
ACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCT
CAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCCGAAA
GGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTG
TAGGATTGGCCGGCGCCTGCCGACGTTTTCCAACCATTCTTTCCAGGTTGACCTCGG
ATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

Source: Laboratory Analysis,2025.

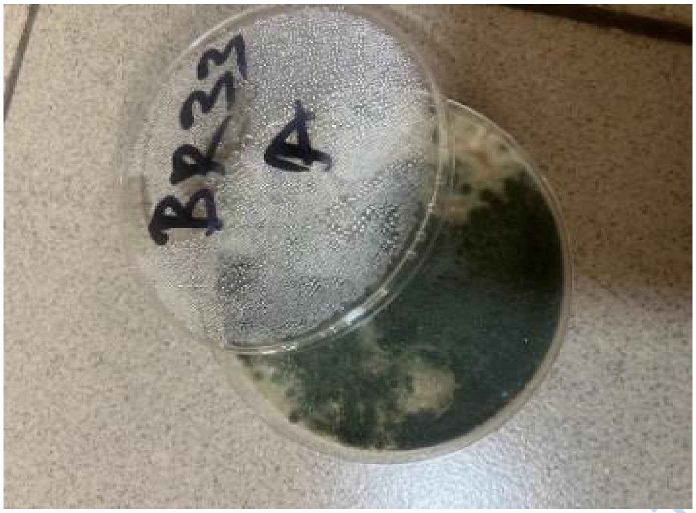

Plate 4. 5**:** Pure culture of predicted organism (*Penicillium oxalicum)* on PDA obtained from group B (Mapo market).

Sequence of *Penicillium oxalicum*

TTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAAC
CTCCCACCCGTGTTTATCGTACCTTGTTGCTTCGGCGGGCCCGCCTCACGGCCGCCG
GGGGGCATCCGCCCCCGGGCCCGCGCCCGCCGAAGACACACAAACGAACTCTTGTC
TGAAGATTGCAGTCTGAGTACTTGACTAAATCAGTTAAAACTTTCAACAACGGATCT
CTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGG
GGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCTC
TCGCCCCCCGGAAGCGGGGGCGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGT
CCTCGAGCGTATGGGGCTTCGTCACCCGCTCTGTAGGCCCGGCCGGCGCCCGCCGGC
GAACACCATCAATCTTAACCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAA
CTTAAGCATATCAATAAGCGGAGGAA

Source: Laboratory Analysis,2025.

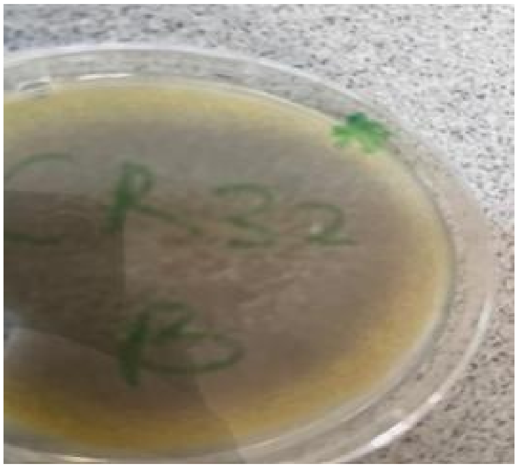

Plate 4.6: Pure culture of predicted organism (*Mucor irregularis)* on PDA obtained from group C (Shasha market).

Sequence of *Mucor irregularis*

TTCCGTAGGTGAACCTGCGGAAGGATCATTAAATAATTTGAGTATGTTTATGCATAT
TTCTAATTTACTGTGAACTGTTTTACTGTTTAGCGTTTTGAGGGATTGCCTAAAGATT
ATAGGGATAGGTCTTCAGGATGTTAACCTAGCTAAAGTCAGGCTTAGGCCTGGTATC
CTAATTCATTATTTACCAAAAGAATTCAGAATTAATATTGTAACATAAGCGTAAAAA
ACTTATAAAACAACTTTTAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGT
AGCAAAGTGCGATAACTAGTGTGAATTGCATATTCAGTGAATCATCGAGTCTTTGAA
CGCATCTTGCGCTCAATGGTATTCCATTGAGCACGCCTGTTTCAGTATCAACAACAA
CCCACATCCACAATTTTGTTGTGAATGGAAGTGAGAGTATCGATGTAAAAATTGAAC
TCTTTAAAACTATTAGGCCTGAACTATTGTTCTATTAGCCTGAACATTTTTTTAATAT
AAAGGAATGCTCTAGTTAAAAGACTATCTTGGGGGCCTCCCAAATAAATCATTTTTT
TAAACTTGATCTGAAATCAGGTGGGATTACCCGCTGAACTTAAGCATATCAATAAGC
GGAGGAA

Source: Laboratory Analysis,2025.

Table 4.5 BLAST Results of the Fungal Isolates in NCBI

S/no	Isolates ID	Identified organisms	Identity (%)	NCBI accession no	E-value	Highest query coverage (%)
1	CR33 B	*Aspergillus fumigatus*	100.00%	PP556163.1	0	100%
2	DR31 B	*Aspergillus tamarii*	98.96%	PV926753.1	0	100%
3	AR11 B	*A. brunneoviolaceus*	99.81%	PP967376.1	0	100%
4	AR12 A	*Aspergillus niger*	100.00%	MG659662.1	0	100%
5	BR33 A	*Penicillium oxalicum*	100.00%	OQ550968.1	0	100%
6	CR32 B	*Mucor irregularis*	99.69%	OP237503.1	0	100%

Source: Laboratory Analysis, 2025.

4.1.3.1 Phylogenetic Result

This analysis involved 6 samples and 12 reference nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1195 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.The phylogenetic tree in (Fig 4.8) showed the relationship within the fungi isolates obtained from four different markets in Ibadan metropolis. *Aspergillus fumigatus* (CR33) *and Penicillium oxalicum* (BR33) showed the highest level of similarity and cluster very closely, indicating potential common origin or highly similar microbial composition. *Aspergillus tamarii (DR31) and Aspergillus* niger (AR12) are closely related and group beneath the (CR33 & BR33) cluster, but are slightly more divergent. *Aspergillus brunneoviolaceus* (AR11) is more genetically distinct compared to the top clusters and branches off separately and *Mucor irregularis* (CR32) form a close cluster at the lower end of the tree. In (Fig 4.9), CR33 B clustered tightly with two identical entries of *Aspergillus fumigatus* (PP556163.1). AR12 grouped with *Aspergillus welwitschiae* (MK841443.1), *Aspergillus niger* (MG659662.1) and PV926753.1 *Aspergillus tamarii.* DR31was close to this group but slightly more divergent, suggesting they are still within *Aspergillus* clade but possibly different strains or species. AR11 grouped near *Penicillium oxalicum* (OQ550968.1), *Aspergillus brunneoviolaceus* (PP967376.1) *and Aspergillus aculeatus* (PQ788767.1). CR32 cluster closely with *Rhizomucor variabilis* (KJ862065.1) and *Mucor irregularis* (OP237503.1). The two entries of *Ganoderma lucidum* (KM375927.1) form a separate, distinct clade.

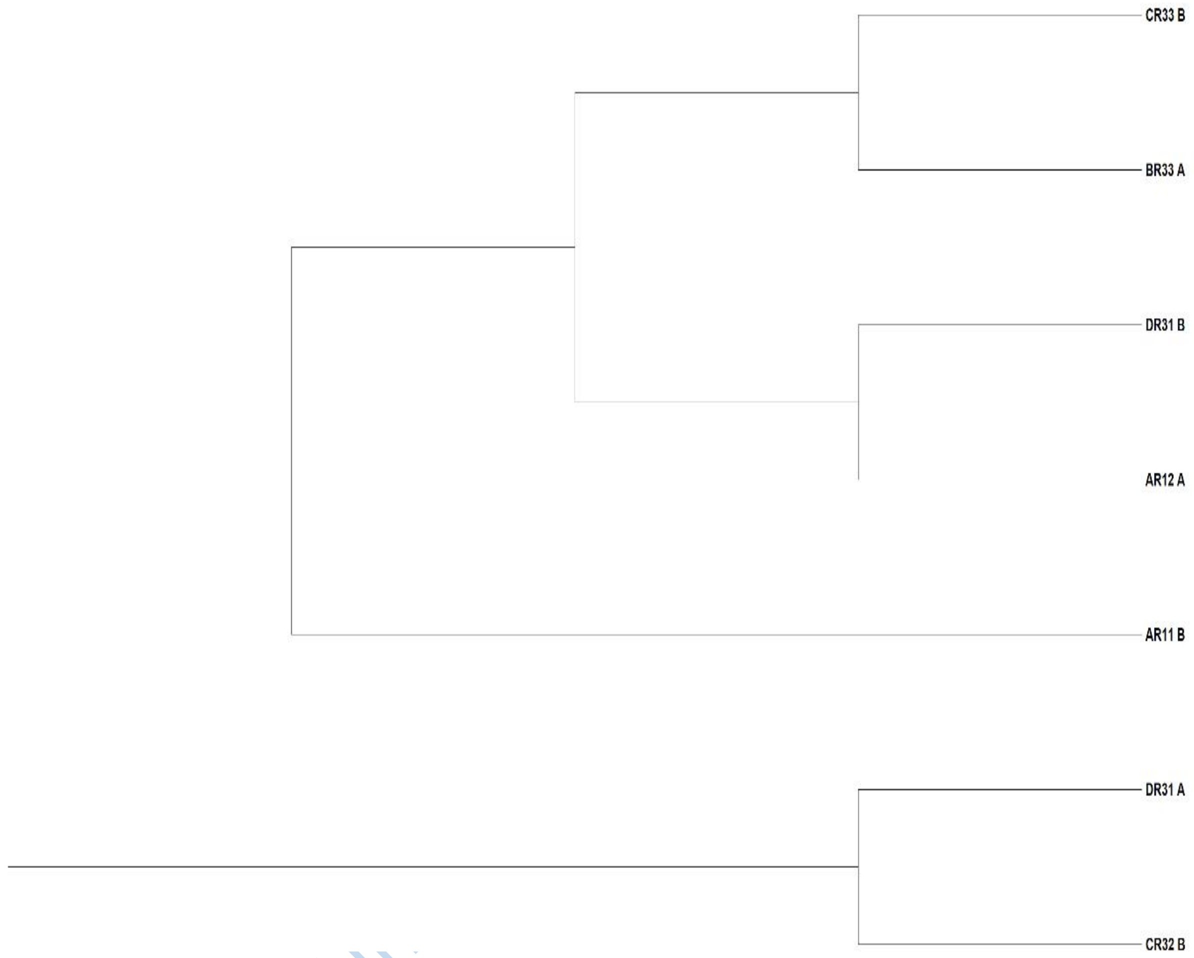

Fig 4.8: Phylogenetic Tree Within Samples.

Source: Laboratory Analysis, 2025.

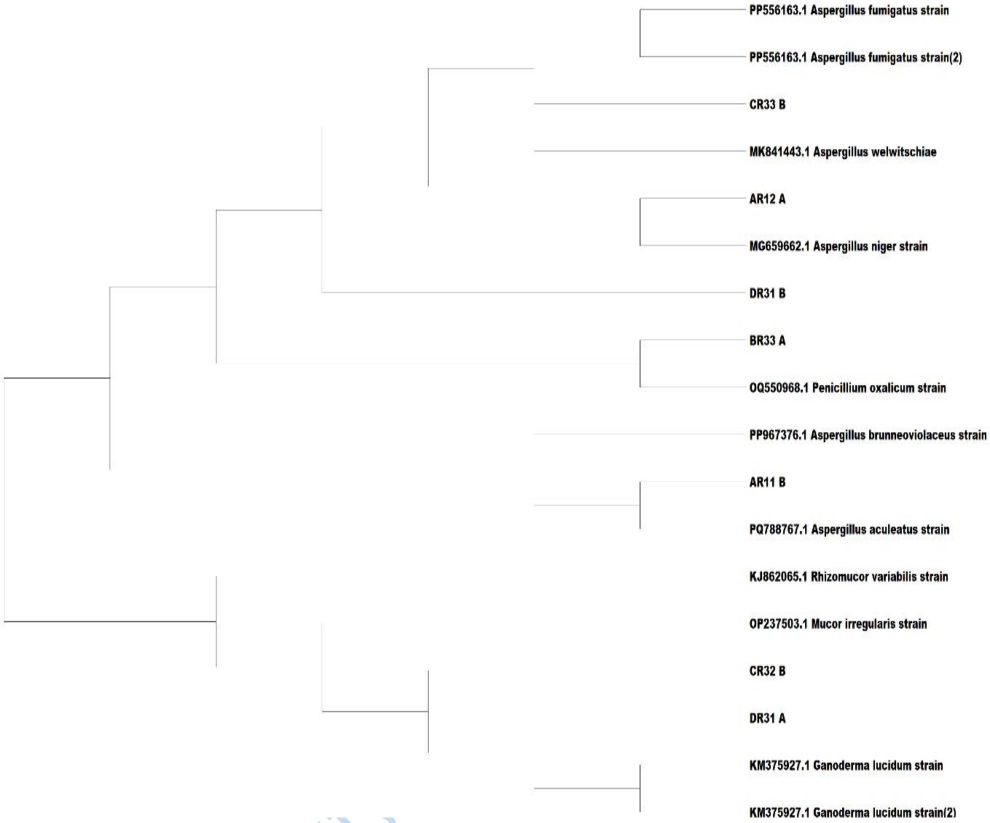

Fig 4.9: Phylogenetic Tree of Samples Using Maximum Composite Likelihood method.

Source: Laboratory Analysis, 2025.

4.1.4 Pathogenicity Result

Symptoms of fungal infection on *Senecio biafrae* leaves following inoculation with the six fungal isolates obtained from the four different markets in Ibadan metropolis was shown in (Fig 4.10). *Aspergillus niger* showed the highest pathogenicity, with large dark necrotic patches covering a significant portion of the leaf. *Aspergillus fumigatus* shows moderate necrosis. *Aspergillus brunneoviolaceus* shows moderate pathogenicity. *Penicillium oxalicum* shows mild symptoms, *Mucor irregularis* also show mild symptoms, presenting localized soft rot symptoms with mild tissue breakdown. *Aspergillus tamarii* has very minimal visible symptoms, weakly pathogenic.

Pure cultures of fungal isolates re-isolated from symptomatic *Senecio biafrae* leaves following pathogenicity testing confirmed that the isolated fungi exhibit similar morphological similarity to the original inoculum in (Fig 4.11). No symptoms were observed on the healthy Senecio biafrae leaves used as control in (Plate 4.7).

4.1.5 Aflatoxin Content Result

The results of the Aflatoxin content from the *Aspergillus* spp. across the four in Ibadan metropolis were presented in (Table 4.5). The aflatoxin concentration of the samples ranged from 0.2-6.8ppb. The sample from Oje market had the highest aflatoxin content (6.8ppb), followed by sample from Orita merin market (2.9ppb). Samples from Shasha has (0.35ppb) and the sample with the least aflatoxin content (0.2ppb) is from Mapo Market.

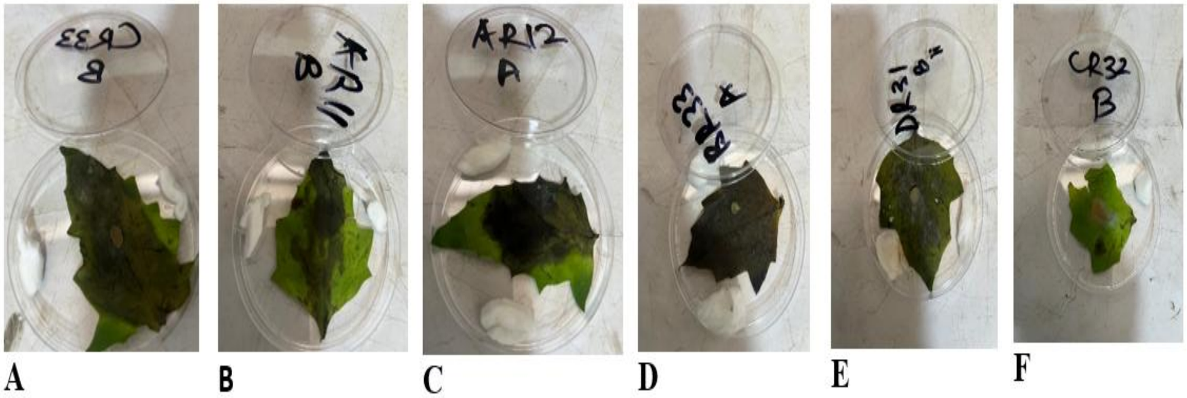

Fig 4.10: Symptomatic *Senecio biafrae* leaves following pathogenicity testing. (A-F represent; *Aspergillus fumigatus*, *A. brunneoviolaceus*, *A. niger*, *Penicillium oxalicum*, *A. tamarii*, and *Mucor irregularis*)

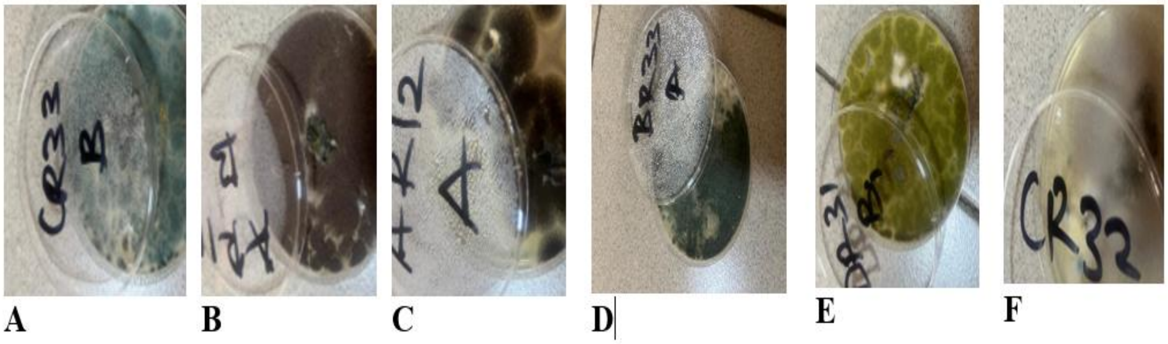

Fig 4.11: Pure cultures of fungal isolates re-isolated from symptomatic *Senecio biafrae* leaves following pathogenicity testing.

Source: Laboratory Analysis, 2025.

Plate 4.7: Controlled experiment for pathogenicity testing.

Source: Laboratory Analysis,2025.

Table 4.6 Total Aflatoxin Concentration of the Samples from Four Markets in Ibadan Metropolis

Sample	Total Aflatoxin Concentration(ppb)
Sample D (Oje Market)	6.8
Sample C (Shasha)	0.35
Sample B (Mapo Market)	0.2
Sample A (Orita merin Market)	2.9
Sample D (Oje market)	0.4

Source: Laboratory analysis,2025.

4.2 Discussion of Findings

This study revealed six fungal isolates associated with *Senecio biafrae* (Wororo) conducted in Orita Merin, Mapo, Shasha, and Oje, four different market concentrated with vegetable retailers in Ibadan metropolis. These fungi include*: Aspergillus niger, Aspergillus brunneoviolaceus, Penicillium oxalicum, Aspergillus fumigatus, Aspergillus tamarii,* and *Mucor irregularis.* This is in agreement with the findings of a researcher who reported *Aspergillus niger, Aspergillus fumigatus, Penicillium spp and Mucor spp* among the fungi isolated from *Abelmoschus esculentus* sold in Abuja[1]. Fungi species isolated in this study are undesirable in food such as vegetables because they are capable of producing mycotoxins which are harmful to both animal and human's health. *Aspergillus niger* was found to be present, it's one of the aflatoxins - producing fungi whose presence in food would constitute health hazards to consumers. *Aspergillus. fumigatus* is thermophilic nature, with a minimum growth temperature of 12.8 °C and a maximum between 40 °C and 42.8 °C; it is considered xerophile due to its minimum growth of 0.82 of water activity[2]. In the post-harvest phase of food production, *A. fumigatus* contamination can occur both during crop growth in the field and storage processes and can be related to surrounding fields or cross-contamination[2].

Aspergillus niger had highest frequency of occurrence (31.71%), aligning with its well-documented prevalence in food and environmental samples due to its adaptability, and prolific spore production. The result corresponds to the findings of a researcher who reported similar fungal isolate with highest frequency of occurrence from selected vegetables sold in Akure Nigeria[3].

Penicillium oxalicum, *A. brunneoviolaceus* and *A.niger* were present across market investigated. Variation in the occurrence of the fungal species across the markets could be explained by variations in handling, storage conditions, and market-specific environmental elements such as humidity. *Aspergillus niger* occurrence in all the markets is in accordance with the study conducted by a researcher who reported isolation of *Aspergillus niger* across all markets in dried Okra and Tomatoes commercialized in Ibadan metropolis[4]. The occurrence of these fungi indicated that they could grow and survive in *Senecio biafrae* which is probably due to the fact that these perishable vegetables contain some nutrients suitable for fungal growth.

Molecular identification and characterization through DNA sequencing validated the morphological identifications of the isolates. The sequences obtained from the fungal isolates were blasted against known sequences on National Centre for Biotechnology Information (NCBI) database and the organisms were identified[5]. The Identified fungi were *Aspergillus fumigatus,Penicillium oxalicum, Aspergillus tamarii, Aspergillus* niger, *Aspergillus brunneoviolaceus*, *Mucor irregularis*. Accession number was assigned to each organism and taxonomic identity were confirmed. DNA sequencing of the internal transcribed spacer (ITS) and large subunit (LSU) regions of rRNA, followed by comparative sequence analysis is the 'gold standard' for molecular identification of most fungi because it is fast, accurate and reliable [6].

The evolutionary relationships among the isolates illustrated by phylogenetic tree revealed five (5) genera of fungi (*Aspergillus, Penicillium, Rhizomucor, Mucor* and *Ganoderma*). Members of the genus *Aspergillus* grouped closely, indicating their genetic relatedness and shared origins. *Mucor irregularis* appeared more distantly related, hence, classified under a distinct fungal order (Mucorales). The *Mucor* spp. are responsible for the majority of human sickness such as, hemoptysis, shortness of breath, vision loss etc[7]. The most common genera isolated was

Aspergillus with four different species namely *Aspergillus niger, Aspergillus tamarii, Aspergillus fumigatus, Aspergillus brunneoviolaceus* which is similar to a research where similar *Aspergillus* species except *Aspergillus fumigatus* were reported[8]. *Aspergillus niger*, a species of the *Aspergillus* genus is multipurpose fungus that may have an impact on food spoiling as well as industrial uses. *A.niger* produces Aflatoxin, highly toxic and carcinogenic mycotoxins. *Penicillium* species are known for their role in antibiotic production and food processing. Mycotoxin which contaminates food products, highly toxic and carcinogenic are produced by *Penicillium spp*.

The genera *Aspergillus, Penicillium and Mucor* are often associated with vegetables as they cause significant damage to the yield, reduce its quality and shelf-life[1.] These genera are extensively found in a wide range of environments and substrates, such as food and soil. *Aspergillus* and other molds have the ability to be poisonous, produce allergic reactions, and cause infections, all of which can have a detrimental effect on human health[8].

The fungal isolates obtained in this study are able to reproduce disease on healthy vegetable samples. The ability of these fungal isolates to exploit the nutrients in *Senecio biafrae* as a substrate for growth and development allowed them to infect healthy samples. Most likely, the fungi or their resistant spores came from the farms where the vegetables were gathered, while some may have spread from the stores as a result of horizontal contamination from the already-spoiled produce. It was noted that the majority of spoilage organisms might be found on farm-grown vegetables during harvesting, which could lead to postharvest contamination and spoilage of these vegetables. Furthermore, because of their low pH, high moisture content, and nutritional makeup, they are particularly vulnerable to pathogenic fungus, which not only cause rots but also render them unsafe for human consumption by generating mycotoxins[9].

Food and vegetable contamination by mycotoxin is a growing global concern that has led to major outbreaks that have impacted both human and animal health and generated economic losses for many countries. It was estimated that mycotoxin-producing fungi contaminate 25% of the world's food crops, resulting in massive losses in agricultural products commerce both domestically and internationally worth billions of dollars. Exposure or direct consumption of Aflatoxin contaminated agricultural produce can lead to cancers, immune system defects, growth retardations, liver diseases and death[10].

This study revealed that *Aspergillus spp* from all the markets tested positive for Aflatoxin. *Senecio biafrae* vegetable from Oje market have the highest total aflatoxin concentration (6.8ppb) which is below the maximum acceptable limit of 10 µg/kg set by NAFDAC. This finding is in contrast to the report which stated high Aflatoxin contamination above the maximum acceptable limit of 10 µg/kg on vegetables. Onion, cabbage, amaranthus, tomato, and cucumber were among the vegetable samples that have been reported with high levels of total aflatoxins (25 µg/kg) beyond the NAFDAC-established maximum permissible limit of 10 µg/kg[11].

The *Aspergillus spp.* is associated with the production of mycotoxins such as Aflatoxins which has been implicated in hepatotoxicity and hepatocellular carcinoma[12].Aflatoxin contamination of *Senecio biafrae* in this study might have occurred right from the farm; during agricultural practices which might increase the moisture content of the vegetable, during harvesting or in the market due to poor hygienic conditions. In open markets, especially in Oje market, vegetables were put on bare ground or dirty containers. The vegetables could easily get contaminated due to proximity to other farm products that contain aflatoxigenic fungi such as *Aspergillus flavus* which was not isolated in this study but commonly associated with aflatoxin production, and storage conditions[13].

Senecio biafrae is one of the African leafy vegetables that contain high moisture content which creates favorable environment for mycotoxin-producing fungi, but drying the vegetable under hygienic and right temperature have been reported not to only extend the shelf-life but to also enhances food safety by lowering the chances of aflatoxin accumulation[13].

Endnotes

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Chapter Five

Conclusion

5.1 Summary of the Findings

Senecio biafrae is one of the indigenous green leafy vegetables, notable for its promising nutritional and economic potentials. The vegetable is called "Wororo" in Ibadan and it grows in large number as undercover in tree crop plantation, majorly in cocoa and kola nut plantations in South-western part of Nigeria. The vegetable is equally known for its therapeutic virtues, notably in Nigeria where it is used in the treatment of diabetes or pulmonary defects, cough, rheumatism, hypertension etc. As a perennial climbing plant, *Senecio biafrae* has the potential to become easily contaminated because its proximity to other agricultural products that contain aflatoxigenic fungi, like *Aspergillus spp.*, which are frequently linked to the synthesis of aflatoxin.

The findings of this study showed that the following fungi *Aspergillus fumigatus, Penicillium oxalicum, Aspergillus tamarii, Aspergillus niger*, *Aspergillus brunneoviolaceus, Mucor irregularis* were associated with *Senecio biafrae* samples obtained from the four selected markets in Ibadan. *Aspergillus niger* had the highest frequency of occurrence among these isolates and was present in every market that was investigated, suggesting that it was widely distributed and dominates the local vegetable microbiome.

Molecular characterization was performed using the Internal Transcribed Spacer (ITS) region of fungal DNA, to accurately identify these fungal species. The ITS sequences that were acquired were compared using BLAST analysis to known sequences in the NCBI GenBank database. In addition to ensuring accurate taxonomic identification, this molecular method made it possible to provide accession numbers to the isolates for data tracking and phylogenetic tree revealed the evolutionary relationship among these isolates.

Pathogenicity tests revealed that all isolated fungi were able to replicate disease symptoms when inoculated onto healthy *Senecio biafrae s*amples, confirming their potential as plant pathogens or spoilage organisms. This has significant implications for food safety, as these fungi may not only reduce yield, its quality and shelf-life of the vegetables but also pose health risks if consumed.

Furthermore, the presence of aflatoxins, secondary metabolites produced by some *Aspergillus spp* were investigated in this study. Aflatoxin analysis was conducted specifically on the *Aspergillus* isolates from all four markets. Vegetable samples from Oje Market had the highest aflatoxin concentration of any market, measuring (6.8ppb). Despite being below the 10ppb maximum allowable limit established by Nigeria's National Agency for Food and Drug Administration and Control (NAFDAC), the cumulative effects of prolonged exposure make its presence concerning.

5.2 Conclusion

This research work showed that the *Senecio biafrae* purchased from the four markets in Ibadan Metropolis contains different fungi species, capable of contaminating the vegetable. The most prominent among these fungal isolates as shown with the results of this study were *Aspergillus spp*. Application of molecular techniques such as ITS sequencing made the identification and characterization of the fungus species present possible. While the aflatoxin levels found do not currently exceed safety limits, the detection of these toxins indicates that consumption of contaminated *Senecio biafrae* can cause health hazard.

5.3 Recommendations

i. Strict hygienic rules should be enforced by regulatory agencies in local markets, particularly with regard to the handling and presentation of fresh food. Regular health inspections, and sanitation upgrades should be given priority in markets with greater levels of pollution.

ii. It is necessary to educate vegetable farmers, sellers, and consumers about the dangers of aflatoxins and fungal contamination. To reduce health hazards, awareness campaigns can encourage safe handling, appropriate vegetable preparation, and hygienic storage practices.

iii. Farmers should be trained on how to explore safe, natural antifungal treatments that can be applied during storage or post-harvest to limit fungal growth and minimize aflatoxin risk.

iv. Agricultural agencies should enforce the use of molecular methods to accurately identify and monitor fungal species affecting *Senecio biafrae.*

5.4 Contribution to Knowledge

The data generated from this study offers essential insights into the use of molecular techniques in characterizing fungal species associated with *Senecio biafrae* and identifying the fungal species capable of deteriorating and contaminating the vegetable with Aflatoxin. Also, these data will enable targeted treatment plans, increase production, control postharvest losses, and evaluate possible food safety hazards related to eating contaminated vegetables.

5.5 Area of Further Research

Research should focus on comprehensive risk assessment and aflatoxin accumulation under various storage and environmental condition in relation to temperature, humidity, and storage duration. Also, selection of appropriate biocontrol measure and developing fungal resistant varieties of *Senecio biafrae* should be area of research concentration.

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Appendices

Appendix I

Collection of samples (*Senecio biafrae)* from Orita merin market

Source: Author's Field Work,2025.

Appendix II

Collection of samples (*Senecio biafrae)* from Oje market

Source: Author's Field Work,2025.

Appendix III

Collection of samples (*Senecio biafrae)* from Mapo market

Source: Author's Field Work,2025.

Appendix IV

Collection of samples (*Senecio biafrae*) from Shasha market

Source: Author's Field Work,2025.

Appendix V

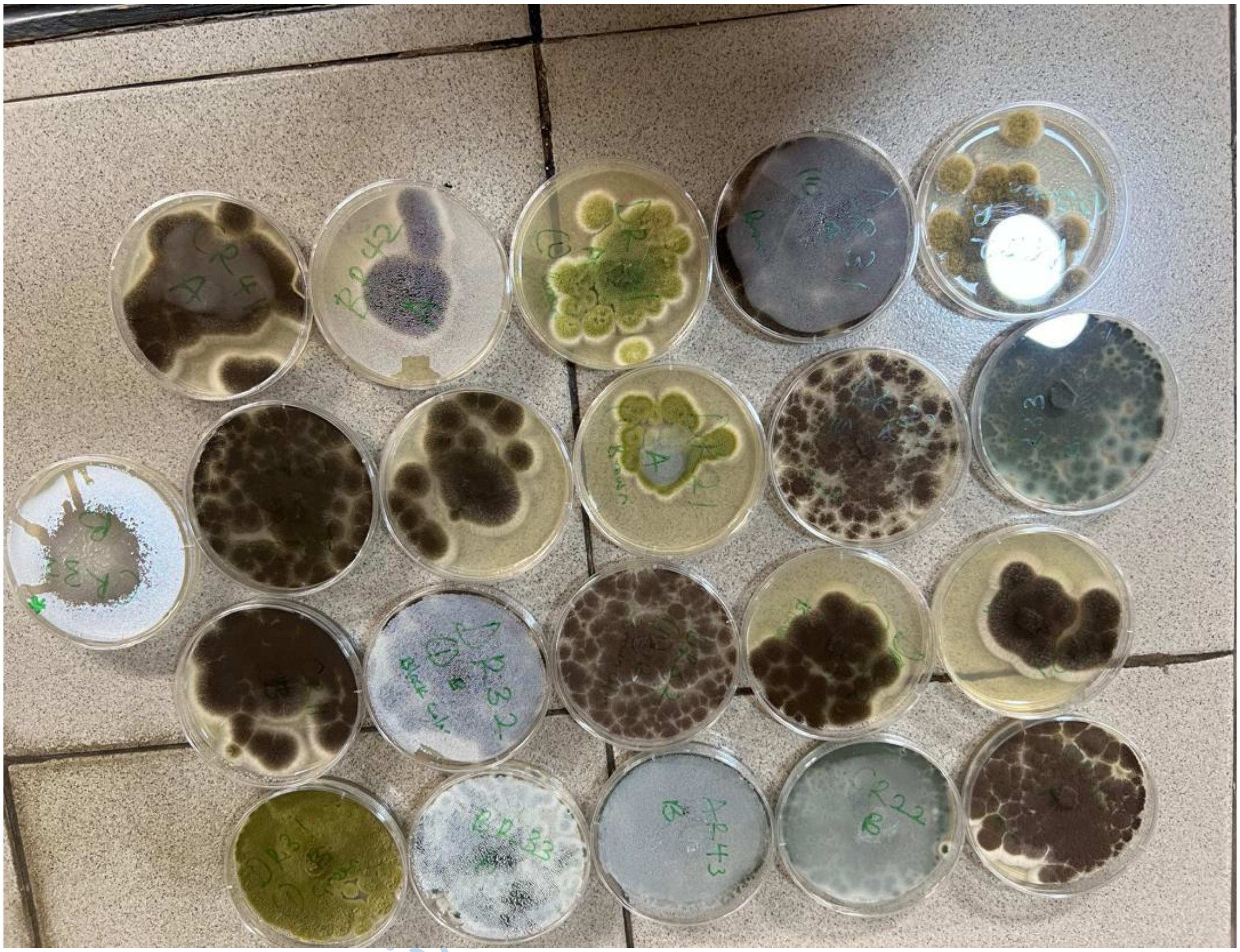
Culture media (PDA) preparation

Source: Author’s Laboratory Work,2025.

Appendix VI

Direct plating of the vegetable samples on the culture media (PDA)

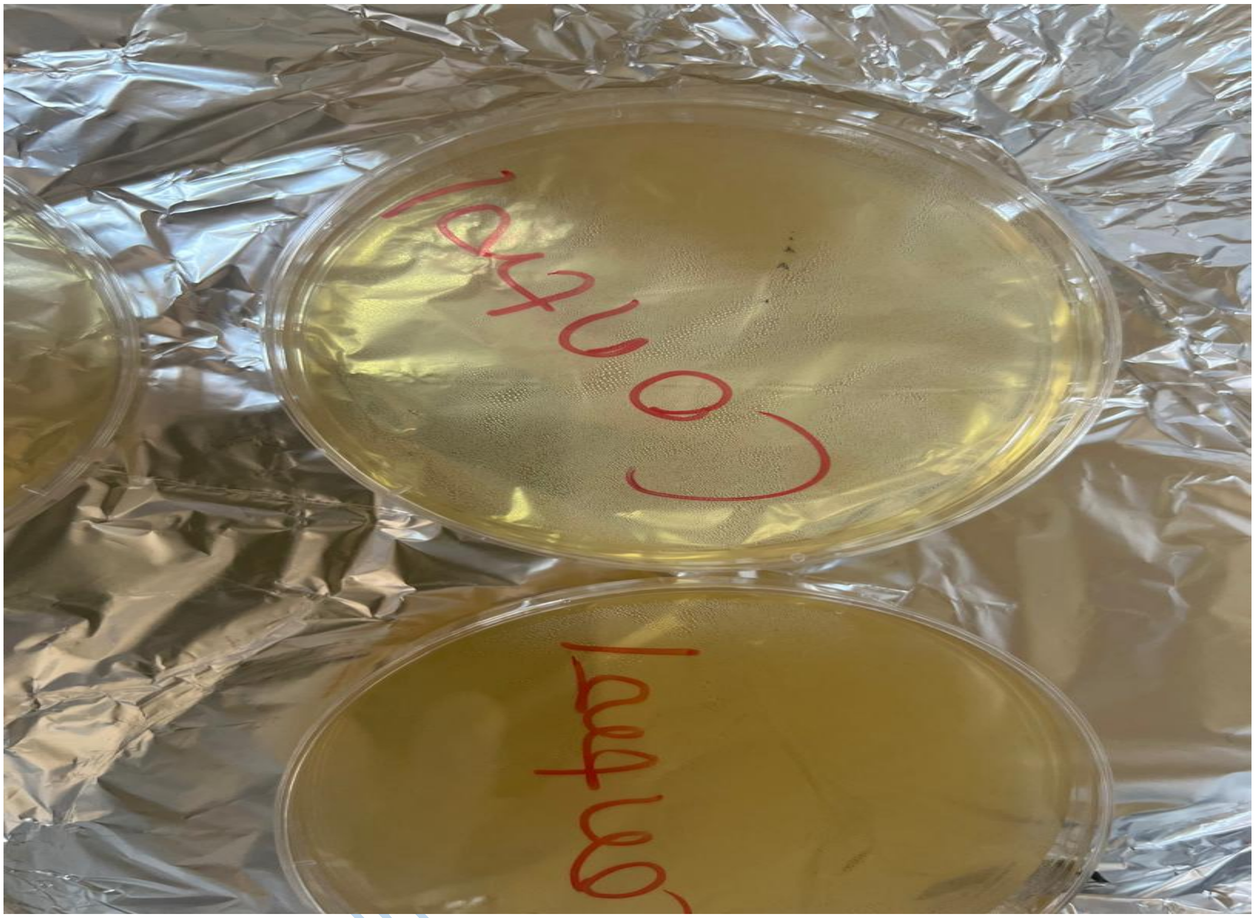
Appendix VII



Fungal Isolates obtained on *Senecio biafrae*

Source: Source: Author's Laboratory work,2025.

Appendix VIII



Culture media used as Control experiment

Source: Author's Laboratory work,2025.

Appendix IX

Fungal Isolate kept McCartney bottles in a slanted position.

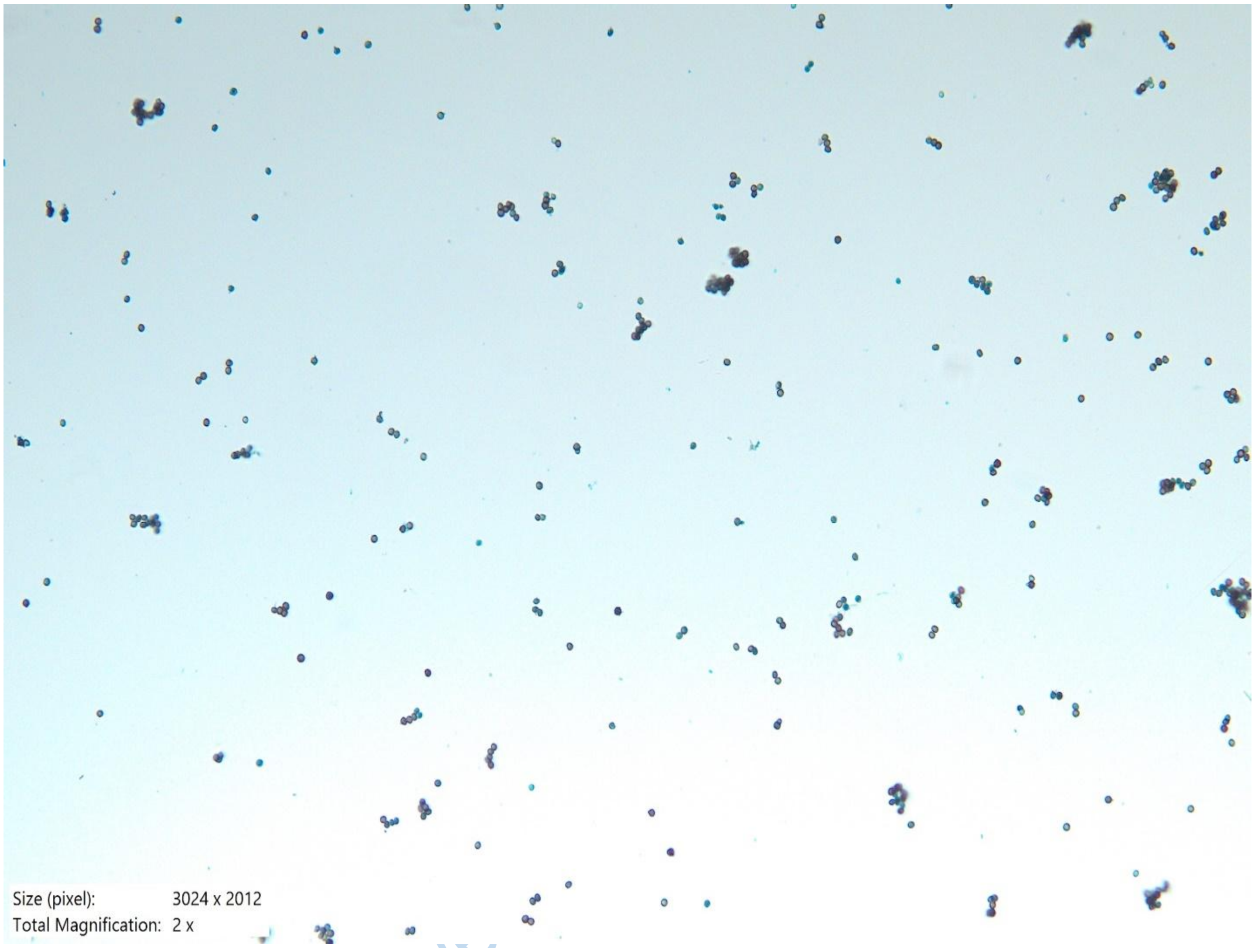
Source: Author's Laboratory Work,2025.

Appendix X

Microscopic Identification of the Fungal Isolates

Source: Author Laboratory Work,2025.

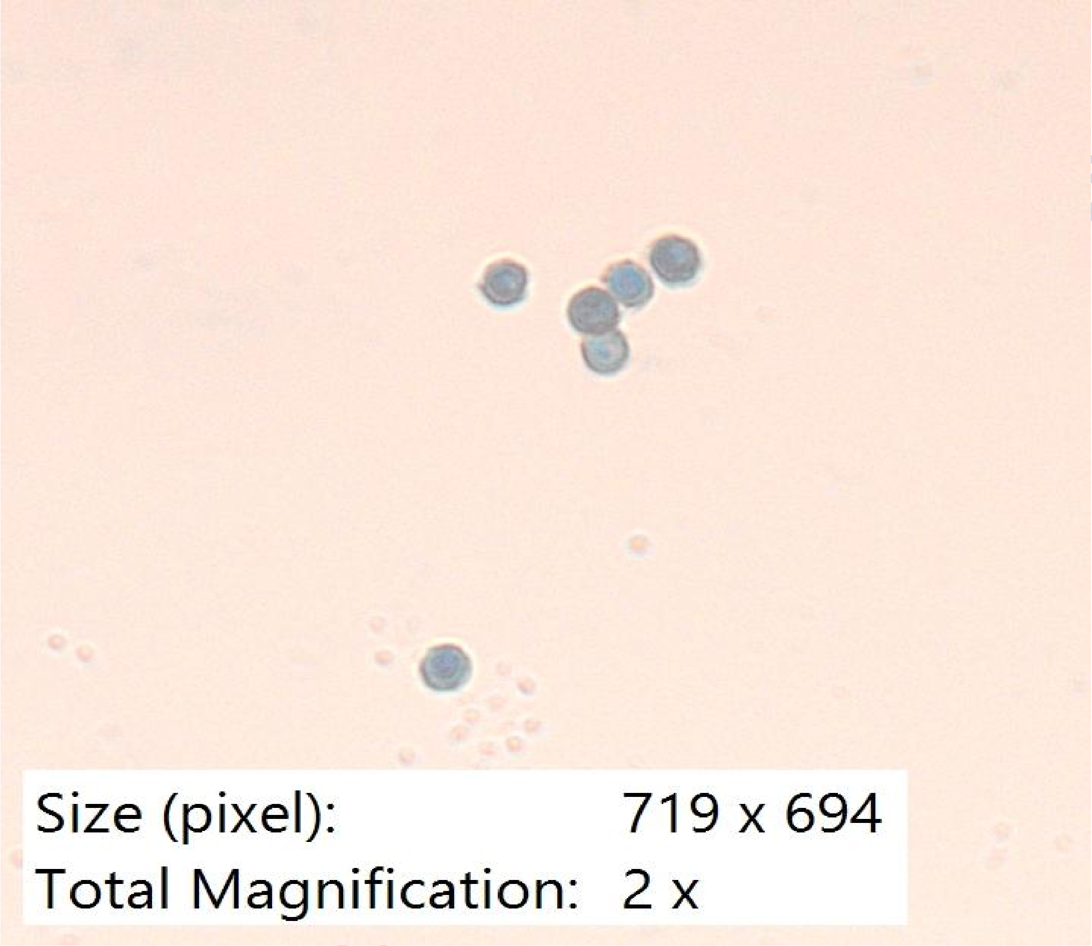
Appendix XI



Photomicrograph of *Aspergillus niger* spores

Source: Author’s Laboratory Work, 2025

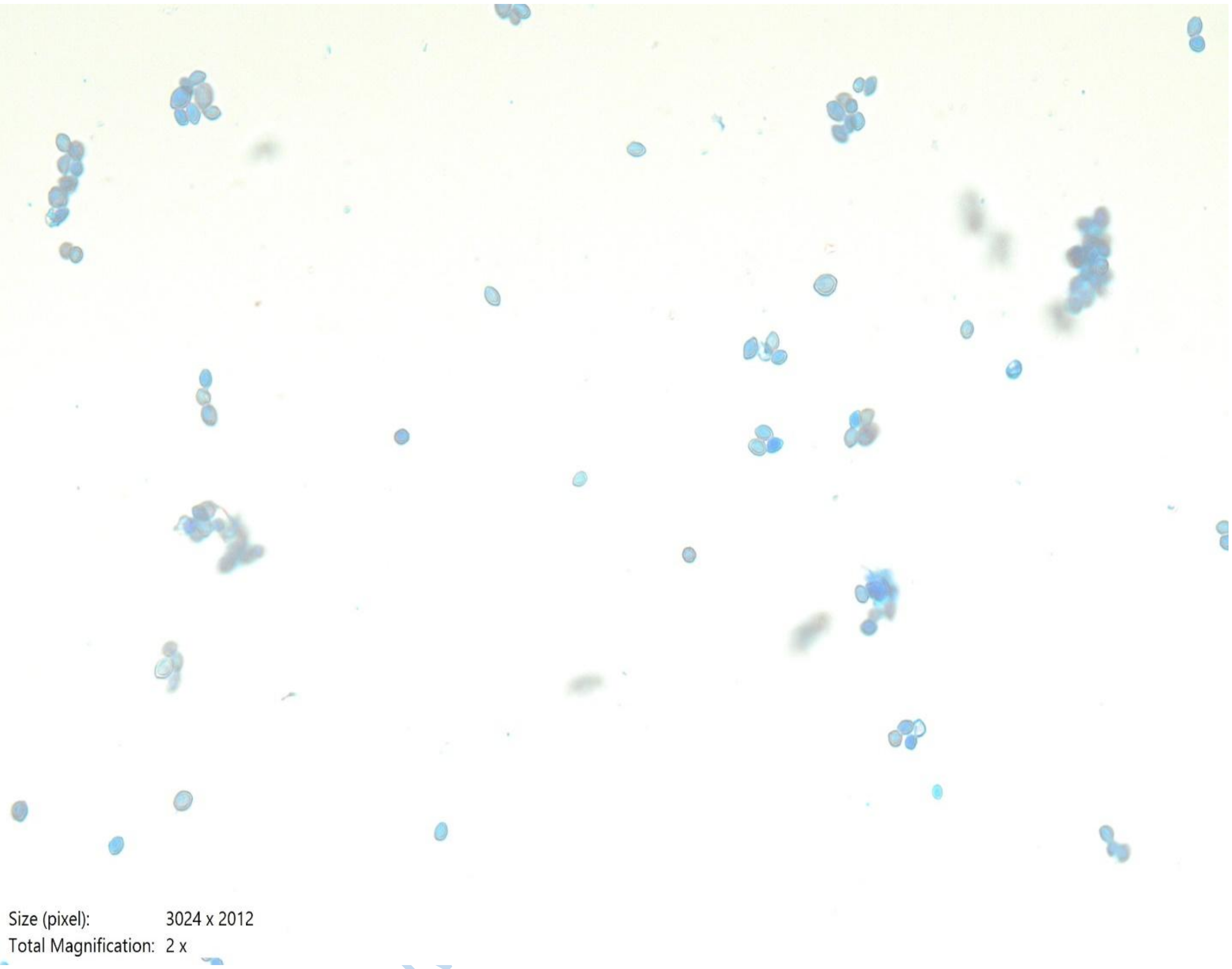
Appendix XII



Photomicrograph of *A. brunneoviolaceus* spores

Source: Author's Laboratory Work, 2025.

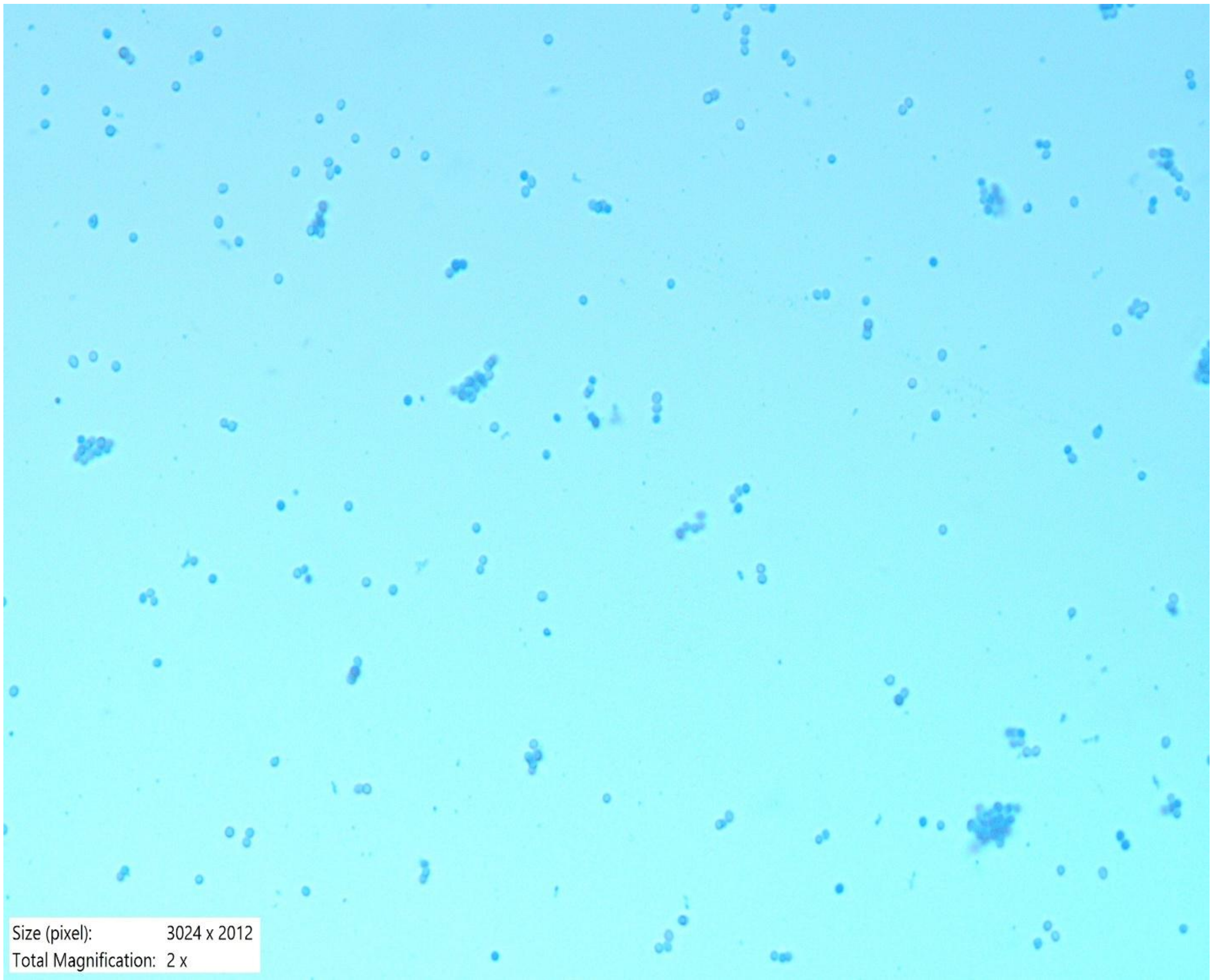
Appendix XIII



Photomicrograph of *Mucor irregularis* spores

Source: Author's Laboratory Work, 2025.

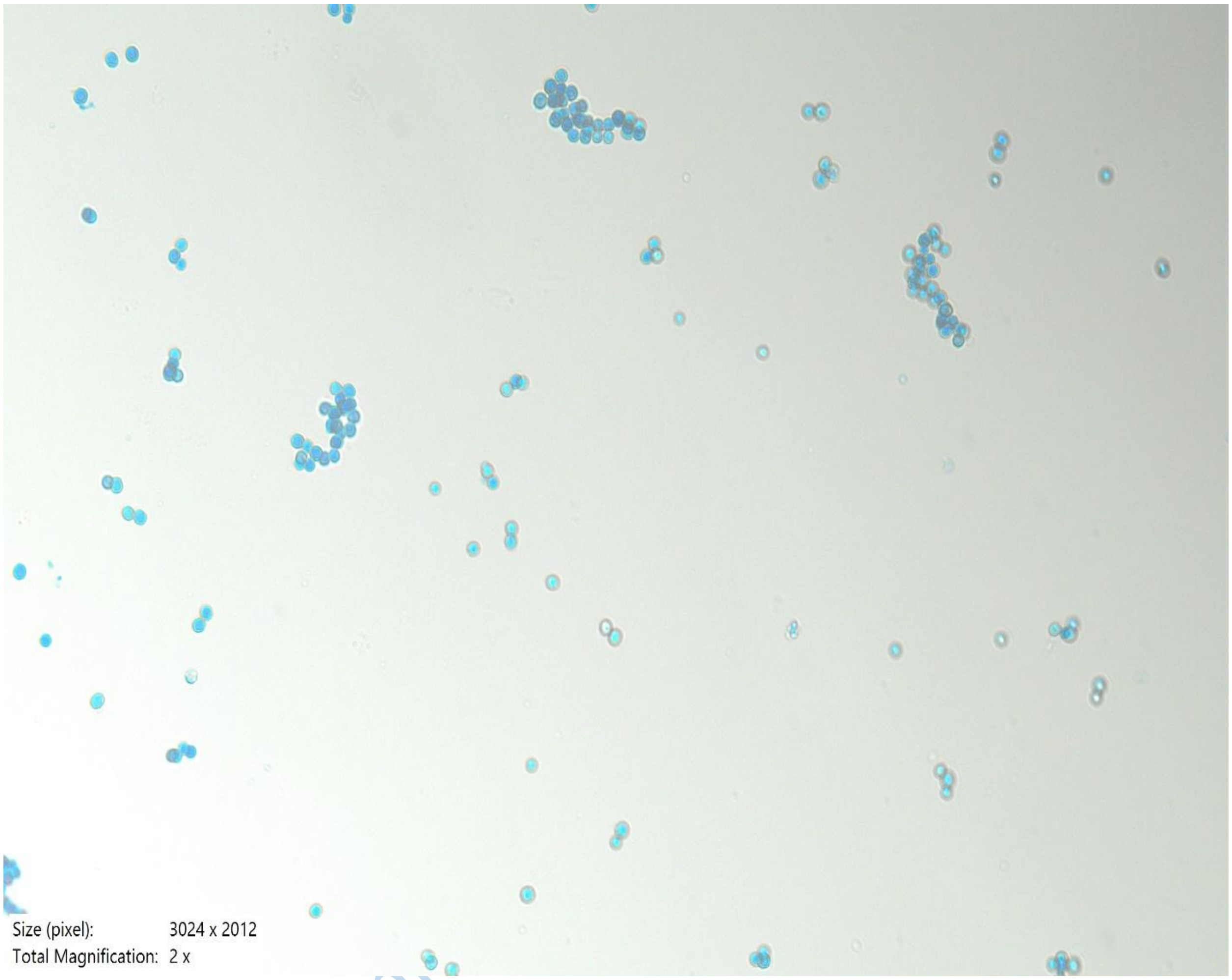
Appendix XIV



Photomicrograph of *Aspergillus fumigatus* spores

Source: Author's Laboratory Work, 2025.

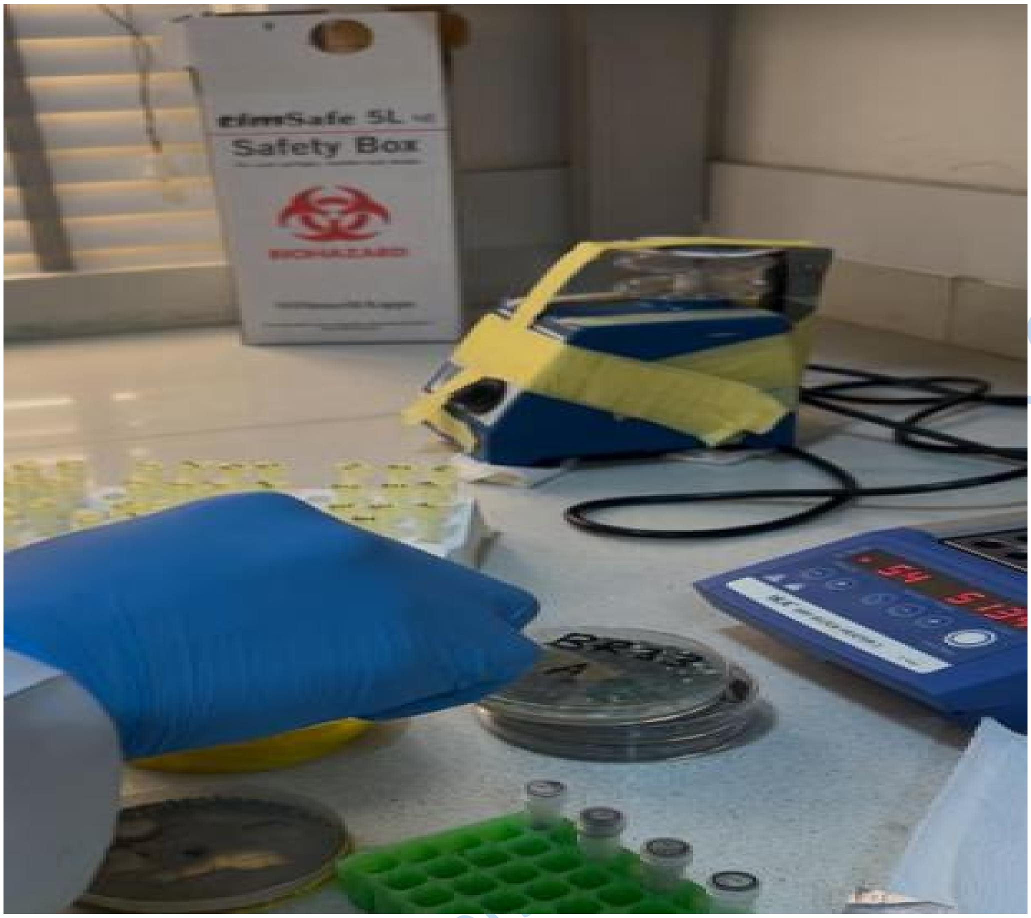
Appendix XV



Photomicrograph of *Aspergillus tamarii* spores

Source: Author’s Laboratory Work

Appendix XVI



DNA Extraction from Fungal Isolates

Source: Author's Laboratory Work,2025.

Appendix XVII

DNA Quantification using Nanodrop spectrophotometer

Source: Author’s Laboratory Work,2025.

Appendix XVIII

Polymerase Chain Reaction for DNA amplification

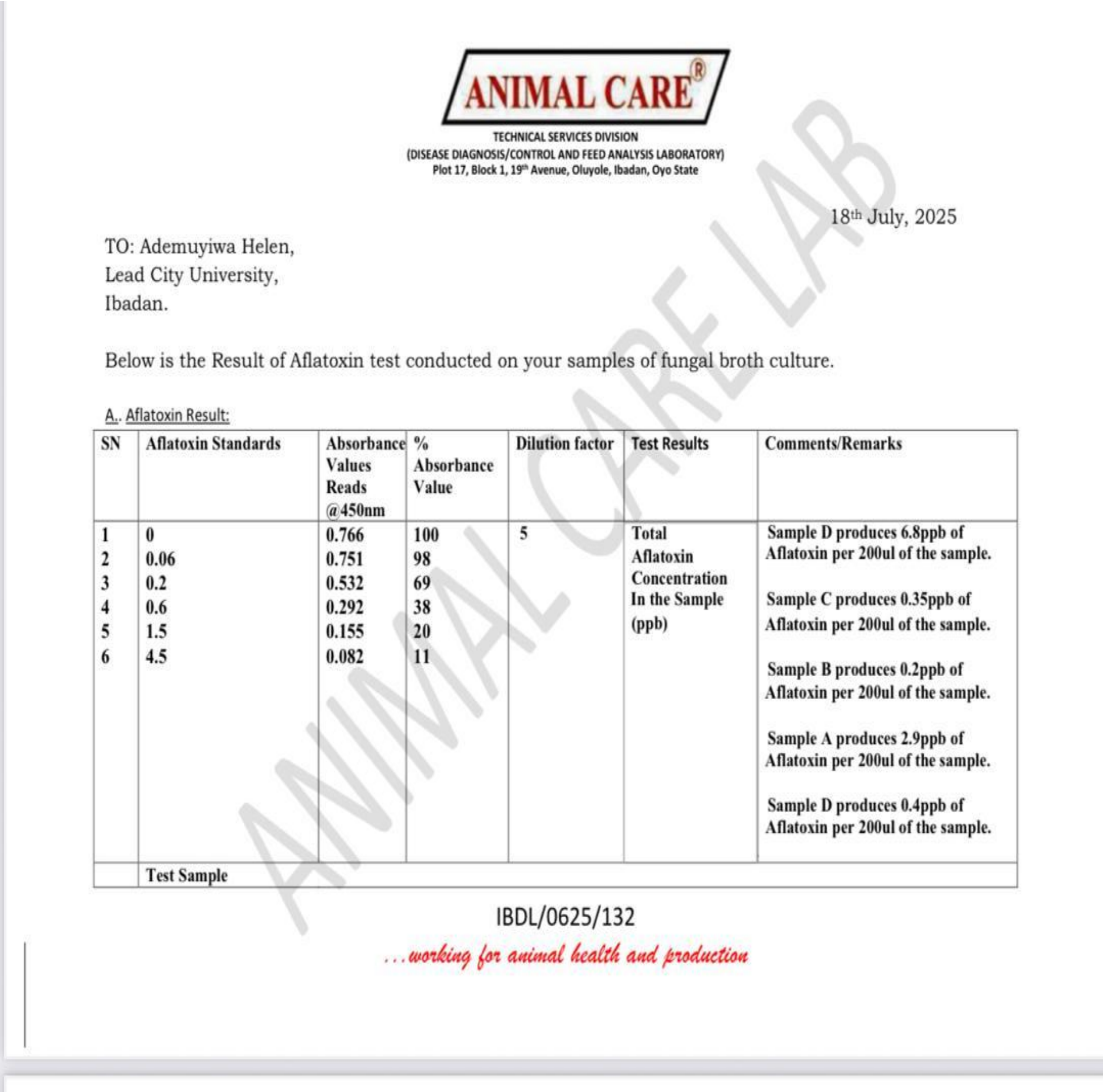
Source: Author's Laboratory Work, 2025.

Appendix XIX

Sequencer

Source: Author's Laboratory Work, 2025.

Appendix XX



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Plot 17, Block 1, 19th Avenue, Oluyole, Ibadan, Oyo State

18th July, 2025

TO: Ademuyiwa Helen,
Lead City University,
Ibadan.

Below is the Result of Aflatoxin test conducted on your samples of fungal broth culture.

A.. Aflatoxin Result:

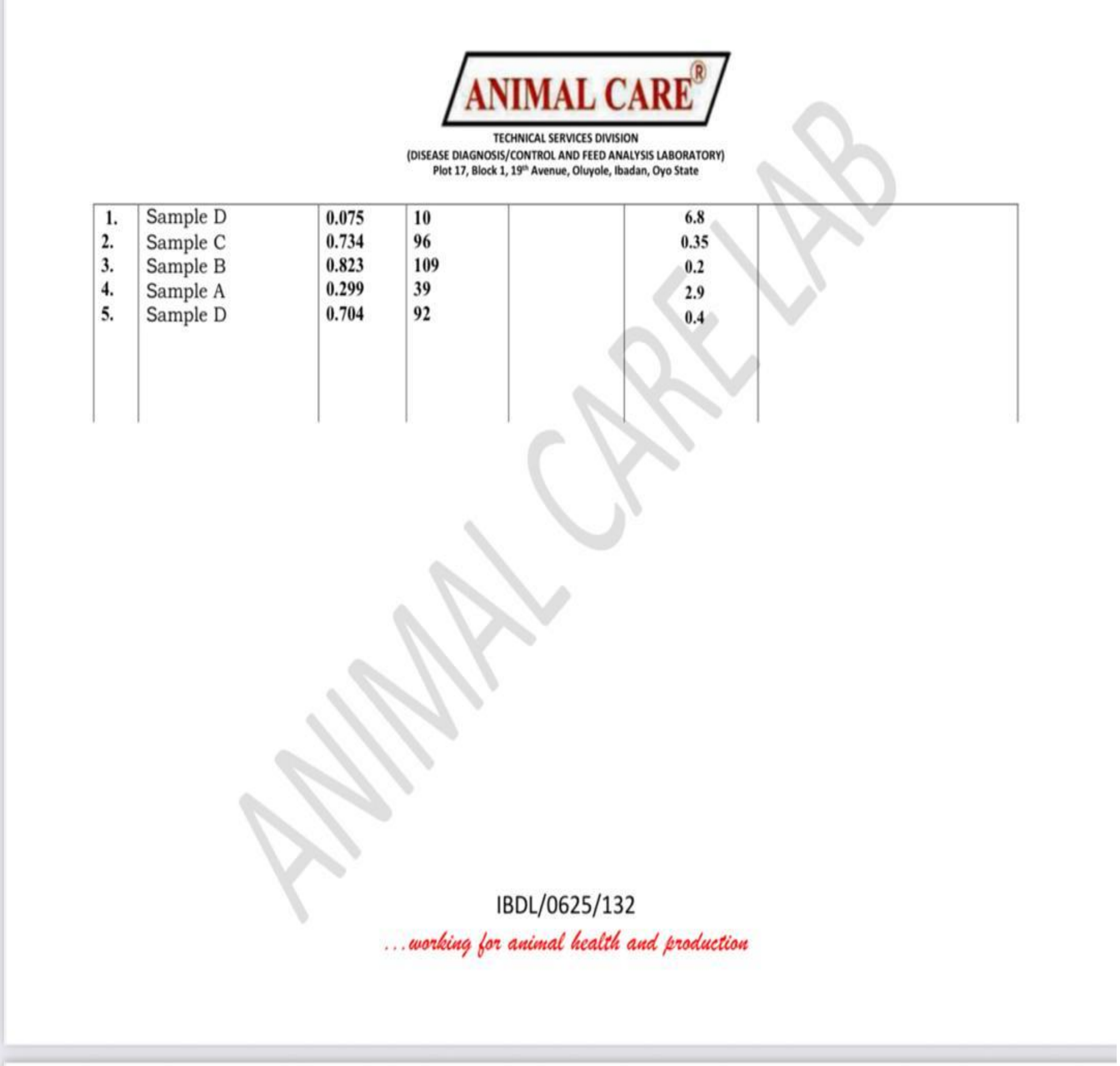
SN	Aflatoxin Standards	Absorbance Values Reads @450nm	% Absorbance Value	Dilution factor	Test Results	Comments/Remarks
1	0	0.766	100	5	Total Aflatoxin Concentration In the Sample (ppb)	Sample D produces 6.8ppb of Aflatoxin per 200ul of the sample. Sample C produces 0.35ppb of Aflatoxin per 200ul of the sample. Sample B produces 0.2ppb of Aflatoxin per 200ul of the sample. Sample A produces 2.9ppb of Aflatoxin per 200ul of the sample. Sample D produces 0.4ppb of Aflatoxin per 200ul of the sample.
2	0.06	0.751	98			
3	0.2	0.532	69			
4	0.6	0.292	38			
5	1.5	0.155	20			
6	4.5	0.082	11			
	Test Sample					

IBDL/0625/132

…working for animal health and production

Source: Author's Laboratory Analysis,2025.

Appendix XXI



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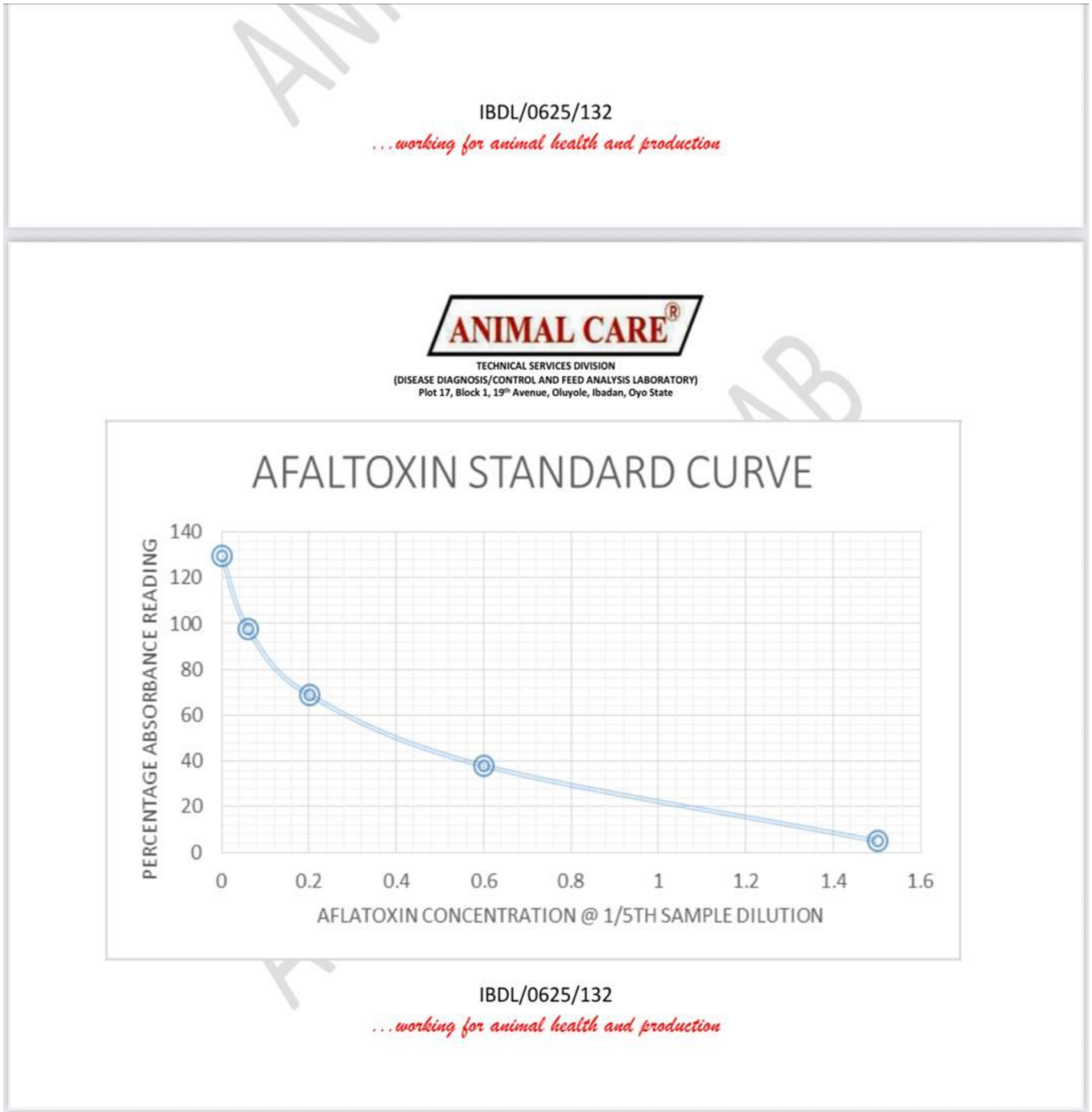
1.	Sample D	0.075	10		6.8	
2.	Sample C	0.734	96		0.35	
3.	Sample B	0.823	109		0.2	
4.	Sample A	0.299	39		2.9	
5.	Sample D	0.704	92		0.4	

IBDL/0625/132

...working for animal health and production

Source: Author's Laboratory Analysis,2025.

Appendix XXII



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AFALTOXIN STANDARD CURVE

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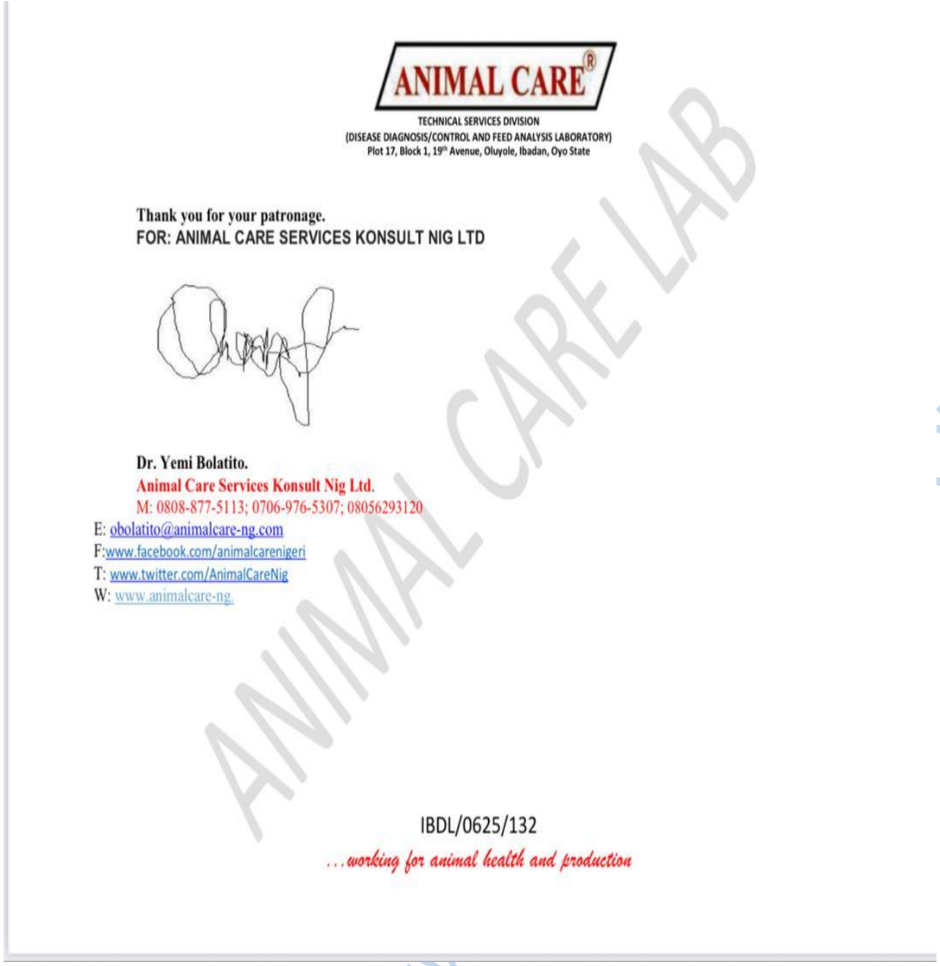
Source: Author’s Laboratory Analysis,2025.

Appendix XXIII



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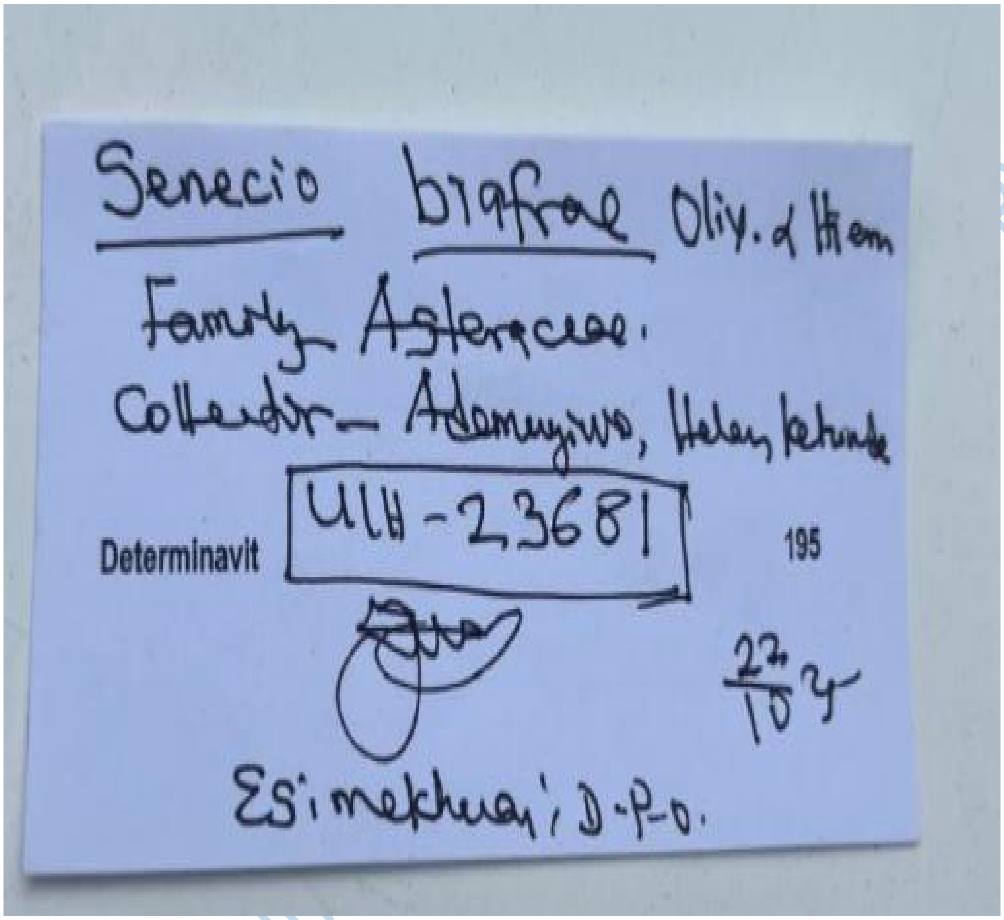
Aflatoxin content results

Source: Laboratory Analysis, 2025.

Appendix XXIV



Senecio biafrae Oliv. & Hiern

Family Asteraceae.

Collector – Adamuyiwo, Helen Kehinde

UIH-23681

Determinavit 195

27/10

Esimekhuai, D.P.O.

Authentication code

Source: Author’s Herbarium work.

Biodata

A. Personal data:

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Phone no +2348067127617

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Nationality: Nigerian

Name and Address of Next of Kin: Lawal Taiwo; 08145316162

B. Educational Background

School Attended	Date	Qualifications
Standard Christian Nusery and Primary School, Ilaje,Ilesa.	(1993-2002)	School Leaving Certificate
Ilesa Grammar School, Okesa, Ilesa.	(2003-2008)	SSCE
Adekunle Ajasin University, Akungba-Akoko,Ondo.	(2012-2016)	Bachelor of Science
National Youth Corps Service	(2016-2017)	NYSC Certificate
Lead City University	(2024-Till Date)	M.Sc. in view

C. Working Experience

- Business Executive Manager (2021- 2024)
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- Customer Support Associate (2020 - 2021
  Inkprint impression, Lagos state.

- Operations / Production Analyst (2018 – 2019)
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- Kebbi State University of Science and Technology (2016 –2017)
  Aliero, Kebbi State.

D. Awards and Fellowships (if any) Nil

E. Professional Membership: Nil

F. Publications (if any)

Ademuyiwa Helen Kehinde, Ologundudu Akinbode Foluso, Fasidi Olutayo, *Propagaton of Black Nightshade (Solanum Nigrum L.): Efect of Light and Shade*, **Eur Exp Biol** Volume. 8 No. 3:20, (2018),19. DOI: 10.21767/2248-9215.100061.

University Compliance Certification

This is to certify that the thesis by Ademuyiwa, Helen Kehinde with matric number LCU/PG/006596 in the Department of Biological Sciences, Faculty of Natural Applied Sciences, Lead City University, Ibadan is in full compliance with the approved University Format and Style.

Signature **Date**