ISOLATION AND CHARACTERIZATION OF L-ASPARAGINASE PRODUCING ENDOPHYTIC FUNGI INHABITING *Prunus africana* AND *Periploca linearifolia*

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I56/28859/2019

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

SEPTEMBER, 2025

DECLARATION

I, Dennis Kipngenoh Cheruiyot, declare that the work presented in this thesis is my original work and has not been submitted for a degree or any other awards in any other university or any other institution.

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DEDICATION

I dedicate this work to my mother, Alice Cheruiyot, father, Stephen Cheruiyot and siblings with sincere gratitude and best wishes for their support, encouragement, and prayers throughout this process.

ACKNOWLEDGEMENTS

First and foremost, I want to express my gratitude to the All-Powerful God for his mercy, constancy, fortitude, health, and discernment. My heartfelt appreciation to Kenyatta University for allowing me to pursue my master's degree, as well as to my supervisors, Dr. George Omwenga and Prof. Eliud Njagi, for their outstanding support and guidance as well as for their intellectual critiques and motivation.

The Department of Biochemistry, Microbiology and Biotechnology (BMB) Laboratory Staff, specifically Mr. Gitonga Daniel, Mr. Alaro Lawrence, and Mr. Waweru Ibrahim, are also greatly appreciated for their invaluable technical guidance and support throughout my laboratory work. My gratitude also extends to my entire family fraternity, which provided me with the inspiration and drive to finish this work.

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ABBREVIATIONS AND ACRONYMS

AIC	Akaike Information Criterion
ALL	Acute Lymphoblastic Anemia
ANOVA	Analysis of Variance
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
CTAB	Cetyl Trimethyl ammonium Bromide
DNA	Deoxyribonucleic acid
EcAII	Escherichia coli type II L-Asparaginase
ITS	Internal Transcribe Spacer
L-ASNase	L-Asparaginase
MCD	Modified Czapex Dots Medium
MCL	Maximum Composite Likelihood
MEGA	Molecular Evolutionary Genetic Analysis
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NACOSTI	National Commission for Science, Technology and Innovation
NCBI	National Center for Biotechnology Information (NCBI)
PCR	Polymerase Chain Reaction
TCA	Trichloroacetic acid
WA	Water agar

ABSTRACT

The clinical use of L-Asparaginase derived from bacterial sources has been hindered by various challenges, including toxicity and repression. This has prompted the exploration of alternative sources, particularly eukaryotic microorganisms like fungi, in an effort to enhance the safety and effectiveness of therapeutic ASNase. In this study, endophytic fungi isolated from medicinal plants, *Periploca linearifolia* (*Apocynacease* family) and *Prunus africana* (*Rosaceae* family), were investigated for their potential as a source of novel ASNase for therapeutic applications. These isolates were screened for L-Asparaginase production using the plate assay method on modified Czapex dots agar medium. L-Asparaginase activity of the fungal endophytes was determined using the nesslerization method. Identification of the fungal endophytes was performed using morphological characteristics and DNA barcoding with ITS sequencing, followed by BLAST analysis. Additionally, a phylogenetic tree was constructed using MEGA version X software. Twenty-four percent of the fungal endophytes exhibited positive reaction for L-ASNase activity and were identified as *Penicillium ubiquetum*, *Penicillium pancosmium, Phoma sp, Penicillium. crustosum, Fusarium sporotrichioides, Cercospora canescens*, *Penicillium commune*, *septoria sp*, *Fusarium solani, and Colletotrichum sydowii*. The fungal endophytes exhibited significant variation in production of L-asparaginase under the inflence of time of incubation and pH. It was observed that the fungal endophytes showed L-asparaginase activity at different day of incubation with *Penicillium ubiquetum* (2.63±0.47UI/mL), *Penicillium pancosmium* (1.44±0.1UI/mL), *Phoma sp* (2.6±0.47UI/mL), *Penicillium crustosum* (3.80±0.37 UI/mL), *Penicillium commune* (2.52±0.29 UI/mL), *Fusarium sporotrichioides* (3.47±0.24 UI/mL), *Cercospora canescen* (2.24±0.12 UI/mL) showed highest enzyme activity on the 6^{th} day of incubation. *Septoria sp* and *Colletotrichum sydowii* exhibited best L-asparaginase activity of 12.6±0.81UI/mL and 4.06±0.23 UI/mL on the 9^{th} day of incubation, respectively. While *Fusarium solani* showed atmost L-asparaginase activity of 12.4±1.12 UI/mL on the 12^{th} day of incubation. In addition, the ten identified fungal endophytes records the highest activity at pH range 5.0-6.0 with *Fusarium solani* recording the highest enzyme activity of (6.14±0.01 UI/mL) at pH 6.0. The study revealed that fungal endophytes inhabiting plants with medicinal properties are potential source of L-Asparaginase. Among the fungal isolates, *Fusarium solani* and *Septoria sp*. showed the highest ASNase activity under optimized conditions (pH 5-6, incubation 9-12 days), indicating their potential as safer alternative to bacterial L-Asparaginase for anticancer therapy.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

L-Asparaginase (ASNase) enzyme is a therapeutic protein utilized in the treatment of acute lymphocytic leukemia, a prevalent type of blood cancer in children (Doriya and Kumar, 2016). It is categorized as an amidohydrolase enzyme and catalyzes the breakdown of L-asparagine substrate to yield aspartate and ammonia (Talluri *et al*., 2023). The amino acid asparagine is a critical substrate for protein synthesis in both cancerous and non-cancerous cells (McCredie *et al*., 1973). However, cancerous hematologic cells rely on external source of L-asparagine, while normal cells possess asparagine synthase, an enzyme that enables them to synthesize L-asparagine and thus be less dependent on external sources (Doriya and Kumar, 2016).

L-Asparaginase, used as antitumor and antileukemic agents, is typically derived from *Erwinia chrysanthemi* and *Escherichia coli* (Parashiva *et al*., 2023). Nonetheless, the pharmacological use of the bacterial L-Asparaginase is linked with potential toxicity and several side effects such as hypersensitivity reactions, difficulty breathing, hyperglycaemia, hepatic dysfunction, nausea, pancreatitis, thrombo-embolysis, skin rashes, fever, nephrotoxicity, loss of consciousness and immunogenic complications (Doriya and Kumar, 2016). Additionally, the cancerous cell resistance against bacterial L-Asparaginases preparations is another challenge throughout cancer management. Recent advanced research has focused on eukaryotic microorganisms such as fungi, as sources of L-Asparaginase to improve efficacy and safety, and minimize issues related to

short half-life and instability associated with bacterial L-Asparaginases (Doriya and Kumar, 2016).

Fungal sources including *Penicillium*, *Aspergillu*s and *Fusarium* sp. isolated from soil and medicinal plants have been reported to synthesize ASNase enzyme with antitumor effect (El-Gendy *et al*., 2018). Given the therapeutic potential of fungal L-Asparaginase (ASNase), fungal endophytes inhabiting plants with medicinal properties should be considered as alternative sources. Endophytic microorganisms reside within plants in mutual relationship and are essential for growth and competitiveness of the host plant by producing signal molecules and bioactive compounds. In return, endophytes receive protection and nutrients from the plant for their own development.

The identification of the antitumor drug Taxol in endophytic fungus *Taxomyes andreanae* shed light on the importance of fungal endophytes (Stierle *et al.,* 1993). This compound was first discovered from the *Taxus brevifolia*, western yew tree, but the endophytic fungus *T. andreanae* was more efficient source due to its ability to produce appreciable amounts of Taxol (Chow and Ting, 2015). This discovery led to the hypothesis that anticancer plants may harbor endophytic fungi capable of producing plant compounds with anticancer effects. In addition, endophytic fungi can perform biosynthesis and biotransformation; hence they can be considered to produce a novel L-Asparaginase.

Prunus africana (*Rosaceae* family) and *Periploca linearifolia* (*Apocynacease* family) are medicinal plants that have been utilized as medicines to cure and manage various

diseases. *Prunus africana* bark extracts are used as an effective herbal remedy for benign prostatic hyperplasia while *Periploca linearifolia* is used traditionally to treat malaria (Belays *et al*., 2018), venereal disease, warts, pneumonia, cancer, antidiarrhea and fertility issues (Jeruto *et al*., 2007).

As a possible source of ASNase, fungal endophytes from medicinal plants such as *Periploca linearifolia* and *Prunus africana* have not been thoroughly investigated. Therefore, the aim of this study was to isolate and characterized L-Asparaginase-synthesizing fungal endophytes inhabiting these plants. The incubation period and pH were optimized to improve the production of L-Asparaginase activity at ambient temperature 28°C under submerged fermentation.

1.2 Statement of Problem

The clinically utilized L-Asparaginase derived from bacteria presents challenges related to potential toxicity and various side effects when administered to human patients. Epidemiological reports showed approximately 30% and 20-30% of treated individuals experience hypersensitivity reactions and hepatotoxicity, respectively. Additionally, other common complications include pancreatitis (3-10%), neurotoxicity and metabolic disturbance such as hyperglycemia (Hijiya and Van Der Sluis, 2015). These side effect significantly limit dosage and long-term therapy, leading to treatment interruptions and reduce survival outcomes among patients with acute lymphoblastic leukemia (ALL). Moreover, bacterial L-Asparaginases (ASNases) preparations are often link to instability in vivo and a short half-life and therefore, necessitate repeated administrations. This further leads to exacerbating immunogenic responses.

1.3 Justification of the study

In contrast Eukaryotic microorganisms such as fungi, which are closely related to humans and have been less studied, may offer a promising alternative. Their ability to carry out post-translational modification could potentially yield L-Asparaginase with improved stability and reduced immunogenicity compared to bacterial-derived enzymes`. Previous studies have reported that fungal genera such as *Penicillium*, *Aspergillus*, and *Fusarium* sp isolated from soil and medicinal plants synthesize L-Asparaginase with antitumor effect that is comparable to or even less toxic compared to bacterial L-Asparaginase enzymes (El-Gendy *et al*., 2018). Therefore, shifting the focus to endophytic fungi found in medicinal plants could potentially provide a unique source of therapeutic ASNase. It is believed that ASNase from endophytic fungi may offer improved efficacy and safety, potentially addressing issues such as its short half-life and instability, which are associated with bacterial L-Asparaginase. This research project aims to isolate and molecularly identified L-Asparaginase-synthesizing endophytic fungi residing in *Prunus africana* and *Periploca linearifolia*.

1.4 Null Hypotheses

i) The L-Asparaginase synthesizing fungal endophytes inhabiting *Prunus africana* and *Periploca linearifolia* cannot be characterized.

ii) Time of Incubation and pH do not influence the production of L-asparaginase by fungal endophytes isolated from *Prunus africana* and *Periploca linearifolia*

1.5 Objectives of the study

1.5.1 General Objective

To isolate, characterize and determine the effect of time of incubation and pH on L-Asparaginase production by endophytic fungi inhabiting *Prunus africana* and *Periploca linearifolia***.**

1.5.2 Specific objectives

i) To isolate and characterized L-Asparaginase producing endophytic fungi inhabiting *Prunus africana* and *Periploca linearifolia***.**

ii) To determine the effect of time of incubation and pH on L-Asparaginase production by endophytic fungal isolates isolated from *Prunus africana* and *Periploca linearifolia***.**

1.6 Significance of the study

The study is of immense significance in the global battle against cancer, as it aims to pave the way for the development of a safer L-Asparaginase-derived anticancer drug by exploring novel sources of L-Asparaginase. This offers promising prospects for the treatment of blood malignancies. By investigating fungal endophytes as potential producers of L-Asparaginase enzymes, the study has the potential to revolutionize pharmaceutical production. Ultimately, the findings of this study are poised to serve as a crucial reference for researchers engaged in similar endeavors, providing valuable insights for the future of cancer treatment.

CHAPTER TWO

LITERATURE REVIEW

2.1 L-Asparaginase (ASNase)

L-asparaginase (ASNase) is a therapeutic protein that catalyzes the breakdown of amino acid asparagine to yield aspartate and ammonia (Brumano *et al*., 2019). It is ubiquitously distributed in various organisms, including plants, rodents' serum, animal tissues, and microbes. Due to the high cost associated with its extraction from plants and animals, microorganism including fungi, bacteria and yeast are frequently used for its production (Doriya and Kumar, 2016). Microbes are often preferred for production of therapeutic L-Asparaginase in large quantities through fermentation process owing to their favorable characteristics, such as ease of culturing and downstream processing (Manasa and Nalini, 2014). Three clinical preparations of ASNase are available: *Erwinia* ASNase, *E. coli* ASNase and PEGylated form of *E. coli* L-Asparaginase (Doriya and Kumar, 2016).

The anti-leukemia activity of L-Asparaginase has been utilized as a first-line therapy for childhood leukemia over the past three decades (Maese and Rau, 2022). Its anti-cancer effect is attributed to the breakdown of the serum asparagine, which is necessary for the growth of certain hematologic malignancies. Depletion of serum asparagine inhibits synthesis of protein, leading to cytotoxicity (El-Nagga *et al*., 2014). However, the application of bacterial L-ASNase to treat cancer patients has encountered issues of hypersensitivity and immunological reactions (Parashiva *et al*., 2023), resulting in antibody production against therapeutic asparaginase in the body, which leads to a loss of efficacy of the administered enzyme drug and anaphylactic shock (El-Nagga *et al*.,

2014). To minimize such cases, improved forms of therapeutic ASNase, such as L-Asparaginase from eukaryotic sources, erythrocyte-loaded ASNase, and PEGylated formulations, have been proposed (Villanueva-Flores *et al*., 2021). L-Asparaginase serves not only medicinal purposes but also finds application in the food processing industry. Its function involves mitigating acrylamide formation in food products subjected to temperatures exceeding 100°C (Jia *et al*., 2021).

2.1.1 Types of L-ASNase

The three distinct categories of L-Asparaginase enzymes encompass bacterial type I and II, plant-based type III, and thermophilic asparaginase from *Rhizobium elti* (*Rhizobium elti* L-asparaginase) (Izadpanah-Qeshmi *et al*., 2018). Bacterial ASNase are classified based on their substrate affinity, with type I localized in the cytoplasm and type II in the periplasmic membrane. These enzymes function as amido hydrolases and employ Thr15 and Thr95 as primary nucleophiles during catalysis (Aghaiypour *et al*., 2001). Type I bacterial L-ASNase has a low affinity for L-asparagine and a high affinity for the amino acid L-glutamine, which it converts to glutamic acid and ammonia (Izadpanah-Qeshmi *et al*., 2018). On the other hand, Type II bacterial L-ASNase is used in clinical settings to treat acute lymphocytic leukemia because of its strong affinity for the amino acid L-Asparagine (Sharafi *et al*., 2017). Furthermore, L-Asparaginases from other microorganisms, such as yeast, share a similar amino acid sequence to bacterial type II L-Asparaginases (Borek and Jaskólski, 2000).

Based on homology, it is evident that plant L-Asparaginases exhibit significant differences from bacterial L-Asparaginases. The primary nucleophile of these hydrolases is located at the N-terminal, and they are distinguished by their low affinity for L-Asparagine (Krishnapura *et al*., 2016). The plant L-Asparaginases are essential in plant metabolic pathways associated with atmospheric nitrogen assimilation (Krishnapura *et al*., 2016). Two distinct types of plant L-Asparaginases, sharing almost identical amino acid sequences, have been identified: potassium-independent and potassium-dependent L-Asparaginases (Dumina *et al*., 2021). Potassium-dependent L-ASNase is primarily localized in higher plants and is used to metabolize asparagine during times of high nitrogen demand; in contrast, potassium-independent L-ASNase metabolizes both β-Aspartyl dipeptides and L-Asparagine (Ajewole *et al*., 2018). *Rhizobium elti* L-asparaginase is utilized by organisms in nitrogen and carbon metabolism and exhibits distinct homology compared to other L-Asparaginases (Krishnapura *et al*., 2016). It is a thermolabile enzyme isolated from *Rhizobium etli*. This enzyme demonstrates two asparaginase activities, specifically thermolabile asparaginase II and thermostable asparaginase I (Izadpanah Qeshmi *et al*., 2018). When compared to L-Asparaginase from *E. coli* and *E. chrysanthemum*, these Asparaginases show different immunological specificities (Izadpanah-Qeshmi *et al*., 2018).

2.1.2 Structure of L-ASNase

L-Asparaginases are classified into several types based on their origin and structural characteristics. These include bacterial types I and II, plant-derived L-Asparaginases, and thermophilic enzymes from Rhizobium species. Among these, bacterial type II L-

Asparaginase, which is located in the periplasmic space, is widely used in clinical treatments due to its high affinity for L-asparagine.

The molecular structure of L-ASNase has been extensively examined in several research studies encompassing microorganisms from prokaryotic to eukaryotic origins (Lubkowski and Wlodawer, 2021). Despite the enzyme's tetrameric nature, alternative forms, such as monomeric, dimeric, and hexameric structures, have been identified from diverse isolate sources. L-Asparaginases sourced from bacteria demonstrate shared biochemical properties and three-dimensional structures, encompassing both quaternary and tertiary configurations (Lubkowski and Wlodawer, 2021). Notably, the molecular structures of bacterial L-ASNase, including those from *E. coli* and *Erwinia carotovora*, have been extensively studied, with comprehensive details provided by Lubkowski *et al*. (2019).

The active form of most bacterial type II L-Asparaginase is a homo-tetramer and consists of 330 amino acid residues. These residues are made up of 8 β-helices and 14 α-strands and constitute two domains: a short C-terminal and a large N-terminal (Aghaiypour *et al*., 2001). Among these beta sheets, β 1 and β 3 are integral to the topological switch points and, in conjunction with alpha-helices 1 and 2 found on either side of the sheet, they form the active site (Lubkowski and Wlodawer, 2021). Moreover, Nα2 and Nα3 are placed contraposition to each other, close to the domain interface pockets, whereas N alpha 1 and 4 in the N-terminal domain are found on the side of the β sheet exposed to solvent (Lubkowski and Wlodawer, 2021). Conversely, the smaller C-terminal is made up of 4α

helices and a four-stranded parallel beta sheet. Cα1 and Cα2 are found on the side of the β sheet that is in contact with the inter-domain, while Cα3 and Cα4 are on the side of the monomer's C-terminal that is exposed to solvent (Pourhossein and Korbekandi, 2014).

The structural features of protomers of ASNase enzymes, particularly those derived from prokaryotes and eukaryotes, exhibit variations in domain arrangements. The N- and C-terminal domains of eukaryotic ASNase are followed by a sequence of ankyrin repeats (Lubkowski and Wlodawer, 2021). ASNase from bacterial sources used in medicine demonstrates almost identical amino acid sequences with highly conserved fundamental active sites (Loch and Jaskolski, 2021). This homology of amino acid sequences determines the high similarity in the tertiary and quaternary structures of ASNase enzymes.

The amino acids present in the dimer determined the active site which lies between two adjacent monomers. Thr15 and Thr95, two active site residues, are essential to the L-ASNase enzyme's catalytic activity (Lubkowski *et al*., 2019). Additionally, other residues such as Thr95, Tyr29, Glu63, Lys168 Ser62, Ala120 Asp96 and Ser62 are present on one monomer, while the adjacent monomer contain residue Ser254 only (Izadpanah Qeshmi *et al*., 2018).

2.1.3 Mechanism of action of L-ASNase

The amide group in the L-asparagine substrate is broken down in part by the enzyme L-Asparaginase. This hydrolytic property of L-ASNase is used to treat acute lymphocytic

leukemia. L-asparagine is a necessary amino acid for regular cell metabolism. While most normal cells can produce L-Asparagine, cancerous cells are unable to do so. Consequently, the growth and advancement of cancerous cells depend on an external supply of L-Asparagine. In contrast, normal cells use the enzyme transaminase to convert oxaloacetate into aspartate, which is then used in a reaction catalyzed by asparagine synthase to make L-Asparagine amino acid (Refer to Figure 2.1). However, neoplastic cells lack the asparagine synthase and rely on serum asparagine for their growth (Batool *et al*., 2016).

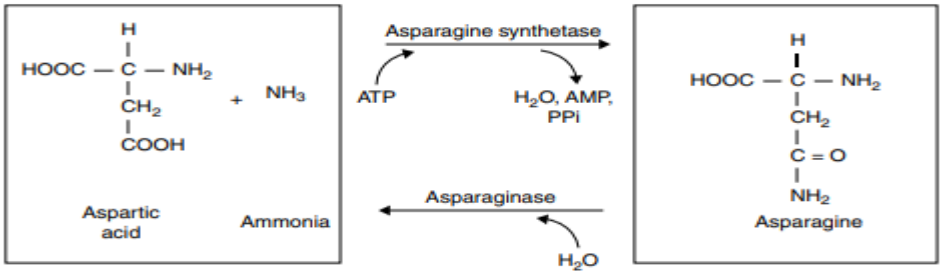


Figure 2.1 Schematic Illustration of Biosynthesis and degradation of L-asparagine (Batool et al., 2016).

2.1.4 **Prokaryotic asparaginase versus Eukaryotic asparaginase**

Bacterial Asparaginases (ASNases), particularly those derived from prokaryotic organisms such as *Escherichia coli* and *Erwinia chrysanthemi*, are widely used in clinical settings; however, their application is often encountered with issues of hypersensitivity, glutaminase activity, immunological reactions, instability *in vivo* and short half-life (Villanueva-Flores *et al*., 2021). In contrast, fungal L-asparaginase (ASNases) shows a closer evolutionary relationship to human enzymes and possesses the ability to carry out post-translational modifications, which may enhance protein folding stability and

mitigate immunological recognition (Moubasher *et al*., 2022). Additionally, the extracellular nature of fungal ASNases facilitates more cost-effective downstream processing compared to intracellularly produce bacterial L-Asparaginase enzymes (Manasa and Nalini, 2014).

2.2 L-ASNase as an anti-cancer agent

The therapeutic drug L-asparaginase was developed after tumor-inhibitory L-asparaginase was identified in guinea pig blood serum (Kidd, 1953; Broome, 1963). The high demand for this anticancer drug for therapeutic purposes has led to its search among microbes, including endophytes. Bacteria were the first microorganisms used to produce therapeutic L-ASNase; for instance, *E. coli* produces L-ASNase enzyme with similar tumor-inhibitory properties as that found in the sera of guinea pig (Jennings and Beacham, 1990). L-Asparaginase I and II are the two ASNase isoenzymes produced by *E. coli*. L-Asparaginase II exhibits greater affinity for L-asparagine, with high antineoplastic properties and therefore clinically effective (Jennings and Beacham, 1990). Other microorganisms that produce L-Asparaginase enzymes used clinically include *Erwinia carotovora* (Keating *et al*., 1993). These discoveries have evoked more interest in other L-asparaginase-producing microorganisms, including endophytic microorganisms.

According to estimates, one-third of all pediatric cancers diagnosed are blood malignancy, particularly Acute lymphoblastic leukemia (ALL), which is more common in children under the age 14 years (Siegel *et al*., 2012). L-Asparaginase was first prescribed as a treatment regimen in the 1970s, and numerous studies have reported and

documented its use as a monotherapy or in combination therapy with other anticancer medications. Extensive research on the toxic effect of L-Asparaginase in ALL malignancy and other cancer cells lines that depend on an external source of L-Asparagine has been reported (Al-jewari *et al*., 2010). Furthermore, a success rate of clinical trials has been achieved when chemotherapeutic drugs are utilized in combination with L-Asparaginase, with reports of remission of certain Hodgkin and non-Hodgkin disease (Kobrinsky *et al*., 2001).

2.3 L-Asparaginase as an acrylamide mitigating agent in food industry

The utilization of ASNase within the food sector has garnered significant interest as a promising food processing agent for mitigating acrylamide formation. Acrylamide is produced during Maillard reactions at elevated temperatures, particularly during the roasting, baking, and frying cereal-based foods rich in free asparagine and reduced carbohydrate content (Gokmen *et al*., 2006). In 1994, acrylamide was designated as a cancer-causing agent by the International Agency for Research on Cancer. Extensive research has linked acrylamide to intrinsic toxic effects, encompassing somatic and germ cell genotoxicity, neurotoxicity, carcinogenicity, and reproductive toxicity (Carere, 2006). Its positive correlation with specific human cancer risks, such as ovarian, breast, renal, and endometrial cancers, is associated with glycidamides formed via the cytochrome P450 mechanism during acrylamide metabolism (Cantrell and McDougal, 2021). Hemoglobin and DNA-glycidamide adducts formed by the epoxide form of acrylamide are linked to DNA damage (Salimi *et al*., 2021). Furthermore, other studies

have reported the genotoxic and cancer-causing effects of acrylamide as a result of glycidamide (Von Tungeln *et al*., 2012).

Acrylamide is created when free asparagine breaks down at a high temperature (Yasuhara *et al*., 2003). Moreover, a considerable amount of acrylamide can be produced at high temperatures when free asparagine reacts with reducing sugars (Muttucumaru *et al*., 2017). A number of strategies, such as blanching and immersion in water to lower the levels of sugar and amino acid (Pedreschi *et al*., 2006), pH adjustment (Rydberg *et al*., 2005), and modification of time and temperature for heating (Boyaci Gunduz, 2023), have been suggested as pre-treatment methods to lessen the production of acrylamide.

Additional strategies include lowering sugar and free asparagine levels in food crops using agronomic and genetic techniques (Curtis *et al*., 2009). It is crucial to remember that these techniques might affect the food's sensory qualities, like flavor and taste (Ciesarová *et al*., 2009). As such, research is being done on L-Asparaginase as a possible acrylamide mitigating agent during the processing of starchy food commodities.

Several researchers have reported pretreatment of food materials as an appropriate solution before heating as it reduce free asparagine content. This assists in minimizing the risk of acrylamide synthesis during heating at high temperatures. Wang *et al*. (2021) found that L-ASNase efficiently lower the synthesis of acrylamide in fresh fries. Gazi *et al*. (2023) concluded that L-Asparaginase effectively reduce asparagine in the dough with water activity value more than 0.75%. Unlike some additives which may help reduce

acrylamide formation and affect the sensory quality of final product, pretreatment of foods substance with L-ASNase lowers the formation of acrylamide content with less effect on the taste and flavor quality in food products (Gazi *et al*., 2023). The commercially available L-ASNase is marketed under the names Acrylway and PrevenAse by Novozyme and DMS respectively (Ciesarova *et al*., 2009).

2.4 Microbial Sources of L-Asparaginase

Prokaryotic organisms, such as gram-positive and gram-negative bacteria, have been reported as the source of L-ASNase preparation (Ebrahiminezhad *et al*., 2011). Examples of gram-negative bacteria from which clinically available ASNase is derived include *Erwinia chrysanthemi* and *E. coli*. The two type of L-ASNase derived from gram-negative bacteria are categorized as Type 1 and Type 2 (Sharafi *et al*., 2017). The distinction between these two types lies in their biological catalysis; Type 1 enzyme breaks down L-asparagine and L-Glutamine amino acids, whereas Type 2 exhibits specific enzymatic activity on L-Asparagine (Sharafi *et al*., 2017). It is worth noting that *E. coli* synthesize these two isoenzymes of L-Asparaginase, with PEGylated *E. coli* ASNase (EcAII-PEG) finding clinical application in the treatment of childhood leukemia (González-Torres *et al*., 2020). The primary limitation of bacterial asparaginase (ASNase) includes hypersensitivity reactions, immunogenic neutralization, hepatic dysfunction and pancreatitis ((Doriya and Kumar, 2016)). Additionally, the repression of intracellular enzyme asparagine synthase in resistance leukemic cells further diminishes the efficacy of bacterial ASNases. This situation underscores the necessity to explore alternative eukaryotic sources that may have reduced toxicity profiles.

Eukaryotic microorganisms, particularly fungi, represent a promising source of therapeutic L-Asparaginase (Moubasher *et al*., 2022). Fungi, being eukaryotic microorganisms, possess the capability to perform post-translational modifications and exhibit a close evolutionary relationship with humans. As a result, they could serve as a unique source of ASNase with reduced toxicity and immunological complications. Despite reports indicating the tumor-inhibitory potential of fungal L-Asparaginase, its commercialization remains unrealized (Moubasher *et al*., 2022). Moreover, the production of extracellular asparaginase by fungi offers cost-effective and streamlined downstream processing (Batool *et al*., 2016).

2.5 Endophytic Fungi interaction with medicinal plants

Several researchers have recorded the habitation of fungal endophytes in every plant species under research so far. Extensive research have frequently uncovered novel taxa of the internal microbiota of plants and predict new distribution of well define fungal species. Endophytes inhabits healthy plant tissue inter- or intra cellular in a mutual relationship and resides within for almost the whole biological life-cycle (Uzma *et al*., 2016). Endophytes relationship with their host has been reported to be primitive due to their present in fossilized tissues of plants (Purushotham *et al*., 2020). The mutualistic interaction of host plant and fungal endophytes is vital for their growth and development. Host plant provides nutrients and protection to fungal endophytes against a biotic factors while in return receive signal molecules that promote their growth and competitiveness (Verma *et al*., 2022).

There is biological mega diversity of endosymbiotic fungi that occur naturally in a wide range of habitat ranging from tropical rainforests ecosystem to temperate region which host more than 300000 terrestrial plant species (Jia *et al*., 2016). Almost all vascular plants are storehouse of more than one endosymbiotic species of fungi. The polyphyletic diversity of endophytic fungi colonizes healthy plant tissues and flourishes without affecting the host plant (Jia *et al.*, 2016). This adaptation may be attributed to favorable physiological and micro ecological conditions existing within plant organ. During the process of isolation, a significant number of endophytes are generally found with only a few host specific isolates dominating. There present are often influenced by the immediate surroundings of the host plant (Fadiji and Babalola, 2020) and distinctive fungal assemblages among plants may be influence by similar niche they share and their immediate environment (Fadiji and Babalola, 2020).

2.6 Medicinal plants selection criteria

The selection of plants of interest is a fundamental step in the examination of endophytic fungi inhabiting medicinal plants. Medicinal plants exhibiting uncommon biological traits, originating from unexplored habitats, and possessing ethnobotanical significance, may hold promise for bioprospecting. They could potentially serve as a source of unique phytochemicals with pharmaceutical relevance. Therefore, the selection of plants should be conducted in disease-free sampling sites.

In order to achieve a significant diversity of fungal establishment, it is vital to consider the number and size of the explant samples. For instance, research by Gamboa *et al*.

(2002) suggests that using 30 to 40 sampling units per tissue of a plant species is adequate to isolate up to 80% of fungal endophytes from a given host. Additionally, it has been observed that utilizing small leaf explants and increasing the number of samples results in a more extensive recovery of endophytic fungi (Gamboa *et al*., 2002). These findings underscore the significance of increasing the size of the explant to achieve the highest establishment of the fungal endophyte population.

The isolation of endosymbiotic fungi is influenced by the age of the explant or host plant, or both. Additionally, season is an important variable that influences the establishment and diversity of endophytes. Hence, age, host plant, and season may act in isolation or in concert to shape fungal colonization. Furthermore, the biochemical conditions within plant parts can influence the colonization frequency fungal endophytes. In comparison to young leaves, mature and old *Calotropis procera* leaves exhibit a higher frequency of endophytic fungal colonization (Nascimento *et al*., 2015). The low colonization frequency of endosymbiotic fungi in young leaves may be attributed to the minimal concentration of structurally important bioactive compounds as compared to old and mature leaves, as noted by Chauhan *et al*. (2019). Additionally, Chauhan *et al*. (2019) also propose that the improved fungal establishment in old leaves may be due to the duration of exposure to the surrounding environments. In a study by Toofanee and Dulymamode (2002), it was found that *Cordemoya intergrifolia*, an endemic plant, harbors a high frequency of fungal endophytes in its mature part of the leaf and petiole compared to young growing parts, with *Pestalotiopsis sp* and *Penicillium* being dominant genera.

The colonization of host plant parts by fungal endophytes exhibits seasonal variation. During the rainy seasons, *Dillenia indica* L. showed a higher diversity of fungal endophytes (Kumar and Prasher, 2021). This occurrence may be ascribed to the elevated moisture content and temperature during the season with high rainfall, which influence endophytic fungi survival and spore dispersal, as suggested by Mishra *et al*. (2012). Conversely, Kumar and Prasher (2021) found a high colonization frequency of endophytic fungi during winter compared to summer and rainy seasons. It is proposed that the fluctuating levels of phytochemicals in the host plant across different seasons may impact the frequency of colonization by the fungal endophyte community (Fang, 2013).

2.7 Distribution and biodiversity of endophytic fungi in medicinal plants

Traditional medicine derived from medicinal herbs is widely utilized in developing countries, with an estimated 80% of the population relying on it. The ethnomedical use of these herbs in treating diseases, ailments, and preventing illnesses indicates the presence of important therapeutic phytochemicals (Pan *et al*., 2013). These bioactive compounds have found application in pharmaceutical industries for cancer therapy, pain relief, antibiotics, antivirals, laxatives, and diuretics (Pan *et al*., 2013). Approximately 8000 different types of plants with medicinal significance have garnered attention as sources of phytochemicals for use in medicines, dietary supplements, biocidal products, and other applications (Pan *et al*., 2013).

Endophytic fungal isolates that yield structurally important phytochemicals have been obtained from various medicinal plants. In a study by Wiyakrutta *et al*. (2004) involving 81 Thai medicinal plant species, 92 endosymbiotic fungal isolates demonstrated inhibitory effects on *Mycobacterium tuberculosis*, while 6 fungal isolates exhibit antimicrobial activity against Herpes simplex virus type 1 by impeding *Plasmodium falciparum*. These findings indicate the synthesis of essential phytochemicals by fungal endophytes and the plant species. Additionally, it was noted by Gangadevi *et al*. (2008) that endosymbiotic fungus *Bartalinia robillardoides*, which was isolated from *Aegle marmelos* produce Taxol. Moreover, prominent taxa like *Acremonium*, *Phomopsis*, and *Pezicula* were explored from *Taxus chinensis* and examined for the ability to produce Taxol, an essential component of anticancer treatment (Liu *et al*., 2009).

Endosymbiotic fungi isolated from *Dendrobium devonianum* and *D. thyrsiflocum* were found to be predominantly comprised of *Fusarium*, *Phoma* sp, and *Epicoccum nigrum*, which exhibited activity against bacterial pathogens (Xing *et al*., 2011). These results imply that fungal endophyte isolates may be a valuable source of novel antibiotics (Xing *et al*., 2011). Moreover, Puri *et al*. (2005) reported that the endophytic fungus *Entrophospora infrequens* isolated from *Nothapodytes foetida* produced camptothecin. Furthermore, significant antimicrobial activity was shown by *Phomopsis* sp. and *Botryosphaeria* sp., which were explored from five distinct species of *Garcinia* plants (Phongpaichit *et al*., 2006). In addition, it was discovered that the main isolates from 21 Chinese medicinal plants, such as *Colletotrichum*, *Phoma*, *Phomopsis*, and *Xylariales*, primarily produced phenolic compounds (Huang *et al*., 2008).

As such, the enzyme L-asparaginase, known for its anticancer properties, has been extracted from fungal endophytes residing in various plants with medicinal properties. Fungal endophytes *Phoma*, *Colletotrichum*, *Penicillium* and *Fusarium* species isolated medicinal plants *P. bleo* and *M. koenigii*, demonstrated high L-ASNase activity (Chow and Ting, 2015). These plants are traditionally recognized for their therapeutic applications in treating diseases and ailments. Additionally, species of *Pletosphaerella*, *Stemphylium*, *Fusarium Septoria*, *Didymella*, *Alternaria*, *Phoma*, *Epicoccum*, *Chaetosphaeronema*, *Cladosporium*, *Nemarria*, *Ulocladium*, and *Sarocladium* isolated from medicinal plants species of the *Asteraceae* family exhibited L-ASNase activity (Hatamzadeh *et al*., 2020). Furthermore, species isolated from *Asclepiadaceae* family, such as *Perigularria tomentosa*, serve as a source of L-ASNase which demonstrates anticancer activities (Al-Said *et al*., 1989).

2.8 Mutualistic and Pathogenic Endophytes

Endophytes are microorganism that live within plant tissues and establish a symbiotic relationship. They can either be mutualistic and pathogenic representing two ends of a spectrum in this kind of relationship. Many mutualistic fungal endophytes establish enhance host plant defense mechanisms and nutrients acquisition, promote metabolite production and deters herbivores; however, some species exhibit opportunistic pathogenic behavior that harms the host by causing diseases. For instance, *Fusarium oxysporum* is known to induce vascular wilt disease in stressed host plants. In addition, an endophyte species can sometimes exhibit both mutualistic and pathogenic lifestyle, a

dynamic phenomenon known as the “endophytic continuum”. Such scenarios have been observed in various species of *Colletotrichum* that can alternate between beneficial and pathogenic behaviour subject upon the host's environmental and physiological conditions. Consequently, distinguishing between pathogenic and beneficial strains is of paramount importance in the bioprospecting of endophytic fungi (Rodriguez *et al*., 2009).

2.9 Isolation of Fungal endophytes inhabiting medicinal plants

The isolation process represents a pivotal step in the comprehensive study of fungal endophyte communities. This process commences with the meticulous surface sterilization of the plant tissue samples under investigation, aimed at eradicating contaminant microorganisms and other epiphytic fungi (Yang *et al*., 2018). Essential considerations encompass the precise concentration and duration of exposure to sterilizing agents, which may permeate the plant tissues and thereby exert influence over the ultimate results. Consequently, it is imperative to verify the success of surface sterilization (Hallmann *et al*., 2006). Moreover, to suppress bacterial proliferation, the supplementation of both culturing and sub-culturing media with antibiotics is recommended (Hallmann *et al*., 2006).

The characterization of endosymbiotic fungi can be achieved through the utilization of standard manual and molecular methods. Morphological and micromorphological characterization involves the assessment of the phenotypic appearance of endophytic fungal isolates, including opacity, colony margin, colony color, colony elevation, and hyphal observation, to determine their taxonomic classes (Seifert and Rossman, 2010).

Nevertheless, it is important to note that morphological identification is considered less reliable when compared to molecular techniques, as the cultural appearance may exhibit variations based on the environmental conditions within their habitat (Schulz and Boyle, 2005).

Endosymbiotic fungal strains are subjected to DNA extraction for molecular identification using either the CTAB method (Cetyl Trimethyl Ammonium Bromide) or a DNA isolating kit (Tejesvi *et al*., 2011, Arnold, 2007). Fungal isolates are frequently identified using DNA barcoding, which specifically targets the ITS region. Polymerase Chain Reaction (PCR) amplification is performed using different ITS primers, including ITS1, ITS2, and ITS4. The PCR products undergo Agarose gel electrophoresis, stained with gel red or SYBR green and examined under a UV transilluminator (Arnold, 2007).

Subsequently, the amplified DNAs are purified and their concentrations are determined using a spectrophotometer prior to sequencing. The investigation of micro fungi heavily relies on molecular identification, which encompasses bioinformatics analysis utilizing the BLAST tool to find sequences with significant matches in the NCBI's Gene Bank database. The accuracy of BLAST matches is validated through taxonomic comparison of similar isolates at the family and genus levels based on the BLAST search.

2.10 Advances in L-Asparaginase production using Modern Biotechnology

The production of L-asparaginase (L-ASNase) has significantly transformed beyond simple microbial fermentation, largely due to advancements in modern biotechnology.

Advance techniques such as recombinant DNA technology, protein engineering, and metabolic pathway optimization are currently being used to increase the enzyme's yield, enhance stability, and specificity while also used to reduce its immunogenicity. These modern innovations aim to address the limitations associated with bacterial ASNase, including immunological reactions, a short half-life, Instability and high production costs. One effective strategy involves cloning ASNase genes from microbial or fungal sources into expression systems like *Escherichia coli, Pichia pastoris,* or *Bacillus subtilis*. This approach can significantly improve both yield and purity through process optimization (Villanueva-Flores *et al*., 2021).

Furthermore, the clinical success of PEG-ASNase formulation shows that PEGylation, which attaches polyethylene glycol chains to ASNase, has been found to reduce immunogenicity and prolongs the enzyme's half-life in vivo (Pan *et al*., 2013). Despite these technological advancements, there are still a number of challenges. Recombinant production systems potentially require costly culture media, and the structural complexity of fungal ASNase may hinder appropriate folding and activity in heterologous hosts. Additionally, obtaining regulatory approval for genetically engineered enzymes continues to be the biggest obstacles in translating laboratory successes into clinical applications (Patel *et al*., 2022).

Nevertheless, there is a lot of promise in using omics technologies (genomics, proteomics, and metabolomics) to identify and develop unique ASNase producers. Furthermore, using synthetic biology techniques can help to restructure bacterial or fungal metabolic circuits,

increasing ASNase expression while reducing harmful effect or by-products (Lefin *et al*., 2023; Shishparenok *et al*., 2023)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of Samples

The plant specimens of *Periploca linearifolia* and *Prunus africana* (leaf, stem and root) were collected from healthy, disease- free plants, with three biological replicates per plant parts. Weather conditions during collection (in the middle of November 2021, wet season) were noted, as moisture and temperature influence fungal colonization. Sampling was restricted to this season to minimize variation in endophytes diversity. The plant samples were obtained from Chepkesui village, Bomet County, Kenya, located between 34.97°E and 35.06°E and between 76°S and 83°S with an elevation range of 1900–3000 m above sea level. Stem/barks samples were removed by cutting at 1.6 m from the base of the plant with a sterilized machete, roots were dug from the soil using a hoe, and both were swabbed with 70% alcohol, whereas leaves were cut using sterile hedged clipper shear scissors. The material samples were then put in a separate well-labeled polyethylene zipper bag and transported to Postgraduate Research Laboratory II (Biochemistry, Microbiology and Biotechnology) at Kenyatta University within 2 days of collection for processing.

3.2 Surface Sterilization of Sample Materials

The sample materials (50 leaves, 50 stems, 50 roots) from each plant species were rinsed using sterile distilled water to remove surface debris and other contaminants. Surface sterilization was performed in a biosafety cabinet (level 2) by dipping the sample materials in 3.5% sodium hypochlorite (NaOCl) for five minutes preceded by 70%

ethanol for three minutes. To eliminate any remnant of the sterilant, the samples were thoroughly rinsed five times with sterile distilled water (Manasa and Nalin, 2014). To evaluate the effectiveness of surface sterilization, the final rinse solution was cultured on a PDA medium plate and incubated for 6-12 days at room temperature with 12-hour light/dark cycles. The PDA medium plate's lack of fungal growth suggests that the sterilization was successful.

3.3 Isolation of Endophytic Fungi

Isolation was performed as described by Manasa and Nalin, (2014). The sterilized samples were blotted dry in a sterilized biosafety cabinet, and small tissue segments of approximately $1cm^2$ were cut using a sterilized scalpel. Fifty (50) tissue segments from stem, root and leaf (five segments per Petri plate) were positioned at equal distances on gentamycin - supplemented potato dextrose agar (PDA) media. Parafilm was used to wrap the prepared plates before they were incubated at 28°C for five to eight weeks and then exposed to 12 h day and 12 h night regimens. To obtain pure cultures of fungal endophyte isolates, fungi colonies that developed on tissue segments in potato dextrose agar (PDA) agar medium were subcultured in potato dextrose agar (PDA) media supplemented with another antibiotic (gentamycin). Forty two purified fungal strains isolated were subjected to qualitative screening for production of L-asparaginase.

3.4 Preliminary screening of L-Asparaginase-synthesizing Endophytic Fungi

A plate assay was used for preliminary screening of endophytic fungi that exhibit L-ASNase as described previously (Theantana *et al*., 2009). Modified Czapex Dox

(McDox) agar was used to screen for fungal isolates that can produce ASNase. A 4 mm disk of mycelia from 4 day PDA agar was used as an inoculum on modified Czapex Dox (McDox) agar media with 2 g/L glucose, 10 g/L L-Asparagine, 1.52 g/L KH_2PO_4, 0.52 g/L $MgSO_4 \cdot 7H_2O$, 0.52 g/L KCl, 0.001 g/L $Cu(NO_3)_2 \cdot 3H_2O$, 0.001 g/ L $ZnSO_4 \cdot 7H_2O$, 0.001 g/L $FeSO_4 \cdot 7H_2O$, 1.8 mL of L 2.5% phenol red in ethanol solution, and 20 g/L agar; at pH 6. Controls were prepared using 10g/l $NaNO_3$ as the sole nitrogen source in a Czapex Dox agar media. Following the incubation of all agar plates at 28°C for a period of 5 days, a pink zone formed a round each fungal colony on the agar plate indicate a positive reaction. Fungal isolates exhibiting a prominent pink zone surrounding the colony were subsequently subcultured in PDA media for morpho-molecular characterization.

3.5 Characterization of L-Asparaginase-synthesizing fungal isolates

3.5.1 Morpho-Cultural characterization of L-Asparaginase producing fungal endophytes

Following successful preliminary screening, pure cultures for morpho-cultural identification were obtained by growing positive fungal isolates on PDA agar medium at 26±2°C. Mycelial plugs (5 mm diameter) from four-day-old pure colonies were cut and placed on new PDA. Each fungal isolate was tested in triplicate. The isolates were stored and kept for five days at 25±2°C.

For macroscopic observation, colony color (surface and reverse characteristic) and margin of mycelia were also documented. On PDA at 26±2°C, conidia production, color

and shape, metulae, phialides, and stripes were analyzed using slide culture methods. A slide containing about 8 mm^2 of PDA was inoculated with spores from an actively growing culture covered with another slide. The prepared slide culture was incubated for five days at room temperature in a sterile Petri dish with wet bottling paper and a Parafilm seal. Using an Lx400 compound microscope (LaboMed, Beijing, China), slide cultures was observed. The endophytic fungal isolates were then subsequently grown for quantitative analysis, DNA extraction and molecular characterization.

3.5.2 Molecular identification of L-Asparaginase producing fungal isolates

3.5.2.1 Isolation of fungal genomic DNA

A 7 mm plug of hyphae from ten distinct fungal endophyte isolates, each representing a different genus based on morphological variations was inoculated on a PDA medium and allowed to incubate for seven days at room temperature. A 300 mg mycelium plug was removed from each PDA medium and washed with sterile distilled water to eliminate the PDA agar. After freeze-drying, the mycelia were crushed in a pestle and mortar filled with liquid nitrogen. The powdered mycelia were then stored at -20°C before use. The nucleic acid was extracted using the Cetyl Trimethyl Bromide (CTAB) method, following the protocol outlined by Arnold (2007).

3.5.2.2 PCR amplification of the target gene

Polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). The amplification process was carried out using the universal primers ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) as the forward

sequence and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as the reverse sequence (Nakahama *et al*., 1973) as shown in Table 3.1. PCR amplification of the purified DNA was performed in a 25 µL reaction mixture containing 1 µL of DNA template, 12.5 µL of master mix (TAG, dNTB, Buffer), and 1.25 µL of each forward and reverse primer and 9.0 µL of PCR water was added to make a final volume of 25 µL. Thermocycler was programmed to carry out the polymerase chain reaction. Preheating for five minutes at 95°C to start the amplification process. This was followed by forty cycles of denaturation at 94°C for one minute, primer annealing at 56°C for thirty seconds, primer extension at 72°C for one minute, and final extension at 72°C for ten minutes before cooled to 4ºC.

After the amplification process, the PCR products were verified through gel electrophoresis using 1% Agarose gel that was stained with gel red (Biotinum ®) and observed under a UV translluminator. After detection of positive band on the gel, all amplified DNA of fungal isolates were purified using GeneJET PCR Purification Kit (KO701) by following the standard protocol of the manufacturer. The concentration was checked by spectrophotometer and outsource to Microgen Netherlands for sequencing in both direction using Sanger's Dideoxy method.

Table 3.1 Primers used for Molecular Identification

Locus	Primers	Primer sequences	Orientation	Reference
ITS	ITS 1	5' TCCGTAGGTGAACCTGCGG 3'	Forward	Nakahama et al, 1973
	ITS 4	5' TCCTCCGCTTATTGATATGC 3'	Reverse	Nakahama et al, 1973

3.6 Bioinformatics analyses of sequences data

A total of 10 endophytic fungal raw sequences (forward 5'-3'and reverse 3'-5' reads) were obtained from macrogen in form of abI file format. Each sequence was analyzed using BioEdit 7.2.5 version software by removing noise and ambiguous peaked followed by creating a consensus sequences. The final fine-tuned sequences were deposited at the National Center for Biotechnology Information's (NCBI) GenBank database and given an accession numbers ON882050, ON879761, ON880417, ON880423, ON880580, ON881143, ON881283, ON881292, *ON882018 except* HQ130716 which was retrieve directly the NCBI database. BLASTn search with default parameter was performed using consensus sequences as a query sequences at NCBI to retrieve the identity with significant match of the endophytic fungal isolates. The closest match sequences with ≥97% identity were downloaded in form of a fasta format for alignment using CLUSTALW tool embedded in MEGA version X software (Kumar *et al*., 2018).

The Kimura 2-parameter model and the Maximum Likelihood statistical method were used to conduct the phylogenetic analysis (Kimura, 1980). According to the Bayesian Information Criterion (BIC) and corrected AIC (Akaike Information Criterion) in the model selection tab of the MEGA version X software, the Kimura 2-parameter model with gamma distributed with invariant site (K2+G+I) was the best fit substitution model used for phylogenetic reconstruction(Kumar *et al*., 2018). In order to analyze the phylogeny test, bootstrap calculations and analyses were based on 1000 replicates (Felsenstein, 1985). The phylogenetic tree that resulted from running the Maximum Likelihood (ML) algorithm on the phylogeny tab of the MEGA version X was viewed in

FigTree v.1.4.3 software. Accession numbers for sequences that were retrieved from GenBank are also shown in the phylogenetic tree rooted with *E. coli*.

3.7 Enzymatic assay of L-Asparaginase

3.7.1 Preparation of ammonia color reagent

Preparation of Nessler reagent was done using a modified procedure by Nxumalo *et al*. (2020). Mercury chloride (2.2g) was dissolved in 45ml distilled water and heated on a hot plate to achieve a complete dissolution. Using two separate conical flasks, 6g of potassium iodide was dissolved with little amount of water by stirring and 5g of NaOH dissolved in 25 ml of distilled water. After preparation of the above reagents, 6g of KI solution was added slowly into 2.2 g of mercury chloride solution and stirred continuously until the red color precipitate formed disappeared or dissolved and a pale yellow solution formed. Sodium hydroxide was added into the solution and topped with distilled water to make a final volume of 100mL.

3.7.2 Enzymatic reaction preparation

A one (1) cm light path quartz cuvette was used to carry out the enzyme reactions in a spectrophotometric stop rate method using a modified method as described by Simas *et al*. (2021). Five hundred (500) µL of 50mM Tris –HCL, pH 8.6 and 400 µL of 100mM of l-asparagine prepared in 50mmTris-buffer, pH 8.6 was transferred to 2.0 mL microtube and equilibrated at 37°C for 2 minutes. After which 200 µL of the fermentation broth containing ASNase and 100 µL of sterile distilled water was added to give a final volume of 1200 µL. The reaction mixture was immediately mixed by inversion and incubated in

a water bath at 37°C for 1 hour. Two hundred (200) µL of TCA was added to stop the reaction. The reaction mixture was subsequently inverted to ensure uniform mixing and then clarified by centrifugation.

Except that the enzyme was added after the TCA was added, the preparation process for the control was identical to that for the reaction mixture. In the blank samples, standards, and controls, the final concentrations of TCA and Tris-HCL were 214.29 5 mmol L^{-1} TCA and 17.86 mmol L^{-1} Tri-HCL. Additionally, 28.57 5 mmol L^{-1} L-Asparagine was present in the controls and enzymatic reactions. Using 7.14-542.87 µmol L^{-1} $(NH_4)_2 SO_4$, a calibrated curve was created. Table 3.2 lists the stock solution volumes that were used to prepare the reaction mixtures.

Table 3.2. Volume of stock solutions (µL) required to prepared blank samples, control and test reactions

Sample (µL)	**Blank (uL)**	**Enzyme control (uL)**	**Enzyme reaction (uL)**
Enzyme solution	0	200	200
50 mmol L^{-1} Tris-HCl, pH 8.6	500	500	500
100 mmol L^{-1} L-Asparagine	0	400	400
1.5 mol L^{-1} TCA	200	200	200
Water	700	100	100
Total volume	1400	1400	1400

Note: TCA-Trichloroacetic acid

3.7.3 Quantification of ammonia by nesslerization

In the subsequent step, 200 microliters of supernatant from the reaction mixture described in section 3.7.2 was transferred to a 2.0 ml microtube containing 200 µL of Nessler's reagent. The volume was then adjusted by adding dH_2O to a final volume of 1400 µL. The solution was immediately mixed and incubated at room temperature for 1 minute, after which the absorbance was recorded at 436 nm. The NH_4^+ concentration was calculated against a standard curve of 7.12-542.87 µmol/L $(NH_4)_2SO_4$. A yellow color indicated the presence of ammonia, while a brown color indicated higher concentrations. The intensity of the yellow color formed at 436 nm was determined using a UV-visible spectrophotometer. A blank was prepared as shown in Table 3.2. Finally, ASNase activity was calculated according to the equation (Imada *et al*., 1973).

$$Units/\ ml = \frac{(\mu mol\ of\ \mathrm{NH3}\ liberate)(1.4)}{(0.2)(60)(0.2)}$$

Where

1.4 = volume of enzyme mixture use in step 1

0.2 = volume of supernatant enzyme mixture from step 1 in ml

60 = incubation time in minutes

0.2 = volume of test solution used in ml

One international unit (U) of ASNase is the volume of enzyme required to release 1µmole of ammonia at 37 °C in one minute.

3.8 Production of L-Asparaginase from endophytic fungi under submerged fermentation

ASNase production by endosymbiotic fungi was carried out using modified Czapek dox (MCD) medium under submerged fermentation process as described by Uzma et al., (2016) The isolated fungal strains were grown on the MCD media plates for 4 days and Cork borer used to transfer each inoculum of 4 mm culture disc from the MCD plates into 20mL of the Modified Czapek dox medium in a 100ml conical flasks. The flasks were incubated at 28°C for 6 days. The broth medium was centrifuged at 10,000 rpm for 10 min at 4°C. Uninoculated medium served as control. The crude enzyme (supernatant) was quantified for L-Asparaginase activity.

3.8.1 Harvesting of extracellular crude L-Asparaginase

The extracellular crude enzyme was harvested at the end of each production phase. This was done by separating the fermentation broth by centrifuging to recover the extracellular fraction. The clarified culture supernatant (extracellular fraction) was retained as the source of crude L-asparaginase.

3.8.2 Effect of Time of incubation on L-Asparaginase production

The fungal endophytes were used to produce L-Asparaginase in 20 mL of medium in 100 mL conical flasks. Each flask was inoculated with a 4 mm culture disc. The fungal cultures were then incubated at different intervals of growth (3, 6, 9 and 12 days) and tested in triplicate flask for each fungal isolate. The supernatant was harvested and

measured every 3 days for a total of 12 days. The L-Asparaginase activity of each sample was then quantified.

3.8.3 Effect of pH on ASNase enzyme production

In order to determine the optimal pH range for L-asparaginase production, a modified Czapek Dox broth medium was utilized and adjusted to varying pH values (ranging from 2 to 6) using 0.1 M NaOH and 0.1 M HCl and each fungal isolate were tested in triplicate flask. The selected pH range was based on previous literature indicating suggesting that a slightly acidic environment is ideal for fungal L-asparaginase production. Since many fungal enzymes tend to exhibit lower activity or stability at higher pH, broader alkaline ranges were not considered (Yap *et al*. (2022). Following this, the fungal culture was inoculated into the medium and allowed to incubate for 6 days at 28°C. Subsequently, the crude enzyme was harvested and quantified to assess the L-Asparaginase activity at each initial pH level.

3.9 Data Management and Statistical Analysis

A spreadsheet application was used to record the quantitative data, which was then imported into R for analysis. The mean ± standard error of mean is the statistical measure used to determine and present descriptive data. Incubation time (3, 6, 9 and 12 days) and each pH treatment (2 to 6) were tested in triplicate flask for each fungal isolate, thereby ensuring the reproducibility of the results. The data are presented as means ±standard error of the mean (SEM) and were subjected to analysis using one-way analysis of Variance (One-factor ANOVA) followed by Tukey's Honest Significant Difference

(HSD) test for multiple comparison. A p-value ≤ 0.05 indicated that the means were statistically different. Tables are used to present quantitative data.

CHAPTER FOUR

RESULTS

4.1 Isolation of endophytic fungi

The study examined 42 fungal endophytic strains that were obtained from *Periploca linearifolia* and *Prunus africana*. The endophytic fungi were isolated from 50 each of fresh leaves, stems, and roots segments of the medicinal plants. Sixty-four percent of fungal endophytes were recovered from *Prunus africana* compared to 36 % from *Periploca linearifolia*. Table 4.1 shows a detailed list of fungal strains isolated from different parts of *Prunus africana* and *Periploca linearifolia*.

Table 4.1 List of endophytic fungal isolates from *Prunus africana (PA)and Periploca linearifolia (L)*

Isolate no	Medicinal Plant	Plant part
PA_2	*Prunus africana*	Leaf
PA_3	*Prunus africana*	Leaf
PA_4	*Prunus africana*	Leaf
PA_5	*Prunus africana*	Leaf
PA_6	*Prunus africana*	Leaf
PA_7	*Prunus africana*	Leaf
PA_8	*Prunus africana*	Leaf
PA_9	*Prunus africana*	Leaf
PA_10	*Prunus africana*	Leaf
PA-11	*Prunus africana*	Leaf
PA_12	*Prunus africana*	Leaf
PA_13	*Prunus africana*	Leaf
PA_14	*Prunus africana*	Leaf
PA_15	*Prunus africana*	Leaf
PA_16	*Prunus africana*	Leaf
PA_17	*Prunus africana*	Leaf
PA_18	*Prunus africana*	Root
PA_19	*Prunus africana*	Root
PA_20	*Prunus africana*	Root
PA_21	*Prunus africana*	Root
PA_22	*Prunus africana*	Root
PA_23	*Prunus africana*	Root
PA_24	*Prunus africana*	Root
PA_25	*Prunus africana*	Root
PA_26	*Prunus africana*	Stem
PA_27	*Prunus africana*	Stem
PA_28	*Prunus africana*	Stem
L_5	*Periploca linearifolia*	Leaf
L_6	*Periploca linearifolia*	Leaf
L_7	*Periploca linearifolia*	Leaf
L_8	*Periploca linearifolia*	Leaf

Isolate no	Medicinal Plant	Plant part
L_9	*Periploca linearifolia*	Leaf
L_10	*Periploca linearifolia*	Leaf
L_11	*Periploca linearifolia*	Leaf
L_12	*Periploca linearifolia*	Leaf
L_13	*Periploca linearifolia*	Leaf
L_14	*Periploca linearifolia*	Stem
L_15	*Periploca linearifolia*	Root
L_16	*Periploca linearifolia*	Root
L_17	*Periploca linearifolia*	Root
L_18	*Periploca linearifolia*	Root
L_19	*Periploca linearifolia*	Root

4.2 Screening of endophytes that produce L-Asparaginase using a qualitative plate assay

Endophytic Fungal strains with the isolation numbers L-6, L_8, L-15, L-18, PA_20, PA_21, PA_24, PA_26, and PA_27 were hyper-producers of ASNase enzyme. A high intensity pink zone formed on modified Czapex Dox media indicate that the endosymbiotic fungal isolates were capable of producing the enzyme L-ASNase, which catalyzes the breakdown of the substrate asparagine to produce ammonia and aspartate (Fig 4.1).

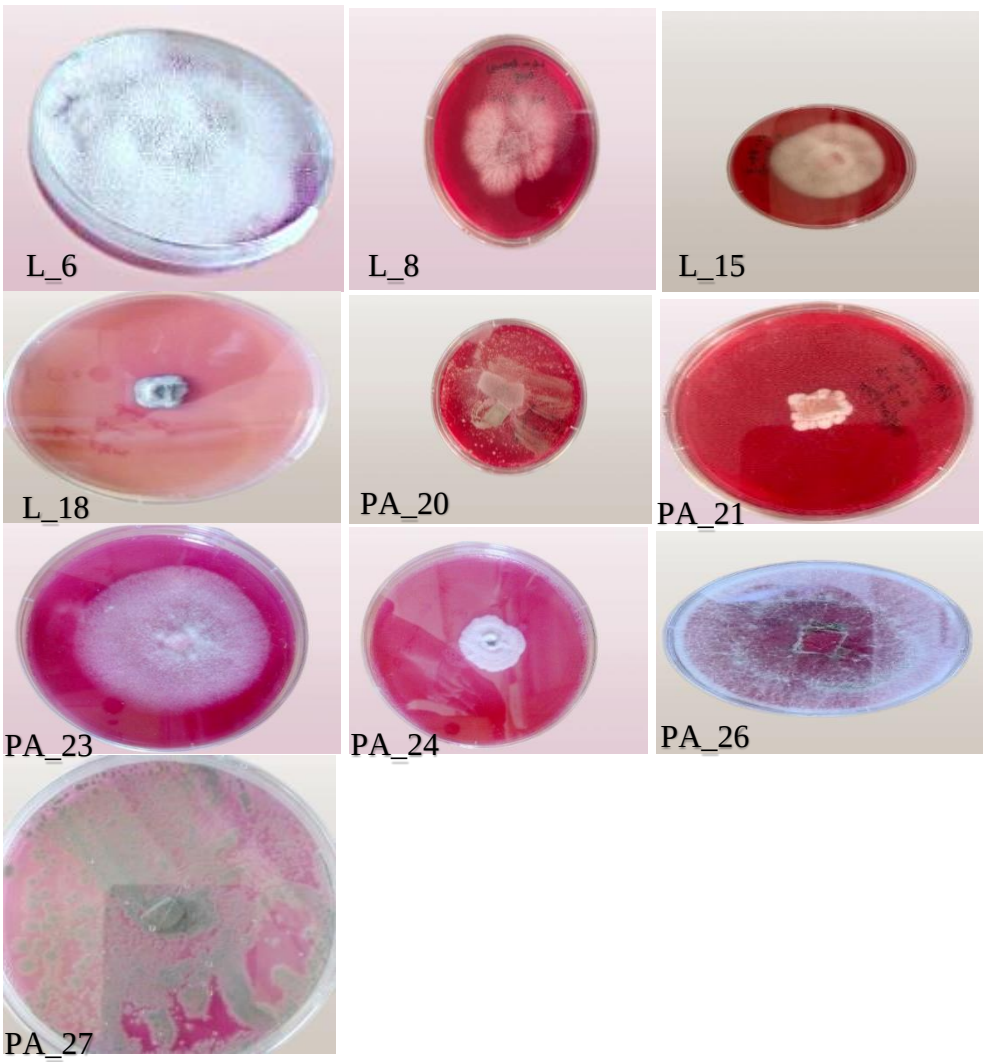


Figure 4.1 L-asparaginase activity detected by plate assay for isolate strains L-6, L_8 ,L_15, L_18, PA_20, PA_21, PA_23, PA_24, PA-26 and PA_27 growing on Modified Czapex Dox media with phenol red as a pH indicator. The isolate code L and PA represent *Periploca linearifolia* and *Prunus africana*, respectively.

4.3 Morpho-cultural Characterization of L-Asparaginase producing fungal endophytes

The data presented in Table 4.2 and Figure 4.2 provide a detailed analysis of the cultural morphology and reproductive structures of ten L-Asparaginase-producing fungal endophyte isolates after being cultivated on potato dextrose agar (PDA) at 28°C for 7

days.. The morphological traits of the colonies, including their hyphal structures and spore arrangements, were examined and recorded in Table 4.2 for identification purposes. The ten fungal isolates had distinct macroscopic and microscopic characteristics, which were carefully observed and documented. For instance, isolate L_6 was a fast grower with cottony olive-grey aerial mycelium on the upper surface and dark brown reverse color. The colony margin was irregular mycelium, with elongated septate acervuli and simple, globose to obovoid appressoria. Isolate L_8 was also a fast grower, initially with an aerial colony that was white and turned yellow-brown on the upper surface. The colony had many aerial mycelia, and the reverse color was light purple to yellow-brown with brown globose chlamydospores.

Isolate L_15 exhibited rapid growth and formed a cottony white colony with creamy to white-greyish coloration and a light yellow reverse side. The colony displayed irregular white margins with cream-yellow edges. The macroconidia were elongated and cylindrical, featuring parallel dorsal and ventral surfaces, three to five septa, and long, thin phialides. Isolate L_18 was a slow grower with a creamy colony color on the upper surface and blackish-gray bottom surface, a serrated edge, septic mycelium, and hyaline cylindrical conidia, with prominent scars. Isolate PA_20 was a moderate grower, forming a fasciculate to crustose colony with dull green to greyish-green color and corrugation. The colony had a thin white margin, and the reverse color was cream to intense yellow on PDA media. The fungal isolate had terverticillate conidiophores with large rough-walled stipes and smooth-walled, globose to subglobose spherical conidia.

Isolate PA_21 showed good sporulation on PDA media, with velvety or floccose mycelium, and dull-green or grey-green conidia. The sporulation was variable with white mycelia and a yellow-orange reverse color. It had a symmetrically biverticillate conidiophore with diverse short branch, long smooth stipe, and finely roughened, globose to subglobose conidia. Isolate PA_23 was a moderate grower with floccose aerial mycelium, smoke-grey olivaceous, and reverse concolorous with some reddish tinges. The colony had regular margins, with black pycnidia scattered over the media. Isolate PA_24 was a good grower with velvety conidia dull green to dark green. The colony had an inconspicuous mycelium, and the reverse color was pinkish-brown with the orange center on PDA media. The isolate had a symmetrically biverticillate conidiophore with a divergent short branch, smooth shorter stipes, and conidia globose to subglobose, finely roughed, and strongly pigmented cell wall. Isolate PA_26 had a white upper surface and grey-blackish bottom surface with a serrated edge. The conidia were septate, elongated shape, hyaline-colored, and needle-shaped. Finally, isolate PA_27 was a good grower with a floccose to fasciculate texture, blue-green to green color with white edge hem, and pale yellow reverse colony color on PDA media. The isolate had terverticillate rough-walled stipes, cylindrical phialides, and smooth-walled, globose to subglobose spherical conidia

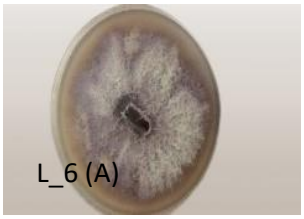
Table 4.2 Mopho-cultural characteristics of L-asparaginase producing fungal endophytes isolated from Prunus africana and Periploca linearifolia, culture on PDA at 28°C for 7 days under initial pH 6.0

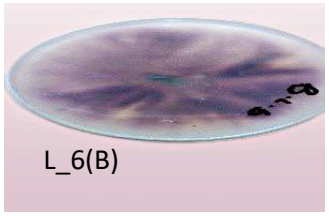
Isolate code	Host plant	Key macrosopic characteristics			Key microscopic characteristics	Isolate Names
		Surface	Reverse	margin		
L_6	*Periploca linearifolia*	cottony olive-grey aerial mycelium	dark brown	Irregular margin mycelium	Hyaline septate conidiophores, Round-ended, smooth walled and non-insulated single cell conidia, simple, globose to obovoid appressoria	*Colletotricum sydowii*
L_8	*Periploca linearifolia*	Fluffy, pinkish to brown red	light purple to yellow brown	Irregular margin mycelium	Abundant microconidia, oval to pyriform, spindle-shaped, thin-walled and hyaline. Macroconidia were thick-walled, sickle-shaped, hyaline and five to seven septate	*Fusarium sporotrichioides*
L_15	*Periploca linearifolia*	Cottony white to light yellow	light yellow	Irregular white margins	Cylindrical to oval microconidia, 3 to 5 septate, fusiform and	*Fusarium solani*

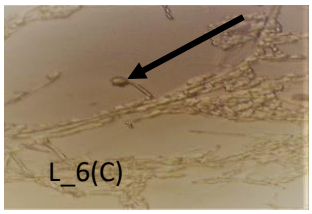
					cylindrical, hyaline, globose, smooth to rough-walled chlamydosphore	
L_18	*Periploca linearifolia*	creamy	blakish gray	Irregular margin mycelium	Cylindrical hyaline conidia with prominent scars, club-tube-shape, 3-7 insulated hypae, simple, straight conidiphores	*Cercospora canescens*
PA_20	*Prunus africana*	Dull green-Grey –green	Cream to yellow brown	Regular magin	Terverticillate conidiophore with rough-walled stripes, cylindrical phialides. Smooth-walled, globose to subglobose conidia	*Penicillium crustosum*
PA_21	*Prunus africana*	greenish	Pale yellow	Regular margin	Terverticillate conidiophore with rough-walled stripes, cylindrical Phialides. Smooth-walled, globose to subglobose conidia	*Penicillium pancosmium*

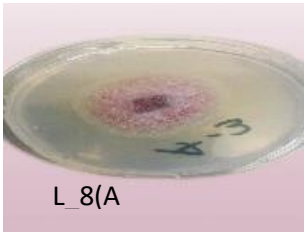
PA_23	*Prunus africana*	Olivaceous to greenish olivaceous	Concolorous with yellow tinges	Regular margin	Conidia are ellipsoidal to ovoid, sub cylindrical, thin and smooth-walled, hyaline, aseptate to septate, large, round to pyriform pycinidia	*Phoma* sp.
PA_24	*Prunus africana*	Dull green to dark green	Pinkish-brown with orange center	Regular margin	Sclerotia absent, conidiophore symmetrically biverticillate with a divergent short branch; smooth shorter stipes; conidia globose to subglobose, finely roughed, strongly pigmented cell wall.	*Penicillium ubiquetum*
PA_26	*Prunus africana*	white	Grey-black		Conidia are septate, elongated shape, hyaline-colored and needle-shaped.	*Septoria* sp

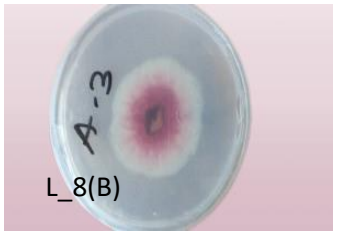
PA_27	*Prunus africana*	Blue-green to green color	creamy yellow	Regular margin	Terverticillate conidiophore with rough-walled stripes, cylindrical phialides. Smooth-walled, globose to subglobose conidia	*Penicillium commune*


L_6 (A)


L_6(B)


L_6(C)


L_8(A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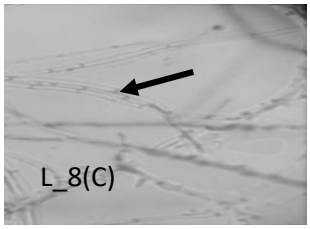
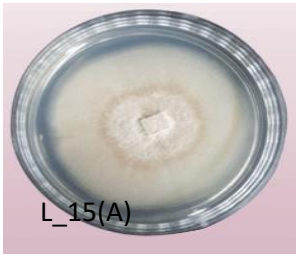
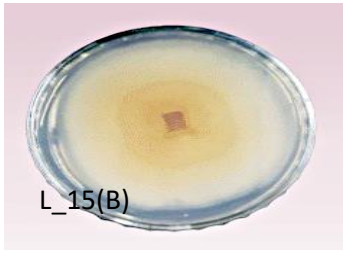
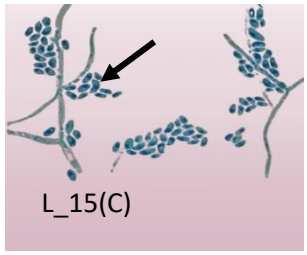
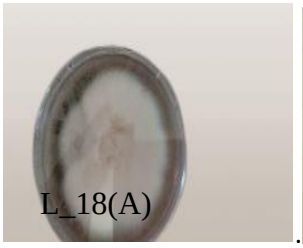
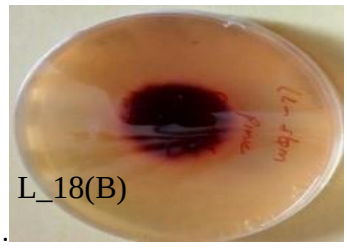

L_8(C)


L_15(A)


L_15(B)


L_15(C)


L_18(A)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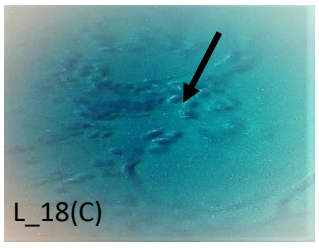
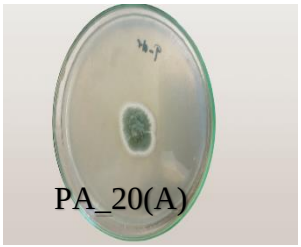
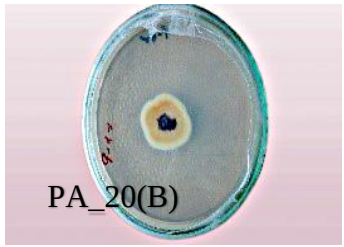
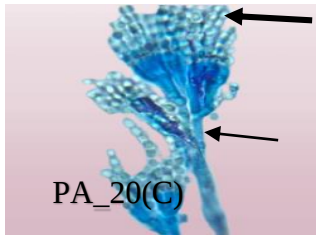
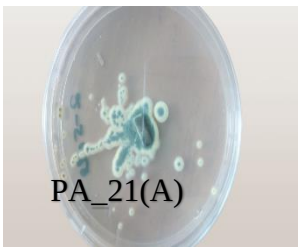

L_18(C)

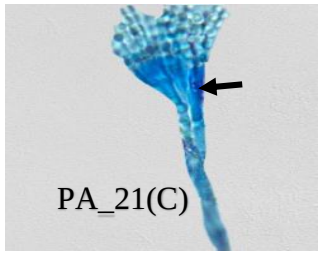

PA_20(A)


PA_20(B)


PA_20(C)


PA_21(A)


PA_21(B)


PA_21(C)

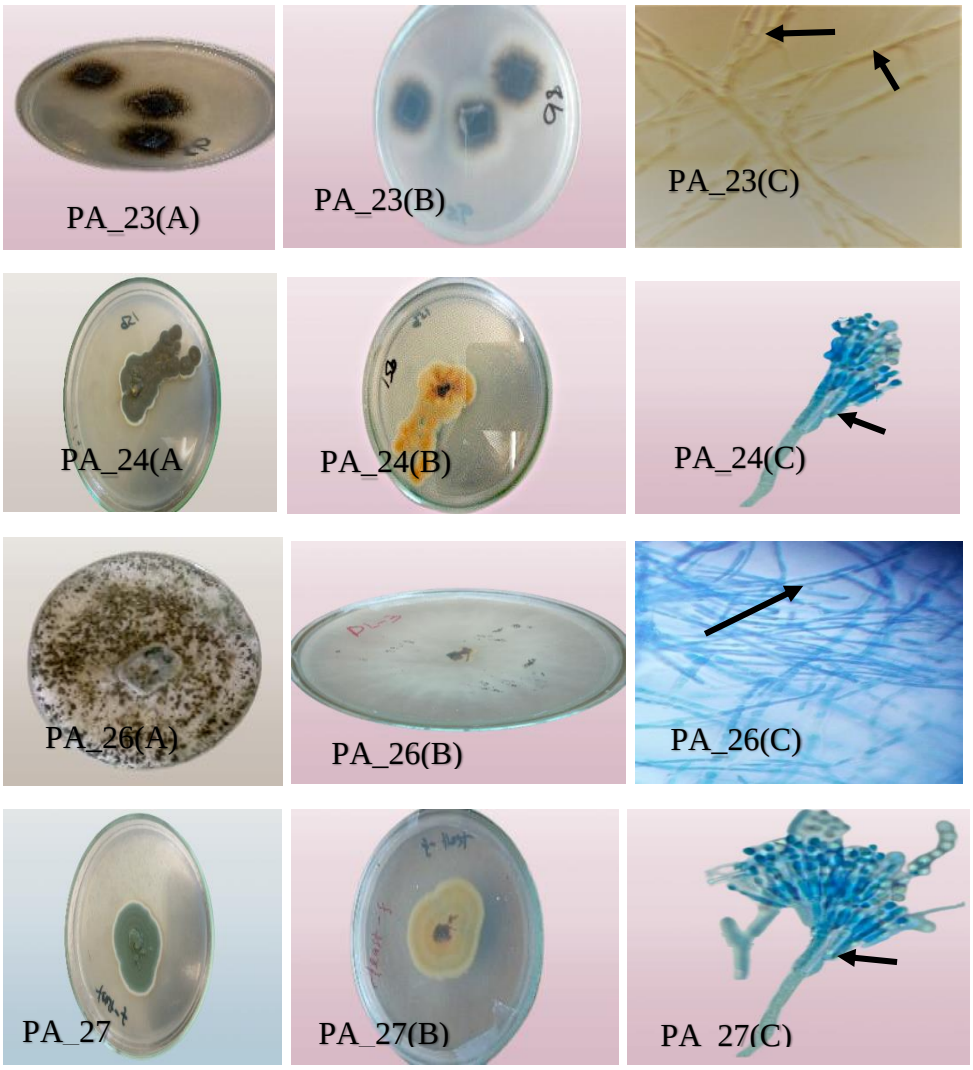


Figure 4.2 Cultural characteristic and micromorphology of the ten fungal endophytes. Letters **A**,**B** and **C** represent surface color, reverse color and micromorphology (magnification, x 400), respectively. *Colletotrichum sydowii* (**L_6**), *Fusarium sporotrichioides* (**L_8**), *Fusarium solani* (**L_15**), *Cercospora canescens* (**L_18**), *Penicillium crustosum* (**PA_20**), *Penicillium pancosmium* (**PA_21**), *Phoma* sp (**PA_23**), *Penicillium ubiquetum* (**PA_24**), *Septoria* sp (**PA_26**), and *Penicillium commune* (**PA_27**).

4.4 Molecular Characterization and Phylogenetic analysis of L-asparaginase-producing fungal endophytes

For molecular characteristics, figure 4.3 shows PCR amplification of the internal transcribed spacer (ITS) region which produces clear single bands of approximately 450-500 bp in all endophytic fungal isolates. ITS region sequences were amplified with the help ITS 1 and ITS4 primers, sequenced and submitted to GenBank with accession numbers ON882050, ON879761, ON880417, ON880423, ON880580, ON881143, ON881283, ON881292, ON882018 except HQ130716 which was retrieve directly from the NCBI database) Table 4.3. The assembled sequences of fungal endophyte isolates and related species were compared to determine their phylogenetic relationships. Data in Table 4.3 shows the closest species and their percentage similarity based on nucleotide Blast with 97-100% sequence similarity with relevant sequence in the NCBI database. Fig 4.4 clarified the phylogenetic analysis obtained using the maximum likelihood protocol. Molecular characteristics and phylogenetic analysis of L-asparaginase producing fungal endophyte strains were in agreement with the description of six genera and they were identified as *Colletotrichum sydowii* (L_6), *Fusarium sporotrichioides* (L_8), *Fusarium solani* (L_15), *Cercospora canescens* (L_18), *Penicillium crustosum* (PA_20), *Penicillium pancosmium* (PA_21), *Phoma* sp (PA_23), *Penicillium ubiquetum* (PA_24), *Septoria* sp (PA_26) and *Penicillium commune* (PA_27) as shown in Table 4.3. *Escherichia coli* was used as the Outgroup in this phylogenetic analysis.

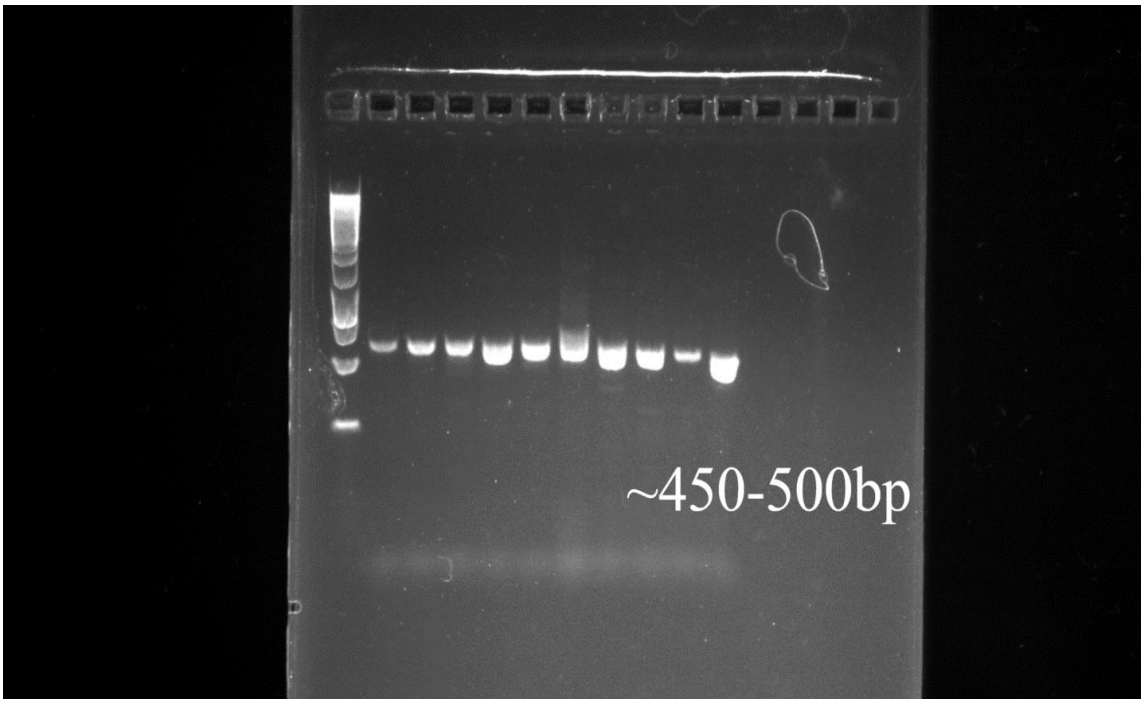


Figure 4.3 PCR gel image showing amplification of the internal transcribed spacer (ITS) region of fungal endophytes

Table 4.3 The species of ten representative of L-asparaginase-producing endophytic fungal isolates inhabiting P. linearifolia and P. africana based on similarities of ITS sequences to the NCBI database

Isolate code	GenBank accession numbers	Closest related species	Number of basepairs analyzed	Percentage similarity
L_6	ON882050	*Colletotrichum sydowii*	416	100
L_8	ON879761	*Fusarium sporotrichioides*	523	99.81
L_15	ON880417	*Fusarium solani*	508	97.66
L_18	ON880423	*Cercospora canescens*	522	99.81
PA_20	ON880580	*Penicillium crustosum*	499	100
PA_21	ON881143	*Penicillium pancosmium*	532	99.81
PA_23	HQ130716	*Phoma sp*	597	99.08
PA_24	ON881283	*Penicillium Ubiquetum*	538	100
PA_26	ON881292	*Septoria sp*	519	99.80
PA_27	ON882018	*Penicillium commune*	539	100

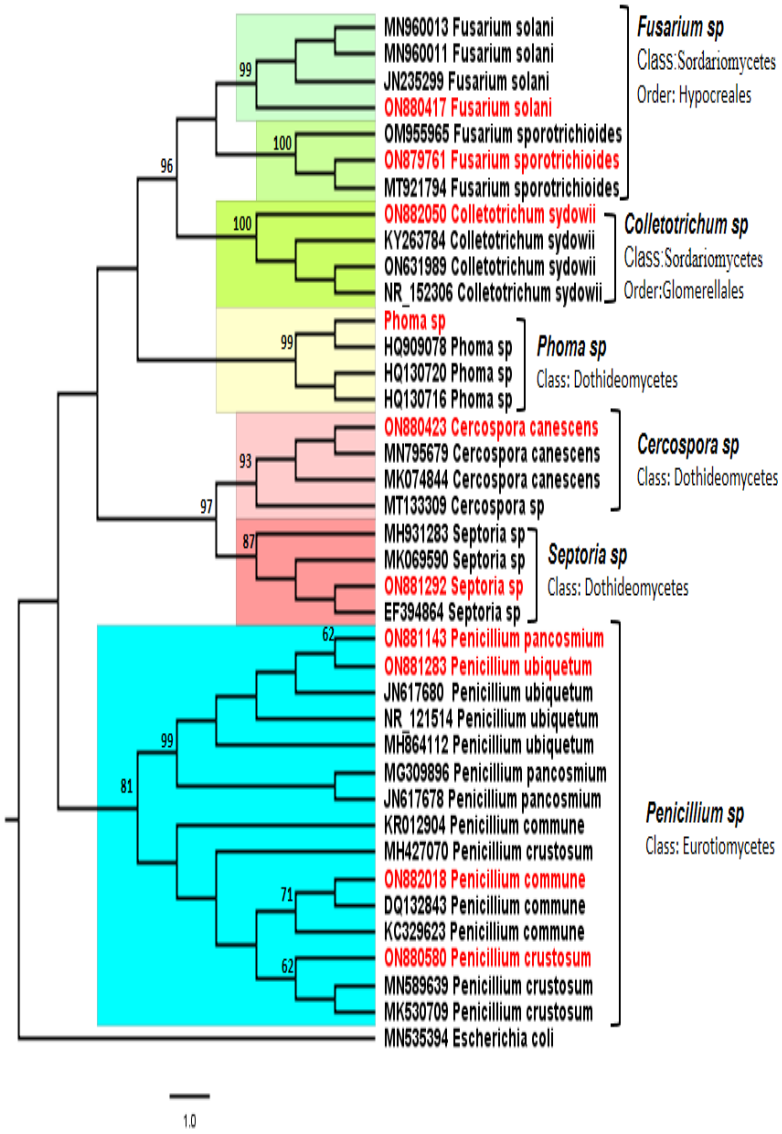


Figure 4.4 Maximum Likelihood (ML) analysis showing the Phylogenetic relation of L-Asparaginase producing fungal endophytes inhabiting *Periploca linearifolia* and *Prunus africana.* The tree has a total of 39 nucleotides sequences in which 10 endophytic fungal sequences came from the present study, 29 as reference sequeces of closely related species retrieve from NCBI database. and rooted with sequence from *E.coli* (MN535394) as an outgroup in this phylogenetic analysis. The type strains of fungal endophytes and their accession numbers from this study are highlited in red colors in bold font and Aerial format. The clades are further highlited in different colors and also represent clusters.

The six clusters shown by the phylogenetic tree in Fig 4.4 represent six different genera that produce L-asparaginase activity as follows: *Fusarium, Colletotrichum, Phoma, Cercospora, Septoria* and *Penicillium* and belong to phylum Ascomycota. The first cluster comprises two sub-clades of *Fusarium* species represented by a single fungal order Hypocreales. The first sub-clade belongs to the species of *Fusarium solani* that grouped with 99% bootstrap to sequences (MN960013 MN960011 JN235299) obtained from NCBI. The second sub-clade showed 100% bootstrapping to *Fusarium sporotrichioides* sequences. The *Colletotrichum* cluster represented by the Glomerellales fungal order grouped with 100% bootstraps to sequences KY263784, ON631989 and NR 152306. The next three clusters: *Phoma, Cercospora,* and *Septoria* were classified as Dothideomycetes grouped with 99%, 83% and 87% bootstrapping respectively with sequences obtained from NCBI as shown in Fig 4.4. The last cluster belongs to the Eurotiomycetes fungal class and was represented by three sub-clades of *Penicillium crustosum, Penicillium commune* and *Penicillium ubiquetum* and *pancosmium* isolates. *Penicillium crustosum* showed 62% bootstrapping while *Penicillium commune* grouped with 71% bootstrapping with sequences from NCBI. The sub-cluster comprising of *Penicillium ubiquetum* and *pancosmium* grouped under one clade showed 62% bootstrapping with sequences. A deeper phylogenetic analysis was performed by using *Escherichia coli* as an Outgroup.

4.5 Effect of time of incubation on L-ASNase production by endophytes

The Table 4.4 shows the effect of fermetation period for optimal production of L-ASNase by fungal endophytes isolated from *Prunus africana* and *Periploca linearifolia* cultured on modified Czapekk Dox broth medium under submerged fermentation for 12 days.

Samples were taken at 3 day interval and the Nessler method was used to quantify the L-ASNase activity. Time of incubation influenced L-ASNase production by fungal endophytes differently. Notably, L-Asparaginase production by *Penicillium ubiquetum*, *Penicillium pancosmium, Phoma sp, Penicillium crustosum, Fusarium. sporotrichiode, Cercospora canescens,* and *Penicillium commune* exhibited an increase from the 3rd day of incubation, reaching peak levels on the 6th day, and subsegently declining from the 9th day to 12th day of incubation.(Table 4.4).

Specifically,the production of L-Asparaginase by *P. ubiquetum* increased by an 8.0-fold from the 3rd day of incubation, reaching a peaked of 2.63±0.47 UI/ml on the 6th day, and the declining by 0.68-fold and 0.72-fold on the 9th and 12th day of incubation, respectively. Similarly, *P. pancosmium* demonstrated a remarkable 13.09-fold increase in L-ASNase production from the 3rd day of incubation, peaking at 1.44±0.1 IU/ml on the 6th day, followed by reductions of 0.9-fold and 0.75-fold from the 6th to the 9th day and from the 9th to the 12th day of incubation, respectively. As for *Phoma* sp, production of L-asparaginase reached a peak of 2.6±0.47 IU/ml on the 6th day of incubation, exhibiting a 3.02-fold increase from the 3rd day of incubation, followed by declines of 0.50-fold from the 6th to the 9th day and 0.81-fold from the 9th to the 12th day of incubation.

L-Asparaginase production by *P. crustosum* exhibited a 2.57-fold increase from the 3rd day of incubation, reaching a peak of 3.80±0.37 IU/ml on the 6th day before subsequently decreasing by 0.42-fold between the 6th and 9th days, and by 0.85-fold between the 9th and 12th days of incubation. Similarly, *F. sporotrichiode* demonstrated a 3.21-fold

increase in L-Asparaginase production from the 3rd day to the 6th day of incubation, with peak activity observed at 3.47±0.24 IU/ml on the 6th day, followed by a decrease of 0.74-fold on the 9th day and 0.85-fold on the 12th day. Meanwhile, *C. canescens* displayed a 2.20-fold increase in L-ASNase production from the 3rd day of incubation, reaching a peak of 2.24±0.12 IU/ml on the 6th day, and subsequently recording a decrease of 0.80-fold on the 9th day and 0.77-fold on the 12th day. Additionally, L-asparaginase production by *P. commune* peaked at 2.52±0.29 IU/ml on the 6th day of incubation, showing a 1.42-fold increase from the 3rd to the 6th day, before decreasing by 0.94-fold on the 9th day and 0.60-fold on the 12th day.

The production of L-Asparaginase by *C. sydowii* exhibited a 1.54-fold increase from the 3rd day of incubation to the 6th day, followed by a 1.61-fold increase from the 6th day to a peak on the 9th day at 4.0±0.13 IU/ml, before decreasing by 0.75-fold on the 12th day. In contrast, *Septoria sp* displayed a lower fold increase of 1.33 from the 3rd day to the 6th day, but a significantly higher fold increase of 5.34 from the 6th day to the 9th day peaking at 12.6±0.81 IU/mI, followed by a decrease of 0.25-fold from the 9th to the 12th day. *F. solani* demonstrated L-asparaginase production increases of 5.96, 1.60, and 1.27-fold between the 3rd to 6th day, 6th to 9th day, and 9th to 12th day, respectively, peaking at 12.4±1.12 IU/ml on the 12th day of incubation.

Table 4.4 Effect of incubation time on *L-Asparaginase* production by endophytes

Fungi	Time of incubation(days)			
	3	6	9	12
Penicillium ubiquetum	0.33 ± 0.12^{cCD}	2.63 ± 0.47^{abCD}	1.79 ± 0.19^{abD}	1.28 ± 0.22^{bcD}
Penicillium pancosmium	0.11 ± 0.03^{bD}	1.44 ± 0.1^{aD}	1.30 ± 0.18^{aD}	0.98 ± 0.21^{aD}
Phoma sp	0.86 ± 0.13^{bBCD}	2.6 ± 0.47^{aBCD}	1.29 ± 0.03^{bD}	1.04 ± 0.1^{bD}
Penicillium crustosum	1.48 ± 0.16^{bAB}	3.80 ± 0.37^{aB}	1.59 ± 0.16^{bD}	1.35 ± 0.03^{bCD}
Fusarium sporotrichioides	1.08 ± 0.14^{cABC}	3.47 ± 0.24^{aBC}	2.57 ± 0.15^{bCD}	2.13 ± 0.1^{bBCD}
Colletotrichum sydowii	1.64 ± 0.29^{cAB}	2.52 ± 0.08^{bcBCD}	4.06 ± 0.13^{aC}	3.05 ± 0.52^{abBC}
Septoria sp	1.77 ± 0.15^{bA}	2.36 ± 0.37^{bBCD}	12.6 ± 0.81^{aA}	3.21 ± 0.51^{bB}
Cercospora canescens	1.02 ± 0.17^{bABC}	2.24 ± 0.12^{aCD}	1.80 ± 0.27^{abD}	1.38 ± 0.31^{abCD}
Fusarium solani	1.03 ± 0.15^{dABC}	6.14 ± 0.15^{cA}	9.78 ± 0.36^{bB}	12.4 ± 1.12^{aA}
Penicillium commune	1.78 ± 0.12^{abA}	2.52 ± 0.29^{aBCD}	2.36 ± 0.28^{abD}	1.42 ± 0.13^{bCD}

Note:Values are the means of 3 replicates±SEM. Means expressed with different superscript capital letters within the same column are significantly different at $\rho \leq 0.05$. Mean expressed with different superscript small letters within the same row are significantly different at $\rho \leq 0.05$

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4.6 Effect of pH on L-Asparaginase production by fungal endophytes

Table 4.5 shows the impact of pH on the production of ASNase enzyme by 10 selected endophytic fungi isolated from *P.africana* and *periploca linearifolia*. Samples were incubated for 7 days and the Nessler method used to quantify the activity of L-ASNase produced. The pH of the culture media significantly influenced the production of ASNase enzyme by the 10 fungal endophytes. A significant increase in ASNase production was observed as the pH levels rose from 2 to 6 for all the fungi. This increase in ASNase production in response to increasing pH levels was found to be statistically significant.

At pH 2.0, *P. ubiquetum, P. pancosmium, Phoma sp, F. sporotrichiode, C. sydowii, C. canescens,* and *P. commune* demonstrated mimimal production of ASNase enzyme, while *P. crustosum Septoria sp* and *F. solani* exhibited low levels of L-Asparaginase activity of 0.06±0.01 IU/ml, 0.49±0.01 IU/ml and 1.18±0.01 IU/ml, respectively. Notably,at pH 3.0, the 10 endophytic fungi displayed significantly varied activities of ASNase, with *Septoria* sp recording the highest activity of 1.62±0.01UI/ml. Additionaly, at pH 4.0 the endophytic fungi exhibited significantly different L-Asparaginase activities, with *F. solani* recording the highest activity of 2.58±0.01UI/ml.

The L-Asparaginase activity varied significantly among the 10 endophytic fungi at pH 5.0, except for *F. solani* and *Septoria* sp, and *P. pancosmium* and *Phoma sp,* which did not exhibit significant differences. At pH 6.0, *F. solani* demonstrated the highest L-Asparaginase activity of 6.14±0.01, while *P. crustosum, Septoria,* and *P. pancosmium,* and *Phoma* sp did not display significant differences.

Table 4.5 Effect of pH on *L-Asparaginase* production

Fungi	pH				
	2	3	4	5	6
Penicillium ubiquetum	-0.15±0.00^{eDE}	0.07±0.01^{dH}	0.38±0.02^{cH}	1.34±0.03^{bH}	2.39±0.02^{aH}
Penicillium pancosmium	-0.24±0.02^{eE}	0.14±0.01^{dGH}	0.60±0.03^{cG}	2.28±0.04^{bF}	3.27±0.03^{aF}
Phoma sp	-0.15±0.01^{eDE}	0.21±0.01^{dG}	0.74±0.03^{cF}	2.32±0.02^{bF}	3.97±0.03^{aD}
Penicillium crustosum	0.06±0.01^{eC}	0.54±0.01^{dD}	1.68±0.03^{cC}	3.53±0.03^{bD}	5.48±0.01^{aB}
Fusarium sporotrichioides	-0.01±0.04^{eCD}	0.75±0.02^{dC}	1.07±0.02^{cE}	2.07±0.03^{bG}	2.71±0.04^{aG}
Colletotrichum sydowii	-0.06±0.08^{eCD}	0.44±0.01^{dE}	1.09±0.02^{cE}	2.54±0.02^{bE}	3.80±0.01^{aE}
Septoria sp	0.49±0.01^{eB}	1.62±0.01^{dA}	2.05±0.01^{cB}	2.57±0.02^{bE}	3.20±0.01^{aF}
Cercospora canescens	-0.16±0.04^{eDE}	0.55±0.01^{dD}	1.72±0.03^{cC}	4.69±0.02^{bB}	5.40±0.05^{aB}
Fusarium solani	1.18±0.01^{eA}	1.45±0.02^{dB}	2.58±0.01^{cA}	5.19±0.03^{bA}	6.14±0.01^{aA}
Penicillium commune	-0.08±0.03^{eCDE}	0.31±0.01^{dF}	1.52±0.01^{cD}	3.74±0.03^{bC}	4.88±0.06^{aC}

Note:Values are the means of 3 replicates±SEM. Means expressed with different superscript capital letters within the same column are significantly different at P<0.05. Mean expressed with different supersript small letters within the same row are significantly different at $\rho \leq 0.05$

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

ASNase has been well-established as an effective antitumor drug use to treat acute lymphocytic leukemia and childhood leukemia (Ali *et al*., 2016). The irreversible hydrolytic reaction of ASNase under physiological conditions highlights its significance as a therapeutic agent in cancer treatment. Fungal isolates capable of producing ASNase enzyme have been found in different environmental sources, including soil, plants, sediments, and water. This study is focused on characterizing fungal endophytes from *Prunus africana* and *Periploca linearifolia* with L-Asparaginase activity, while also assessing the impact of pH and fermentation period on the synthesis of ASNase enzyme. The present study revealed that nineteen percent of fungal endophyte isolates from medicinal plants exhibited significant L-asparaginase production capabilities.

The qualitative plate assay method was employed as an effective approach for the rapid screening of L-Asparaginase enzymes produced by endophytic fungal isolates. This method can be readily executed, allowing for direct visualization of the enzyme activity of fungal strains without the need for complex assays or reagents. The semi-quantitative method employs phenol red or bromothymol blue as pH indicators in the screening media (Doriya and Kumar, 2016). This study uses phenol red to screen ASNase enzyme production. The ASNase enzyme hydrolyses substrate L-Asparagine, yielding aspartate and ammonia (Chow and Ting, 2015). The resultant ammonia alters the media pH from acidic to alkaline. The phenol red exhibits a yellow color in acidic media and transitions

to pink in alkaline media. Consequently, a pink zone formed around the colonies resulted from the alkalinity from ammonia production (Doriya and Kumar, 2016).

The morpho-cultural characteristics were regarded as taxonomically significant for identification of endophytic fungal isolates. The study unveiled distinct macroscopic and microscopic traits that facilitated the identification of fungal isolates at the genus level (Wanget *et al*., 2016). The endophytic fungi were classified under the genera *Fusarium*, *Colletotrichum*, *Cercospora*, *Penicillium*, *Phoma*, and *Septoria*, aligning with the findings of Hatamzadeh *et al*. (2020). Moreover, sequencing of the ITS regions allowed for the classification of endophytic fungal isolates at the species level based on nucleotide conservation. This region is widely acknowledged as a fungal barcode and is instrumental in identification, phylogenetic, and systematics. The 5.8S-ITS region has previously been utilized for the genus-level identification of endophytic fungi (Khalmuratova *et al*., 2021).

According to the present study, 24% of the endosymbiotic fungal strains obtained from medicinal plants *Periploca linearifolia* and *Prunus africana* exhibited positive response for ASNase enzyme activity. These observations are in line with previous studies that have also found fungal endophytes with L-Asparaginase activity. Arumugam *et al*. (2021) found that 33% of the twenty-seven isolated endophytic fungi demonstrated L-Asparaginase activity. Additionally, Hatamzadeh *et al*. (2020) documented that 45% of the 84 endophytic fungal isolates from *Asteraceae* family members recorded ASNase activity. In a separate study, Singh and Sao (2021) reported four fungal endophytes from

Ocimum santum, exhibiting moderate to high L-Asparaginase activity. Furthermore, Araújo-Magalhães *et al*. (2021) screened fourteen isolates from the leaves of *Mandevilla catimbauensi* for L-Asparaginase activity and found that all fourteen were positive for L-asparaginase production. Similarly, Elangovan and Gnanadoss (2023) reported that all thirty-five fungal endophyte isolates from plants of the *Rutaceae* family tested positive for the L-Asparaginase enzyme.

The findings in this study indicate that most endophytic fungi achieved optimum incubation period for maximum enzyme activity at different days of incubations (6, 9 and 12). These observations suggest that endophytic fungi may display varying growth rates, exponential phases, and fermentation periods to attain maximal growth and L-Asparaginase production (Yap *et al*., 2021). A study by Yadav and Sarkar (2014) reported that *Fusarium oxysporum* achieved its maximum ASNase activity of 182.5 U/mL on the 5^{th} day of the incubation period. Similarly, Yap *et al*. (2021) documented that the fungal endophyte *Fusarium proliferatum* exhibited the best L-ASNase activity of 16.75 U/mL on the 5^{th} day of incubation. Furthermore, Elangovan and Gnanadoss (2023) noted that *Fusarium* sp. reached its optimal L-ASNase activity of 8.84±0.35 UI/mL on the 5^{th} days of incubation, which is in close agreement with *Fusarium sporotrichioides*, an endophytic fungus isolated in this study.

Similarly, the endophytic fungus *Fusarium solani* examined in this study exhibited slow growth, a prolonged exponential phase, and fermentation period to achieve maximum L-Asparaginase enzyme activity of 12.4±1.12 UI/mL at 12 days under submerged

fermentation. In contrast, Isaac and Abu-Tahon, (2016) isolated *Fusarium solani* from soil sample and reported an optimal 5-day incubation period to attain the best ASNase activity of 192.3 U/mL under solid-state fermentation. This difference may be due to the fundamental differences between the two fermentation methods. Submerged fermentation (SmF) offers better control over environmental factors such as pH, temperature, and oxygen transfer, making it suitable for studying enzyme kinetics.

While, solid state fermentation (SSF) typically enhances the production of secondary metabolites because it mimics the organism's natural habitat, which may result in higher yields of L-Asparaginase. Additionally, factors like substrate type, moisture content, aeration, and the variability of fungal strains can significantly affect the results. Thus, while SmF is optimal for reproducibility and easier downstream processing, SSF may be more advantageous for maximizing enzyme yield. This potential for higher yield in SSF could be the focus of future optimization studies. Moreover, Manasa and Nalini (2014) noted significantly lower L-Asparaginase activities of the endosymbiotic fungus *Fusarium* sp. on the 5th day of incubation.

On the other hand, *Colletotrichum sydowii* exhibited moderate growth, reaching its peak L-asparaginase activity on the 9^{th} day, with recorded activity of 4.06±0.13 IU/mL, which is consistent with the findings reported by (Yap *et al*., 2022). In contrast, Manasa and Nalini (2014) observed significantly lower L-asparaginase activity on the 5^{th} day. *Septoria* sp. also showed moderate growth, achieving its maximum L-ASNase activity of 12.6±0.18 IU/mL on the 9th day, contrasting with the considerably lower ASNase

enzyme activity of 0.208 Unit/mL at 5 days reported by Hatamzadeh *et al*. (2020) for a similar endophytic fungus. *Penicillium* species were fast growers, reaching their maximum L-Asparaginase activity within 6 days, ranging from 1.44±0.1 IU/mL to 3.80±0.37 IU/mL, was in agreement with the findings observed by (Soniyamby *et al*., 2011).

Moreover, *Phoma* sp. and *Cercospora canescens* in the present study exhibited fast growth, reaching the stationary phase at day 6, after which a decline in the production of ASNase was observed. The decrease in L-asparaginase activities after reaching the peak may be attributed to various factors, including limited nutrient availability, the accumulation of inhibitory end products such as ammonia altering the pH of the culture media, and enzyme inhibitors hindering the growth of fungal isolates and the synthesis of L-ASNase enzyme (Papagianni, 2004). Additionally, the varying L-Asparaginase activity produced by different species of fungal endophytes may be attributed to other factors including the substrate used in the synthesis of enzymes, the solid or liquid culture media, and the different species of fungal endophytes belonging to the same genus, additive nutrients, and other cultural conditions (Chow and Ting, 2015).

The pH of the culture broth (2-6) had an adverse effect on the production of ASNase enzyme activity by fungal endophytes under submerged fermentation. The findings of this study revealed that a pH difference in the culture media (2-3) significantly influenced the production of L-ASNase activity. The low L-ASNase production observed within the pH range of 2.0-3.0 may be ascribed to the adverse impact of pH on microbial enzyme

production (Yap *et al*., 2022). This is because a highly acidic environment affects the fungal metabolic processes, membrane permeability of the cell, and causes hydrolysis of the peptide bond leading to irreversible damage to the enzyme (El-Gendy *et al*., 2015; Pallem, 2019b).

Fungal endophytes were able to adapt to a slightly acidic pH (5-6), thus producing a significant amount of L-ASNase enzyme. This shows the capability of most fungi to withstand a wide range of pH (4-6) with maximum growth and sporulation observed at slightly acidic conditions. Similar observations were reported by Mohsin *et al*. (2012), Yadav and Sarkar (2014), Yap *et al*. (2021), and Yap *et al*. (2022).

The study was limited by the fact that sampling was conducted in only one season, which may not accurately reflect the year –round diversity of fungal endophytes. Furthermore, the enzyme assays were limited to crude extracts, and their kinetic characterization was not undertaken. This impedes the comprehensive understanding of the enzyme properties. These limitations underscore the need for follow-up studies that incorporate sampling across multiple seasons and employ purification and biochemical assays.

5.2 Conclusions

i) Endophytic fungi isolated and identified from *Prunus africana* and *Periploca linearifolia* may be used as potential candidates for production of L-Asparaginase

ii) Time of incubation and pH have significant influence on the production of L-Asparaginase enzyme, by endophytes isolated from *Prunus africana* and *Periploca linearifolia*.

5.3 Recommendation

5.3.1 Recommendation from the Study

i) Fungal endophytes isolated from *Prunus africana* and *Periploca linearifolia* can be used as sources for the production of L-asparaginase.

ii) The time of incubation and pH for optimum production of L-asparaginase from fungal endophytes isolated from *Prunus africana* and *Periploca linearifolia* should be determined individually from each fungus.

5.3.2 Recommendation for future Studies

i) Further studies should focus on the purification and biochemical characterization of L-asparaginase enzyme from the different fungal isolates inhabiting *Periploca linearifolia* and *Prunus africana*.

ii) The purified L-asparaginase should be tested in animal model to determine the immunogenic activities and its safety profile

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