Characterisation of *Ganoderma* Species Using Morphological, Molecular and Biochemical Markers and Evaluation of Substrate Enhancement Influence on Their Development and Biochemical Profile

By

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DECLARATION

I, Sydwell Mcebo Sihlangu, duly declare that the entirety of the work contained therein is my original work. This dissertation has not been previously in its entirety or in part tendered by me or any other person for obtaining a qualification at this institution or any other university. I further declare that all designs, structures, materials, and sources contained herein have been acknowledged.

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SUMMARY

Ganoderma, also known as Reishi mushroom, is used for its potential health benefits in several countries. The current study characterised *Ganoderma* species using molecular and biochemical markers and evaluated the substrate enhancement influence on its development and biochemical profile. The overall research study consisted of four objectives. The first objective focused on the isolation and characterisation of fifteen fungal specimens collected from the three provinces of South Africa, namely, Mpumalanga, KwaZulu-Natal, and North-West. Five fungal specimens were collected in each province and growth media potato dextrose agar (PDA), malt extract agar (MEA), and sabouraud dextrose agar (SDA) were used to grow sample isolates. After 8 days of incubation, MEA recorded the highest mycelial diameter followed by PDA and SDA. Samples were identified using comparative morphology traits and supported by internal transcribed spacer region (ITS) and phylogenetic analyses. Based on the ITS of ribosomal DNA, fungal samples KG3SY219 and MG1SY119 were found to be closely related to *Ganoderma resinaceum* and *Ganoderma austroafricanum*, respectively. The species were further characterised by biochemical compounds, including antioxidants, proteins, essential elements, and heavy metals. The antioxidant capacity exhibited a higher radical scavenging activity in *G. austroafricanum* compared to *G. resinaceum*. The concentrations of total phenolics, flavonoids, proteins, essential elements and heavy metals were more abundant in *G. austroafricanum* compared to *G. resinaceum*.

This study also evaluated the effect of different growth conditions on mycelial growth and development of *Ganoderma austroafricanum* and *Ganoderma resinaceum*. The experimental treatments included three levels of pH (4, 6 & 8), temperature (20, 25 & 30 °C), and different types of plant residues namely; beech sawdust (BS), sugarcane bagasse (SB), and buffalo grass (BG). Three independent *in vitro* experiments were conducted, PDA and MEA were used as standard growth media to grow each fungal species. Mycelial growth and development were measured over 9 days where they reached maximum growth. The culture media pH results demonstrated that the maximum growth for mycelia was reached on day 9 for both species. Typically, *G. resinaceum* showed the highest mycelial growth for both cultures except for days 6 and 9 where the mycelial growth of the species was decreased by low levels (pH 4) and high levels (pH 8), respectively. The addition of BG to the growth media delayed the mycelial growth of *G. resinaceum* for both growth media (PD+MEA).

Experiment three investigated the effect of different substrates on the development, total biomass, and biochemical profile of *Ganoderma* species. The experiment involved growing *G. austroafricanum* (GA) and *G. resinaceum* (GR) on different substrates; beech sawdust (BS), sugarcane bagasse (SB), and buffalo grass (BG), and suspension of all substrates (BSSBBG). The growth parameters such as pileus size, weight, total biomass, and biological efficiency were measured. Additionally, each substrate was analysed for biochemical composition. The biochemical composition of the harvested samples was also analysed to determine the levels of biochemical compounds such as minerals, antioxidants, and protein. The substrate pH levels demonstrated that all substrates were within the optimal growth pH range (5-6). SB exhibited greater levels in the majority of essential elements such as Zn and K, also, heavy metals Pb and Hg. The results on the development and total biomass production of *Ganoderma* species revealed significant variations across different substrates. In terms of development, GRBS was faster to reach the 100% rate of all production parameters in 40-52 days after inoculation. However, GASB exhibited higher quantities in total yield and biological efficiency. In addition, pileus from GASB demonstrated higher concentrations of all evaluated biochemical compounds. GASB also yielded higher levels of DPPH, phenolic compounds, flavonoids, and protein.

Experiment four examined the impact of substrate fortified with essential elements on the development, total biomass, and biochemical compounds of *Ganoderma* species. The experiment involved growing *G. austroafricanum* (GA) and *G. resinaceum* (GR) on beech enhanced with elements; no element (Control), $Zn(NO_3)_2.6H_2O$ (Zn), Fe_2SO_4 $7H_2O$ (Fe), Na_2SeO_3 (Se), and suspension of all essential elements (ZnFeSe). The growth parameters such as the pileus size, weight, total biomass, and biological efficiency were measured. In addition, the biochemical profile of *Ganoderma* spp. was analysed to evaluate the concentration of compounds. The development and total biomass production findings for the substrate fortified with essential elements exhibited significant differences. GRZn developed expeditious, reaching 100% of all production parameters in 52 days after inoculation. In comparison to all treatments, GAFe showed larger quantities in total yield and biological efficiency. The substrate enhancement with Zn had a significant increase in the majority of minerals. GAZn exhibited higher concentrations of essential elements such as Zn, K, and Mg. Higher levels of heavy metals such as Cd, Pb, and As were recorded from GAControl. GASe produced higher

levels of DPPH, phenolic compounds, flavonoids, and protein reading. These findings demonstrate the variability of morphological characteristics, biochemical compounds, and growth conditions requirements between *Ganoderma* species. These findings provide valuable insights into the diversity, taxonomy, and potential therapeutic applications of *Ganoderma* species in South Africa. Further investigation is required to identify *Ganoderma* species and its pharmaceutical properties.

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DEDICATION

I dedicate this dissertation to both my parents Mr. Irvine A. Sihlangu and Mrs. Sitani Magaret Sihlangu who never got the opportunity to go to school. I remain eternally grateful for being the greater source of support to pursue my dream of studying further despite poverty. Also, to all my not-yet-born children “Everything great comes with a great price in this life, always be tenacious and never succumb to any form of pressure”.

PREFACE

This dissertation is presented as a compilation of manuscripts with each chapter serving as an independent entity. Some repetition between chapters has, therefore, been unavoidable. This dissertation represents the culmination of years of hard work, dedication, and passion for the subject matter. It is with great pleasure and a sense of accomplishment that I present this research to the academic community. The journey towards completing this thesis has been both challenging and rewarding. It has allowed me to delve deep into my chosen field of study, exploring its intricacies, and expanding my knowledge and understanding. Throughout this process, I have encountered numerous obstacles and setbacks, but I have also experienced moments of inspiration and breakthroughs that have propelled me forward.

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LIST OF PEER REVIEW PAPERS

1 Determination of optimum temperature and pH for the mycelial establishment of *Ganoderma* fungal species under different growth media – Plant-Environment Interactions Journal (Manuscript ID: PEI3-2024-0011)

2 Morphological, molecular and biochemical characterisation of *Ganoderma* species from selected provinces in South Africa – Journal of Ethnopharmacology (Manuscript ID: JETHNO-D-24-00404)

CHAPTER 1
GENERAL INTRODUCTION AND RESEARCH AIMS

1 Introduction

Ganoderma species is one of the oldest and well-known fungal-based medicine (Nikšić et al., 2022; Adewole, 2023). It has been extensively used for over 4,000 years in many traditional bioremediation practices (Ahmad et al., 2021a; Lilburn, 2022). In Asian countries such as China, Japan, and Korea, the *Ganoderma* species is still frequently used as a traditional medicine (Singh et al., 2021; El Sheikha, 2022; Raman et al., 2022). *Ganoderma* is a genus of polypore mushrooms that belong to the kingdom of Fungi, phylum of Basidiomycota, class of Agaricomycetes, order Polyporales, and the family of *Ganodermataceae* (Richter et al., 2015; Basnet et al., 2017). The genus has species ranging from 250 to 400 and mostly originates from tropical regions (Kirk et al., 2008; Richter et al., 2015; Suldbold, 2017; Peng and Qiu, 2018; Yalcin et al., 2020). However, other reports reveal records of more than 400 species including synonyms (Baby et al., 2015; Ranadive and Jagtap, 2016; Basnet et al., 2017). The recent search for "Ganoderma" in the database Index Fungorum reveals 421 species records (Cabi Databases, 2023). This fungus grows as mycelia within the woods of living and dead plant material (Bishop et al., 2015; Jandaik and Gupta, 2022). They are commonly lignicolous and consist of leathery pileus with double-walled, truncate spores with yellow to brown ornamented inner layers (Mawar et al., 2020). It forms a pileus which is approximately 10 to 100 centimetres across, hard, woody-textured, and inedible (Erkel, 2009; Mohammadifar et al., 2020).

There are several claims reported on the substantial role that *Ganoderma* plays in medicine worldwide to address numerous human illnesses (Benzie and Wachtel-Galor, 2011; Gonul et al., 2015; Richter et al., 2015). These species consist of several nutritional values and pharmaceutical properties that are important for the human body (Ma et al., 2015; Hu et al., 2018; Khatian and Aslam, 2018; Bhat et al., 2019; Sudheer et al., 2019; Xu et al., 2019b; Yang and Yang, 2019; Li et al., 2020; Ahmad et al., 2021b). *Ganoderma* extracts are also reported to contain biochemical compounds and essential elements (Cör et al., 2018; Bulam et al., 2019; Ahmad et al., 2022). Approximately 400 biochemical compounds are reported from its mycelia, pileus, and spores (Wu et al., 2013; Ahmad, 2018; Sudheer et al., 2019; Ahmad et al., 2021b). The prolific biochemical compounds include *Ganoderma* triterpenes (GTs), polysaccharides, proteins, steroids, peptides, amino acids, alkaloids, nucleotides, lactones, and

unsaturated fatty acids (Dou et al., 2014; Xia et al., 2014; Shah and Modi, 2018; Sudheer et al., 2019; Liu et al., 2020; Sułkowska-Ziaja et al., 2022). In addition, about 90% of *Ganoderma* pileus weight contains water and the remaining 10% is made of other various constituents (Khatian and Aslam, 2018). These constituents include protein, carbohydrates, fibre, ash, minerals such as Zn, Fe, Ca, P, K, Mg, Cu, and Se, and vitamins such as B1, B2, B6, choline, and inositol (Zhou et al., 2007; Cör et al., 2018; Bulam et al., 2019). The biochemical compounds are responsible for its pharmaceutical properties such as anti-viral, anti-microbial, anti-cytotoxic, anti-cancer, anti-oxidant, anti-staphylococcal, anti-inflammatory, anti-diabetic, and anti-ageing properties (Liu et al., 2014; Linnakoski et al., 2018; Wang et al., 2020; Ahmad et al., 2022).

However, the literature suggests that the nutritional quality and pharmaceutical properties vary within species (Bishop et al., 2015; Sharma et al., 2019; Sudheer et al., 2019; Venturella et al., 2021). This variation is attributed to the different climatic conditions or environments from which they grow (Islam et al., 2018). A wide range of factors such as the species type, substrate, body part, pileus maturity, temperature, pH, and postharvest handling are also known to have a direct influence on their biochemical composition (Fang and Zhong, 2002; Colak et al., 2009; Wandati et al., 2013; Sudheer et al., 2018; Sudheer et al., 2019; Cho et al., 2021). As a result, the production/cultivation of *Ganoderma* species has been met with so many challenges such as maintaining good quality (Dong et al., 2021; Singh et al., 2021; Suansia and John, 2021). Initiatives on various techniques that have the potential to enhance yield quantity, nutritional quality, pharmaceutical properties, and ensure purity have been explored (Bishop et al., 2015; Singh et al., 2021). Previous studies have used several plant growth regulators (PGRs) to enhance various mushroom production activities (Khandakar, 2004; Maniruzzaman, 2004; Ramachela and Sihlangu, 2016; Kumar et al., 2017; Vi et al., 2018; Xu et al., 2019a; Vedenicheva et al., 2021). The approach to enriching substrate with essential elements has also been used not only to improve mushroom yield and quality but also for functional food and diet supplement production (Zhao et al., 2004; Nunes et al., 2012; Gąsecka et al., 2016; Rzymski et al., 2016; Rathore et al., 2019; Velez et al., 2019; Zięba et al., 2020).

Despite all the current achievements and ongoing efforts made to improve the yield and quality of *Ganoderma* species, proper identification of the species remains a challenge, globally. This tends to hinder the authenticity claims of commercials, patents, and publications (Bishop et al., 2015). The classification of *Ganoderma* is primarily based on morphological characteristics (Seo and Kirk, 2000; Chen et al., 2017). Pileus features such as colour, texture, size, and

basidiospore wall are often used to characterise the morphology of *Ganoderma* species (Erkel, 2009; Richter et al., 2015; Jargalmaa et al., 2017). However, this technique of identification or characterisation of species is considered as inconclusive for accurate classification (Kwon et al., 2016; Suldbold, 2017). The difficulties in accurately identifying these species is due to the high similarities of their pileus features (Kinge et al., 2015). In Africa, more than forty-nine various species have been identified (Kinge et al., 2015), with twenty species being reported to have originated from Southern Africa (Ueitele, 2021). However, these species were morphologically identified, their phylogenetic position is unknown and there are no DNA sequences provided for samples (Coetzee et al., 2015; Ueitele, 2021).

1.1 Problem statement and justification

An estimated 2.2 to 3.8 million fungal species exist in the biosphere of the fungal kingdom with only 146 150 identified (Wanasinghe et al., 2022b), and 96% of these species remain unknown (Maraz and Khan, 2021; Wanasinghe et al., 2022a). Approximately 140 000 fungal species are considered to be mushroom species, yet only 10% have been formally described (Jo et al., 2009; Bhat et al., 2021). In general, a larger number of mushroom species are reported to contain numerous nutritional quality and pharmaceutical properties (Romorosa et al., 2017). However, from the identified 10% of world macro-fungi, 5 000 species are described to be edible and just over 1 800 species are considered to have pharmaceutical properties (Roberts, 2004). In South Africa, a total of thirteen *Ganoderma* species have been reported. Most of these species were mainly identified in Gauteng, Western Cape, KwaZulu-Natal, Eastern Cape, Mpumalanga, Free State, and Limpopo regions. However, there is no sufficient information on whether these species represent the whole gene pool of *Ganoderma* species present in South Africa. Previous reports reveal that there is insufficient information regarding the identity of macro-fungi in South Africa's natural ecosystem. Recent studies suggest that there is a continuous discovery of new *Ganoderma* species in South Africa. Therefore, it is likely that a great number of other species are yet to be discovered. Furthermore, there is very little scientific knowledge of whether there is a variation of biochemical compounds of *Ganoderma* species occurring in various parts of South Africa. As a result, the taxonomic status, and biochemical compounds of *Ganoderma* species in South Africa is currently uncertain.

In addition, only a few of these species are intensively investigated whereas the chemistry and bioactivities of many other species remain unknown. The biochemical composition and pharmaceutical properties of macro-fungal species vary in terms of their efficacy, depending

on the type of substrate in which they grow. It is also known that the use of substrate enhancement, particularly, the use of plant growth regulators plays a significant role in the growth and development stages of macro-fungal development. Although interesting scientific results have been reported from this macro-fungal, their use and acceptance tend to be localised to areas where the research has been carried out. Therefore, there is a need to investigate the use of morphological, molecular, and biochemical markers for the identification of *Ganoderma* species, particularly, in South Africa. Furthermore, the effect of substrate enhancement on the growth and quality of these characterised *Ganoderma* species also needs to be evaluated so that sound cultivation systems can be developed.

1.2 Study aims and objectives

1.2.1 Study aims

The overall aim of this study was to characterise *Ganoderma* species using morphological, molecular, and biochemical markers and evaluate the influence of substrate enhancement on their development and biochemical composition.

1.2.2 Study specific objectives

(i) To isolate and characterise *Ganoderma* species from selected provinces in South Africa using morphological, molecular, and biochemical markers.

(ii) To determine the optimum temperature and pH for the mycelial establishment of *Ganoderma* fungal isolates under different growth media.

(iii) To assess the effect of using different substrates for the development, total biomass, and biochemical profile of *Ganoderma* species.

(iv) To examine the effect of substrate enhanced with selected essential elements on the development, total biomass, and biochemical levels of *Ganoderma* species.

1.2.3 Research questions

(i) How the identified South African *Ganoderma* species are characterised using morphological, molecular, and biochemical markers?

(ii) What is the optimum temperature and pH for the mycelial establishment of *Ganoderma* fungal isolates under different growth media?

(iii) How does the use of different substrates affect the development, total biomass, and biochemical profile of *Ganoderma* species?

(iv) What is the impact of substrate enhanced with selected essential elements on the development, total biomass, and biochemical levels of *Ganoderma* species?

1.3 Hypotheses

1.3.1 Null hypothesis:

The use of different substrates and substrate enhancement with essential elements will not influence the development, total biomass, and biochemical profile of *Ganoderma* species.

1.3.2 Alternative hypothesis:

The use of different substrates and substrate enhancement with essential elements will influence the development, total biomass, and biochemical profile of *Ganoderma* species.

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CHAPTER 2

ECOLOGY AND DISTRIBUTION OF *GANODERMA* SPECIES IN SOUTHERN AFRICA – A REVIEW

Abstract

Ganoderma species are widely distributed in Asia, North America, and Africa, they have a long rich history of medicinal use in various Asian countries. The genus is well-known with over 400 species spread across several regions of the world, mostly found in tropical and subtropical areas. Its recognised medicinal properties and nutritional qualities provide this genus with an exorbitant economic market value. Despite this, their taxonomic status remains unclear due to basidiocarp similarities that affect morphological characterisation and accurate identification. Southern Africa has about twenty species identified and most of them have been found in South Africa. However, their phylogenetic position is unknown and there is little information regarding their distribution and DNA sequences. Therefore, characterisation techniques such as molecular, biochemical, and biotechnological are recommended to accurately identify *Ganoderma* species. In addition, growth parameters such as environmental conditions and the substrate's chemical composition influence the genus development and quality. This review discusses the ecology and distribution of *Ganoderma* species focusing on Southern Africa. Also, the characterisation techniques used to identify *Ganoderma* species and the factors affecting their characterisation and development are highlighted.

***Keywords*:** Mushrooms, classification, basidiospores, basidiocarp, environment conditions

1 Introduction

Ganoderma species were first identified thousands of years ago in Asian countries including, China, Korea, and Japan (Sanodiya et al., 2009; Thawthong et al., 2017). Over the years, the genus has been identified in various parts of the world, including Africa, Europe, and America (Kwon et al., 2016; Hapuarachchi et al., 2018). *Ganoderma* belongs to the family *Ganodermataceae* (Polyporale, Basidiomycota), and was introduced to the scientific world by Finnish mycologist, Peter Adolf Karsten in 1881, with *Ganoderma lucidum* (Curtis:Fr) P.Karst from England as the type of species (Karsten, 1881; Wang et al., 2020). *Ganodermataceae* family has eight genera that are distinguished by their distinctive doubled-walled basidiospores (Palanna et al., 2020). *Ganoderma* grows in tropical and subtropical regions, however, their distribution continues to intensify in temperate regions (Jargalmaa et al., 2017). The species

can grow well on living woods and dead trees (Upadhyay et al., 2014), and they survive as saprophytes, causing white rot by decomposing lignin and cellulose (Coetzee et al., 2015).

In recent reports, the genus consists of over 400 species worldwide (Peng and Qiu, 2018; Yalcin et al., 2020). Even though more species have been recorded worldwide, China has a large number, with a few species cultivated (Chen et al., 2017). The inability of species to grow under artificial conditions is attributed to poor growth environment resistance, resulting in low yields (Chen et al., 2017; Cao et al., 2018; Ngo et al., 2019). Yalcin et al. (2020) stated that even though *Ganoderma* is extensively researched, the majority of studies focused on a few species such as *Ganoderma lucidum* and *Ganoderma applanatum*. In addition, *Ganoderma* has a huge economic value primarily due to its medicinal and cultural significance (Dong et al., 2019). Their basidiocarps contain nutritional quality and pharmaceutical properties that are essential to the human body (Romorosa et al., 2017; Shah and Modi, 2018; Berovic and Podgornik, 2019). Despite these reports, the misperception relating to poor species identification tends to hinder the commercial, patents, and publications authenticity claims (Bishop et al., 2015). One of the major challenges highlighted in numerous reports is the difficulty in defining their identity (Kües et al., 2015; Umroong et al., 2021). Thus far, no review has reported the ecology, distribution patterns, and taxonomic status of *Ganoderma* species in the natural ecosystem of South Africa. Therefore, this review seeks to highlight these issues and also explore characterisation techniques used to identify *Ganoderma* species and the factors influencing their characterisation and development.

2 Taxonomic status and distribution patterns of *Ganoderma* species in South Africa

Reports reveal that Mr. C.O Farquharson was the first mycologist to identify the genus *Ganoderma* in Africa, the species identified was *Ganoderma lucidum* found in West Africa (Wakefield, 1920). Since then, more than 49 species have been identified in Africa (Kinge et al., 2015). Approximately twenty species are identified to have originated from Southern Africa and this has led to the advancement of *Ganoderma* research in Africa (Ise et al., 2021). Even though the discovery of these species in Southern Africa, their phylogenetic position is unknown and there are no DNA sequences provided for samples (Coetzee et al., 2015; Ise et al., 2021). Notwithstanding advances in taxonomic techniques, the diversity of *Ganoderma* and other polypores in Africa has received very little attention (Kinge et al., 2015). In South Africa, a total of thirteen *Ganoderma* species (Table 2.1) have been reported thus far, mainly found in Gauteng, Western Cape, KwaZulu-Natal, Eastern Cape, Mpumalanga, Free State, and Limpopo regions (Muthelo, 2011; Coetzee et al., 2015; Xing et al., 2016; Tchotet Tchoumi et

al., 2017; 2018; 2019). Previous reports indicate that there is no sufficient information regarding the identity of macro-fungi in the natural ecosystem of South Africa (Tchotet Tchoumi et al., 2017). Therefore, the dearth of information about macro-fungi suggests that more species including *Ganoderma* are yet to be identified in several parts of South Africa.

Table 2.1 List of previously identified and documented *Ganoderma* species in South Africa.

Species	GenBank accession number		Voucher number	Province	Geographical origin/GPS Coordinates	References
	ITS	EFI-α				
Ganoderma applanatum	AJ608709	–	–	–	Australia	Tchotet Tchoumi et al. (2017)
Ganoderma aridicola	KU572491	KU572502	Dai 12588 (holotype)	KwaZulu-Natal	Durban	Xing et al. (2016)
Ganoderma austrate	–	–	CMW 25877	Free State	South Africa	Coetzee et al. (2005)
Ganoderma austrate	–	–	CMW 25897	Gauteng	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma austrate	MH571686	MH567276	CMW 47785	–	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma austrate	MH571685	MH567283	CMW 48146	–	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma austrate	MH571687	MH567277	CMW 49694	Western Cape	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma austrate	MH571688	MH567280	CMW 49697	Mpumalanga	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma austrate	–	–	CMW 50311	Western Cape	South Africa	Tchotet Tchoumi et al. (2017)
Ganoderma austrate	AY884180	–	–	–	United Kingdom	Tchotet Tchoumi et al. (2017)
Ganoderma austrate	KF605665	–	–	–	Unspecified	Tchotet Tchoumi et al. (2017)
Ganoderma austroafricanum	MH571693	MH567296	CMW 25884	–	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma austroafricanum	KM507324	–	CMW 41454	–	South Africa	Xing et al. (2016)
Ganoderma cupreunm	MH571696	MH567291	CMW 48134	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma cupreunm	JN105701	–	–	–	Cameron	Tchotet Tchoumi et al. (2017)
Ganoderma cupreunm	KX055560	–	–	–	Unspecified	Tchotet Tchoumi et al. (2017)
Ganoderma dunense	MGR0248	MG020226	CMW 42149	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma dunense	MGR0249	MG020227	CMW 42150	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma dunense	MGR0255	MG020228	CMW 42157 (type)	Western Cape	34° 03.242' S, 22° 22.711' E	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	KR183856	KR183860	CMW 43670	Gauteng	25° 45.65' S, 25° 14.50' E	Coetzee et al. (2015)
Ganoderma destructans	NR132919	–	CBS 139793 (type)	Gauteng	Pretoria	Xing et al. (2016)

Ganoderma destructans	KR183856	KR183860	CMW 43670	Gauteng	25° 45.65' S, 28° 14.50' E	Coetzee et al. (2015)
Ganoderma destructans	KR183857	MG020220	CMW 43671	–	25° 45.65' S, 28° 14.21' E	Coetzee et al. (2015)
Ganoderma destructans	MGR0232	MG020191	CMW 42129	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0233	MG020192	CMW 42130	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0234	MG020193	CMW 42131	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0235	MG020216	CMW 42134	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0236	MG020214	CMW 42135	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0237	MG020194	CMW 42136	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0238	MG020195	CMW 42137	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0239	MG020196	CMW 42138	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0240	MG020197	CMW 42139	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0241	MG020215	CMW 42140	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0242	MG020198	CMW 42141	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0243	MG020199	CMW 42142	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0244	MG020221	CMW 42143	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0245	MG020200	CMW 42146	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0246	MG020201	CMW 42147	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0247	MG020202	CMW 42148	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0250	MG020203	CMW 42151	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0251	MG020204	CMW 42152	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0252	MG020205	CMW 42153	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0253	MG020206	CMW 42154	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0254	MG020217	CMW 42155	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0256	MG020224	CMW 42158	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0257	MG020223	CMW 42159	–	South Africa	Tchotet Tchoumi et al. (2018)

Ganoderma destructans	MGR0258	MG020207	CMW 42160	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0259	MG020208	CMW 42161	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0260	MG020209	CMW 42162	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0261	MG020218	CMW 42163	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0262	MG020210	CMW 42164	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0263	MG020211	CMW 42165	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0266	MG020225	CMW 45109	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0267	MG020222	CMW 45110	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0268	MG020212	CMW 45113	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	MGR0269	MG020219	CMW 45114	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma destructans	–	–	CMW 25883	Limpopo	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma destructans	–	–	CMW 25902	Gauteng	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma destructans	–	–	CMW 49707	Limpopo	South Africa	Tchotet Tchoumi et al. (2017)
Ganoderma destructans	MH571694	MH567303	CMW 49708	Limpopo	South Africa	Tchotet Tchoumi et al. (2017)
Ganoderma destructans	–	–	CMW 49713	Gauteng	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma destructans	–	–	CMW 49714	Gauteng	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma destructans	–	–	CMW 49715	Gauteng	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma destructans	–	–	CMW 49716	Gauteng	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma destructans	MH571695	MH567299	CMW 49717	Gauteng	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma destructans	–	–	CMW 49719	Gauteng	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma destructans	–	–	CMW 49720	Gauteng	South Africa	Tchotet Tchoumi et al. (2017)
Ganoderma destructans	–	–	CMW 49721	Gauteng	South Africa	Tchotet Tchoumi et al. (2017)
Ganoderma destructans	–	–	CMW 49722	Gauteng	South Africa	Tchotet Tchoumi et al. (2017)
Ganoderma destructans	–	–	CMW 49723	Gauteng	South Africa	Tchotet Tchoumi et al. (2017)
Ganoderma destructans	–	–	CMW 49724	Gauteng	South Africa	Tchotet Tchoumi et al. (2017)

Ganoderma eickeri	MH571690	MH56287	CMW 49692 (type)	Mpumalanga	25° 26.38' S, 30° 58.14' E	Tchotet Tchoumi et al. (2017)
Ganoderma eickeri	MH571689	MH56290	CMW 49705	Limpopo	South Africa	Tchotet Tchoumi et al. (2017)
Ganoderma eickeri	–	–	CMW 50313	Western Cape	South Africa	Tchotet Tchoumi et al. (2017)
Ganoderma eickeri	–	–	CMW 50325	KwaZulu-Natal	South Africa	Tchotet Tchoumi et al. (2017)
Ganoderma enigmaticum	NR132981	MG020231	CBS 139792 (type)	–	South Africa	Xing et al. (2016)
Ganoderma enigmaticum	KR183855	KR183859	CMW 43669	Gauteng	25° 45.47' S, 28° 13.87' E	Coetzee et al. (2015)
Ganoderma enigmaticum	MH571697	MH567297	CMW 50318	Gauteng	South Africa	Tchotet Tchoumi et al. (2017)
Ganoderma fornicatum	JX840347	–	–	–	China	Tchotet Tchoumi et al. (2017)
Ganoderma fornicatum	JX840348	–	–	–	China	Tchotet Tchoumi et al. (2017)
Ganoderma knysnamense	MH571681	MH567261	CMW 47755	Western Cape	33° 56.82' S, 23° 35.20' E	Tchotet Tchoumi et al. (2019)
Ganoderma knysnamense	MH571684	MH567274	CMW 47756	Western Cape	33° 56.82' S, 23° 35.20' E	Tchotet Tchoumi et al. (2019)
Ganoderma knysnamense	MH571683	MH567266	CMW 49688	Western Cape	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma knysnamense	–	–	CMW 49689	Western Cape	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma knysnamense	–	–	CMW49690	Western Cape	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma knysnamense	MH571682	MH567267	CMW 49691	Western Cape	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma mutabile	JN383977	–	–	–	China	Tchotet Tchoumi et al. (2017)
Ganoderma pfeifferi	AY884181	–	–	–	United Kingdom	Tchotet Tchoumi et al. (2017)
Ganoderma pfeifferi	AM906059	–	–	–	Czech Republic	Tchotet Tchoumi et al. (2017)
Ganoderma resinaceum	–	–	CMW 25881	Gauteng	South Africa	Coetzee et al. (2005)
Ganoderma resinaceum	–	–	CMW 25895	Gauteng	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma resinaceum	–	–	CMW 25900	Eastern Cape	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma resinaceum	MH571691	MH567295	CMW 49711	Gauteng	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma resinaceum	MH571692	MH567294	CMW 50326	KwaZulu-Natal	South Africa	Tchotet Tchoumi et al. (2019)
Ganoderma ryvardenii	HM138671	–	HKAS 58053 (type)	–	South Africa	Xing et al. (2016)
Ganoderma ryvardenii	HM138672	–	HKAS 58054	–	South Africa	Xing et al. (2016)

Ganoderma ryvardenii	HM138670	–	HKAS 58055	–	South Africa	Xing et al. (2016)
Ganoderma sp.	MG020264	MG020229	CMW 45100	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma sp.	MG020265	MG020230	CMW 45101	–	South Africa	Tchotet Tchoumi et al. (2018)
Ganoderma sp	–	–	CMW 29570	Gauteng	25° 45.76' S, 28° 13.76' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29571	Gauteng	25° 45.49' S, 28° 14.06' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29572	Gauteng	25° 45.48' S, 28° 14.00' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29573	Gauteng	25° 45.40' S, 28° 14.38' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29574	Gauteng	25° 45.41' S, 28° 13.55' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29575	Gauteng	25° 45.45' S, 28° 14.11' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29576	Gauteng	25° 45.53' S, 28° 14.46' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29577	Gauteng	25° 45.53' S, 28° 14.19' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29578	Gauteng	25° 45.52' S, 28° 14.18' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29579	Gauteng	25° 45.48' S, 28° 14.01' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29580	Gauteng	25° 46.04' S, 28° 14.46' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29581	Gauteng	25° 45.51' S, 28° 14.40' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29582	Gauteng	25° 45.32' S, 28° 14.04' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29583	Gauteng	25° 45.44' S, 28° 14.05' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29584	Gauteng	25° 45.46' S, 28° 13.53' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29585	Gauteng	25° 45.50' S, 28° 14.11' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29586	Gauteng	25° 45.45' S, 28° 13.45' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29587	Gauteng	25° 45.59' S, 28° 14.27' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29588	Gauteng	25° 46.02' S, 28° 14.27' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29589	Gauteng	25° 45.51' S, 28° 14.14' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29590	Gauteng	25° 45.54' S, 28° 14.47' E	Muthelo (2011)
Ganoderma sp	–	–	CMW 29591	Gauteng	25° 45.50' S, 28° 14.33' E	Muthelo (2011)

3 Characterisation of *Ganoderma* species

More than 290 taxonomic names in the genus have been published (Kinge et al., 2015). Basidiocarp features such as colour, texture, size, and basidiospore wall are often used to characterise their morphology (Erkel, 2009; Richter et al., 2015; Jargalmaa et al., 2017). However, due to the high similarities of basidiocarp features, this characterisation technique is inconclusive for accurate identification and classification (Kinge et al., 2015). Hence, the morphological taxonomy of the genus is described to be in a chaotic state (Wang et al., 2014; Coetzee et al., 2015; Jargalmaa et al., 2017; Loyd et al., 2018b). Even though the taxonomic complex, biological species concepts, and molecular tools have provided alternatives to resolve *Ganoderma* taxonomical questions (Loyd et al., 2017; Ise et al., 2021). Microscopy, deoxyribonucleic Acid (DNA) sequencing, spectroscopy, chromatography, thin-layer chromatography (TLC), and chemical fingerprinting are some of the molecular tools commonly used to identify *Ganoderma* (Cao et al., 2018). Though the value of conventional macro-and micro-morphological characters should be infrageneric classified, basidiocarp morphology, basidiospores, and pilocystidia should be reviewed on the background of molecular result accuracy (Papp et al., 2012).

3.1 Factors affecting the characterisation/identification of *Ganoderma* species

Predominantly, species are identified based on cultural and morphological characterisation such as basidiocarps, tree host, and geographical distribution (Zakaria et al., 2009; Mukhtar, 2019). These morphological characteristics vary with changes in environmental factors (Ekandjo, 2012), thus considered not to be used as the sole identification technique. Several factors such as environmental variability, morphological inclination, and interhybridisation contribute to the inaccurate identification of the genus (Kwon et al., 2016). Therefore, this suggests that the diverse locations where the fungus is found might have contributed to the scepticism concerning the genus's accuracy in taxonomic classification. Their identification remains not well established, particularly in tropical species (Ekandjo, 2012; Bishop et al., 2015). Hapuarachchi et al. (2018) suggested the importance of establishing a combination of morphological, chemotaxonomic, and molecular techniques that would develop a firmer taxonomy position.

3.2 Morphological characterisation of *Ganoderma* species

Basidiocarps of *Ganoderma* have many tiny holes underside which contain reproductive spores (Roberts, 2004). Basidiospore morphology and surface texture of the pileus are the most imperative discriminative features for their segregation (Richter et al., 2015). Basidiocarps are

the source of spores often used for different purposes (Chen, 1999a; Douanla-Meli and Langer, 2009), including *in vivo* culture development (Mukhtar, 2019), and DNA extraction (Mercière et al., 2015). These basidiocarps and mycelia are used for several biochemical extracts such as anti-oxidant, anti-tumour, anti-microbial activities, nutritional value, etc. (Barros et al., 2008; Halliwell, 2008; Heleno et al., 2012; Kalogeropoulos et al., 2013; Xu et al., 2017; Cör et al., 2018; Islam et al., 2018; Dong et al., 2019). However, the morphological characteristics variability often imposes challenges in their identification, most species are usually mistakenly identified (Mohanty et al., 2011). Moreover, *Ganoderma* morphological characterisation is discouraged as it may be affected by environmental conditions during development. Muthelo (2011) noted that morphological plasticity in some species resulted from these environmental conditions. Due to the phenotypic plasticity and morphological stasis, the DNA sequence data is recommended for characterisation accuracy (Yang and Feng, 2013).

3.2.1 Micro-morphological

Micro-morphology of *Ganoderma* contains staghorn hyphae, thin- and thick-walled round-shaped brownish cuticular cells that are organised from generative hyphae, hyphal rosettes, and wrinkled hyphae (Badalyan et al., 2019; Cabarroi-Hernández et al., 2019). Micro-morphological characteristics include the presence and form of asexual spores, chlamydospores, and hyphal clamps known to be valuable in the characterisation of Agaricomycetes (Badalyan and Sakeyan, 2004; Tsivileva et al., 2016). Most micro-morphology features are often used for characterising the differentiation rate. However, for effective characterisation, cultures not more than two weeks are used (Szedlay et al., 1996; Mohanty et al., 2011). On the contrary, some reports reveal that these micro-morphology features can only form six weeks after inoculation and longer on other strains (Wang and Hua, 1991). Macroscopically basidiocarps have a high degree of consistency across different species (Adaskaveg and Gilbertson, 1986). Conversely, microscopical and cultural characters vary within species depending on geographical distribution (Szedlay et al., 1996; Loyd et al., 2018a).

3.2.2 Macro-morphological

Dissimilar to edible mushrooms, *Ganoderma* basidiocarps are thick, tough, corky, and have a woody or leathery texture, they do not consist of fleshy texture characteristics (Roberts, 2004; Mukhtar, 2019). Their basidiocarps are sexual structures and they are comprised of various morphological characteristics such as stipitate, sessile, imbricate, and non-imbricate (Muthelo, 2011). These basidiocarps consist of a pileus shape, at maturity, the size can be up to 10-16 cm

in length, 4-9 cm in width, and 0.7-1.2 cm thick (Luangharn et al., 2019). However, Smith and Sivasithamparam (2003) described the basidiocarps to typically vary from 5-50 cm wide and consist of a crust greater than 0.2 mm. At a mature stage, their surface accumulates a red-brown thick layer often very similar among various species and thus challenging to distinguish (Muthelo, 2011; Chen et al., 2017). Therefore, a need to consider the application of molecular techniques for accurate identification. To further neutralise inaccuracies and clarify the taxonomic challenges, the application of nucleotide sequences for informative DNA markers is imperative (Hennicke et al., 2016; Oyarzún et al., 2021).

3.3 Biochemical and biotechnological characterisation

Biochemical and biotechnological characterisation are used to distinguish mushroom species (Zhao et al., 2010; Bilal et al., 2015; Gauna et al., 2021). *Ganoderma* can synthesise valuable molecules, including anti-oxidants anti-biotics, anti-inflammatory, and anti-tumour compounds as well as the efficiency to produce laccases, xylanases, and other industrially critical enzymes (Smânia et al., 2003; Zhou et al., 2012; Yu et al., 2015). Recent studies revealed an increase in biotechnology techniques used on natural raw materials (Sułkowska-Ziaja et al., 2022), this technique is often used in *Ganoderma* characterisation (Ospina Álvarez et al., 2014). Although biochemical and biotechnological characterisation of *Ganoderma* has been reported, further research due to newly discovered species is recommended (He et al., 2015; Deveci et al., 2019; Tchotet Tchoumi et al., 2019; Gauna et al., 2021).

3.3.1 Utilisation of scanning electron microscope on the characterisation *Ganoderma* spp.

Prediction of the organism's secondary structure can be done using various techniques like the electron microscope, chemical and structure probing, and computer software programs such as mfold and sfold (Valappil et al., 2009; Goyal et al., 2015; Singh et al., 2020). Scanning electron microscope (SEM) has been widely used in several morphology, biochemical, and chemical composition studies of *Ganoderma* (Xu et al., 2019; Elumalai et al., 2021; Ganapathy et al., 2021; Umroong et al., 2021). According to Umroong et al. (2021), the SEM is applied to study *Ganoderma* morphology characteristics such as the structures of cell wall pattern, thickness, shape, and size of spores, hyphae, and hypha-clamp connection of culture cells (Table 2.2). Even though the SEM has been successful in explaining inconsistencies in the existing descriptions of species, limitations such as dirt or debris often settling on the external surface of the specimen have been reported (Kownacki et al., 2015). SEM users encounter poor contrast and resolution of a certain specimen without metal coating (Ensikat et al., 2010). Therefore,

ultrastructural studies utilising microscope techniques are recommended for studying the morphology of *Ganoderma* as the basis for further research.

Table 1.2 Morphological characteristics of *Ganoderma* species using various microscopic techniques

Species	Type of microscope	Size in diameter		References
		Spores	Hyphae	
Ganoderma lucidum	Transmission Electron Microscope (TEM)	82.90-508.00 (nm)	59.90-508.00 (nm)	Umroong et al. (2021)
Ganoderma lucidum	Scanning Electron Microscope (SEM)	1.68-10.85 (µm)	1.22-4.7 (µm)	Singh et al. (2020)
Ganoderma sichuanense	Stereo Dissecting Microscope (Motic SMZ 168 series)	1.1-1.3 (µm)	3.0-3.1 (µm)	Thawthong et al. (2017)
Ganoderma lucidum	Scanning Electron Microscope (SEM)	10.04 (nm)	756-919 (nm)	Goyal et al. (2015)
Ganoderma tsugae	Scanning Electron Microscope (SEM)	10.9-12.5 (µm)	20-25 (µm)	Adaskaveg and Gilbertson (1986)

3.4 Molecular characterisation of *Ganoderma* spp.

The chaotic taxonomic state of *Ganoderma* from previous reports is evident, therefore this has led to various uses of well-established molecular techniques to characterisation these species (Ekandjo, 2012; Chen et al., 2017; Loyd et al., 2019; Mukhtar, 2019). DNA sequences in the internal transcribed spacer (ITS) region, mitochondrial small-subunit ribosomal (mRNAs), restriction amplified fragment length polymorphism (RFLP), random amplified polymorphic (RAPD), sequence-related amplified polymorphism (SRAP), and amplified fragment length polymorphism are used as molecular markers (AFLP) (Sun et al., 2006; Osuji et al., 2016). These molecular makers were initially designed to detect naturally occurring polymorphism at DNA levels. However, molecular markers that have been utilised in the past to study the structure of fungal pathogens are limited to the use of vegetative incompatibility groups (VCG) analysis (Jiat et al., 2019). Molecular markers have numerous advantages over morphological markers in a manner that are not influenced by environmental factors, physiological phase of an individual and they are not tissue-specific (Wu et al., 2007; Zhou et al., 2007). However, very limited works are being reported on the identification and application of molecular markers on macro-fungi, particularly in South Africa (Tchotet Tchoumi et al., 2017).

3.4.1 DNA extraction for *Ganoderma* spp.

Fungal spores from mycelia cultures are used for DNA analysis, and DNA results are obtained by comparing them to the non-redundant nucleotide database at GenBank (Osuji et al., 2016; Madihah et al., 2018; Jiat et al., 2019; Nagappan et al., 2021). DNA-based characters are widely used in the taxonomy of fungi at various levels (Muthelo, 2011). Ribosomal ribonucleic acid (rRNA), RFLP, and ITS are extensively used to distinguish the taxa of *Ganodemataceae* at the generic and sub-generic levels (Smith and Sivasithamparam, 2000; Smith and Sivasithamparam, 2003; Llorens et al., 2006). However, other than RFLP, all DNA markers are based in one way or another upon the use of polymerase chain reaction (PCR) (Osuji et al., 2016). The currently used DNA detection techniques protocols are time-consuming and subject to cross-reactivity with other fungal species (Madihah et al., 2018). Smith and Sivasithamparam (2000) report revealed that even though these techniques are used, *Ganoderma* isolates are sometimes misidentified based on sequence variation and phylogenetic analysis. The results of Sun et al. (2006) reported that the polymorphism of species is closely related to their complex genetic background and environment. Therefore, the DNA technique application provides authentication identification of *Ganoderma*. However, DNA sequencing limitations arise when polymorphisms and DNA heterogeneity are discovered, particularly when dealing with intraspecies diversity of more than one gene copy in the genome (Muthelo, 2011; Leigh et al., 2021).

3.4.2 Internal transcribed spacer (ITS) sequencing

Genetic relationships at lower taxonomic levels involving sequence diversity can be determined with ITS (Coetzee et al., 2005; Dizkirici et al., 2010; Liu et al., 2010; Rodrigues, 2021). The ITS has been widely used in fungal studies such as taxonomy and phylogeographic studies and also to assess fungal composition in different environments by deep sequencing (Chen et al., 2016; Yang et al., 2018). At the fourth International Barcode of Life Conference, the ITS region was proposed as a global DNA barcode sequence for fungi identification (Zhang and Guo, 2012). ITS has a high success rate in PCR amplification and a high degree of interspecific variation in distinguishing between most of the closely related fungal species (Chen et al., 2016). When compared with other regions of the ribosomal cistron, the ITS region has the highest probability of successfully identifying the wide-ranging fungi, with the most clearly defined barcode gap between inter- and intraspecific variation (Schoch et al., 2012). In a case whereby the fungal taxa is identified to have a low ITS interspecific variability, therefore, secondary markers must be used for an accurate genetic report (Gazis et al., 2011).

Careful documentation is advised due to ITS sequence limitations in identifying species in some groups and the failure of universal ITS primers to work in a minority of other groups (Nilsson et al., 2008; Voigt and Kirk, 2011; Schneider et al., 2021). Even though studies of genome diversity on fungi using the ITS region are increasing, the vast majority of fungal species remain unknown (Schoch et al., 2012). Despite these challenges, ITS is reported to combine the highest resolving standard for discriminating closely related species with a high PCR and sequencing success rate across a wide range of fungi (Al Daccache et al., 2020; Ogbonna et al., 2021).

3.4.3 Phylogenetic analysis of *Ganoderma* species

Ganoderma taxonomy encounters great challenges due to several species being poorly identified (Mendoza et al., 2011). Therefore, various molecular techniques have been used in the attempt to understand the nucleotide variation among isolates and this has been key in the fungi phylogenetic and systemic studies (Hong et al., 2002). Despite reports regarding limitations on the use of DNA and ITS sequencing, these sequences provide data that assist in resolving the taxonomy by using DNA barcoding of several fungal groups in most molecular phylogenetic studies (Jargalmaa et al., 2017; Al Daccache et al., 2020; Leigh et al., 2021; Ogbonna et al., 2021). According to Smith and Sivasithamparam (2000), the ITS region is a suitable molecular marker for inferring phylogenies of species within *Ganoderma*. Muthelo (2011) highlighted that phylogenetic analysis was used to resolve the misidentification of *Ganoderma*. However, the infrageneric taxonomy of *Ganoderma* and the suitable characters for the species identification such as those that can be related to the molecular phylogenetic results still need clarification (Papp et al., 2012). Therefore, the taxonomy of *Ganoderma* has been convoluted, and their phylogenetic relationships among taxa are currently still being actively investigated (Loyd et al., 2018b; Yalcin et al., 2020).

4 Factors influencing the development of *Ganoderma* spp.

Substrate, climatic conditions (temperature and relative humidity), pH, and biochemical are the major factors influencing the development of various mushroom species (Khandakar, 2004; Shah and Modi, 2018). These growth parameters have a significant role in mushroom growth and development (Ramachela and Sihlangu, 2016). Different substrates have diverse structures, textures, and chemical compositions thus influencing chemical availability and release from the substrates (Buah et al., 2010). This is attributed to its potential to provide a high conversion efficiency ratio and chemical efficiency transfer. Siwulski et al. (2019) described the conversion efficiency ratio as the total rate of mycelia to convert substrate into

mushrooms and chemical efficiency transfer to be the ability of a substrate to transfer accumulation of biochemical and nutritional value to basidiocarps, respectively.

4.1 Substrate selection for *Ganoderma* production

Most mushrooms are cultivated by the use of substrates such as straws, paddy, and grass plants (Hanafi et al., 2018). *Ganoderma* species grow well within the woods of living and dead trees and agro-waste material, therefore several materials are being used as a substrate (Bishop et al., 2015; Richter et al., 2015; Loyd et al., 2018a; Meng et al., 2019). Substrates such as sawdust, wheat bran, rice bran, sugarcane bagasse, banana leaves, peanut hulls, rice husks, and coconut fibre are used for successful artificial *Ganoderma* cultivation (Wagner et al., 2003; Erkel, 2009; Peksen and Yakupoglu, 2009; Lakshmi, 2013; Meng et al., 2019). Several substrate mixtures used as substrate supplements have been investigated for the cultivation of *Ganoderma lucidum* (Roy et al., 2015). Typically, these mixtures consist of 75-80 % of the substrate with the supplement of 20% wheat bran, 1 % gypsum, 1 % sucrose, 60-65 % moisture content, and a pH of 5.5-6.5 (Chen, 1999b; Stamets, 2005; Erkel, 2009). Peksen and Yakupoglu (2009) established that tea waste can be used as a substrate supplement for the production of *Ganoderma lucidum.* Generally, the selection of main and co-substrate is influenced by geographic location and the ready availability of substrate. However, it is important to identify and select the best substrate possible.

4.1.1 Role of the substrate on total biomass and biological efficiency

The type of substrate used has a significant role in the development, quality, biomass, and nutritional value of the mushroom (Chitamba et al., 2012). Therefore, this is attributed to various biochemical properties and nutrient content of a substrate that influences mycelia to convert biomass into mushrooms (Ramachela and Sihlangu, 2016). The biological efficiency/conversion efficiency ratio is calculated using the below equation as described by Meng et al. (2019).

$$BE = \frac{total\ weight\ fresh\ mushroom\ (g)}{total\ dry\ weight\ of\ substrate\ (g)}\ X\ 100$$

Several researchers have investigated the effect of various types of substrates on the yield and biological efficiency of *Ganoderma* species (Table 2.3). Erkel (2009) examined the effect of three various types of sawdust (oak, poplar, and beech) and three various types of bran (corn, wheat, and rice) on the yield and biological efficiency of *Ganoderma* (Table 3). The findings

of Erkel (2009) indicated that the yield and biological efficiency of *Ganoderma* varied depending on the kind of substrate used.

Table 2.3 Effect of substrates on yield (g kg^{-1}) and biological efficiency (%) on the production of *Ganoderma* species.

Type of substrate	Specific substrate	Yield (g kg^{-1})	Biological Efficiency (%)	References
Sawdust	Beech	142.44	7.6	Ngo et al. (2019)
Sawdust	Swietenia mahagoni	235.2	7.6	Roy et al. (2015)
Rice	Rice straw	17.20	6.01	Magday Jr et al. (2014)
Other	Rice husks	82.4	16.4	Postemsky et al. (2014)
Other	Sugarcane bagasse	64.78	12.95	Lakshmi (2013)
Sawdust	Sawdust	17.75	19.37	Gurung et al. (2012)
Bran	Rice bran	17.15	19.37	Gurung et al. (2012)
Bran	Wheat bran	63.66	18.63	Erkel (2009)
Sawdust	Oak	60.24	17.48	(Erkel, 2009)
Other	Tea waste	99.88	39.62	Peksen and Yakupoglu (2009)

4.2 Chemical composition of the substrate

It is critical to identify optimal substrate and physical, chemical, and biological factors due to their importance in the *Ganoderma*'s production cycles and biological yield (Nguyen et al., 2019). *Ganoderma* can grow on different substrates but due to variations in the chemical composition of substrates (Table 2.4), growth and yield greatly vary (Postemsky et al., 2017; Loyd et al., 2018a; Nguyen et al., 2019; Siwulski et al., 2019). Substrate characteristics, cultivation techniques, and postharvest conditions influence the chemical composition, nutritional value, fungal and cell development, also metabolites production of mushrooms (Heleno et al., 2012; Valverde et al., 2015). Moreover, substrate pH, EC, organic carbon, nitrogen (N), macro-and micro-elements, and carbon-to-nitrogen (C:N) ratios are some of the chemical constituents identified to influence *Ganoderma* growth (Peksen et al., 2011). Peksen et al. (2011) reported that the content of these chemicals changes before and after *Ganoderma* cultivation. The results of Nunes et al. (2012) indicated that substrates with nitrogen supplementation increase the mushroom's productivity and nutritional value, particularly the β-glucan content. The β-glucan found in most mushrooms is considered to be responsible for

some of the medicinal properties (Nunes et al., 2012). Considering the various use substrates as one of the major factors influencing *Ganoderma* chemical composition. Therefore, there is need to examine the substrate's chemical composition before use is warranted.

Table 2.4 Chemical composition of substrate used in the cultivation of mushrooms.

Species	Substrate	Chemical composition					References
		OC (%)	C:N	Total N (%)	P (ppm)	K (ppm)	
Agrocybe cylindracea	Almond and walnut shells	62.9	–	–	12.10	346	Hanafi et al. (2018)
Auricularia auricula	Rice husk	43.67	40.93	1.07	2.33	4.19	Meng et al. (2018)
Ganoderma lucidum	Oak sawdust	56.79	162.26	0.35	149.08	728.78	Peksen et al. (2011)
Pleurotus ostreatus	Sugarcane bagasse	–	–	0.33	1.14	2.63	Nunes et al. (2012)
Pleurotus ostreatus	Cotton stalk	51.24	150.70	0.34	–	–	Abdurrahman et al. (2009)

4.3 Role of production techniques and growth parameters on the development of *Ganoderma* spp.

Naturally, *Ganoderma* grows and is harvested from the wild (Bishop et al., 2015). However, with the high market demand, a large production takes place through artificial cultivation (Cao et al., 2018). Artificial cultivation of the genus was first attempted in 1937 (Perumal, 2009), with the first successful artificial cultivation of *Ganoderma* executed in 1969 by a Chinese technician at the Institute of Microbiology, Chinese Academy of Sciences, Beijing (Hapuarachchi et al., 2018). Following successful artificial cultivation, the genus has become increasingly popular due to its valuable medicinal properties and spent substrate used as organic material (Peksen et al., 2011). The ancient technique of cultivating medicinal mushrooms is still practised mainly in Asia where logs of hardwood trees are used (Peksen and Yakupoglu, 2009; Veena and Pandey, 2012). However, wood logs, short wood segments, tree stamps, sawdust bags, and bottles or containers are widely adopted cultivation techniques for medicinal mushroom production.

4.3.1 Production techniques

Over the years many researchers have had a great interest in mycelia cultivation production as a systematic technique for the production of various agro-industrial by-products and industrial production of valuable metabolites (Song et al., 2007). The total mushroom mycelia and

biomass are determined by the production techniques. Hence understanding the dynamics within production techniques will lead to their increase. Production techniques for *Ganoderma* are divided into two types namely; solid-state fermentation (SSF) and liquid-state fermentation (LSF) techniques (Zhou et al., 2012). These fermentation techniques are considered to have been modernised to enable the production of both mycelia and metabolic products in a shorter time, with less production space requirement, and low contamination probabilities (Wagner et al., 2003). Both techniques have advantages and disadvantages that may differ according to species, the technology employed, and mushroom purpose (Letti et al., 2018). However, Wagner et al. (2003) indicated that usage of the solid-state fermentation technique has an advantage over other techniques and has been mostly used to produce various fungal species. The less time and labour of SSF than LSF have resulted in the frequent utilisation in preliminary tests for cultivating microorganisms under experimental conditions (Song et al., 2007). On the contrary, other authors stated that the period to produce *Ganoderma* fruiting bodies is often long when the SSF cultivation technique is used (Dong et al., 2021; Singh et al., 2021; Suansia and John, 2021).

One of the great advantages of the SSF technique is the affordable production material such as a substrate. In addition, Malarvizhi et al. (2003) reported the SSF technique can be employed for the production of various groups of enzymes during the growth of *Ganoderma*. The usage of these enzymes is known to have several economic and engineering advantages. Despite the advantages reported, this technique has several disadvantages such as the long time required to produce basidiocarps and the difficulties to control mushroom quality (Yang and Liau, 1998; Lee et al., 1999). The difficulties in controlling mushroom quality are due to the larger number of variables and growth parameters involved in the process of mushroom development and its metabolite production (Letti et al., 2018). Although several researchers indicate the successful use of these fermentation techniques to obtain cultures and effective products, the process depends on several key factors including temperature, pH value, relative humidity, level of oxygen, carbon dioxide, nutritional components, and nitrogen requirements (Malarvizhi et al., 2003). Therefore, it is imperative to have a concise understanding of these factors as they have a direct influence on the total biomass and conversion efficiency ratio for the fungus.

4.3.2 Growth parameters requirement

The morphology and cultural characteristics of species are affected by various growth parameters (Roberts, 2004; Maszlavér and Balázs, 2008). The growth and quality of mycelia

production depend on factors such as culture components and environmental conditions (Roberts, 2004; Kadowaki et al., 2010; Gurung et al., 2012; Shah and Modi, 2018). These growth parameters have an impact on the qualitative/quantitative profile of the chemical composition of *Ganoderma* basidiocarps (Ćilerdžić et al., 2018). Due to the diversity of geographical distribution, tree host/substrate, and environmental factors, these species are influenced by the variety of growth parameters found in these locations (Zakaria et al., 2009; Kinge et al., 2015). Therefore, optimisation of growth conditions such as temperature, relative humidity, pH substrate, and light are crucial, especially under artificial production (Table 5).

4.3.2.1 pH

The pH range for growth and development of *Ganoderma* is reported to be 5 to 9, with an optimum of 5.5 to 6.0 (Jo et al., 2009). Mycelial growth is described to be significantly higher at pH 5.0 and is considered the best pH value (Kapoor and Sharma, 2014). Other research results highlighted a similar pH range of 4 to 7, with optimum growth at pH 5.0 for *Ganoderma* mycelial growth (Chen et al., 2017). However, Kapoor and Sharma (2014) the fungus can grow over a wide range of pH values of 3.0 to 11.0. Chen et al. (2017), further alluded that even though *Ganoderma* mycelial can grow at a pH higher than 8, the mycelial growth rate can significantly decrease. Therefore, *Ganoderma* may be considered as an acidic environment preferable species.

4.3.2.2 Temperature

Temperature is one of the most critical environmental factors influencing mycelial growth (Fletcher, 2019). *Ganoderma* mycelia can grow at temperatures ranging from 9-40 °C, however, they have optimal growth at 25-30 °C (Magday Jr et al., 2014; Badalyan et al., 2019). Similarly, Jayasinghe et al. (2008) examined the influence of cardinal temperatures (15-35 °C) on mycelial growth and density of *Ganoderma*, the best mycelial growth was obtained at 30 °C followed by 35 °C. Notably, the temperature does not only influence mycelial growth but also primordium development and the total biomass of *Ganoderma* (Magday Jr et al., 2014). Findings by Magday Jr et al. (2014) indicated that mycelial development was higher at 32 °C with an average biomass of 50.67 g in 5 days. Even though they can grow under such temperatures, the exposure of mycelia at 9 °C and 35 °C is reported to suppress the growth (Magday Jr et al., 2014). The suppression of mycelial growth is caused by the denaturation and inactivation of essential enzymes that catalyse metabolic processes (Jayasinghe et al., 2008).

4.3.2.3 Relative humidity

Relative humidity (RH) is another important environmental factor that influences the development of *Ganoderma* (Kien et al., 2019). Different RH levels (%) are required during the different growth and development stages of *Ganoderma* such as preparation of spawn, substrate, inoculation, spawn running, pinning, and fruiting (Table 2.5) (Ye et al., 2018). However, RH should be maintained at 60-70% or more during growth stages (Zhou et al., 2015; Ye et al., 2018; Du et al., 2019). Incubation of these species at 80-85% RH increases mycelial development by 80% (Kim et al., 2001; Lisiecka et al., 2015). However, the primordial initiation of *Ganoderma* is known to be optimal at RH of 90-95%.

4.3.2.4 Aeration

Aeration is another important growth parameter and its effect on fungal development has been studied by several investigators. Aeration rate variation is reported to influence the shape of mycelia morphology from filamentous to pellet (Park et al., 2002). An increase in aeration rate leads to a change in morphological form from rough pellet to smooth pellet and results in mycelia roughness, however, pellet size is not affected by aeration (Lee et al., 2011). Poor aeration can result in high levels of carbon dioxide (CO_2) and fermentation (Seo and Suzuki, 2004; Wan et al., 2016). Even though CO_2 is one of the important environmental factors that affect the growth and morphogenesis of Basidiomycetes, excess CO_2 prohibits basidiocarp development and often causes basidiocarp abnormalities (Seo and Suzuki, 2004). Aeration rates are also known to regulate the production process of medicinal properties and the metabolism of microbial cells (Kim et al., 2006; Feng et al., 2021).

Table 2.5 Growth requirements for the cultivation and development of medicinal mushrooms.

Species	Mycelia colonisation			Primordia formation					Basidiocarp development					References
	Temp	Light	Duration	Temp	R.H%	Duration	Light	CO_2	Temp	R.H%	Duration	Light	CO_2	
Ganoderma lucidum	25 °C (optimum)	Not required	3-4 weeks	28-30 °C	90-95%	1 week after mycelia colonisation	Reduce light (100 lux)	>0.1%	28-30 °C	80-85%	25-30 days after primordial formation	150-200 lux	>0.1%	Sudheer et al. (2019)
Pleurotus eryingii	25 °C	Not required	12 weeks	25 °C	80-85%	–	500 lux	1000 ppm	18 ± 1 °C	85-90%	–	500 lux	1000 ppm	Siwulski et al. (2019)
Ganoderma lucidum	25 °C	Not needed	1-2 weeks	25 ± 1 °C	80-90%	1 week after mycelia colonisation	100 lux	>0.1%	25-30 °C	85-95%	20-30 days after primordial formation	15 000 -50 000 lux	>0.1%	Zhou (2017)
Trametes versicolor	25 ± 2 °C	Not required	2-3 weeks	25 ± 2 °C	8-85%	2-3 weeks after colonisation	12 hrs/day	–	25 ± 2 °C	80-85%	–	12 hrs/day	–	Veena and Pandey (2012)
Ganoderma applanatum	25-30 °C	Not required	1 week	25 °C	80-85%	2-3 weeks after mycelia colonisation	–	2%	25-30 °C	–	–	–	2%	Jo et al. (2009)
Ganoderma lucidum	30 °C	Not required	–	20-25 °C	85-95%	–	200-500 lux	100-500 ppm	20-22 °C	80-85%	–	500-100 lux	–	Maszlavér and Balázs (2008)
Ganoderma spp.	25-30 °C	Not required	2-8 weeks	25-30 °C	90-95%	7-8 weeks after mycelia colonisation	100-200 lux	>0.1%	25-30 °C	85-95%	25-30 days after primordial formation	150-200 lux	>0.1%	Chen (1999a)
Ganoderma bonsai	27-30 °C	Not required	2-3 weeks	27-30 °C	85-95%	2-3 weeks after mycelia colonisation	150-200 lux	0.03%	30 °C	50-60%	–	150-200 lux	0.1-1%	Chen and Miles (1996)

Note: Temp = Temperature; R.H% = Relative humidity percentage; CO_2 = Carbon dioxide; ppm = Parts per million; °C = Degrees Celsius

5 Conclusion

Evidently, the distribution and continual discovery of new species in the natural ecosystem of South Africa from recent studies suggest a large number of other *Ganoderma* species are likely to be discovered. However, a comprehensive mechanism to characterise these species remains to be elucidated in the future as this will clearly illustrate the differences between species. Although there are reports on the ecology, distribution, and development of these species, their identification, and characterisation remain a challenge. Morphological and cultural characterisation are discouraged from being used as a sole technique used to identify these species. Therefore, to ensure accurate identification, an integrated characterisation technique approach should be considered. The unknown phylogenetic position and lack of DNA sequence information for samples in Southern Africa might be the result of poor documentation. Hence, a concise record-keeping practice can also assist in the effective characterization of these species and this information could be kept safe in the GenBank records. This will provide a piece of readily available information to be used and stimulate more research toward the extensive characterisation techniques for various species. In addition, various factors such as environmental conditions (temperature, relative humidity, light, and carbon dioxide), pH, and substrate's chemical composition have a direct effect on the development and quality of *Ganoderma* species. Therefore, there is a need to optimise the growth parameters to maximise production and enable successful artificial cultivation of *Ganoderma*.

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CHAPTER 3

BIOCHEMICAL QUALITY AND PHARMACEUTICAL PROPERTIES OF *GANODERMA* SPECIES: A REVIEW

Abstract

Ganoderma is recognised worldwide as an important medicinal mushroom because of its pharmaceutical properties, including anti-tumour, anti-fungal, anti-viral, anti-bacterial, anti-oxidant, anti-diabetic, anti-parasitic, anti-hypercholesterolemia, immunomodulating, radical scavenging, detoxification, and hepatoprotective abilities. Biochemical compounds and essential elements are two major direct contributors to these pharmaceutical properties. This has led to high market demand and intense research activities investigating the nutritional quality of *Ganoderma* species. The total biochemical compounds and nutritional value of fruiting bodies vary within species. However, various techniques have been used to enhance the biochemical properties and nutritional value of fruiting bodies from these species. The present review provides recent research development over the past 10 years on the biochemical quality and pharmaceutical properties of *Ganoderma*. This review further highlights the safety and techniques used to enhance the biochemical properties and nutritional value of *Ganoderma*.

Keywords: Fungus, biomedicine, nutraceutical, bioactive compounds, enrichment, plant growth regulators

1 Introduction

Ganoderma species are widely cultivated and consumed with high global market demand (Kwon et al., 2016; Cao et al., 2018). These species have a significant economic value mainly for their medicinal and cultural importance, such as nutritional quality and pharmaceutical properties (Shah and Modi, 2018; Raman et al., 2022). The genus has become a popular dietary supplement with an annual global market worth over 2.5 billion USD (Dong et al., 2019). The global food market demand for *Ganoderma* extracts has been estimated to reach 34.3 billion USD by the year 2024 (Ahmad et al., 2021b). These mushrooms consist of therapeutic and nutritional value imparted by the plethora of chemical classes present with approximately 400 biochemical compounds reported from its mycelia, fruiting body, and spores (Wu et al., 2013; Ahmad, 2018; Sudheer et al., 2019; Ahmad et al., 2021b). However, several reports identified

most of these biochemical compounds to be dominant in fruiting bodies (Gurung et al., 2012; Li et al., 2016) and recognised as critical for the human body (Kalogeropoulos et al., 2013).

These biochemical compounds include *Ganoderma* triterpenes (GTs), proteins, polysaccharides, steroids, peptides, amino acids, alkaloids, nucleotides, lactones, and unsaturated fatty acids (Dou et al., 2014; Xia et al., 2014; Shah and Modi, 2018). The identified biochemical compounds are known to play a vital role in the pharmaceutical properties, such as prophylactic and therapeutic of miscellaneous aliments (Richter et al., 2015). Therefore, a better understanding of these biochemical compounds on macro-fungal has the potential to be incorporated into the global efforts to address the extensive nutrient deficiencies, particularly, in developing countries (Bhutta, 2004; Stein, 2010; Bharaniidharan and Reshmi, 2019; Mani et al., 2019; Zięba et al., 2020). Notwithstanding these scientific claims, a quantifiable dose range must be established for both beneficial and safe doses of active ingredients for each disease type and their pharmacological mechanisms (Bishop et al., 2015; Wang et al., 2020). Until now, no review has reported the factors affecting the biochemical compound levels of *Ganoderma* and techniques used to enhance biochemical quality and pharmaceutical properties. This present review presents a concise update on the biochemical quality and pharmaceutical properties of *Ganoderma* species based on the review publications over the past decade (Table 3.1). Also, it highlights the safety and techniques used to enhance nutritional quality and pharmaceutical properties.

Table 3.1 Review publications on nutritional quality and pharmaceutical properties of *Ganoderma* species since 2010.

References	Scope of the review
Ahmad et al. (2022)	*Ganoderma lucidum*: A potential pleiotropic approach of ganoderic acids in health reinforcement and factors influencing their production
Tran et al. (2022)	Secondary metabolites from higher fungi in Vietnam: discovery, chemodiversity, and bioactivity
Ahmad et al. (2021a)	*Ganoderma lucidum*: A potential source to surmount viral infections through β-glucans immunomodulatory and triterpenoids antiviral properties
Ahmad et al. (2021b)	*Ganoderma lucidum* (Reishi) an edible mushroom; a comprehensive and critical review of its nutritional, cosmeceutical, mycochemical, pharmacological, clinical, and toxicological properties
Bhat et al. (2021)	Major bioactive properties of *Ganoderma* polysaccharides: A review

Ise et al. (2021)	*Ganoderma* research activities and development in Namibia: A review
Venturella et al. (2021)	Medicinal mushrooms: bioactive compounds, use, and clinical trials
Xu et al. (2021)	Effect of selenium on mushroom growth and metabolism: A review
Abebaw (2020)	Review on: Nutritional Value and Health Benefits of Edible Mushroom
Lu et al. (2020a)	Macrofungi: A review of cultivation strategies, bioactivity, and application of mushrooms
Lu et al. (2020b)	Molecular mechanisms of bioactive polysaccharides from *Ganoderma lucidum* (Lingzhi), a review
Wang et al. (2020)	Traditional uses, chemical components and pharmacological activities of the genus *Ganoderma* P. Karst.: a review
Bhat et al. (2019)	Major bioactive triterpenoids from *Ganoderma* species and their therapeutic activity: a review
Sudheer et al. (2019)	Bioactive compounds of the wonder medicinal mushroom "*Ganoderma lucidum*"
Rathore et al. (2019)	Medicinal importance of mushroom mycelium: Mechanisms and applications
Ahmad (2018)	*Ganoderma lucidum*: Persuasive biologically active constituents and their health endorsement
Cao et al. (2018)	*Ganoderma*: A cancer immunotherapy review
Cör et al. (2018)	Antitumour, Antimicrobial, Antioxidant and Antiacetylcholinesterase Effect of *Ganoderma Lucidum* Terpenoids and Polysaccharides: A Review
Hapuarachchi et al. (2018)	Current status of global *Ganoderma* cultivation, products, industry, and market
Khatian and Aslam (2018)	A review of *Ganoderma lucidum* (Reishi): A miraculous medicinal mushroom
Bryant et al. (2017)	Anticancer activity of ganoderic acid DM: current status and future perspective
Gargano et al. (2017)	Medicinal mushrooms: Valuable biological resources of high exploitation potential
Bishop et al. (2015)	From 2000 years of *Ganoderma lucidum* to recent developments in nutraceuticals
Delzenne and Bindels (2015)	*Ganoderma lucidum*, a new prebiotic agent to treat obesity?
Kozarski et al. (2015a)	Antioxidants of edible mushrooms
Ma et al. (2015)	Anti-diabetic effects of Ganoderma lucidum
Xia et al. (2014)	A comprehensive review of the structure elucidation and biological activity of triterpenoids from *Ganoderma spp*.
Wu et al. (2013)	Anti-cancer properties of triterpenoids isolated from *Ganoderma lucidum*–a review
Ma et al. (2011)	Triterpenoids from the spores of *Ganoderma lucidum*
Wasser (2010)	Medicinal mushroom science: history, current status, future trends, and unsolved problems

2 Biochemical compounds present in *Ganoderma*

Generally, mushroom biochemical compounds are found in fruit bodies, mycelia, and broth, these include phenolics, flavonoids, polysaccharides, ascorbic acid, glycosides, carotenoids, ergothioneine, and tocopherols (Klaus et al., 2011; Chen et al., 2012; Kozarski et al., 2015b; Suabjakyong et al., 2015). These biochemical compounds from mushrooms are described to have two main types, namely; primary and secondary (Barros et al., 2008; Wei et al., 2008; Cheung et al., 2012). *Ganoderma* species contain different levels of these biochemical compounds. The biochemical compounds from *Ganoderma* species are known to exhibit protective properties at different stages of the oxidation process and by various mechanisms (Zhou et al., 2014; Kozarski et al., 2015a). Fresh mushroom fruiting bodies are known to contain both insoluble and soluble fibre which is a component of the cell walls (Roy et al., 2015). Triterpenoids, phenols, flavonoids, water-soluble polysaccharides, and ganoderic acid are some of the major biochemical compounds found in *Ganoderma* fruiting bodies (Kao et al., 2013; Sudheer et al., 2018).

Although there are various biochemical compounds found in *Ganoderma* species, triterpenoids, and polysaccharides are considered to be the major biochemical compounds present (Xia et al., 2014; Bhat et al., 2021; Sułkowska-Ziaja et al., 2022). Other than the above-mentioned constituents, a significant number of studies reveal that about 90% of the fruiting body weight contains water (Khatian and Aslam, 2018). While the remaining 10% is rich in various constituents including protein 10-40%, carbohydrates 3-28%, fat 2-8%, fibre 3-32% and ash 8-10% (Zhou et al., 2007), together with minerals, such as Zn, Fe, Ca, P, K, Mg, Cu, and Se (Bulam et al., 2019) as well as vitamins, such as B1, B2, B6, choline, and inositol (Cör et al., 2018). Even though extensive research has been conducted on *Ganoderma* and its influence on pharmaceutical properties (Table 3.2), most of these studies report on the effects of biochemical compounds, and only a few report on the pathways and mechanisms of action (Elkhateeb et al., 2018; Liang et al., 2019; Lu et al., 2020b; Luo et al., 2022). Therefore, there is still a lot of scope for further research, especially on pathways and the mechanisms of various biochemical properties. The research scope needs to focus on different species with a high emphasis on agents containing anti-tumuor, anti-human immunodeficiency (HIV), and neurotrophic properties.

Table 3.2 Studies on potential biochemical compounds found in *Ganoderma* species and their pharmacological properties effect.

***Ganoderma* spp.**	**Biochemical compounds associated**	**Biochemical properties**	**Research findings (Effect of biochemical compounds)**	**References**
G. applanatum	Kojic acid	Anti-oxidant activity	Exhibited significant anti-oxidant activities	Sułkowska-Ziaja et al. (2022)
G. lucidum	Ganoderic acid B, β, C1, Ganodermanondiol and lucidumol B	Anti-cancer activity	Bioactive compounds were shown to have a positive effect through modulating cytokines on the Coronavirus	Al-Jumaili et al. (2020); Ahmad et al. (2021a)
G. lucidum	Ganoderic acid B, A, α, β, C1, H. GS-2	Anti-HIV activity	Inhibited HIV-protease	Kang et al. (2015); Cai et al. (2020)
G. carnosum	Phenolic acid, 2.5-dihydroxybenzoic acid, vanillic acid	Anti-oxidant activity	Exhibited anti-oxidant activities and enzyme inhibition activities	Yalcin et al. (2020)
G. pfeifferi	Phenolic acid, 2.5 - dihydroxybenzoic acid, vanillic acid	Anti-oxidant activity	Comprising anti-oxidant and enzyme inhibition properties	Yalcin et al. (2020)
G. lucidum	Polysaccharides peptides	Anti-obesity	Improved lipid metabolism disorders	Lv et al. (2019)
G. lucidum	Triterpenes, Polysaccharides	Anti-oxidant activity	*G.lucidum* was found to be absorbed quickly after ingestion, resulting in an increase in plasma total anti-oxidant activity	Sudheer et al. (2019)
G. applanatum	Terpene (Deoxyherqueinone, Myrocin C, Trichol C, Xylariacin B, Erinacine H Sphaeropsidin D, Presiccanochrominic acid, Comazaphine D, Zeylasteral, Applanoxide acid C - H)	Anti-cancer activity	Induced apoptosis in cancer cells, increased glutathione level in treated cells, and increased Bax/Bcl-2 ratio significantly	Elkhateeb et al. (2018)
G. lucidum	Enriched with triterpenoids	Anti-obesity	Enhanced lipid metabolism disorders and alleviate dyslipidemia	Hu et al. (2018)

G. lucidum	Triterpenes	Anti-cancer activity	GLT inhibits the growth of prostate cancer cells, suppresses migration, invasion and induces apoptosis through the inhibition of MMP	Qu et al. (2017)
G. atrum	Polysaccharides (PSG-1)	Anti-inflammatory activity	Results indicated that PSG-1 protected organs against CTX-induced immune dysfunction in mice and provided evidence that PSG-1 improved immune dysfunction by ameliorating ROS generation and apoptosis in the immune organs	Li et al. (2017)
G. hainanense	Lanostane-type, triterpenoids, ganoderenses A – E, laostane, nor-triterpenoids, ganoderenses F – G	Anti-cancer activity	None of the compounds evaluated for inhibitory activity against TrxR had inhibition activities (inactive)	Li et al. (2016)
G.lucidum	Polysaccharides, Gonoparticels	Anti-microbial activity	Gonoparticels and GLP had a significant on cell growth inhibition and were found to increase with increasing concentration of gonoparticels, while cell viability was decreased with increasing concentration of gonoparticels	Bhardwaj et al. (2016)
G. lucidum	HMW polysaccharides	Anti-obesity	Reduced weight and fat, inflammatory and insulin resistance	Chang et al. (2015)
G.lucidum	Triterpenoids, Polysaccharide, proteoglycans, and proteins (LZ-8)	Anti-diabetic effects	Hypoglycemic effects, promising, therapeutic target diabetic and inhibiting enzyme. Inhibitory activity an aldose reductase and α-glucosidase that can suppress postprandial hyperglycemia	Ma et al. (2015)
G. sichuanense	Meroteroenoids, Lingzhilactone B	Anti-inflammatory activity	Metabolites were noted to promote the proliferation of (NSCs, and as such, they constitute a class of NSC stimulators	Yan et al. (2015)
G. cochlear	(+)- and (-)-cochlearols A and B	Anti-inflammatory; Anti-cancer activity	(-)-cochlearols B has a strong p-Smad inhibitor, exhibiting renoprotective activities in TGF-β1 induced rat renal proximal tubular cells	Dou et al. (2014)
G. capense	*G. capanse* Glycopeptide (GCGP)	Anti-inflammatory activity	GCGP had inflammatory modulation effects on macrophage cells to maintain nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression at the normal level. Provided new evidence for the therapeutic value of GCGP in the treatment of different kinds of inflammatory disorders	Zhou et al. (2014)

G. theaecolum	Lanostane triterpenoids, ganoderic acid XL, ganoderic acid AM, ganoderic acid B, ganoderic acid C, ganoderisin C, lucidone B	Anti-cancer activity	Exhibited hepatoprotective activities against DL-galactosamine-induced cell damage in HL-770 cells	Liu et al. (2014)
G.atrum	Polysaccharides (PSG)	Anti-diabetic effects	PSG-1 may have an anti-apoptotic effect by inhibiting the expression of Bax and improving the expression of Bcl-2 protein in pancreatic tissues of type 2 diabetic rats	Zhu et al. (2013)
G. orbiforme	Ganoderic acid T	Anti-mycobacterial activity	Exhibited significant antimycobacterial activity against MIC H37Ra (MIC 1.3µM)	Isaka et al. (2013)
G.applanatum	Phenolic acid	Anti-oxidant activity	Effective concentration (EC_{50}) of extracts increased with a decrease in total phenol content	Kozarski et al. (2012)
G. sinense	Ganodermatetraol; ganolucidate F; ganolucidic acid B, ganolucidic acid C	Anti-cytotoxic activity	Compounds exhibited induction ability of hPXR-mediated CYP3A4 expression	Liu et al. (2012)
G. zonatum	Ganoderic acid Y	Anti-cytotoxic activity	Cytotoxic activity against liver and lung cancers	Kinge and Mih (2011)
G.colossum	Colosolactones, hydroquinone, ganomycin I, B	Anti-HIV activity	Compounds significantly inhibited HIV-1 protease with IC_{50} values of 7.5 and 1.0 µg/mL	El Dine et al. (2009)
G.japonicum	Oxygenated monoterpenes, sesquiterpenes	Anti-microbial activity	Essential oil of *G. japonicum* had significant inhibitory activity against isolates of disease-causing clinical pathogens and it is a potential medicinal resource that can be used as a natural antibiotic	Liu et al. (2009)
G.amboinense	Lanostanoid triterpenes	Anti-diabetic	Consumption of 2% *G. amboinense* significantly decreased glucose, triglyceride, and total cholesterol plasma level	Hsu et al. (2008)
G. sinense	Triterpenoid-enriched lipids	Anti-tumour activity	*Ganoderma* whole fruiting body, stipe, and sporoderm-broken spores possessed stronger inhibitory activities on sarcoma growth when compared with pileus extract	Yue et al. (2008)

G. tsugae	Water-soluble polysaccharides, lanostanoids, 24-dien-21-oic acid, tsugae acid	Anti-inflammatory activity	Compounds showed a significant inhibitory effect on the release of β-glucuronidase from rat neutrophils stimulated with fMLP/CB and also exhibited a potent inhibitory effect on NO production LPS/IFN-γ stimulated N9 microglial cells	Ko et al. (2008)
G. resinaceum	3α-(3-Hydroxy-5-methoxy-3methyl-1,5-dioxopentyloxy)-24-methyle-5α-lanost-8-en-21-oic acid	Anti-cytotoxic activity	Projected significant cytotoxic activity with IC_{50} values of 2.5 µg/mL in Hep-2 cell line	Niu et al. (2007)
G. australe	Austrolactone, australic acid	Anti-cytotoxic activity	Inhibited the viability and growth of the HL-60 cell line	Leon et al. (2003)
G. lucidum	Protein bond polysaccharides	Anti-virus activity	Inhibited the HSV multiplication	Eo et al. (1999)

Note: GLT = *Ganoderma lucidum* triterpenes; MMP = Matrix metalloproteinase; MIC = Mycobacterium tuberculosis; HSV = Herpes simplex virus; Bax/Bcl-2 = Protein Coding gene; IC_{50} = Inhibitory concentration; fMLP = Formly-Met-Leu-Phe; CB = Cytochalasin B; LPS = Lipopolysaccharide; IFN-γ = Interfence-γ; GLP = *Ganoderma lucidum* polysaccharides; HIV = Human immunodeficiency virus; NSCs = Neural stem cells; TrxR = Thioredoxin Reductase; CTX = Cyclophospharide; ROS = Reactive oxygen species; TGF-β1 = Transforming growth factor beta 1; Smads = Mothers against decapentaplegic; hPXR= Human pregnane X receptor; CYP3A4 = Cytochrome P450 3A4; HMW = High molecular eight;

2.1 *Ganoderma* biochemical compounds attribute to pharmaceutical activities

2.1.1 Preclinical studies

Medicinal mushrooms and fungi are reported to produce a total of 126 medicinal functions, including anti-tumour, anti-fungal, anti-viral, anti-oxidant, anti-parasitic, anti-metastatic, anti-bacterial, anti-diabetic, detoxification, hepatoprotective, radical scavenging, anti-hypercholesterolemia, immunomodulating, and immunomodulatory effects (Wasser, 2010; Gargano et al., 2017). The *Ganoderma* genus mostly consists of these pharmaceutical activities deriving from its rich biochemical compounds (Ahmad et al., 2022; El Sheikha, 2022; Lilburn, 2022; Sułkowska-Ziaja et al., 2022). *Ganoderma* species are widely used to address a variety of diseases, such as inflammation, treatment of cancer, hypertension, hepatitis, leukopenia, neurasthenia, and hyperlipidemia (Liu et al., 2009; Bhardwaj et al., 2016). Delzenne and Bindels (2015) reported that *Ganoderma* extracts can be used as a potential traditional remedy that can reduce obesity. Some of the macro-fungi biochemical compounds display pharmaceutical activities that can work as inducers and/or cell signals, driving changes in gene expression, which result in the activation of enzymes that dispense with reactive enzyme species (Yu et al., 1997; Chang et al., 2007; Jia et al., 2009; Elkhateeb et al., 2018).

Biochemical compounds such as phenols and flavonoids extensively contribute to the ingredients of dietary supplements that have been investigated for the prevention of various ailments (Kozarski et al., 2012). These natural anti-oxidants have been preferred for food applications and this is due to the increasing demand for natural additives and ingredients by consumers (Abebaw, 2020). *Ganoderma* triterpenoids and polysaccharides extracts supplemented with other drugs exhibit potential action in the prevention and treatment of various diseases, such as HIV, cancer, and neuraminidase (Ahmad et al., 2021a). The absence of potential toxicity allows patients to use *Ganoderma* as an adjunctive to chemotherapy at lower concentrations (Gill and Rieder, 2008). However, caution should be exercised when planning studies on the use of *Ganoderma* extracts in the therapy of patients with cancer. Ahmad et al. (2021b) argued that even though studies are conducted on the biochemical effect of compounds present in *Ganoderma*, most of these studies are preclinical studies. Therefore, this suggests that often these preclinical studies may not be appropriately translated into clinical studies.

2.1.2 Clinical studies

A recent report reveal that twenty-two clinical studies have been conducted on several preparations of *Ganoderma* biochemical compounds in patients and healthy volunteers (Ahmad et al., 2021b). At least five cases of adverse events associated with the use of *Ganoderma* supplements have been reported thus far without any casualties reports (Organization, 2002; Wanmuang et al., 2007; Ma et al., 2015). A case was presented in which fatal fulminant hepatitis occurs after taking *Ganoderma* powder extract for 1–2 months (Wanmuang et al., 2007). However, no deaths or abnormalities in clinical symptoms and significant differences in body weight have been reported (Cao et al., 2018). Despite the concerning reports regarding diverse effects and toxicity, it is required to validate the biologically active compounds to verify their efficacy and safety (Ahmad et al., 2021a). There is insufficient data to determine which biochemical compounds are more effective and have a higher safety profile, purified mushroom extracts, and fractions thereof (Kozarski et al., 2015a). Lu et al. (2020a) suggested that this has led to most of the biochemical ingredients in macro-fungi being classified as healthcare products rather than drugs for the adjunctive treatment of various diseases. Therefore, further experiments including *in vitro*, *in vivo*, and clinical should be encouraged to identify any potential biochemical compound side effects of *Ganoderma* (Xia et al., 2014; Ahmad et al., 2022).

2.2 Major biochemical compounds present in *Ganoderma*

2.2.1 Triterpenoids

Triterpenoids consist of the molecular formula $C_{30}H_{48}$ and these compounds are reported to play a significant role in food, health, and industrial biotechnology (Garg et al., 2020). These triterpenoids are found in the spores which are the reproductive cells ejected from *Ganoderma* after the fruiting bodies are matured (Jong and Birmingham, 1992; Boh et al., 2007). A series of more than 150 triterpenoids have been reported from the fruiting bodies of *Ganoderma* representing five major structural classes and this has drawn the attention of many chemists and pharmacists (Ma et al., 2011; Cör et al., 2018). These classes of organic compounds include triterpenes, steroids, quassinoids, limonoids, and steroidal saponins (Ayad and Akkal, 2019). These biochemical compounds have received substantial attention because of their diverse, promising biological and pharmaceutical, including anti-viral, anti-tumour, anti-fungal, and anti-inflammatory activity as well as cell cycle and epigenetic regulation (Ko et al., 2008; Petronelli et al., 2009; Li et al., 2020). In a study conducted on the anti-cancer effect of triterpenes in human prostate cancer cells, triterpenes were identified as the biologically active

compounds that may be responsible for the anti-tumour effect (Niu et al., 2007; Qu et al., 2017). Several studies have reported diverse pharmaceutical promising results attributed to these triterpenoids. However, details of these essential attributes mechanisms involved, and the active components of *Ganoderma* triterpenes need to be further clarified (Wu et al., 2013; Ahmad et al., 2021a).

Furthermore, the bitter taste found in most *Ganoderma* species is due to the presence of triterpenoids, especially the ganoderic acids (GAs) which are the major type of triterpenoid present in the fungus (Sudheer et al., 2019). More than 150 GAs derivatives are acquired from *Ganoderma* species (Ahmad et al., 2022). However, about 140 subtypes of GAs have been reported from *Ganoderma* and represent a subtype of triterpenes with four cycling and two linea isoprene units (Bhat et al., 2019). GAs are oxygenated lanostane-type triterpenoids from *Ganoderma* and are known to have substantial biological activities (Leon et al., 2003; Kinge and Mih, 2011; Cai et al., 2020). GAs contain a carboxyl group that is described to be the most commonly studied terpenes of *Ganoderma* and mainly contain 24, 27, or 30 carbon atoms in the molecules (molecular formula – $C_{30}H_{44}O_7$) (Gill and Rieder, 2008; Satria et al., 2019). Diverse types of GAs are reported and these include ganoderic acids A (GA-A), ganoderic acids C (GA-C), ganoderic acids D (GA-D), ganoderic acids DM (GA-DM), ganoderic acids F (GA-F), ganoderic acids H (GA-H), ganoderic acids Me (GA-Me), ganoderic acids T (GA-T), ganoderic acids TQ (GA-TQ), ganoderic acids X (GA-X), and ganoderic acids Y (GA-Y) (Paterson, 2006; Tang et al., 2006; Zhang et al., 2011; Liu et al., 2014; Hapuarachchi et al., 2018). GA-A, GA-C2, GA-D, GA-F, GA-DM, GA-X, and GA-Y are however the most well-studied ganoderic acids (Liang et al., 2019). This might be the result of the high level of pharmaceutical properties, the phase level reached, and clear mechanism of action such as anti-proliferation and inducing apoptosis. These GAs are observed as the most prominent bioactive metabolites resulting from the significant effects against various diseases (Bhat et al., 2019; Ahmad et al., 2022). It is reported that approximately 100 isoforms of GAs were screened and reviewed for their pharmaceutical activities and characterised by a molecular mass, high lipophilicity, and complex structure (Liu et al., 2012; Yang and Yang, 2019). Given the variation of biochemical compounds within *Ganoderma* species and the continuous discovery of new species, there is a need to investigate which species consist of high GA at optimum production conditions.

2.2.2 Polysaccharides

The majority of mushrooms are known to produce various types of polysaccharides. *Ganoderma* extracts are reported to enhance the immune system without considerable side effects due to their polysaccharides (Szedlay, 2002; Li et al., 2017; Lv et al., 2019). These polysaccharides have been studied and used for pharmaceutical purposes resulting from their diverse bioactive activities (Eo et al., 1999; Lee et al., 2007; Hu et al., 2018). These activities are described to include immunomodulating and anti-tumour (Tokunaka et al., 2000; Han et al., 2020). A large number of these polysaccharides (molecular formula – $C_6H_3O_5$, $40 \leq n \leq 3000$) such as exopolysaccharides (EPS) and endopolysaccharides (PPS) are obtained from mycelia as they are reported to be efficient in mycelia compared to the fruit bodies (Song et al., 1998; Cheung, 1999; Lee et al., 1999; Kim et al., 2005; Cheung, 2010). Generally, each fungal polysaccharides consist of different structural characteristics, such as composition, molecular weight, glycosidic bond type, and degree of branching (Ruthes et al., 2015). The difference in structure causes a variation in the bioactive properties of each polysaccharide, hence the continuous research to link the pharmaceutical properties of polysaccharides and the structural component (Gong et al., 2020).

Ganoderma polysaccharides (GPs) are reported to be relatively constant and mostly consist of D-fructose, D-galactose, D-mannose, D-xylose, L-fructose, L-rhamnose, and L-arabinose (Lu et al., 2020b). A report from Yue et al. (2008) demonstrated that the GPs were able to inhibit the growth of a cancer tumour. The GPs crude extract used on human lung cancer cell lines indicated that GPs may potentially serve as a chemopreventive agent for cancer therapy (Bhardwaj et al., 2016). One of the main effective constituents found to be responsible for these pharmaceutical properties is the β-D-glucan. Choong et al. (2018) described the main chain of β-D-glucan to consist of β-(1→3) linkages with some β-(1→6) branches together with chitin, galactans, xylans, and mannons. A study by Hsu and Cheng (2018) established that the β-1,3-D-glucan can activate macrophage and natural killer cells. Although the pharmaceutical properties of several fungal polysaccharides have been reported repeatedly, only a few fungal polysaccharides have been commercialised thus far. This has been partially due to high production or purification costs, erratic, and unstable chemical characteristics (Giavasis, 2014; Liaqat and Eltem, 2018). The effects of GPs on the prevention of various diseases still need to be determined (Zhu et al., 2013). Despite the intensive research on GPs, signalling has been the commonly proposed mechanism. A recent report by Luo et al. (2022) found several mechanisms and network pathways. However, more in-depth investigations on GPs still need

to be conducted to determine the precise mechanisms and pathways (Luo et al., 2022). The discovery of the relationship between the structural characteristics and bioactive properties of fungal polysaccharides will contribute to the understanding of its mode of action against diseases. Furthermore, a better understanding of the structure-bioactive relationship will improve the quality control and commercialisation of the GPs.

2.2.3 Protein

Multiple proteins have been found in *Ganoderma* (Li et al., 2019). However, very few studies have been reported on its protein components (Szedlay, 2002; Wang and Ng, 2006). The proteins from *Ganoderma* hold all of the essential amino acids, especially leucine and lysine representing the high percentages (Al-Jumaili et al., 2020). These amino acids target cancer signalling factors, such as plasma membrane receptor proteins and adaptor molecules (Gullett et al., 2010). Zhao et al. (2010) suggested that some of the biological properties were low due to the presence of low-molecular-weight compounds, such as protein and triterpenoid. *Ganoderma* proteins, such as fungal immunomodulatory proteins (FIPs), ribosome-inactivating proteins (RIPs), antimicrobial proteins, ribonucleases, lectins, and laccases possess special immunogenicity that led to immunomodulatory and anti-tumour (Zhou et al., 2012). Several researchers tend to focus more on the FIPs due to their substantial role in anti-allergy, anti-tumour, anti-transplant rejection activities, inducing the expression of cytokines and promoting the proliferation of lymphocytes (Li et al., 2011; Zhou et al., 2012; Li et al., 2018).

The differences between FIPs structures of *Ganoderma* species influence the type of pharmaceutical activities (Huang et al., 2009). Huang et al. (2009) alluded that the biological structures and functions of FIPs can be maintained by the several common conserved motifs such as C-terminal and N-terminal found in FIPs. However, there have been challenges in the natural production capacity of FIPs. Hence, to have a sufficient production capacity for functional studies and industrial applications, Ejike et al. (2020) suggested the use of recombinant technology. Furthermore, the consideration of substrate enhancement with protein agents might increase the amount of total protein content on the fruiting bodies of *Ganoderma*.

2.2.4 Phenols

The main phenolic compounds found in mushrooms are phenolic acids (Reid et al., 2017; Velez et al., 2019). Their effect on dietary polyphenols on human health has developed significantly in the last 20 years (Scalbert et al., 2005; Ferreira et al., 2009). Kozarski et al. (2015a) mentioned that most of these compounds are present in a form of glycosides, polymers, or

esters. These compounds may be classified into different groups as a function of the phenol rings large number and the structural elements (molecular formula – C_6H_5OH) binding these rings to each other (Kozarski et al., 2015a). Phenolic acids can be divided into two major groups namely; hydroxybenzoic acids and hydrocinnamic acids, which are derived from the non-phenolic molecules benzoic and cinnamic acid (Manach et al., 2004; Halliwell et al., 2005; Ferreira et al., 2009; Lojek et al., 2014). It is reported that some of these naturally occurring phenolic acids inhibit various anti-viral activities (El Dine et al., 2009). However, the major challenge in elucidating the health effects of polyphenols is the large number of phenolic compounds identified in food (Scalbert et al., 2005; Vujovic et al., 2016), yielding differing biological activities (Kuntz et al., 1999). Recent studies reveal that the commercial production of secondary metabolites from medicinal mushrooms is limited mainly due to less information (Yan et al., 2015; Tran et al., 2022). Therefore, further research on secondary metabolites and their regulation should be investigated.

2.2.5 Flavonoids

Diverse classes of flavonoids are described based on the differences in their generic structure of the heterocycle C ring and can be classified into flavanols, flavones, flavanones, anthocyanins, and isoflavonoids (Ferreira et al., 2009). Flavonoids [molecular formula – C_6-C_3-C_6] are reported to be a strong anti-oxidant essential for most organisms (Yao et al., 2016). In general, natural flavonoid compounds exist in the form of glycosides and different flavonoids glycosides can be formed resulting in different connections of locations and modes of sugars (Yao et al., 2016). The flavonoids not only improve the circulation of blood but are also reported to effectively reduce the occurrence of cardiovascular and cerebrovascular diseases and alleviate their symptoms (Liu et al., 2017). It has more ability to prevent oxidation in comparison to vitamin E, which can prevent the degrading of cells and ageing and cancer (Yao et al., 2016). Although macro-fungi have been reported to contain these phytochemicals, their levels have been noted to be highly variable, possibly influenced by the type of substrates used and climatic conditions which it is grown under (Valverde et al., 2015). Su et al. (2016) stated that the information regarding the chemical and biological compounds of macro-fungi remains limited in various parts of the world. Further research studies focusing on flavonoid content from different species will increase and contribute to the currently existing information.

2.3 Consumption and safety of *Ganoderma*

2.3.1 Production

Despite the high global consumption reported for these species, their production rate is lower than the demand (Ma et al., 2011; Sudheer et al., 2019). Ma et al. (2011) alluded that there are still several challenges to be overcome before *Ganoderma* spores can become a modern drug. These challenges include the cost of *Ganoderma* spores being high, and the extraction rate being too low as it is often not more than 5%. To meet the market demand and consumer demands, therefore, a practical high-yield macro-fungus cultivation technology is urgently needed (Lu et al., 2020a). Also, quantifiable doses range for both beneficial and safe doses of active ingredients for each disease type and their pharmaceutical pathway must be established.

2.3.2 Safety

A great number of synthetic chemicals such as synthetic phenolic compounds are strong radical scavengers (Gąsecka et al., 2016), but normally they have side effects (Abebaw, 2020). The low rate of side effects on consumption of *Ganoderma* exacts reported and presumed health benefits have provided a reputation for these species to be used as herbal medicine (Sanodiya et al., 2009; Sudheer et al., 2019). The long-term safety and tolerance have led to this fungus being admitted as a nutritional supplement across the world (Bhat et al., 2019). Generally, the consumption of *Ganoderma* exacts is safe when used according to intended doses or instructions for health care (Yuan et al., 2020; Ahmad et al., 2021a; Ahmad et al., 2021b). The use of oral doses of up to 9 g/day is reported to produce temporary side or adverse effects, including abnormal sweating, frequent urination, nausea, and hepatotoxicity (Chang et al., 2015; Wang et al., 2015; Zhang et al., 2020). The findings of Ise et al. (2021) suggested that the *Ganoderma* total aerobic and total coliform counts were above the acceptable limits of <1 cfu/g set by the European Union Commission Recommendation (Directive 2004/24/EC). However, wild and cultivated *Ganoderma* mushrooms have different microbial qualities, therefore wild *Ganoderma* may require further processing before they are of suitable microbial quality and safe to consume (Ise et al., 2021). Even though macro-fungi consist of numerous biochemical compound activities, neither the efficacy nor the safety of their constituents has been validated at the clinical trial level (Lu et al., 2020a). Therefore, the efficacy and safety of *Ganoderma* warrant further investigation.

2.4 Factors affecting the biochemical compound levels of *Ganoderma*

Literature suggests that the amount and percentage of biochemical compounds *Ganoderma* can be diverse (Wachtel-Galor et al., 2011; Sudheer et al., 2019). Such diversity can occur due to several factors, including preharvest conditions (Cao et al., 2018; Ćilerdžić et al., 2018b; Letti et al., 2018), and postharvest conditions (Dong et al., 2019; Zhang et al., 2020; López-Hortas et al., 2022). Therefore, it is critical to have an overview of preharvest and postharvest conditions as factors influencing the total content of biochemical compounds in these species.

2.4.1 Preharvest conditions

The total biochemical compound content of *Ganoderma* species is influenced by diverse factors, such as the type of species, substrate, pH, temperature, relative humidity, mushroom body part, and maturity of the basidiocarp (Fang and Zhong, 2002; Colak et al., 2009; Wandati et al., 2013; Sudheer et al., 2018; Sudheer et al., 2019; Cho et al., 2021). Growing conditions, various substrates used, temperature, presence of air, and extraction technique used are described to be some of the factors affecting the pharmacologically active compounds yields and composition of *Ganoderma* species (Sudheer et al., 2019; Ahmad et al., 2021a).

2.4.1.1 Production techniques

The production cultivation techniques for *Ganoderma* are divided majorly into two patterns based on the growth medium used for cultivation (Zhou et al., 2012). Solid solid-state fermentation (SSF) and liquid-state fermentation (LSF) are two commonly used production cultivation techniques. The SSF production cultivation technique is used for the production of basidiocarp, spores, and mycelia biomass, while, the LSF is used for mycelia biomass cultivation only (Zhou, 2017). Biochemical compounds can be extracted in 2-3 weeks through the LSF as compared to SSF which takes 3-5 months (Bijalwan et al., 2020). Nonetheless, the SSF cultivation technique is often used to produce *Ganoderma*. This might be because the technique is cost-effective and high production of basidiocarps can take place on a larger scale given a comprehensive well-controlled environment providing optimum growth conditions. Despite this, the SSF technique can still be affected by a wide range of factors, such as growth parameters. Also, the period to produce basidiocarps under SSF is long (Dong et al., 2021; Singh et al., 2021; Suansia and John, 2021), and during this period it is difficult to control the quality (Yang and Liau, 1998; Lee et al., 1999). The quality of basidiocarps determines the quality of biochemical compounds present. Therefore, the LSF technique might be an effective

technique to be used for obtaining and extracting high-quality biochemical compounds that can be used for commercial purposes.

2.4.1.2 Growing conditions

The quality of mycelia produced is influenced by several factors such as carbon, nitrogen, and inorganic salts (Kadowaki et al., 2010; Berovic and Podgornik, 2019). Also, growth parameters such as temperature, pH, relative humidity, and aeration play a vital role in mycelia's quality (Roberts, 2004; Gurung et al., 2012; Shah and Modi, 2018). These growth parameters have a significant effect on the qualitative/quantitative profile of biochemical composition for *Ganoderma* basidiocarps which determines their bioactivities (Ćilerdžić et al., 2018a). The findings by Bidegain et al. (2019) indicated that the biochemical composition of *Ganoderma* can be influenced by the formulation of a cultivation substrate. Sudheer et al. (2018) highlighted that the amount of light and ventilation causes an increase in the level of carbon dioxide gas in the air due to the respiration of *Ganoderma*. Hence, this led to a significant impact on the quantity of these biochemical compounds. However, it is imperative to consider that biochemical compounds' activity measured by *in vitro* techniques may not reflect *in vivo* effect of the biochemical compounds as many other factors may be involved (Sini et al., 2011). Therefore, irrespective of the production technique used, optimising the growth conductions is imperative as they directly impact the quality of biochemical compounds in the basidiocarps of these species.

2.4.2 Postharvest conditions

In general, the shelf-life of mushrooms is relatively short due to diverse factors, such as respiration rate, microbial activity, and water activity (El Hage et al., 2021). During this period most of the biochemical compounds available in the fruiting body deteriorate continuously (Karimirad et al., 2018). The biochemical composition of mushroom species is influenced by postharvest conditions (Valverde et al., 2015). Several reports note the postharvest conditions of *Ganoderma* as one of the major factors influencing the total content of biochemical compounds. Hence, it is necessary to have a fundamental understanding of postharvest conditions, such as the storage, extraction, and processing of biochemical compounds on fruiting bodies.

2.4.2.1 Processing/preservation/storage techniques

Conventional heat-drying and freeze-drying techniques are considered the most efficient and effective for use in phytochemical-related industries (Sadiq et al., 2021). The drying techniques

are commonly used for the postharvest processing of *Ganoderma* species. In general, these drying techniques are used to dry fruiting bodies, these techniques are classified into different groups: heating (ovens, stoves, microwaves, baking, sun, and infrared) and air-drying (hot-air, vacuum drying) and freeze-drying (Liu, 2011; Kubra and Rao, 2012; El Hage et al., 2021). However, choosing a suitable drying technique is limited by the type of desired bio-components and their physical properties (Sudheer et al., 2019). A report reveals that different drying techniques have positive and negative effects on the physical properties, bioactive ingredients, and anti-oxidant activity of *Ganoderma* (Dong et al., 2019). Freeze-drying is one of the most efficient and effective techniques used in preventing the loss of bioactive compounds in dried plant materials as compared to conventional drying techniques, such as sun, room temperature, and shade drying (Barimah et al., 2017). A report by Bhatta et al. (2020) however suggested that the use of freeze-drying can lead to the loss of essential nutritional contents, such as minerals and vitamins.

The hot air-drying technique is also reported to be economically cost-effective but it might have a physical effect due to the probability of oxidation damage occurring during air stream exposure (Fan et al., 2012). Anti-oxidant components such as ascorbic acid, carotenoids, tocopherols, and phenolics are highly sensitive to heat, thus freeze-drying is usually recommended (Marques et al., 2009). Furthermore, the short time it takes when using microwave drying prevents oxidation and enzyme reactions leading to significantly higher anti-oxidant phenolic content in comparison to hot air drying and sun drying (Kubra and Rao, 2012; Zhao et al., 2017; Dong et al., 2019). To avoid thermal degradation of bioactive compounds such as triterpenes and polyphenols, it is recommended to maintain the drying temperature below 40 °C (Kim and Lee, 2002; Krishnaswamy, 2010). A temperature range of 50-60 °C is widely used for drying herbal material as most of the bioactive compounds are retained at these temperatures (Chin et al., 2009; Liu et al., 2011). Despite several factors mentioned, more detailed studies on the chemical composition of mushroom extracts, as well as *in vivo* assays are necessary to characterise them as bioactive compounds (Prathima, 2022). The type of bioactive compound intended for extraction will determine the choice of technique to be used for processing. The current existing techniques necessitate further development into cost-effective, scalable, viable, and standardised methods for specific bioactive compounds.

2.4.2.2 Extraction techniques

Several extraction techniques have been developed and investigated intensively, with the sole purpose of obtaining extracts of *Ganoderma* with higher yields and lower costs (Do et al., 2021). The literature points out that most bioactive compound requires a specified extraction technique. Although these extraction techniques have been developed and used, extraction of individual components is difficult, time-consuming, complex, expensive, and some have accuracy limitations (Muniroh et al., 2014). Before extraction, it is recommended that the mushroom size from the raw material is reduced to a smaller particle size, this increases the diffusion of bioactive compounds in the solvent and thus increases the total mass transfer rate (Sudheer et al., 2019). According to Cho et al. (2021), extraction of freeze-dried *Ganoderma lucidum* between 64.2 and 70 °C for 1.2 hrs maximised the anti-oxidant activity and ganoderic acid concentration, whereas the polysaccharides content and anti-diabetic activity were maximised by extraction between 66.8 and 70 °C for more than 2.8 hrs. The results suggested that extraction conditions might be a limiting factor for target-oriented investigations (Cho et al., 2021). Therefore, extraction processes with purity remain a demanding and important task for future research work. Optimised extraction techniques may improve the potential effect and quality of extracted bioactive compounds.

3 Techniques used to enhance biochemical quality and pharmaceutical properties of mushroom fruiting bodies

Mushroom species contain high of levels biochemical compounds (Kalogeropoulos et al., 2013; Roy et al., 2015). However, there is scientific evidence that the total biochemical compounds of fruiting bodies vary within species (Bishop et al., 2015; Sharma et al., 2019; Sudheer et al., 2019; Venturella et al., 2021). Also, the occurrence of this phenomenon has been stipulated to be due to diverse factors. These factors include the production period it takes to produce the fruiting bodies of *Ganoderma*. It is highly challenging to regulate the quantity of biochemical compounds produced during the development of *Ganoderma* (Bryant et al., 2017). This results from the use of different agricultural residues for fruiting bodies production. hence the use of mycelia as a source for these biochemical compounds may be an economic and safe alternative for the production of raw materials for different purposes in different industries (Zięba et al., 2020). Therefore, it is imperative to explore various techniques that have the potential to enhance biochemical compounds and ensure purity.

3.1 Plant growth regulators

3.1.1 Significance of plant growth regulators

Generally, plant growth regulators (PGRs) are known to have a pivotal role in the development and behavioural processes of plants (Vedenicheva et al., 2018). They influence cell division, elongation, and differentiation (Kaur, 2016), and are reported to have a greater impact on the final shape and function of cells and tissue in all higher plants (Valentino and Galvez, 2015). The influence of PGRs on plants has led most researchers interested to investigate whether their influence can apply to macro-and micro-fungus development (Khandakar, 2004; Guo et al., 2009). Mushrooms are plants with no chlorophyll and they do not require photosynthesis to produce their food (Santner and Estelle, 2009). PGRs influence various production stages of mushrooms such as growth and development (Maniruzzaman, 2004; Mukhopadhyay et al., 2005; Ramachela and Sihlangu, 2016). PGRs can work together or independently to influence the development, biomass, nutritional value, biochemical composition, and pharmaceutical properties of macro-fungi (Mukhopadhyay et al., 2005; Guo et al., 2009; Maciel et al., 2013; Yang et al., 2013; Sarker and Chowdhury, 2014; Kaur, 2016; Ong et al., 2018; Vedenicheva et al., 2018; Vedenicheva et al., 2021).

3.1.2 Plant growth regulator's enhancement

Several PGRs are commonly used for the enhancement of mushrooms, including auxins (indole acetic acid IAA) [molecular formula $C_{18}H_{32}O_5$], gibberellin (A_3/GA/GA_3) [molecular formula $C_{19}H_{22}O_6$], cytokinins (CK) [molecular formula $C_{10}H_9N_5O$], ethylene (ethane) [molecular formula C_2H_4], and abscisic acid (ABA) [molecular formula $C_{15}H_{20}O_4$] (Dey et al., 2007; Sood, 2011; Cheng et al., 2013; Maciel et al., 2013; Vi et al., 2018). However, IAA, CK, and GA_3 are the commonly used plant growth regulators in most of the research work on macro-fungal species (Kaur, 2016). PGRs play a major role in the growth media of mushrooms to enhance biomass (Yang et al., 2013; Sarker and Chowdhury, 2014). PGRs also play an important role in *Ganoderma* species by enhancing protein and anti-oxidants such as phenols (Kumar et al., 2017; Xu et al., 2019). Even though the PGRs have been investigated for the enhancement of bioactive compounds on mushrooms (Table 3.3), other techniques for the extraction or enhancement of PGRs on fungal raw material for the preparation of pharmacologically active drugs still need further investigation (Vedenicheva et al., 2021).

Table 3.3 Influence of plant growth regulators on nutritional quality and pharmaceutical properties of fungal species.

Fungal species	Plant growth regulators	Research scope of work	Key research findings	References
Hericium coralloides	Cytokinin	Effect of cytokinin-containing extracts from some medicinal mushroom mycelia on HepGR cells *in vitro*	Demonstrated a cytotoxic effect on HepGR cells	Vedenicheva et al. (2021)
Inonotus obliquus	Tween 80	Synergistic effects of surfactant-assisted biodegradation of wheat straw and production of polysaccharides by Inonotus obliquus under submerged fermentation	Increased in EPS by 142.9%, the EPS had higher contents of protein, uronic acid, sugar, and mannose ration, and also higher anti-oxidant activity. Increased the activities of ligninolytic enzymes MnP, Lip, and Lac.	Xu et al. (2019)
Hericium erinaceus	Gibberellin	Improvement of nutritional and bioactive compound production by lion's mane medicinal mushroom, Agaricomycetes, by spraying growth regulators	Results showed that GA_3 increased (108%), polyphenols (26%), and free amino acid (100%).	Vi et al. (2018)
Ganoderma boninense	Salicylic acid; jasmonic acid	A preliminary study on the effects of salicylic and jasmonic acids on *Ganoderma boninense* growth, mycelial hydrophobicity, and media pH under *in vitro* assays	Anti-fungal effect, triggering fungal defense mechanisms against various pathogens	Ong et al. (2018)
Hericium erinaceus	Nephthyl acetic acid	Improvement of nutritional and bioactive compound production by lion's mane medicinal mushroom, Agaricomycetes, by spraying growth regulators	Increased polysaccharides with 4.37 g/100g and triterpenoids with 17.27 g/100g	Vi et al. (2018)
Ganoderma lucidum	Cytokinin	Cytokinins comparative analysis in mycelial biomass of medicinal mushrooms	Increased the production of pharmacological compounds	Vedenicheva et al. (2018)

Calocybe indica	Auxin	Effect of growth regulators on biomass production and yield potential of milky mushroom (P & C)	Fruiting bodies treated with auxin increased protein content	Kaur (2016)
Calocybe indica	Gibberellin	Effect of growth regulators on biomass production and yield potential of milky mushroom (P & C)	Increased various nutritional components from fruiting bodies treated with gibberellin	Kaur (2016)
Ganoderma lucidum	Auxin	Response of *Ganoderma lucidum* and Trametes sp. to the herbicide picloram: Tolerance, antioxidants, and production of ligninolytic enzymes	Auxin low concentrations (ppm) promoted the stimulation of the metabolism of nitrogenous compounds and produced fruiting bodies with high protein content	Maciel et al. (2013)
Ganoderma lucidum	Coix lacryma-jobi oil	Stimulatory effects of Coix lacryma-jobi oil on the mycelial growth and metabolites biosynthesis by the submerged culture of *Ganoderma lucidum*	Triterpenoids, EPS, and IPS increased by 2.76 fold, 2.2 fold, and 2.23 fold respectively	Yang et al. (2013)
Asperyillus umbrosus	Gibberellin	Cultural Physiology: effect of plant growth hormones on the growth and sporulation of *Aspergillus umbrosus*	Stimulatory effect on mycotoxin synthesis	Sood (2011)
Phellinus linteus	indole-3-acetic acid; indole-3-butyric acid; naphthaleneacetic	Effects of phytohormones on mycelial growth and exopolysaccharide biosynthesis of medicinal mushroom *Phellinus linteus*	Increased EPS synthesis	Guo et al. (2009)
P. sajor-caju	indole-3-acetic acid	Effect of hormone, media and variety on mycelial growth of mushroom	Increased protein content of mycelia	Dey et al. (2007)
P. sajor-caju	Cytokinin	Effect of hormone, media and variety on mycelial growth of mushroom	Triggered an appropriate response as protein synthesis	Dey et al. (2007)

Note: HepGR = Hepatama GR; ppm = Parts per million; EPS = Exopolysaccharide; IPS = Intracellular polysaccharides

3.2 Essential elements

3.2.1 Mushrooms essential elements

There is a constant need to explore potential sources of various nutritional value, essential elements, and biochemical components as they can assist in the fight against malnutrition (Roy et al., 2015; Rathore et al., 2019; Oyetayo et al., 2021). Generally, fruiting bodies of mushroom species are characterized by high mineral constituents such as K, P, Na, Ca, Mg, Cu, Zn, Fe, Mo, and Se and vitamins (Dundar et al., 2008; Nunes et al., 2012a). *Ganoderma* is identified to be a rich source of protein, various vitamins, carbohydrates, dietary fibre, fat, oils, coumarin-glycosides, and inorganic ions such as Mg, Zn, Fe, Ca, Cu, P, K, Na, and Ge (Patocka, 1999; Roy et al., 2015). Micro-nutrients such as Zn are known to have anti-microbial properties and *Ganoderma* species are described to have high levels of this mineral content (Kalogeropoulos et al., 2013). However, the quality of these essential elements is determined by the production activities, hence the importance to develop new or integrated production techniques that can maintain or improve quality should be considered.

3.2.2 Essential elements importance

The importance of essential elements in the human body is boosting the immune system and managing various ailments, such as using K to lower high blood pressure (Sun and Liu, 2004). It has been reported that micro-nutrients such, as Fe, Zn, and Mg control important biological processes, facilitating the binding of molecules to receptor sites on cell membranes (Miles and Chang, 2004). Some of these micro-nutrients donate or accept electrons in the reactions of reduction and oxidation which results in the generation and utilisation of metabolic energy (Chiu et al., 2000; De Silva et al., 2013). These mushrooms are known to assist in providing essential amino acids for humans, especially for vegetarians' dietary needs and this is due to the rich source of protein it contains (Shah et al., 1997; Zhao et al., 2004; Wani et al., 2010). Even though there are several reports on various essential elements, only a few macro-fungal essential elements derived components have been commercialised (Lu et al., 2020a). Therefore, the techniques to improve the quality and control of these elements with the intention of commercialisation still require further investigation.

3.2.3 Essential elements enhancement

The essential element content of mushrooms is reported to vary due to the substrate composition on which the fungus is produced (Parashare et al., 2013). Zięba et al. (2020) reported that a new approach for enriching substrate with essential elements employed for

mushroom cultivation not only improves their agronomic potential in terms of yield and quality but also functional food and diet supplement production. *Ganoderma* can grow on various lignocellulosic substrates and substrate enrichment with innumerable additives use has been one of the strategies used to increase mushroom productivity (Bidegain et al., 2019; Xu et al., 2021). The use of additives such as micro-nutrients and vegetative oils for the enrichment of substrate has been reported to have a significant increase in mushroom production in terms of yield and reduction production cycle (Postemsky et al., 2014; Bidegain et al., 2015; Bidegain et al., 2019). *Ganoderma* can absorb, accumulate, and transform inorganic compounds into organic compounds (Nunes et al., 2012b; Rzymski et al., 2016). However, the chemical forms of essential elements used for enrichment can affect mycelia growth, and fruiting body development, together with its chemical composition (Da Silva et al., 2019).

Even though cultivation techniques may influence various activities including chemical composition and nutritional value of *Ganoderma* production (Chen and Miles, 1996; Chen, 1999; Wagner et al., 2003; Miles and Chang, 2004; Maszlavér and Balázs, 2008; Zhou et al., 2012; Kamra and Bhatt, 2013; Hapuarachchi et al., 2018; Berovic and Podgornik, 2019; Du et al., 2019; Lu et al., 2020a), enriching the substrate with essential elements increase the accumulation of these elements in fruiting bodies of *Ganoderma* regardless of the cultivation technique applied (Rzymski et al., 2016). The results of several studies highlight that *Ganoderma* can incorporate various essential elements from a substrate, and that, when enriched its dried fruiting bodies may be used as a nutritional source for the essential elements (Table 3.4). It is scientifically evident that mushroom extracts from fruiting bodies and mycelia can be used as a raw material that has a pivotal role in dietary supplements and the pharmaceutical industry. Therefore, the need for a continuous exploration of diverse mushroom enrichment techniques with various essential elements.

Table 3.4 Influence of essential elements on nutritional quality and pharmaceutical properties of fungal species.

Fungal species	Essential elements	Research scope of work	Key research findings	References
Ganoderma lucidum	Selenium	Effect of selenium on mushroom growth and metabolism: A review	An increase in phenolics, flavonoids, basic amino acids, cordycepin, and adenosine were enhanced in mushrooms enriched at a moderate level of Se	Xu et al. (2021)
Pleurotus pulmonarius	Zinc and Iron	Evaluation of biological efficiency, nutrient contents and antioxidant activity of *Pleurotus pulmonarius* enriched with Zinc and Iron	Mushrooms from substrate enriched with FeSO4 and $ZnSO_4$ had the highest iron content of 417.6 mg/100g and the highest zinc of 349.5 mg/100g, respectively. Also, the scavenging activity of extract from mushrooms enriched with zinc (96.8%) and butylated hydroxytoluene (97.1%) were observed	Oyetayo et al. (2021)
Pleurotus eryngii	Selenium; Zinc	Selenium and zinc biofortification of *Pleurotus eryngii* mycelium and fruiting bodies as a tool for controlling their biological activity	Significantly increased iron content in fruiting bodies and increased anti-oxidant ability	Zięba et al. (2020)
Pleurotus djamor	Sodium selenite	Production of bioactive compounds by the mycelial growth of *Pleurotus djamor* in whey powder enriched with selenium	Na_2SeO_3 promoted an increase in the anti-oxidant activity as well as the ergosterol and β-glucan content	Velez et al. (2019)
Lentinula edodes	Selenium	Medicinal importance of mushroom mycelium: Mechanisms and applications	The study explained that Se enrichment of the mycelia preparation *L. edodes* species can significantly improve the ergosterol contents	Rathore et al. (2019)
Pleurotus ostreatus & *Pleurotus eryngii*		Phenolic composition and antioxidant properties of *Pleurotus ostreatus* and *Pleurotus eryngii* enriched with selenium and zinc	After Se and Zn enrichment, substrates possess higher mineral availability for fruiting bodies and it influenced micro-nutrients accumulation in both mushrooms	Gąsecka et al. (2016)

Ganoderma lucidum	Copper; Selenium; Zinc	Potential of cultivated *Ganoderma lucidum* mushrooms for the production of supplements enriched with essential elements	Increased bioaccumulation of Cu, Se, and Zn in fruiting bodies from substrate enriched with Cu + Se + Zn	Rzymski et al. (2016)
Pleurotus ostreatus & *Pleurotus eryngii*		Phenolic composition and antioxidant properties of *Pleurotus ostreatus* and *Pleurotus eryngii* enriched with selenium and zinc	Enrichment of substrate with Se and Zn resulted in a significant increase in TPC, TFC, and AAC	Gąsecka et al. (2016)
Pleurotus ostreatus	Nitrogen	Nitrogen supplementation on the productivity and the chemical composition of oyster mushroom	Substrates supplemented with nitrogen increased the mushroom's productivity and nutritional value, particularly the β-glucan content	Nunes et al. (2012a)
Lentinula edodes	Selenium	Selenium bioaccumulation in shiitake mushrooms: a nutritional alternative source of this element	Se concentration in mushrooms enhanced the function of the dose of Na2SeO3, the shiitake mushrooms enriched with Se can be a source of nutritional alternative for this element	Nunes et al. (2012b)
Ganoderma lucidum	Selenium	Effect of selenium on increasing the antioxidant activity of protein extracts from a selenium-enriched mushroom species of the *Ganoderma* Genus	Se-GLPr exhibited higher activities of scavenging superoxide and hydroxyl radicals, Se increased the anti-oxidant activities of protein extracts	Zhao et al. (2004)

Note: Cu = Copper; Se = Selenium; Zn = Zinc; Na_2SeO_3 = Sodium selenite; Se-GLPr = Se-enriched *Ganoderma lucidum*; TPC = Total phenolic content; TFC = Total flavonoid content; AAC = Ascorbic acid content; $FeSO_4$ = Iron sulphate; $ZnSO_4$ = Zinc sulphate

4 Conclusion and future prospects

The present review provides the most up-to-date biochemical quality and pharmaceutical properties of *Ganoderma* species research over 10 years. This review establishes that the number of studies on nutritional quality and pharmaceutical properties of *Ganoderma* and commercial demand has significantly increased over the years. Though artificial techniques have been developed to increase the yield of the bioactive compounds, it takes a long period for some of the production cultivation techniques of *Ganoderma* and the amount of active compounds within the fungus is difficult to manage. Therefore, bioactive compounds must be integrated into the quality control system for *Ganoderma* production. The nutritional quality and pharmaceutical potential of *Ganoderma* species warrant further investigation. The research currently available is promising, though further research is greatly encouraged to better understand *Ganoderma* bioactive compounds in conventional medicine.

Ganoderma species have been demonstrated to contain several pharmaceutical properties under numerous scientific studies. However, there are research gaps that necessitate further investigation before the transition from nutraceutical to pharmaceutical applications takes place. Clinical research studies on the use of *Ganoderma* extracts in treating various diseases are very limited. Even though no deaths or abnormalities in clinical symptoms from the twenty-two clinical studies have been reported thus far, five cases of adverse events associated with the use of *Ganoderma* supplements have been reported. Therefore, further clinical trials are needed in order to provide convincing scientific evidence of benefits against both chronic and infectious diseases. Furthermore, the enrichment of these species with plant growth regulators and essential elements to enhance their nutritional quality and pharmaceutical properties may be of commercial interest. Therefore, further exploration of diverse techniques that have the potential to enhance bioactive compounds and ensure purity is recommended.

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CHAPTER 4

MORPHOLOGICAL, MOLECULAR AND BIOCHEMICAL CHARACTERISATION OF *GANODERMA* SPECIES FROM SELECTED PROVINCES IN SOUTH AFRICA

Abstract

This study focused on the isolation and characterisation of fifteen fungal specimens collected from the three provinces of South Africa, namely, Mpumalanga, KwaZulu-Natal, and North-West. The research methodology involved sample collection and molecular techniques, such as deoxyribonucleic acid (DNA) sequencing and phylogenetic analysis, were employed to determine the genetic relationships and species diversity within the collected samples. Also, conducting morphological analyses to identify the macroscopic and microscopic features of the *Ganoderma* species. In addition, biochemical analyses were performed to assess the presence and concentration of biochemical compounds. Potato dextrose agar (PDA), malt extract agar (MEA), and sabouraud dextrose agar (SDA) were used to grow isolates. After an 8-day incubation period, MEA consistently had the highest mycelial diameter, reaching 85.00 mm followed by PDA at 83.44 mm and SDA at 77.83 mm. Identification was based on comparative macro- and micro-morphological traits, complemented by internal transcribed spacer region (ITS) analysis and phylogenetic assessment. Analysis of the ribosomal DNA ITS revealed that fungal samples KG3SY219 and MG1SY119 were closely related to *Ganoderma austroafricanum* and *Ganoderma resinaceum*, respectively. Further analyses delved into various biochemical aspects, including antioxidants, proteins, essential elements, and heavy metals. In terms of antioxidant capacity, *G. austroafricanum* exhibited a higher radical scavenging activity at 48.01% compared to *G. resinaceum* at 38.48%. The concentration of total phenolics in *G. austroafricanum* was higher (156.3 mg GAE/g DM) compared to *G. resinaceum* (146.1 mg GAE/g DM). *G. austroafricanum* had a higher flavonoid concentration at 94.7 mg QE/g DM than *G. resinaceum* at 81.5 mg QE/g DM. Protein concentrations were 24.04 µg/g and 12.69 µg/g for *G. austroafricanum* and *G. resinaceum*, respectively. Furthermore, *G. austroafricanum* exhibited a more abundant presence of essential elements and heavy metals compared to *G. resinaceum*. These findings underscore the substantial variability in morphological and biochemical characteristics among different *Ganoderma* species. Therefore, the findings of this study provide valuable insights into the diversity, taxonomy, and potential therapeutic applications of *Ganoderma* species in South Africa.

***Keywords*:** Growth media, pileus, biodiversity, macro-fungal, morphology, pharmaceutical

1 Introduction

Ganoderma is an important genus among polypore mushrooms (Bleha et al., 2022). It contains a hard fruiting body that grows saprobically or parasitically on woody plant material (Richter et al., 2015; Bhat et al., 2021). Previous reports documented the genus with more than 290 taxonomic names identified in various parts of the world, including Asia, Africa, Europe, and America (Kinge et al., 2015; Kwon et al., 2016; Hapuarachchi et al., 2018). Conversely, current reports highlighted that the genus has over 400 species widely distributed across various tropical and subtropical regions worldwide (Peng and Qiu, 2018; Yalcin et al., 2020). However, the recent search for "*Ganoderma*" in the database Index Fungorum divulges 421 species records including synonyms (Cabi Databases, 2023). Generally, the taxonomy of *Ganoderma* is primarily characterised by the justification of pileus morphology (Mawar et al., 2020), specific host, and geographical distribution (Chen et al., 2017). Several reports highlighted the complexity of their identity which remains a challenge (Kües et al., 2015; Umroong et al., 2021). Due to the striking similarities in morphological characteristics, the taxonomic classification of the genus is uncertain (Jargalmaa et al., 2017). Most species are often misidentified as either *Ganoderma lucidum* or *Ganoderma applanatum* (Mohanty et al., 2011). Since the taxonomic status remains unclear, only a few of the species within this genus have been the subject of phylogenetic studies (Bishop et al., 2015). Relying only on morphological features is therefore regarded as insufficient for identification and characterisation.

In Africa, more than forty-nine different species have been identified (Kinge et al., 2015), with twenty morphological species reported to have originated from Southern Africa (Ueitele, 2021). Although these species are reported in Southern Africa, their phylogenetic position is unknown and there were no DNA sequences provided for the samples (Coetzee et al., 2015; Ueitele, 2021). A total of thirteen *Ganoderma* spp. have previously been identified and published in South Africa, mainly located in Gauteng, Western Cape, KwaZulu-Natal, Eastern Cape, Mpumalanga, Free State, and Limpopo regions (Muthelo, 2011; Coetzee et al., 2015; Xing et al., 2016; Tchotet Tchoumi et al., 2017; 2018; 2019). Tchotet Tchoumi et al. (2019) argued that the available data does not represent all the species or specify whether these species were not previously identified. Thus, the taxonomic state and geographical location of these species is not well documented as supported by the report from Tchotet Tchoumi et al. (2017). These reports suggest that there is insufficient information regarding the identity of macro-fungi in the South African natural ecosystem. The continuous discovery of new *Ganoderma* spp. in South Africa implies that there could be a greater number of species than previously

documented (Tchotet Tchoumi et al., 2019). Understanding the specific characteristics of *Ganoderma* spp. in South Africa is crucial for their identification, classification, and potential therapeutic applications. In addition, there is a dearth of information on the biochemical compound concentrations of *G. austroafricanum* and *G. resinaceum*. Though there are few reports on the antioxidant activity of *G. resinaceum* (Saltarelli et al., 2009; Saltarelli et al., 2015; Sipping et al., 2022; Sułkowska-Ziaja et al., 2022), reports on mineral levels are insufficient. Furthermore, information regarding the biochemical compound levels in *G. austroafricanum* is inadequate, thus making this study one of the few to examine biochemical compounds in this species. This study, therefore, sought to investigate the morphological, molecular, and biochemical characteristics of *Ganoderma* spp. found in selected provinces in South Africa.

2 Materials and methods

2.1 Sample collection

2.1.1 Collection sites

A total of fifteen fungal specimens with pileus morphology resembling that of *Ganoderma* spp. were gathered from diverse host plant materials. The precise location of each specimen was documented using the Global Positioning System (GPS) coordinates and their pileus dimensions were measured (Figures 4.1A–O & Table 4.1). Specifically, five pileus samples were collected from each of the three provinces of South Africa, namely; KwaZulu-Natal (KZN) – encompassing the local Municipalities of Ubuhlebezwe, UMlalazi, and Msunduzi; Mpumalanga (MP) – covering local municipalities of Nkomazi, Mbombela, and Thaba Chweu; North-West (NW) – including the local municipalities of Kgetlengrivier, Mahikeng, and Ditsobotla (Figure 2). These samples were harvested during their vegetative stage within their respective natural habitats in each province. After collection, each sample was individually placed in paper bags and stored at a temperature of 26 °C. These samples were then transported and preserved within the Horticultural Science Laboratory facilities of the University of KwaZulu-Natal in Pietermaritzburg, South Africa. The samples were clearly labelled and identified (ID) based on its province of origin as follows: KZN (KG1SY219, KG2SY219, KG3SY219, KG4SY219, KG5SY219); MP (MG1SY119, MG2SY119, MG3SY119, MG4SY119, MG5SY119); NW (NG1SY319, NG2SY319, NG3SY319, NG4SY319, NG5SY319).

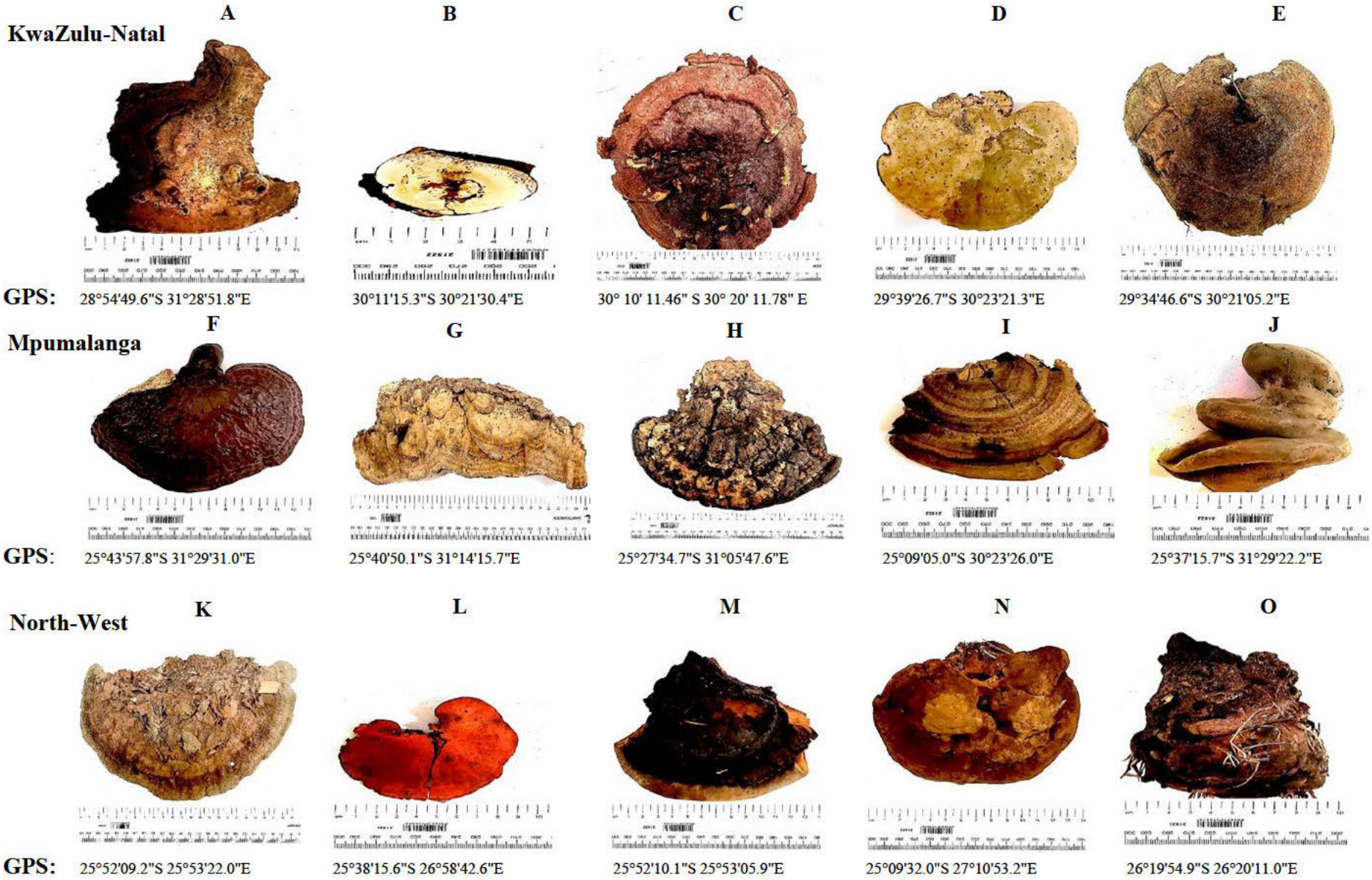

Figure 4.1 Fungal pileus of specimen collected from KwaZulu-Natal (A–E), Mpumalanga (F–J), and North-West (K–O). Sample ID: **A** (KG1SY219); **B** (KG2SY219), **C** (KG3SY219), **D** (KG4SY219), **E** (KG5SY219); **F** (MG1SY119), **G** (MG2SY119), **H** (MG3SY119), **I** (MG4SY119), **J** (MG5SY119); **K** (NG1SY319), **L** (NG2SY319), **M** (NG3SY319), **N** (NG4SY319), and **O** (NG5SY319).

Figure 4.2 Map illustrating the locations/sites in South African provinces namely; KwaZulu-Natal, Mpumalanga, and North-West where the fungal pileus samples were collected.

2.1.2 Ecology and pileus size

The ecological data of fifteen fungal pileus samples was documented and the pileus size was measured and recorded (Table 4.1). The largest pileus was noted from isolate MG2SY119, followed by isolate NG1SY319 and KG3SY219, with pileus sizes of 29.2, 23.5, and 23.0 cm, respectively. The smallest pileus was recorded from isolates KG2SY219 (5.5 cm), NG2SY319 (9.1 cm), and MG5SY119 (9.5 cm).

Table 4.1 Ecological data and pileus size of fungal pileus samples.

Isolate	Habitat	Growth type	Forest type	Pileus size (cm)	
				Diameter	Thickness
KG1SY219	Decomposed leaves	Colony	Coniferous	11.2	4.4
KG2SY219	Wood logs	Scattered	Mixed	5.5	2.1
KG3SY219	Deadwood logs	Scattered	Broad leaves	23.0	2.0
KG4SY219	Dry tree	Colony	Mixed	14.2	1.0
KG5SY219	Wood logs	Scattered	Coniferous	19.5	3.2
MG1SY119	Dead trunk	Colony	Coniferous	13.5	3.0
MG2SY119	Sprouting stumps	Solitary	Mixed	26.5	5.2
MG3SY119	Wood logs	Colony	Coniferous	24.1	3.7
MG4SY119	Vegetation debris	Scattered	Broad leaves	11.0	1.3
MG5SY119	Dead trunk	Solitary	Mixed	9.5	4.5
NG1SY319	Dry tree	Solitary	Mixed	23.5	2.9
NG2SY319	Dead trunk	Colony	Coniferous	9.1	0.9

NG3SY319	Deadwood logs	Scattered	Broad leaves	14.1	3.6
NG4SY319	Wood logs	Colony	Mixed	13.9	4.8
NG5SY319	Decomposed leaves	Scattered	Mixed	9.6	4.3

*All isolates samples beginning with letters K, M and N represent collection sites KwaZulu-Natal, Mpumalanga, and North-West province, respectively.

2.2 Experimental description

2.2.1 Isolation

The collected samples were cleaned and sun-dried, following the recommendations outlined by Mohanty et al. (2011). Subsequently, samples were placed in individual paper bags and stored at 26 °C. To process the samples, approximately 5 mm of spores located underneath each pileus were isolated. Each isolate was replicated three times and sequentially sterilised for 10 min, following a liquid phases technique; involving successive immersion in deionised water, 70% (v/v) ethanol, and deionised water, respectively. Following sterilisation, all isolates were dried by placing them on a paper towel for 30 min.

2.2.2 Growth media preparation

Potato dextrose agar (PDA), malt extract agar (MEA), and sabouraud dextrose agar (SDA) were used to culture the isolates. All the growth media were prepared and weighed accordingly to accommodate a 500 mL Erlenmeyer flask at PDA (19.5 g), MEA (25 g), and SDA (32.5 g). Deionised water was added to the flasks and sterilised for 30 min, using an autoclave with a temperature set at 121 °C, and poured into 80 mm Petri dishes. Each isolate was placed in separate dishes and incubated for 8 days. The incubation conditions maintained a temperature of 26 °C, along with exposure to a light intensity of 1000 lux. Fifteen isolates were cultured in media, with each isolate having three replicates, resulting in a total of 135 treatment combinations. The petri dishes were observed every other day to monitor changes in morphological characteristics and mycelial growth.

2.2.3 Deoxyribonucleic acid (DNA) extraction and sequencing

All respective cultures were sent to the Inqaba Biotechnical Industries (Pty) Ltd Laboratory for sample sequence repeat (SSR) analysis. Genomic DNA was extracted from cultures (Table 1) using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). Samples from the agro-morphological and physiological characterisation were used for diversity analysis using SSR markers.

2.2.4 Internal transcribed spacer (ITS) sequencing

The ITS target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers presented in Table 4.1. The polymerase chain reaction (PCR) products were run on gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in a forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500XL Genetic Analyser (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample, as shown in Figure 1. CLC Bio Main Workbench v7.6 was used to analyse the .ab1 files generated by the ABI 3500XL Genetic Analyser and results were abstained by a BLAST search (NCBI). The nuclear ribosomal ITS regions of the samples were amplified using ITS primers – 600bp (sense, 5′-TCCGTAGGTGAACCTGCGG-3′; antisense, 5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The BLAST was carried out following the BLASTIN 2.2.31+ protocol described by Altschul et al. (1997).

2.2.5 Amplicon sequencing

2.2.5.1 PCR amplicon purification

To obtain the finest and most fundamental level of genetic detail, the combination of PCR and well-designed primers was used. Fragments were enzymatically purified using the ExoSAP procedure (NEB M0293L; NEB M0371). The PCR Parameters were NEB OneTaq® 2X Master Mix with Standard Buffer (Catalogue No. M0482S); Genomic DNA (10-30 ng/μL); Forward primer (10 μM); Reverse primer (10 μM); and Nuclease free water (Catalogue No. ZnFeSe76).

2.2.5.2 Agarose gel analysis

The integrity of the PCR amplicons was visualised on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye. The NEB Fast Ladder was used on all gels (N3238) as a size standard.

2.2.5.3 Sanger sequencing

All samples were subjected to molecular identification using molecular markers sanger sequencing. The amplicons were purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050), and sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) using the ABI 3730XLGenetic Analyser (Applied Biosystems, Thermo Fisher Scientific).

2.2.6 Phylogenetic analyses

The nucleotide sequences were aligned using MAFFT and BioEdit software (Hall, 1999; Katoh et al., 2002). The isolates from both samples were examined in combination with reference sequences from Genbank for closely related species downloaded from the National Centre for Biotechnology (NCBI) database. The sequences included various South African isolates with accession numbers (MH571696, KU572491, MG020227, MH567261, MG020231, MH567295, MH567294, MH571690, MH571688, KR183856, MG020196, MZ220443, MZ220444, KM507324, and MH571693) from previous studies by Xing et al. (2016); Tchotet Tchoumi et al. (2018); (2019). The phylogenetic tree construction was performed using Mega 11 (Tamura et al., 2021). The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8596)). The bootstrap consensus tree was inferred from 1000 replicates and branches with bootstrap values less than 70% were collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach and thereafter selecting the topology with superior log likelihood value. This analysis involved 76 nucleotide sequences. All positions with less than 95% site coverage were eliminated, *i.e.*, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 360 positions in the final dataset.

2.2.7 Macro-morphological characterisation

The macro-morphological characteristics of identified species were compared against each other in terms of colour, size, and shape.

2.2.8 Micro-morphological characterisation

2.2.8.1 Growing of spores

The two growth media PDA and MEA were used to grow the isolates. Both media were prepared and weighed in accordance to accommodate a 500 mL Erlenmeyer flask and poured into 80 mm diameter Petri dishes. Petri dishes containing isolates were placed and incubated at 26 °C and observed for 9 days before preparing for viewing.

2.2.8.2 Isolates preparation for scanning electron microscope (SEM)

Isolates of 5 mm were made from Petri dishes containing the mycelial. Isolates were placed in a 3% buffered glutaraldehyde for 1-3 h and thereafter in sodium cacodylate buffer 2 x 5 min

for buffer wash. Secondary fixation was executed by placing isolates in a 2% buffered osmium tetroxide (10 mL 4% OsO_4, 5 mL 0.2M sodium cacodylate buffer, and 5 mL deionised water) for 1 h. Each of the samples was dehydrated inside a fume cupboard for 10 min in an ethanol series (10 min each in 10%, 30%, 50%, 70%, 90%, and 3 x 10 min in 100% ethanol, respectively). The specimens were carefully transferred into critical point dryer baskets under 100% ethanol and placed in a pre-cooled critical point dryer. All isolates were thereafter subjected to gold-palladium sputter coating before viewing their surface with SEM at an acceleration voltage of 5.00 keV. The micro-morphological characteristics of species were classified focusing on the following micrographs and bars; staghorn hyphae (bar = 30 µm); staghorn hyphae (bar = 20 µm); chlamydospores (bar = 20 µm); chlamydospores (bar = 10 µm); basidiospores (bar = 2 µm).

2.2.9 Biochemical compound quantification

2.2.9.1 Sample preparation

The pileus of the samples were oven-dried at 50 °C for 8 h using the protocol described by Hayati et al. (2016) with some modifications. When the oven drying process was terminated, the final moisture content achieved was 8%.

2.2.9.2 Extraction

Dried samples were milled and 0.5 g of the material was extracted using 25 mL of deionised water. The suspension was placed at 26 °C and allowed to stand for 1 h in the dark. To obtain an aqueous extract, a Whatman No. 1 filter paper was used to filter the suspension. Thereafter, the suspension was poured into 50 mL centrifuge tubes and centrifuged at 10 000 rpm for 15 min at 4 °C. Glass wool was used to filter the samples, and the extracts were stored in specimen bottles at 4 °C without further treatment until used for analyses.

2.2.10 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay

Samples were spectrophotometrically determined for DPPH free radical-scavenging activity as described by Wong et al. (2006), with minor modifications. Briefly, a 0.1 mM solution of DPPH in methanol was prepared and an aliquot of 40 µL of an extract was added to 3 mL of methanolic DPPH solution, thereafter incubated for 30 min at 26 °C. The absorbance of the DPPH in methanol was measured at 515 nm. The DPPH radical-scavenging capacity (%) of the samples was calculated using Eq. 1 described by Fang et al. (2018).

$$DPPH\ Value\ (\%) = \frac{\Delta A_{515}\ of\ control - \Delta A_{515}\ of\ treatment}{\Delta A_{515}\ of\ control}\ X\ 100 \qquad 1$$

2.2.11 Total phenolics

The total phenolic content was spectrophotometrically determined using the Folin-Ciocalteu procedure as described by Siangu et al. (2019), with some modifications. An aliquot extract of 1 mL was mixed with 5 mL of deionised water and 1 mL of Folin-Ciocalteu's reagent, and incubated at 26 °C for 5 min. A 10 mL solution of 7% Na_2HCO_3 was added and allowed to stand at 26 °C for 2 h. All extracts were triplicated, and the absorbance was read at 725 nm. The readings were expressed in milligrams of gallic acid equivalents per gram (mg GAE/g).

2.2.12 Total flavonoids

Total flavonoids were determined by a colorimetric method as described by Marnewick et al. (2011). Briefly, 600 µL sample/standard was mixed with 2% of $AlCl_3$ in ethanol. Subsequently, the solution was thoroughly mixed and incubated for 1 h at 25 °C. The change of colour on samples was measured at 420 nm using a spectrophotometer. Samples were triplicated, analysed, and expressed as milligrams of quercetin equivalents per gram (mg QE/g).

2.2.13 Total protein content

Protein content was determined using the procedure described by Bradford (1976). Bradford dye reagent of 900 µL was added to each of the 100 µL fungal extract samples and vortexed. The samples were left without disturbing for 5 min before reading the absorbance at 595 nm using a spectrophotometer. Samples were triplicated and readings were expressed in micrograms per gram (µg/g).

2.2.14 Essential elements analyses

The dry ash procedure described by Agrilasa (1998) was used to analyse the essential elements. Isolates were dried at 26 °C for 72 h and milled. The process involved placing 1 g of the dried isolates into a crucible and further subjected to additional drying for 24 h. The crucibles containing dry isolate were heated in an ashing oven at 600 °C for a duration of 8 h. The inductively coupled plasma (ICP) 4:1 plant extract procedure was used, 8 mL Nitric acid (HNO_3) and 2 mL hydrochloric acid (HCI) were used as reagents and incubated in a microwave reaction system for 45 min. Digested samples were transferred into volumetric flasks (100 mL) and topped up with deionised water and left standing for 24 h. Samples were carefully transferred to McCartney bottles without disturbing the sediment and an ICP mass spectrometer was used. Zinc (Zn), iron (Fe), selenium (Se), manganese (Mn), magnesium (Mg), sodium (Na), calcium (Ca), aluminium (Al), potassium (K) and phosphorus (P) were quantified and expressed in milligrams per kilogram of dry matter (mg/kg DM).

2.2.15 Heavy metals analyses

Samples were prepared and analysed for heavy metals content using the same analysis procedure for essential elements (Agrilasa, 1998). A protocol described by Stihi et al. (2011) was used to quantify the heavy metals content. The levels of nickel (Ni), copper (Cu), cobalt (Co), cadmium (Cd), chromium (Cr), palladium (Pd), lead (Pb), mercury (Hg), silver (Ag), and arsenic (As) were quantified and presented as mg/kg DM.

2.3 Statistical analyses

The collected data were subjected to analysis of variance (ANOVA) using Genstat statistical software (Genstat® 20th edition, VSN International UK). All means were separated according to Duncan's multiple range test at 5% level of significance. Data for all DNA analyses sequences; FinchTV (https://finchtv.software.informer.com/1.4/) was used to view the raw chromatogram files (.abi). CLC Bio Main Workbench was used to assemble the forward and reverse sequencing reads to form a consensus sequence for each sample. BLASTn analysis (with default parameters) was performed on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al. (1997) to determine if a sequence in the database matches the query sequence above a certain threshold (99% query coverage; 99% identity).

3 Results and Discussion

3.1 Growth media effect on mycelial development

Growth media had a significant effect on the mycelial growth of all fungal pileus samples after 8 days of incubation (Figure 4.3). The samples in the MEA growth media had the highest mycelial growth for most treatments at 85.00±0.57 mm followed by PDA at 83.44±0.72 mm and SDA with the lowest growth value at 77.83±0.61 mm. Based on these results, PDA and MEA were established to be the two best growth media. Therefore, were later used to grow the isolates for the remainder of the experiments (DNA extraction cultures and micro-morphology characteristics) in this study. The current results corresponded with the findings from Mahadevan and Shanmugasundaram (2018) where MEA was reported to have superior mycelial growth followed by PDA and SDA. Contrary to the present results, the findings from Abdullah and Saadullah (2018) identified PDA to have a dominant fungal mycelial growth followed by MEA and SDA. Findings from Ali et al. (2016) concluded that SDA was the best growth media for fungal species, followed by PDA and MEA. The different deduction in findings to the current results could probably be that the fungal species used in these studies

were not necessarily macro-fungal. Based on these findings, it can be deduced that fungal species prefer different growth media but macro-fungal species favour MEA.

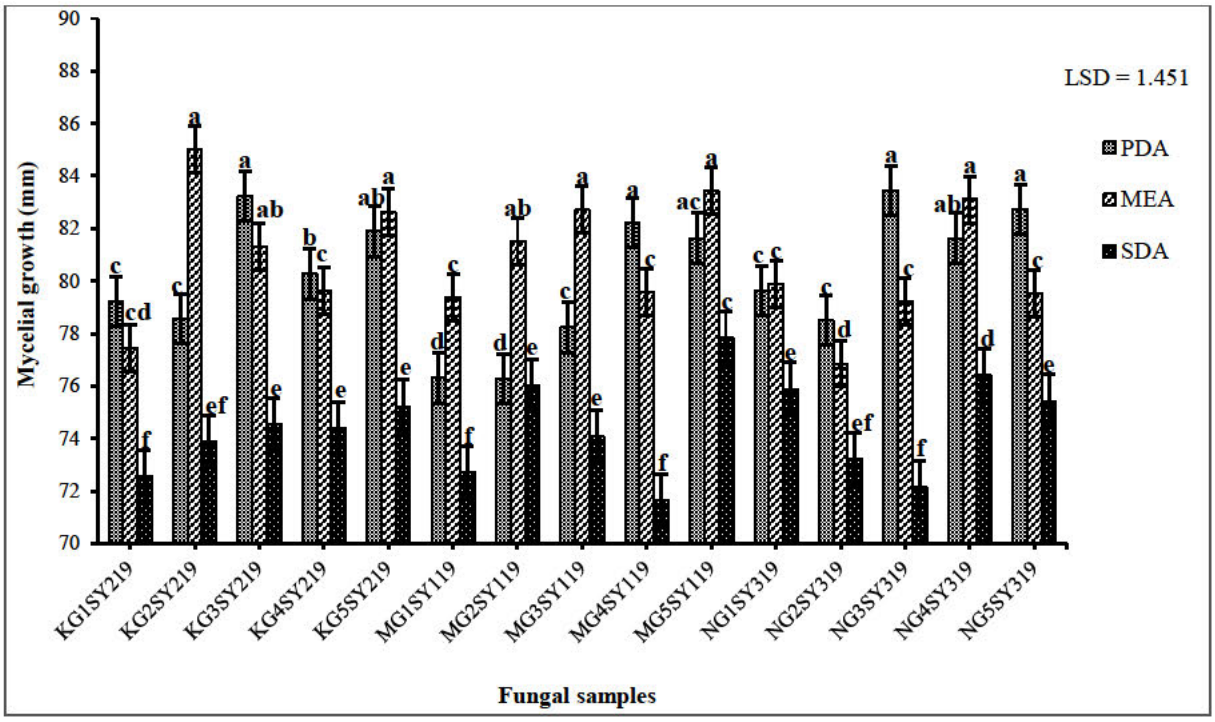

Figure 4.3 Influence of different growth media on mycelial development (mm) of fifteen collected fungal pileus samples after incubation for 8 days. * Sample isolates beginning with letters K, M, and N represent collection sites KwaZulu-Natal, Mpumalanga, and North-West province, respectively. The results were expressed as mean ± SE, $n = 3$. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.2 Molecular characterisation

3.2.1 Internal transcribed spacer (ITS) sequencing and gene amplification

The molecular identification results demonstrated that from the sampled 15 fungal isolates, only two fungal isolates were identified as *Ganoderma* spp. The BLASTn search results in the GeneBank nucleotide sequence database confirmed that isolates KG3SY219 (KZN) and MG1SY119 (MP) were taxonomically affiliated with *Ganoderma*. The BLAST results corresponded to the similarity between the sequence queried and the biological sequences with the NCBI database. Isolates KG3SY219 and MG1SY119 were classified to be *Ganoderma resinaceum* and *Ganoderma austroafricanum*, respectively. These findings were supported by the results from the phylogenetic tree (Figure 4.4). Previously, species *G. austroafricanum* have been identified in Gauteng and Limpopo provinces (Coetzee et al., 2015; Tchotet Tchoumi et al., 2019) and *G. resinaceum* in KwaZulu-Natal, Gauteng and Eastern Cape province (Coetzee et al., 2005; Tchotet Tchoumi et al., 2019). Therefore, with these distinct findings and considering their geographical location, *G. austroafricanum* species is newly

identified in Mpumalanga. Even though the North-West samples resembled the morphology of *Ganoderma* spp., all isolates were established to have no taxonomic affiliation with *Ganoderma*. Hence demonstrating the morphological traits should be incorporated with molecular identification technology to accurately identify fungal species. In addition, *Ganoderma* spp. originate and grow in tropical and subtropical regions, however, some of these species continue to intensify their spread into the temperate regions (Jargalmaa et al., 2017). MP and KZN receive an estimated rainfall per annum ranging between 650-775 mm and 70-89 % relative humidity (South African Weather Services, 2023). This elucidates the *Ganoderma* spp. that were found in these provinces known to be temperate regions. In contrast, NW experiences relative humidity of 15-40%, and an average of 450-550 mm per annum, therefore, considered a semi-arid region. The major part of the NW province might be not favourable for the development of these species due to the climatic conditions. This explicates wherefore none of the fungal samples collected from NW had any taxonomic affiliation with *Ganoderma*.

3.2.2 Phylogenetic

The phylogenetic analysis showed that isolates KG3SY219 and MG1SY119 were related to *Ganoderma* isolates (Figure 4.4). The tree topology and branch lengths demonstrated that isolate KG3SY219 was closely related to isolates from Korea and the United States of America with a bootstrap value of 73%. The close relation with species from other countries could be the similarity in environmental factors. The phylogenetic relationships among fungal species are primarily driven by environmental factors (Vasar et al., 2022). Although isolate MG1SY119 had no close relationship with any of the isolates, it had a relationship with Brazilian and other South African isolates. This can be deduced from *G. austroafricanum* species was first identified in South Africa hence the relationship (Coetzee et al., 2015; Tchotet Tchoumi et al., 2019).

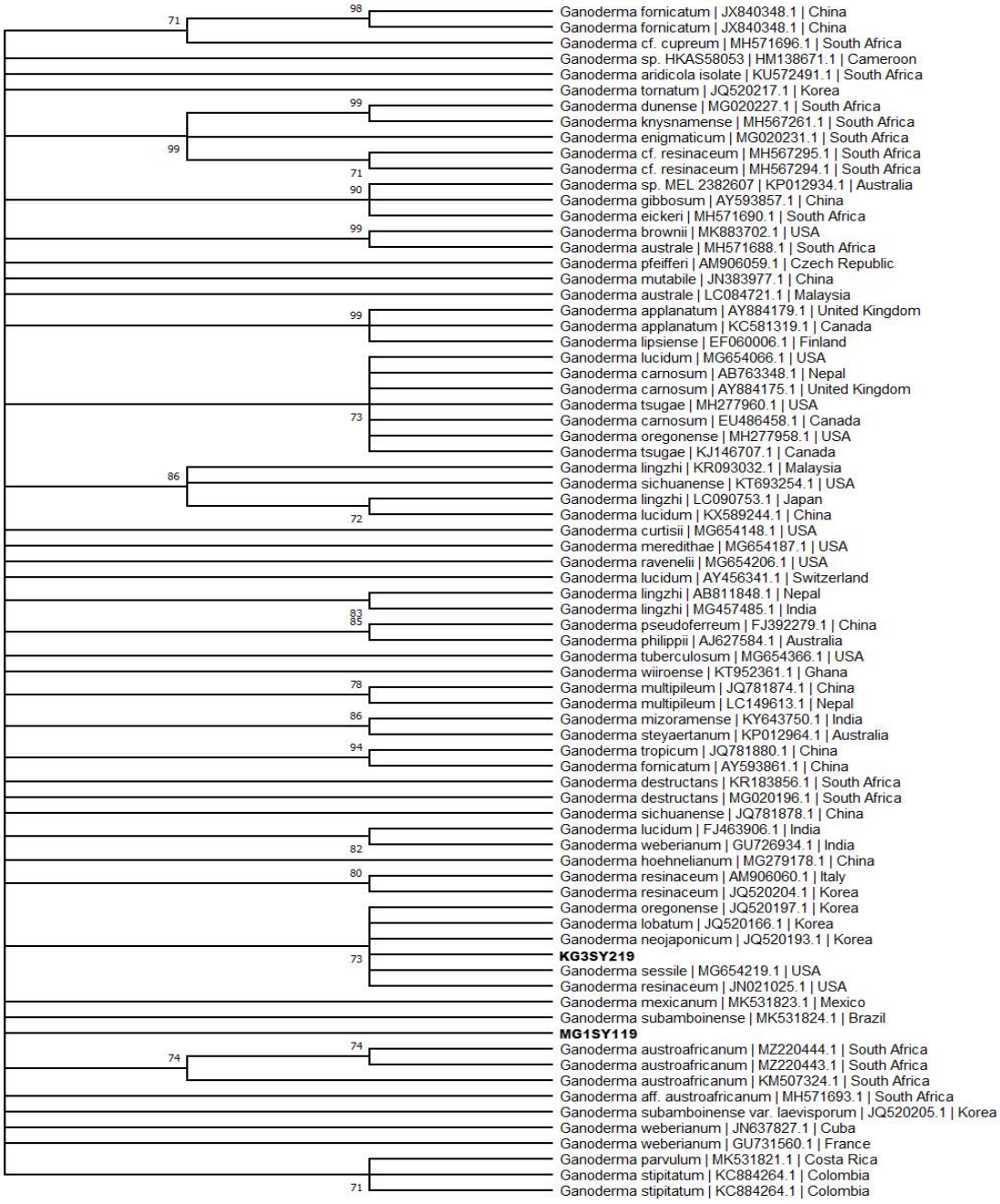

Figure 4.4 Phylogenetic relationship between *Ganoderma* spp. inferred from nucleotide sequence data. Evolutionary genetic relationships of two *Ganoderma* isolates collected from KwaZulu-Natal (KG3SY219), Mpumalanga (MG1SY119) province, South Africa, and other fifteen isolates of *Ganoderma* identified from different areas in South Africa, and fifty-nine from other countries. The values above and below branches are confidence levels estimated by 1000 bootstrap replicates.

3.3 Morphological characteristics

3.3.1 Macro-morphological

The results of macro-morphological analysis for *G. austroafricanum* (MG1SY119) indicated that the pileus had a kidney-like shape with a diameter of 13.5 cm and a 3 cm thick base (Figure 4.5a). It had a corky to light woody thick shiny body surface with a dark red-brown layer colour.

The pileus of *G. resinaceum* (KG3SY219) had a round-like shape with a hard corky to woody pileus surface, convex diameter projecting up to 23 cm, and a 2 cm thick base (Figure 4.5b). It had a broad round-like shape and distinctive reddish-brown colour. The pileus of both species had differences in shape size and surface texture. Both pileus, however, had a similar reddish-brown colour and corky to woody surface. Although both species had a phylogenetic relationship divergence, they had similar macro-morphological characteristics to previously described *G. resinaceum* (Chen et al., 2017; Hassan and Al-Qiassi, 2022) and *G. austroafricanum* (Crous et al., 2014; Coetzee et al., 2015).

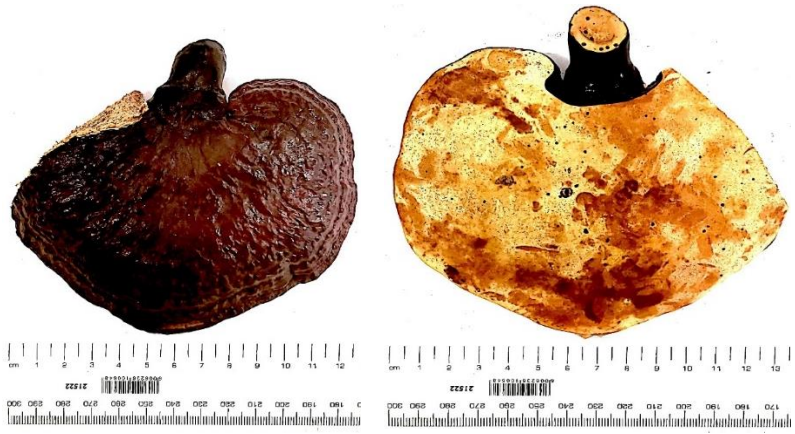

Figure 4.5(a). Pileus morphological characteristics of *G. austroafricanum* collected in Mpumalanga province (MP) at Nkomazi local municipality in Jeppes Reef area (GPS coordinates: 25°43'57.8"S, 31°29'31.0"E).

Figure 4.5(b). Pileus morphological characteristics of *G. resinaceum* collected in KwaZulu-Natal province (KZN) at Ubuhlebezwe local municipality in Mnyanyabuzi area (GPS coordinates: 30°10'11.46"S, 30°20'11.78"E).

3.3.2 Micro-morphological

The micro-morphological characteristics of *G. austroafricanum* and *G. resinaceum* micrographs are shown in Figures 4.5c and 4.5d. The difference in distinctive characteristics was observed in both species where the micrographs showed smooth basidiospores from *G. austroafricanum* and rough from *G. resinaceum*. The variation could have been because the fungal basidiospores differ with each species. Fungal identification is reported to be based on spore structure, membrane fatty acid composition, and morphology (Maraz and Khan, 2021). Also, the results demonstrated a firm structure with no nanofibres on the surface of *G. austroafricanum* and exhibited approximately a smooth morphological surface. While *G. resinaceum* had a uniform nanofiber structure. The surface morphology of fungal mycelia is one of the crucial factors used to characterise *Ganoderma* spp. (Miller et al., 2000).

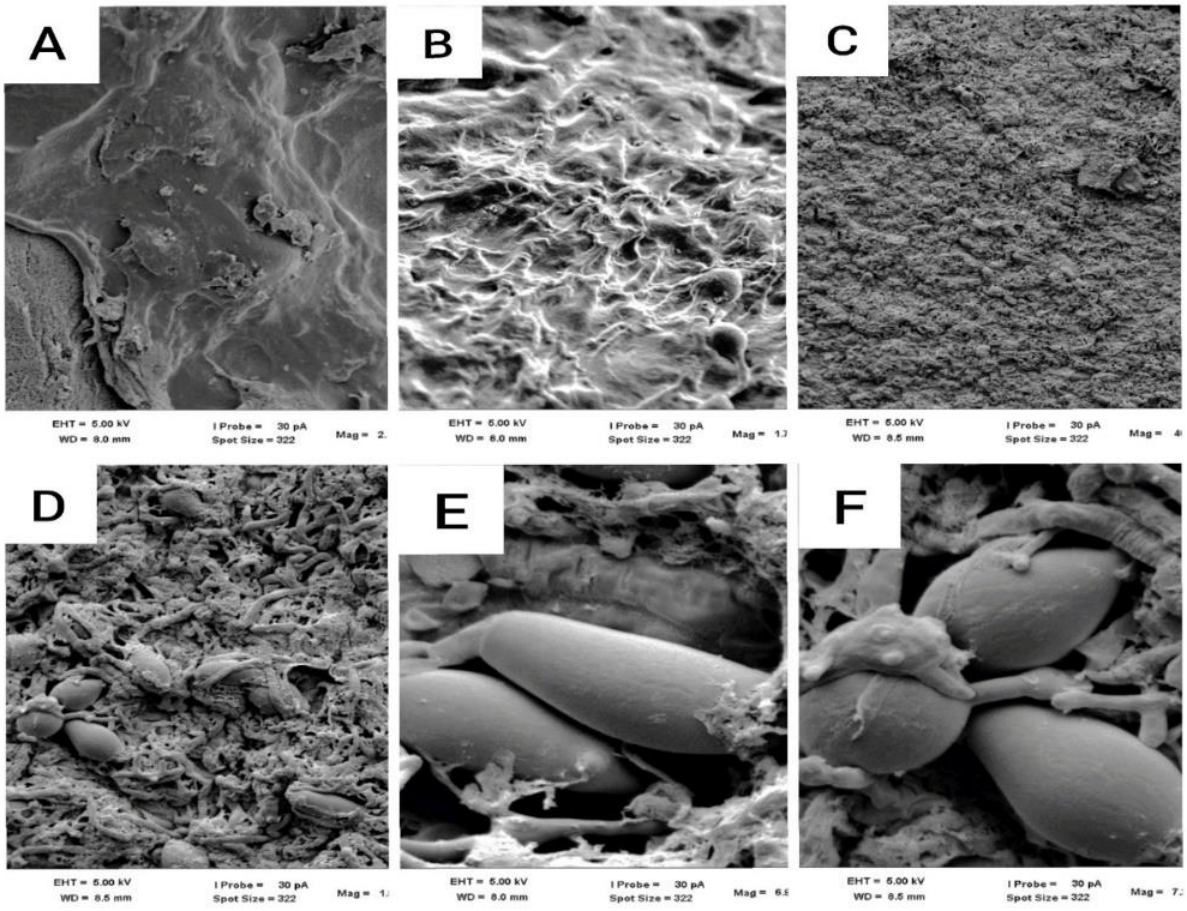

Figure 4.5(c) Scanning electron micrographs of *G. austroafricanum* mycelia morphological characteristics. A – staghorn hyphae (bar = 30 µm); B – staghorn hyphae (bar = 20 µm); C – chlamydospores (bar = 20 µm); D – chlamydospores (bar = 10 µm); E – basidiospores (bar = 2 µm); F – basidiospores (bar = 2 µm).

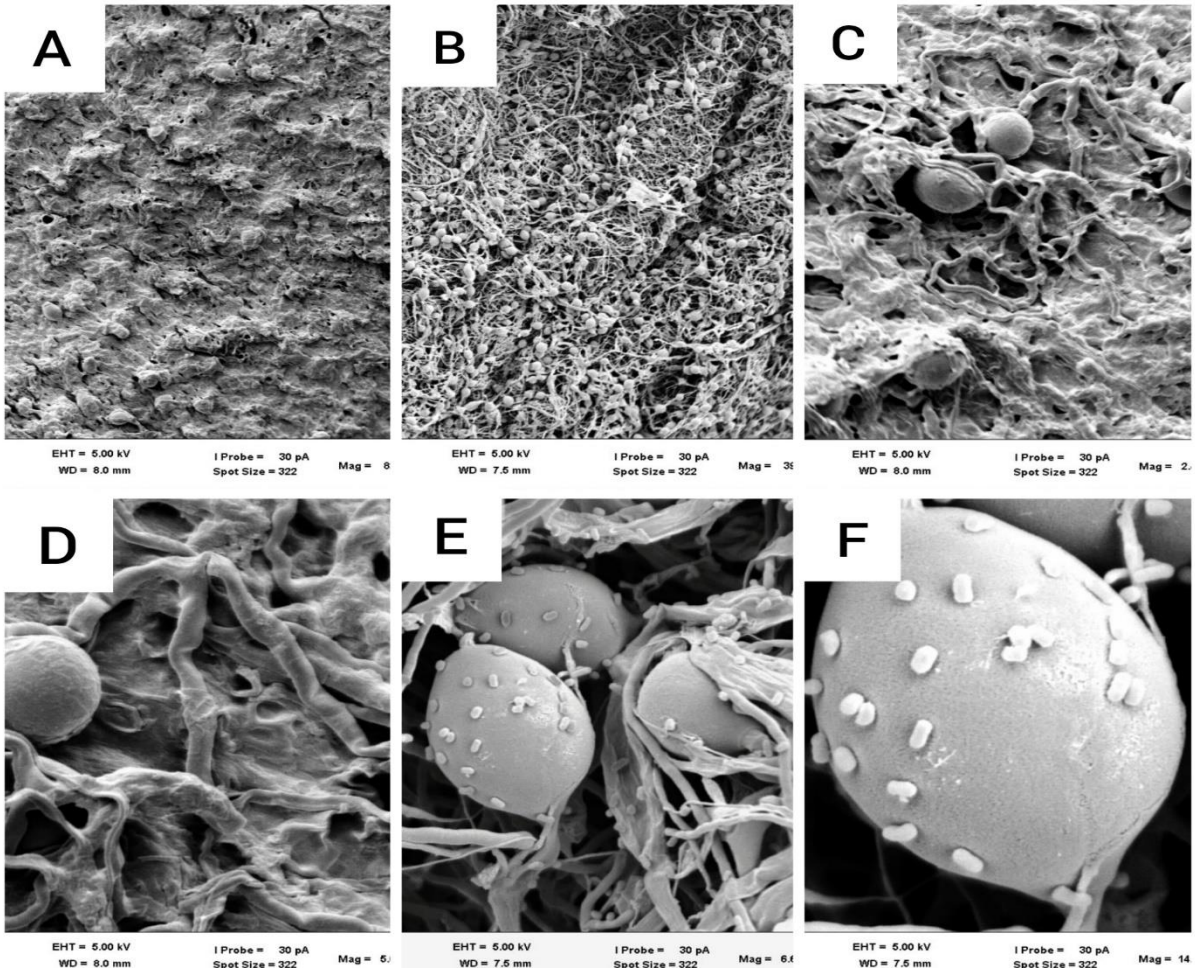

Figure 4.5(d) Scanning electron micrographs of *G. resinaceum* mycelia morphological characteristics. A – staghorn hyphae (bar = 30 µm); B – staghorn hyphae (bar = 20 µm); C – chlamydospores (bar = 20 µm); D – chlamydospores (bar = 10 µm); E – basidiospores (bar = 2 µm); F – basidiospores (bar = 2 µm).

3.4 Biochemical compounds

3.4.1 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay

The DPPH free radical scavenging activity of two *Ganoderma* spp. extracts is shown in Figure 4.6. Significant differences in DPPH free radical scavenging activity were observed between the two species. The scavenging activity percentage of *G. austroafricanum* and *G. resinaceum* was observed at 48.01±0.41 and 38.48±0.68%, respectively. The DPPH results of the present study were consistent with the findings of Sipping et al. (2022) where *G. resinaceum* extracts exhibited scavenging activity at 24.20 to 58.51%. However, the current results were different from the findings of Saltarelli et al. (2015) where *G. resinaceum* extracts ranged between 60 to 85%. This could be linked to the different concentrations (1-5 mg/mL) used in their study as the DPPH free radical scavenging activity percentage was observed to rise with an increase in concentration. The radical scavenging activity increased with concentrations of *Ganoderma*

extracts (Saltarelli et al., 2009). The relationship between DPPH and antioxidants lies in the ability of antioxidants to donate electrons or hydrogen atoms to the DPPH radical, neutralizing its free radical nature. The antioxidant capacity is a quantity measure of a certain free radical captured by an antioxidant sample (Santos-Sánchez et al., 2019). Therefore, the discrepancies in DPPH free radical scavenging activity findings could be linked to the different antioxidant concentrations such as total phenolics and flavonoids from the two species.

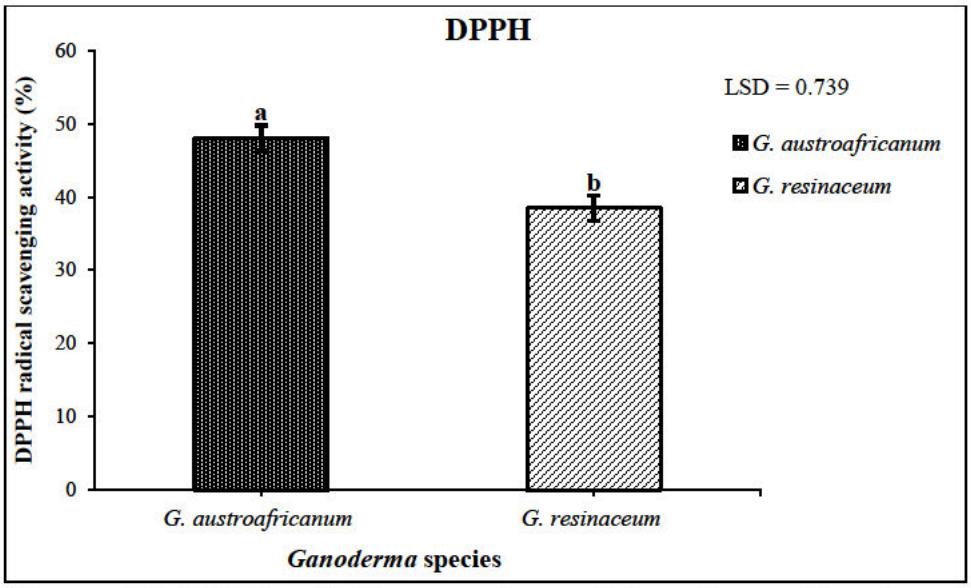

Figure 4.6 DPPH radical scavenging activity of two *Ganoderma* spp. namely; *G. austroafricanum* and *G. resinaceum*. The results were expressed as mean ± SE, $n = 3$. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.4.2 Total phenolics

The two species were observed to have significant differences ($p = 0.026$) in total phenolics. *G. austroafricanum* had the highest phenolic content of 156.3±6.97 mg GAE/g while *G. resinaceum* had the lowest content of 146.1±5.19 mg GAE/g (Figure 4.7). The phenolics results of the present study were within the phenolics range of other *Ganoderma* spp. Siangu et al. (2019) findings observed the phenolics readings of *Ganoderma lucidum* and *Ganoderma applanatum* at 156.07 and 127.23 mg GAE/g, respectively. However, the findings of Saltarelli et al. (2009) observed the lowest amount of phenolics in *G. resinaceum* at 16.25 mg/g. The variation with the current study could have been the sodium carbonate percentage (20%) used in their phenolic assay. Sodium carbonate concentration in the Folin-Ciocalteu assay has significant discrepancies in total phenolics (Lawag et al., 2023). Also, the use of gallic acid instead of cafferic acid as a standard solution could have led to the finding's variation. In a study by Contato et al. (2020), phenolic concentrations were higher in pileus extract where

gallic acid was used compared to cafferic acid. Gallic acid may therefore be the component responsible for the antioxidant activity of the pileus extract.

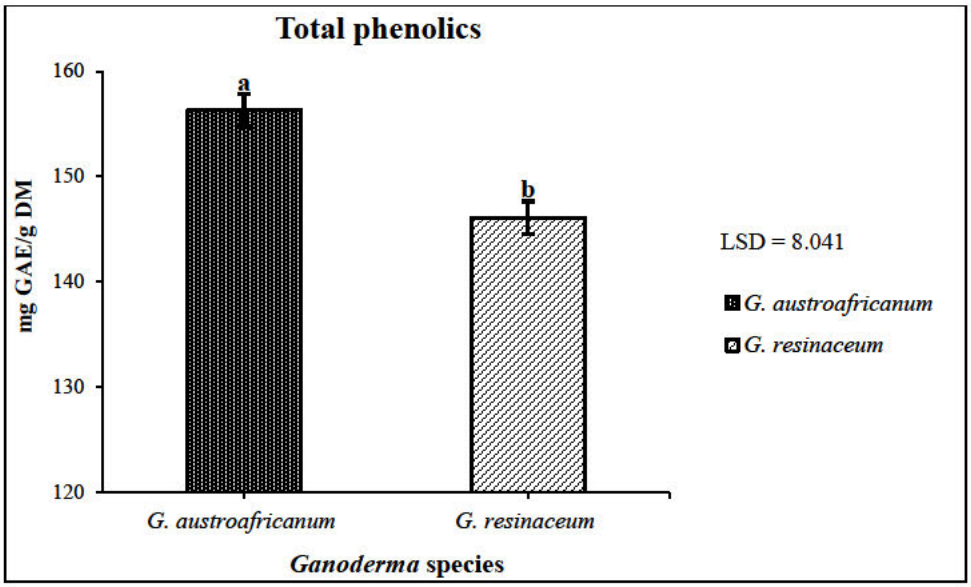

Figure 4.7 Total phenolics of *G. austroafricanum* and *G. resinaceum*. The results were expressed as mean ± SE, $n = 3$. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.4.3 Total flavonoids

Significant differences ($p = 0.033$) in flavonoid content were observed between the two species (Figure 4.8). Fungal extracts from *G. austroafricanum* and *G. resinaceum* were recorded at 94.7±12.30 and 81.5±1.96 mg QE/g, respectively. The current results had inconsistencies with the findings by Sułkowska-Ziaja et al. (2022) where the flavonoid content of *G. resinaceum* was 0.57 mg RE/g. This could be attributed to the methods used during the extraction, preparation, and conditions. The extraction yield and antioxidant activity not only depend on the extraction technique but also on the solvent used for extraction (Do et al., 2014). The flavonoid content in the extract depends on the polarity of the extraction solvents used (Stankovic, 2011). Therefore, it the important to select a sustainable technique with a lower consumption of solvent which is known to provide positive results on pileus and mycelia such as colorimetric methods.

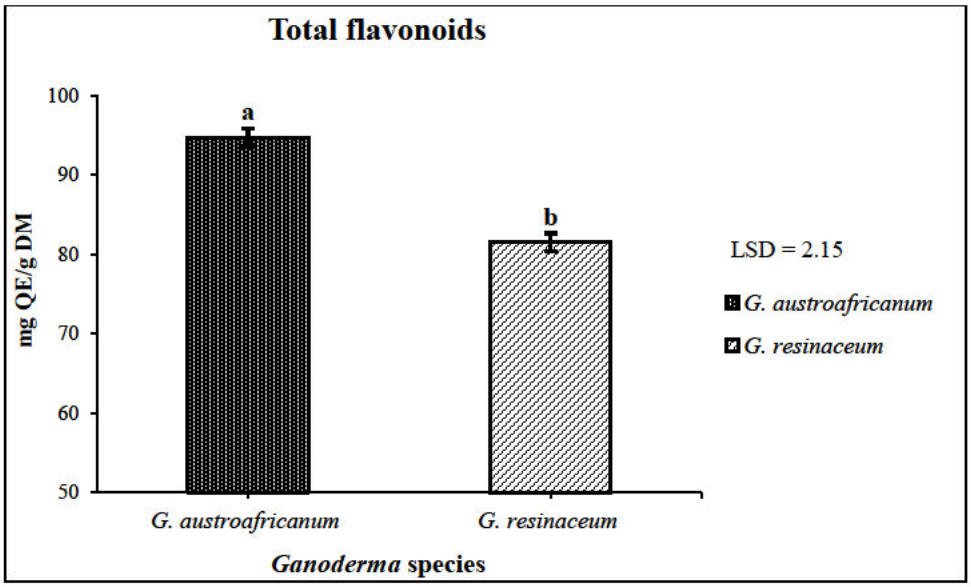

Figure 4.8 Total flavonoids content of *G. austroafricanum* and *G. resinaceum*. The results were expressed as mean ± SE, $n = 3$. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.4.4 Total protein

The results on total protein content indicated significant differences between the two *Ganoderma* spp. (Figure 4.9). *G. austroafricanum* had a high protein content compared to *G. resinaceum* recorded at 24.04±0.97 and 12.69±1.41 µg/g, respectively. The protein content of *Ganoderma* can vary depending on factors such as the species, growth conditions, and processing methods. The distinctions could be attributed to the different substrates where these species grew. Even though both species were collected from decaying plant material hosts, there was variation in vegetation (Table 4.1). The forest types of *G. austroafricanum* and *G. resinaceum* were broad leaves and coniferous, respectively. Substrates vary in structure, texture, and chemical composition thus influencing nutrient availability and release (Nguyen et al., 2019; Siwulski et al., 2019). Therefore, these substrates might have had variations in physical and chemical composition resulting in biochemical compound content distinctions.

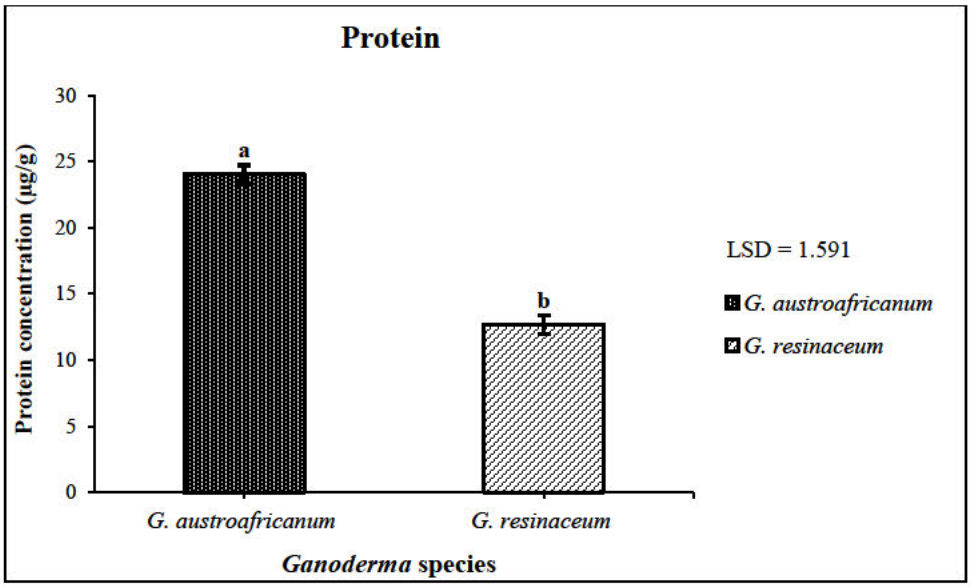

Figure 4.9 Total protein content of *G. austroafricanum* and *G. resinaceum*. The results were expressed as mean ± SE, $n = 3$. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.4.5 Essential elements

Essential elements results indicated significant differences ($p < 0.05$) in both species (Table 4.2). Phosphorus was observed to be the most abundant element found on both species, reaching 8458.67±104.97 and 8112.58±48.01 mg/kg DM for *G. austroafricanum* and *G. resinaceum*, respectively. Potassium was also notably high at 6880.22±118.48 and 5451.09±100.84, together with calcium readings at 982.02±13.89 and 857.42±14.57 mg/kg DM for *G. austroafricanum* and *G. resinaceum*, respectively. Zinc was observed to be the major element amongst the microelements at 56.18±3.82 mg/kg DM for *G. austroafricanum* and 43.57±2.8 mg/kg DM for *G. resinaceum*. Even though the levels of the essential elements contained in both species varied, the element levels were not too distinct between the species. The diversity of geographical location and environmental factors of these species could have influenced their essential element levels. *Ganoderma*'s essential elements vary between species, origin, and growing conditions, as a result, these lead to variations in quantity and quality (Cortina-Escribano et al., 2020). Furthermore, the variability in these findings could have been the type of species, pileus size, and maturity. Factors such as pileus size, type of species, and harvest time influence mushroom biochemical compositions (Kortei, 2015; Obodai et al., 2017).

3.4.6 Heavy metals

Heavy metals significantly differed between the *G. austroafricanum* and *G. resinaceum*. For instance, the levels Co, Cu, Cr, Pb, and As were higher in *G. austroafricanum* than in *G.*

resinaceum (Table 4.3). Notably, Cr and Co were the most abundant heavy metals in *G. austroafricanum* and *G. resinaceum*. Furthermore, *G. resinaceum* had higher levels of Mercury at 1.04±0.04 compared to 0.17±0.01 mg/kg DM for *G. austroafricanum*. The variations could be attributed to the soil type, water, air, and possible human/industrial activities such as mining in the areas where these species were collected. Another attribute could be the environmental habitat properties that may have influenced the heavy metal levels. Environmental habitat properties related to pollution can be the major factors to influence the levels of metal content in fungal pileus (Rašeta et al., 2016). The total biochemical compounds and mineral levels of pileus vary within species (Sudheer et al., 2019; Venturella et al., 2021). The variation in heavy metals can also be linked to the variation of species.

Table 4.2 Essential element levels (mg/kg DM) of *G. austroafricanum* and *G. resinaceum*.

Species	Zn	Fe	Se	Mn	Mg	Na	Ca	Al	K	P
G. austroafricanum	56.18±3.82[a]	20.29±0.62[a]	1.12±0.16[a]	5.34±0.64[a]	439.40±27.42[a]	133.41±10.02[a]	982.02±13.89[a]	1.22±0.06[a]	6880.22±118.48[a]	8458.67±104.97[a]
G. resinaceum	43.57±2.88[b]	15.90±1.97[a]	0.91±0.06[a]	4.35±0.69[a]	366.71±11.02[b]	110.05±7.55[b]	857.42±14.57[b]	0.97±0.05[b]	5451.09±100.84[b]	8112.58±48.01[b]

The results were expressed as mean ± SD, $n = 3$. Means followed by the same letter within columns are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

Table 4.3 Heavy metal levels (mg/kg DM) of *G. austroafricanum* and *G. resinaceum*.

Species	Ni	Cu	Co	Cd	Cr	Pd	Pb	Hg	Ag	As
G. austroafricanum	0.39±0.03[a]	13.67±0.55[a]	2.71±0.91[a]	0.73±0.03[a]	14.49±0.50[a]	0.11±0.03[a]	3.02±0.67[a]	0.17±0.01[b]	0.061±0.02[a]	0.741±0.01[a]
G. resinaceum	0.28±0.02[a]	11.83±1.45[b]	1.04±0.31[b]	0.62±0.06[a]	10.64±0.82[b]	0.05±0.01[a]	1.09±0.14[b]	1.04±0.04[a]	0.030±0.01[a]	0.432±0.00[b]

The results were expressed as mean ± SD, $n = 3$. Means followed by the same letter within columns are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

4 Conclusion

In conclusion, this study represents the identification and comparative characterisation of *Ganoderma* spp. from selected provinces in South Africa. The molecular identification results demonstrated that only two collected fungal samples tested positive as *Ganoderma* spp. The morphological characterisation allowed for the identification and classification of different *Ganoderma* spp. based on their physical characteristics, such as shape, colour, and size. This information contributes to our understanding of the biodiversity and distribution of *Ganoderma* spp. in South Africa. The molecular characterisation, which involves DNA analysis and sequencing, provides a deeper understanding of the genetic makeup and relationships between different *Ganoderma* spp. This knowledge is crucial for taxonomy, species identification, and evolutionary studies. Furthermore, the biochemical characterisation explored the chemical composition and bioactive compounds present in *Ganoderma* spp. This analysis aids in uncovering the potential medicinal and therapeutic properties of these species, contributing to the development of natural products and pharmaceutical applications. Overall, the comprehensive approach employed in this study lays a foundation for the molecular systematics of *Ganoderma* spp. and other important mushroom species in South Africa. Further research on the identification of these species in other parts of South Africa and their possible pharmaceutical properties and applications is recommended.

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CHAPTER 5

DETERMINATION OF OPTIMUM TEMPERATURE AND pH FOR THE MYCELIAL ESTABLISHMENT OF *GANODERMA* FUNGAL SPECIES UNDER DIFFERENT GROWTH MEDIA

Abstract

This study evaluated the effect of different growth conditions on mycelial growth and development of *Ganoderma austroafricanum* and *Ganoderma resinaceum*. The experimental treatments included three levels of pH (4, 6 & 8), temperature (20, 25 & 30 °C), and different types of plant residues namely; beech sawdust (BS), sugarcane bagasse (SB), and buffalo grass (BG). The study consisted of three independent *in vitro* experiments and evaluated over 9 days. Potato dextrose agar (PDA) and malt extract agar (MEA) were used as standard growth media to grow each fungal species. With regard to culture media pH, the study results demonstrated that the maximum growth (85.00 mm) for mycelia was reached on day 9 for both species. Generally, *G. resinaceum* showed the highest mycelial growth for both PDA and MEA except for days 6 and 9 where mycelial growth of the species was decreased by low levels (pH 4) and high levels (pH 8), respectively. However, *G. austroafricanum* has shown to be more sensitive to culture pH at early stages of mycelial growth, with pH 6 being the optimum level. In terms of temperature, the effect on mycelial growth was more prevalent than the latter for both species. Increased levels of temperature adversely reduced the mycelial growth of *G. austroafricanum* species for all the media used. On the other hand, *G. resinaceum* responded positively to increased levels of temperature across all the growth stages, reaching a maximum growth of 85 mm earlier than *G. austroafricanum*. The effect of plant residues as a growth medium showed varying responses for mycelial development for the species across all growth stages. *G. resinaceum* exhibited the lowest (0.0 mm) and highest mycelial growth (85 mm) for both the earlier and later growth stages, respectively. The addition of BG to the growth media delayed the mycelial growth of *G. resinaceum* for both PDA and MEA. A different response was observed on *G. austroafricanum* which showed improved mycelial growth. The study findings have shown that differences in growth media pH and temperature, as well as the type of growth media, can greatly influence the mycelial growth of *Ganoderma* species.

***Keywords*:** Fungi, agar, agro-industrial wastes, environmental conditions, growth medium

1 Introduction

Ganoderma species from the family *Ganodermataceae* are well-known for their health-promoting and therapeutic properties in traditional Chinese medicine (Li et al., 2020; Lilburn, 2022). The mycelia of these species can degrade various agro-industrial wastes such as sawdust, sugarcane bagasse, bran, banana leaves, peanut hulls, rice husks, and coconut fibre (Loyd et al., 2018b; Ab Rhaman et al., 2021). However, the mycelial growth of *Ganoderma* spp. is subject to multifaceted influences from a variety of factors including, environmental conditions and cultural characteristics (Maszlavér and Balázs, 2008; Shah and Modi, 2018). As our understanding of these influences grows, it becomes increasingly clear that *Ganoderma* spp. mycelial growth and development is a complex process that responds dynamically to a range of variables. Environmental conditions such as temperature, pH, relative humidity, and aeration have emerged as critical determinants of mycelial growth (Roberts, 2004; Gurung et al., 2012; Nguyen et al., 2023). However, little attention from researchers has been given to the significant role played by these factors.

In addition, the type of growth media used to culture *Ganoderma* spp. is also an important factor that influences its growth and development. The amount of inoculum, stirring illumination, and components of the culture are also reported to have a direct influence on the growth and quality of mycelial production (Kadowaki et al., 2010; Shah and Modi, 2018). Furthermore, the diverse geographical distribution, tree host/plant residue, and environmental contexts in which *Ganoderma* spp. thrive further accentuate the variability in mycelial growth rates across different settings (Zakaria et al., 2009; Kinge et al., 2015). Under optimal conditions, the mycelial growth of *Ganoderma* spp. follows a typical trend of approximately 9 days to achieve full development (Fahimeh et al., 2013; Ngo et al., 2019). However, variations exist, with reports by Cortina-Escribano et al. (2020) indicating that the optimal range can extend from 10-18 days. This stands in contrast to other mushroom species, some of which can reach full mycelial development in as little as 4 days while others take 6 days (Furlan et al., 1997).

Within the sphere of these influences, the growth media pH emerges as a critical factor that controls *Ganoderma* mycelial development. This genus has shown remarkable adaptability, thriving over different levels of pH ranging from 3-11 (Kapoor and Sharma, 2014; Zhou et al., 2015). Nonetheless, optimal mycelial development is reported to be achieved within the pH range of 5-6, reflecting a preference for a slightly acidic growth environment (Jo et al., 2009; Kapoor and Sharma, 2014; Chen et al., 2017; Nguyen et al., 2023). On the other hand,

temperature is a key determinant of mycelial growth (Fletcher, 2019), with *Ganoderma* spp. demonstrating a growth range of 9-40 °C. Furthermore, an optimum temperature zone of 25-30 °C has been identified, showing their capacity to endure extreme temperature variations (Magday Jr et al., 2014; Badalyan et al., 2019). Notably, reduced mycelial growth has been observed at both low (9 °C) and high (35 °C) temperatures (Magday Jr et al., 2014).

In addition to pH and temperature, the chemical composition of the growth medium, encompassing soluble inorganic and organic compounds such as carbon, exerts a substantial influence on mycelial development (Vilas et al., 2020). The relationship among these factors contributes to the complexity of the *Ganoderma* mycelial growth process. As a result, ensuring optimal growth conditions is crucial, especially under artificial production (Chen and Miles, 1996; Chen, 1999; Cao et al., 2018; Shah and Modi, 2018; Sardar et al., 2020). This study investigated the specific requirements for optimising the mycelial development of *G. austroafricanum* and *G. resinaceum* under various growth media, shedding light on the intricate relationship between temperature, pH, and growth media in shaping the growth dynamics of these remarkable fungal species.

2 Materials and methods

2.1 Study area

All experiments conducted in this study were performed at Plant Pathology laboratory facilities, at the University of KwaZulu-Natal, Pietermaritzburg campus (South Africa). This study consisted of three independent *in vitro* experiments.

2.2 Fungal source

2.2.1 Strains

The two *Ganoderma* fungal species, namely, *Ganoderma austroafricanum* (GA) and *Ganoderma resinaceum* (GR) were sourced from the Nkomazi Local Municipality, Jeppes Reef (25°43'57.8"S 31°29'31.0"E), Mpumalanga Province (MP) and Ubuhlebezwe Local Municipality, Mnyanyabuzi (30°10'11.46"S 30°20'11.78"E), KwaZulu-Natal Province (KZN). The identification of species was carried out using morphological techniques and further confirmed by molecular characterisation.

2.3 Fungal preparation

2.3.1 Fungal isolation

The sampled fungal pileus was cleaned and dried in sunlight, placed in paper bags, and stored at room temperature as described by Mohanty et al. (2011). The fungal species were isolated by cutting pieces of approximately 5 mm from underneath each pileus. Each species was replicated three times and sequentially sterilised with deionised water, 70% (v/v) ethanol, and deionised water, respectively. All the samples were thereafter allowed to dry by placing them on a paper towel for 30 min. Potato dextrose agar (PDA) and malt extract agar (MEA) were prepared and autoclaved at 121 °C for 15 min at 121 kPa and 121 °C for 10 min at 121 kPa, respectively. The media was removed and allowed to cool and thereafter poured into 90 mm Petri dishes. After the preparation of culture media, each isolate/species was placed in the middle of the Petri dishes containing PDA and MEA. The Petri dishes were thereafter incubated for 5 days at 25 °C. The pure cultures were obtained by cutting 5 mm mycelial plugs from the edges of 5-day-old cultures followed by incubation into fresh media, and incubated for 12 days at 25 °C. Pure cultures were then stored in 70% (v/v) glycerol at -80 °C until further used.

2.4 Effect of pH on mycelial growth of *G. austroafricanum* and *G. resinaceum*

The study was laid out as a factorial experiment arranged in a completely randomised design (CRD). The experimental treatment comprised three treatment factors namely; pH at three levels (4, 6, and 8), species at two levels (GA and GR), and culture media at two levels (PDA and MEA). The treatments were replicated three times resulting in 36 experimental units (3 x 2 x 2 x 3). To obtain the specified culture media pH levels, PDA and MEA were adjusted using the method described by Chen et al. (2017). Hydrochloric acid – HCl (1 mol L^{-1}) and sodium hydroxide – NaOH (1 mol L^{-1}) were used to adjust the pH of both media. Each species was placed in Petri dishes with different pH values and incubated for 12 days at 25 °C. The mycelial growth diameter (mm) was measured using a vernier caliper on days 3, 6, 9, and 12.

2.5 Effect of culture media temperature on mycelial growth and development of *G. austroafricanum* and *G. resinaceum*

The study experiment was laid out in a factorial design and was arranged in a CRD. The experimental treatment comprised three treatment factors namely; temperature at three levels (20, 25, and 30 °C), species at two levels (GA and GR), and culture media at two levels (PDA and MEA). The treatments were replicated three times resulting in 36 experimental units (3 x 2 x 2 x 3). Each species was cultured in PDA and MEA and incubated for 12 days at 20, 25,

and 30 °C. The mycelial growth diameter (mm) was measured using a vernier caliper on days 3, 6, 9, and 12.

2.6 Effect of different growth media on mycelial growth and development of *G. austroafricanum* and *G. resinaceum*

2.6.1 Types of growth media and source

Plant residues namely; beech sawdust (BS), and buffalo grass (BG) were purchased at Animal Smith Feeds, Pietermaritzburg, KwaZulu-Natal, and sugarcane bagasse (SB) at Hullet Sugar Maidstone Mill, Tongaat, KwaZulu-Natal. Their selection was informed by availability, especially in MP and KZN. These plant residues were introduced in this study with the consideration of the natural growth media state where these species normally grow or are commercially produced. PDA and MEA were also used as standard growth media. The chemical composition of plant residues is presented in Table 5.1. The study was laid as a factorial experiment arranged in a CRD. The experimental treatment comprised two treatment factors namely; fungal species, at two levels (*G. austroafricanum* and *G. resinaceum*) and growth media, at 10 levels (PDA, PDA+BS, PDA+SB, PDA+BG, PDA+BS+SB+BG, MEA, MEA+BS, MEA+SB, MEA+BG, and MEA+BS+SB+BG). The treatments were replicated three times resulting in 60 experimental units (2 x 10 x 3).

2.6.2 Growth media preparation

A suspension of culture media and plant residues was developed as described by Cortina-Escribano et al. (2020), with minor modifications. Culture media were prepared by using 1% of PDA at 10 g/500 mL, and 1% of MEA at 10 g/500 mL. Plant residues were prepared by adding 10 g/500 mL dry weight of BS, SB, and BG. These plant residues were also prepared by combining them with 3.33 g/500 mL of each on both culture media. Each culture media and plant residue was combined in a 500 mL Borosilicate glass reagent bottle and thereafter topped with deionised water. The suspension was then autoclaved at 121 °C for 15 min at 121 kPa and the culture media without plant residues was considered a control. The suspension was removed and allowed to cool and thereafter poured into 90 mm Petri dishes. The dishes were then inoculated with GA and GR species and incubated for 12 days at 20 and 30 °C. Each treatment was observed and measured for mycelial growth using a vernier caliper (mm) from day 3 until day 12.

2.6.3 Plant residue pH

The pH levels of all three individual plant residues (Table 5.2) were determined as described by Jones Jr (1999). The plant residues were dried and milled into smaller homogenous sizes. A 100 mL graduated cylinder was used to measure 50 mL of deionised water and poured into a 100 mL glass beaker. A mass of 5 g of each plant residue was weighed and added into a glass beaker. The suspension was left undisturbed for 1 h. To obtain a homogenous mixture, a 20 mm magnetic stirrer bar was used for 30 min. A buffer solution of pH 4 and 7 was used to calibrate the pH meter. Each treatment was replicated three times and measured for pH level using a benchtop pH meter.

2.6.4 Plant residue essential elements

The dry ashing procedure was used to measure the essential elements (Agrilasa, 1998). Plant residues were dried at 26 °C for 72 h and milled. The process involved placing 1 g of each dried plant residue into a crucible and further subjected to additional drying for 24 h. The crucibles containing dry plant residues were heated in an ashing oven at 600 °C for a duration of 8 h. The inductively coupled plasma (ICP) 4:1 plant extract procedure was used, 8 mL Nitric acid (HNO_3) and 2 mL hydrochloric acid (HCI) were used as reagents and incubated in a microwave reaction system for 45 min. Digested samples were transferred into volumetric flasks (100 mL) and topped up with deionised water and left standing for 24 h. Samples were carefully transferred to McCartney bottles without disturbing the sediment and an ICP mass spectrometer was used. Zinc (Zn), iron (Fe), selenium (Se), manganese (Mn), magnesium (Mg), sodium (Na), calcium (Ca), aluminium (Al), potassium (K) and phosphorus (P) were quantified and expressed in milligrams per kilogram of dry matter (mg/kg DM) (Table 4.1).

2.6.5 Plant residue heavy metals

Samples were prepared and examined for heavy metal content using the same procedure for essential elements (Agrilasa, 1998). A protocol described by Stihi et al. (2011) was used to quantify the heavy metals content. The levels of nickel (Ni), copper (Cu), cobalt (Co), cadmium (Cd), chromium (Cr), palladium (Pd), lead (Pb), mercury (Hg), silver (Ag), and arsenic (As) were quantified and presented as mg/kg DM (Table 5.3).

Table 5.1 Essential elements levels (mg/kg DM) of beech sawdust (BS), sugarcane bagasse (SB), and buffalo grass (BG).

Substrates	Zn	Fe	Se	Mn	Mg	Na	Ca	Al	K	P
BS	17.56	11.32	0.35	5.04	541.03	64.22	16.12	5.51	1523.37	1318.35
SB	28.93	26.56	1.30	9.89	935.62	95.48	46.11	3.29	3520.29	5529.81
BG	22.15	43.31	0.80	8.74	729.14	82.78	35.33	2.34	2808.68	3666.11

Table 5.2 pH levels of plant residues

Substrates	pH values
BS	5.25
SB	6.15
BG	5.52

Table 5.3 Heavy metal levels (mg/kg DM) of beech sawdust (BS), sugarcane bagasse (SB), and buffalo grass (BG).

Substrates	Ni	Cu	Co	Cd	Cr	Pd	Pb	Hg	Ag	As
BS	0.40	4.98	0.94	27.13	10.81	0.03	0.50	0.32	0.014	0.032
SB	1.94	9.32	1.49	33.47	8.04	0.15	5.79	1.34	0.025	0.219
BG	0.27	2.90	4.19	15.89	14.62	0.06	2.45	0.29	0.014	1.044

2.7 Statistical analysis

The data was subjected to the analysis of variance (Three-Way-ANOVA) using GenStat statistical software (Genstat® 20th edition, VSN International, Hemel Hempstead, UK). Means were separated according to Duncan's multiple range test at 5% level of significance.

3 Results and Discussion

3.1 Effect of culture media pH on mycelial development of *G. austroafricanum* and *G. resinaceum*

The study results indicated significant differences ($p \leq 0.001$) with regard to the effect of culture media pH on mycelial development for the selected *Ganoderma* species for 12 days (Table 5.4). At day 3 the mycelial development had significant variations at different pH levels. pH 6 (18.36±1.12) showed the highest development, followed by pH 8 (17.67±0.69), and pH 4 (14.28±0.43) for the GA species cultured on PDA at day 3. A similar trend was observed in GR species, with the highest development at pH 8 (22.68±0.87), followed by pH 6 (21.62±1.79), and pH 4 (20.68±1.36). With regard to growth media, the fungal species showed increased mycelial growth in MEA compared to PDA. However, their response to pH varied significantly, with GA species obtaining the highest mycelial growth at pH 6 and GR species at both pH 6 and 8. As the fungal species continued to grow, the different levels of culture media pH started to show instability in the mycelial growth of the two species. Though differences were observed in the culture media pH, GA+MEA reached maximum mycelial growth (85.00 mm) on day 9 from all pH levels. Similarly, on day 9, GR+MEA obtained maximum (85.00 mm) mycelial development at pH 4 and 6, whilst the treatment combination had 80.40 mm at pH 8. Even though between days 3-6 species GA recorded the lowest mycelial growth in the majority of treatments on both culture media PDA and MEA, it had a doubled growth rate on day 9. In comparison with all other treatment combinations throughout the experiment, GR+PDA at pH 8 failed to reach maximum mycelial growth recording 80.40 mm on day 12. Current results could be attributed to GR growing well at a pH range of 4-7, with optimum growth at pH 5 (Chen et al., 2017). The mycelial growth of *Ganoderma* species tends to decline significantly at a pH higher than 7 (Adaskaveg and Gilbertson, 1986; Jayasinghe et al., 2008; Zhou et al., 2015). Therefore, it can be deduced that a pH higher than 7 could decrease mycelial growth.

Table 5.4 Effect of pH on the mycelial development for *G. austroafricanum* (GA) and *G. resinaceum* (GR) from culture media PDA – potato-dextrose agar and MEA – malt-extract agar and observed for 12 days. The results were expressed as mean ± SD, $n = 3$.

Days after inoculation	Species	Culture media	Mycelial growth (mm)		
			pH 4	pH 6	pH 8
Day 3	GA	PDA	14.28 ± 0.43^{a}	18.36 ± 1.12^{b}	17.67 ± 0.69^{b}
	GR	PDA	20.68 ± 1.36^{cd}	21.62 ± 1.79^{cd}	22.68 ± 0.87^{d}
	GA	MEA	20.54 ± 1.14^{c}	21.13 ± 1.04^{cd}	17.65 ± 1.85^{b}
	GR	MEA	25.67 ± 2.22^{e}	32.67 ± 2.04^{g}	30.55 ± 1.65^{f}
Day 6	GA	PDA	35.56 ± 2.30^{h}	41.28 ± 1.22^{j}	50.58 ± 1.66^{n}
	GR	PDA	43.34 ± 2.13^{k}	45.01 ± 2.76^{kl}	38.32 ± 1.37^{i}
	GA	MEA	55.75 ± 1.71^{o}	50.84 ± 1.56^{n}	46.14 ± 2.32^{l}
	GR	MEA	48.35 ± 0.58^{m}	73.34 ± 2.40^{r}	58.35 ± 1.42^{p}
Day 9	GA	PDA	85.00 ± 0.00^{t}	81.33 ± 2.08^{s}	85.00 ± 0.00^{t}
	GR	PDA	85.00 ± 0.00^{t}	85.00 ± 0.00^{t}	66.74 ± 1.56^{q}
	GA	MEA	85.00 ± 0.00^{t}	85.00 ± 0.00^{t}	85.00 ± 0.00^{t}
	GR	MEA	85.00 ± 0.00^{t}	85.00 ± 0.00^{t}	80.40 ± 0.95^{s}
Day 12	GA	PDA	85.00 ± 0.00^{t}	85.00 ± 0.00^{t}	85.00 ± 0.00^{t}
	GR	PDA	85.00 ± 0.00^{t}	85.00 ± 0.00^{t}	80.46 ± 0.00^{s}
	GA	MEA	85.00 ± 0.00^{t}	85.00 ± 0.00^{t}	85.00 ± 0.00^{t}
	GR	MEA	85.00 ± 0.00^{t}	85.00 ± 0.00^{t}	85.00 ± 0.00^{t}
P Value	0.001				
LSD ($p \geq 0.05$)	1.8943				
CV %	2.5%				

* Means followed by the same letter within the table are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.2 Effect of temperature on the mycelial development of *G. austroafricanum* and *G. resinaceum* grown in two culture media

In the current study, temperature had a significant effect on the mycelial development of *Ganoderma* species from two culture media. After 12 days of assessment, Table 5.5 shows mycelial development in response to various temperatures under PDA and MEA. On day 9, treatment combination GR+MEA mycelial growth (85.00 mm) performed better at 30 °C, however recording the lowest (70.11±1.23) at 20 °C. Concurrently, a different trend was

observed for GA+MEA reaching the highest mycelial growth (80.61 mm) at 20 °C, nevertheless, mycelial growth was notably slow (8.11±0.94) as temperature increased (30 °C). The overall results indicated that the mycelial development of *Ganoderma* species varies at different temperatures after 12 days of evaluation. Therefore, deduced that the mycelial growth of GR increased as temperatures increased while it was inversely for GA. The results on GR corroborate the assertions that this species is a high-temperate fungus (Schwarze and Ferner, 2003; Chen et al., 2017). The current results indicated an optimal mycelial growth temperature for GR to be 30 °C, therefore, this explains why these species are mostly found in hot climates and tropical regions. GR is a temperate species commonly grown in Europe and Northern America with an optimum growth temperature ranging between 22–35 °C (Mendoza et al., 2011). However, it is reported that temperatures exceeding 35 °C inhibit the mycelial growth of GR (Chen et al., 2017). The growth of mycelia was high on GA at 20 °C, with a decline as temperatures increased (25-30 °C). This could be justified by the reported temperature growth range of 15-25 °C for GA (Crous et al., 2014; Coetzee et al., 2015). The overall results revealed huge variations among the temperatures in both species, therefore the findings proved that temperature may influence the mycelial growth of *Ganoderma* species.

Table 5.5 Effect of temperature on the mycelial development of *G. austroafricanum* (GA) and *G. resinaceum* (GR) from two culture media observed for 12 days. * PDA – potato-dextrose agar; MEA – malt-extract agar. The results were expressed as mean ± SD, $n = 3$.

Days after inoculation	Species	Culture media	Mycelial growth (mm)		
			20 °C	25 °C	30 °C
Day 3	GA	PDA	3.33 ± 0.27^{b}	3.31 ± 0.49^{b}	1.42 ± 0.44^{ab}
	GR	PDA	16.36 ± 1.26^{e}	22.61 ± 1.33^{g}	34.76 ± 1.77^{ij}
	GA	MEA	3.33 ± 0.64^{b}	3.40 ± 0.67^{b}	1.09 ± 0.19^{a}
	GR	MEA	22.67 ± 1.02^{g}	58.65 ± 1.17^{op}	62.46 ± 2.56^{q}
Day 6	GA	PDA	21.68 ± 2.11^{fg}	15.79 ± 2.08^{e}	7.86 ± 0.25^{c}
	GR	PDA	39.41 ± 1.13^{k}	36.35 ± 1.01^{j}	53.19 ± 1.81^{n}
	GA	MEA	22.76 ± 2.05^{g}	11.68 ± 0.75^{d}	3.39 ± 0.69^{b}
	GR	MEA	50.26 ± 1.54^{m}	69.52 ± 2.36^{r}	79.36 ± 0.72^{v}
Day 9	GA	PDA	57.57 ± 1.13^{op}	33.76 ± 1.70^{i}	23.33 ± 1.45^{g}
	GR	PDA	50.33 ± 0.98^{m}	56.69 ± 1.32^{o}	72.12 ± 1.67^{st}
	GA	MEA	80.61 ± 1.45^{v}	26.61 ± 1.78^{h}	8.11 ± 0.94^{c}

	GR	MEA	70.11±1.23^{rs}	75.34±2.30^{u}	85.00±0.00^{w}
Day 12	GA	PDA	72.50±2.17^{t}	62.05±2.40^{q}	59.23±0.94^{p}
	GR	PDA	72.81±1.61^{t}	71.44±2.18^{rst}	85.00±0.00^{w}
	GA	MEA	85.00±0.00^{w}	48.23±1.03^{l}	19.94±0,10^{f}
	GR	MEA	80.19±0.30^{v}	85.00±0.00^{w}	85.00±0.00^{w}
P Value	0.001				
LSD ($p \geq 0.05$)	1.986				
CV %	3.5%				

* Means followed by the same letter within the table are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.3 Influence of different growth media on the mycelial development of *G. austroafricanum* and *G. resinaceum*

The results in Table 5.6 show the significant differences in the mycelial development treatment combinations. The majority of treatments under media MEA had the highest mycelial development on both species compared to other treatments. On day 9, MEA reached maximum mycelial growth (85.00 mm) on GR. Under GR, a similar trend was observed on day 9 from treatment combinations MEA+BS and PDA+SB where mycelial growth was recorded at 81.67 and 80.11 mm respectively. In contrast, the mycelia for GA managed to reach maximum development (85.00 mm) on day 12 under growth media MEA and MEA+BS. Whilst on day 12 GR had the maximum mycelial growth from growth media PDA, PDA+SB, MEA, and MEA+BS. Even though GR had most treatment combinations with high mycelial growth, the slowest growth was observed on PDA+BG (27.09 mm) and MEA+BG (29.09 mm). Furthermore, the comprehensive results of this study indicated that the majority of treatment combinations with MEA had an expeditious mycelial growth on both species compared to those with PDA.

Current results are consistent with those of Suansia and John (2020), where MEA had a rapid mycelial growth of *Ganoderma* species. Similarly, Cortina-Escribano et al. (2020) reported that MEA was suitable for the growth of *Ganoderma* spores in comparison to PDA. Also, the current results showed a superior mycelial development on culture media without plant residues compared to other treatment combinations. This could be due to the nutrient composition of carbon and nitrogen readily available in the commercial media (PDA and MEA). Agar culture media contains different carbon sources such as glucose, sucrose, fructose, dextrose, and molasses that influence mycelial development (Hoa and Wang, 2015). The slow

mycelial growth observed on treatments with plant residues added might be due to the saprophytic ability of fungal enzymes to synthesise the substrates into carbons (Challacombe et al., 2019; Kumar et al., 2023). *Ganoderma* is a facultative (parasitic and saprophytic) mushroom species. Saprotrophic mushroom species can decompose lignocellulosic biomass by producing fungal enzymes (Sánchez, 2009). These fungi produce extracellulase hydrolysing enzymes (cellulose, ligninase, and laccase) to hydrolyse cellulose, hemicelluloses, and lignin present in the substrate to simple sugars for growth (Hoa and Wang, 2015). Therefore, the slow growth rate on culture media with plant residues could have been due to the synthesis time takes to convert the substrate into simple sugars.

In addition, the variation in mycelial development under BS, SB, and BG could be linked to the variation in their physical properties and chemical composition. The plant residues used in this study had a substantial amount of chemical composition such as K, P, Zn, and Fe (Table 5.1). Plant residues used for mushroom production contain chemical compositions such as N, P, K, and organic carbon that are favourable and essential for mycelial development (Nguyen et al., 2019; Siwulski et al., 2019). Even though BS had the lowest chemical composition levels compared to SB and BG, BS+MEA had the highest (85.00 mm) mycelial growth in both species on day 12. This could be due to *Ganoderma* species growing well on woody plant material (Loyd et al., 2018a). Cortina-Escribano et al. (2020), reported that sawdust plant residue from different lignocellulosic biomass sources interferes with the growth ability of the media. The high mycelial growth on BS could, therefore be attributed to the plant residue's chemical composition and physical properties. Therefore, the usage of plant residue on culture media for fungal growth may influence mycelial development.

Table 5.6 Effect of culture media and plant residues on the mycelial development for *G. austroafricanum* (GA) at 20 °C and *G. resinaceum* (GR) at 30 °C using ten growth media observed for 12 days. * PDA – potato-dextrose agar; MEA – malt-extract agar; BS – beech sawdust; SB – sugarcane bagasse; BG – buffalo grass. The results were expressed as mean ± SD, $n = 3$.

Days after inoculation	**Growth media**	**Mycelial growth (mm)**	
		GA	**GR**
Day 3	PDA	3.33 ± 0.09^{b}	34.78 ± 2.04^{p}
	PDA+BS	8.67 ± 0.97^{cde}	8.05 ± 1.01^{cd}
	PDA+SB	10.76 ± 0.86^{ef}	12.41 ± 0.71^{fgh}

	PDA+BG	3.43±0.44^{b}	0.00±0.00^{a}
	PDA+BS+SB+BG	7.62±0.79^{c}	6.81±0.36^{c}
	MEA	3.33±0.09^{b}	62.06±1.03^{w}
	MEA+BS	16.33±0.9^{ij}	20.51±0.87^{kl}
	MEA+SB	8.52±0.81^{cde}	14.06±0.98^{hi}
	MEA+BG	3.43±0.51^{b}	0.00±0.00^{a}
	MEA+BS+SB+BG	10.54±1.07^{def}	11.33±0.46^{fg}
Day 6	PDA	22.67±1.45^{lm}	53.20±2.42^{u}
	PDA+BS	21.59±1.16^{lm}	18.69±1.66^{jk}
	PDA+SB	22.10±1.96^{lm}	42.73±1.38^{r}
	PDA+BG	27.33±1.81^{n}	0.00±0.00^{a}
	PDA+BS+SB+BG	23.64±0.82^{m}	20.45±1.58^{kl}
	MEA	21.67±1.87^{lm}	79.11±1.02^{D}
	MEA+BS	40.18±2.39^{q}	46.52±1.09^{s}
	MEA+SB	30.06±1.19^{o}	42.42±0.72^{qr}
	MEA+BG	33.38±2.46^{p}	0.00±0.00^{a}
	MEA+BS+SB+BG	34.46±0.10^{p}	29.54±2.53^{no}
Day 9	PDA	57.19±3.06^{v}	72.13±1.08^{zA}
	PDA+BS	49.34±2.08^{t}	35.10±1.38^{p}
	PDA+SB	53.37±1.94^{u}	80.11±1.68^{DE}
	PDA+BG	56.70±3.07^{v}	16.72±2.07^{j}
	PDA+BS+SB+BG	53.26±2.48^{u}	43.92±2.49^{r}
	MEA	80.09±2.06^{DE}	85.00±0.00^{F}
	MEA+BS	76.68±2.13^{C}	81.67±2.95^{DE}
	MEA+SB	55.06±3.63^{uv}	75.04±1.99^{BC}
	MEA+BG	61.72±1.49^{w}	13.37±0.86^{gh}
	MEA+BS+SB+BG	61.56±2.18^{w}	56.69±2.75^{v}
Day 12	PDA	72.11±1.51^{zA}	85.00±0.00^{F}
	PDA+BS	70.00±2.44^{yz}	56.82±1.60^{v}
	PDA+SB	61.67±2.07^{w}	85.00±0.00^{F}

	PDA+BG	71.67±1.86^{zA}	27.09±1.97^{n}
	PDA+BS+SB+BG	67.80±2.33^{y}	56.27±2.56^{v}
	MEA	85.00±0.00^{F}	85.00±0.00^{F}
	MEA+BS	85.00±0.00^{F}	85.00±0.00^{F}
	MEA+SB	68.34±1.89^{y}	81.67±2.05^{E}
	MEA+BG	73.40±0.08^{AB}	29.13±1.98^{no}
	MEA+BS+SB+BG	72.78±0.89^{AB}	65.27±0.95^{x}
P Value	0.001		
LSD ($p \geq 0.05$)	2.3385		
CV %	4.3%		

* Means followed by the same letter within the table are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

4 Conclusion

The study provides data on various growth conditions of *G. austroafricanum* and *G. resinaceum*. The findings have shown that *Ganoderma* spp. may grow well on woody plant material and prefer a pH range of 4-6 but their temperature requirement varies. Plant residue and culture media combinations may be suitable for the development of *Ganoderma* spp. but the mycelial growth can still be slow. Also, MEA is the best growth media for the mycelial development of *Ganoderma* spp. The results of this study contribute to the ongoing research on the behaviour of *Ganoderma* spp. under different growth conditions. These growth conditions results will enable the domestication and commercial production of these species, particularly *G. austroafricanum* due to a lack of information concerning this species. In addition, information regarding growth parameters and biochemical composition of *G. austroafricanum* is very limited, therefore future research should focus on these aspects.

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CHAPTER 6

EFFECT OF DIFFERENT SUBSTRATES ON THE TOTAL BIOMASS AND BIOCHEMICAL PROFILE OF *GANODERMA* SPP.

Abstract

This study investigated the effect of different substrates on the development, total biomass, and biochemical profile of *Ganoderma* species. The experiment involved growing *Ganoderma austroafricanum* (GA) and *Ganoderma resinaceum* (GR) on beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG), and suspension of all substrates (BSSBBG). Growth parameters, such as mycelial development, pinning, pileus development, and pileus maturity rate were assessed. Furthermore, the pileus, stipe size, total pileus, weight, total biomass production, and biological efficiency were measured. Additionally, each substrate was analysed for biochemical composition, including pH and minerals. The biochemical composition of the harvested *Ganoderma* samples was also analysed to determine the levels of biochemical compounds such as minerals, antioxidants, and protein. The results on substrate pH levels demonstrated that all substrates were within the optimal growth pH range (5-6). Among the substrates tested, SB had higher concentrations of Zn (28.93±1.97 mg/kg DM) and K (3520.29±57.00 mg/kg DM), also, heavy metals Pb (5.79±0.54 mg/kg DM) and Hg (1.34±0.10 mg/kg DM). The results on the development and total biomass production of *Ganoderma* species revealed significant variations across different substrates. In terms of development, GRBS was faster to reach the 100% rate of all production parameters in 40-52 days after inoculation. However, this did not translate to a high total biomass and biological efficiency, in contrast, GASB exhibited higher quantities of 81.77±1.84 g/kg and 9.09±0.82%, respectively. In addition, GASB yielded higher levels of DPPH, phenolics, flavonoids, and protein of 66.94±1.04%, 180.58±1.36 mg GAE/g, 84.43±2.31 mg QE/g and 24.01±0.54 µg/g, respectively. These findings demonstrated that the physical properties and chemical composition of a substrate could influence the development, total biomass, and biochemical profile of *Ganoderma* spp.

***Keywords*:** Medicinal mushrooms, agro-waste, total yield, conversion efficiency, bioactive compounds

1 Introduction

The cultivation of mushrooms is an eminent commercial agribusiness that uses various agro-industrial wastes as a substrate (Gurung et al., 2012; Chang and Wasser, 2018). Agro-industrial waste material has always been used in many various ways. These include its use as a source of energy, biofuel, carbon as well as a substrate for mushroom cultivation (Lal, 2005; Kumla et al., 2020; Koul et al., 2022; Leong et al., 2022). A wide range of mushroom species can be cultivated by using substrates such as straws, paddy, grass plants, sawdust, corn, cottonseed hull, wood chips, livestock litter, manure, etc. (Carrasco et al., 2018; Hanafi et al., 2018; Leong et al., 2022). However, the type of substrate used has a major influence on mushroom production including development phases and total biomass (Khandakar, 2004; Chitamba et al., 2012; Shah and Modi, 2018; Carrasco et al., 2020). In recent years, there has been growing interest in cultivating *Ganoderma* on different substrates to optimise its growth and maximise its bioactive compound production. These various substrates include sawdust, sugarcane bagasse, bran, banana leaves, peanut hulls, rice husks, and coconut fibre (Loyd et al., 2018b; Meng et al., 2019; Ab Rhaman et al., 2021; Nikšić et al., 2022; Shrikhandia et al., 2022).

Substrates vary in structure, texture, and chemical composition thus influencing nutrient availability and release (Buah et al., 2010; Ćilerdžić et al., 2018; Nguyen et al., 2019; Siwulski et al., 2019). Therefore, the chemical composition of a substrate influences the conversion efficiency rate of mycelial to convert biomass into mushrooms (Ramachela and Sihlangu, 2016). This suggests that the substrate's chemical composition has an effect on the production phases and yield of mushrooms. To achieve good production, it is also important to maintain growth parameters such as relative humidity (RH), temperature, moisture content, light intensity, and pH. In addition, to optimise mycelial growth, several supplements such as gypsum, wheat bran, and sucrose are used as substrate mixtures (Magday Jr et al., 2014; Bidegain et al., 2015; Shah and Modi, 2018). A good substrate must be suitable both chemically and physically, as well as have conducive conditions for microbial activities (Chang and Wasser, 2018). Even though the selection of main and co-substrate for mushroom production is generally influenced by geographic location, it is important to identify and select the best substrate possible. Therefore, this study evaluated the effect of using different substrates on the development, total biomass, and biochemical profile of *G. austroafricanum and G. resinaceum*.

2 Materials and methods

2.1 Experimental description

2.1.1 Study area

All research production activities and sample preservation were carried out in the facilities of Mushroom Guru Pty Ltd in Cape Town (South Africa). Sample preparation, extraction, and antioxidant activity were conducted at the Horticultural Science laboratory facilities of the University of KwaZulu-Natal – Pietermaritzburg campus (South Africa). Protein content was determined at the Biochemistry Department of the University of KwaZulu-Natal. Essential elements and heavy metals from various substrates and pileus were analysed at Cedara College of Agriculture.

Fungal species used in the present study were grown and obtained from Eco-Agro Enterprise Pty Ltd in Mbombela (South Africa). The two *Ganoderma* fungal spp., namely, *Ganoderma austroafricanum* (GA) and *Ganoderma resinaceum* (GR) were sourced from the Nkomazi Local Municipality, Jeppes Reef (25°43'57.8"S 31°29'31.0"E), Mpumalanga Province (MP) and Ubuhlebezwe Local Municipality, Mnyanyabuzi (30°10'11.46"S 30°20'11.78"E), KwaZulu-Natal Province (KZN). The identification of species was carried out through morphological technique and further confirmed by molecular characterisation.

2.2 Experimental layout

The production experiment was laid out in a 2 x 4 factorial experimental combination with factors being: two *Ganoderma* spp. namely; *G. austroafricanum* (GA) and *G. resinaceum* (GR) and four substrates namely; beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BS) and suspension of all substrates (BSSBBG). The treatment combinations were coded as follows: GABS, GASB, GABG, and GABSSBBG; GRBS, GRSB, GRBG, and GRBSSBBG making a total of 8 treatment combinations. Respective treatment combinations were replicated three times making a total of 24 observations. These treatment combinations were laid out in a Completely Randomised Design (CRD).

2.3 Preparation

2.3.1 Spawn

Sorghum (*Sorghum bicolour*) grain was used for the spawn production of both species. The grain was soaked for 12 h and dried for 1 h to allow water drainage. Spawn was prepared using the protocol described by Matute et al. (2002), with minor modifications. Bottle sizes of 1 L were used to place the wheat grain mixed with 0.1% (w/w) calcium carbonate ($CaCO_3$), 0.8%

(w/w) cobalt sulfate ($CoSO_4$), and 40% (v/v) water. The grain was thereafter pasteurised at 121 °C for 30 min using an autoclave at 121 kPa. The bottles were removed and allowed to cool for 1 h before inoculation with fungal species. To minimise contamination, preparation of the spawn was carried out within a laminar flow enclosure where the wheat grain was inoculated with GA and GR. The spawn suspension was packed and incubated in the dark at 26 °C, RH 53%, and a carbon dioxide (CO_2) level of 1000 parts per million (ppm) for 20 days up until ready for inoculating the substrate treatment combinations.

2.3.2 Substrates

Beech sawdust – *Fagus sylvatica* (BS), sugarcane bagasse – *Saccharum officinarum L.* (SB), and buffalo grass – *Stenotaphrum secundatum* (BG) were used. BS and BG were sourced from Animal Smith Feeds, Pietermaritzburg, KwaZulu-Natal, and SB was sourced from Hullet Sugar Maidstone Mill, Tongaat, KwaZulu-Natal. Their selection was informed by availability, especially in these two provinces.

All substrates were milled into small homogenous particles. The substrates were autoclaved for 30 min at 121 °C at 121 kPa. After autoclaving, the substrates were removed and cooled in sterilised containers for 1 h. Before inoculation with *Ganoderma* spp. spawn, the substrates were weighed as described by Ramachela and Sihlangu (2016).

2.3.3 Inoculation

The growth medium was prepared by mixing each substrate and supplement at a ratio of 9:1 (w/w) based on their dry weight. A modified substrate suspension procedure described by Enriquez (2015) was followed. Dry substrates were prepared before mixing with water. The four designated different substrates were composed as follows: all substrates were prepared at 88% (w/w) mixed with supplements namely - wheat bran 8% (w/w), gypsum 2% (w/w), and 2% (w/w) sucrose (brown sugar). Adequate moisture content was set at 68% (v/v) field capacity level of the substrate. The inoculation ratio of 1:9 (w/w) spawn-substrate suspension was used for each treatment as described by Sofi et al. (2014). A 1 kg suspension inoculation ratio was quantified as follows: 10% (w/w) – 100 g of spawn per bag and 90% (w/w) – 900 g of substrate. To ensure high-quality mycelial development, the suspension of 1 kg was placed in 250 mm x 460 mm transparent filter patch autoclave bags and placed in the incubation room at 25°C, RH 53%, and CO_2 at 2000 ppm for 18 days (Figure 6.1). Thereafter, transferred to controlled production growth chambers at 23°C, RH 92%, and CO_2 levels 750 ppm.

Figure 6.1 A – B spawn (*Ganoderma austroafricanum* and *Ganoderma resinaceum*); C – substrates; D – E incubation; mycelial development (after 12-18 days).

2.3.4 Growth conditions

The growth conditions such as micro-climate, carbon dioxide, and light intensity were monitored inside the growth chambers (Figure 6.2). Temperature (°C) and relative humidity (%) were assessed and recorded using a thermostat. The CO_2 levels were observed and measured using a CO_2 level transmitter and expressed in ppm. Also, light intensity was observed and recorded using a digital light meter and was expressed in lux. All micro-climate, carbon dioxide, and light intensity data were recorded at three-day intervals from the first day of inoculation to the last day of harvest.

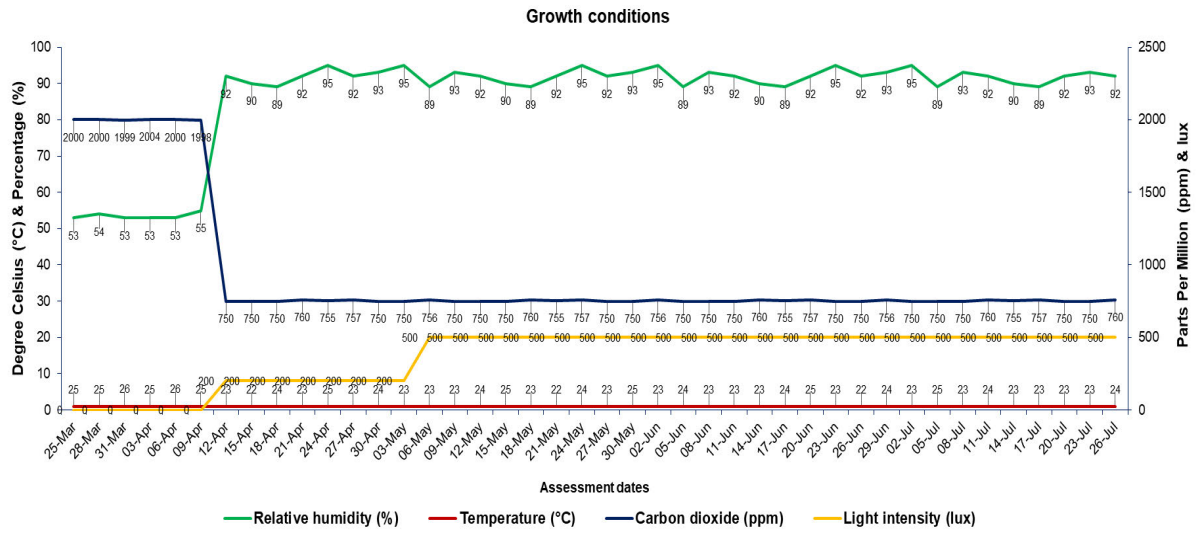


Figure 6.2 Growth conditions recorded in 123 days (from 25 March 2022 to 26 July 2022) inside the growth chambers during the production of *Ganoderma austroafricanum* and *Ganoderma resinaceum*. Mean temperature (°C), relative humidity (%), carbon dioxide (ppm), and light intensity (lux).

2.3.5 Sample preparation

Individual substrates and harvested pileus samples were oven-dried at 50 °C for 8 h using the protocol described by Hayati et al. (2016) with some modifications. When the oven drying process was terminated, the final moisture content achieved was 8%.

2.3.6 Extraction

Dried samples were milled and 0.5 g of the material was extracted using 25 mL of deionised water. The suspension was placed at 26 °C and allowed to stand for 1 h in the dark. To obtain an aqueous extract, a Whatman No. 1 filter paper was used to filter the suspension. Thereafter, the suspension was poured into 50 mL centrifuge tubes and centrifuged at 10 000 rpm for 15 min at 4 °C. Glass wool was used to filter the samples, and the extracts were stored in specimen bottles at 4 °C without further treatment until used for analyses.

2.4 Treatments description

2.4.1 Experiment one: Substrates biochemical compound concentration

2.4.1.1 pH

The pH levels of all three individual substrates were determined as described by Jones Jr (1999). The substrates were dried and milled into smaller homogenous sizes. A 100 mL graduated cylinder was used to measure 50 mL of deionised water and poured into a 100 mL glass beaker. A mass of 5 g of each substrate was weighed and added into a glass beaker. The suspension was left undisturbed for 1 h. To obtain a homogenous mixture, a 20 mm magnetic stirrer bar was used for 30 min. A buffer solution of pH 4 and 7 was used to calibrate the pH meter. Each treatment was replicated three times and measured for pH level using a benchtop pH meter.

2.4.1.2 Evaluation of essential elements on substrates

Prior to the establishment of the experiment, all substrates were evaluated for essential elements zinc (Zn), iron (Fe), selenium (Se), manganese (Mn), magnesium (Mg), sodium (Na), calcium (Ca), aluminium (Al), potassium (K) and phosphorus (P) concentrations. The element concentrations were expressed in milligrams per kilogram of dry matter (mg/kg DM). The dry ashing procedure was used to analyse the essential elements from each substrate the procedure by Agrilasa (1998). Approximately 1 g of the milled substrate was placed into a crucible and further dried for 24 h by placing the crucibles with dry substrate into an ashing oven at 600 °C for 8 h. The inductively coupled plasma (ICP) 4:1 plant extract procedure was used, 8 mL Nitric acid (HNO_3) and 2 mL hydrochloric acid (HCI) were used as reagents. The suspension

was incubated in a microwave reaction system for 45 min. Samples were transferred into volumetric flasks (100 mL) and topped up with deionised water and left standing for 24 h. Samples were carefully transferred to McCartney bottles without disturbing the sediment and an ICP mass spectrometer was used.

2.4.1.3 Heavy metals analyses on substrates

Each sample was prepared and analysed for heavy metals using the same procedure for essential elements analysis (Agrilasa, 1998). The protocol described by (Stihi et al., 2011) was used to quantify the heavy metals content. The heavy metal concentrations of nickel (Ni), copper (Cu), cobalt (Co), cadmium (Cd), chromium (Cr), palladium (Pd), lead (Pb), mercury (Hg), silver (Ag) and arsenic (As) from the substrates were analysed and expressed in mg/kg DM.

2.4.2 Experiment two: Assessment of mycelial growth, pinning, pileus development, and pileus maturity rate on treatment combinations

The growth and development on different substrates were evaluated daily (from the first day of inoculation to the last day of harvest) by assessing mycelial growth, pinning, and pileus development rate. Mycelial development data was assessed until full primordia initiation from all treatment combinations for both species was achieved. The mycelial growth cover was closely monitored, measured, and recorded at two-day intervals on each of the 1 kg transparent plastic bags. All data collected were expressed in percentages based on visible growth cover. Pinning initiation was assessed, measured, recorded at two-day intervals, and expressed in percentages. The pileus development rate was assessed from all treatment combinations on both species until maturity.

2.4.3 Experiment three: Measurement of pileus diameter, stipe size, total pileus, weight, yield, and biological efficiency

The freshly harvested pileus from each respective treatment combination was assessed by measuring the following parameters: diameter, thickness, and stipe length using a 150 mm digital Vernier caliper and expressed in mm. The number of pileus accumulated was counted. Samples were gravimetrically determined for moisture content based on the weight. The moisture content was calculated as the water percentage removed from the initial sample weight using Eq. 1 described by Doymaz (2014); Tran et al. (2020).

$$M = \frac{(W_0 - W) - W_1}{W_1} \qquad 1$$

Where *M* is the moisture content (kg water/kg dry matter), W_0 is the initial weight of the sample (kg), *W* is the amount of water evaporated moisture (kg) and W_1 is the dry matter content of the sample (kg).

Also, the total biomass accumulated from all treatment combinations was measured by weighing harvested pileus using an electronic balance scale. The total biomass was expressed in grams (g). The recorded harvested total biomass was used to calculate the biological efficiency (BE) using Eq. 2 described by Meng et al. (2019).

$$BE = \frac{total\ weight\ fresh\ mushroom\ (g)}{total\ dry\ weight\ of\ substrate\ (g)}\ \text{X}\ 100 \qquad 2$$

2.4.4 Experiment four: Quantification of pileus biochemical composition

2.4.4.1 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

Samples were spectrophotometrically determined for DPPH free radical-scavenging activity as described by Wong et al. (2006), with minor modifications. Briefly, a 0.1 mM solution of DPPH in methanol was prepared and an aliquot of 40 µL was added to 3 mL of methanolic DPPH solution, thereafter incubated for 30 min at 26 °C. The absorbance of the DPPH in methanol was measured at 515 nm. The DPPH radical-scavenging capacity (%) of the samples was calculated using Eq. 3 described by Fang et al. (2018).

$$DPPH\ Value\ (\%) = \frac{\Delta A_{515}\ of\ control - \Delta A_{515}\ of\ treatment}{\Delta A_{515}\ of\ control}\ \text{X}\ 100 \qquad 3$$

2.4.4.2 Total phenolics

The total phenolic content was spectrophotometrically determined using the Folin-Ciocalteu procedure as described by Siangu et al. (2019), with some modifications. An aliquot extract of 1 mL was mixed with 5 mL of deionised water and 1 mL of Folin-Ciocalteu's reagent, and incubated at 26 °C for 5 min. A 10 mL solution of 7% (v/v) Na_2HCO_3 was added and allowed to stand at 26 °C for 2 h. All extracts were replicated three times and the absorbance was read at 725 nm. The readings were expressed in milligrams of gallic acid equivalents per gram (mg GAE/g).

2.4.4.3 Total flavonoids

Total flavonoids were determined by a colorimetric method as described by Marnewick et al. (2011). Briefly, a 600 µL sample/standard was mixed with 2% (v/v) of $AlCl_3$ in ethanol. Subsequently, the solution was thoroughly mixed and incubated for 1 h at 25 °C. The change

of colour on samples was measured at 420 nm using a spectrophotometer. Samples were replicated three times, analysed, and expressed as milligrams of quercetin equivalents per gram (mg QE/g).

2.4.4.4 Total protein

Protein content was determined using the procedure described by Bradford (1976). Bradford dye reagent of 900 µL was added to each of the 100 µL fungal extract samples and vortexed. The samples were left without disturbing for 5 min before reading the absorbance at 595 nm using a spectrophotometer. Samples were replicated three times and readings were expressed in micrograms per gram (µg/g).

2.5 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using Genstat statistical software (Genstat® 20th edition, VSN International, Hemel Hempstead, UK). Means were separated according to Duncan's multiple range test at 5% level of significance.

3 Results and Discussion

3.1 Substrates pH analysis

The results indicated that there was a statistical difference ($p < 0.05$) in the pH levels of the different substrates (Figure 6.3). Substrate SB had a higher (6.15±0.98) pH value compared to BG (5.52±0.64), BSSBBG (5.46±0.82), and BS (5.25±1.22). Based on these results, the pH levels of all substrates used in this study were within the optimal pH range (5-6) for the development of *Ganoderma* (Chen et al., 2017; Nguyen et al., 2023). Therefore, the deduction was that substrate pH did not influence the production aspects of both *Ganoderma* spp.

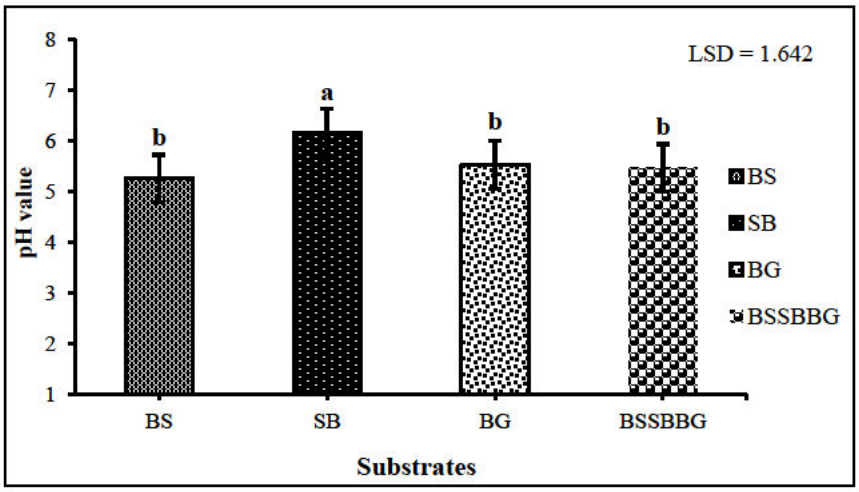


Figure 6.3. Graphical presentation of pH levels for different substrates. * BS – beech sawdust, SB – sugarcane bagasse, BG – buffalo grass, and BSSBBG – suspension of all substrates. The

results were expressed as mean ± SE, $n = 3$. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.2 Essential elements on substrates

The results showed that there was a statistically significant difference ($p < 0.05$) in the essential elements content of different substrates (Table 6.1). SB had higher levels of Mg, K, and P compared to all substrates, reaching 935.62±4.17, 3520.29±57.00, and 5529.81±24.38 mg/kg DM, respectively. Essential elements Se, Mn, Na, and Ca were also notably high on SB reading at 1.30±0.10, 9.89±0.11, 95.48±1.53, and 46.11±1.14 mg/kg DM, respectively. Fe was observed to be higher on BG recording at 43.31±1.36 followed by SB at 26.56±1.17, BSSBBG at 22.62±0.95 and BS at 11.32±0.96 mg/kg DM. Amongst the essential elements determined, BS had high levels of Al compared to SB, BSSBBG, and BG, reading at 5.51±0.34, 3.29±0.00, 3.04±0.03 and 2.34±0.10 mg/kg DM, respectively. Even though there was a variation in essential element levels in all four substrates, SB and BG were not too distinct in the majority of element levels. The higher levels could be that both substrates are inherently biological to have higher capacity that sequestrate these elements than others (Negrão et al., 2021). Therefore, their high levels can be attributed to their genotype which contains higher concentrations of essential elements. Hence, these two substrates are commonly used for commercial mushroom production.

3.3 Heavy metals on substrates

Results obtained for the heavy metals content of four substrates are presented in Table 6.2. Heavy metal levels significantly differed ($p < 0.05$) between the substrates. The levels of Ni, Cu, Cd, Pd, Pb, Hg, and Ag were higher in SB, reading at 1.94±0.01, 9.32±1.45, 33.47±4.76, 0.15±0.00, 5.79±0.54, 1.34±0.10 and 0.025±0.00 mg/kg DM, respectively. The high concentration of heavy metals in SB could be linked to various factors such as the soil conditions (Miner et al., 2018), cultivation practices (Nguyen et al., 2023), pH (Kapoor and Sharma, 2014), temperature (Badalyan et al., 2019), and industrial processes involved in its production (Lakshmi, 2013). pH and temperature play a significant role in the adsorbent capacity of heavy metals in sugarcane bagasse (Iwuozor et al., 2022). Notably, Cd and Cr were the most abundant heavy metals in all substrates. In addition, BG had higher levels of Co at 4.19±0.55 and As at 1.044±0.03 mg/kg DM compared to all substrates. Heavy metal contamination can occur in most crops especially if it is grown in areas with high levels of heavy metal pollution or become into contact with contaminated materials during processing.

Table 6.1 Essential element levels (mg/kg DM) of beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG), and suspension of all substrates (BSSBBG) for the production of *G. austroafricanum* and *G. resinaceum*.

Substrates	Zn	Fe	Se	Mn	Mg	Na	Ca	Al	K	P
BS	17.56±2.13^{c}	11.32±0.96^{c}	0.35±0.05^{c}	5.04±0.99^{b}	541.03±0.58^{d}	64.22±1.25^{d}	16.12±0.99^{d}	5.51±0.34^{a}	1523.37±18.48^{c}	1318.35±52.36^{d}
SB	28.93±1.97^{a}	26.56±1.17^{b}	1.30±0.10^{a}	9.89±0.11^{a}	935.62±4.17^{a}	95.48±1.53^{a}	46.11±1.14^{a}	3.29±0.00^{b}	3520.29±57.00^{a}	5529.81±24.38^{a}
BG	22.15±1.40^{b}	43.31±1.36^{a}	0.80±0.09^{b}	8.74±0.37^{a}	729.14±11.53^{b}	82.78±2.23^{b}	35.33±1.98^{b}	2.34±0.10^{c}	2808.68±115.60^{b}	3666.11±112.61^{b}
BSSBBG	20.37±1.62^{b}	31.62±0.95^{b}	0.61±0.07^{b}	6.93±0.45^{b}	663.02±7.13^{c}	75.35±1.89^{c}	28.76±2.04^{c}	3.04±0.03^{b}	2674.68±101.17^{b}	2851.28±89.45^{c}

The results were expressed as mean ± SD, $n = 3$. Means followed by the same letter within columns are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

Table 6.2. Heavy metal levels (mg/kg DM) of beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG), and suspension of all substrates (BSSBBG) used for the production of *G. austroafricanum* and *G. resinaceum*.

Substrates	Ni	Cu	Co	Cd	Cr	Pd	Pb	Hg	Ag	As
BS	0.40±0.03^{b}	4.98±0.19^{b}	0.94±0.05^{c}	27.13±0.52^{b}	10.81±1.18^{b}	0.03±0.00^{c}	0.50±0.10^{c}	0.32±0.10^{b}	0.014±0.00^{b}	0.032±0.01^{c}
SB	1.94±0.01^{a}	9.32±1.45^{a}	1.49±0.15^{b}	33.47±4.76^{a}	8.04±0.01^{c}	0.15±0.00^{a}	5.79±0.54^{a}	1.34±0.10^{a}	0.025±0.00^{a}	0.219±0.00^{b}
BG	0.27±0.05^{c}	2.90±0.59^{c}	4.19±0.55^{a}	15.89±1.74^{d}	14.62±0.60^{a}	0.06±0.02^{b}	2.45±0.02^{b}	0.29±0.00^{b}	0.014±0.00^{b}	1.044±0.03^{a}
BSSBBG	0.32±0.01^{bc}	3.33±0.12^{bc}	1.19±0.23^{bc}	20.77±0.81^{c}	9.34±0.22^{b}	0.05±0.01^{b}	2.20±0.03^{b}	0.25±0.03^{b}	0.013±0.00^{b}	0.030±0.02^{c}

The results were expressed as mean ± SD, $n = 3$. Means followed by the same letter within columns are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.4 Effect of different substrates on mycelial growth, pinning, pileus development, and pileus maturity rate of *G. austroafricanum and G. resinaceum*

3.4.1 Mycelial growth rate

The results on the mycelial growth rate of two *Ganoderma* spp. growing on four substrates assessed for 40 days after inoculation are shown in Figures 6.4 (a & b). There was a statistically significant difference ($p \leq 0.001$) in mycelial growth between different treatments. The highest mycelial growth rate reaching 100% on days 24, 28, 30, 32, 34, 36, and 40 after inoculation was observed from treatment combination GRBS, GABS, GRSB, GRBSSBBG, GASB, GRBG, GABSSBBG and GABG, respectively. Even BS had the lowest essential elements composition compared to BS, BSSBBG, and BG (Table 6.1), however, it had an expeditious mycelial growth rate on both species (Figures 6.4a & 4b). The rapid mycelial growth rate observed in BS could be linked to its physical characteristics known to be favourable and crucial for mycelial growth for *Ganoderma* spp. (Loyd et al., 2018a). The physiological features of a woody plant material play a significant role in the success of mycelial growth and degradation ability of *Ganoderma* spp. (Cortina-Escribano et al., 2020). Therefore, the type of substrate used for *Ganoderma* cultivation growth may influence mycelial development. In addition, among the two species, GR was observed to have a prompt mycelial growth rate in all substrates compared to GA. Furthermore, all treatment combinations under GR managed to reach 100% mycelial growth rate on day 36 as opposed to GA reaching 100% on day 40. This could be because different *Ganoderma* spp. can exhibit variations in mycelial growth due to the preference of substrate state. Some prefer to grow on living plant material and others on non-living material (Nussbaum et al., 2023). *Ganoderma* is a facultative fungi, it can grow as both parasitic and saprophytic macro-fungal species.

3.4.2 Pinning rate

The results on the pinning rate of two *Ganoderma* spp. growing on four substrates are presented in Figures 6.5 (a & b). There was a statistically significant difference ($p \leq 0.001$) in pinning rate between different treatments. BS was observed to have reached the highest pinning rate on both species compared to SB, BSSBBG, and BG. This could be because *Ganoderma* spp. in nature grows on woody plant materials. Sawdust from hardwood trees, such as beech and oak is often preferred due to its favourable composition for *Ganoderma* cultivation (Loyd et al., 2018a; Amiri-Sadeghan et al., 2022). Treatment combinations GRBS, GRSB, GABS, GRBSSBBG, GASB, GRBG, GABSSBBG, and GABG reached 100% pinning rate on 24, 28, 30, 32, 34, 36, and 40 days after inoculation, respectively. A 100% pinning rate was achieved

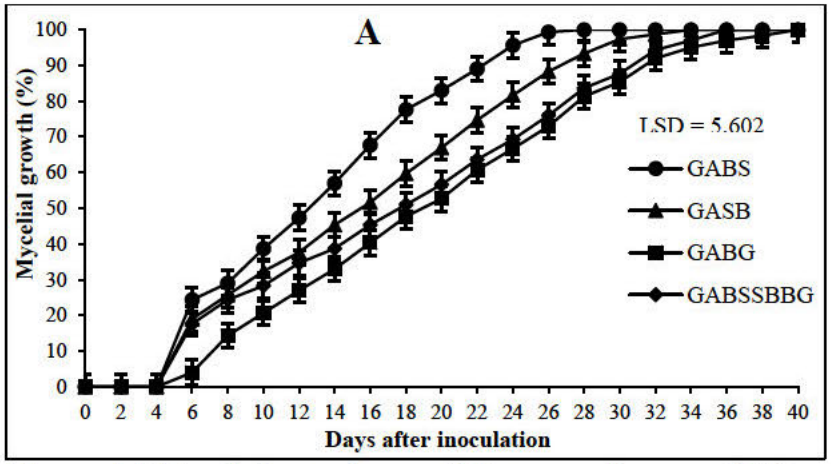
on all treatment combinations under GR on day 36 whereas on GA it was observed on day 40 after inoculation. The overall results of the current study on the effect of substrates on pinning rate followed a similar trend as the mycelial growth rate findings.

3.4.3 Pileus development rate

The result in pileus development had significant differences ($p \leq 0.001$). The pileus development rate of two *Ganoderma* spp. growing on different substrates is shown in Figures 6.6 (a & b). Treatment GRBS reached a 100% pileus development rate in 26 days and GRSB in 30 days after inoculation. On day 32, GRBS and GRBSSBBG all achieved a 100% pileus development rate. Furthermore, GRBG, GASB, GRBSSBBG, and GABG were observed at 100% pileus development rate in 34, 36, 38, and 40 days after inoculation, respectively. The variation in pileus development rate between treatment combinations can be attributed to the differences in substrate composition and physical conditions that can impact the rate at which the pileus develops. Different types of substrates may provide more favourable conditions and nutrients resulting in a faster pileus development rate of the *Ganoderma* (Atila, 2020).

3.4.4 Pileus maturity rate

The pileus maturity rate for *Ganoderma* spp significantly differed ($p \leq 0.001$) among the different substrates. The pileus maturity rate reached 100% in GRBS on 28 days and GRSB on 32 days after inoculation (Figures 6.7a & 6.7b). Treatment GABS and GRBSSBBG obtained 100% pileus maturity on day 34 whilst GRBG and GASB were on day 38 after inoculation. Notably, GABSSBBG and GABG attained full pileus maturity on days 40 and 44 after inoculation, respectively. The results of the pileus maturity rate followed the overall findings on growth and development where GRBS reached the maximum on all growth phases faster compared to other treatments. In addition, the use of BS substrate provided high pileus rates on both species. These results were consistent with the findings of Azizi et al. (2012); Cortina-Escribano et al. (2020) where BS promoted the mycelial growth rate subsequently increasing yield, pileus maturity rate, total biomass and biological efficiency. These findings demonstrate that BS stimulate the development of *Ganoderma* species.



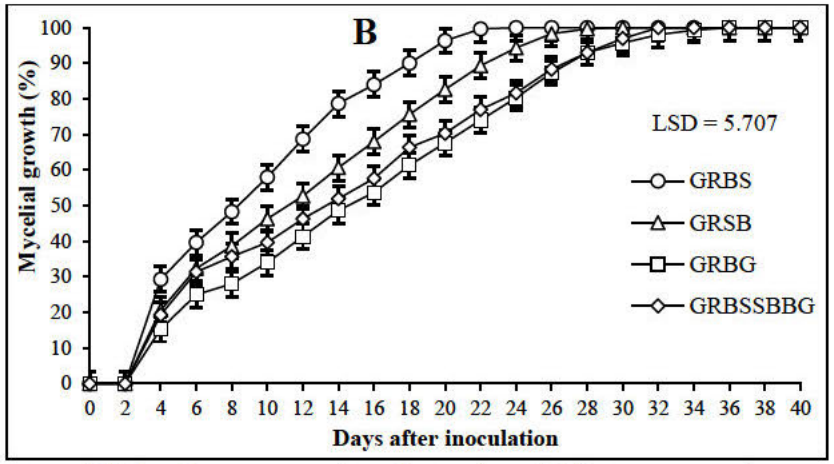
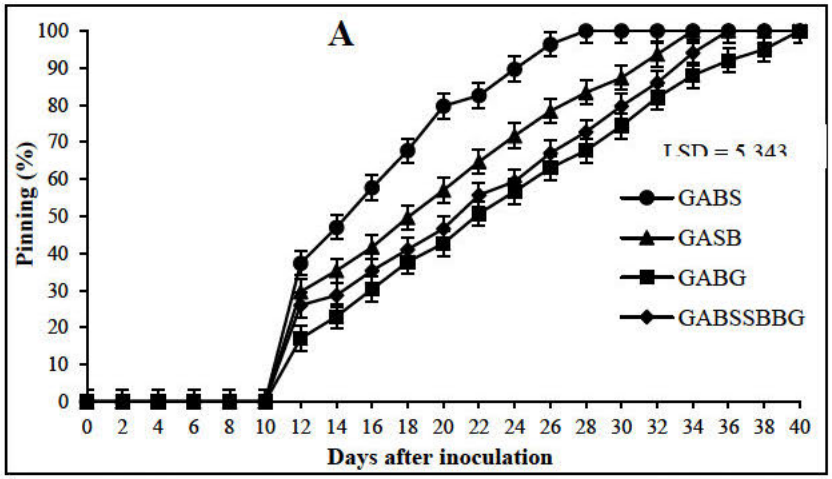


Figure 6.4 Mycelial growth rate of *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** grown on beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG), and suspension of all substrates (BSSBBG) assessed over 40 days after inoculation. Values were expressed as mean ± SE, $n = 3$. Means differences were according to Duncan's multiple range test ($p \geq 0.05$).



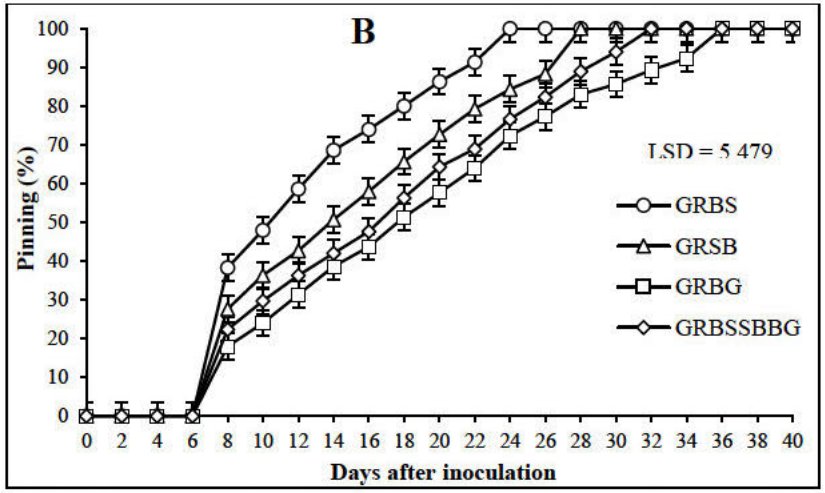


Figure 6.5 The pinning rate of *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** grown on beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG), and suspension of all substrates (BSSBBG) assessed over 40 days after inoculation. Values were expressed as mean ± SE, $n = 3$. Means differences were according to Duncan's multiple range test ($p \geq 0.05$).

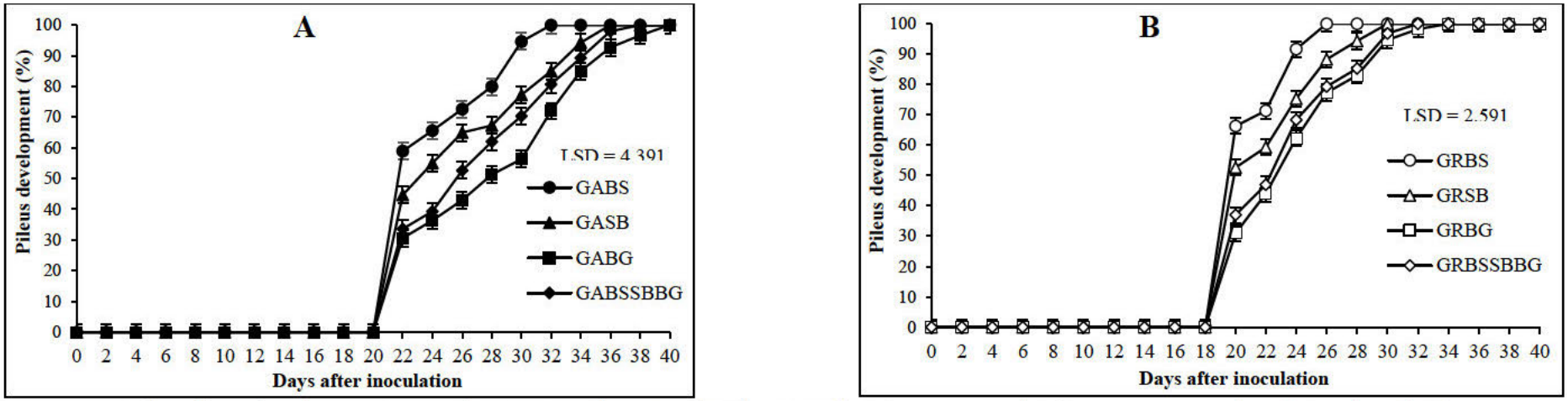


Figure 6.6 The pileus development rate of *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** grown on beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG), and suspension of all substrates (BSSBBG) assessed over 40 days after inoculation. Values were expressed as mean ± SE, $n = 3$. Means differences were according to Duncan's multiple range test ($p \geq 0.05$).

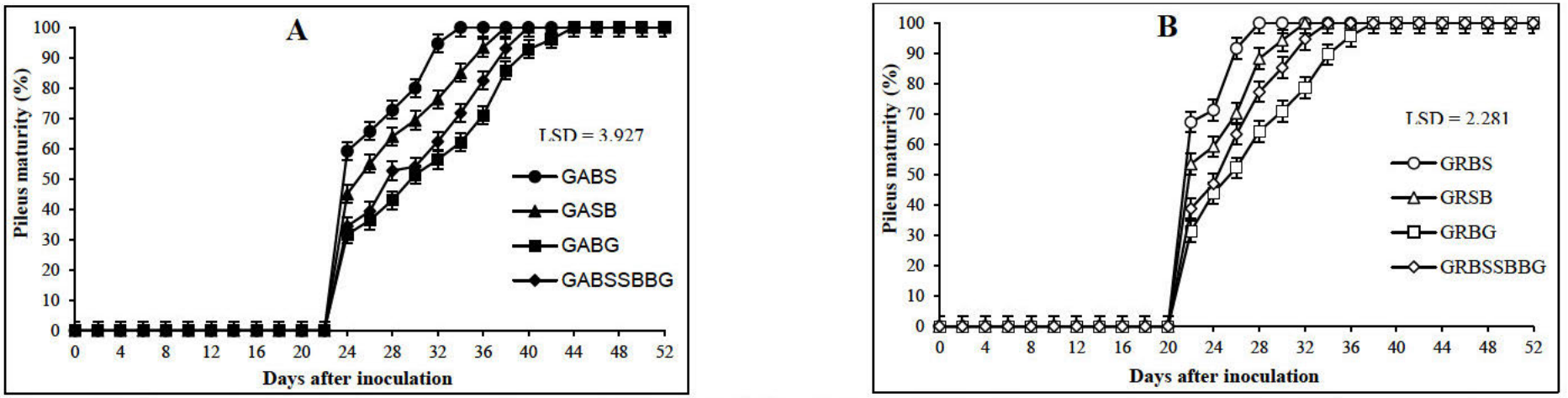


Figure 6.7 Pileus maturity rate of *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** grown on beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG), and suspension of all substrates (BSSBBG) assessed over 52 days after inoculation. Values were expressed as mean ± SE, $n = 3$. Means differences were according to Duncan's multiple range test ($p \geq 0.05$).

3.5 Effect of substrate on the pileus diameter, stipe size, total pileus, weight, total yield, and biological efficiency of *G. austroafricanum and G. resinaceum*

Results on the effect of different substrates on the pileus diameter, thickness, stipe length, total pileus, weight, total yield, and biological efficiency from treatment combinations of *Ganoderma* spp. obtained in 123 days are presented in Table 6.3. Significant differences ($p < 0.05$) were observed in variables from treatment combinations. The fresh pileus diameter, thickness, and stipe length from treatment combinations ranged from 17.26±0.62 to 59.77±1.15, 8.44±1.17 to 23.57±0.96, and 11.01±2.38 to 58.49±1.04 mm, respectively. While the fresh total number of pileus, weight, total yield, and biological efficiency ranged from 17.98±0.38 to 36.67±0.51, 1.04±0.16 to 2.23±1.08 g/kg, 18.70±2.74 to 81.77±1.84 g/kg, and 1.87±0.35 to 9.09±0.82%, respectively. Among the different substrates tested, treatment combinations with SB performed better in all variables from both species followed by BS, BSSBBG, and BG. The variation between substrates could have been that different substrates offer varying chemical compositions. The substrate's chemical composition can be sequestered and used by the mushrooms during their growth phases, subsequently determining the size of the mushroom harvested, yield, and biological efficiency (Bellettini et al., 2019; Siwulski et al., 2019; Atila, 2020). In addition, GASB exhibited higher variable quantities compared to other treatment combinations. Even though there were significant differences observed in treatment combinations, the pileus from both species did not present any type of deformations or abnormal appearance in all substrates. The variation in treatment combinations could have been the substrate composition and genotype. Substrate composition and mushroom genotype might influence the biological efficiency of mushrooms (Jin et al., 2018). Treatment combination GRBS had the highest mycelial growth, pinning, pileus development, and pileus maturity rate, however, this did not translate to a high total yield and biological efficiency. Therefore, the findings of this study can be used to deduce that total yield and biological efficiency depend on the type of species and substrate used.

Table 6.3 The pileus diameter, thickness, stipe length, total pileus, weight, total yield, and biological efficiency of *G. austroafricanum* and *G. resinaceum* measured in 123 days after inoculation.

Treatments	Size of pileus						Number of pileus	Pileus weight (g/kg)		Total mushroom yield (g/kg)		Biological efficiency (%)
	Diameter (mm)		Thickness (mm)		Stipe length (mm)							
	Fresh	Dry	Fresh	Dry	Fresh	Dry		Fresh	Dry	Fresh	Dry	
GABS	58.46±2.72^{a}	53.18±1.84^{a}	20.26±1.90^{ab}	18.36±1.16^{ab}	53.91±1.37^{b}	48.53±1.74^{b}	27.33±0.22^{d}	2.08±0.95^{a}	1.87±0.10^{ab}	56.85±2.19^{d}	51.17±1.22^{c}	6.32±1.11^{bc}
GASB	59.77±1.15^{a}	55.20±0.08^{a}	23.57±0.96^{a}	21.37±0.68^{a}	58.49±1.04^{a}	53.28±0.88^{a}	36.67±0.51^{b}	2.23±1.08^{a}	2.01±0.00^{a}	81.77±1.84^{a}	73.59±0.87^{a}	9.09±0.82^{a}
GABG	42.44±2.12^{c}	39.05±2.12^{c}	13.47±0.93^{d}	12.13±0.76^{bc}	35.57±1.31^{d}	32.19±1.59^{d}	22.63±0.41^{e}	1.52±0.15^{b}	1.37±0.27^{b}	34.39±1.72^{g}	30.95±0.78^{e}	3.82±1.12^{d}
GABSSBBG	51.77±2.37^{b}	46.49±0.54^{b}	17.82±1.50^{c}	15.26±0.25^{b}	43.73±1.80^{c}	39.59±2.06^{c}	21.75±0.43^{e}	1.87±0.06^{ab}	1.98±0.53^{ab}	40.67±1.34^{f}	36.60±0.62^{f}	4.52±1.05^{cd}
GA Total Average	**53.11±2.09**	**48.48±1.15**	**18.78±1.32**	**16.78±0.71**	**47.93±1.38**	**43.40±1.57**	**27.10±0.39**	**1.93±0.56**	**1.81±0.23**	**53.42±1.77**	**48.08±0.87**	**5.94±1.03**
GRBS	32.14±1.29^{d}	29.68±0.15^{d}	11.36±1.26^{e}	10.90±1.62^{c}	16.77±2.11^{g}	15.27±0.67^{g}	32.24±0.53^{c}	1.89±0.03^{ab}	1.70±0.26^{ab}	60.93±0.46^{c}	54.84±1.16^{bc}	6.77±.0.44^{bc}
GRSB	41.31±1.77^{c}	37.83±0.94^{c}	19.98±0.58^{b}	15.43±0.77^{b}	25.67±1.13^{e}	23.09±2.02^{e}	41.39±0.73^{a}	1.63±0.49^{b}	1.47±0.44^{b}	67.47±0.22^{b}	60.72±0.38^{b}	7.50±1.34^{b}
GRBG	17.26±0.62^{f}	15.22±0.99^{f}	8.44±1.17^{f}	7.22±2.75^{d}	11.01±2.38^{h}	10.42±0.92^{h}	17.98±1.38^{f}	1.04±0.16^{c}	0.95±0.07^{c}	18.70±2.74^{h}	16.83±1.31^{g}	1.87±0.35^{e}
GRBSSBBG	23.53±0.69^{e}	21.42±1.28^{e}	9.89±1.01^{ef}	8.61±0.96^{cd}	20.97±0.99^{f}	18.71±1.10^{f}	26.49±0.87^{d}	1.74±0.10^{ab}	1.56±0.31^{b}	46.09±0.31^{e}	41.48±1.28^{d}	5.12±1.01^{c}
GR Total Average	**28.56±1.09**	**26.04±0.84**	**12.42±1.01**	**10.54±1.51**	**18.61±1.65**	**16.87±1.18**	**29.51±0.89**	**1.58±0.20**	**1.42±0.27**	**48.30±0.93**	**43.47±1.03**	**5.32±079**

The results are presented as means of pileus diameter, thickness, stipe length, and the number of pileus from each treatment combination from week six to seventeen of harvest. Only the largest pileus on each treatment was the focal point. Values were expressed as mean ± SD, $n = 3$. Means followed by the same letter within columns are not significantly different according to Duncan's multiple range test ($p \geq 0.05$). **Keys**: GA – *Ganoderma austroafricanum*; GR – *Ganoderma resinaceum*; BS – Beech sawdust; SB – Sugarcane bagasse BG – Buffalo grass; BSSBBG – suspension of all substrates.

3.6 Effect of different substrates on the biochemical composition of *G. austroafricanum and G. resinaceum*

3.6.1 Total essential elements content on pileus

Different substrates had a statistically significant ($p < 0.05$) effect on essential elements in the harvested pileus from *Ganoderma* spp. (Table 6.4). SB exhibited higher levels of essential elements compared to BSSBBG, BG, and BS in both species. Substrates provide varying amounts of biochemical composition that can be absorbed by the pileus during growth (Ab Rhaman et al., 2021). GASB was observed to have higher concentrations of Zn, Se, Mn, Mg, Na, Ca, K, and P at 75.69±0.39, 1.37±0.07, 8.44±0.25, 549.61±15.87, 158.37±0.62, 1221.55±34.30, 7142.30±150.74 and 8705.01±164.17 mg/kg DM, respectively. The high levels of essential elements in both species from treatment combinations with SB could be linked to the ability of pileus to extract its nutrients from the substrate used (Bellettini et al., 2019). These nutrients are then utilised by the mushrooms to synthesise their chemical compounds (Rathore et al., 2017). Following GASB, elements Zn, Fe, Se, Mg, Na, Ca, Al, K, and P were also notably high on GABSSBBG, reaching 71.98±0.66, 35.74±0.47, 1.31±0.03, 512.07±2.64, 148.75±1.15, 1145.07±26.15, 2.08±0.00, 7048.49±131.36, 8645.78±117.74 mg/kg DM, respectively. However, GRBS recorded lower concentrations of Zn, Fe, Se, Mn, Mg, Na, Ca, K, and P at 45.30±0.90, 18.25±1.28, 0.68±0.06, 4.53±0.35, 367.11±13.24, 110.12±3.00, 879.77±10.41, 5450.97±180.59, and 7053.30±114.99 mg/kg DM, respectively. Even though the essential element levels had variations between treatment combinations, P and K were the two most abundant elements found in all treatments. Other than substrate, factors such as species, cultivation techniques, environmental conditions, and post-harvest handling can also have an impact on pileus chemical composition (Wagner et al., 2003; Hoa and Wang, 2015; Sadiq et al., 2021). Therefore, the presence of specific minerals in the substrate, production conditions, and post-harvest techniques can affect the mineral composition of the pileus.

3.6.2 Heavy metals composition on pileus

Different substrates had a statistically significant effect ($p < 0.05$) on essential elements in the harvested pileus from treatment combinations. Among all detected heavy metals, Cu and Cr were present in the largest amounts in all treatments (Table 6.5). SB had higher levels of heavy metals in the majority of treatment combinations compared to BSSBBG, BG, and BS. The higher levels of heavy metals observed on pileus harvested from SB could have been the bioaccumulation of these metals that occurred during the growth of the substrate (Stihi et al.,

2011). Heavy metals are naturally occurring elements that can be present in the environment, including in the substrate on which mushrooms grow (Isildak et al., 2007). Therefore, mushrooms cultivated on substrates that contain heavy metals have a high probability for these metals to be sequestered by the mycelium and subsequently accumulate in the pileus. In addition, higher levels of Ni, Cu, Cd, Pd, Pb, and Ag were observed on GASB, reaching 1.02±0.02, 13.51±0.75, 2.27±0.65, 2.50±0.36, 0.24±0.03, 4.22±0.84, and 0.050±0.01 mg/kg DM, respectively. Furthermore, Co, Cr, and As were notably higher on GABG reading at 3.43±0.73, 14.74±0.58, and 0.756±0.04 mg/kg DM, respectively. Even though heavy metals were detected in both species (Table 5), the overall finding indicated that both species were able to successfully execute the mycorestoration or mycoremediation process considering the substrates in Table 2. *Ganoderma* can be cultivated on substrates that require remediation, such as contaminated soil or waste materials. The mycelia of *Ganoderma* spp. can colonise and break down these substrates, potentially reducing the levels of pollutants or contaminants present (Ipeaiyeda et al., 2020; Shourie and Vijayalakshmi, 2022).

Table 5.4 Essential element levels (mg/kg DM) of *G. austroafricanum* (GA) and *G. resinaceum* (GR) harvested from beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG) and suspension of all substrates (BSSBBG).

Treatments	Zn	Fe	Se	Mn	Mg	Na	Ca	Al	K	P
GABS	62.87±0.89^{b}	25.78±0.88^{c}	1.08±0.04^{a}	5.42±0.31^{d}	467.73±20.50^{a}	138.41±0.58^{c}	988.38±10.02^{cd}	2.64±0.43^{a}	6730.57±174.49^{b}	8437.40±177.02^{a}
GASB	75.69±0.39^{a}	32.17±0.78^{b}	1.37±0.07^{a}	8.44±0.25^{a}	549.61±15.87^{a}	158.37±0.62^{a}	1221.55±34.30^{a}	1.74±0.20^{b}	7142.30±150.74^{a}	8705.01±164.17^{a}
GABG	68.56±0.52^{b}	41.75±1.67^{a}	1.23±0.01^{a}	7.48±0.33^{b}	496.74±1.52^{ab}	147.43±4.94^{b}	1070.44±17.78^{c}	1.93±0.17^{b}	6845.39±145.80^{ab}	8530.06±116.63^{a}
GABSSBBG	71.98±0.66^{a}	35.74±0.47^{ab}	1.31±0.03^{a}	6.31±0.37^{c}	512.07±2.64^{a}	148.75±1.15^{b}	1145.07±26.15^{b}	2.08±0.00^{ab}	7048.49±131.36^{a}	8645.78±117.74^{a}
GRBS	45.30±0.90^{d}	18.25±1.28^{d}	0.68±0.06^{c}	4.53±0.35^{e}	367.11±13.24^{c}	110.12±3.00^{e}	879.77±10.41^{e}	1.89±0.01^{b}	5450.97±180.59^{c}	7053.30±114.99^{b}
GRSB	62.03±0.69^{b}	22.60±1.30^{cd}	0.93±0.02^{b}	7.40±0.39^{b}	477.11±19.06^{b}	145.19±1.22^{b}	977.68±15.17^{d}	0.94±0.03^{c}	6734.09±140.28^{b}	8147.16±145.73^{a}
GRBG	49.65±1.05^{cd}	29.31±0.68^{bc}	0.84±0.05^{b}	6.61±0.25^{c}	378.97±8.14^{c}	125.95±2.10^{d}	905.89±14.04^{de}	1.04±0.03^{bc}	6054.03±162.63^{b}	7429.93±139.71^{b}
GRBSSBBG	56.34±1.24^{c}	25.80±0.81^{c}	0.91±0.01^{b}	5.40±0.30^{d}	411.35±14.33^{b}	136.36±1.14^{c}	937.74±13.07^{d}	1.50±0.06^{b}	6393.03±171.10^{b}	7709.32±143.70^{b}

The results were expressed as mean ± SD, $n = 3$. Means followed by the same letter within columns are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

Table 6.5 Heavy metal levels (mg/kg DM) of *G. austroafricanum* (GA) and *G. resinaceum* (GR) harvested from beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG), and suspension of all substrates (BSSBBG).

Treatments	Ni	Cu	Co	Cd	Cr	Pd	Pb	Hg	Ag	As
GABS	0.49±0.03^{c}	10.08±0.66^{b}	0.91±0.08^{c}	1.21±0.06^{b}	11.07±0.99^{b}	0.15±0.01^{b}	0.88±0.06^{d}	0.27±0.05^{d}	0.032±0.01^{b}	0.295±0.02^{c}
GASB	1.02±0.02^{a}	13.51±0.75^{a}	2.27±0.65^{b}	2.50±0.36^{a}	9.28±1.01^{c}	0.24±0.03^{a}	4.22±0.84^{a}	1.05±0.04^{a}	0.050±0.01^{a}	0.392±0.03^{b}
GABG	0.40±0.00^{c}	6.78±0.84^{c}	3.43±0.73^{a}	0.68±0.15^{d}	14.74±0.58^{a}	0.07±0.01^{d}	2.77±0.58^{b}	0.29±0.03^{d}	0.018±0.00^{c}	0.756±0.04^{a}
GABSSBBG	0.64±0.10^{b}	7.74±0.91^{c}	1.52±0.06^{bc}	1.30±0.37^{b}	10.41±0.58^{bc}	0.11±0.00^{c}	1.74±1.07^{c}	0.85±0.04^{b}	0.029±0.00^{b}	0.417±0.03^{b}
GRBS	0.34±0.04^{d}	9.13±1.03^{b}	0.75±0.04^{d}	0.82±0.09^{c}	7.77±0.56^{d}	0.05±0.00^{d}	0.63±0.08^{e}	0.89±0.02^{b}	0.027±0.00^{b}	0.159±0.01^{d}
GRSB	0.90±0.06^{a}	10.60±0.81^{b}	1.50±0.20^{bc}	1.17±0.03^{b}	6.44±0.55^{de}	0.10±0.00^{c}	1.01±0.05^{cd}	1.12±0.08^{a}	0.047±0.00^{a}	0.360±0.05^{b}
GRBG	0.31±0.01^{d}	2.60±0.31^{d}	2.09±0.03^{b}	0.46±0.03^{e}	11.41±0.57^{b}	0.08±0.00^{d}	0.81±0.01^{d}	0.76±0.06^{c}	0.007±0.00^{d}	0.432±0.15^{b}
GRBSSBBG	0.43±0.02^{c}	3.65±0.28^{d}	0.98±0.04^{c}	0.86±0.01^{c}	7.35±0.44^{d}	0.09±0.01^{cd}	0.72±0.02^{de}	1.03±0.04^{a}	0.035±0.00^{b}	0.251±0.01^{cd}

The results were expressed as mean ± SD, $n = 3$. Means followed by the same letter within columns are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.6.3 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay

The DPPH free radical scavenging activity of two *Ganoderma* spp. harvested on four substrates is shown in Figures 6.8 (a & b). Different substrates had a statistically significant effect ($p \leq 0.001$) in DPPH free radical scavenging activity results. SB recorded the highest scavenging percentage in both species. Different substrates provide distinct levels of biochemical compounds to the mushroom which can influence its antioxidant activity (Otieno et al., 2022). Treatment combinations GASB followed by GRSB had the highest percentage compared to other treatments, reading at 66.94±1.04 and 60.23±0.95%, respectively. The variation in DPPH free radical scavenging activity findings from treatment combination might be interconnected with biochemical compounds of substrate and species (Ferreira et al., 2009). Even though most treatment combinations ranged between 50-60% of scavenging activity, treatment combination GRBS recorded the lowest scavenging activity at 38.76±1.33%. Based on the results presented in Table 6.1, BS had the lowest essential element compared to other substrates. Hence the DPPH radical scavenging activities of pileus were influenced by the substrate. Furthermore, the poor scavenging activity on BS could have been that beech sawdust can vary depending on factors such as the age and quality of the wood, processing methods, and storage conditions (Roy et al., 2015; Siwale et al., 2022). In addition, the specific effect of the substrate on the DPPH activity of the pileus can vary depending on the mushroom species (Radulescu et al., 2019). Even though the DPPH activity of GA has not been extensively studied or documented in scientific literature, it had the highest DPPH activity compared to GR.

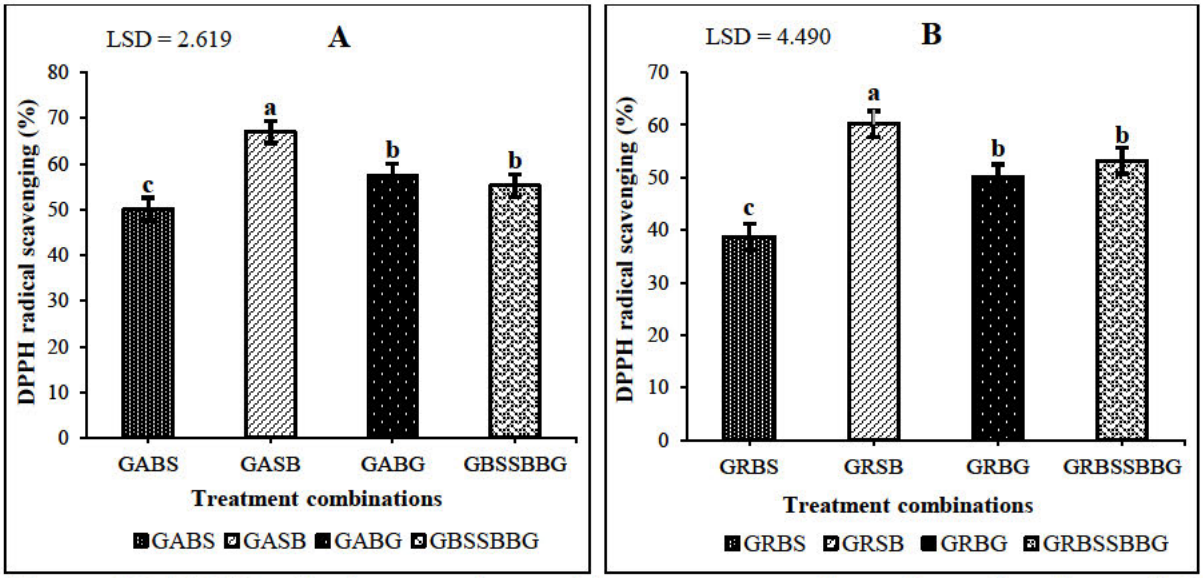


Figure 6.8 DPPH radical scavenging activity percentage release from the pileus of *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** harvested from beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG), and suspension of all substrates (BSSBBG). The

results were expressed as mean ± SE, n = 3. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.6.4 Total phenolics

The total phenolic results of two *Ganoderma* spp. extracts harvested from four different substrates are shown in Figures 6.9 (a & b). The different substrates had a significant effect ($p \leq 0.001$) on total phenolic levels. The highest phenolic content was detected on treatment combination GASB at 180.58±1.36 mg GAE/g followed by GABG at 171.72±2.14 mg GAE/g. These discrepancies could be linked to the substrate phenolic levels that may lead to a higher accumulation of phenolic compounds present in the pileus (Atila et al., 2023). The overall results indicated that GA had the highest total phenolic levels compared to GR, also, SB performed better on both species compared to other substrates. The level of phenolic compounds in *Ganoderma* spp. can be influenced by several factors, including the type of species (Obodai et al., 2017). This suggests that each species contains a unique profile of phenolic compounds, which can contribute to variations in their levels. Furthermore, variations in phenolic levels were also observed in treatment combinations GRSB, GABS, GRBG, and GABSSBBG recording at 159.90±2.54, 156.79±0.89, 148.62±1.76 and 139.89±0.97 mg GAE/g, respectively. However, the lowest phenolic levels were noted from GRBSSBBG reading at 114.69±0.51 and GRBS at 124.81±2.14 mg GAE/g. Therefore, the substrate and cultivation method on which *Ganoderma* spp. are cultivated can influence their phenolic composition.

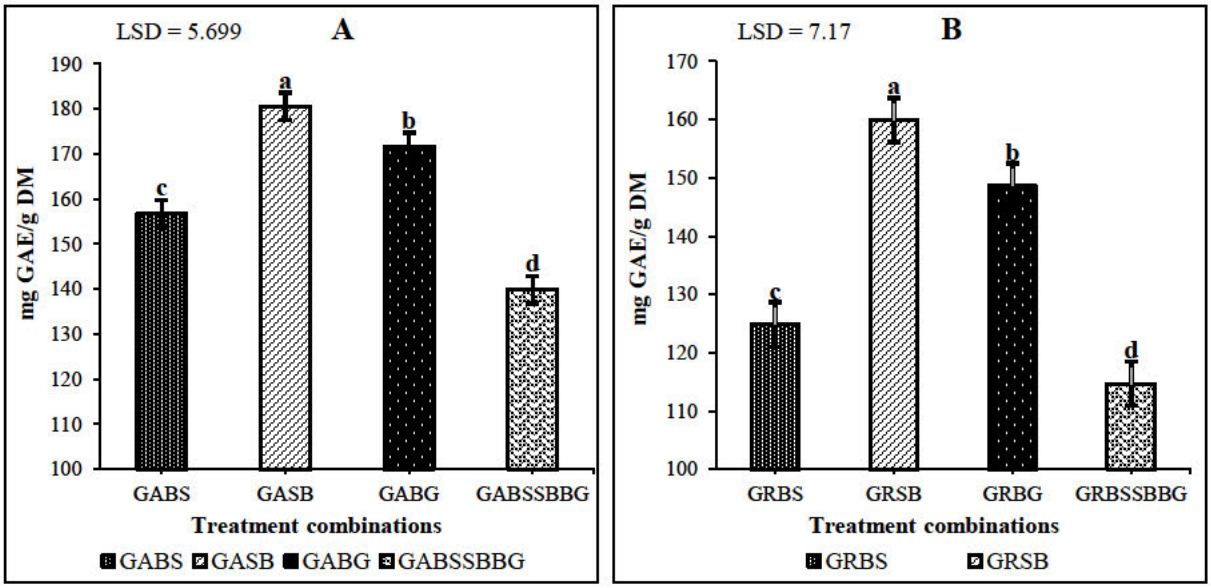


Figure 6.9. Total phenolic compounds extracted from the pileus of *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** harvested from beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG), and suspension of all substrates (BSSBBG). The results were expressed as

mean ± SE, $n = 3$. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.6.5 Total flavonoids

The flavonoid concentration levels were significantly different ($p \leq 0.001$) from *G. austroafricanum* and *G. resinaceum* extracts harvested from different substrates (Figures 6.10a & 6.10b). The results indicated that flavonoid concentration was dominant in treatment combination GASB reading at 84.43±2.31 followed by GRSB at 78.99±1.82 and GABG at 78.83±1.37 mg QE/g. Other than substrate composition, the discrepancies among treatment combinations could have been influenced by the time of maturity and harvest (Ren et al., 2020). This suggests that flavonoid levels may vary during different growth stages, and the optimal time for harvesting to obtain the highest flavonoid content may differ depending on the species. The results on pileus maturity rate exhibited GRBS reached 100% compared to other treatments on day 28 and GASB on day 38. However, the results below show abundant (84.43±2.31 mg QE/g) levels of flavonoid concentration on GASB, whilst GRBS had the lowest (53.35±1.03 mg QE/g) levels compared to all treatments. Therefore, the stage at which *Ganoderma* spp. is harvested can affect the flavonoid content. In addition, GABS had the lowest concentration followed by GRBSSBBG at 62.21±2.45 and 64.94±0.78 mg QE/g, respectively. These variations could be linked to the genetic makeup of *Ganoderma* spp. which may have played a role in determining the flavonoid content (Hamwenye, 2020). Therefore, variations in the genes responsible for flavonoid synthesis and metabolism may influence the overall flavonoid levels in different species.

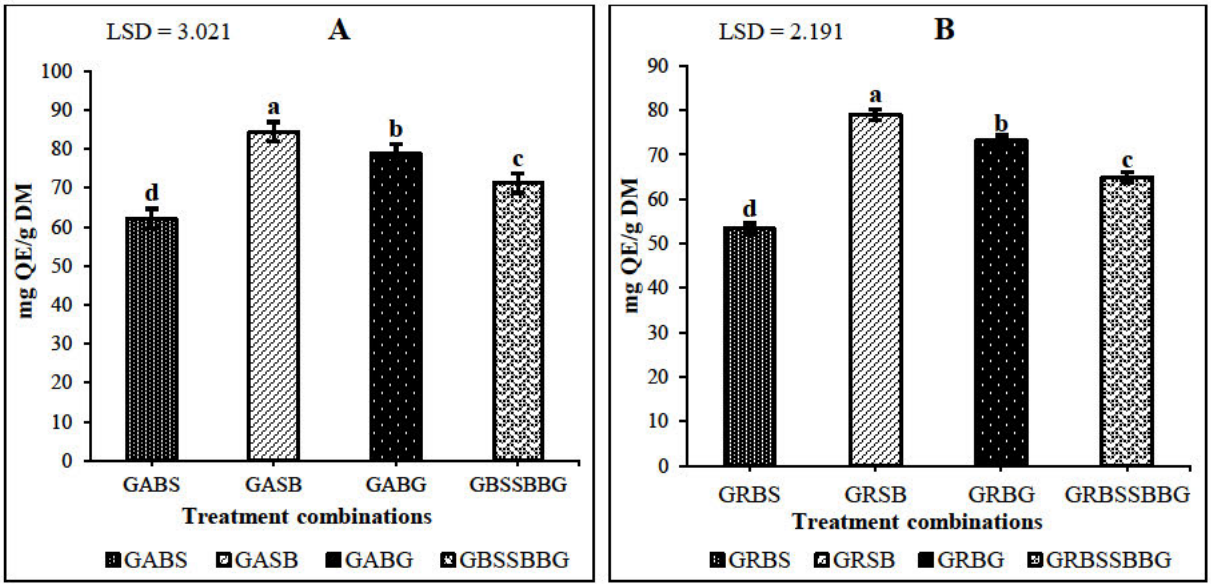


Figure 6.10 Total flavonoid content extracted from the pileus of *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** harvested from beech sawdust (BS), sugarcane bagasse (SB),

buffalo grass (BG), and suspension of all substrates (BSSBBG). The results were expressed as mean ± SE, $n = 3$. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.6.6 Total protein content

The different treatment combinations had a significant ($p < 0.05$) influence on the total protein content of the pileus (Figures 6.11a & 6.11b). GASB had a higher protein concentration at 24.01±0.54 µg/g followed by GABSSBBG at 19.30±2.12 µg/g and GABG at 18.21±1.62 µg/g. The top three highest protein levels were in GA. Typically, treatment combinations from Figure 6.11a had greater protein concentration compared to B. These findings indicate that *Ganoderma* spp. may produce different protein levels depending on the species. Some *Ganoderma* species may have higher protein content than other species (Kozarski et al., 2012). Hence, the results of the current study suggest that regardless of the treatment combinations (species x substrate), GA had greater protein concentration compared to GR.

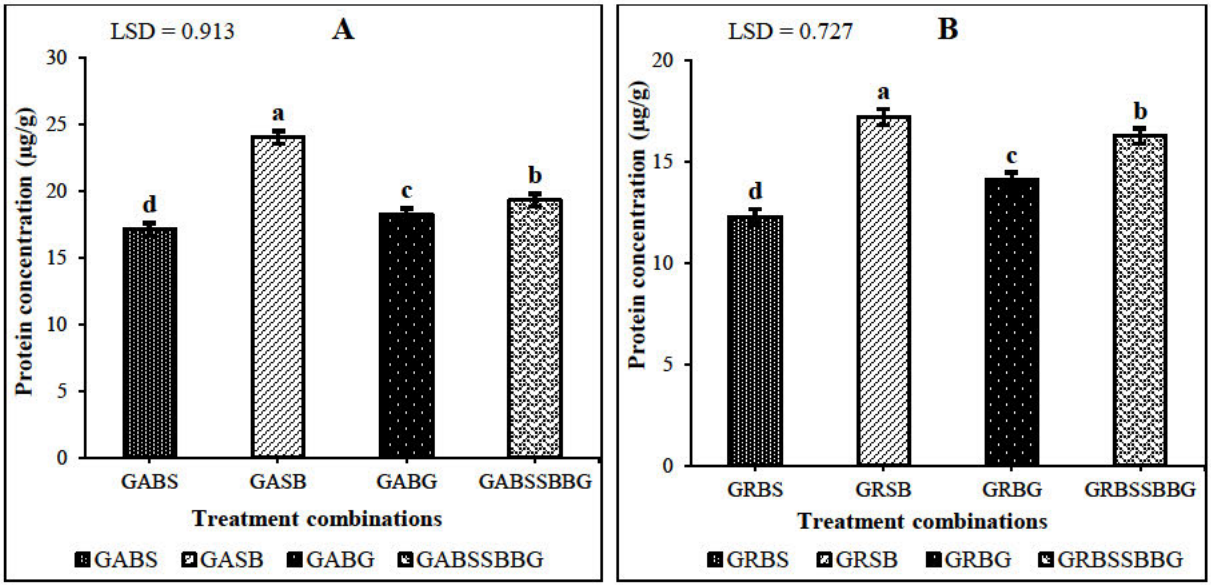


Figure 6.11 Total protein concentration extracted from the pileus of *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** harvested from beech sawdust (BS), sugarcane bagasse (SB), buffalo grass (BG), and suspension of all substrates (BSSBBG). The results were expressed as mean ± SE, $n = 3$. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

4 Conclusion

The research findings demonstrated that the choice of substrate significantly influences the development, total biomass, and biochemical profile of *Ganoderma* spp. Overall, the effect of different substrates used for the production of *Ganoderma* involves a wide range of factors, including development, biochemical compounds, physical characteristics, and yield. The study reveals that different substrates can have varying effects on the development and biochemical

composition of *Ganoderma* spp. The current study established that *G. resinaceum* grown on beech sawdust had a rapid development rate (mycelial, pinning, pileus, and pileus maturity rate) compared to *G. austroafricanum* grown on sugarcane bagasse. However, *G. austroafricanum* grown on sugarcane bagasse exhibited the highest pileus, stipe size, total pileus, weight, total biomass production, and biological efficiency. In addition, *G. austroafricanum* grown on sugarcane bagasse also had the highest concentration of biochemical compounds. Furthermore, factors such as nutrient availability, substrate composition, and physical characteristics play an important role in determining the overall performance of the fungi. Hence, understanding the significance of different substrates and their influence can assist in optimising *Ganoderma* cultivation for specific objectives such as improving quality and enhancing specific medicinal compounds. The utilisation of agricultural waste as a substrate can be a sustainable and cost-effective approach for cultivating *Ganoderma* spp. Further research is warranted to explore the underlying substrate enhancement mechanisms, additional substrate options, and optimised cultivation conditions for *Ganoderma* spp, ultimately contributing to the advancement of its medicinal applications.

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CHAPTER 7

IMPACT OF SUBSTRATE FORTIFIED WITH ESSENTIAL ELEMENTS ON TOTAL BIOMASS, AND BIOCHEMICAL COMPOUNDS OF *GANODERMA* SPP.

Abstract

This study investigated the impact of substrate fortified with essential elements on the development, total biomass, and biochemical compounds of *Ganoderma* species. The experiment involved growing *Ganoderma austroafricanum* (GA) and *Ganoderma resinaceum* (GR) on beech enhanced with elements; no element (Control), $Zn(NO_3)_2.6H_2O$ (Zn), Fe_2SO_4 $7H_2O$ (Fe), Na_2SeO_3 (Se), and suspension of all essential elements (ZnFeSe). The development and total biomass production findings for the substrate fortified with essential elements exhibited significant differences. GRZn developed expeditiously, reaching 100% in all production parameters in 52 days after inoculation. Nevertheless, this did not result in a high total yield and biological efficiency in comparison to GAFe which showed larger quantities at 85.69±0.75 g/kg and 9.52±1.23%, respectively. The substrate enhancement with Zn had a significant increase in the majority of minerals. GAZn exhibited higher concentrations of essential elements such as Zn (74.19±0.15 mg/kg DM), K (7260.63±143.42 mg/kg DM), and Mg (550.45±11.90 mg/kg DM). Higher levels of heavy metals such as Cd, Pb, and As were recorded from GAControl at 1.20±0.08, 0.88±0.03, and 0.284±0.03 mg/kg DM, respectively. The results further revealed that pileus from GASe produced higher levels of DPPH, phenolic compounds, flavonoids, and protein reading at 74.79±2.35%, 196.10±3.05 mg GAE/g, 101.66±2.07 mg QE/g and 23.41±1.21 µg/g, respectively. These findings provide evidence that the enhancement of substrates with essential elements may improve the production and biochemical attributes of *Ganoderma* species.

***Keywords*:** Fungal species, enhancement, basidiocarp, bioaccumulation, minerals, antioxidants

1 Introduction

Ganoderma is one of the most widely used herbal fungi and there is continuous interest in its biochemical properties (Rzymski et al., 2016; Jandaik and Gupta, 2022). Its mycelia, fruiting bodies, and spores contain various biochemical compounds (Ahmad, 2018; Sudheer et al., 2018; Ahmad et al., 2021). These biochemical compounds are recognised to be essential to the human body (Kalogeropoulos et al., 2013). *Ganoderma* triterpenes (GTs), proteins, steroids, polysaccharides, triterpenoids, peptides, amino acids, alkaloids, nucleotides, lactones, and unsaturated fatty acids are some of the biochemical compounds reported (Dou et al., 2014; Xia

et al., 2014; Shah and Modi, 2018). The genus is also identified to be a rich source of carbohydrates, dietary fibre, protein, various vitamins, fat, oils, coumarin-glycosides, and inorganic ions such as Zn, Fe, Ca, Mg, Cu, P, K, and Na (Patocka, 1999; Roy et al., 2015; Alamgir, 2018). This has led to an increasing number of study reports providing substantial scientific evidence of their nutritional and therapeutic value (Valverde et al., 2015; Shaito et al., 2020; Tran et al., 2022).

These species have the potential to be developed into fungal-based drugs as they are instrumental in contributing to medicinal properties, in the fight against malnutrition and chronic diseases (Deshpande and Arya, 2022; Kumar and Satpathy, 2022; Raman et al., 2022). However, the mineral composition in mushroom fruiting bodies is reported to be species-specific and depends on the substrate used for cultivation (Siwulski et al., 2019; Mleczek et al., 2021). The approach of enriching substrate with essential elements is used to improve mushroom yield and quality (Nunes et al., 2012; Gąsecka et al., 2016; Velez et al., 2019; Zięba et al., 2020). Substrate enrichment with essential elements also increases element accumulation in the fruiting bodies of *Ganoderma* and significantly reduces the production cycle (Postemsky et al., 2014; Bidegain et al., 2015; Rzymski et al., 2016; Bidegain et al., 2019). Furthermore, essential elements enrichment is also for food and diet supplement production (Zhao et al., 2004; Rzymski et al., 2016; Rathore et al., 2019). The type of essential elements used for enrichment has an enormous influence on mycelial growth, pileus development, and chemical composition (Da Silva et al., 2019; Oyetayo et al., 2021; Xu et al., 2021). This study investigated the impact of substrate fortified with zinc iron and selenium on the development, total biomass, and biochemical compounds of *G. austroafricanum* and *G. resinaceum*.

2 Materials and methods

2.1 Experimental description

2.1.2 Study area

All research production activities and sample preservation were carried out in the facilities of Mushroom Guru Pty Ltd in Cape Town (South Africa). Sample preparation, extraction, and antioxidant activity were conducted at the Horticultural Science laboratory facilities of the University of KwaZulu-Natal – Pietermaritzburg campus (South Africa). Protein content was determined at the Biochemistry Department of the University of KwaZulu-Natal. Essential elements and heavy metals from pileus were analysed at Cedara College of Agriculture in the laboratory of the Plant department. This study consisted of four sequential experiments.

2.1 Experimental layout

The experiment was laid out in a 2 x 4 factorial experimental combination with factors being; two various *Ganoderma* isolates and four essential elements. The following isolates were used and coded as follows: *G. austroafricanum* (GA), *G. resinaceum* (GR); and essential elements were: Zinc nitrate hexahydrate [$Zn(NO_3)_2.6H_2O$] – Zn; Iron sulphate heptahydrate (Fe_2SO_4 $7H_2O$) – Fe; Sodium selenite (Na_2SeO_3) – Se and suspension of all essential elements (ZnFeSe). All treatment combinations without essential elements were considered as control (Control). The treatment combinations were as follows: GAControl, GAZn, GAFe, GASe, and GAZnFeSe; GRControl, GRZn, GRFe, GRSe, and GRZnFeSe making a total of 10 treatment combinations. These respective treatment combinations were replicated three times making a total of 30 observations. The treatment combinations were laid out in a Completely Randomised Design (CRD). Substrate beech sawdust was used to grow all treatment combinations.

2.2 Preparation

2.2.1 Spawn

Spawn was produced using the sorghum grain method as described in Chapter 6.

2.2.2 Substrate

The substrate was prepared following the method described in Chapter 6.

2.2.3 Essential elements

Essential elements Fe_2SO_4 $7H_2O$, $Zn(NO_3)_2.6H_2O$, and Na_2SeO_3 were all supplied by Sigma-Aldrich (South Africa). The essential elements were prepared following the protocol described by Rzymski et al. (2016). A quantity of 0.4 mM from all essential elements was weighed before dissolving it in sterile deionised H_2O thereafter, added to the substrate with H_2O content reaching approximately 60% (v/v). Suspension of all essential elements using the same quantity was prepared and substrate without elements was considered as a control.

2.2.4 Inoculation

The inoculation was done following the method described in Chapter 6.

2.2.5 Growth conditions

The growth conditions such as micro-climate, carbon dioxide, and light intensity were monitored inside the growth chamber. Temperature (°C) and relative humidity (%) were assessed and recorded using a thermostat. The CO_2 levels were observed and measured using

a CO_2 level transmitter and expressed in ppm. Also, light intensity was observed and recorded using a digital light meter and was expressed in lux. All micro-climate, carbon dioxide, and light intensity data was recorded at three-day intervals from the first day of inoculation to the last day of harvest (Figure 7.1).

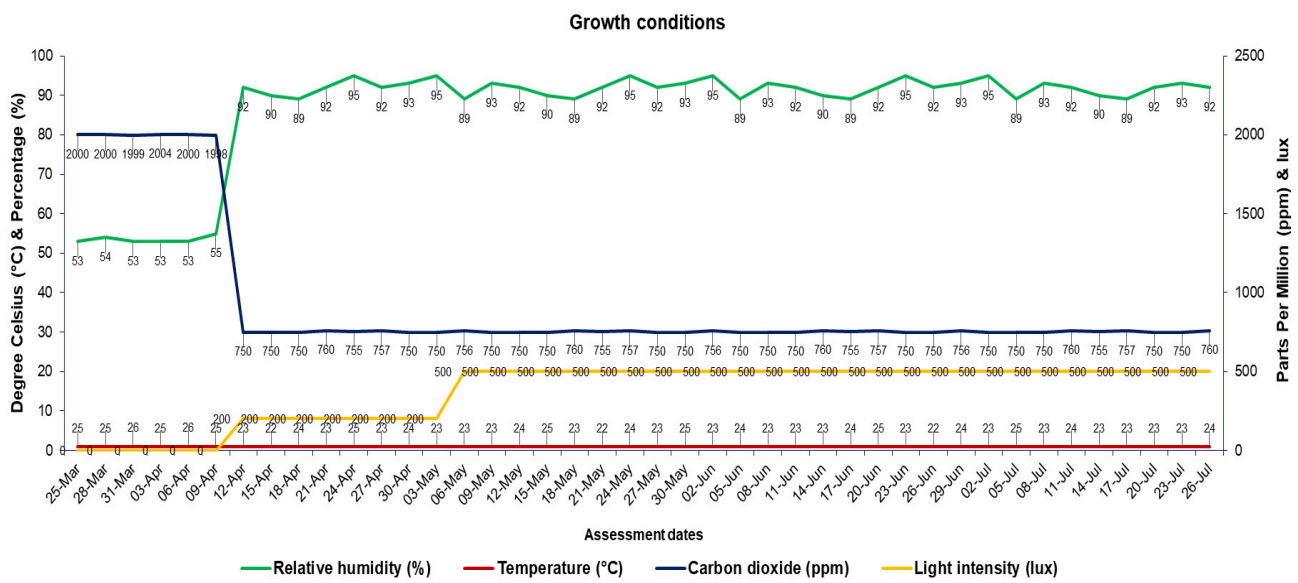

Figure 7.1 Growth conditions recorded in 123 days (from 25 March 2022 to 26 July 2022) inside the growth chambers during the production experiment. Mean temperature (°C), relative humidity (%), carbon dioxide (ppm), and light intensity (lux).

2.2.6 Sample preparation and extraction

The samples were prepared and extracted using the method described in Chapter 6.

2.3 Treatments description

2.3.1 Measurement of pileus diameter, stipe size, total pileus, weight, yield, and biological efficiency

The pileus diameter, thickness, and stipe length were measured using a 150 mm digital Vernier caliper and expressed in mm as described in Chapter 6.

2.3.2 Quantification of biochemical compound composition

2.3.2.1 Essential elements

The essential elements were quantified following the method described in Chapter 6.

2.3.2.2 Heavy metals analyses on pileus

The heavy metals were quantified following the method described in Chapter 6.

2.3.2.3 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

The DPPH value was spectrophotometrically determined using the method described in Chapter 6.

2.3.2.4 Total phenolics

The total phenolic content was spectrophotometrically determined using the Folin-Ciocalteu procedure as described in Chapter 6.

2.3.2.5 Total flavonoids

Total flavonoids were determined by a colorimetric method as described in Chapter 6.

2.3.2.6 Total protein

The protein content was determined using the Bradford dye reagent method as described in Chapter 6.

2.4 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using Genstat statistical software (Genstat® 20th edition, VSN International, Hemel Hempstead, UK). Means were separated according to Duncan's multiple range test at 5% level of significance.

3 Results and Discussion

3.1 Effect of substrate enhanced with essential elements on the pileus diameter, stipe length, total pileus, weight, yield, and biological efficiency of *G. austroafricanum and G. resinaceum*

The results had statistically significant differences ($p < 0.05$) observed between treatment combinations. The pileus diameter, thickness, stipe length, total pileus, weight, yield, and biological efficiency of *Ganoderma* spp. were measured after 123 days of the production cycle (Table 7.1). The fresh pileus diameter, thickness and stipe length on treatment combinations ranged from 30.17±1.21 to 62.35±1.63, 10.18±0.86 to 26.81±1.93, and 17.00±0.37 to 57.55±1.80 mm, respectively. Whilst the fresh total number of pileus, weight, total yield, and biological efficiency ranged from 18.53±0.34 to 37.87±1.36, 1.31±0.27 to 3.67±0.88 g/kg, 46.70±0.78 to 85.69±0.75 g/kg, and 5.19±1.33 to 9.52±1.23%, respectively. Even though the results on growth production phases exhibited the Zn element and GR species to have the fastest rate, this did not translate to large pileus size, higher yield quantity, and biological efficiency rate. Instead, Fe element and GA species had large numbers throughout the measurement of pileus diameter, thickness, stipe length, total pileus, weight, yield, and biological efficiency. The higher quantities from treatments enhanced with Fe in both species

could be linked to the vital role it has on fungi. Iron plays a significant role in enzyme activity and electron transport within fungal cells (Kosman, 2003). These enzymes are involved in critical cellular processes such as the breakdown of organic matter, nutrient uptake, and the synthesis of important molecules (Das and Varma, 2011). Therefore, the larger pileus, higher yield, and biological efficiency rate observed in treatment enriched with Fe might be due to improved breakdown of organic matter and nutrient uptake. Furthermore, substrate enriched with Se had the lowest variable quantities, this could be attributed to the dosage used in the current study. Rzymski et al. (2016) highlighted that in order to obtain high yield and adequate biomass, the substrate enrichment should not exceed 0.4 mM of elements such as Se, Cu, and Zn. Even though selenium-enriched substrate may alter the yield and their chemical composition, there is a lack of standardisation that is still an obstacle to scaling up the production process (De Souza et al., 2023). The overall findings of this study revealed that total yield and biological efficiency may be influenced by the substrate enhanced with essential elements.

Table 7.1 The pileus diameter, thickness, stipe length, total pileus, weight, yield, and biological efficiency *G. austroafricanum* and *G. resinaceum* measured in 123 days after inoculation.

Treatments	Pileus size						Number of pileus	Pileus weight (g/kg)		Total mushroom yield (g/kg)		Biological efficiency (%)
	Diameter (mm)		Thickness (mm)		Stipe length (mm)							
	Fresh	Dry	Fresh	Dry	Fresh	Dry		Fresh	Dry	Fresh	Dry	
GAControl	55.63±1.45^{b}	48.12±1.52^{bc}	16.32±0.96^{bc}	13.55±1.43^{b}	50.78±2.41^{b}	43.65±0.87^{bc}	22.33±0.67^{e}	1.89±0.64^{c}	1.11±0.25^{de}	62.63±1.22^{cd}	55.23±1.35^{d}	6.96±1.03^{bc}
GAZn	57.89±0.56^{ab}	50.32±2.15^{b}	17.89±0.64^{b}	12.78±2.12^{bc}	51.16±0.64^{b}	45.10±1.01^{b}	25.70±1.21^{d}	2.02±0.55^{bc}	1.64±0.34^{c}	64.76±1.41^{c}	58.24±2.76^{cd}	7.20±0.83^{b}
GAFe	62.35±1.63^{a}	54.34±1.78^{a}	26.81±1.93^{a}	22.11±1.48^{a}	57.55±1.80^{a}	51.07±1.65^{a}	37.87±1.36^{a}	3.67±0.88^{a}	2.89±0.36^{a}	85.69±0.75^{a}	76.81±2.50^{a}	9.52±1.23^{a}
GASe	50.42±1.75^{c}	42.35±0.81^{d}	12.53±1.52^{d}	10.47±1.57^{c}	40.38±0.58^{d}	35.41±0.43^{d}	18.53±0.34^{f}	1.34±0.62^{e}	0.89±0.54^{f}	55.75±1.33^{e}	44.27±0.92^{fg}	6.19±0.89^{c}
GAZnFeSe	53.04±1.66^{bc}	46.30±2.05^{c}	15.38±1.29^{c}	13.02±0.36^{b}	48.25±1.11^{c}	42.61±2.16^{c}	21.98±0.22^{ef}	1.62±0.25^{cd}	0.98±0.22^{ef}	60.52±1.61^{cd}	52.54±0.34^{e}	6.72±0.42^{c}
GA Total Average	**55.87±1.41**	**48.29±1.66**	**17.79±1.27**	**14.39±1.93**	**49.62±1.31**	**53.47±1.22**	**25.28±0.76**	**2.11±0.59**	**1.33±0.34**	**65.87±1.26**	**57.42±1.57**	**7.32±0.88**
GRControl	33.21±1.58^{e}	30.42±0.29^{f}	10.84±0.78^{f}	9.01±1.25^{cd}	17.36±0.32^{h}	16.71±0.52^{g}	28.62±1.04^{c}	1.41±0.51^{d}	1.01±0.40^{e}	59.31±0.32^{d}	57.45±1.24^{cd}	6.59±2.01^{c}
GRZn	40.18±1.06^{d}	36.11±0.74^{e}	11.03±0.62^{e}	10.16±0.27^{b}	30.41±1.31^{f}	24.88±1.53^{e}	32.58±0.34^{b}	1.55±0.09^{c}	1.20±0.67^{d}	63.28±0.26^{c}	60.01±0.77^{c}	7.03±0.36^{b}
GRFe	56.82±2.29^{b}	47.92±1.13^{bc}	14.40±3.05^{d}	13.03±0.81^{b}	37.63±1.91^{e}	32.05±1.17^{d}	35.08±1.33^{ab}	2.87±0.11^{b}	2.11±0.89^{b}	72.82±1.61^{b}	68.15±1.06^{b}	8.09±0.78^{ab}
GRSe	30.17±1.21^{e}	29.12±0.39^{f}	10.18±0.86^{f}	8.89±0.63^{d}	26.07±1.18^{g}	20.31±0.44^{f}	25.54±0.69^{d}	1.31±0.27^{e}	0.86±0.35^{f}	46.70±0.78^{g}	42.85±0.91^{g}	5.19±1.33^{d}
GRZnFeSe	31.62±0.80^{e}	29.62±2.12^{f}	10.69±0.66^{f}	8.97±0.49^{d}	17.00±0.37^{h}	16.64±1.00^{g}	27.18±0.55^{cd}	1.38±0.00^{de}	0.97±0.64^{ef}	51.72±0.87^{f}	48.63±1.85^{f}	5.75±0.65^{cd}
GR Total Average	**38.40±1.39**	**34.64±0.93**	**11.43±1.19**	**10.01±0.69**	**25.69±1.08**	**22.12±1.04**	**29.80±1.00**	**1.70±0.30**	**1.23±0.67**	**58.77±0.83**	**55.42±1.41**	**6.53±1.43**

The results are presented as means of pileus diameter, thickness, stipe length, and the number of pileus from each treatment combination from week six to seventeen of harvest. Only the largest pileus on each treatment was the focal point. * GA – *Ganoderma austroafricanum*; GR – *Ganoderma resinaceum*; and Essential elements were: Control – Control (no essential elements); Zn – Zinc nitrate hexahydrate [Zn(NO3)2.6H2O]; Fe – Iron sulphate heptahydrate (Fe2SO4 7H2O); Se – Sodium selenite (Na2SeO3) and ZnFeSe – suspension of all essential elements. Values were expressed as mean ± SD, $n = 3$. Means followed by the same letter within columns are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.1 Effect of substrate enhanced with essential elements on the biochemical composition of *G. austroafricanum and G. resinaceum*

3.1.1 Essential elements

Substrate enhanced with different essential elements had a significant effect ($p < 0.05$) on essential element levels in the harvested pileus of *Ganoderma* spp. (Table 7.2). Zn exhibited higher levels of essential elements compared to Fe, ZnFeSe, Se, and Control in both species. The variation in element levels could have been that different elements may have different uptake rates and preferences by fungi (Johanson et al., 2004; Falandysz, 2008). GAZn was observed to have higher concentrations of Zn, Mn, Mg, Na, Ca, Al, K, and P at 74.19±0.15, 7.86±0.11, 550.45±11.90, 164.95±0.72, 1267.81±15.44, 2.81±0.13, 7260.63±143.42, and 8756.11±105.55 mg/kg DM, respectively. These results were consistent with findings by Matute et al. (2011) where the enhancement of substrate with Zn had a significant increase in the mineral concentration of *Ganoderma* spp. Furthermore, various essential element levels on a substrate are shown in Table 3, the pileus was able to sequestrate these elements during growth (Bellettini et al., 2019; Ab Rhaman et al., 2021). Mushrooms can absorb and accumulate higher levels of essential elements when a substrate is enhanced with zinc in a soluble and easily accessible form (Figlas et al., 2010; Oyetayo et al., 2021). Despite differences in the essential element levels between treatment combinations, P and K were the two most abundant elements present in all treatments. In addition, the enhancement of substrate with Fe and Se managed to increase the specified element levels (Fe & Se) on both species. A significant increase in Fe and Se was observed on treatment combination GAFe, GRFe, GASe, and GRSe recording at 40.15±0.50, 37.25±0.84, 1.46±0.01, and 1.16±0.01, respectively. This increase can be attributed to the enhancement of substrate with specified elements that can increase the levels of the same elements in mushrooms (Rzymski et al., 2016). Therefore, these findings revealed that substrate enhanced with element Zn may increase various elements in the pileus of *Ganoderma* spp.

3.1.2 Heavy metals

The heavy metal composition results from two *Ganoderma* spp. harvested on substrate enhanced with essential elements are presented in Table 7.3. Substrate enhanced with different essential elements had a significant effect ($p < 0.05$) on the heavy metal levels between treatments. Cu and Cr had the highest concentrations across all treatments. Compared to Se, Zn, ZnFeSe, and Fe, Control had greater amounts of heavy metal concentrations in most treatment combinations. The higher concentrations of heavy metals found on pileus harvested

from Control may have resulted from these metals' bioaccumulation during the substrate's development (Table 7.4) (Stihi et al., 2011; Golian et al., 2021). Some studies highlighted that the accumulation of heavy metals in mushroom pileus is noted to be influenced by the mushroom species, substrates composition, and bioavailability of the metals (Tüzen et al., 1998; Isildak et al., 2007; Ab Rhaman et al., 2021). However, the results of the current study reveal that substrate enhanced with essential elements may have increased the heavy metal composition of pileus. GAControl higher levels of Cu, Cd, Pb, and As reading at 10.13±0.34, 1.20±0.08, 0.88±0.03 and 0.284±0.03 mg/kg DM, respectively. Treatment GASe also exhibited higher levels of elements Ni, Cr, Pb, Ag, and As observed at 0.62±0.05, 15.44±0.61, 0.79±0.05, 0.046±0.00 and 0.236±0.01 mg/kg DM, respectively. In addition, GRControl had higher levels in Hg (0.86±0.02 mg/kg DM), and Ag (0.033±0.00 mg/kg DM) whilst GAZn had higher levels in Co (1.04±0.02 mg/kg DM). Even though toxic metals such as Cd, Pb, and As are known to cause serious illness to organisms (Ndimele et al., 2017), the current study results detected below toxic levels. Furthermore, according to Ipeaiyeda et al. (2020); Shourie and Vijayalakshmi (2022) the mycelia of *Ganoderma* spp. can colonise and break down a substrate that leads to potentially reducing the levels of pollutants or contaminants present. Given that Control had higher concentrations of heavy metals than other treatments, the overall finding suggested that both species were able to complete the mycoremediation process.

Table 7.2 Essential element levels (mg/kg DM) of *G. austroafricanum* (GA) and *G. resinaceum* (GR) harvested from substrate enhanced with zinc nitrate hexahydrate (Zn), iron sulphate heptahydrate (Fe) and sodium selenite (Se) and suspension of all essential elements (ZnFeSe).

Treatment	Zn	Fe	Se	Mn	Mg	Na	Ca	Al	K	P
GAControl	63.18±1.12^{b}	26.46±0.87^{c}	1.11±0.02^{b}	5.70±0.34^{c}	482.80±6.90^{b}	145.45±0.56^{c}	981.95±5.46^{c}	2.24±0.16^{a}	6778.75±122.79^{b}	8415.71±159.22^{a}
GAZn	74.19±0.15^{a}	33.91±0.30^{b}	1.38±0.01^{a}	7.86±0.11^{a}	550.45±11.90^{a}	164.95±0.72^{a}	1267.81±15.44^{a}	2.81±0.13^{a}	7260.63±143.42^{a}	8756.11±105.55^{a}
GAFe	69.10±0.94^{a}	40.15±0.50^{a}	1.24±0.01^{ab}	6.80±0.16^{ab}	501.11±1.01^{ab}	156.16±0.99^{b}	1183.02±2.83^{b}	2.67±0.07^{a}	7062.37±251.04^{a}	8659.49±122.62^{a}
GASe	64.76±0.65^{b}	28.47±0.24^{bc}	1.46±0.01^{a}	5.81±0.05^{c}	532.02±3.62^{a}	147.83±0.57^{c}	1060.73±4.17^{bc}	2.34±0.06^{a}	6847.70±140.31^{b}	8530.13±116.99^{a}
GAZnFeSe	71.02±0.79^{a}	31.33±0.78^{b}	1.29±0.01^{a}	6.29±0.17^{b}	503.11±0.97^{ab}	152.33±0.86^{b}	1154.14±15.59^{b}	2.64±0.05^{a}	6971.78±67.52^{ab}	8614.74±113.68^{a}
GRControl	47.73±1.61^{d}	17.50±0.66^{d}	0.64±0.06^{d}	4.30±0.19^{d}	376.65±10.88^{c}	116.14±2.07^{e}	878.14±15.40^{d}	1.53±0.14^{b}	5363.86±170.98^{c}	7001.63±128.80^{b}
GRZn	62.66±1.49^{bc}	28.26±0.49^{bc}	0.90±0.01^{c}	6.60±0.16^{ab}	536.65±15.70^{a}	153.95±0.57^{b}	1053.54±9.47^{bc}	1.85±0.03^{b}	6963.87±110.48^{ab}	8337.54±132.95^{a}
GRFe	58.25±0.67^{bc}	37.25±0.84^{ab}	0.75±0.02^{cd}	6.05±0.01^{bc}	452.08±2.00^{b}	142.43±2.54^{c}	958.09±6.02^{c}	1.65±0.02^{b}	6738.65±124.28^{b}	8074.10±156.84^{a}
GRSe	53.66±1.40^{cd}	21.92±0.68^{cd}	1.16±0.01^{b}	4.92±0.06^{dc}	392.90±0.59^{c}	127.34±0.57^{d}	905.35±0.57^{cd}	0.85±0.07^{c}	6196.61±117.10^{bc}	7363.50±161.47^{b}
GRZnFeSe	57.64±0.64^{c}	25.20±0.46^{c}	1.01±0.05^{bc}	5.44±0.39^{c}	425.40±2.08^{bc}	131.69±1.15^{cd}	931.08±8.00^{cd}	1.61±0.01^{b}	6566.95±135.66^{b}	7611.68±190.84^{b}

The results were expressed as mean ± SD, $n = 3$. Means followed by the same letter within columns are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

Table 7.3 Essential element levels (mg/kg DM) of beech sawdust

Substrate	Zn	Fe	Se	Mn	Mg	Na	Ca	Al	K	P
Beech sawdust	17.56±2.13	11.32±0.96	0.35±0.05	5.04±0.99	541.03±0.58	64.22±1.25	16.12±0.99	5.51±0.34	1523.37±18.48	1318.35±52.36

The results were expressed as mean ± SD, $n = 3$

Table 7.4 Heavy metal levels (mg/kg DM) of *G. austroafricanum* (GA) and *G. resinaceum* (GR) harvested from substrate enhanced with zinc nitrate hexahydrate (Zn), iron sulphate heptahydrate (Fe) and sodium selenite (Se) and suspension of all essential elements (ZnFeSe).

Treatments	Ni	Cu	Co	Cd	Cr	Pd	Pb	Hg	Ag	As
GAControl	0.48±0.02^{b}	10.13±0.34^{a}	0.86±0.10^{b}	1.20±0.08^{a}	11.08±0.00^{b}	0.14±0.00^{b}	0.88±0.03^{a}	0.24±0.02^{d}	0.031±0.01^{b}	0.284±0.03^{a}
GAZn	0.41±0.01^{bc}	8.81±0.33^{b}	1.04±0.02^{a}	0.96±0.04^{a}	9.12±0.04^{d}	0.09±0.00^{e}	0.57±0.04^{c}	0.14±0.01^{e}	0.026±0.00^{c}	0.211±0.01^{b}
GAFe	0.37±0.02^{bc}	7.24±0.32^{c}	0.61±0.04^{c}	0.56±0.06^{d}	12.35±0.44^{b}	0.13±0.01^{c}	0.37±0.01^{d}	0.19±0.01^{de}	0.027±0.00^{bc}	0.169±0.02^{dc}
GASe	0.62±0.05^{a}	4.82±0.31^{cd}	0.42±0.05^{d}	0.77±0.05^{b}	15.44±0.61^{a}	0.11±0.00^{dc}	0.79±0.05^{a}	0.11±0.00^{f}	0.046±0.00^{a}	0.236±0.01^{a}
GAZnFeSe	0.43±0.00^{b}	7.00±1.32^{c}	0.55±0.02^{cd}	0.66±0.10^{c}	10.08±0.00^{c}	0.23±0.18^{a}	0.64±0.01^{b}	0.12±0.00^{ef}	0.023±0.00^{c}	0.197±0.03^{bc}
GRControl	0.33±0.05^{cd}	8.17±0.28^{b}	0.73±0.03^{b}	0.54±0.07^{d}	7.46±0.59^{e}	0.05±0.01^{f}	0.61±0.05^{b}	0.86±0.02^{a}	0.033±0.00^{b}	0.177±0.00^{c}
GRZn	0.27±0.03^{d}	5.92±0.32^{d}	0.90±0.01^{a}	0.28±0.01^{f}	4.12±0.01^{g}	0.02±0.00^{g}	0.34±0.05^{d}	0.47±0.01^{c}	0.012±0.00^{d}	0.136±0.01^{e}
GRFe	0.17±0.02^{e}	4.59±0.35^{de}	0.54±0.03^{cd}	0.48±0.01^{de}	6.08±0.00^{f}	0.05±0.00^{f}	0.21±0.02^{e}	0.65±0.02^{b}	0.021±0.00^{c}	0.091±0.01^{f}
GRSe	0.44±0.02^{b}	2.12±0.09^{f}	0.25±0.01^{e}	0.75±0.04^{b}	10.24±0.04^{bc}	0.03±0.00^{fg}	0.52±0.01^{c}	0.19±0.02^{de}	0.032±0.00^{b}	0.156±0.01^{d}
GRZnFeSe	0.34±0.03^{c}	3.54±0.18^{e}	0.34±0.03^{de}	0.40±0.01^{e}	7.07±0.01^{e}	0.03±0.00^{fg}	0.34±0.07^{d}	0.41±0.01^{c}	0.022±0.01^{c}	0.128±0.01^{e}

The results were expressed as mean ± SD, $n = 3$. Means followed by the same letter within columns are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

Table 7.5 Heavy metal levels (mg/kg DM) of beech sawdust.

Substrate	Ni	Cu	Co	Cd	Cr	Pd	Pb	Hg	Ag	As
Beech sawdust	0.40±0.03	4.98±0.19	0.94±0.05	27.13±0.52	10.81±1.18	0.03±0.00	0.50±0.10	0.32±0.10	0.014±0.00	0.032±0.01

The results were expressed as mean ± SD, $n = 3$.

3.1.3 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay

The DPPH free radical scavenging activity of two *Ganoderma* spp. harvested on substrate enhanced with essential elements is shown in Figures 7.2 (a & b). There was a notable significant effect ($p \leq 0.001$) in DPPH free radical scavenging activity results between the treatment combinations. In both species, essential element Se had the highest scavenging activity percentages followed by Zn. In comparison to other treatment combinations, the combinations GASe and GRZn had the greatest percentages, reading 74.79±2.35 and 68.51±0.84%, respectively. The highest scavenging activity percentages in element Se could be linked to selenium's enhancement of antioxidant capacity and antioxidant characteristics (Rathore et al., 2018; Fasoranti et al., 2019). A study by Zięba et al. (2020) stated that the addition of Se to substrate increased DPPH free radical scavenging activity on pileus from 33.15±1.03 to 39.22±0.47%. In addition, even though Zn is a strong antioxidant because it acts as a cofactor of superoxide dismutase and many other enzymes (Greenough et al., 2011), its scavenging activity did not supersede that of Se. The element Se increased the scavenging activity percentage on GA and GR from 50.04±1.64 to 74.79±2.35 and 39.13±0.71 to 67.40±2.61%, respectively. The finding of the current study suggests that Se may contribute to the overall antioxidant capacity of the pileus.

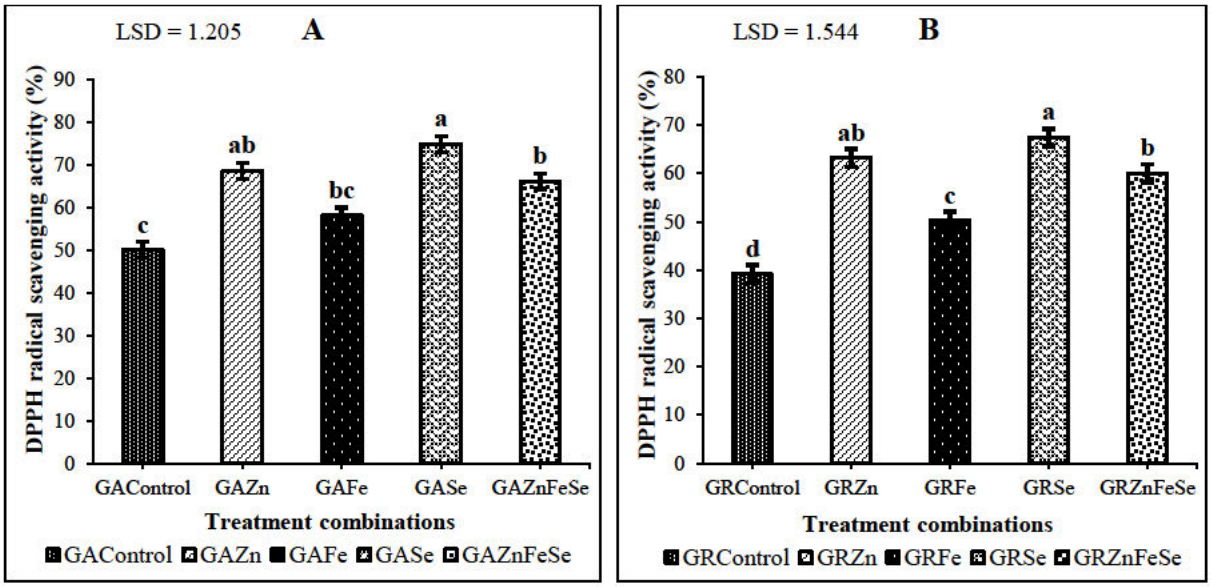

Figure 7.2 DPPH radical scavenging activity percentage release from the pileus *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** harvested on substrate enhanced with zinc nitrate hexahydrate (Zn), iron sulphate heptahydrate (Fe) and sodium selenite (Se) and suspension of all essential elements (ZnFeSe). The results were expressed as mean ± SE, n = 3. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.1.4 Total phenolics

The total phenolics results of two *Ganoderma* spp. harvested on substrate enhanced with essential elements are shown in Figures 7.3 (a & b). The substrate enhanced with different essential elements had a significant effect ($p \leq 0.001$) on the total phenolic levels of treatments. The highest phenolic content was detected on treatment combination GASe at 196.10±3.05 mg GAE/g followed by GAZn at 184.50±1.66 mg GAE/g. These findings were consistent with the report by De Souza et al. (2023) highlighted an increase in the total phenolic content of *Ganoderma* spp. from 29 to 40 mg GAE/g where selenium was added to the substrate. In addition, discrepancies in phenolic concentrations were also observed in treatment combinations GRSe, GAZnFeSe, GAFe, and GRZn recorded at 171.41±0.88, 170.60±2.33, 160.60±0.91 and 159.72±1.56, mg GAE/g, respectively. Even though numerous factors can affect *Ganoderma* spp. levels of phenolic compounds, Se outperformed other elements in both species. These results correlate with the findings from Zięba et al. (2020) that demonstrated mushroom pileus from substrate enhanced with selenium increased the phenolic concentration by 13.68 mg GAE/g. Furthermore, the lowest phenolic levels were detected from GAControl reading at 156.40±1.73, GRZnFeSe at 150.82±1.67, GRFe at 145.77±1.02, and GRControl at 127.26±0.92 mg GAE/g. Overall, the data showed that GA had higher total phenolic levels than GR. This could be attributed to the unique phenolic compound profile of each *Ganoderma* spp. possesses (Obodai et al., 2017). This suggests that the phenolics makeup of different species of *Ganoderma* can differ, and each species has a distinct profile of phenolic compounds that can cause differences in their concentrations. Therefore, the phenolic composition of *Ganoderma* species can be influenced by the substrate enhanced with essential elements and the type of species used.

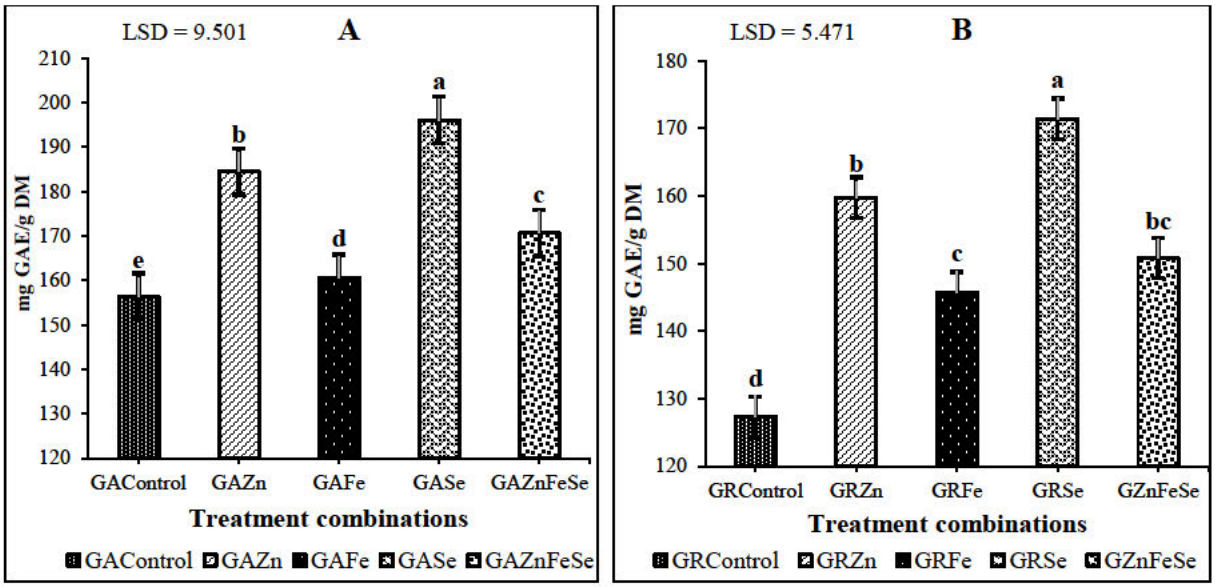

Figure 7.3 Total phenolic compounds extracted from the pileus *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** harvested on substrate enhanced with zinc nitrate hexahydrate (Zn), iron sulphate heptahydrate (Fe) and sodium selenite (Se) and suspension of all essential elements (ZnFeSe). The results were expressed as mean ± SE, $n = 3$. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.1.5 Total flavonoids

The flavonoid concentration levels were significantly different ($p \leq 0.001$) from *G. austroafricanum* and *G. resinaceum* extracts harvested from substrate enhanced with essential elements (Figures 7.4 a & b). Treatment combination GASe, GAZn, GAZnFeSe and GRSe had the highest flavonoid concentration in reading at 101.66±2.07, at 95.69±0.93, at 85.32±1.37 and 78.91±1.12 mg QE/g, respectively. These findings were consistent with the report from Gąsecka et al. (2016) which concluded that the enrichment of mushroom substrate with Se and Zn increased the antioxidant contents including flavonoids. The findings of the current study proved that substrate enhanced with essential elements may influence the flavonoid levels of *Ganoderma* spp. In addition, GAFe, GRZn, GRZnFeSe, GRFe, GAControl, and GRControl, had the lowest concentrations measuring 77.33±2.11, 73.29±1.01, 67.32±1.51, 66.05±0.43, 64.27±1.25 and 53.87±0.88 mg QE/g, respectively. Furthermore, the results revealed that Se followed by Zn had abundant flavonoid levels in both species. The discrepancies in flavonoid levels between species could be linked to the genetic composition of the *Ganoderma* spp. (Hamwenye, 2020). Thus, differences in the genes involved in the synthesis and metabolism of flavonoids may impact the total amount of flavonoids in various species.

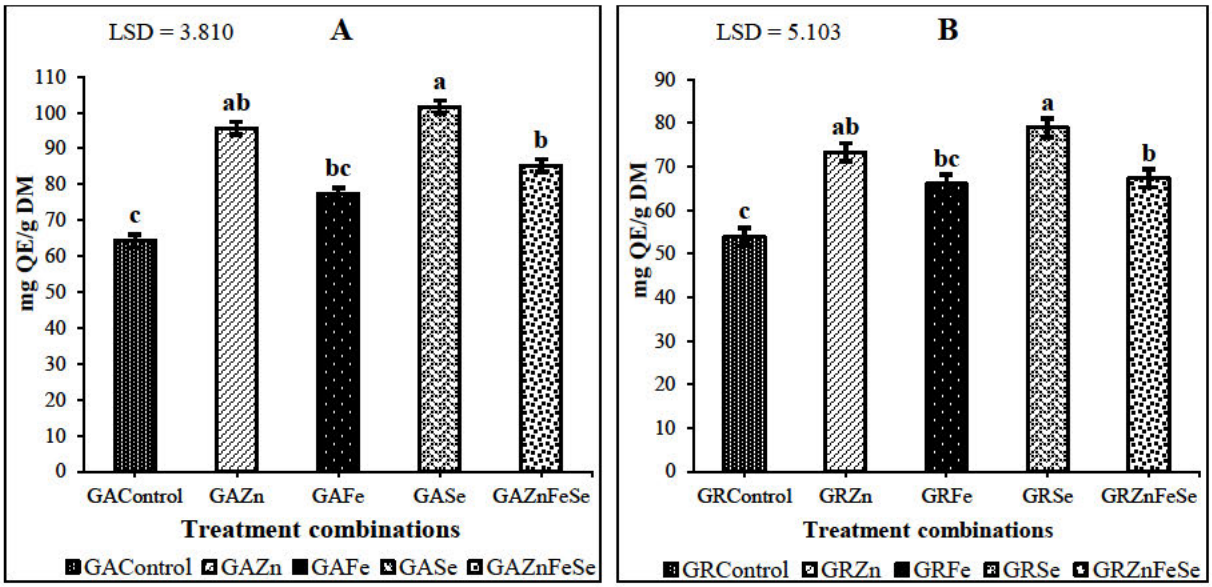

Figure 7.4 Total flavonoid content extracted from the pileus *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** harvested on substrate enhanced with zinc nitrate hexahydrate (Zn), iron sulphate heptahydrate (Fe) and sodium selenite (Se) and suspension of all essential elements (ZnFeSe). The results were expressed as mean ± SE, $n = 3$. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

3.1.6 Total protein

There were statistically significant differences in the total protein content results amongst the treatment combinations (Figures 9a & 9b). At 23.41±1.21 µg/g, GASe exhibited a higher protein concentration than GAZn (21.46±1.38 µg/g), GAZnFeSe (20.69±2.17 µg/g), GRSe (18.65±054 µg/g), and GAFe (18.14±2.25 µg/g). On both species, element Se performed better than Zn, ZnFeSe Fe, and Control, respectively. This could be linked to certain enzymes involved in protein metabolism, promoting the production and accumulation of proteins enhanced by Se (Min-Chang et al., 2014; Zoidis et al., 2018). Selenium is involved in the biosynthesis of selenoenzymes and selenoproteins such as glutathaione, peroxidase, iodothyronone 5'-deiodades, and thioredoxin reductases (Zięba et al., 2020). The results of the current study were consistent with findings from previous studies where substrate enhanced with Se increased the protein concentration of mushrooms (Fasoranti et al., 2019; De Souza et al., 2023). Also, the higher levels of Zn compared to Fe corresponded with the findings of Oyetayo et al. (2021) where substrate enhanced with Zn had higher protein at 16.8% compared to Fe at 11.4%. In addition, lower protein concentrations were detected in treatment GRZn, GAControl, GRZnFeSe, GRFe, and GRControl logged at 17.36±1.40, 16.66±1.86, 16.37±0.99, 15.07±1.11 and 11.96±0.39, respectively. These findings elucidate that substrate enhanced with Se and Zn can increase the protein concentration of *Ganoderma* spp.

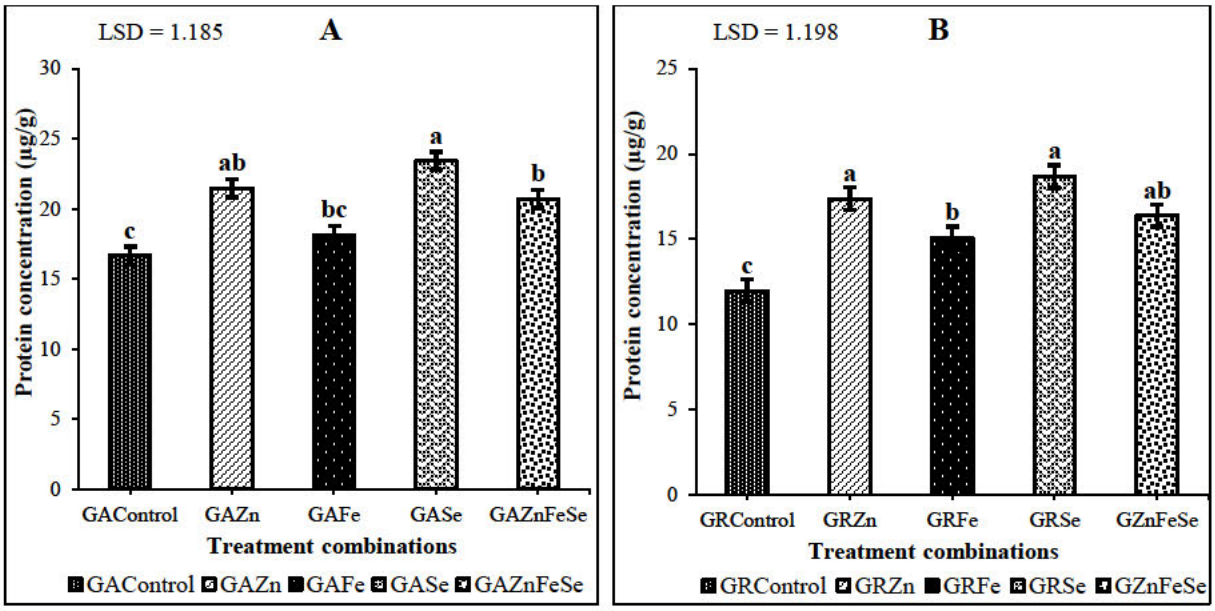

Figure 7.5 Total protein concentration extracted from the pileus of *G. austroafricanum* (GA) – **A** and *G. resinaceum* (GR) – **B** harvested from substrate enhanced with zinc nitrate hexahydrate (Zn), iron sulphate heptahydrate (Fe) and sodium selenite (Se) and suspension of all essential elements (ZnFeSe). The results were expressed as mean ± SE, n = 3. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p \geq 0.05$).

4 Conclusion

The results of the study showed that the development, total biomass, and biochemical profile of *G. austroafricanum and G. resinaceum* are influenced by substrate enhanced with Zn, Fe, and Se. Further research and experimentation are warranted to fully understand the mechanisms underlying the impact of substrate fortification on *Ganoderma* spp. This will assist in identifying the optimal combination and concentration of essential elements that can maximise the development, total biomass, and biochemical compounds of these species.

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CHAPTER 8
GENERAL DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

8.1 Introduction

Ganoderma has been long regarded as one of the most important medicinal fungi. However, it is known to be a complex genus with some challenges. Some of these challenges related to *Ganoderma* species include identification and distinguishing between different species, understanding their complex biochemical compounds and potential health benefits, as well as addressing issues related to cultivation. Characterisation complexity of *Ganoderma* species has been observed resulting from their genetic makeup, which can make it difficult to accurately identify and differentiate between different species. According to earlier reports, not enough is known about the identity of macro-fungi found in the natural ecosystem of South Africa. Because of this, it is currently unknown what the taxonomic status and biochemical composition of *Ganoderma* species are in South Africa. Furthermore, there is a dearth of information on the production and biochemical compound concentrations of these species in South Africa, particularly, *G. austroafricanum*. Therefore, the current study characterised *Ganoderma* spp. using different characterisation markers and investigated the influence of different substrates and substrates fortified with essential elements on the development and biochemical profile. This multifaceted approach has enhanced our ability to identify and differentiate between different species, as well as to explore the influence of substrate enhancement on their development and biochemical profile. The four experimental objectives proved that deploying various markers in characterising these species can be effective, also, substrate composition has a significant role in the overall production and biochemical profile.

8.2 Literature review focusing on the ecology and distribution of *Ganoderma* species in Southern Africa

The main objective of this review was to discuss the taxonomic status, distribution patterns, characterisation techniques, production techniques, and growth parameters of *Ganoderma* species. *Ganoderma* has medicinal properties and nutritional qualities hence providing this genus with an exorbitant economic market value. Notwithstanding this, their taxonomic status remains unclear due to similarities in basidiocarp that affect morphological characterisation and accurate identification. Previous reviews on the taxonomic status of *Ganoderma* species revealed that twenty species have been identified in Southern Africa with a large number found in South Africa. It further discussed the lack of information regarding species distribution,

DNA sequences, and the phylogenetic position that is unknown in Southern Africa. In addition, production techniques, substrate's chemical composition, and growth parameters such as pH, temperature, relative humidity, and aeration influence on the genus development and quality were discussed. According to previous reviews, the chemical composition of the substrate is the major component responsible for the development and quality of this genus.

8.3 Literature review focusing on biochemical quality and pharmaceutical properties of *Ganoderma* species

A review of the biochemical quality and pharmaceutical properties of *Ganoderma* species was discussed. The present review focused on biochemical compounds and essential elements as several reports revealed them as the two major direct contributors to these pharmaceutical properties. Previously, approximately 400 biochemical compounds were reported from its mycelia, fruiting body, and spores of *Ganoderma*. However, *Ganoderma* triterpenes and polysaccharides are identified to be the two major constituents that play a vital role in the pharmaceutical properties of this genus. Literature suggests that the biochemical quality and pharmaceutical properties differ within species, this distinction is attributed to the diverse climatic conditions or environments from which they grow. Factors such as the species type, substrate, body part, pileus maturity, temperature, pH, and postharvest handling are also known to have a direct influence on their biochemical composition. This review further highlighted the safety and techniques used to enrich the biochemical properties and nutritional value of *Ganoderma*. This review established that the number of studies on biochemical quality and pharmaceutical properties of *Ganoderma* and commercial demand has significantly increased over the years. Nonetheless, there are research gaps that necessitate further investigation before the transition from nutraceutical to pharmaceutical applications takes place. Even though there are clinical research studies on the use of *Ganoderma* extracts in treating various diseases are very limited, the research currently available is promising for these biochemical compounds to be used in conventional medicine.

8.4 Morphological, molecular and biochemical characterisation of *Ganoderma* species from selected provinces in South Africa

This study focused on the isolation and characterisation of fifteen fungal specimens collected from the three provinces of South Africa, namely, Mpumalanga, KwaZulu-Natal, and North-West. The characterisation of *Ganoderma* spp. using a combination of morphological, molecular, and biochemical markers has provided valuable insights into the taxonomy and diversity of these fungi. Through using multiple characterisation markers, the study was able

to distinguish between different species with greater accuracy and precision. The molecular identification results demonstrated that only two collected fungal samples tested positive as *Ganoderma* spp. The morphological characterisation provided the identification and classification of different *Ganoderma* spp. based on their physical characteristics, such as shape, colour, and size. Furthermore, this approach has also highlighted the genetic and biochemical variations within *Ganoderma* spp. used in this study. Therefore, the integration of morphological, molecular, and biochemical markers in the characterisation of *Ganoderma* spp. provided an understanding of the biological, genetic characteristics and evolutionary relationships within the *Ganoderma* genus. In addition, his information contributes to our understanding of the biodiversity and distribution of *Ganoderma* spp. in South Africa.

8.5 Determination of optimum temperature and pH for the mycelial establishment of *Ganoderma* fungal species under different growth media

Data on the different growing conditions for *G. austroafricanum* and *G. resinaceum* are provided by the study. The results have demonstrated that *Ganoderma* spp. may grow well on woody plant material and prefer a pH range of 4-6. However, they also have different temperature requirements with *G. austroafricanum* at 20 °C and *G. resinaceum* at 30 °C. The study findings have shown that differences in growth media pH and temperature, as well as the type of growth media, can greatly influence the mycelial growth of *Ganoderma* species. Furthermore, *Ganoderma* spp. may develop well in combination with plant residue and culture conditions, although it might lead to slow mycelial growth. Additionally, MEA is the ideal growth medium for *Ganoderma* spp. mycelial development. The results of the study demonstrated that variations in growth media type, pH, and temperature can have a significant impact on *Ganoderma* species' mycelial growth.

8.6 Effect of different substrates on the total biomass and biochemical profile of *Ganoderma* spp.

The utilisation of agricultural waste as a substrate can be a sustainable and cost-effective approach for cultivating *Ganoderma* spp. Factors such as substrate nutrient composition and physical characteristics have a significant impact on determining the overall performance of the fungi. The results of the study showed that the development, total biomass, and biochemical profile of *Ganoderma* spp. are strongly influenced by the substrate selection. The impact of various substrates utilised in *Ganoderma* production encompasses a broad spectrum of parameters, such as development, biochemical composition, physical attributes, and yield. The results on the development and total biomass production of *Ganoderma* species revealed

significant variations across different substrates. Both species grew well on beech sawdust, however, sugarcane bagasse had the highest concentration of biochemical concentration, total biomass production, and biological efficiency. The study showed that the growth and biochemical profile of *Ganoderma* spp. can be affected differently by a variety of substrates. Therefore, these findings demonstrated that the physical properties and chemical composition of a substrate could influence the development, total biomass, and biochemical profile of *Ganoderma* spp.

8.7 Impact of substrate fortified with essential elements on total biomass, and biochemical compounds of *Ganoderma* spp.

The manipulation of substrate composition with essential elements managed to optimise the cultivation conditions for enhanced fungal growth parameters, pileus size, biomass yield, biological efficiency, and biochemical compounds production. This study investigated the impact of substrate fortified with essential elements on the development, total biomass, and biochemical compounds of *Ganoderma* species. The substrate enhancement with zinc, iron, and selenium demonstrated a significant impact on the overall production including the development and biochemical profile of *Ganoderma* spp. Element zinc developed expeditiously, reaching 100% in all production parameters in 52 days after inoculation and significantly increased the majority of mineral concentrations. Nevertheless, iron yielded higher quantities in total biomass and biological efficiency. In addition, element selenium improved the DPPH free radical scavenging activity percentage, total phenolics, flavonoids, protein, and the majority of heavy metal concentrations. These findings provide evidence that the enhancement of substrates with essential elements may improve the production and biochemical attributes of *Ganoderma* species.

8.8 Recommendations

- The identification of *Ganoderma* species in other parts of South Africa and their possible pharmaceutical properties and applications is recommended.

- The integration of molecular, biochemical, and biotechnological characterisation techniques are recommended to accurately identify *Ganoderma* species.

- The use of beech sawdust and sugarcane bagasse is recommended as production substrates as this may increase the quality and quantity of *Ganoderma* species. Other substrates readily available in other regions of South Africa should be investigated.

- Substrate enhancement with Zn, Fe, and Se may be used to improve the development, total biomass, and biochemical profile of these species. The usage of other minerals such as nitrogen, potassium, etc. should be investigated for these effects.

- Further research and experimentation are warranted to fully understand the mechanisms underlying the impact of substrate fortification on *Ganoderma* spp. This will assist in identifying the optimal combination and concentration of essential elements that can maximise the development, total biomass, and biochemical compounds of these species.

- Further exploration of diverse techniques that have the potential to enhance biochemical compounds and ensure purity is recommended.

- The underlying substrate enhancement mechanisms, and optimised cultivation conditions for *Ganoderma* spp, need further research to ultimately contribute to the advancement of its medicinal applications.

- *G. austroafricanum* consists of higher biochemical compound concentrations, total biomass, and biological efficiency, however, there is very limited work conducted on this species. Therefore, future research should focus on the production of this species for a better understanding of its growth parameters and biochemical composition.

- The behaviour of other *Ganoderma* spp. under different growth conditions should be determined. These growth conditions results will enable the domestication and commercial production of these species

- Collaborative efforts between researchers from different disciplines, such as mycology, genetics, and biochemistry, will be essential to harness the full potential of *Ganoderma* spp. for sustainable and innovative biotechnological applications.

Probability of *Ganoderma* sites

Province	Municipality	Location	GPS coordinates
Mpumalanga	Nkomazi	Jeppes Reef	25°43'11.0"S 31°29'04.3"E
Mpumalanga	Nkomazi	Jeppes Reef	25°42'33.1"S 31°29'32.0"E
Mpumalanga	Nkomazi	Jeppes Reef	25°43'09.7"S 31°29'44.0"E
Mpumalanga	Nkomazi	Jeppes Reef	25°43'20.3"S 31°29'18.0"E
Mpumalanga	Nkomazi	Schoemansdal	25°42'34.3"S 31°30'07.7"E
Mpumalanga	Nkomazi	Buffelspruit	25°38'60.0"S 31°32'30.8"E
Mpumalanga	Nkomazi	Sincobile	25°38'48.1"S 31°30'46.5"E
Mpumalanga	Nkomazi	Lows Creek	25°38'42.1"S 31°18'41.9"E
Mpumalanga	Nkomazi	Matsulu	25°32'08.6"S 31°18'37.0"E
Mpumalanga	Mbombela	Tekwane	25°28'05.0"S 31°07'32.7"E
Mpumalanga	Mbombela	Karino	25°27'30.6"S 31°05'36.5"E
Mpumalanga	Mbombela	Matafeni	25°28'32.5"S 30°54'33.4"E
Mpumalanga	Thaba chweu	Mashishing	25°05'11.9"S 30°24'32.7"E
Mpumalanga	Thaba chweu	Sabie	25°06'43.9"S 30°46'45.0"E
Mpumalanga	Thaba chweu	Hendriksdal	25°11'14.5"S 30°46'32.8"E
KwaZulu-Natal	Ubuhlebezwe	Mnyanyabuzi	30°09'50.4"S 30°20'53.0"E
KwaZulu-Natal	Ubuhlebezwe	Mnyanyabuzi	30°10'45.7"S 30°23'43.6"E
KwaZulu-Natal	Ubuhlebezwe	Mnyanyabuzi	30°10'02.7"S 30°20'34.8"E
KwaZulu-Natal	Ubuhlebezwe	Sangcwaba	30°11'12.8"S 30°24'42.2"E
KwaZulu-Natal	Ubuhlebezwe	Sangcwaba	30°11'17.7"S 30°24'06.7"E
KwaZulu-Natal	Ubuhlebezwe	Qumeni	30°11'54.9"S 30°22'08.8"E
KwaZulu-Natal	Ubuhlebezwe	Qumeni	30°12'19.7"S 30°21'16.9"E
KwaZulu-Natal	Ubuhlebezwe	Nhlazanyoni	30°11'46.2"S 30°25'20.1"E
KwaZulu-Natal	UMlalazi	Nyanini	28°54'31.8"S 31°29'01.9"E

KwaZulu-Natal	UMlalazi	Eziqwaqwani	28°51'39.0"S 31°31'09.5"E
KwaZulu-Natal	UMlalazi	Emaqeleni	28°49'35.9"S 31°30'39.2"E
KwaZulu-Natal	Msunduzi	Phayiphini	29°37'35.8"S 30°20'47.5"E
KwaZulu-Natal	Msunduzi	Hazelmere	29°39'19.6"S 30°23'04.6"E
KwaZulu-Natal	Msunduzi	Scottville	29°36'59.9"S 30°23'32.0"E
KwaZulu-Natal	Msunduzi	Scottville	29°37'15.2"S 30°23'35.6"E
North-West	Mahikeng	Mmabatho	25°49'21.2"S 25°37'15.2"E
North-West	Mahikeng	Tsetse	25°43'52.7"S 25°40'45.4"E
North-West	Mahikeng	Malapo ogg	25°52'06.7"S 25°53'21.6"E
North-West	Mahikeng	Slurry	25°51'41.0"S 25°50'37.8"E
North-West	Mahikeng	Buhrmannsdrift	25°49'13.4"S 25°47'17.2"E
North-West	Mahikeng	Tloung	25°52'47.7"S 25°36'40.5"E
North-West	Ditsobotla	Blydeville	26°10'15.1"S 26°09'25.5"E
North-West	Ditsobotla	Boikhutso	26°10'36.9"S 26°08'19.7"E
North-West	Ditsobotla	Boikhutso	26°10'35.5"S 26°07'58.9"E
North-West	Ditsobotla	Lichtenburg	26°07'45.0"S 26°09'27.4"E
North-West	Ditsobotla	Lichtenburg	26°08'29.0"S 26°10'20.4"E
North-West	Kgetlengrivier	Moedwill	25°37'48.1"S 26°58'08.4"E
North-West	Kgetlengrivier	Borelelo	25°38'08.3"S 26°40'34.7"E
North-West	Kgetlengrivier	Swartruggens	25°38'39.1"S 26°41'26.1"E
North-West	Kgetlengrivier	Mabiskraal	25°37'19.2"S 27°08'26.3"E

PHOTOGRAPHS

Sample collection and isolation

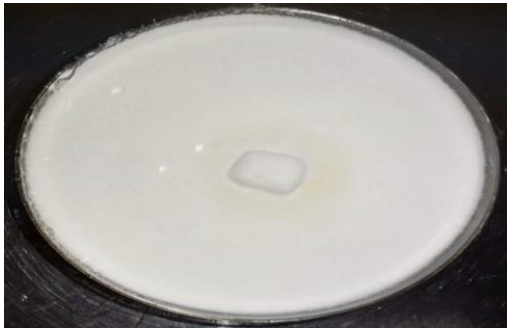


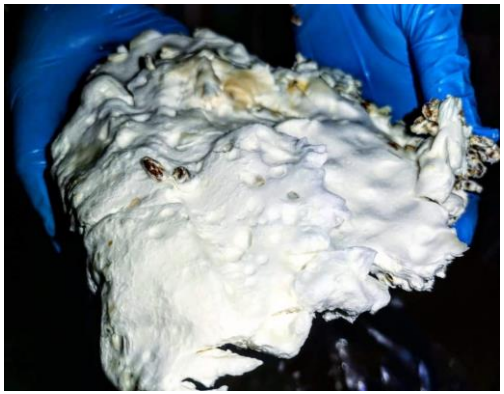







Spawn production and inoculation





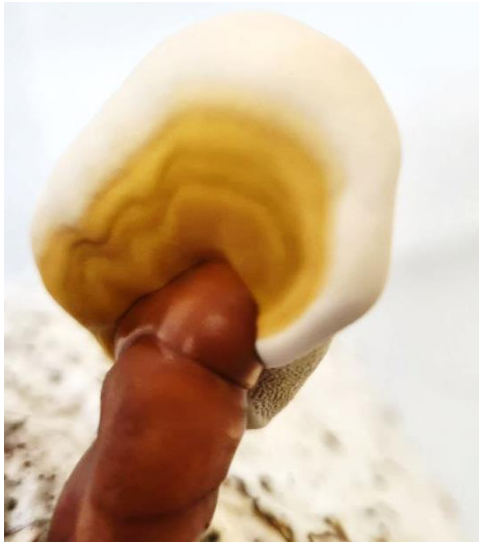
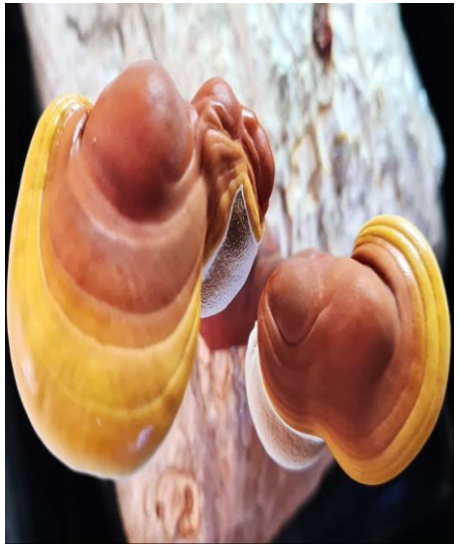
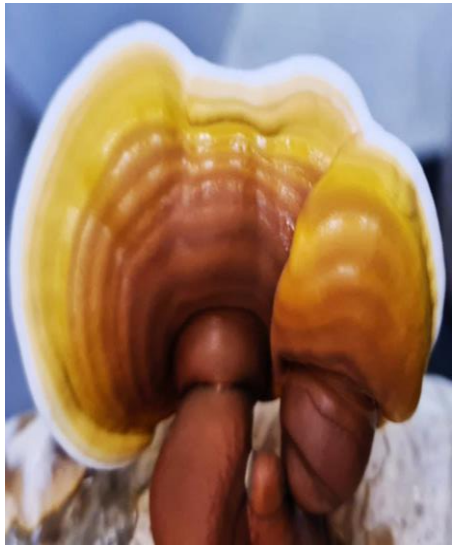



Growth production

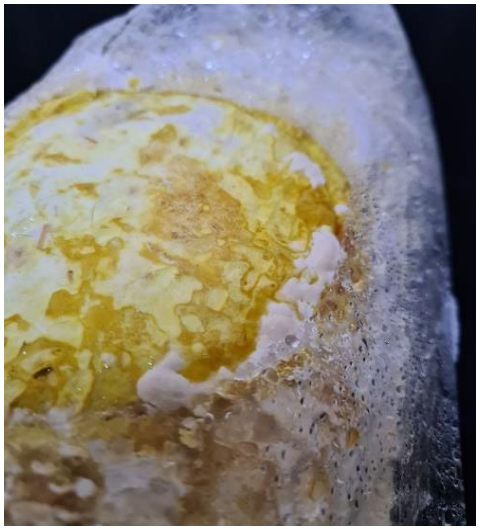
Ganoderma austroafricanum

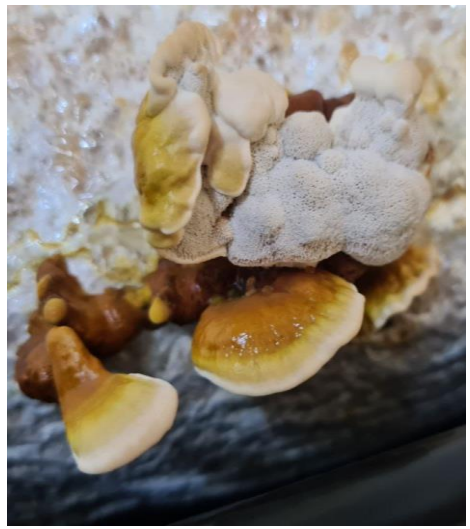
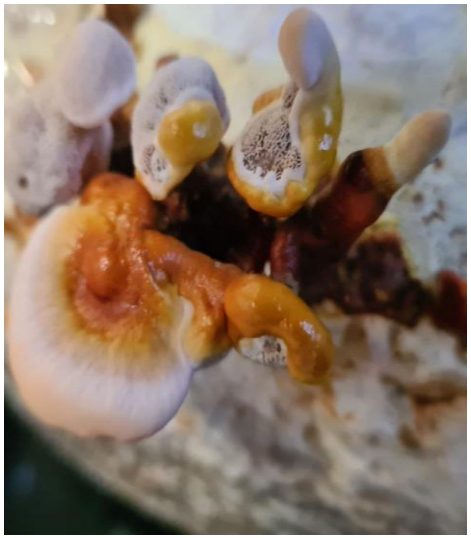


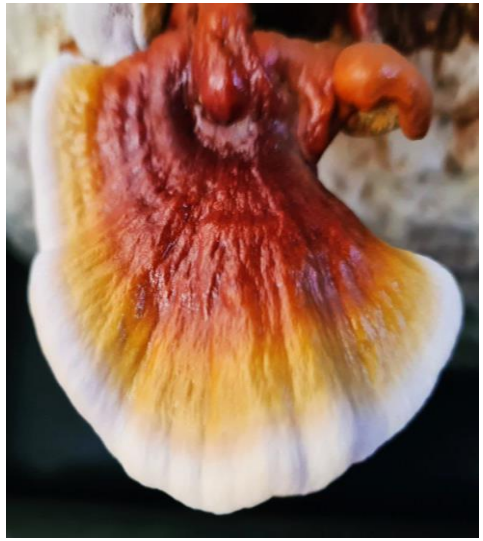



Ganoderma resinaceum






Measurement of oven dried

Ganoderma austroafricanum

Ganoderma resinaceum

Biochemical compounds analysis preparation