Degradation of glyphosate using environmental lignocellulolytic fungal isolates and extracellular enzymes

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ABSTRACT

Glyphosate is the most used herbicide in the world. Having been detected in food and water systems, glyphosate raises several concerns around food safety and its environmental impact. Recent studies have found an increase in environmental levels of glyphosate in agricultural soils as well as in food. Glyphosate is reported to be likely carcinogenic and potentially poses a great risk to human health. In this study, environmental fungal isolates capable of degrading glyphosate were chosen from a previous study, along with environmentally acquired fungal isolates. The study aimed to evaluate these fungal isolates as well as their extracellular enzymes for the degradation of glyphosate *in vitro*. Fungal species were isolated from soil samples (0.5 g) and sub-cultured until pure cultures were obtained. Isolates were grown in a liquid media consisting of brewery spent grain (BSG), yeast nitrogen base (YNB) and distilled water. The extracellular fluid was used to test the degradation of Roundup through thin-layer chromatography plates (TLC plates). Isolates capable of glyphosate degradation were sequenced and identified, tested in spiked microcosms and analysed using enzyme-linked immunosorbent assay (ELISA). The results showed *Penicillium adametzioides* to be the most promising isolate through screening tests (52.06% ± 3.96% degraded). *Trichoderma virens* showed the largest level of degradation in soil with 22.03% ± 6.88% glyphosate remaining after 10 days. Nutrient analysis of soil was used as an indication of potential degradation pathways the isolates could follow to build on ELISA and TLC tests. According to the pure Roundup degradation test *Cladosporium cladosporioides* (3.69% ± 1.07% glyphosate remaining) and *Aspergillus niger* (4.15% ± 0.18% glyphosate remaining) showed the best results in using Roundup as a sole source of nutrients. In conclusion, the fungal isolates in this study can potentially degrade glyphosate in soil, and some even use Roundup as the nutrient source. In future studies, the viability of these isolates should be tested in soil conditions and also against a range of different pesticides.

Keywords: Glyphosate, Roundup, Thin-layer chromatography (TLC), lignin, brewery spent grains (BSG), lignocellulolytic fungi, fungi, ELISA

DECLARATION

I declare that the dissertation submitted by me for the degree Magister Scientiae in Environmental Studies at the North-West University (Potchefstroom Campus), Potchefstroom, North West, South Africa, is my own independent work and has not previously been submitted by me at another university.

Signed in Potchefstroom, South Africa

Signature:

S. W. Combrinck

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CHAPTER 1 – INTRODUCTION

1.1 History of glyphosate

Herbicides have been a critical component in agriculture for many years to control unwanted weeds that negatively impact agricultural productivity. Weeds are any type of plant deemed undesirable for food production in cultivated areas that directly competes with plants for nutrients, sunlight, space and water. This competition is what causes weeds to be such a hindrance for people growing food and can lead to losses in food production (Chauhan, 2020). A study done by Karim *et al*. (1998) determined that Bangladesh loses an estimated $1 383 million per year or 9.66 million tons of crops per year at that time. More recently India's agricultural production losses over $11 billion each year due to the influence of weeds in the agricultural sector (Chauhan, 2020). The global impact of weeds is a massive hindrance in the agricultural sectors across the world. Although these losses are substantial, herbicides such as Roundup have been used since the 1970s to help increase the yields on food production and control weeds.

In the 1970s, Monsanto developed a herbicide compound known as Roundup. Roundup was introduced to the public in 1976, as a safer alternative to other herbicides of the time. Roundup promised a safe, easy and quick solution to weeds and grasses impacting food production without damaging any of the crops (Seralini, 2020; McHenry, 2018). This promise saw the number of people using Roundup rise rapidly and Roundup became the most used herbicide in the world with over 130 countries using Roundup (Cai *et al.,* 2020). The active ingredient of Roundup, which has made it such an effective product, is glyphosate.

Glyphosate was originally discovered by Swiss chemist Henri Martin, and because of the effectiveness of glyphosate, it became a staple of herbicides which led to an increase in production rates of up to 20% in average yield (McHenry, 2018).

1.2 Mechanism of glyphosate

Glyphosate is an herbicide that targets broadleaf plants and grasses (McHenry, 2018), but Monsanto developed glyphosate tolerant crops. Roundup does not kill such plants. It kills only the target weeds (Torretta *et al*., 2018). Roundup does this by using glyphosate which interferes with a plant's ability to produce essential proteins by inhibiting the function of the shikimic acid pathway (Feng *et al*., 2018). This pathway is a seven-step metabolic route that is present in various organisms, such as certain fungi, bacteria, protozoan parasites, algae and plants (Gomes *et al*.,

2015). In this context, the shikimic acid pathway is needed for the production of a variety of aromatic compounds that have been the focus of several genetic engineering projects (Brückner *et al*., 2018). The inhibition of 5-enopyruvyl shikimate 3-phosphate synthase (EPSPS) (Keeling *et al*., 1999) prevents the pathway from completing its cycle and the effect is the inhibition of protein production. Even though glyphosate has a fatal effect on plants as well as microbial species, certain microorganisms have been unaffected or evolved to exploit these compounds (Bai & Ogbourne, 2016). It was through these species that crops have been synthetically manipulated to resist the effects of glyphosate through either 1) the incorporation of a soil bacterial gene that can produce a glyphosate-resistant form of EPSPS or 2) the use of a soil bacterial gene that can produce the enzymes needed for glyphosate degradation (Han *et al*., 2017).

1.3 Risk of glyphosate

Although considered safe, glyphosate has recently been detected in various food sources (Smit, 2017). It is a concern that the use of glyphosate along with various other ingredients can cause food to become harmful (Gill *et al*., 2016). Potential side effects have become evident, such as skin irritation and burns, but only in very extreme cases, and as a result, glyphosate was never seen as a problem, until 2015. In 2015 the International Agency for Research on Cancer (IARC), which is linked to the World Health Organization (WHO), deemed glyphosate to be a possible carcinogenic product (a substance that can cause cancer) (Samsel and Seneff, 2015; McHenry, 2018; Kogevinas, 2019). This has raised concerns about the use of glyphosate as a herbicide, because glyphosate has been detected in water, soil and food in recent years (Lescano *et al.*, 2018).

In 2018, Bayer bought Roundup's mother company, Monsanto, for $63 billion. The reclassification of glyphosate as a possible carcinogenic has led to numerous court cases since 2015. Bayer settled an agreement in 2020 to pay $10.1 billion in court settlements for claims against Roundup and more specifically glyphosate (Centner, 2020). Though the court cases cost Bayer a lot of money, it still produces and sells Roundup.

1.4 Aims and objectives

This study aimed to evaluate lignocellulolytic fungal isolates from the environment and isolates from a previous study to degrade glyphosate using whole fungal isolates and their extracellular enzymes. This was achieved through:

- The screening and identification of fungal isolates with the ability to degrade glyphosate.
- Determination of the degradation rate and yield of glyphosate removal by selected fungal isolates.
- The establishment of microcosms to test glyphosate degradation in a simulated environment.
- The evaluation of degradation rates in pure Roundup.

CHAPTER 2 – LITERATURE REVIEW

Roundup refers to a broad-spectrum, systemic glyphosate-based herbicide (Kanabar *et al.*, 2021; Cai *et al*., 2020; Hosseinpour *et al.*, 2021). Monsanto developed Roundup and it became the most widely used herbicide worldwide (Centner, 2020). Even though the patent issued by the US patent office expired for the use of glyphosate as a herbicide in 2000, Roundup still contributed to roughly 10% of Monsanto's income by 2018. Because of the expiration of the patent, many companies have made their versions of glyphosate-based herbicides, but Roundup remains the dominant herbicide. Along with the herbicide, Monsanto has also released a line of varieties of Roundup products, including many glyphosate-resistant seeds and this constituted the largest source of income for Monsanto (Haugo, 2014; McHenry, 2018).

Although Monsanto initially claimed that Roundup was safe to use, in 1996 consumers suspected this might not be the case. Several lawsuits were filed against Monsanto for false advertising relating to the claims that Roundup was completely biodegradable and left no traces in soil. Later years showed that glyphosate, polyoxyethylene-alkylamine (POEA) and aminomethylphosphonic acid (AMPA), especially when used improperly, do accumulate in soil and can even accumulate in food sources to a lesser degree (Cai *et al*., 2020). Further lawsuits about false advertising were filed in France in 2001. In 2015 many lawsuits on the topic of the carcinogenic properties of Roundup were filed, accumulating in 125 000 lawsuits against Bayer (the newly acquired owners) concerning the safety of Roundup (Centner, 2020).

2.1 Roundup

Roundup is a type of herbicide that targets broadleaf plants and grasses that can interfere with crop productions. The main mechanism for this is through the inhibition of the shikimic acid pathway which stops the production of aromatic compounds and inhibits photosynthesis (Feng *et al.*, 2018). Due to the presence of glyphosate (the active ingredient in Roundup) in food sources, fears have grown over the safety of the herbicide, but companies that use glyphosate still insist that it is safe (Smit, 2017; Zhang *et al.*, 2019; Cai *et al*., 2020). It is feared that the use of glyphosate in Roundup along with various other ingredients could cause food crops treated with glyphosate to negatively impact human health (Gill *et al*., 2016; Kogevinas, 2019). The added ingredients in the Roundup formulation help the product to stick to the surface of broadleaf plants. A wide range of formulations is available today, depending on the needs of the application. Surfactants such as POEA may or may not be present, depending on the glyphosate formulation

and function. The function can differ based on the desired plant types (weeds, ferns, grasses, etc.). Originally added to aid in the application of Roundup, POEA is deemed toxic and can come into contact with aquatic environments where it can cause negative effects. Roundup also contains the isopropylamine salt of glyphosate as its active ingredient (Perego *et al*., 2017). Wetting agents can be added but are normally not included in general Roundup products, and if needed are often added. Thus, 10 ml of cooking oil may be added to the Roundup mixture before application, but most plants do not require a wetting agent.

2.1.1 Glyphosate

Glyphosate is the active ingredient in Roundup (in the form of "Isopropylamine salt of glyphosate") and the main reason behind the herbicide's effectiveness (Kanabar et al., 2021). This ingredient targets both broadleaf plants and grasses by inhibiting its metabolic pathways (as discussed in section 2.1.2) (Kanabar et al., 2021). If used on its own, glyphosate is not a selective herbicide and can kill most kinds of plant species (Alonso et al., 2011). The surfactant used in the original composition of Roundup is POEA, which has toxic effects on aquatic systems (Perego et al., 2017; Cai *et al*., 2020). This was allowed as very small amounts of Roundup entered aquatic systems. Roundup seemed to be environmentally friendly at the time, because, unlike other herbicides, Roundup did not wash away into the water systems, which made it very effective and a favourite among farmers. This was because of the added surfactant, POEA, which served the function of clinging to plant surfaces to avoid the herbicide from falling to the ground. This clinging action makes it possible for the active ingredient (glyphosate) to enter the and increased the efficiency of the product, by increasing the penetrability of the plant tissues (Székács *et al*., 2014). Monsanto also developed seeds that were resistant to the application of glyphosate (Carlson *et al*., 1997).

2.1.2 Mechanism of glyphosate

Glyphosate-based herbicides move systematically from a plant's leafy surface and enter the plant through its leaf cuticles. The herbicide struggles with waxy surfaces, however, and thus requires surfactants such as POAE to penetrate the protective layer. Once the plant has been penetrated, glyphosate interferes with a plants ability to produce essential proteins by interfering with the shikimic acid pathway (Feng *et al*., 2018). The shikimic acid pathway is a seven-step metabolic route that is present in various organisms such as certain fungi, bacteria, protozoan parasites, algae and plants (Fig. 2-1) (Gomes *et al*., 2015). Glyphosate can therefore have a profound effect on the microbial community in soil or water. This is essential as the specificity towards the

shikimic acid pathway removes the threat to mammals that consumed glyphosate due to their contact with minimal amounts of food produced in this way, although there may still be negative effects when the food is consumed in larger quantities. The shikimic acid pathway produces a variety of aromatic compounds required for amino acids synthesis in green plants (Bai & Ogbourne, 2016; Wood, 2019) and has been the focus of several genetic engineering projects (Brückner *et al*., 2018).

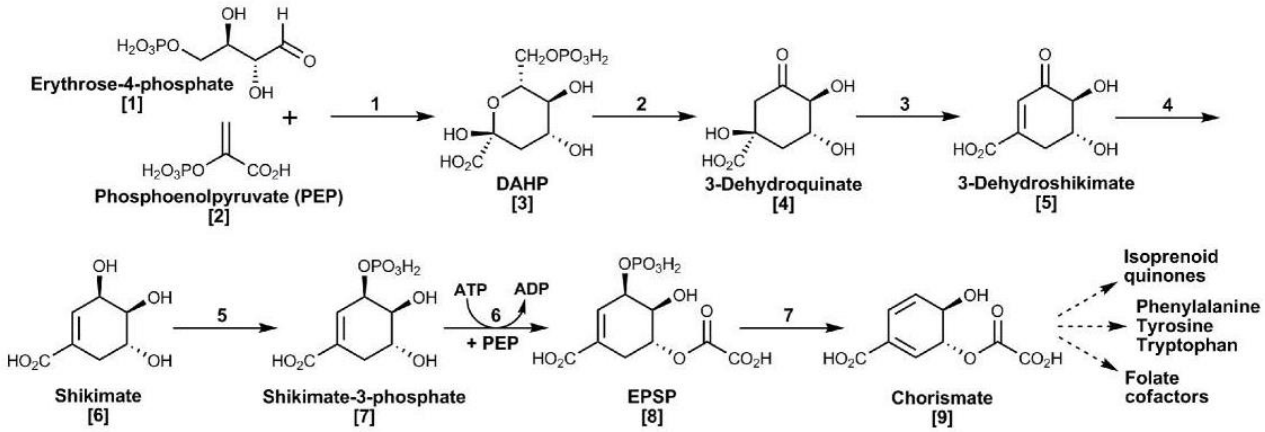

Figure 2-1: Schematic representation of the shikimic acid pathway (Zucko et al., 2010).

Glyphosate affects the shikimic acid pathway at stage 6 (Fig. 2-1). The inhibition of the 5-enopyruvyl shikimate 3-phosphate synthase (Keeling *et al*., 1999; Kanabar *et al*., 2021; Tyczewska *et al.*, 2021) prevents the pathway from completing its cycle and the effect is the termination of production of aromatic amino acids by the organism involved (Hosseinpour *et al*., 2021; Cai *et al.*, 2020). Glyphosate has a fatal effect on plants, while certain microorganisms are unafected (Bai & Ogbourne, 2016; Wood, 2019).

2.1.3 Effect of Roundup on humans

When Roundup encounters human skin, it can cause irritation when applied in high concentrations contrary to previous statements by the Environmental protection agency (EPA) and the National pesticide information centre (NPIC) (Costas-Ferreira *et al*., 2022; Madani & Carpenter, 2022). If consumed in high volumes it can cause membrane burns to internal organs and can be fatal if large amounts are ingested. The body will fight this by causing gastrointestinal symptoms, such as diarrhoea and vomiting (Bradberry *et al.,* 2004).

According to the EPA, glyphosate is safe to use when the instructions (as labelled by the manufacturer) are followed. However, in 2015 the WHO stated that glyphosate is a probable carcinogen (Tarone, 2018). This conflict in statements has led many to question the safety of glyphosate in relation to human health and as a result of this several court cases have been opened and have been successful against Bayer and Monsanto (Samsel and Seneff, 2015). The $2bn pay-out decreed by a California court was the third case to be won against the Roundup product (Staff, 2019). Other successful cases include the $78 million compensation paid to Lee Johnson who was diagnosed with non-Hodgkins Lymphoma (Taylor, 2019). Contrary to these court cases and despite mounting evidence, Bayer and the EPA have reported that glyphosate is not linked to cancer. Several other authorities also support the claims of the EPA, such as the Canadian health authorities and the European Food Safety Authority (EFSA) (Williams *et al*., 2016). The argument that glyphosate is associated with cancer has been argued against and even though glyphosate does cause mild effects, carcinogenic properties are being disputed (Williams *et al*., 2016; Williams *et al*., 2016). The main argument against the carcinogenic properties of glyphosate is the lack of plausible mechanism and inconclusiveness of studies (Greim *et al.*, 2015).

2.1.4 Environmental fate of glyphosate

Glyphosate can be broken down in the environment. If an organism can grow in the presence of glyphosate, the organism is considered to be glyphosate resistant. Glyphosate can also be actively broken down by certain organisms to be used as a source of carbon (C), phosphorous (P) or nitrogen (N) (Zhan *et al*., 2018) with glyphosates main degradation components being NH_4^+ (Ammonium) (also referred to as NH_3) and PO_4 (phosphate) Fig. 2-2.

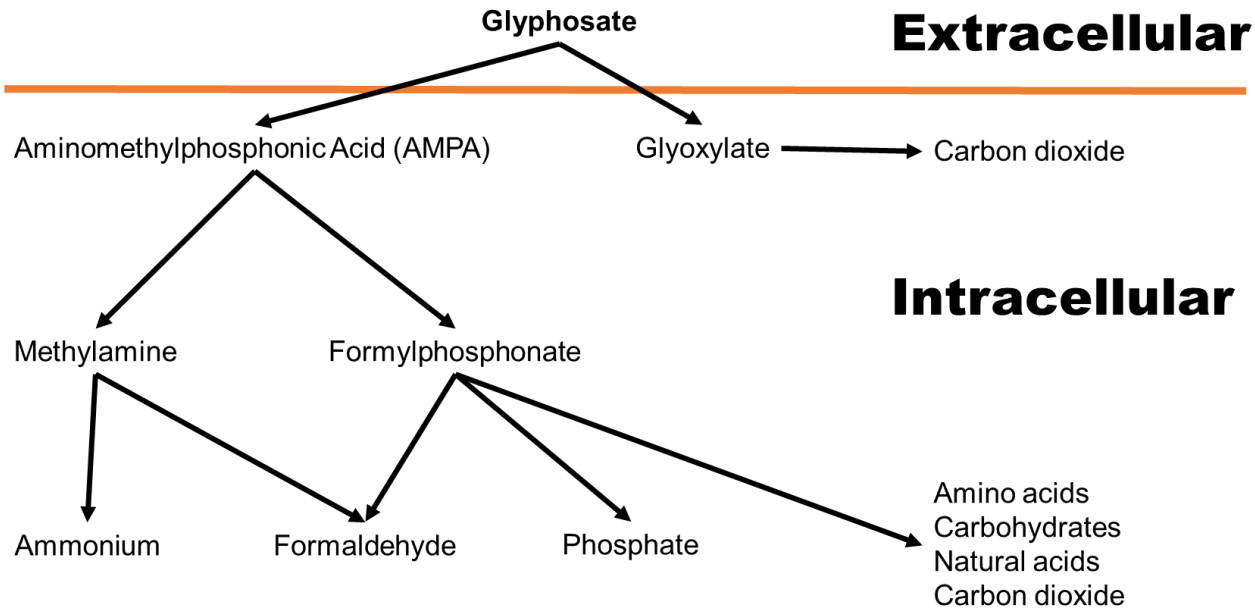

Figure 2-2: Breakdown of glyphosate to AMPA and further to compounds that can be used as building blocks by certain organisms (Schuette, 1998).

Figure 2-2 shows that degradation of glyphosate occurs intracellularly. Thus, glyphosate needs to be transported into a cell to be degraded (la Cecilia and Maggi, 2018). The extracellular enzymes as discussed in section 2.3 break the C-P bonds of glyphosate. Two pathways of glyphosate degradation occur ounce it is inside the cells of resistant organisms. The sarcosine pathway that can occur aerobically or anaerobically is the first degradation pathway which produces PO_4 as a degradation product (la Cecilia and Maggi, 2018). The AMPA pathway is the second degradation pathway that can occur only aerobically Fig. 2-3 (Lescano *et al*., 2018).

Glyphosate has active C-P bonds these allow glyphosate to be very resistant to acid or alkaline hydrolysis (Sviridov *et al*., 2011). Glyphosate is also a synthetic phosphonate compound that relies on the C-P bond for stability (Sun *et al*., 2019). This bond is also the focal point for glyphosate degradation. Glyphosate can be broken down by lignocellulosic enzymes (lignin peroxidase, manganese peroxidase, laccase and versatile peroxidase) to form intermediates and breakdown products (Lescano *et al.*, 2018; la Cecilia and Maggi, 2018). AMPA is a very characteristic intermediate of glyphosate hydrolysis. AMPA has properties similar to glyphosate (Wood, 2019), but has a higher toxicity level. It is also harder to degrade under environmental conditions (Sun *et al*., 2019).

2.2 AMPA as a degradation product of glyphosate

Glyphosate is typically degraded into AMPA and glyoxylate (Schuette, 1998). The glyoxylate is broken down to form CO_2 while AMPA can be degraded further through various metabolic processes (la Cecilia and Maggi, 2018). This is done aerobically Fig. 2-3. AMPA can be found in many different places other than glyphosates, such as cleaning products and artificial sweeteners (Struger *et al.*, 2015).

As a degradation product of glyphosate, AMPA is an aminophosphonate with a primary amine group and is considered a weak organic acid (Carretta *et al*., 2019). The toxicity of AMPA can be compared to glyphosate as it is of similar toxicological concern (Wood, 2019). *In vitro*, AMPA can be broken down through the use of manganese oxides. The problem with this approach is the fact that in soil conditions, manganese oxides are present in only trace amounts, which limits the level of AMPA degradation. Because of this, the more likely route of degradation is through microorganisms that utilise AMPA as a source of nutrients (Sun *et al*., 2019).

Much like glyphosate, AMPA clings to soil particles and with the displacement of soil (rain, crop rotations, wind, etc.), the soil and the AMPA ends up in water systems and have even been reported in wastewater in urban areas. It is possible to detect AMPA without the presence of glyphosate as was shown by Battaglin *et al.* (2014). This might be because glyphosate has been degraded to a point where it is no longer detectable. The introduction is mainly through cleaning products that spill into the environment, but because AMPA is harder to break down in soil (2 years in some cases) it can accumulate (Wood, 2019). Glyphosate on the other hand is rarely detected without the presence of AMPA (Lescano *et al*., 2018). Other glyphosate degradation products include sarcosine, glycine, methylamine, phosphonoformaldehyde, ammonia (NH_3) and phosphate ions (PO_4^{3-}) (Fig. 2-3).

The presence of AMPA indicates the possibility of glyphosate degradation along with the formation of glyoxylate (Simonsen *et al*., 2008). As glyphosate degrades, the amount of AMPA typically rise to a point and then decreases, as it is broken down further (Farragher, 2013). Glyphosate has a half-life of weeks to months and in some cases, years depending on the pesticide mixture, the surfactants used, the soil composition, microbial community, the rainfall, temperature and several other factors (Kanabar *et al.,* 2021). The concentration and amount of glyphosate deposited into the soil are directly linked to the half-life of glyphosate. AMPA, on the other hand, is much more difficult to degrade than glyphosate and thus has a much longer half-life (Samghani & Hossein, 2016; Zhang *et al.*, 2019). AMPA can also be used by organisms, but it tends to build up in the environment. When glyphosate and AMPA degrade they can release orthophosphates as soon as AMPA and glyphosate are degraded (Barrett and McBride, 2005).

Originally, it was believed that despite the high solubility of AMPA and glyphosate in water, very small amounts of AMPA and glyphosate enter into water systems because of the surfactant's ability to cling to organic and soil surfaces (Motekaitis and Martell, 1985). Despite this, it has been found that glyphosate can be remobilised, which causes offsite movement (Zhang *et al.*, 2019). This has led to several reports of the presence of glyphosate in water, as well as in food, bore holes and several other areas.

Glyphosate and AMPA have a strong capacity to bind to soil particles as well as the leaves of plants. By binding to these surfaces, glyphosate is absorbed rather quickly into plants through leaves and later through roots (Wood, 2019). Glyphosate has a high initial degradation rate that systematically reduces over time (Pizzul *et al*., 2009). Regular accumulation can occur in soil through constant application as well as high levels of metal cations. Cations can bind to glyphosate to form insoluble complexes. These complexes settle in soil or sediments and accumulation can occur in soil over time (Cakmak *et al*., 2009). The degradation rate is determined by the organic (microbes, plants, animals etc.) and the inorganic (soil, air, water, etc.) matter (Wood, 2019). It is this accumulation that has led to several plant species, considered to be weeds, becoming glyphosate-resistant. Microorganisms are an important part of the degradation process of glyphosate and this study will focus on fungal isolates and their ability to degrade glyphosate in soil.

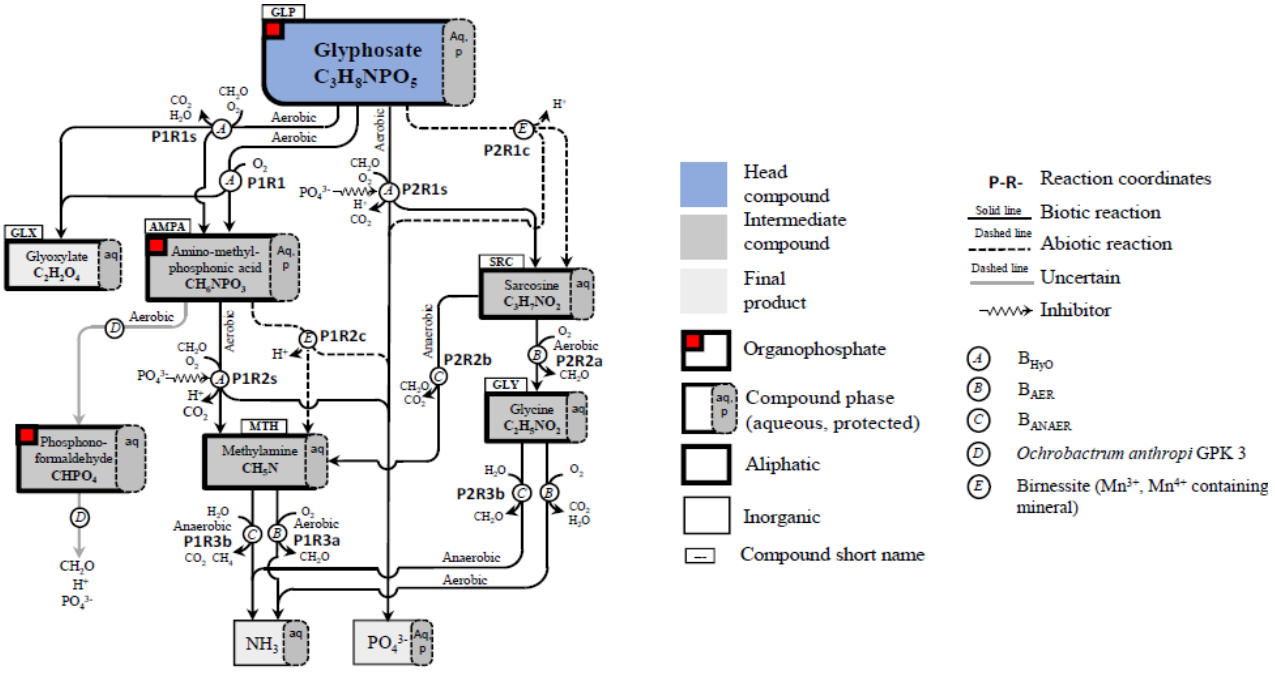

Figure 2-3: Degradation pathways of glyphosate in soil conditions (la Cecilia and Maggi, 2018).

Glyphosate can be degraded either aerobically or anaerobically in soil. This can lead to several intermediate compounds (AMPA, phosphonomethaldehyde, methylamine, glycine and sarcosine) as well as certain end products (phosphonate ions, ammonia and glyoxylate) (Fig. 2-3). The main by-product of this degradation pathway is CO_2 emissions as found in several aerobic and anaerobic steps (Fig. 2-3).

2.3 Enzymes and their role in the degradation of glyphosate

The enzymes targeting glyphosate for degradation are produced as extracellular enzymes. Microorganisms produce enzymes that cleave the C-P bonds which form sarcosine and glycine or the C-N bonds that form AMPA (Sun *et al*., 2019). Fungal species such as white-rot fungi (and several other fungal species and bacteria) have been known to produce the enzymes necessary for glyphosate degradation because of their ability to break down lignin, a polyphenolic compound in plant material (Pizzul *et al*., 2009). The main enzymes associated with lignin breakdown (by association with glyphosate) are lignin peroxidase (LiP) (EC 1.11.1.14), manganese peroxidase (MnP) (EC 1.11.1.13) and laccase (EC 1.10.3.2) (Saez-Jimenez *et al.* 2015). The mechanism for LiP involves oxidation of the heme iron by hydrogen peroxide. LiP forms FeIV=O which is a radial cation at the active site. In order to form a FeIV=O non-radial cation a single one-electron derived from a substrate which is followed by a second one-electron transfer to return it to a ferric oxidation state. After this, it can act on a wide variety of molecules which includes lignin and glyphosate as it can break down the large molecule into smaller molecules. It degrades in the presence of veratyl alcohol.

Versatile peroxidases (VP) work in synergy with LiP to also break down glyphosate (Shraddha *et al.,* 2011). Each enzyme has a unique mechanism of degradation, but they work together to successfully degrade glyphosate in a complex multienzyme system. VP combines with LiP (EC 1.11.1.14) and MnP (EC 1.11.1.13) and is able to oxidize phenols, hydroquinones redox potential dyes. The hybrid molecular architecture has multiple binding sites.

The above-mentioned enzymes are known as Class II Heme Peroxidases and are mainly produced by fungal and bacterial species. These are the main enzymes associated with lignin degradation and due to their non-specific nature, they can degrade glyphosate as well in a manner similar to lignin (Zámocký *et al*., 2015). All enzymes pertaining to this class of enzymes are known to be extracellular in nature (Hiner *et al*., 2002). This is because the structure of lignin is large,

and complex, and it must be degraded before it can be transported into a cell for further degradation.

Among the important enzymes are MnP and LiP. MnP and LiP cause the oxidation of lignin structures and act in synergy alongside laccase (Mishra *et al*., 2017) to effectively degrade organic components, while MnP acts upon phenolic compounds. It uses a complex Mn3+ that is released from the active site in the pressance of hydrogen peroxide to diffuse into the cell and degrade the phenolic compounds. It inactivates in the presence of veratyl alcohol. LiP acts upon the non-phenolic compounds (Fig. 2-4). Together these enzymes break down lignin successfully. A versatile peroxidase (VP) combines the catalytic qualities of MnP and LiP to break down phenolic- and non-phenolic compounds (Fig. 2-4) (Datta *et al*., 2017). The levels of AMPA can be measured to determine the success or failure of an organism's ability to degrade glyphosate. An accumulation of AMPA shows that glyphosate is degraded, but AMPA is much more difficult to degrade (Sviridov *et al*., 2011).

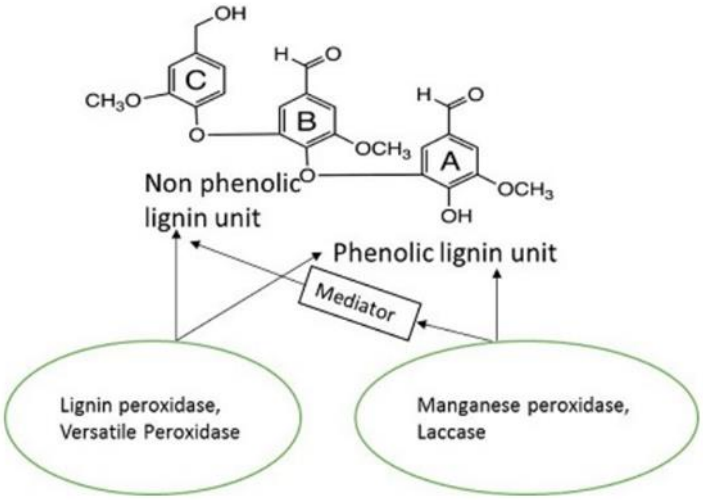

Figure 2-4: The activity of lignin degrading enzymes on lignin (Datta et al., 2017).

Laccase is widely found in nature and is also used in industrial applications (Couto and Herrera, 2006). Oxidation through laccase usually results in the loss of a single electron. An example of this is that when laccase interacts with lignin it catalyses the cleavage of two C-bonds by oxidizing one carbon atom and splitting the aryl alkyl bond. This releases a free radical, that can either undergo further oxidation or non-enzymatic reactions. This enzyme is produced by several bacterial (*Aquisalibacillus elongatus, Bacillus subtilis, Pseudomonas extremorientalis* - Chauhan *et al.* (2017)) and fungal (*Phanerochaete chrysosporium*, *Theiophora terrestris* - Datta *et al.*

(2017)) species. White-rot fungi, which are associated with lignin degradation have also been known to produce laccases along with LiP and MnP.

The production of laccase is triggered when the environmental nitrogen is low or depleted. This is simulated in this study by using a yeast nitrogen base (YNB) during the screening tests (Pizzul *et al*., 2009). Laccase production seems unaffected by the carbon-nitrogen ratio as several studies have found the production of laccase at both high and low concentrations. Temperature studies on laccase production suggest that the optimal temperature needed is 25 °C – 30 °C (Snajdr and Baldrian, 2007).

Table 2-1: Enzymes involved in lignin hydrolysis that has been shown to be active on glyphosate

Enzymes	***Description***	***General applications***	***Sources***
Lignin Peroxidase (LiP)	These enzymes use hydrogen peroxide-dependant oxidative degradation to degrade compounds. Mainly associated with lignin degradation, it is a key factor in glyphosate degradation through the presence of AMPA. It is a non-specific enzyme also associated with non-phenolic degradation.	Biorefinery dermatology, energy bioremediation, textile, cosmetology	(Falade *et al.*, 2016) (Pizzul *et al*., 2009) (Saez-Jimenez *et al*. (2015) (Falade *et al*., 2016)
Manganese Peroxidase (MnP)	MnP is involved in the breakdown of glyphosate It was found that it can also transform glyphosate on its own. As with LiP, the catalytic cycle is triggered by the binding of hydrogen peroxidase to the ferric enzyme where MnP acts as a mediator in the process to form an iron-peroxide complex. MnP is mostly associated with phenolic degradation.	Degradation of recalcitrant organic pollutants (chlorophenols, hydrocarbons etc.)	(Pizzul *et al*., 2009) (Hofrichter, 2002) (Saez-Jimenez *et al*. (2015) (Xu et al., 2017)
Laccase	Laccase can transform glyphosate, but only in the presence of substances such as $MnSO_4$ and Tween 80. This enzyme is mainly associated with white-rot fungi such as *Phanerochaete chrysosporium*. It is also known for direct phenolic degradation.	Detoxification of industrial effluents mainly in the paper, pulp and petroleum industries.	(Pizzul *et al*., 2009) (Shraddha *et al*., 2011) (Couto and Herrera, 2006)
Versatile Peroxidase (VP)	These enzymes are not well studied for glyphosate degradation; however, VP has a direct impact on lignin degradation. VP is known for non-phenolic degradation and also works with LiP, MnP and laccases.	Industrial biocatalyst, delignification processes	(Shraddha *et al*., 2011) (Busse and Czermak, 2016)

2.4 Fungal species and their role in the degradation of glyphosate

Although this study focusses on fungal isolates, there are several bacterial species that has been shown to degrade glyphosate very effectively (Hallas, 1992). These bacteria such as *Bacillus subtillis* (Yu *et al*., 2015)*, Rhizobium sp., Bacillus megaterium* and *Azotobacter sp* (Santos and Flores, 1995) have shown sigficant degradation over 30 – 60 days with over 90% degradation. Although this study is aware of the degradation properties of bacteria, the understanding of fungal species potential to degrade glyphosate remains limited. This study attempted to broaden the understanding of how fungal isolates can degrade glyphosate as well as the rate of glyphosate degradation.

Fungal species play an important role in degrading various organic materials found in soil. An example of this is how fungal species found in the gut of a dung beetle can assist in the degradation of plant material (Rudakiya and Gupte, 2019). Most of the degradation of plant material, especially hard material such as lignin (found in woody structures), is degraded using combinations of extracellular enzymes (Mori *et al*., 2019). Lignocellulosic fungi can degrade glyphosate through the extracellular enzymatic reactions used for the hydrolysis of plant material. The polymer of lignin is too big to be transported into the cell without it being partially degraded.

Glyphosate molecules have a weakness between the carbon-phosphorous (C-P) bonds Fig. 2-5 which are exploited by these extracellular enzymes. The breakage of these bonds allows microorganisms to utilise organophosphorus as a phosphorous source if the AMPA pathway is followed (Adelowo *et al*., 2018). If the Sarcosine pathway is followed, then glyoxalic acid and ammonia (NH_3) are formed (Jaisi *et al.*, 2016). The enzymes mainly associated with the degradation of glyphosate are laccases, manganese peroxidases and lignin peroxidases (Goodell *et al*., 2003).

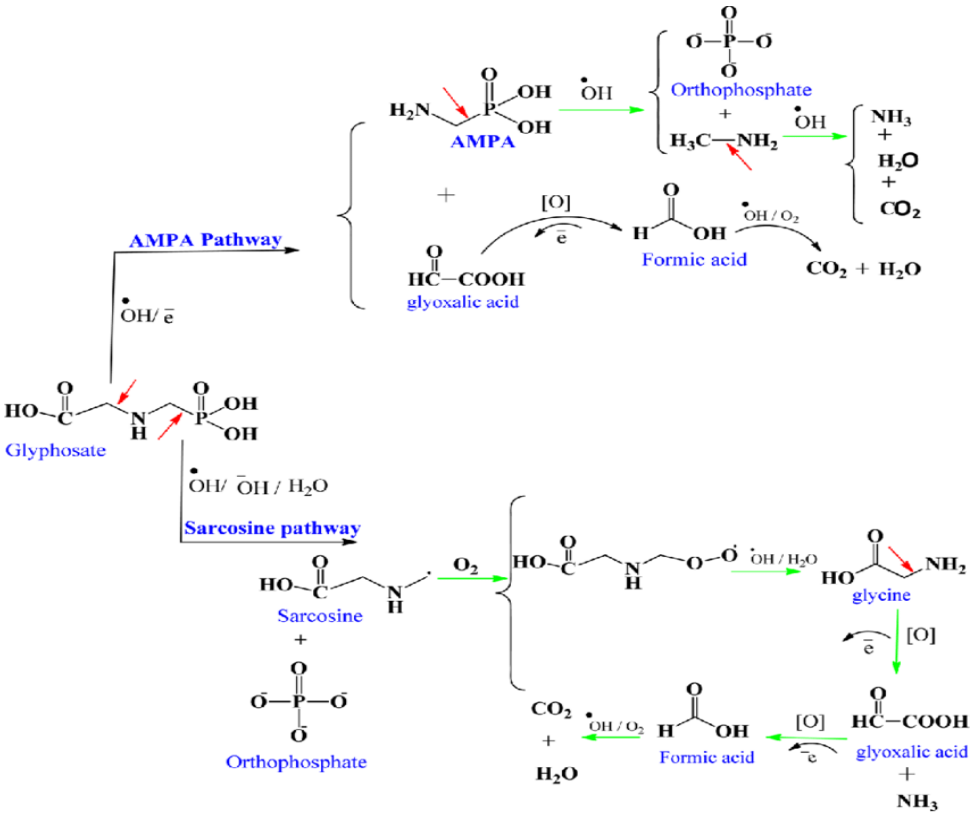

Figure 2-5: The two potential pathways where glyphosate degradation can occur. Weaknesses of C-N bonds and C-P bonds are indicated with red arrows and indicate the bond cleavage points (Jaisi et al., 2016).

2.4.1 Lignocellulolytic fungi

Degradation of lignin is mostly associated with white-rot fungi, but some brown-rot and soft-rot fungi also produce extracellular enzymes capable of oxidizing lignin and the other plant material components, cellulose and hemicellulose (Manavalan *et al*., 2014; Rudakiya and Gupte, 2019). When this group of fungi degrades glyphosate using extracellular enzymes, the degradation products are transported through hyphae and are then further degraded internally (Silva, 2017). Natural materials such as woody plants and fibres can be found in soil and are the inducers for the expression of extracellular enzymes. Brewery spent grain serve the same purpose in this project as it consists mainly of cellulose, lignin and hemicellulose (arabinoxylan) (Mussatto *et al*., 2006).

2.4.2 White-rot fungi

White-rot fungi are known to cause a bleaching effect on wood structures (Nakazawa *et al*., 2017). The reason for the bleaching effect is that white-rot degrades the lignin in the woody structures

without degrading most of the cellulose (Mori *et al*., 2019). Cellulose is thus left behind and leaves a white pigmentation on the wood structure. Lignin is classified as a biopolymer and as a result, it possesses multiple molecular configurations, but the main function of lignin is to serve as a strengthening element for wood. As the lignin is degraded by white-rot fungi, the wood becomes soft and almost sponge-like through the action of extracellular enzymes (Leonowicz *et al*., 1999).

Some white-rot fungi tend to be selective towards lignin degradation while others are non-selective. Fungal species such as *Phanerochaete spp.* are examples of non-selective species that secrete several peroxidase enzymes to perform multiple forms of degradation (Zhao *et al*., 2015; Datta *et al*., 2017). However, there are several other lignin-degrading species such as *Pleurotus ostreatus, Coriolus versicolor, Cyathus stercoreus* and *Ceriporiopsis subvermispora*. Some species attack lignin directly (*C. subvermispora, Phellinus pini, Phlebia spp*. and *Pleurotus spp*.) leaving behind enriched cellulose through delignification, while other species focus on attacking cell wall components (*Trametes versicolor, Heterobasidion annosum* and *Irpex lacteus*).

2.4.3 Brown-rot fungi

Brown-rot fungi target the structure of wood composed of cellulose and hemicellulose primarily (softwoods). These types of fungal species (such as *Fomitopsis palustris, Gloeophyllum trabeum, Lenzites trabea, Poria cocos, Aspergillus niger, Fusarium oxysporum*, etc.) are found mostly in wet environments and in some cases in soils, where they produce hydrogen peroxide (H_2O_2) to degrade cellulose (Mahmood, 2017). The H_2O_2 molecule is very small and because of this, it can diffuse rapidly through woody structures, which causes degradation to occur around the cell and not at specific regions (Castaño *et al*., 2018). A brown discolouration occurs during this rotting process along with cubical fractures (the wood breaks in small cubical structures). This type of rot, commonly referred to as dry rot, thrives in areas that contains large amounts of moisture necessary to this kind of rot process (Łucejko *et al*., 2018). Where white-rot forms, low yields of demethylated functional groups forms when while degrading lignin. Brown-rot creates extensive demethylation of lignin (Arantes *et al*., 2012). The third type of lignocellulosic fungi, soft-rot fungi, contains a different mode of action compared to brown- or white-rot fungi.

2.4.4 Soft-rot fungi

Soft-rot fungi are incredibly resistant to climatic conditions. They often persist to survive in regions that would be inhospitable to brown- and white-rot fungi. Soft rot is often associated with fruit decomposition, but it can also decompose cellulose structures in wood (Rajala *et al*., 2011). Soft-

rot fungi degrade cellulose structures in wood through extracellular enzyme production within the cells. The degradation of cellulose and hemicellulose has been scrutinised in previous studies such as a study by Falkoski *et al*.'s (2013) study on *Chrysoporthe cubensis* for lignocellulosic biomass degradation. One method in which soft-rot fungi can do this is through pH changes caused by the excretion of substances such as H_2O_2 from soft rot fungi. The change in pH allows exo-1,4-β-glucanases and endo-1,4-β-glucanases to activate and work more efficiently.

One noticeable trait of soft-rot fungal species is the resistance to biological attacks (Gao *et al*., 2018). Some biological attacks include tannin and suberin found in tree bark that acts as a barrier against fungal species such as white- and brown rot. Soft rot can overcome these defences (Anttila *et al*., 2013) and cause microscopic pores and cracking patterns in wood structures similar to brown-rot fungi, which cause the softening of wood (Schwarze, 2007). Soft-rot fungi such as *Chaetomium* spp*., Ceratocystis* spp*.*, and *Kretzschmaria deusta* often attack wood exposed to water, extreme heat or cold. Degradation products all have different compounds that form during the process of degradation. All of these compounds can be separated using chromatography. Many different types of chromatography can be used, but thin-layer chromatography was used in this study.

2.5 Effects of growth media

The growth media were specifically selected to grow fungal isolates, as was the case with potato dextrose agar (PDA). The growth of a wide variety of fungal isolates was essential to this study as many fungal species were isolated from soil sampled at the Agricultural Research Centre (ARC) in Potchefstroom. For these isolates to have been selected for further study, a growth medium was required that would not only offer nutrients for growth but also stimulate the release of extracellular enzymes (Rudakiya and Gupte, 2019). The growth media to fill this need was brewery spent grain (BSG) and yeast nitrogen base (YNB). Due to the nutrient makeup of BSG, it was the main ingredient in stimulating extracellular enzymes (Mussatto *et al*., 2006; Manavalan *et al.*, 2014), while YNB was used, due to its high nutritional value, to trigger the initial growth of the fungal isolate.

2.5.1 Potato dextrose agar

PDA is one of the most frequently used media to maintain fungal strains. PDA is also very versatile. Made from dextrose (which acts as a carbohydrate source) and potato infusion (Which provides a nutrient base) in water with added agar powder (solidification at 25 °C) it can grow

wide ranges of microorganisms such as fungi and bacteria (Song *et al.*, 2004; Latha *et al*., 2009). It can also be infused with many water-soluble substances such as antibiotics if the growth of bacteria is undesired (Beuchat, 1979). Since antibiotics have little to no effect on the growth of fungal species and are water-soluble, they were added to the PDA mixture to inhibit the growth of any possible bacteria.

2.5.2 Brewery spent grain and the yeast nitrogen base

Brewery spent grain (BSG) is a frequently used and abundant beer by-product. It is used in many applications, from pig feed to the rehabilitation of soils, baking or creating compost, or even as a medium for growing mushrooms (Lynch *et al*., 2016). In the brewing of beer, the grain is mashed and boiled and what is left is the spent grain. This grain contains many nutrients that can be utilised as the sources of nutrients. These nutrients are ash (2%-5%), lipids (10%), hemicellulose (20%-25%), cellulose (12%-25%), proteins (19%-30%) and lignin (12%-28%) (Lynch *et al*., 2016). It is the lignin and cellulose content that was of interest to this study. Lignin and cellulose have been studied to stimulate the release of LiP and cellulase. The same enzymes have previously been connected to the degradation of glyphosate (Mussatto *et al*., 2006; Manavalan *et al*., 2014; Rudakiya and Gupte, 2019). However, BSG alone might not cultivate all the fungal isolates without the addition of yeast nitrogen base (YNB). YNB served as an initial nutrient base as it consists of essential vitamins and inorganic salts. Mixed with the BSG, YNB served as the base growth for the screening tests (thin-layer chromatography) in this study. Therefore, the addition of BSG serves purely as a condensed biomass to stimulate the production of LiP, MnP, laccase and VP. Although it is known that fungi can grow on grains and BSG, studies to use it as a source of lignin to trigger enzyme production on fungal isolates related to glyphosate degradation have not been found at the time of writing.

2.6 Thin-layer chromatography

Although Gas Chromatography-Mass Spectrometry (GC-MS) is the primary test to determine glyphosate in soil, thin layer chromatography (TLC) was used as it is a quick, easy and rapid test. The speed and affordable nature of TLC makes it an ideal candidate to scan the degradation potential of a large range of isolates. GC-MS is a more accurate and sensitive test, making it better suited to low level detection of glyphosate.

TLC can be used to monitor the progress of reactions to identify compounds and to determine the purity of a substance (Skowron *et al*., 2018). TLC has been used for the detection of pesticides

in food. The size, non-volatile nature, high levels of polarity and several other factors makes detecting glyphosate and AMPA a challenge (Zhang *et al*., 2019).

TLC consists of two phases, namely a stationary phase (solid) and a mobile phase (liquid). The stationary phase is supported on a sheet of glass, plastic, or aluminium. A liquid sample is applied through spotting at the base of the plate and the mobile phase separates different compounds based on capillary action. The stationary phase utilises silica gel, cellulose or aluminium as an absorbent layer (Fried & Sherma, 1999). Different mobile phase compositions, as well as different stationary phases, are needed for different sample types to effectively separate the chemical elements (Klimek-Turek *et al.*, 2017).

The stationary phase consists of absorbent materials such as silica gel, aluminium oxide or cellulose. A sheet of glass, aluminium foil or plastic acts as a “backbone” for a thin coating of the stationary phase. This layer of absorbent materials (Kul’kov & Vorob’ev, 2017) can be used for solid-phase extractions (Medina & Unruh, 1995).

2.6.1 Stationary and mobile phases

The mobile phase involves a solvent mixture in which the stationary phase is placed. Capillary action absorbs the mobile phase and this action separates various compounds in the samples based on their chemical properties such as their polarity (Zhang *et al*., 2009). Various different mobile phases can be used to improve the separation of compounds (Sowa *et al*., 2018). Silica gel is a polar absorber with relation to the mobile phase. Water has a relative polarity of 1 compared to ethanol that has a relative polarity of 0.654 and acetic acid has a polarity of 0.648 Thus ethanol and acetic acid have similar levels of relative polarity. Glyphosate has been described as “very polar”, which contributes to its high solubility in water. It is because of these polarity levels that the mobile phase, as well as glyphosate, can be absorbed through the silica gel and the separation of the compounds can occur (Crisanto *et al*., 1994). A mobile phase can be any combination of liquid chemicals, although it is important to note how a mobile phase will affect a TLC plate.

Ultraviolet (UV) light is often used to identify different spots on TLC plates (Ko *et al*., 2014), but when spotting organic materials stained with ninhydrin it is not necessary. Organic materials combined with this stain reagent produces a coloured spot on the TLC plate (Akram *et al*., 2015). The mobile phase used in this study was a combination of ethanol (EtOH), H_2O and Acetic acid (80: 15: 5). This mobile phase was adapted from Babić *et al*. (2005) and separates compounds

on the principle of capillary action and the polarity of substances. The stronger a substance is bound to the solid phase (polar compounds), the slower it will move. Therefore, non-polar compounds will move faster than polar compounds.

2.6.2 Separation principle

Capillary action is the separation principle on which TLC is based (Sowa *et al.*, 2018). It separates compounds based on their adhesive and cohesive forces. This is a process where a liquid (mobile phase) can move through small pores in the stationary phase without external forces affecting the process (Zhang *et al*., 2009). Intermolecular forces between the stationary- and mobile phase to separate substances can then be identified through two methods:

1. Using a control and comparing its migration distance with the different compounds present in the sample.
2. Determining the *Rf* values for the various compounds in the samples as shown in equation 1.

$$\boldsymbol{Rf} = \frac{\boldsymbol{Rs}}{\boldsymbol{Rt}} \quad (1)$$

In equation 1, *Rt* is the total distance the mobile phase moved and *Rs* is the distance different compounds moved. This is useful if the capillary action is not symmetrical (Sajewicz *et al*., 2006).

The polarity of the stationary phase is very important for the separation of compounds. On silica gel, less polar substances tend to move further with the mobile phase than polar compounds. Compounds then move different distances on the TLC plates. The further a compound moves on the TLC plate, the higher the *Rf* values will be.

2.6.3 Ninhydrin

Ninhydrin (2, 2-dihydroxyindane-1, 3-dione) is a staining agent used for the identification of organic materials and ammonia, along with primary and secondary amines. Free amines are shown with a deep blue to purple colour (Ruhemann's purple) (Sinhababu *et al*., 2013). A common application for ninhydrin is in the detection of amino acids, amino sugars and various other organic materials (Churms, 2004).

Ninhydrin can hydrolyse most amino acids except proline. Most of the different amino acid identification is done based on colour differences that can range from a deep blue to purple to a dark red (Akram *et al.*, 2015) applied after separation on a TLC plate. AMPA shows a deep blue to dark purple colour without a baking process. To detect glyphosate with ninhydrin, it is necessary to bake the plate at 110 °C until glyphosate shows as a dark red to deep purple spot on the TLC plate (Xu *et al*., 2018).

2.7 Glyphosate enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) tests are commonly used in the medical field to detect illnesses such as the human immunodeficiency virus (HIV) by using anti-bodies. The glyphosate ELISA test modifies this principle in order to detect glyphosate. In order to study the degradation of glyphosate in soil, glyphosate was extracted from soil which were inoculated with fungal isolates and studied using glyphosate specific ELISA tests.

2.7.1 Microcosms

Microcosms are areas set up to replicate environments but also to keep constant many variables that would otherwise be impossible to control (such as wind, temperature, light, water content). In this instance, the microcosm replicated the agricultural soil in which glyphosate is found. Studying the degradation rates in soil means involves monitoring for selective pressures while eliminating some of the environmental variables (wind, rain, unwanted microbial activity etc.) (Barra Caracciolo *et al*., 2013; Orellana *et al*., 2019). Using microcosms eliminates many different variables but also reduces the possibility of cross-, aerial- or environmental contamination of microorganisms and aids in the reproducibility of tests (Barra Caracciolo *et al*., 2013; Mattana *et al.*, 2019).

2.7.2 Soil conditions

The soil for this project was a nutrient-rich soil that incorporated some twigs and leaves to simulate fertile soil for agricultural use. This soil is best suited for plants as it is high in natural carbon, nitrogen and phosphorous levels (C: N: P). Furthermore, twigs and leaves act as a lignin source (Wang *et al*., 2017) that allows the fungal isolates to best produce the extracellular enzymes

needed to degrade glyphosate. Lignin is also abundant in natural soils and can consist of up to 30% of soil organic carbon (Zobiole *et al.*, 2010). Lignin has been found in roots, stems and leaves of plants and is also responsible for the long term, stable carbon sources in soil (Rasse *et al.*, 2006). The high level of organic material in the composition of the soil simulates a cultivated agricultural topsoil layer.

2.7.3 Glyphosate ELISA tests

Enzyme-linked immunosorbent assay (ELISA) is best known for its role in the medical field. Commonly it is used to detect HIV as well as several other infectious conditions. Although it is seen as a rapid sample test (Selvi *et al.*, 2011; Vicini *et al.*, 2021) it is very sensitive to changes detected throughout the testing samples and can thus analyse both quantitively and qualitatively. The technique is known as a “wet lab” technique as well as a biochemical analytical assay (Selvi *et al.*, 2011). ELISA uses an analyte in a liquid sample during the analysis. The analysis is carried out using a spectrophotometer and is based on colour change by the analyte.

The reaction separates some components of a heterogeneous mixture by adding specialised components to a solid phase (as in section 2.6). These specialised components (rabbit anti-glyphosate antibody solution in this instance) are designed to signal the presence of specific components such as glyphosate. When the glyphosate is attached several steps occur to purify the sample. After purification, the sample can be analysed at wave lengths (450 nm) that are specific to the glyphosate-based sample tested based on enzyme-linked reactions.

CHAPTER 3 - MATERIALS AND METHODS

Fungi capable of growing on pre-treated thatch grass were isolated from the gut of dung beetles at the University of Limpopo, South Africa. All these isolates were identified and tested for their ability to break down lignocellulosic biomass. Twenty-four of the best lignocellulolytic fungi were kindly donated by researchers at the University of Limpopo and fifteen of these were used in the present study as well as several fungi isolated from an agricultural environment.

3.1 Isolation and preservation of fungal isolates

Fungal isolates were obtained using a composite sampling method of soil over three different fields from the Agricultural Research Centre (ARC) in Potchefstroom. Each field was treated for a year with several different glyphosate products leading to an accumulation of glyphosate in the soil. The soil was treated with glyphosate-related products as part of ongoing research conducted by the ARC in Potchefstroom and as such could not disclose the specific glyphosate products used. The large amount of glyphosate added to the soil increased the chances of finding glyphosate-resistant fungal species. Each field that were sampled were labelled using the following:

1) Composite samples were taken from field 1, 2 and 3 labelled as F1, F2, F3.
2) Soil (0.5g) was placed on a plate containing nutrient agar infused with 100 mg/L chloramphenicol.
3) Samples were taken from each plate to isolate the fungal species and marked as F1A, F1B, F2A, F2B etc.
4) This process was repeated until singular samples were isolated on individual plates.

Composite sampling was done by adding several small (2 grams each) volumes of surface soil (up to 10 centimetres deep) to a 100 ml bottle (sterilised beforehand). This was done by sterilising a spoon with a 70% (v/v) ethanol concentration, then disturbing the top layer and retrieving a spoon sized sample of soil and adding it to the 100 ml bottle. The composite sampling method gave a much broader area in which to isolate fungal isolates and also increased the chance of finding glyphosate-resistant fungal isolates (Sharma *et al*., 2021).

The fungal species were isolated using PDA and 0.5 g of soil per plate. The agar plates were prepared according to the manufacturer's instructions, with the addition of 100 mg/L chloramphenicol as antibiotic to avoid bacterial contamination on the agar plates. Incubation for

the PDA plates was between 25-30 °C with incubation periods largely dependent on the type of organism. The fungal growth was observed every 12 hours. As soon fungal growth started showing, the fungi isolates were transferred to a new agar plate until a pure culture was obtained. Yeast cells were incubated for 24 hours, whereas fungal isolates were incubated between 7 and 14 days due to differences in growth rate. After incubation, the PDA plates were sealed with parafilm and stored at 2 °C until needed. For long term storage, the fungal isolates were stored in glycerol as described by Jadhav *et al*. (2015).

3.2 Growth mediums

Potato dextrose agar (PDA) from Merck/Sigma-Aldrich was used in this study as the general growth medium. It was used to grow and isolate fungal isolates from the soil collected from the ARC. Chloramphenicol (100 mg/L) was added to the PDA plates to prevent bacterial growth and contamination. Yeast nitrogen base (YNB) (Thermo-fisher, Waltham, Massachusetts, United States) is a minimal, synthetic defined yeast media. YNB contains the elements needed for fungal species to grow apart from carbon, which in this case, is added in the form of brewery-spent grain (BSG) (Krzyściak, 2013). The main purpose of using BSG is to trigger the release of extracellular enzymes including lignin peroxidase, manganese peroxidase and laccase (Ravindran *et al*., 2018).

3.3 Screening tests

3.3.1 Growth of fungal isolates

The fungal isolates were placed in a medium composed of a yeast nitrogen base (3.04 g/L) dissolved in distilled water and 1.25 g BSG per flask. Erlenmeyer flasks (100 mL) were used for the liquid growth medium and each flask contained 25 ml of liquid medium in total, with a cotton ball as a plug for the flask and covered with aluminium foil. Each isolate was inoculated in triplicate, including controls and incubated at 30 °C. One set of controls consisted of three flasks without Roundup and fungal isolates and a second set of controls consisted of three flasks with Roundup (4%) but no fungal isolate. Seven fungal isolates were tested at a time, each grown for fourteen days, after which the culture supernatant was extracted.

3.3.2 Extracellular enzyme extraction and glyphosate degradation

Standard extraction of culture supernatant was performed in order to remove any unwanted solid material from the extracellular fluid (Lang *et al*., 1997). This prevented any unwanted spots on the

TLC plate and gave a clearer reading on glyphosate and the subsequent degradation (Asif & Hai, 2020). Culture supernatant was suspected to contain extracellular enzymes necessary to degrade lignocelluloses (as lignin was the primary nutrient available) and by association glyphosate (Rojas-Verde *et al*., 2010). After fourteen days of growth, 1.5 ml of supernatant was collected and used as a source of enzymes. Each supernatant sample was pipetted into an Eppendorf tube and centrifuged at 13 200 RPM for 90 seconds. After transferring the supernatant to new Eppendorf tubes (400 µl) without disturbing the pellet, the supernatant was centrifuged again as before. Once the supernatant was a clear fluid (without visual fungal matter) the 400 µl Eppendorf tube was inoculated with 16 µl of 5% Roundup concentration which provided the glyphosate source.

3.3.3 TLC plates

The Eppendorf tubes containing both the supernatant and Roundup mixtures were incubated at 37 °C for 48 hours, with samples being extracted (100 µl) at 0-hours, 12-hours, 24-hours and 48-hours. Each supernatant tube of 100 µl was spotted onto a TLC plate after the 48-hour incubation. The TLC plates from Merck (tlc silica gel 60 f254) were spotted with 3 µl of each supernatant mix (0-, 12-, 24- and 48-hours) as well as a 3 µl of 2%, 3%, 4% and 5% Roundup concentration. Roundup concentrations were diluted using distilled water and pure Roundup. Each spot was air-dried before the TLC plate was placed inside a 250 ml beaker containing the mobile phase and a petri dish was placed over the opening for the mobile phase to saturate the atmosphere in the beaker.

The mobile phase used was adapted from the mobile phase used by Babić *et al*. (2005). Babić's mobile phase consisted of ethanol: dH_2O: acetic acid (glacial) in a ratio of [6:3.5:0.5]. The mobile phase that was used in this study consisted of ethanol: dH_2O: acetic acid (glacial), but the ratio was changed to [8:1.5:0.5]. This ratio was used as it showed a better separation of glyphosate than the original mobile phase on TLC plates. After the TLC plates were allowed to separate the components of the supernatant mix, it was air-dried and stained.

A ninhydrin solution from Sigma-Aldrich was used as a colouring agent to stain and visualise the components on the TLC plate. Ninhydrin (0.2 g) was dissolved in a mixture of 0.5 ml acetic acid, 100 ml of n-butanol and 4.5 ml of dH_2O. The visualisation was done by covering the air-dried TLC plate with the ninhydrin solution and allowing it to air dry again. Once dry, the TLC plates were baked at 110 °C until glyphosate spots were visible, varying in colour from light pink to dark red. This was confirmed to be glyphosate spots as initial testing was done with a glyphosate dilution series in order to determine the effectiveness of TLC plates. AMPA was also tested, however the

extremely low concentration of AMPA present in the degradation of glyphosate did not show on the TLC plates and therefore, only glyphosate was measured.

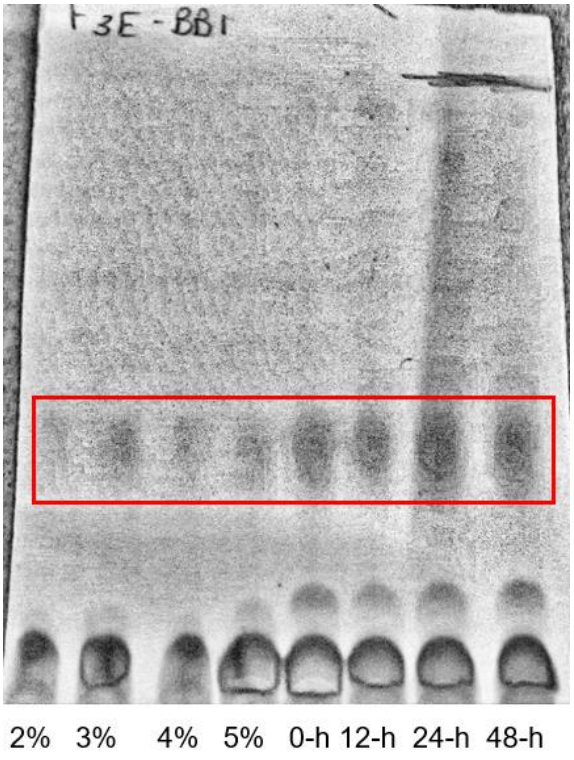

Figure 3-1: Visual image of the TLC plate after GIMP was used, 2%, 3%, 4%, and 5% Roundup dilutions were used for the calibration curve (Fig. 3-2), while 0-h, 12-h, 24-h and 48-h was used to determine the average degradation (Fig. 3-3)

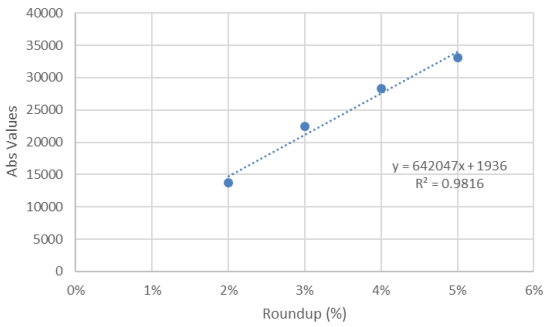

Figure 3-2: Calibration curve example of Roundup dilutions 2%, 3%, 4% and 5% on the TLC Plate (left)

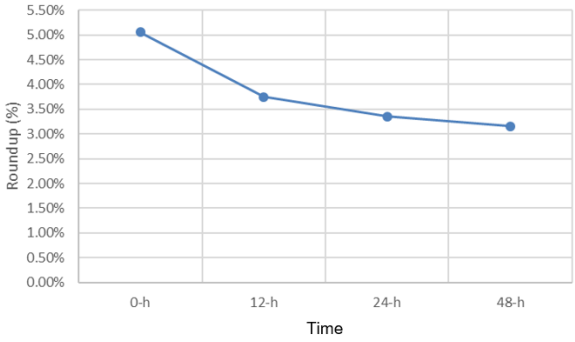

Figure 3-3: Average degradation of Roundup over 48-hours

The dilution of Roundup (2% - 5%) was used because of the linear nature that the results showed (Fig. 3-2). This made it possible to establish a calibration curve which could determine the minimum concentrations that TLC could detect a dilution. It was found that under a 2% Roundup concentration detection of glyphosate became difficult while 5% Roundup concentration was as per the supplier the highest recommended application concentration.

3.3.4 Analysis of TLC

The results were analysed using photographic methods. The images were desaturated (grey scaled), and the shadow levels enhanced using a GNU image manipulation programme called GIMP (https://www.gimp.org/). This was done to produce a highly detailed image in black and white to get a clear reading in the ImageJ programme as it can read only grey-scaled images. The shadow enhancement was done to have a clear reading with little possibility of misreading or

misinterpretation. After the TLC-plates were finished, baked and photographed, and grey scaled in GIMP, the photographs were loaded into ImageJ (Schneider *et al.*, 2012). ImageJ was used for densitometric analyses of photographs to determine the spatial and density fields of each glyphosate spot. The determination of the density fields of each spot yielded a numerical value (in other words the darker the spot, the higher the value, the higher the glyphosate concentration) which was entered into Microsoft Excel (MS Excel) to create a calibration curve which was used to calibrate the degradation rates of glyphosate by various fungal isolates (Fig. 3-1). A curve was created to show the stability of glyphosate under the conditions of the assay. In MS-Excel, the 2% - 5% dilutions of glyphosate were used to establish a calibration curve which was used to determine how much glyphosate was degraded over the 48 hours.

3.4 Molecular identification of fungal isolates

An internal transcribed spacer region (ITS) of the rRNA was used to identify fungal species. The results of these sequences were compared to the GenBank database to identify the isolates. The primers used were ITS 1 (5'–TCCGTAGGTGAACCTGCGG–3') for the forward primer and ITS 4 (5'–TCCTCCGCTTATTGATATGC–3) for the reverse primer (Bellemain *et al.*, 2010). All primers were acquisitioned from Inqaba biotec SA as standard primers.

3.4.1 DNA extraction

The DNA extraction method used was adapted from Hoffman & Winson (1987). Adaptations to the method were made by combining the above-mentioned with the nuclease tissue kit from Machery-Nigel, Germany. Culture broth samples (2 ml) were centrifuged for 90 seconds at 13 200 rpm and the supernatant was removed. The cell pellet was suspended in 500µL lysis buffer (100mM Tris HCL at pH8; 50Mm EDTA; 1% SDS, 10 µl Proteinase K) and incubated for 15 min at room temperature. Glass beads (200µL) were added and the suspension was mixed on a vortex shaker at maximum speed for 4 minutes and immediately placed on ice. The suspension was centrifuged at 13 200 rpm for 1 minute. The liquid phase was transferred to a clean microcentrifuge tube and 275µL of ammonium acetate (pH 7) was added before vortex mixing for 5 minutes. The reaction was incubated at 65 °C for 5 minutes before cooling on ice. 500µL of chloroform was added and mixed. The reaction was centrifuged at 13 200 for 2 minutes at 4 °C. The top layer was transferred to a clean micro-centrifuge tube and 750µL of isopropanol was added. The mixture was incubated at room temperature for 5 minutes. The DNA was further purified by a Nucleospin Tissue Kit (Machery-Nigel, Germany) – following the manufactures instruction from step 5.

3.4.2 Polymerase chain reaction and sequencing

DNA amplification primers, ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) were purchased from Inqaba Biotech, SA. Polymerase chain reaction (PCR) amplification was performed using the DreamTaq® Master Mix (Promega Corporation, Madison, WI, USA) protocol. The purchased primers were made up according to the manufacturer's instructions. Nuclease free water was used to adjust the final volume of the mixture to a total volume of 25µl. PCR was purified using 70% and 80% ethanol. PCR amplification was carried out using a PCR Systems Thermocycler (Applied Biosystems, Waltham, Massachusetts, United States). PCR included an initial denaturation of 94 °C for 1 minute and afterwards included 35 cycles consisting of 94 °C (denaturation) for 1 minute, 51 °C (annealing) for 1-minute 72 °C (elongation) for 1 minute. The final steps were 72 °C for 8 min and a storing step at 10 °C. PCR products were separated by electrophoresis on 2.5% agarose gels. The gels were photographed with a gel documentation system (Gel Doc 1000, BioRad) and the sizes of the PCR products were calculated by comparison with internal mol. wt standards (Siahmard *et al.*, 2017).

3.4.3 First clean-up for sequencing

PCR was added to a 96-well plate to remove primers and primer dimers. AMPure beads were used after it was vortexed to ensure homogenous dispersion. Twenty µl of AMPure beads was added to the PCR products in the 96-well plate and mixed thoroughly by pipetting up and down. The 96-well plate was incubated for 5 minutes at room temperature and placed on a magnetic stand. After the beads had been separated the supernatant was removed and discarded. The beads were washed by adding 200 µl 80% ethanol and incubated for 30 seconds at room temperature. The supernatant was removed and discarded. The beads were air-dried for 10 minutes on the magnetic stand. After they had been air-dried, the plate was removed from the magnetic stand and 52.5 µL of 10 mM Tris (pH 8.5) were added and mixed by pipetting up and down. The 96-well plate was incubated for two minutes at room temperature and placed back on the magnetic stand. After the beads had been separated by the magnetic plate, the 50µl supernatant was collected and placed in a sterile PCR tube.

The final preparation for sequencing PCR was done by using new PCR tubes. Four µl of 1:10 dilution Ready Reaction Premix (2.5x) was added, along with 2µL BigDye Sequencing Buffer (5x) and 1µl of ITS-1 forward primer (Inqaba Biotech, SA). The nuclease-free water (Fermentas Life

Sciences, US) and DNA template that were added varied based on the DNA concentration after the first PCR. The amount of DNA added was aimed at 20 ng by adapting the amount of nuclease-free water added to a final volume of the PCR tube was 20µl.

3.4.4 Second Cleanup

The Agencourt CleanSEQ dye-terminator removal protocol (Beckman Coulter) was used to purify the PCR products to prepare the DNA for sequencing. The only adaption of this protocol was at step 10 where the elution buffer was replaced with nuclease-free water. Amplicons were sequenced in-house, using an ABI 3130 Genetic Analyser (Applied Biosystems, UK). Chromatograms were obtained and viewed in Geospiza Finch TV software (version 1.4; http://www.geospiza.com/ftvdlinfo.html). The DNA sequence information was compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) to determine the identity of the amplified sequences (J Mol Biol, 1990).

3.5 Microcosms and ELISA

3.5.1 Microcosms

Numerous physicochemical properties of soils including phosphorus, total organic carbon, total nitrogen as well as several exchangeable cations, such as calcium (Ca^{2+}), magnesium (Mg^{2+}) and potassium (K^{+}), ammonium (NH_4^{+}) and phosphate anion (PO_4^{3-}) content, were measured". The goal of monitoring these levels was to monitor the effects of glyphosate degradation in the soil.

The soil used for the microcosm set was potting soil from Culterra (10g per sample, weighed and excessive foliage removed) that contained coconut fibre that acted as a lignin source. Because this study focussed on the possibility of fungal isolates to degrade glyphosate in soil conditions, the microbial community was removed through autoclaving. This was done to evaluate the effectiveness of the fungal isolates individually for ELISA testing purposes and to better monitor glyphosate levels.

3.5.2 ELISA soil sample extraction

The fungal organisms are listed in chapter 4 – Table 4-1. Soil of ten grams (10 g) were added to a 100 ml Erlenmeyer flask. Fungal organisms of 1 g were added, after they were identified and screened, where each isolate was added to a separate flask containing soil in triplicates. A plug was made by enclosing the opening of the flask with cotton in the flasks neck, covered with tightly

pressed aluminium foil. Five percent Roundup solution (5%) was added (1 mL) to the soil and left to incubate at room temperature in a dark environment (24 °C) for 10 days within an incubator. After 10 days of incubation 10 g of soil per flask was placed in a 50 ml Falcon tube and suspended in 20 ml of 40 mM sodium tetraborate, pH 8.5. The soil was vigorously shaken for 60 min and centrifuged at 3000 rpm for 20 minutes. The glyphosate extracted from the soil was then tested using the Glyphosate-ELISA tests. The soil was sent to Eco-Analytica Laboratory at the NWU (North-West University) and tested for soil nutrient content by using the 1:2 water extract analysis for all water-soluble elements. This was compared to soil before the addition of glyphosate, after the addition of glyphosate and after incubation periods.

3.5.3 Glyphosate-ELISA tests

The glyphosate ELISA test was carried out according to the manufacturer's instruction (Abraxis BioScience, Los Angeles, California). Each well of the test were prepared by adding 50 µL of the given derivatized standards, control or samples into the test strips. Antibody solution of 50 µL were added and the wells covered with tape and the contents mixed in slow circular motions for 60 seconds. After 30 minutes incubation 50 µL of enzyme conjugate was added to the wells. The wells were covered and mixed as before and incubated for 60 minutes. The tape was removed, and the contents decanted into a sink. The plate was inverted and blot dried. Strips were washed using diluted wash buffer that was diluted as instructed. The plate was inverted and blot dried. Substrate solution of 150 µL was added to each well, the covered and mixed as before and incubated for 20 – 30 minutes in a dark area. After incubation 100 µL of stop solution was added and absorbance was read at 450 nm using a microtiter plate ELISA photometer within 15 minutes of adding stopping solution.

Figure 3-4: Image of a completed glyphosate ELISA test prior to spectrophotometry at 450 nm

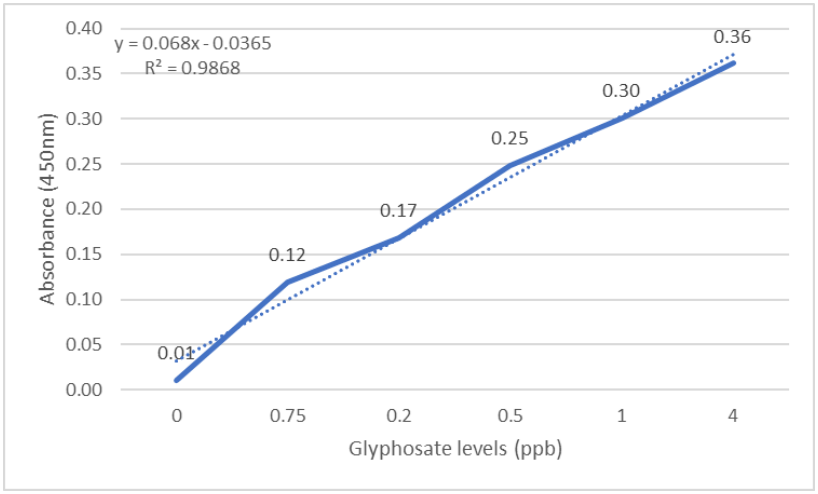

Figure 3-5: : ELISA test calibration curve used for comparison with incubated fungal isolates

3.6 Long-term Roundup degradation

Each flask was sterilised in an autoclave at 121 °C for 15 minutes and had a plug made from cotton and aluminium foil. For long term degradation, each isolate (1 cm^2 agar piece containing the isolate) was placed in a 100 ml flask of 6% Roundup concentration (50 ml) for 28-days at 25 °C. The purpose of this was to determine if the isolates can use the Roundup mixture as a nutrient source.

After the 28-day incubation period, the Roundup fluid was extracted (1 ml) in an Eppendorf tube. The tube was centrifuged at 13 200 rpm twice. Each time the top layer of fluid (the fluid above the formed pellet) was removed. The TLC plates were spotted (3 µl) along with the positive control of 6%, 5%, 4% and 3% Roundup that was used to establish a calibration curve. Analysis of the TLC plates was done as described in section 3.3.4.

CHAPTER 4 - RESULTS

Environmental soil samples collected at the ARC were spread on PDA containing chloramphenicol (100 mg/L) to inhibit bacterial growth while promoting fungal growth. The isolates were transferred to fresh PDA plates until pure colonies were obtained. All isolates were screened for glyphosate degrade as described above (section 3.3). The isolates were separated into 3 groups based on their ability to break down glyphosate, group 1 less than 10% hydrolysis (not studied further), group 2 10%-25% hydrolysis (isolates were identified, but not used in microcosm studies) and group 3 more than 25% (isolates identified and evaluated with ELISA in microcosms study).

The ITS gene regions were used to identify isolates from group 2 and group 3 using PCR with ITS-1 for the forward primer and ITS-4 for the reverse primers. The DNA was acquired and compared using the NCBI database to achieve the identification of the fungal isolates. Genetic information was compared using MEGA X to create a phylogenetic tree. The phylogenetic tree compared the isolates in terms of how closely their genetic material matched.

Isolates from group 3 were inoculated in triplicate for the microcosm studies described in Table 4-3 After inoculation the microcosms were incubated for 10 days at room temperature and glyphosate was extracted (as described in section 3.5.3) and analysed using a calibration curve as shown in Fig. 4-3. The ELISA test results are summarised in Table 4-3. ELISA test results were then compared to the changes in soil contents (specifically NH_4^+ and PO_4^{3-}) with comparisons being made between soil that contained Roundup and soils that contains both Roundup and an isolate, to determine if an isolate was capable of degrading glyphosate. The isolates were finally incubated for one month in a strong (6%) Roundup solution with distilled water to determine if the isolates could use Roundup as a nutrient source (through measurement of glyphosate as described in section 3.3.3).

4.1 TLC plate screening test

All the isolates purified from ARC soil samples are listed in Table 4-1. All of these were tested for their ability to break down glyphosate using the TLC method described in section 3.3.3.

Table 4-1: Fungal isolates from agricultural soil tested for glyphosate degradation using a Roundup dilution (%) with TLC-plates over 48 hours.

Isolate ID	12-h (%)	24-h (%)	48-h (%)
18	28.36 ± 2.74	41.15 ± 5.62	52.06 ± 3.96
F3B-BC	12.50 ± 7.15	35.41 ± 15.38	45.96 ± 8.79
8	20.80 ± 5.78	35.21 ± 3.84	45.22 ± 1.18
16	22.25 ± 3.14	35.73 ± 5.11	43.49 ± 5.31
22	14.35 ± 7.41	33.78 ± 10.89	40.85 ± 8.16
F3B-EE	25.92 ± 2.82	33.42 ± 6.67	37.34 ± 9.21
11	10.97 ± 4.08	25.60 ± 2.44	36.45 ± 2.78
10	4.47 ± 7.08	9.49 ± 3.90	32.32 ± 2.09
F1U-B	18.68 ± 6.43	24.76 ± 3.33	28.25 ± 3.47
25a	7.74 ± 5.42	13.47 ± 2.42	22.01 ± 2.07
8C	18.46 ± 1.66	20.91 ± 2.84	19.27 ± 2.09
F1A - AB	10.49 ± 6.18	12.09 ± 5.84	19.05 ± 9.36
13	25.42 ± 4.96	10.99 ± 6.03	16.71 ± 8.34
10B	18.62 ± 8.04	10.65 ± 3.26	14.52 ± 7.84
26B	3.13 ± 2.43	5.98 ± 2.44	14.16 ± 3.17
25b	15.34 ± 4.28	14.82 ± 8.44	12.36 ± 9.74
F1I-B	4.60 ± 4.07	1.04 ± 4.12	10.90 ± 8.31
17	11.01 ± 5.17	9.25 ± 3.54	8.82 ± 2.76
23	2.71 ± 12.53	3.76 ± 15.76	6.36 ± 12.80
30	5.17 ± 3.15	0.83 ± 4.96	4.00 ± 0.84
21	3.49 ± 2.00	6.26 ± 2.49	3.74 ± 2.71
52	1.61 ± 10.43	2.52 ± 10.58	2.18 ± 10.96
6	0.23 ± 5.78	-0.76 ± 4.96	2.17 ± 6.15
50	0.83 ± 3.75	3.01 ± 3.77	2.13 ± 3.16
3a	3.33 ± 2.62	2.00 ± 2.54	0.53 ± 0.82
F1H - B	-3.28 ± 2.11	-2.36 ± 1.52	-1.62 ± 2.72
31	-0.55 ± 2.85	0.22 ± 0.94	-1.87 ± 3.01
5a	-6.86 ± 1.51	-3.03 ± 3.29	-9.58 ± 6.36
2c	-10.42 ± 5.97	-11.02 ± 12.39	-13.30 ± 7.41
14a	0.17 ± 11.07	-1.84 ± 3.69	-14.05 ± 12.80

Nine of the soil isolates (Table 4-1 and Fig. 4-1) were able to degrade more than 25% (marked in green) of the glyphosate in the medium within 48 hours. Isolate 18 was the most effective, being capable of breaking down 52.06% ± 3.96% of the glyphosate present. These fungal isolates were

identified using the ITS primers and further evaluated using ELISA tests to determine degradation of glyphosate in soil and were classified as group 3, as described previously.

An additional 8 isolates (25a, 8C, 10B, 13, F1A-AB, 26B, 25b and F1I-B) were able to degrade between 10%-25% of the glyphosate in the medium Fig. 4-1. These isolates were only identified using ITS primers, but not used in ELISA studies due to their low degradation values. The purpose of the identification of these 8 isolates was to allow for comparison with other studies as potential degraders of glyphosate.

Isolates that degraded less than 10% of glyphosate within 48 hours were not studied further. The following isolates showed negative degradation (experimental error) values 31, F1H-B, 5a, 14a and 2c. The reason for this can be attributed to a combination of human and experimental error and they were thus discarded as zero values. The fungal isolates used in the study showed the following results (Fig. 4-1 and Fig. 4-2) in the screening test:

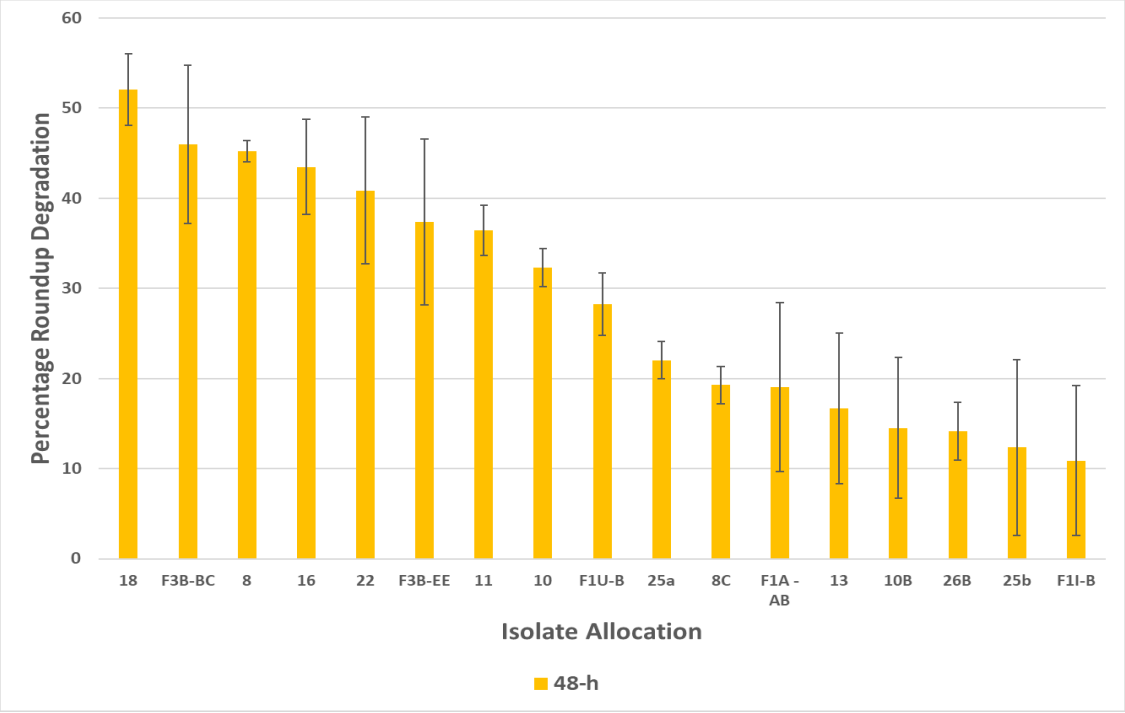

Figure 4-1: Glyphosate degradation over 48-hours estimated through TLC. The ability to degrade glyphosate was divided into three categories. Two categories were displayed on Fig. 4-1 (> 25% and 10% - 25%). Degradation levels: >25% good degradation; 10%-25% intermediate and <10% non-degrading (Fig. 4-2)

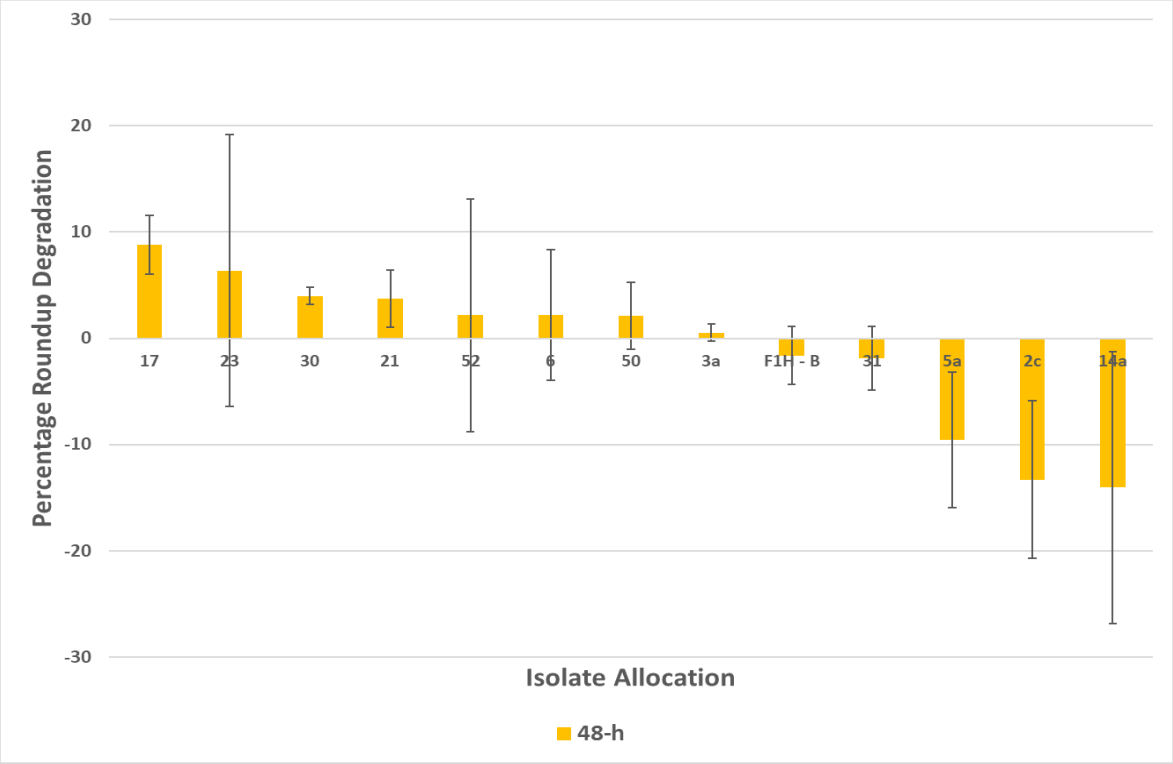

Figure 4-2: Glyphosate degradation over 48-hours estimated through TLC. Contains 13 isolates that were under 10% degradation and were not used further in this study and thus discarded as non-viable isolates.

Several fungal isolates were classified as good: *18*, *F3B-BC*, *8*, *16*, *22*, *F3B-EE*, *10*, *11* and *F1U-B*. Of these, the following were of a previous study: *18, 8, 16, 22, 10* and *11*. The following were environmental samples collected from the ARC: *F3B-BC, F3B-EE* and *F1U-B*.

The following isolates were considered intermediate: *25a, 8C, 10B, 13, F1A – AB, 26B, 25b* and *F1I-B*. Of these isolates, the following were from a previous study: *8C, 10B* and *13*. The following were environmental samples collected from the ARC: *25a, F1A-AB, 26B, 25b* and *F1I-B*.

The following isolates were considered non-degrading: *17, 52, 23, 30, 21, 50 (F3E-C), 6, 3a, 31, F1H – B, 5a, 14a* and *2c*. Of these isolates, the following were of a previous study: *17, 6, 3a, 5a, 14a* and *2c*. The following were environmental samples collected from the ARC: *52, 23, 30, 21, 50 (F3E-C),* 31 and *F1H – B.* All isolates that showed more than 25% degradation over 48 hours was used in the microcosm tests. Any deviations on the degradation values that differs from the control value can be attributed to deviations within the TLC plates which can cause data deviations.

4.2 Molecular identification

The ITS region was amplified from genomic DNA extracted from each fungal isolate before SANGER sequencing. The sequenced data were analysed as FASTA data using Finch TV. The FASTA files were used to BLAST the genomic DNA to identify the isolates. Using MEGA X, the identified genomic DNA of each isolate was compared in a phylogenetic tree to establish DNA similarities. After the DNA was analysed, the results were recorded in Table 4-2. Only intermediate- and good glycogen degrading fungal isolates were sequenced.

Table 4-2: Molecular identification of fungal isolates that showed glyphosate degradation above 10%. The NCBI strains indicate the closest association strain to the isolate used (and is thus used as an indication of similarity not particular strain)

Strain	Closest identification	NCBI Strain	Query cover	E value	Percentage Identified
18	*Penicillium adametzioides*	MH777425.1	98%	2.00E-164	100%
F3B-BC	*Trichoderma virens*	MT530036.1	100%	0	99.25%
8	*Mucor circinelloides*	MN452824.1	100%	0	100%
16	*Rhizopus microsporus*	MG250464.1	100%	0	100%
22	*Hypocrea virens*	GU365894.1	100%	0	100%
F3B-EE	*Aspergillus niger*	MT609916.1	98%	0	96.83%
11	*Trichoderma harzianum*	MT584872.1	100%	0	100%
10	*Trichoderma harzianum*	MT613650.1	98%	2.00E-155	98.17%
F1U-B	*Cladosporium cladosporioides*	MN341230.1	100%	5.00E-157	100%
25a	*Trichoderma harzianum*	AF030395.1	100%	4.00E-171	99.70%
8C	*Penicillium glabrum*	MT582777.1	100%	0	100%
10B	*Trichoderma harzianum*	MN644782.1	99%	0	98.92%
13	*Penicillium glabrum*	MT582777.1	100%	2.00E-174	99.43%
F1A - AB	*Aspergillus oryzae*	MT529829.1	100%	1.00E-120	91.35%
26B	*Penicillium spinulosum*	KF588647.1	100%	3.00E-18	97.08%
25b	*Trichoderma harzianum*	MG575471.1	100%	0	99.27%
F1I-B	*Trichoderma simmonsii*	MT613650.1	100%	0	99.72%

After each of the isolates had been identified in Table 4-2, the genetic DNA was compared using MEGA X with a bootstrap value of 1 000. The comparison presented in Fig. 4-3 showed the correlation between DNA obtained from SANGER sequencing of base pairs (bp) between 350 bp and 650 bp.

4.3 Microcosms

For the microcosm tests only the 9 isolates which showed degradation levels >25% (Group 3) in the initial screening tests were selected for testing. Microcosms were established by adding fungal isolates to potting soil. Potting soil was chosen as it contains coco peat and bark that provides lignin as a nutrient source, which improves the production of extracellular enzymes (LiP, MnP, laccase and versatile peroxidase) linked to glyphosate degradation. Fungal isolates were grown, and spores were added to autoclaved soil. The soil was prepared with a 5% Roundup concentration and soil nutrients were measured both before isolates were added to the soil and compared to the soil after the isolates have been added to determine the effect of the fungal isolates and Roundup with regards to changes in glyphosate levels and changes in nutrient levels. Table 4-3 shows the total amount of glyphosate left after 10 days of incubation.

Table 4-3: Total amount of glyphosate left after 10 days incubation

Identification	**Total glyphosate left**
Positive control	100.00% ± 8.18%
Cladosporium cladosporioides	95.23% ± 6.20%
Mucor circinelloides	66.13% ± 15.86%
Trichoderma harzianum (MT584872.1)	58.31% ± 8.67%
Trichoderma harzianum (MT613650.1)	49.72% ± 11.32%
Penicillium adametzioides	35.78% ± 4.04%
Aspergillus niger	32.48% ± 13.98%
Rhizopus microsporus	25.48% ± 8.07%
Trichoderma virens	24.66% ± 14.29%
Hypocrea virens	22.03% ± 6.88%
Negative control	27.25% ± 3.78%

The positive control refers to a soil that has been inoculated with glyphosate from Roundup but has not been given time to degrade. Glyphosate levels were highest in the positive control and were used to compare the changes in glyphosate concentration in soil with the glyphosate concentration of the other isolates. The closer the isolates came to having glyphosate levels the same as the negative control (no added glyphosate), the more glyphosate was degraded. Therefore, for this study, it was optimal for the isolates to be closer to the negative control, as it showed higher degradation levels.

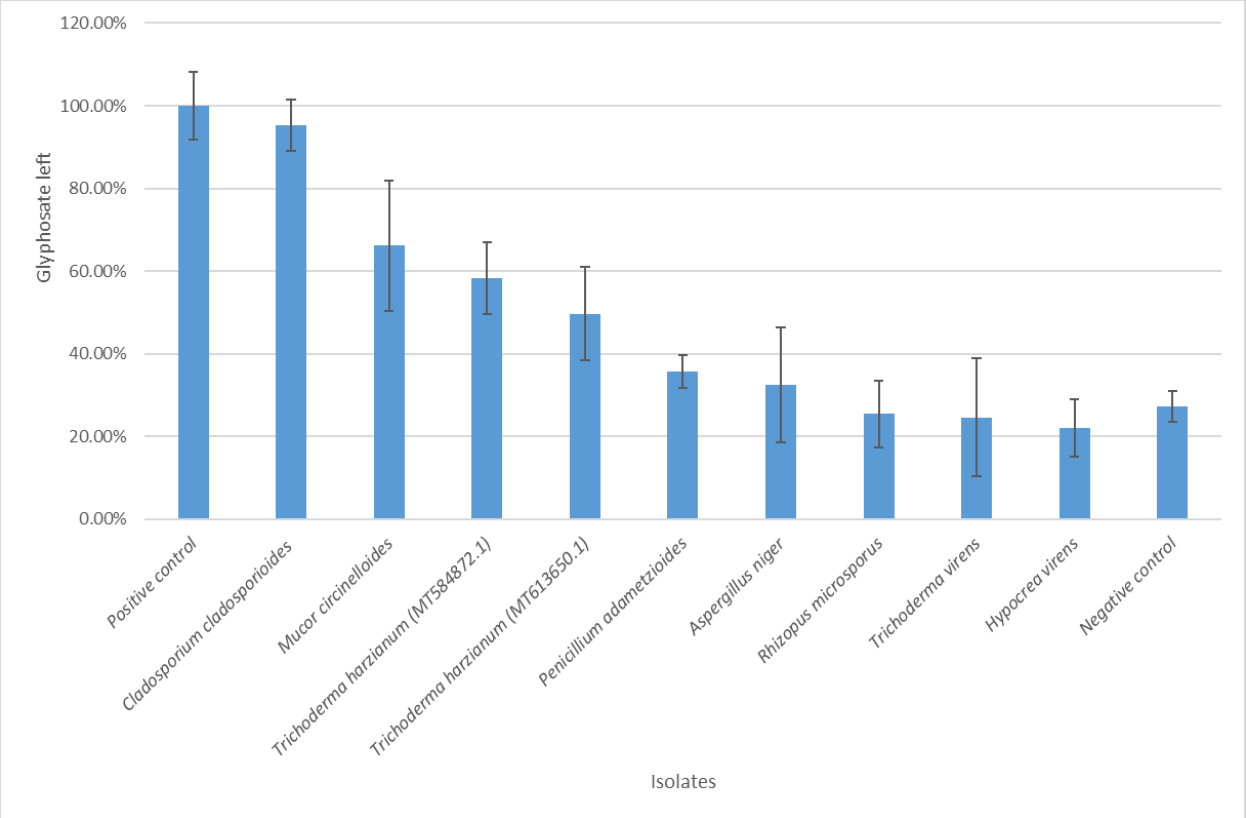

Figure 4-3: Degradation of glyphosate in soil tested with ELISA compared to positive control which had the highest level of glyphosate as no degradation took place and the negative control which had no added glyphosate.

C. cladosporioides had 95.23% ± 6.20% glyphosate levels left showing very little degradation (Fig. 4-4). *M. circinelloides* had more glyphosate left (66.13% ± 15.86%) but failed to degrade more than half the glyphosate levels. Both the *T. harzianum* (MT584872.1 and MT613650.1) isolates had glyphosate levels of 58.31% ± 8.67% (MT584872.1) and 49.72% ± 11.32% (MT613650.1) left showing degradation with both isolates. *P. adametzioides* had 35.78% ± 4.04% glyphosate left and *A. niger* had 32.48% ± 13.98%. Both these isolates degraded most of the

glyphosate levels and showed significant degradation levels. *R. microsporus, T. virens* and *H. virens* showed the highest levels of degradation and as such only had 25.48% ± 8.07%, 24.66% ± 14.29% and 22.03% ± 6.88% of glyphosate remaining. This is very comparable with the negative control (27.25% ± 3.78%) which had no added glyphosate to the soil.

4.4 Changes in soil nutrients

Changes in the soil levels were monitored to determine changes in the nutritional levels of soil as Roundup is degraded. Samples were taken before and after Roundup was added to determine the changes in soil nutrient levels such as Calcium (Ca_2^+), Magnesium (Mg^{2+}), Potassium (K+), Phosphate (PO_4^{3-}), Ammonium (NH_4^+), the amount of nitrogen (%N) and the carbon contents (%C). The general trends were determined and are shown in Table 4-4 on the levels of degradation.

Table 4-4: Fungal isolates degradation of soil nutrients.

Fungal isolate	Ca (mg/L)	Mg (mg/L)	K (mg/L)	PO_4 (mg/L)	NH_4 (mg/L)	LECO (%N)	LECO2 (%C)
Negative control	13.25	15.62	116.2	9.84	0.16	0.14	31.8
Positive control	30.21	30.54	285.7	61.75	340.87	0.45	33.15
Penicillium adametzioides	28.71	29.59	264.1	50.57	405.28	0.45	39.25
Cladosporium cladosporioides	34.41	34.8	270.6	61.95	856.89	0.45	34.94
Trichoderma virens	33.64	33.71	251.8	62.57	867.30	0.34	32.64
Hypocrea virens	39.49	39.1	316.4	67.75	717.80	0.49	38.07
Rhizopus microsporus	43.06	43.47	347.4	68.42	774.86	0.64	34.31
Aspergillus niger	42.89	42.1	310.9	69.91	970.78	0.45	33.83
Trichoderma harzianum (MT613650.1)	41.41	42.08	317	71.96	906.57	0.6	37.53
Mucor circinelloides	33.69	33.7	306.6	74.2	331.39	0.18	33.1
Trichoderma harzianum (MT584872.1)	47.88	47.49	334	75.19	982.58	1.04	67.09

Table 4-4 and Fig. 4-5 to 4-8 illustrate the changes in soil nutrients. Changes in soil nutrients were used to identify the potential of fungal isolates to degrade glyphosate introduced in soil, through cross-examination of the results obtained from using ELISA tests. Cross-examination was done by using the values shown through ELISA of glyphosate degradation along with the soil nutrient values (specifically NH_4 and PO_4). As shown in section 2.1.4 Fig. 2-2 and section 2.2 Fig. 2-3 NH_4 and PO_4 are known products of glyphosate degradation. Therefore, the more glyphosate was degraded by an isolate as shown by ELISA, the higher the levels of NH_4 and PO_4 should be. The negative control had no Roundup added to the soil sample along with no isolate, and the positive control had Roundup of a 5% concentration added and no isolate. Thus, the negative control was "clean" soil and the positive control was inoculated with Roundup, but no organisms.

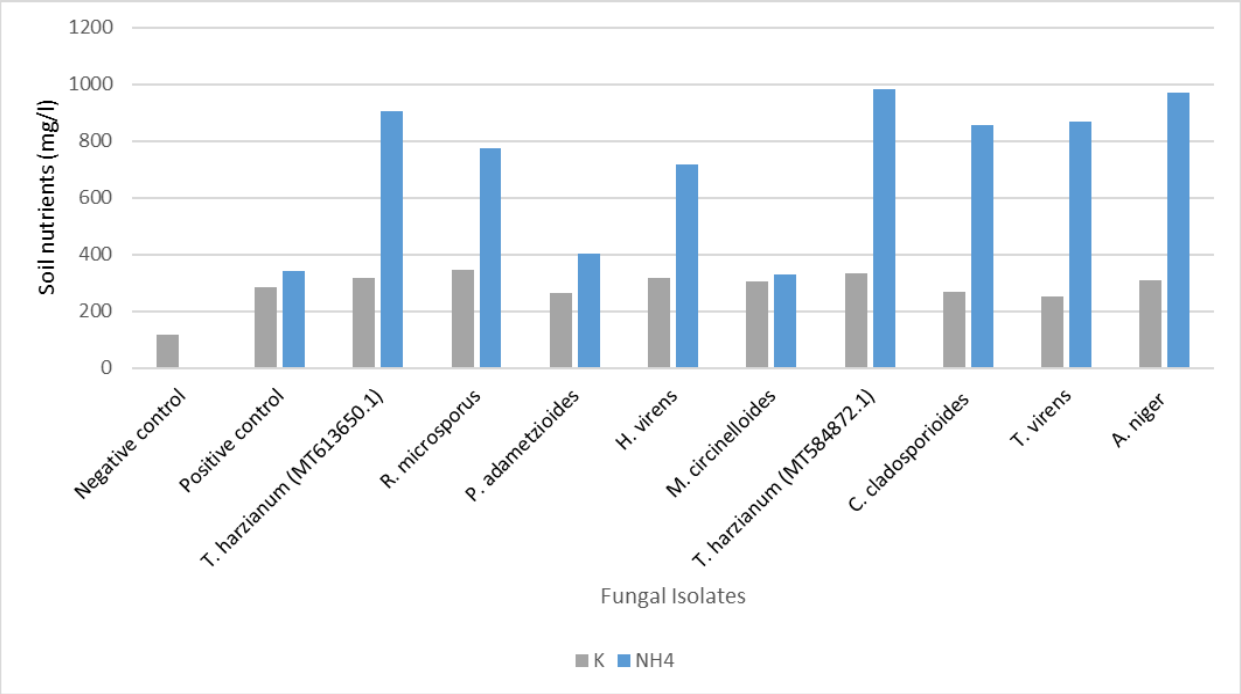

Figure 4-4: Soil extraction of potassium (K) and ammonium (NH4).

Figure 4-5 represents the levels of potassium (K) and ammonium (NH_4^+) present in soil samples that contained each of the fungal isolates that displayed a good ability to degrade glyphosate (>25%). The negative control sample, with no glyphosate present, showed low potassium levels (116.2 mg/L) and almost no NH_4^+ (0.16 mg/L). With Roundup present in the soil (positive control), significant levels of both potassium and NH_4^+ (285.7 mg/L potassium and 340.87 NH_4^+) were measured.

Rhizopus microsporus soil contained the highest potassium concentration at 347.4 mg/L while *Trichoderma virens* contained the lowest potassium concentration of 251.8 mg/L. *T. virens* had a lower value than the positive control. *M. circinelloides* was the only isolate to show a decrease in NH_4^+ (331.4 mg/L) levels from the positive control (340.87 mg/L). This small margin indicates minimal changes in NH_4^+ levels from the positive control. *T. harzianum* (MT584872.1) showed the largest increase in NH_4^+ levels alongside *A. niger* and *T. harzianum* (MT613650.1) with 982.58 mg/L, 970.78 mg/L and 906.57 mg/L.

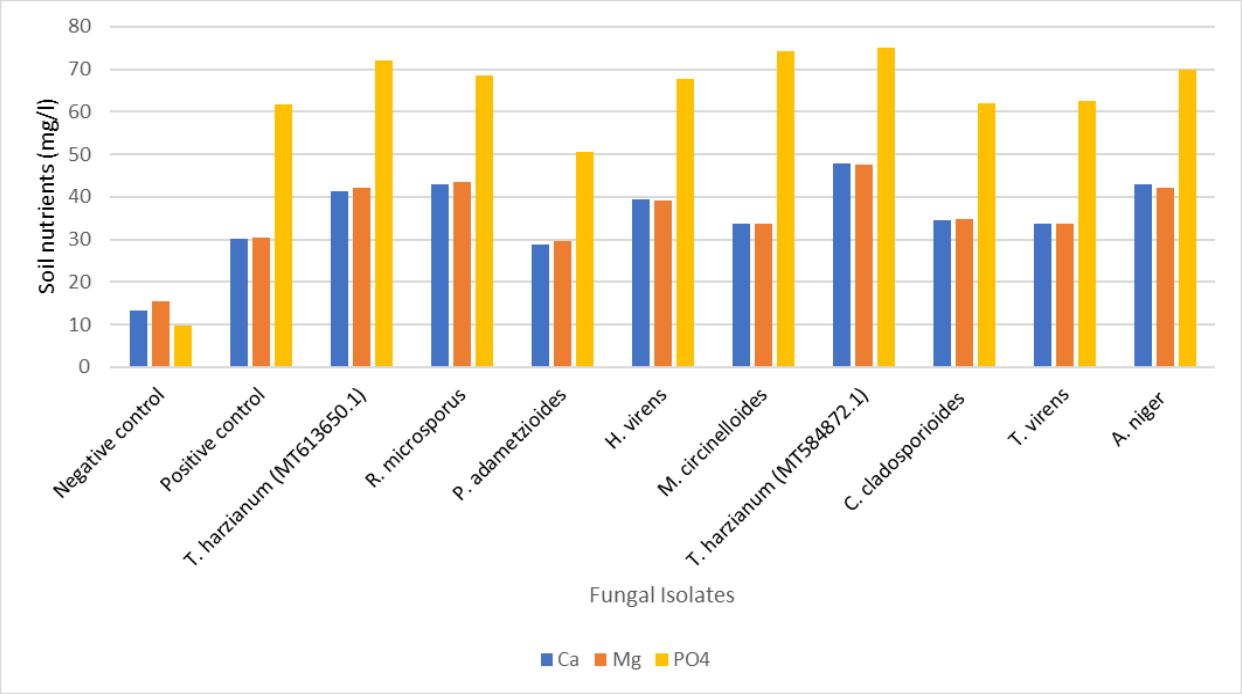

Figure 4-5: Soil extraction of calcium (Ca), magnesium (Mg) and phosphate (PO4).

Figure 4-6 shows that there was an increase in all soil nutrients Ca, Mg and PO_4 after the addition of Roundup. Ca increased from 13.25mg/L to 30.21 mg/L, Mg increased from 15.62 mg/L to 30.54 mg/L and PO_4 increased significantly from 9.84 mg/L to 61.75 mg/L. The only fungal isolate that showed a small decrease in Ca levels was *P. adametzioides* that had a Ca level of 28.71 mg/L. *P. adametzioides* also had a slight decrease in Mg levels from the positive control (30.54 mg/L) to 29.59 mg/L. It also decreased the PO_4 levels from the positive control (61.75 mg/L) to 50.57 mg/L.

T. virens, *M. circinelloides* and *C. cladosporioides* had roughly the same Ca concentration as the positive control with only a small increase resulting in 33.64 mg/L, 33.69 mg/L and 34.41 mg/L. There was also a very slight increase from the positive control in terms of Mg concentration from 30.54 to 33.71 mg/L (*T. virens*), 33.7 mg/L (*M. circinelloides*) and 34.8 mg/L (*C. cladosporioides)*. Only *T. virens* and *C. cladosporioides* did not show an increase in PO_4 levels by remaining fairly consistent with the positive control sample at 62.57 mg/L and 61.95 mg/L. *M. circinelloides* showed an increase in PO_4 concentration to 74.2 mg/L, but did not show an increase when compared to the NH_4^+ concentration in Fig. 4-5 and glyphosate degradation in Fig. 4-4.

Except for *P. adametzioides* which showed a decrease and *C. cladosporioides* which stayed similar to the positive control, most isolates showed increases in PO_4 nutrient levels in the soil. This could be a result of glyphosate degradation with glyphosate degrading through the sarcosine pathway. The trends that are shown in Fig. 4-6 roughly correlate with those of Fig. 4-5, as both PO_4 and NH_4^+ are degradation products of the sarcosine metabolic pathway. These degradation products are the indicators of glyphosate degradation in soil.

H. virens, *T. harzianum* (MT613650.1) and *A. niger* showed an increase in Ca concentrations from the positive control to 39.49 mg/L, 41.41 mg/L and 42.89 mg/L. The same level of increase was seen in Mg concentrations that showed *H. virens* a 39.1 mg/L, *T. harzianum* (MT613650.1) at 42.08 mg/L and *A. niger* at 42.1 mg/L. The PO_4 rose with *H. virens* as well showing a concentration of 67.75 mg/L, *T. harzianum* (MT613650.1) 71.96 mg/L and *A. niger* at 69.91 mg/L.

R. microsporus and *T. harzianum* (MT584872.1) showed the highest values of Ca and Mg at 43.06 mg/L and 47.88 mg/L Ca, respectively along with 43.47 mg/L and 47.49 mg/L for Mg concentrations. In terms of PO_4 *R. microsporus* showed a high concentration of 68.42 mg/L while *T. harzianum* (MT584872.1) showed a concentration of 75.19 mg/L.

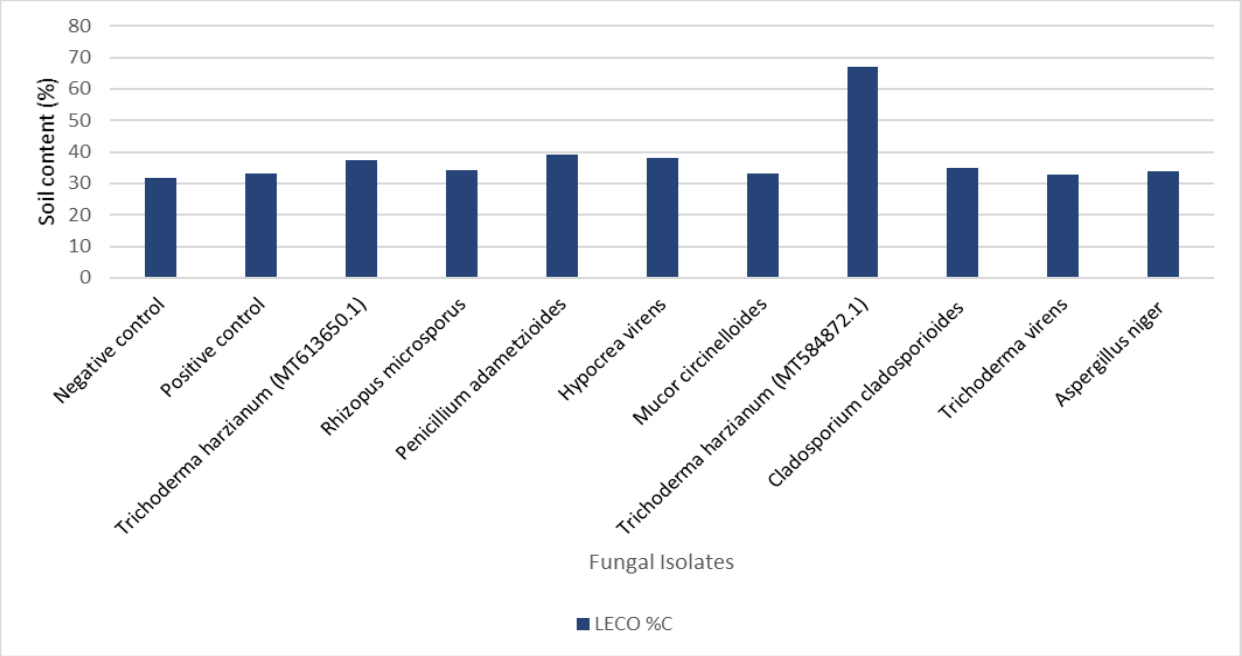

Figure 4-6: : Soil extraction on levels of carbon (LECO %C).

Figure 4-7 shows the levels of carbon (%C) present in the soil. The negative control again had no Roundup added to the soil, whereas the positive control had Roundup added. The only standout result is that of *T. harzianum* (MT584872.1), that had a soil carbon content of 67.09%. This could be attributed to there being more foliage (twigs, leaves etc.) in this sample compared to other samples. The same result may be observed in Fig. 4-8 where *T. harzianum* (MT584872.1) showed a major increase in nitrogen levels (%N) which strengthens the assumption of an increase of foliage.

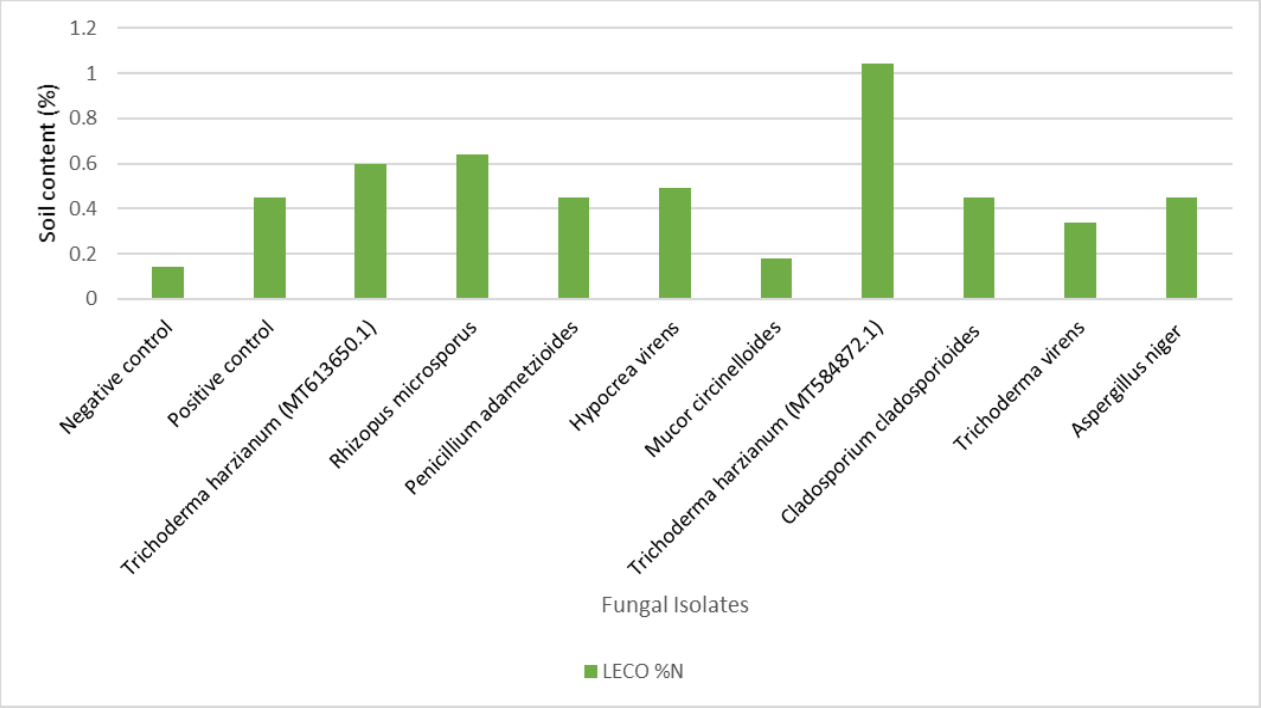

Figure 4-7: Soil extraction on levels of nitrogen (LECO %N).

Figure 4-7 and 4-8 represent the levels of Carbon content (%C) and Nitrogen (%N) content present in the soil samples, respectively that contained the fungal isolates used in the ELISA tests. The negative control sample, which contained no glyphosate, showed a low percentage of N (0.14 %N) and carbon content of 31.8 %C. After Roundup was added to the soil in the positive control sample the %N increased slightly to 0.18 %N and 33.1 %C for carbon levels. The levels across all fungal isolates remained fairly stable, with %C remaining between 30 %C and 40 %C. The only exception to this was *T. harzianum* (MT584872.1) which showed a rise in %C to 67.09 %C. The %N rose from 0.14 %N in the control to 0.45 %N after the addition of Roundup. Two fungal isolates reduced the %N in the soil to 0.34 %N (*T. virens*) and 0.18 %N (*M. circinelloides*). *P. adametzioides, C. cladosporioides* and *A. niger* showed a %N level of 0.45 %N. *H. virens* showed 0.49 %N while *R. microsporus* and *T. harzianum* (MT584872.1) showed 0.64 %N and 1.04 %N.

Except for the control sample, *M. circinelloides* had the lowest %N at 0.18 %N and *T. virens* had the lowest %C at 32.64 %C. *T. harzianum* (MT584872.1) had the highest value in both cases with 1.04 %N and 67.07 %C.

4.5 Long-term pure Roundup degradation

A higher concentration of Roundup was used to identify which fungal isolates could survive and utilize Roundup and glyphosate as an energy source. Extended incubation times were used to determine if the Group 3 isolates can use glyphosate as a nutrient source. A final concentration of 6% Roundup was used. An uninoculated control was used to determine the spontaneous degradation of glyphosate under the conditions of the experiment.

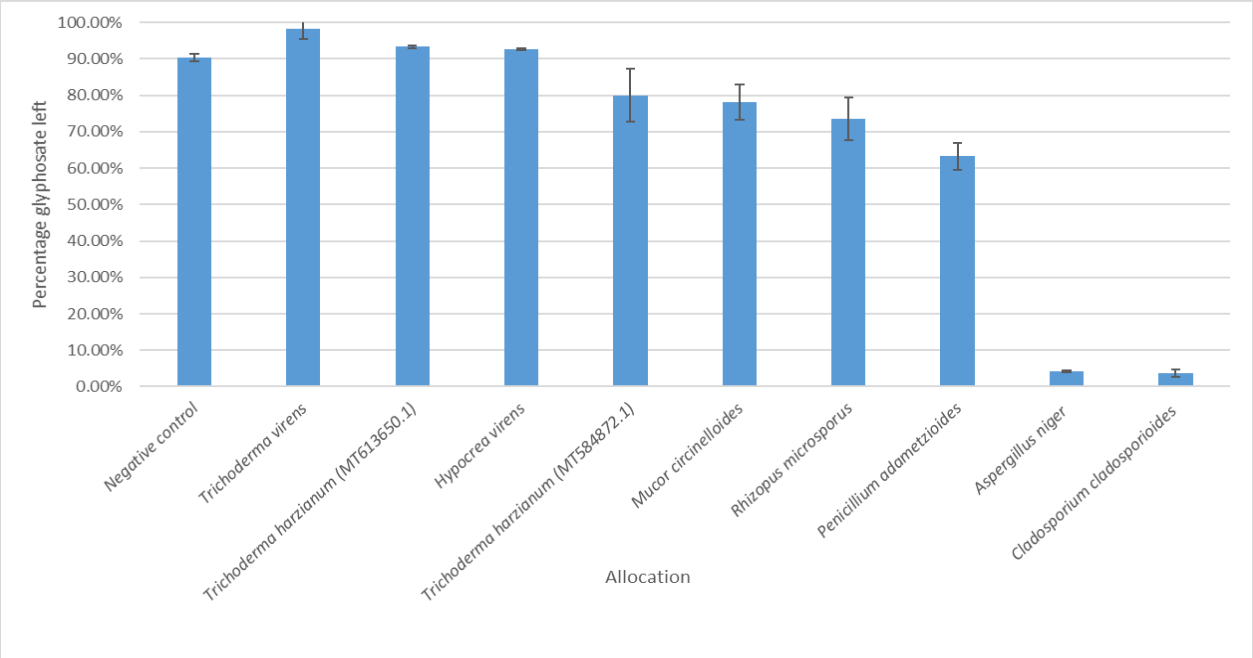

Figure 4-8: Degradation of glyphosate over 28 days through inoculated fungal isolates used in ELISA tests.

Group 3 isolates were grown for 28 days in a 6% concentration of Roundup (Roundup and distilled water). The glyphosate levels were analysed using TLC-plates in the same way as in the screening test as described in section 3.3.4 and displayed in Fig. 4-1 and Fig. 4-2. Fig. 4-9 values are shown in Table 4-5. The long period of 28 days meant that a higher Roundup concentration would have been needed as glyphosate and Roundup does naturally degrade over time.

Table 4-5: Degradation of glyphosate for pure roundup degradation.

Allocation	Glyphosate left
Negative control	90.41% ± 1.02%
Trichoderma virens	98.15% ± 2.80%
Trichoderma harzianum (MT613650.1)	93.38% ± 0.36%
Hypocrea virens	92.72% ± 0.31%
Trichoderma harzianum (MT584872.1)	80.00% ± 7.28%
Mucor circinelloides	78.10% ± 4.80%
Rhizopus microsporus	73.54% ± 5.77%
Penicillium adametzioides	63.23% ± 3.73%
Aspergillus niger	4.15% ± 0.18%
Cladosporium cladosporioides	3.69% ± 1.07%

Cladosporium cladosporioides (3.69% ± 1.07%) and *Aspergillus niger* (4.15% ± 0.18%) showed the greatest degradation to the extent that a TLC-test struggled to detect the glyphosate. *Penicillium adametzioides* (63.23% ± 3.73%), *Mucor circinelloides* (78.10% ± 4.80%), *Rhizopus microspores* (73.54% ± 5.77%) and *Trichoderma harzianum* (MT584872.1) (80.00% ± 7.28%) (MT584872.1) showed intermediate degradation which showed either a good reuse of nutrients, a slow growth rate, or an inhibition of growth by Roundup. Fungal isolates such *as Hypocrea virens* (92.72% ± 0.31%), *Trichoderma harzianum* (MT613650.1) (93.38% ± 0.36%%) (MT613650.1) and *Trichoderma virens* (98.15% ± 2.80%) were inhibited by the high levels of Roundup. The small levels of degradation of glyphosate in the control can be explained through light exposure and the natural degradation rate of glyphosate over the degradation rate of other samples and represented a more natural degradation than the negative control (90.41% ± 1.02%). Light exposure at any point could have increased the natural degradation rate of glyphosate.

CHAPTER 5 – DISCUSSION

5.1.1 Isolation and screening

In this study, a total number of 30 isolates were obtained from the isolation and screening tests (Refer to Table 4-2 in Chapter 4). The isolates obtained were grouped into 3 distinct groups based on the Roundup degradation observed during the screening test. A total of 30 isolates were obtained during the isolation and screening step of this project where 9 isolates were able to achieve greater than 25% Roundup degradation, 8 isolates achieved between 10% and 25% Roundup degradation, and 13 of the isolates achieved less than 10% Roundup degradation. Isolates were given allocations based on where the isolates originated from. Isolates starting with "F" (F3B-BC, F3B-EE, F1U-B, F1A-AB, F1I-B, F3E-C and F1H-B) were isolated from glyphosate spiked agricultural soil from the ARC-Potchefstroom. The remaining isolates were given a numerical designation (2c, 3a, 5a, 6, 8, 8C, 10, 10B, 11, 13, 14a, 16, 17, 18, 21, 23, 25a, 25b, 26B, 30, 31, 52) and were obtained from a previous study, as stated before.

Table 5-1: Summary of isolates from screening tests

Percentage Roundup degradation	**Isolate number(s):**
Greater than 25% degradation (Group 3)	18, F3B-BC, 8, 16, 22, F3B-EE, 11, 10, F1U-B
Between 10% and 25% degradation (Group 2)	25a, 8C, 10B, 13, F1A-AB, 26B, 25b, F1I-B
Less than 10% degradation (Group 1)	17, 52, 23, 30, 21, 50 (F3E-C), 6, 3a, 31, F1H-B, 5a, 14a, 2c

Isolates achieving less than 10% degradation of glyphosate were not identified and not taken further in this study. Low degradation isolates (17, 52, 23, 30, 21, 50 (F3E-C), 6, 3a, 31, F1H-B, 5a, 14a, 2c) were classified as non-degrading isolates as the small deviations in data could be attributed to experimental and human error. It was also theorized that the isolates that degrade these small amounts of glyphosate do not, under the lab conditions in this study, excrete the desired enzymes necessary for glyphosate degradation. This could have been due to low growth rates, undesired incubation temperature or the isolates degradation capabilities and as such the isolates were discarded and classified as non-degrading isolates (Brockett *et al*., 2012).

The specific isolates achieving more than 10% degradation and less than 25% degradation were classified as potential degraders of glyphosate and were identified but not used in the soil degradation tests of glyphosate. These isolates (25a, 8C, 10B, 13, F1A-AB, 26B, 25b, F1I-B) were theorised to be able to degrade glyphosate, but low growth rates, the partial excretion of the desired enzymes or the lab conditions caused these isolates to only partially degrade glyphosate. Due to budgetary constraints and the high cost involved in executing soil tests (multiple ELISA runs and nutrient analyses) these isolates were only theoretically compared to the data in previous studies to determine the viability of in these isolated capabilities to degrade glyphosate. Further research will have to be left for future studies.

All isolates degrading more than 25% of glyphosate, determined using TLC tests, were identified and studied for their degradation capabilities in soil analysis tests (ELISA) and long-term degradation of glyphosate in Roundup (28-days) studies. These isolates (18, F3B-BC, 8, 16, 22, F3B-EE, 11, 10, F1U-B) were theorised to excrete the desired enzymes necessary for glyphosate degradation.

5.1.2 Molecular ID

A total of 12 distinct species of fungi were obtained in this study. These included species such as *Aspergillus niger, Aspergillus oryzae, Cladosporium cladosporioides, Hypocrea virens, Mucor circinelloides, Penicillium adametzioides, Penicillium glabrum, Penicillium spinulosum, Rhizopus microspores, Trichoderma harzianum, Trichoderma simmonsii* and *Trichoderma virens* identified in Table 4-3.

5.1.3 ELISA

Nine isolates were used for glyphosate degradation in soil. ELISA tests were used to determine which isolate were most successful in degrading glyphosate in soil conditions. The degradation in soil showed how an isolate would perform in a high nutrient environment where nutrients would be easy to come by. Therefore, in the event the isolate struggled to degrade glyphosate in soil, the degradation pathways and nutrient preference might be the main causes. If an isolate did degrade glyphosate in soil (screening test more than 25%), the residue of glyphosate was

measured. Each isolate is discussed in the sections below and the results compared with those reported in other studies, where applicable.

5.1.4 Soil nutrient analysis

5.1.4.1 NH_4^+

According to Feng *et al*., (2020) a degradation product of both AMPA and glyphosate, is NH_4^+. This was also mentioned in section 2.1.4, which showed a possible intercellular degradation of glyphosate to be NH_4^+. NH_4^+ is formed from glyphosate when $CH_2NH_3^+$ reacts to free OH radicals and therefore forms NH_4^+ and formaldehyde. Another method involves adding molecular oxygen to the $CH_2NH_3^+$ to form formaldehyde, NH_4^+ and HO_2 (Manassero *et al.*, 2010).

Thus, the formation of high levels of NH_4^+ can indicate the potential that one or both of these metabolic pathways were used to increases in NH_4^+ compared with the positive control. Also, NH_4^+ was classified as one of the “possible inorganic degradation by-products of glyphosate” (Feng *et al*., 2020). As seen in all isolates except *M. circinelloides,* the NH_4^+ content in the soil spiked in the presence of Roundup and the isolates, indicating the possible degradation of glyphosate or AMPA.

5.1.4.2 PO_4

PO_4 is a degradation product of glyphosate as shown in section 2.2 Fig. 2-3. It occurs through the sarcosine pathway when glyphosate is broken down at the C-P bond (Fig. 2-5) in section 2.4 (Jaisi *et al.*, 2016). This is also the most common degradation pathway fungal species used to degrade glyphosate. The result of this degradation is sarcosine and PO_4 where the sarcosine undergoes further degradation.

PO_4 has been shown in previous studies to have a direct impact on the uptake rate of glyphosate in the root systems of plants. The PO_4 also serves as a nutrient source for fungal isolates (Adelowo *et al.*, 2014). Soil organic matter has a direct impact on the sorption rate of glyphosate as glyphosate competes with the soil contents. In the case of PO_4, soil with high levels of PO_4 will compete with the breakdown products of glyphosate for use as a nutrient source. Previous studies have shown that this can cause the retention of glyphosate. PO_4 is thus a direct indicator of glyphosate sorption or retention in soils and can be used to manipulate the sorption rate (Singh

et al., 2020). In PO_4 rich soils, such as agricultural soils, this can hinder the degradation of glyphosate.

5.1.4.3 Potassium (K)

Very few studies have linked potassium to glyphosate degradation at the time of writing. However, it has been speculated that the addition of glyphosate sources that contain potassium as a salt of glyphosate, can exert pressure on microbial communities and directly influence the nutrient dynamics of potassium (Lane *et al*., 2012). In poorly drained soils fungal species can use potassium as a nutrient source through uptake, especially when high amounts of glyphosate are added (Lane *et al*., 2012). With this being the case, each fungal isolate uses potassium uptake at different rates based on their growth and metabolic requirements (Dick *et al.*, 2010).

Some studies have also suggested that constant applications of glyphosate may immobilise the soil potassium contents and change the nutrient dynamics of potassium by directly impacting the uptake rate of microbial communities (Dick *et al.*, 2010). Though there were fluctuations in the potassium contents in the soil, the fluctuations were a result of experimental variables and changes in the metabolic activity of the isolates. The glyphosate may have affected the uptake and use of potassium, but more studies would have to be done to verify this.

5.1.4.4 Calcium (Ca) and Magnesium (Mg)

Calcium has a direct influence on the soil adsorption of glyphosate in soils along with Mg, soil inorganic phosphates, soil organic matter and pH (Gunarathna *et al*., 2018). The metal complexes (Ca, Mg, Iron(III) and copper(III)) that binds to glyphosate and forms insoluble glyphosate complexes in soil can lead to the inactivation of glyphosate. These insoluble complexes can precipitate in groundwater but can form only when the metal complex concentration is high (Subramaniam and Hoggard, 1988).

These concentrations of metal complexes, when applied to seeds in the soil, can significantly reduce when exposed to glyphosate. This suggests that glyphosate can interfere with the retranslocation of metal complexes through the formation of insoluble complexes (Cakmak *et al.*, 2009) as stated in section 2.2. The fluctuations in the levels of Ca and Mg can be attributed to the different soil samples as well as trace amounts that might be present in the Roundup mixture.

Neither Ca nor Mg has been reported in the past as an indicator of glyphosate degradation, but in large quantities, it can bind to glyphosate to make it less effective and harder to degrade.

5.1.4.5 Soil organic carbon content

Soil organic carbon content could be attributed to the addition of Roundup, which contains organophosphates (carbon and phosphorous). Also, the slight increases could be because of differing levels of foliage content and could have a direct link to lignin in the soil. Lignin does make up the majority of soil organic carbon (Rasse *et al.*, 2006) as stated in section 2.7.2.

Due to the minor fluctuation across most isolates, the addition of Roundup does not appear to have any significant effects on the soil carbon content. Carbon sources in glyphosate degrade to form CO_2 as shown with the degradation of glyoxylate (Schuette, 1998) in section 2.1.4 and AMPA degradation when formic acid binds with free OH radicals in the presence of O_2 (Jaisi *et al.*, 2016) in section 2.3.

5.1.4.6 Soil nitrogen content

Fluctuations in soil nitrogen are attributed to a variety of reasons. According to the national library of medicine (Pubchem), glyphosate and the Roundup surfactant (such as POAE) contains nitrogen as part of their chemical composition. The addition of Roundup shows a rise in the %N levels from the negative- to the positive control. Most of the fluctuations of the isolates can be attributed to the natural metabolic processes of the isolates.

Soil moisture and temperature therefore also play major roles, as they have a direct impact on the isolates' metabolic processes (Haney *et al*., 2002). Other factors that can affect the metabolic processes are pH and the type of soil (Liu *et al*., 2020). Therefore, %N alone is not a good representation of glyphosate degradation as too many factors influence the outcome in this particular study.

5.1.4.7 Roundup pure water

The concept behind Roundup pure water degradation was to determine if isolates can degrade glyphosate from Roundup in high concentrations, without the addition of any other nutrients. It

was based on the knowledge that certain isolates (for example *Aspergillus spp*. and *Trichoderma spp*.) can use glyphosate as a sole phosphorous source. For an isolate to use glyphosate as a sole nutrient source it must be able to use both the AMPA and sarcosine pathway as described in section 2.2 and 2.3. The degradation of glyphosate using these pathways was evaluated in relation to the soil nutrient analysis to confirm whether an isolate can degrade glyphosate. No other studies to date have been found to test Roundup utilisation in this manner.

5.2 Genera and species

5.2.1 Aspergillus spp.

Aspergillus is the genus name for a large group of filamentous fungi. Most of these fungi occur in an asexual state and reproduce by producing conidia that can spread into many different environments. This causes this type of fungus to be widespread, and of the 185 different species only about 20 species cause infections in humans. *Aspergillus spp*. were extensively studied for their bioremediation qualities. *A. niger* have been studied for its remediation of soils contaminated with heavy metals (Wasay *et al*., 1998). *Aspergillus* have also been studied for the remediation of crude oil contamination with *A. flavus* (Maruthi, 2013) and Triton X-100 (Abu-Ghunmi *et al*., 2015), amongst other contaminants.

5.2.1.1 Aspergillus niger

The fungus *A. niger* is a fungus, which can sometimes be thought to be the cause of some cases of pneumonia. It is also the causative agent of 'black mould' on the outsides of certain foods, such as apricots, onions, grapes, etc. Therefore, making *A. niger*, a food 'spoilage' organism. Industrial use of *A. niger* fungus takes advantage of its bio absorption abilities (Schuster *et al*., 2002). In certain areas such as wastewater, *A. niger* can be used to remove certain dyes (Congo red or Blue 9) from the water. It is also well studied and used for its production of citric acid in the industrial sector (Baker, 2006). *A. niger* is a brown-rot fungus capable of degrading cellulose and hemicellulose through the production of H_2O_2 (Person *et al*., 2010).

In a study conducted by Carranza *et al*., (2017) *A. niger* was tested for its ability to utilise glyphosate as a sole carbon source. The studies found that *A. niger* could utilise glyphosate as a sole carbon, nitrogen or phosphorous source at 10, 1.0- and 1.5-mM glyphosate. This was

theorised through literature studies done by Singh *et al.* (2020) which suggested potential similarities between these studies. *A. niger* also showed a shorter adaptation time than other organisms with regard to changing environments. Also, *A. niger* was shown in that study to be very resilient in the presence of multiple herbicides (Baker, 2006).

In the initial screening test *A. niger* (F3B-EE) degraded 37.34% ± 9.21% of glyphosate in a 48-h period Fig. 4-1. A study done by Hu *et al*., 2011 showed that *A. niger*, and *A. oryzae*, can degrade lignin and wood structures and thus produce the extracellular enzymes to do so. It can be seen in this study as well that the degradation rate was well above the minimum set limit of 25% degradation in 48-hours. Carranza *et al*. (2017) showed that *A. niger* has the capability to degrade glyphosate under simulated conditions.

In Table 4-3 it is shown that *A. niger* degraded glyphosate with only 32.48% ± 13.98% of glyphosate remaining. This again shows the potential for *A. niger* to degrade and utilise glyphosate as a nutrient source through degradation in soil. The growth conditions of *A. niger* were also supported in other studies, which, as in this study, grew the isolate in the presence of glyphosate (Carranza *et al.*, 2017). This showed that in both studies *A. niger* was resistant to the effects of glyphosate on the isolate. Carranza *et al.* (2017) suggest as well that in vitro, *A. niger* can grow even when glyphosate is the only phosphorous source.

Figure 4-5 shows *A. niger* to make very little change to the potassium levels in soil through only a slight increase from 285.7 mg/L to 310.9 mg/L. This amount of change is negligible. However, the level of NH_4^+ was very high compared with the level of the control with a rise in concentration from 340.84 mg/L to 970.78 mg/L. This sharp rise in concentration is probably the result of the degradation pathway (sarcosine and AMPA degradation) used by *A. niger* to utilise glyphosate as a nutrient source, as previously mentioned, NH_4^+ being the by-product of the degradation pathway, as explained in Fig. 2-3.

In Fig. 4-6 *A. niger* is shown to have a sharp increase in calcium-, magnesium- and phosphorous levels. The increase in calcium (from 30.21 mg/L to 42.89 mg/L) and magnesium (from 30.54 mg/L to 42.1 mg/L) levels could be attributed to metabolic pathways of *A. niger*, although the rise in PO_4 (from 61.75 mg/L to 69.91 mg/L) levels coincide with the rise in NH_4^+ levels in Fig. 4-6

which again supports the previous statement of using glyphosate as a nutrient source. No noticeable differences were observed in the %N and %C sources in Fig. 4-7 and 4-8.

In Fig. 4-9 *A. niger* shows extensive degradation of glyphosate after one month in a diluted Roundup mixture. The glyphosate was degraded to such an extent that almost all of the glyphosate was degraded with only 4.15% ± 0.18% glyphosate left. This showed that *A. niger* can grow and utilise glyphosate as a nutrient source using both the sarcosine and AMPA degradation pathways, a finding which correlates with those of Carranza *et al.* (2017) and Singh *et al*. (2020). Due to its rapid growth, it is also well suited for field studies and can potentially be used to remediate soil with herbicides other than glyphosate.

The data indicates that *A. niger* does possess the capability to degrade glyphosate from Roundup through the potential use of extracellular enzymes *in vitro* and in soil. A potential future study could include *A. niger* to be paired up with an isolate such as *T. virens* or *H. virens* which showed great degradation potential as well. *T. virens* and *H. virens* cannot use glyphosate as a sole nutrient source but can degrade glyphosate in soil. *A. niger* could potentially utilize the degraded components as a nutrient source and together the fungal species may degrade glyphosate effectively.

5.2.1.2 Aspergillus oryzae

A. oryzae is also known as kōji mould. Found in soil and decaying plant material this filamentous fungus is used in Japan to saccharify rice (Barbesgaard *et al*., 1992), sweet potato and barley in the making of alcoholic beverages such as sake and shōchū, and to ferment soybeans for making soy sauce and miso (Machida *et al.,* 2008). However, in the production of fermented foods of soybeans such as soy sauce and miso, *Aspergillus sojae* is mainly used instead of *A. oryzae*. *A. oryzae* is also used to produce rice vinegar (Machida *et al*., 2008). Barley kōji (an enzyme complex used in the production of soy sauce, miso and Japanese wine “sake”) or rice koji are made by fermenting the grains with *A. oryzae* hyphae and *A. oryzae* has been known even to grow on various types of grain other than barley (Chang *et al*., 2009).

Identified initially as F1A – AB, this isolate degraded 19.05% ± 9.36% over 48-hours. As with numerous other isolates, this showed that a probable degradation of glyphosate under simulated

circumstances is possible but may not be viable with this isolate. *A. oryzae* also has quite a high optimal growth temperature of 32 °C – 36 °C, which contributed to reduced levels of glyphosate degradation (Barbesgaard *et al*., 1992; Machida *et al.,* 2008). The incubation temperature of 30 °C may not have been sufficient for this isolate to produce the necessary enzymes to degrade glyphosate effectively. This was contradicted by studies by Carranza *et a*l. (2019), that showed that *A. oryzae* did possess the capability of glyphosate degradation. A study by Singh *et al.* (2020) reached the same finding but, the growth conditions differed. The afore mentioned grew the isolate in complete darkness for 7 days, while this study did not grow the isolate in complete darkness. This could have affected the growth rate of *A. oryzae*. *A. oryzae* was not selected for further studies, as the soil tests were done at room temperatures.

5.2.2 Cladosporium spp.

Fungi belonging to this type of genus are common in- and outdoor fungi (Grinn-Gofroń and Rapiejko, 2009). When growing indoors, this genus grows mainly on surfaces that contain moisture while outdoors this genus prefers living on dead plant tissue and contribute to many different allergens when spores are released. This makes these species hard to avoid and, in some cases, difficult to treat (Grinn-Gofroń and Rapiejko, 2009). *Cladosporium spp*. are described as olive-green and darker, due to the dark pigmented conidia that *Cladosporium spp*. form (Zhang *et al.*, 2009). The spores are dispersed in wind and are therefore very abundant in outdoor air, such as agricultural areas for example (Zhang *et al.*, 2009). Some species such as *C. cladosporioides* and *C. herbarum* are the main causes of grapevine rot (Latorre *et al.*, 2011), while *C. fulvum* causes tomato leaf mould.

5.2.2.1 Cladosporium cladosporioides

C. cladosporioides is a darkly pigmented fungus that occurs worldwide on a wide range of materials both outdoors and indoors and belongs to the family ascomycetes (Halaburgi *et al*., 2011). It is a common fungus in outdoor air and in marshlands (Balaji *et al*., 2009) where its spores are important in seasonal allergenic disease. While this species rarely causes invasive disease in animals, it is an important agent of plant disease, attacking both the leaves and fruits of many plants (Wang *et al*., 2013). This species produces asexual spores in delicate, branched chains that break apart readily and drift in the air. It can grow under low water conditions and at very low

temperatures. *C. cladosporioides* is also used as an agricultural biocontrol agent of *Venturia inaequalis* (apple scab fungus) (Wang *et al*., 2013).

Identified as F1U – B this fungal isolate degraded 28.25% ± 3.47% of glyphosate over 48 hours. Although *C*. *cladosporioides* showed high levels of degradation in this study, a study by Wardle and Parkinson (1990) showed that the growth of *C. cladosporioides* gets inhibited at high concentrations of glyphosate. This was confirmed by Aluffi *et al.* (2020) in a study which showed that *Cladosporium spp.* can have up to a 4-hour adjustment period before growth can occur. In this study, growth conditions were not monitored. Comparing the 12-hour and 24-hour degradation of *C. cladosporioides* it is seen that as time goes by, the degradation slows, which may suggest a fast growth period between 0-hours and 12-hours that caused a rapid degradation period of glyphosate. This high level of degradation meant that this isolate was further identified as *C. cladosporioides* and also as a probable degrader of glyphosate.

Table 4-3 reflects a comparison that was drawn between the TLC-plates and the ELISA tests. This showed that *C. cladosporioides* could degrade glyphosate under controlled lab conditions (30.24%) (a screening test) but failed to degrade glyphosate in soil conditions. In soil conditions, *C. cladosporioides* did not show much deviation from the positive control and had 95.23% ± 6.20% glyphosate was left. This shows the probability that *C. cladosporioides* can produce the enzymes required for glyphosate degradation in the presence of Roundup but might not degrade glyphosate in soil due to metabolic processes and preferences or the inhibition of metabolic pathways as discussed in section 2.1.2. High concentrations of glyphosate could also have suppressed the growth period or prevented the adjustment period for the isolate (Wardle and Parkinson, 1990; Aluffi *et al.,* 2020).

The soil levels in Fig. 4-6 showed no increase in PO_4 (61.95 mg/L) levels, but a substantial increase in NH_4 (856.89 mg/L) Fig. 4-5. The rise in levels from the positive control suggests that glyphosate underwent degradation as part of the sarcosine pathway, but not the AMPA degradation pathways. This suggests that *C. cladosporioides* can degrade glyphosate, but not use it as a phosphorous source. It also shows that *C. cladosporioides* could have been inhibited in growth leading to an elevated degradation product but a lack of further degradation. This correlates with the findings of Wardle and Parkinson (1990). The rapid rise in NH_4 can also be attributed to the low and negligible changes in Ca and Mg levels as shown Fig. 4-6, as the small

rise compared to the positive control should have had minimal influence on glyphosate adsorption and degradation. Potassium levels showed only a small decrease in levels compared to the positive control. This could be attributed to human error or to a small reaction to the glyphosate, but it would also have minimal influence on the degradation of glyphosate.

It is possible that *C. cladosporioides* growth could be inhibited due to high glyphosate levels in soil, as in studies done by Wardle and Parkinson (1990). In this study, however, *C. cladosporioides* in soil failed to degrade glyphosate. Glyphosate may have been marginally degraded as shown with a rise in NH_4 levels. The two tests, therefore, confirmed suspicions that *C. cladosporioides* can potentially degrade glyphosate under controlled conditions. When faced with no additional nutrients, *C. cladosporioides* can degrade and utilise glyphosate. Fig. 4-9 when faced with long-term degradation, with no additional nutrients, *C. cladosporioides* can degrade glyphosate with 3.69% ± 1.07% glyphosate left. This strengthens the argument that *C. cladosporioide*s prefers different nutrients sources to maintain growth and survivability but can adapt and adjust to the environment.

Therefore, this fungal isolate can potentially degrade glyphosate but cannot be recommended for future studies as the degradation of glyphosate requires the isolate to choose unfavourable metabolic pathways. However, it does potentially produce some of the enzymes required to degrade glyphosate and might have applications in other fields of study related to herbicide degradation.

5.2.3 Mucor species

Most *Mucor spp*. are commonly found in soil, plant surfaces, digestive systems and several kinds of cheese (Lebreton *et al*., 2020). These fungal species are fast-growing ranging from white to grey. *Mucor spp*. forms spores to sporulate and extend their reach to many areas in natural environments. Some *Mucor spp*. such as *Mucor circinelloides* can produce asexually, whereas *Mucor racemosus* can produce both asexually and sexually. Further, *Mucor spp*. Can have either a positive or a negative impact on humans. Some species such as *Mucor indicus*, *Mucor ramosissimus* and *Mucor circinelloides* have proven to be pathogenic in nature as well as thermotolerant (Zhang and Zhao, 2010). However, in contrast to this, some *Mucor* species have

been used in industrial processes because of their fast-growing nature and ability to produce enzymes (Lebreton *et al*., 2020).

5.2.3.1 Mucor circinelloides

M. circinelloides is a dimorphic fungus belonging to the order Mucorales and grows as either a budding yeast (anaerobically) or as a filamentous fungus (aerobically) (Li *et al.*, 2011). M. circinelloides is also known for producing carotene (Nicolás *et al*., 2018). Carotene is a substance known for absorbing ultraviolet, violet, scatter orange and blue light. This aids in photosynthesis in plants and also gives some fruits their bright colours (Silva et al., 2006). *M. circinelloides* is also an opportunistic pathogen, infecting people with a lowered immune system to cause a condition known as mucormycosis which can be fatal in extreme cases (Li *et al*., 2011; López-Fernández *et al*., 2018). It has a worldwide distribution, and is found mostly in soil, dung and root vegetables. This species is described as unable to produce mycotoxins (Wagner *et al*., 2020). Ketoacidotic patients are particularly at risk of infection by *M. circinelloides*. *Mucor* species have also been found to grow in the presence of glyphosate and even to utilise it as a nutrient source, such as the *Mucor IIIR* used in a study by Singh *et al.* (2020). It is also an isolate that bears a unique genetic structure among the fungal isolates of this study, as seen in Fig. 4-3. It shows a 100% similarity to *Rhizopus microsporus*. This was because a small area of DNA was compared (345 bp), when the DNA length of *Rhizopus microsporus* was much longer (567 bp). If a longer DNA strand had been compared, there would have been fewer similarities.

Identified as isolate 8, this fungal isolate showed a 45.22% ± 1.18% degradation over 48-hours in table 4-1. According to a study done by Aluffi *et al*. (2020) *Mucor spp*. have been isolated many times in the past from glyphosate contaminated soils and have shown a clear resistance to the inhibition effects of glyphosate. It is similar in this study, as *M. circinelloides* was isolated in glyphosate contaminated soil. This high level of degradation meant that this isolate was identified as *M. circinelloides* and used in soil testing conditions. The high level of degradation hinted at the production of the necessary enzymes capable of degrading glyphosate.

According to table 4-3 *M. circinelloides* degraded glyphosate with 66.13 ± 15.86% of glyphosate left in soil conditions. This intermediate level of degradation correlates with the findings of the

study previously mentioned by Singh *et al.* (2020). However, studies done in the past found that *Mucor spp.* produces fewer mycelia in the absence of glyphosate related herbicides. This suggests that in the presence of herbicides, *Mucor spp*. can perform their function (beneficial plant and water interactions that produce CO_2 better and show improved growth. Thus, *M. circinelloides* can degrade glyphosate in controlled soil conditions and does secrete the necessary enzymes for glyphosate degradation in soil conditions.

Figure 4-6 does partially support the statement that glyphosate can be degraded in soil by a rise of PO_4 levels. This rose rapidly to 74.2 mg/L, but the NH_4 in Fig. 4-5 levels remained low at 331.39 mg/L. This could indicate a weak or lack of sarcosine metabolic pathway but a strong AMPA degradation pathway. Mg and Ca in Fig. 4-7 did not seem to have had a great effect on the results showing mid-range values of 33.7 mg/L of Mg and 33.69 mg/L of Ca.

M. circinelloides showed only 78.10% ± 4.80% in long term degradation. However, it also showed that, like several other isolates, it does not use Roundup as a primary nutrient source effectively. Considering the low changes in PO_4 and NH_4 levels in soil and the high screening test degradation, it can be concluded that *M. circinelloides* can degrade glyphosate initially, but more long-term exposure to glyphosate hampers growth and ultimately inhibits the isolate. It would therefore not be recommended for future studies.

5.2.4 Penicillium species

A genus of ascomycetous fungi, *Penicillium* plays a large part in natural ecosystems as well as in industry. *Penicillium* species are known decomposers of organic material and produce a wide variety of mycotoxins while leading to food spoilage in the industry (Frisvad *et al*., 2004; Visagie *et al.*, 2014). Best known to produce penicillin (one of the most widely used antibiotics) these species are held in high regard all over the world and have also been very well studied (Müller *et al.*, 1991; Meijer *et al*., 2010). *Penicillium* species are also very diverse in their areas of growth. Where some species grow in soil, others can grow indoors and be transported through the wind (Visagie *et al*., 2014). These fungi are therefore present in most places around the world and have thus been used in many ways. Most interestingly these fungi produce various enzymes such as lipase, cellulase, protease, citric etc. (Mendes *et al*., 2011)

5.2.4.1 Penicillium adametzioides

This fungus was first isolated in Cheongsoo (South Korea) from decaying grapes (Deng *et al.*, 2012). The report was made in 2012 and as such *P. adametzioides* has not been studied extensively for biocontrol and remediation studies. A suggestion was made in 2015 that *P. adametzioides* might be used as a biocontrol agent for ochratoxin-producing fungus, showing that it has potential (Ahmed *et al*., 2015). In this study contains the first instance in which this fungus is to be used as a glyphosate bioremediation agent.

P. adametzioides has been identified as an emerging biocontrol agent in recent years. *P. adametzioides* has shown the production of peniciadametizine A, which is a known fungicide against *Alternaria brassicae* (Bovio *et al*., 2019). Some *P. adametzioides* have also been reported to produce cellulase and ligninase as enzyme products, though not all samples showed the same levels of enzyme production as they were influenced by seasonal changes (Lucretia *et al.,* 2014). The presence of cellulase showed that *P. adametzioides* is partially a brown rot fungus that can degrade cellulose as well as lignin. The lignin degradation is closely linked to glyphosate degradation and thus explains why *P. adametzioides* was able to degrade glyphosate. *P. adametzioides* was closely related to *A. oryzae* in this instance with a 97% similarity according to Fig. 4-3 and was also closely related to the other *Penicillium* species with a 94% similarity. It is important to note that this species was first found only in 2012 (Deng *et al.*, 2012) and has been classified as its own species. Therefore, there is a significant lack of information on this species in this study, and as far as can be ascertained, this is one of the first studies for glyphosate degradation fir this species.

As seen in Fig. 4-1 and table 4-1 under the allocation of 18, the degradation rate of glyphosate in a controlled environment showed the most promise by degrading 52.06% ± 3.96% of glyphosate after 48 hours. *Penicillium spp.* have been found in many glyphosate contaminated soils and have been shown to be resistant to glyphosate in terms of growth. This shows the potential in screening tests that this fungus has for bioremediation. According to the TLC-test, this is the best glyphosate degradation isolate in this study.

However, with soil degradation in Table 4-3 *P. adametzioides* showed 35.78% ± 4.04% of glyphosate was left according to the ELISA tests. However, comparisons between studies are not possible as there is a lack of evidence of *P. adametzioides'* ability to degrade glyphosate. Some

studies have shown that other Penicillium species do in fact possess the ability to degrade glyphosate such as *P. spinulosum* (Eman *et al.*, 2013). This shows that this fungal isolate does possess the ability to effectively degrade glyphosate in soil, but other fungal isolates performed better.

P. adametzioides showed, as can be seen in Fig. 4-5 an increase in the levels of NH_4^+ and in Fig. 4-6 a slight decrease in levels of PO_4 compared to those of the positive control. This indicates that *P. adametzioides* may use glyphosate in its metabolic system with a specific notion towards the AMPA degradation pathway. This is further supported when compared to the 28-day degradation period mentioned, as well as the TLC-plates in Fig. 4-1. It does show, however that *P. adametzioides* struggles to use glyphosate as a primary nutrient source over a long period but can degrade significant levels of glyphosate (63.23% ± 3.73%) according to Fig. 4-9. This shows that *P. adametzioides* has the potential to degrade glyphosate. However, it also shows that the life of this fungal isolate is limited and that it will possibly not survive in high concentrations for an extended period.

5.2.4.2 Penicillium glabrum

P. glabrum is a species of fungi distributed worldwide (Nevarez *et al*., 2009). This species belongs to the subgenus *Aspergilloides*. It is well known as a spoilage species especially to strawberries. It also affects margarine and cheese, which makes it one of only a handful of monoverticillate organisms capable of doing this. *P. glabrum* has been reported to produce anti-fungal substances which show an aptitude for it to be a biocontrol agent. This ability was first reported by de Cal *et al*. (1988) by the presence of *P. glabrum* on twigs affected by *Monilinia laxa* peach twig blight. *P. glabrum* has also been tested for bioleaching of coal ash in the past as another application as a biocontrol agent.

A recent research project studied *P. glabrum* for the ability to degrade phenolic compounds. This requires the same enzymes needed to degrade glyphosate. In this particular study Isik *et al*. (2020) found that *P. glabrum* had a high degradation rate for phenolic compounds and thus the enzymes were theorised to be present. This fugal species is also fairly similar to *P. spinulosum* with a similarity of 99% Fig. 4-3.

In Fig. 4-1 and Table 4-1, *P. glabrum* is labelled 13. It was found that this fungal isolate degrades glyphosate by 16.71% ± 8.34% over 48-hours in the initial screening (TLC-plates) test. Initially, at 12- hours there was a high degradation value of 25.42% ± 4.96%. The decline in glyphosate degradation from 12-hours to 48-hours showed a possible experimental error in the extraction of the extracellular enzymes. Compared with the other fungal isolates present this was not enough degradation to warrant further examination.

Although it was found in previous studies that strains of this organism excreted the desired enzymes (Lip, MnP, VP and laccase), this strain did not do so with enough capacity. Unfavourable growth conditions such as temperature (Nevarez *et al*., 2009) may have been the influencing variable. The optimal growth temperature of *P. glabrum* is 25 °C while the universal temperature for the fungal isolates submitted for screening was 30 °C (Nevarez *et al.*, 2009). Despite this, the isolate still functioned like *P. spinulosum*. As seen in Fig. 4-3, the genetic structure of *P. glabrum* is 99% similar to *P. spinulosum*. This could explain the similarities in degradation rates, even though *P. spinulosum* has a higher enzyme production capacity. However, *P. glabrum* is sensitive to temperature change and therefore, under favourable conditions, *P. glabrum* may degrade more glyphosate out of Roundup than in this study.

5.2.4.3 Penicillium spinulosum

P. spinulosum is a hardy fungal species that can grow in areas that have both low water availability and low temperatures. Above this, it is also acid-tolerant and can often be found widely distributed in soil. *P. spinulosum* is therefore classified as a xerophile and a psychroptroph. This fungal isolate is also a non-mycotoxin producing fungal isolate (Pitt and Hocking, 1997). *P. spinulosum* has a high similarity to *P. glabrum* and is differentiated through the rougher exterior found on the conidia of *P. spinulosum*.

P. spinulosum has been extensively studied for its secretion of cellulase (it secretes a larger amount of cellulase than other fungal species) and its bio remedial application in landfills. *P. spinulosum* is also a model organism when applied to the evolutionary classification of fungal organisms (Kim *et al.*, 2014). This species is also known to release spores that may cause inflammatory disorders in the respiratory system (Jussila *et al.*, 2002).

Identified as 26b in Fig. 4-1 and Table 4-1 this fungal isolate degraded 14.16% ± 3.17% of glyphosate over 48-hours. This outcome is fairly similar to that of *P. glabrum*. The similarities between the genetic structures are highlighted in Fig. 4-3 as the isolates were found to be within 99% similar. Due to the low degradation value of *P. spinulosum,* it was not further investigated through the use of ELISA and in soil degradation. This corresponds with the findings of a study done by Eman *et al*. (2013) which showed that *P. spinulosum* was capable of tolerating a glyphosate rich environment, with the possibility of the assimilation of glyphosate, although similar degradation rates were recorded in the study by Eman *et al*. (2013) that after 16 days only 13.9% of the glyphosate was degraded. In both studies, *P. spinulosum* was classified as a slight degrader of glyphosate. Although *P. spinulosum* has industrial applications through the use of cellulase excretion, in this study the degradation of glyphosate was too poor, and it would thus not be recommended for glyphosate degradation.

5.2.5 Rhizopus species

Rhizopus is a saprophytic fungus commonly found on plants and some parasites. These fungal species can be found on a wide array of food substances such as fruits and vegetables (spoiled), syrup, jellies, bread, leather etc. The growth of *Rhizopus spp*. is filamentous with branching hyphae. An interesting feature is the lack of cross-walls which makes these species coenocytic. Reproduction occurs sexually and asexually. In some cases, *Rhizopus* species can be pathogenic, such as *R*. *delemar* which causes mucormycosis, which can sometimes be lethal (Sephton-Clark *et al.*, 2018). *Rhizopus spp*. are widely used in the industrial sector such as *R. microsporus* for making tempeh (fermented food type from soybeans), *R. oryzae* is used in alcohol and *R. stolonifera* is used to produce fumaric acid.

5.2.5.1 Rhizopus microsporus

This fungal isolate is a plant pathogen infecting mainly maize, rice and sunflowers. *R. microsporus* can infect plants and food products but have been known to infect humans with compromised immune systems leading to conditions such as lung diseases (Cheng *et al*., 2009; Dolatabadi *et al.,* 2013). Domesticated variants have been used in the preparation of soy fermentation (Dolatabadi *et al.,* 2013). However, *R. microsporus* has been known to produce rhizoxin, which is

an antitumor drug (Lackner *et al*., 2011). This is also a common soil fungus found in most debris, soil and foodstuff (Jennessen, 2005). Because it can affect a wide variety of plants and crops, it is often found in a diverse range of environments. Its optimum growth temperature is 28 °C, but it can survive in temperature ranges between 25 °C and 55 °C (Dolatabadi *et al*., 2013). Due to its tough outer shell, *R. microsporus* could not undergo DNA extraction using the available methods, but because this fungal isolate was received from a previous study, that identification was taken.

Originally allocated as 16 as an isolate from the gut of a dung beetle, *R. microsporus* showed degradation of 43.49% ± 5.31% in the screening tests. Rhizopus species have been tested for glyphosate degradation in past studies. In studies such as Tapere (2019) *Rhizopus spp*. showed reduced levels of glyphosate degradation after 91 days. This led to this organism being tested further as it indicated possible glyphosate degradation.

Very few studies link *R. microsporus* to glyphosate degradation in soil. Studies have tested some *Rhizopus* species, but the results were contradictory with some being able to degrade glyphosate and some not. One reason *Rhizopus* species are not well studied is that competition can easily inhibit the growth of *Rhizopus* species (Tapere, 2019). Therefore, in soil with more than one isolate, *Rhizopus* would struggle to degrade glyphosate effectively. In this study, however, according to Table 4-3 *R. microsporus* degraded glyphosate with 25.48% ± 8.07% of glyphosate left in soil showing that this isolate could degrade glyphosate under soil conditions *in vitro*.

It can be seen that *R. microsporus* showed increases in both PO_4 in Fig. 4-6 and NH_4 in Fig. 4-5, which again indicated that *R. microsporus* can degrade glyphosate in Roundup. The NH_4^+ levels were lower than in the other fungal isolates but higher than in the positive control. This indicates that an AMPA degradation pathway was present, but not a sarcosine degradation pathway. Potassium levels rose Fig. 4-5 to 347.4 mg/L. This was the highest rise in potassium levels of all the isolates which could have affected the uptake rate of the isolate which would influence the degradation rate of glyphosate. Calcium and Mg also rose to the second-highest levels of all the isolates. This could also have caused the glyphosate to become insoluble and contributed to the degradation rates of glyphosate.

However effective under controlled conditions (TLC-plates and ELISA), *R. microsporus* did not degrade a lot of glyphosate in the long-term test with 78.10% ± 4.80% glyphosate left (Fig. 4-9). Although this particular strain of *R. microsporus* does degrade glyphosate under controlled conditions, it does not use glyphosate from Roundup effectively as a nutrient source. This corresponds with the findings of other studies that tested *Rhizopus spp*. for glyphosate degradation. It was found that only a limited number of *Rhizopus* strains could degrade glyphosate and use it as a nutrient source (Carranza *et al*., 2017). In contrast with this, a study done by Kanat (2018) found that *Rhizopus spp*. can utilise glyphosate well as a nutrient source and recommended it for further studies.

5.2.6 Trichoderma species

Trichoderma is a ubiquitous type of fungus that is prevalent in soils and is found in the *Hypocreaceae family* (Schuster and Schmoll, 2010). Most of the *Trichoderma* species are opportunistic avirulent plant symbionts. *Trichoderma* species also form mutualistic endophytic relationships with several plant species (Vinale *et al*., 2008). Furthermore, several species of *Trichoderma* have been used in biocontrol such as *T. harzianum*, *T. viride* and *T. hamatum* which shows the potential that these fungi possess. Some species have been reported to excrete a fungicide to protect plant species, particularly affecting root diseases, but they can also be effective against foliar diseases (Harman, 2006). *Trichoderma* also attacks other fungi species to gain nutrition (Schuster and Schmoll, 2010). Therefore, the development of almost all of the fungal control products was possible because of the quantities of the *Trichoderma* species. Studies have shown that *Trichoderma* species such as *T. harzianum* can grow in the presence of glyphosate and even utilise it as a carbon source (Singh *et al*., 2020).

5.2.6.1 Trichoderma harzianum

T. harzianum is a fungus used as a fungicide in foliar application, seed- and soil treatment (Kleifeld & Chet, 1992; Perazzolli *et al.*, 2008). Very closely related to *T. afroharzianum* the two species were originally split in 2015. This makes it possible that studies prior to 2015 contain both strains and thus was discussed together. *T. harzianum* was reported to give systemic protection to the foliar disease, grey mould (Ahmad, 1987). It does this through hyper parasitizing other fungal species (Elad, 1980). *T. harzianum* is one of the best-characterised *Trichoderma* species used in

products to promote the suppression of plant diseases (Yedidia *et al.*, 2001; Perazzolli *et al.*, 2008). Previously this fugal isolate has been isolated from *Fiscus elastica* (the rubber tree) and soil from northeast China and parts of Australia. This shows that *T. harzianum* Is a widely spread species of fungus, as it has been found in South African soil by De La Torre *et al*. (2020) as well in this study. It has been tested and studied for its secondary metabolic profile. *T. harzianum* species were isolated, and the genetic code in all five isolates was found to be similar to within 99% according to Fig. 4-3.

T. harzianum was isolated more than once as species 11, 10, 10B, 25a and 25b but species 11 (MT584872.1), 10 (MT613650.1) and 25a (AF030395.1) showed the most promise with glyphosate degradation values of 36.45% ± 2.78%, 32.32% ± 2.09% and 22.01% ± 2.07% over a 48-hour period. The species 25b showed 12.36% ± 9.74% degradation over 48-hours, while 10B showed 14.52% ± 7.84% degradation. While *T. harzianum* showed the capability to potentially degrade glyphosate with a multitude of different strains, as has been shown in other studies, the most promising were selected to undergo soil testing. The isolates used for further studies were 11, 10 and 25b.

In soil *T. harzianum* (MT613650.1 and MT584872.1) degraded glyphosate with 49.72% ± 11.32% and 58.31% ± 8.67% glyphosate left effectively. This showed an effective degradation pattern when compared to the TLC-plates as seen in Table 4. It also shows that *T. harzianum* has the potential to degrade glyphosate in soil under lab conditions. *T. harzianum* is affected by the glyphosate level in soil that can slow its growth and thus affect its ability to degrade glyphosate shown in a study by De La Torre *et al*. (2020). The stunted growth is caused by damage to the fungi spores (conidia) (De La Torre *et al.*, 2020). However, *T. harzianum* can overcome the stunted growth when given enough time.

T. harzianum showed among the highest spikes in soil NH_4 concentrations, which indicates probable degradation of glyphosate and AMPA in soil through the sarcosine and AMPA degradation pathways. It also furthers the argument of *T. harzianum* utilizing glyphosate or Roundup as a sole nutrient source as indicated in Fig. 4-9 and confirmed by Singh *et al.* (2020). *T. harzianum* was also among the highest spikes when looking at PO_4 levels. This is again a good indication that *T. harzianum* can degrade glyphosate out of Roundup through the AMPA pathway (Bouchiat *et al*., 2016) Degradation through the AMPA pathway leaves an organophosphate (PO_4)

as a degradation by-product as well as NH_3/NH_4^+ which can contribute the rise in NH_4^+ and PO_4 over both *T. harzianum* species (Bouchiat *et al*., 2016). Thus, when used in combination with other fungal isolates *T. harzianum* may be an effective fungal isolate for degradation studies of glyphosate.

When compared to the long-term degradation, however, 80.00% ± 7.28% and 93.38% ± 0.36% of glyphosate were left, which showed that *T. harzianum* struggled to use glyphosate as a metabolic nutrient. This was further suggested in a study by Singh *et al*. (2020) which showed *T. harzianum*'s ability to use glyphosate as a sole phosphorous source. It may have an initial degradation, but not long-term which suggested that *T. harzianum* may have to be used in combination with other isolates to be effective.

5.2.6.2 Trichoderma virens and *Hypocrea virens*

This soilborne filamentous fungus *T. virens* is known as a biocontrol agent with known abilities such as the production of antibiotics. It can also parasitize pathogenic fungi (Howell, 2006). *T. virens* can also induce systemic resistance in plants and can help with resistance to fungal infections (Perazzolli *et al*., 2008). *T. virens* is known to be a mycoparasite fungus and this species is known to be the only Trichoderma species capable of producing halogenated terpenes. *T. virens* is also beneficial to plants, as it interacts with plant roots and boosts plant immune systems. Furthermore, it targets fungal species, many of which are plant pathogens, which raises a plants survival capability. It does this by parasitizing the hyphae and it can also penetrate some resting structures to reduce their inoculum potential.

T. virens is known for the production of extracellular chitinase, which contributes to mycoparasitism in *T. virens. T. virens* has also been reported to produce antibiotics (Howell, 2006). In addition, this isolate produces the herbicidal molecule viridiol. Because of this, *T. virens* has been used as a biocontrol agent in the past (for example against *Rhizoctonia solani*) and as a bio-fungicide (Howell *et al*., 2000). Applications of *T. virens* in previous studies have shown an increase in higher xylanase and cellulase activities beneficial to the degradation of plant components such as cellulose and hemicelluloses. *H. virens* is an organism very closely related to *T. virens*. It was classified in the early 2000s as a teleomorph of *T. virens*. The two were at a stage often identified as the same organism due to their similarity (Chaverri *et al*., 2001). When

compared to the other isolates, *T. virens* showed that *H. virens* was the closest genetic relative and not the other *Trichoderma* species. This can be explained as *H. virens* and *T. virens* were, until the early 2000s, seen as the same species and *H. virens* was later classified as a teleomorph species of T. virens. Their similarity in genetic structure is shown in Fig. 4-3, with the two species showing a 96% similarity.

According to studies done by Szabo *et al*. (2015), *T. virens* (F3B-BC) has shown secretion of lignin peroxidase, manganese peroxidase, 1,4-b-D-endoglucanase, polygalacturonase and laccase. In the study by Szabo *et al*. (2015), *T. virens* was the best enzyme producer of all the organisms tested. This is confirmed by this study as *T. virens* was the second-highest degrader of glyphosate in the screening test (Fig. 4-1), showing a 45.96% ± 8.79% degradation over 48 hours. This may have been caused by the probability of high levels of extracellular enzymes produced by *T. virens*.

In soil, *T. virens* was also the second-highest degrader of glyphosate as 24.66% ± 14.29% of glyphosate was left and *H. virens* was the best, with only 11.03% ± 6.88% glyphosate left. This illustrates these isolates' ability to degrade glyphosate in soil (within a controlled environment) and the potential for degradation in an open field. These two isolates are commonly found in soil and can protect plants due to the excretion of fungicides (Howell, 2006). This raises the profile of both these isolates. *T. virens* and *H. virens* have been extensively studied for their bio control capabilities (Howell, 2002), but evidence that they can directly degrade glyphosate is rare.

In Fig. 4-5 it is shown that *T. virens* had a very high level of NH_4^+ which again correlates with the previous findings. Due to the inability of *T. virens* to use glyphosate as a nutrient source, the degradation product of glyphosate (NH_4^+) rose significantly from 340.87 mg/L (positive control) to 867.30 mg/L. *H. virens* showed a similar trend of raising NH_4 levels to 717.80 mg/L. This rise in NH_4^+ levels could be a result of degrading glyphosate, however other factors may also have affected these values. Nevertheless, it does coincide with the other results obtained and thus *T. virens* and *H. virens* can indeed degrade glyphosate in soil conditions.

The only negative aspect of both of these isolates is that they cannot use glyphosate as a nutrient source and thus will have to be paired up with other isolates to work properly in field studies. This is shown in Fig. 4-9, where *T. virens* showed next to no degradation with 98.15% ± 2.80%

glyphosate left in Roundup and *H. virens* showed 92.72% ± 0.31% glyphosate left, showing no degradation. Both were below the control degradation level, and therefore no degradation of glyphosate in Roundup had occurred.

5.2.6.3 Trichoderma simmonsii

Both *T. harzianum* and *T. simmonsii* are found in temperate North America and Europe, except for one Colombian soil isolate. *T. simmonsii* was found in soil or on decaying wood, clearly or cryptically parasitizing other fungi (Kubicek *et al.*, 2019). *T. simmonsii* has not been extensively studied and thus very little information is available with relation to glyphosate degradation. As far as could be ascertained, this is the first occasion on which *T. simmonsii* has been studied in this way. *T. simmonsii* was isolated from soil and identified as F1I-B. With a total of 10.90% ± 8.31% of degradation over 48-hours, it was identified as a possible degrader of glyphosate. However, due to the low degradation rate, this species was not studied further in favour of better glyphosate degraders.

CHAPTER 6 – CONCLUSION AND RECOMMENDATIONS

Even though products containing glyphosate also contain a surfactant to help prevent run-off and improve product efficiency, glyphosate has been reported in water samples, over-accumulated soils and food products. This has led to investigations regarding the safety of glyphosate from an environmental and human health perspective. The investigations yielded that glyphosate was a probable carcinogen that has been detected in food samples in many areas across the world. This poses a serious health risk to people that come into long-term direct contact with glyphosate related products (farm workers, gardeners, manufacturers etc.) and consumers of food that contains glyphosate. Soil accumulation of glyphosate occurs due to malpractices (excessive use of glyphosate-based herbicides) and is responsible for glyphosate accumulation in food as plants try to excise all accumulated glyphosate taken up through the root systems and leaves by moving the glyphosate to fruits, flowers and decaying areas of plants. Another threat that glyphosate can cause is environmental harm, as it can affect non-target organisms (water plants, freshwater shrimps, etc.).

However, since most of these problems persist due to an accumulation of glyphosate in soil, this study attempted to find lignocellulolytic fungal isolates that can degrade glyphosate in soil. This was done through the evaluation of many fungal isolates through TLC-plate screening tests to evaluate the potential of glyphosate degradation. After fungal isolates were established as potential degraders of glyphosate, the fungal species were identified. Of these identified species of isolates, the best species (<25% degradation over 48 hours) were selected to be tested in soil microcosms and nutrient availability testing was conducted. Finally, long-term Roundup degradation was investigated to establish whether an isolate could utilise glyphosate as a sole nutrient source.

Through TLC tests, it was found that *Penicillium adametzioides, Trichoderma virens, Mucor circinelloides, Rhizopus microsporus* and *Hypocrea virens* showed the most promise in degrading glyphosate in the screening tests with more than 40% degradation. These isolates showed great extracellular enzyme production when grown in the presence of lignin and also showed rapid degradation rates. Interestingly, the results showed a genetic diversity in fungal species capable of degrading glyphosate which can be focussed on in future studies.

ELISA tests showed *Hypocraea virens, Trichoderma virens, Rhizopus microsporus, Aspergillus niger* and *Penicillium adametzioides* as the strongest candidates with each showing over 60% glyphosate degradation over 10 days. After it had been established that these isolates can produce extracellular enzymes under simulated conditions, it was shown these isolates likely produce these enzymes within glyphosate contaminated soils and degrade glyphosate within soils. This suggested that it might be productive to conduct soil tests in future studies in agricultural soils contaminated with high levels of glyphosate.

Long-term glyphosate degradation in pure water was done to see which isolates can utilise glyphosate as a sole nutrient source. *Cladosporium cladosporioides, Aspergillus niger, Penicillium adametzioides, Rhizopus microsporus* and *Mucor circinelloides* showed the highest degradation values. However, only *Cladosporium cladosporioides* and *Aspergillus niger* showed near complete degradation of glyphosate over 28-days. These two isolates showed utilisation of glyphosate as a nutrient source than any other isolate. *Penicillium adametzioides* and *Rhizopus microsporus* showed the most consistent degradation over each test. *Aspergillus niger* was also an isolate that performed well over each test.

Penicillium adametzioides is a newly classified fungal species, which is why this study is the first to test *P. adametzioides* for glyphosate degradation. In previous studies *P. adametzioides* has been reported to produce fungicidal products which could help in field studies with relation to survivability in the presence of nutrient competition. It has also been reported to produce cellulase and ligninase, which was suspected to be present in this study as well through the degradation of glyphosate over 48-hours. This isolate is recommended for further study.

Rhizopus microsporus is also not often referenced in literature for glyphosate degradation, but other *Rhizopus* species have shown the ability to degrade glyphosate. However, it is a group of species that can easily fail when natural competition is introduced. Therefore, although this isolate has shown to be able to degrade glyphosate effectively, more studies will have to be conducted on this isolate with regards to how it can overcome natural competition to effectively degrade glyphosate in a natural environment.

Aspergillus niger was not the best performing in any of the tests performed but was consistently degrading glyphosate and thus was the best overall consistent isolate. It is an isolate that grows

fast and occurs in soil naturally. It showed significant degradation rates in each test and would be the best candidate for future studies. Also, due to its fast-growth rate *A. niger* should not suffer from nutrient competition organisms in the same way that *Rhizopus* species do. The danger associated with *A. niger* is aspergillosis which can be hazardous to human health and can also cause several respiratory tract infections. However, according to this study *A. niger* has shown the best potential for future studies with regards to glyphosate degradation.

In conclusion, what this study has shown is that TLC can be used as a screening tool to detect glyphosate degradation and that the results of TLC gave a successful indication as to which isolates could potentially degrade glyphosate in soil through ELISA. It has also shown that even though white-rot fungi have been the benchmark for glyphosate degradation, this study has shown several isolates that can degrade glyphosate at an acceptable rate (not falling withing White-, brown- or soft rot fungi) that may rival most white-rot fungal species. Also, this study has shown that lignocellulolytic fungal isolates do excrete extracellular enzymes needed for glyphosate degradation as shown in all test areas. This led to the successful characterisation of fungal isolates based on degradation rates from screening tests as their degradation rates remained consistent throughout the study with only a few deviations. Microcosms provided much-needed insight into how each of the fungal species could potentially degrade glyphosate in simulated soil environments. Simulated soil environments showed the potential of this study to branch out in several directions in future studies with studies based on practical applications and genetic research. Long term degradation showed which of these isolates could survive in the presence of high glyphosate levels and use glyphosate for nutrient absorption.

Future studies with these organisms can concentrate on their real-life practical application in agricultural fields or study the genetic traits of these fungal isolates to determine if the genetic code could be of use in the future. These fungal isolates could be developed in a way to degrade glyphosate accumulated in the soil through the use of spores or enzymatic applications in soil.

With court cases and future bans on glyphosate products, this research is a small step in the direction of solving the glyphosate problem without banning the product. Glyphosate is the most widely produced herbicide in the world and if banned the herbicide industry could be facing great losses and can have a substantial negative economic impact. With glyphosate being at the heart of agriculture, pressure on the industry continues to grow and forces many countries to take

drastic steps to remove glyphosate from the farming industry. This study contributes but a small part to lifting the pressure on the agriculture sector to offer solutions to these problems and not the exodus of the product.

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