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Exploration and Optimization of Potential Fungi to Degrade Herbicides with Active Ingredient Isopropylamine Glyphosate from Shallot Plantations by *In Vitro*

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ABSTRACT

This research was conducted by isolating potential fungi from soil samples exposed the herbicides with the active ingredient of isopropylamine glyphosate (IAG) and then testing the degradation ability of the obtained fungal isolates. The results showed 15 isolates fungi obtained from the soil to a depth of 10 cm including Aspergillus *sp. with black, green and yellow colonies,* Cladosporium *sp.,* Penicillum *sp.,* Rhizopus *sp.,* Mucor *sp.,* Cephalosporium *sp.,* Phymatotrichum *sp.,* Pytopthora *sp.,* Curvularia *sp.,* Microsporum *sp.,* Colletothricum *sp.,* Acremonium *sp.,* Tricophyton *sp. and 4 isolates from soil samples with a depth of 20 cm, namely* Chrysosporium *sp., Geotrichum sp.,* Aspergillus *sp. with black and green colonies. However, after testing for the presence of herbicides with concentrations of 10 ppm, 20 ppm, and 30 ppm, only 2 isolates were able to survive, namely* Aspergillus *sp with black and green colonies. Further testing on the ability to degrade the IAG was found to reduce the concentration of herbicide. The optimum biodegradability to IAG of* Aspergillus *sp. green and black found in P20 (20 ppm) treatment at the 5th day of incubation, namely 98.69 ppm for* Aspergillus *sp. green, and 96.11 ppm for* Aspergillus *sp. black.*

1. INTRODUCTION

The use of herbicides in agriculture has increased significantly and has become an important part of modern agricultural systems, including the use of herbicides that contribute to increasing agricultural productivity (Ratnaningsih *et al*., 2020). The intensity of herbicide use by shallot farmers is very high, usually used before planting, after planting, and after harvesting. In addition, information was obtained that the amount of shallot production decreased every year, one of the causes was the decline in soil fertility. The type of herbicide used so far is herbicide with the active ingredient isopropyl amine glyphosate 165 g/L equivalent to glyphosate (N-Phosphonomethyl-glycine) 122.26 g/L (Menteri Pertanian RI., 2019).

The isopropylamine glyphosate (IAG) is a herbicide used by farmers in the shallot plantation, Baraka District, Enrekang Regency, South Sulawesi. The application of the IAG herbicide is carried out every day, even if the rainy season arrives the herbicide is given twice a day. It is known that excessive use of herbicides can cause environmental damage, especially to water and soil. Research by Kremer & Means (2009) indicated that the noted alterations in the compositions of soil ecosystem are due to application of glyphosate. The presence of IAG residues in the soil can affect the abundance of microbes, this can have an impact on decreasing soil fertility. Soils treated with glyphosate upsurge in the population of certain fungal species which trigger diseases in the plant, has been observed (Zobiole *et*

al., 2011). Microbes have an important role as a provider of nutrients. Research by Kuklinsky-Sobral *et al*., (2005) reported that glyphosate interfere the equilibrium of beneficial bacteria community for plant growth such as endophytic bacterial. Thus, glyphosate existence in the soil may alter the equilibrium of bacteria and fungi, thereby modifying the functions of soil ecosystems and plant health. In fact, the IAG herbicide is still being used intensively with increased doses, and no effort is made by farmers to reduce the residue of this herbicide in shallot plantations. As a result, it will affect the production of shallots (Tabuni, 2017).

Singh *et al*., (2020*a*; 2020*b*) reported glyphosate has three main metabolic intermediates namely AMPA, acetyl-glyphosate, and sarcosine, which are degraded by bacteria through different metabolic pathways. Glyphosate is systemic and non-selective for weed control, repeated use of herbicides over a long period in an area can lead to two possibilities, namely the dominance of herbicide-resistant weed populations or the dominance of herbicide-tolerant weeds (Widowati *et al*., 2017). The glyphosate contained in the herbicide is not only toxic to weeds but can also affect the activity of soil biota (Fan *et al*., 2012). The high intensity and the amount of IAG herbicide application raises concerns about the dangers of pollution from herbicide residues left in the environment because it can affect the abundance of microorganisms (Jatsiyah *et al*., 2019). Therefore, efforts to restore environmental conditions by using biological activities to degrade and reduce the toxicity of various pollutant compounds are needed.

Bacteria have the ability to decompose complex organic matter into simpler ones, through a degradation process (Sahribulan *et al*., 2019). Several studies have been conducted on the ability of fungi to degrade herbicides. *Basillus sp.* was reported have a high ability to degrade glyphosate compounds (Triwahyuni, 2019; Fan et al., 2012). Spinelli *et al*., 2021 isolated several saprotrophic fungi from agricultural environments and the ability *Purpureocillium lilacinum* to break down and utilize glyphosate as a P source in a liquid medium. *P. lilacinum* was reported for its ability to degrade glyphosate to a considerable extent (80%) and to utilize it as a P source, without showing dose-dependent negative effects on growth. The results of research by Ayu (2022) indicated that *Fusarium falciforme*, *Neocosmospora falciformis*, and *Aspergillus foetidus* was able to degrade chlorpyrifos (100 ppm) biotically with the highest reduction value among other treatments, namely 9.33% with the highest dry weight biomass in a single isolate, namely 185 mg.

Therefore, this research aims to isolate and identify indigenous fungi that utilize glyphosate as the only source of growth and energy. This research is different from other studies because the fungus was isolated from soil that had long been contaminated with glyphosate. The fungus that has been isolated has a high ability to break down contaminants such as glyphosate compared to bacteria isolated from uncontaminated areas. The novel aspect of this article is that it provides the latest information about soil fungi that can degrade glyphosate. Therefore, this will provide information about glyphosate-degrading indigenous fungi and may be useful for the degradation of soil contaminated with herbicide pollutants.

2. MATERIALS AND METHODS

2.1. Materials

The materials used in this study included soil samples were taken from shallot farms in Baraka District, Enrekang Regency, South Sulawesi. Soil sampling point was latitude 03°24’48.63” S, longitude 119°51’50.81” E (Figure 1).

The materials included Supremo 480 SL and Antracol 70 WP pesticides, 1000 ml of distilled water, 10 grams of soil, spirit, Potato Dextrose Agar (PDA) medium, Chlorampenicol and alcohol. The tools used in this study included autoclaves, refrigerators, spoons, diluent bottles, 48 petri dishes, incubators, 1000 ml measuring cup, 500 ml Erlenmeyer flasks, laminar air flow, dropper pipettes, loop needles (round and straight loops), bunsen, object glass, deck glass, match, vortex, microscope, analytical balance, one piece spatula, test tube, label paper, syringe and general equipment used in the laboratory. Soil drills and rulers were used to take soil samples with a depth of 10 and 20 cm.

2.2. Methods

Soil samples were taken from shallot planting land in Baraka District using a soil drill and measured using a ruler to take soil at a depth of 10 and 20 cm. The soil samples were used to isolate fungi that were exposed to herbicides containing the active ingredient glyphosate.

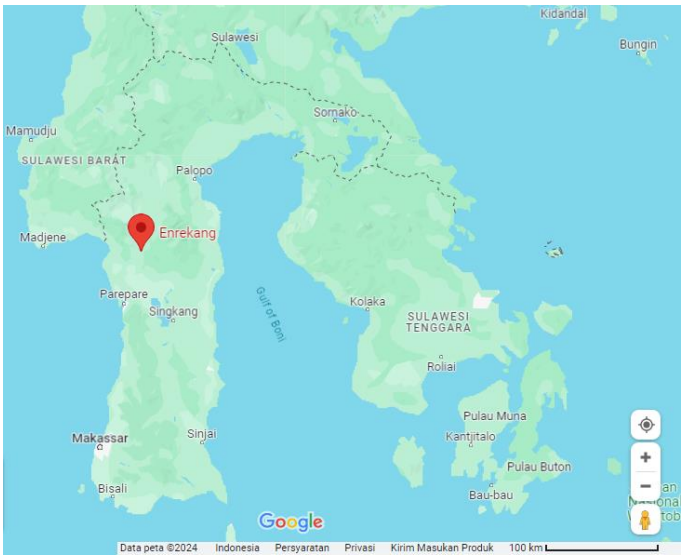


Figure 1. Research location, Enrekang, South Sulawesi from the Google Earth

2.2.1. Exploration and Isolation of Potential Fungi

This stage begins with isolating the fungus through soil dilution by weighing 10 grams of the soil sample and grinding it using a mortar and pistillum then adding sterile water after which 1 ml is suspended into a vial bottle containing 9 ml of sterile distilled water in a 10 diluent bottle homogenized using a vortex. 1 ml is taken and put into a 10 diluent bottle, then homogenized. This is done until diluent bottles 10-4. The results of the dilutions are 10-3 to 10-4 then 1 ml is taken and put into a petri dish. After that, PDA medium is poured into a petri dish and each is given pesticide with a treatment that is the result of a soil dilution of 1 ml (depth 10 cm and 20 cm) + Medium PDA + Pesticide Supremo 480 SL concentration of 10 ppm, 20 ppm and 30 ppm.

2.2.2. Characterization Isolat

2.2.2.1. Macroscopic Characteristics

The macroscopic characteristics of the treatment and control media were based on the shape and color of the colonies on PDA culture media and then observed under a microscope. Fungal colonies were inoculated by taking a quarter of the media overgrown with fungi and transferred to new media and then put in an incubator for 2 - 3 days.

2.2.2.2. Microscopic Characteristics

Microscopic identification of the fungus was carried out by observing under a microscope. In the first stage, the object glass was cleaned with 96% alcohol then distilled water was placed on a glass preparation 1-2 drops, then the active isolate was taken aseptically and placed on the object glass and then covered with a deck glass and then observed under a microscope. This observation was carried out by looking at the shape of the cells and endospores under a microscope with 100x magnification.

2.2.3. Biodegradability Test of Indigenous Fungi

The fungus that has been isolated is then grown in 50 mL mineral salt (MSM) media containing 10 ppm, 20 ppm, and 30 ppm glyphosate. The number of colonies grown for each fungus isolate was determined with an Optical Density (OD) value of 0.5. The OD value was calculated using a UV-Vis Spectrophotometer with a wavelength of 600 nm, and then the mushrooms were incubated on a shaker at a speed of 125 rpm at room temperature for 5 and 7 days. Test for the biodegradability of isopropylamine glyphosate (IAG) by indigenous fungi which have been identified at concentrations of 10 ppm, 20 ppm and 30 ppm, incubation time of 5 and 7 days with optimum growth of the fungus.

Observations on the biodegradation ability of the fungus included a decrease in the IAG (ppm) and fungal biomass (g). The degradation ability was calculated experimentally with a Randomized Factorial Design, using 24 treatments (2 indigenous fungi, tested with IAG concentrations of 0 ppm, 10 ppm, 20 ppm, and 30 ppm) and repeated 3 times for each treatment. Percentage of biodegradation was calculated according to (Yu *et al*., 2005).

$$\text{Bodegradation} = \frac{Control\ Residue - Treatment\ Residue}{Control\ Residue}\ x\ 100\% \quad (1)$$

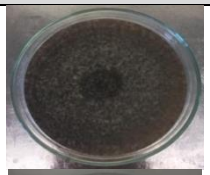
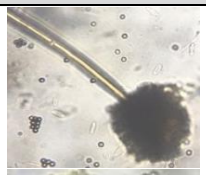
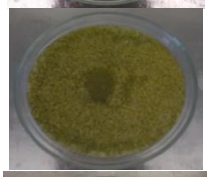
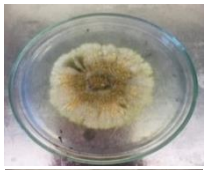
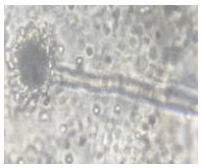
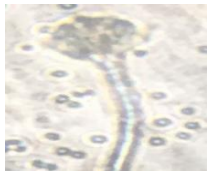
2.3. Data Analysis Techniques

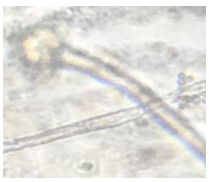
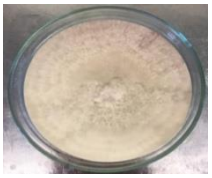
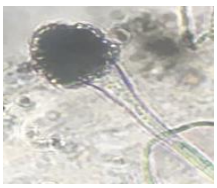
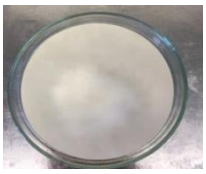
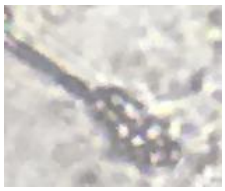
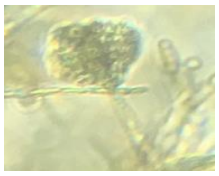
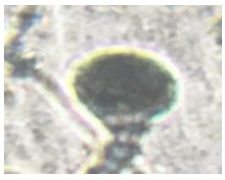
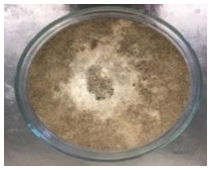
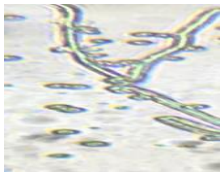
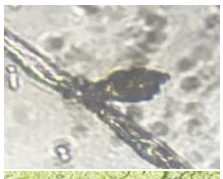
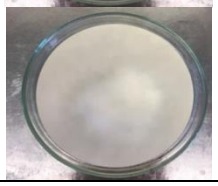
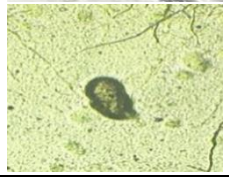
The fungus isolates obtained were then matched with the characteristics of the fungus contained in the book Illustrated Genera of Imperfect Fungi (3rd Ed.) (Barnett & Hunter (1972)) to determine the fungus genus. Analysis of the degradation ability of the fungus isolates obtained on the IAG (ppm) measured using a UV-Vis spectrophotometer, the data were displayed in image form and analyzed statistically. Biomass and degradation data were tested using ANOVA with a further test the DMRT (Duncan Multi Range Test).

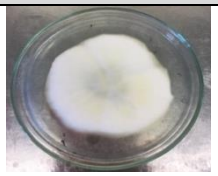
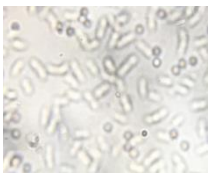
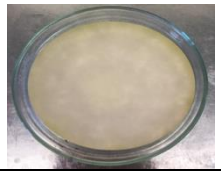
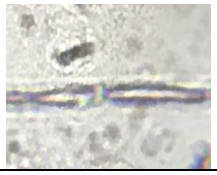
3. RESULTS AND DISCUSSION

Based on the research that has been done, 15 isolates were obtained from the soil sample at a depth of 10 cm and 4 isolates from the soil sample at a depth of 20 cm. The isolates found were identified for microscopic and microscopic characteristics and then the type of fungus was determined, which can be seen in Tables 1 and 2.

Table 1. Fungi isolates found at a depth of 10 cm

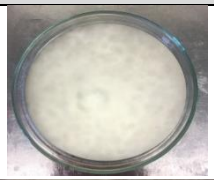
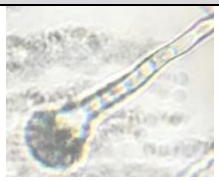
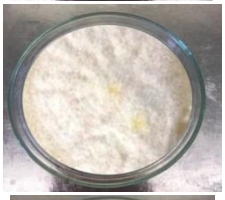
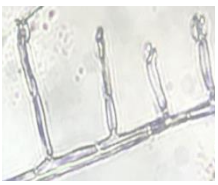
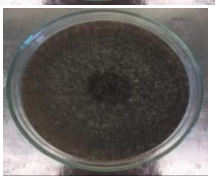
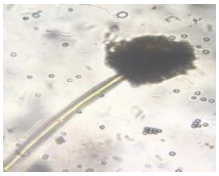
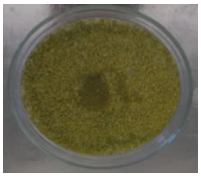
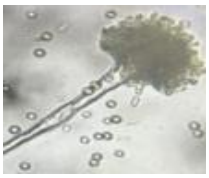
Macroscopic Features	Microscopic Features		Macroscopic Image	Microscopic Image	Type of Fungi
Colony Surface Color	Hyphae	Conidia			
Black	Partition	Round			*Aspergillus* sp
Green	Partition	Fan shaped			*Aspergillus* sp
Yellow	Partition	Fan shaped			*Aspergillus* sp
Yellowish-brown	Partition	Round			*Cladosporium* sp
White	Partition	Chain			*Penicillum* sp

Macroscopic Features	Microscopic Features		Macroscopic Image	Microscopic Image	Type of Fungi
Colony Surface Color	Hyphae	Conidia			
White	Partition	Round			*Rhizopus* sp
Grayish White	Insulated	Round			*Mucor sp*
White	Partition	Oval			*Cephalosporium sp*
White	Partition	Round			*Phymatotrichum sp*
Grayish White	Insulated	Round			*Pytopthora* sp
Dark Chocolate	Partition	Oval			*Curvularia* sp
Yellowish White	Partition	Oval			*Microsporum* sp
White	Partition	Round			*Colletothricum* sp

Macroscopic Features	Microscopic Features		Macroscopic Image	Microscopic Image	Type of Fungi
Colony Surface Color	Hyphae	Conidia			
White	Partition	Ellipse			*Acremonium* sp
White	Partition	Chain			*Tricophyton* sp

Note: Macroscopic images according to diameter size of Petri dish used (80 mm) and microscopic images using a 100x magnification microscope.

Table 2. Fungi isolates found at a depth of 20 cm

Macroscopic Features	Microscopic Features		Macroscopic Image	Microscopic Image	Type of Fungi
Colony Surface Color	Hyphae	Colony Surface Color			
White	Partition	Round			*Chrysosporium* sp
White	Partition	Ellipse			*Geotrichum* sp
Black	Partition	Round			*Aspergillus* sp
Green	Partition	Round			*Aspergillus* sp

Note: Macroscopic images according to diameter size of Petri dish used (80 mm) and microscopic images using a 100x magnification microscope

Based on the research results, it was found that two fungal isolates had the potential to degrade Isoprofil amine glyphosate by treating soil samples from a depth of 10 cm with the addition of herbicide Supremo 480 SL concentrations of 10 and 20 ppm. In the growth medium, *Aspergillus sp* isolates were found with macroscopic characteristics of black and green colonies while at concentrations of 30 ppm found *Aspergillus sp* isolates with macroscopic characteristics of black colonies. In the treatment of soil samples from a depth of 20 cm, Supremo 480 SL herbicide with a concentration of 10 ppm was added to the growth medium. In the growth medium, *Aspergillus sp*

isolates were found with macroscopic characteristics of black and green colonies, at 20 ppm, *Aspergillus* isolates were found with macroscopic characteristics of black colonies, while at a concentration of 30 ppm, isolates were found *Aspergillus sp* with macroscopic characteristics of green colonies, black *Aspergillus sp* has characteristics of black surface color, black back color of colonies, visible hyphae and conidia round. As for *Aspergillus sp* green, it has the characteristics of a green surface color, green back color of the colony, visible hyphae forms insulated and fan-like conidia. This is in accordance with the theory of Barnett & Hanter (1972), that the characteristics of the *Aspergillus* fungus are black, sometimes green, white or yellow, conidiophores erect, simple, rounded ends, radiating from the tip or the entire surface: conidia round, often colorful colorful. The isolated data found is presented in Table 3.

Table 3. Potential Fungi Isolates for Herbicide Degradation.

Soil Depth	Herbicide Concentration	Type of Fungi	Colour of Fungi
10 cm	10 ppm	*Aspergillus* sp	Black
		Aspergillus sp	Green
10 cm	20 ppm	*Aspergillus* sp	Black
		Aspergillus sp	Green
10 cm	30 ppm	*Aspergillus* sp	Black
20 cm	10 ppm	*Aspergillus* sp	Green
		Aspergillus sp	Black
20 cm	20 ppm	*Aspergillus* sp	Black
20 cm	30 ppm	*Aspergillus* sp	Green

Table 4. *Aspergillus* degradation capability test against concentration isoprofile amine glyphosate (%)

Treatment	**Green *Aspergillus***			***Black Aspergillus***			**Control**		
Observation day	5	7	Δ	5	7	Δ	5	7	Δ
P0 (Control)	40.83^{a}	16.16^{a}	24.67	32.43^{a}	4.50^{b}	27.93	8.07^{c}	4.17^{b}	3.90
P10 (10 ppm)	43.72^{a}	9.29^{b}	34.43	93.54^{b}	7.84^{b}	85.70	7.00^{c}	5.87^{bc}	1.13
P20 (20 ppm)	98.69^{b}	22.57^{a}	76.12	96.11^{b}	7.74^{b}	88.37	2.47^{c}	1.86^{c}	0.61
P30 (30 ppm)	88.22^{b}	10.48^{a}	77.74	94.07^{b}	7.05^{b}	87.02	7.19^{c}	2.68^{c}	5.51

Note: Treatments accompanied by the same letter in the column are not significantly different, whereas treatments accompanied by different letters are significantly different, based on the results of the Duncan test with a confidence level of $\alpha = 0.05$.

Based on the observations that have been made, it is known that *Aspergillus* sp. has ability to degrade isoprofile amine glyphosate. The degradation data of *Aspergillus sp*. on to isoprofile amine glyphosate is presented in Table 4. On the 5th day the highest degradation ability was shown by green *Aspergillus* at a P20 concentration (20 ppm) of 98.69%. The degradation ability of green *Aspergillus* was not significantly different at the P30 concentration (30 ppm). Apart from that, it was not significantly different from the degradation ability of black *Aspergillus* at concentrations of P10 (10 ppm), P20 (20 ppm) and P30 (30 ppm). However, it was significantly different from the control treatment.

On the 7^{th} day, the highest degradation ability was observed by green *Aspergillus* at a P20 concentration (20 ppm) of 22.57%. The degradation ability of green *Aspergillus* was significantly different from the degradation ability of black and control *Aspergillus*. All treatments showed a reduction so that this could indicate the biodegradation activity carried out by *Aspergillus* sp. It degradation ability against isoprofil amine glyphosate herbicide is shown in Figure 2.

Based on the results of observations, it showed that both isolates had the ability to degrade glyphosate isoprofile amine. However, there was a visible difference in the concentration of degradation between days 5 and 7. The concentration of degradation on day 5 was higher than on day 7 because on day 5 the peak activity of *Aspergillus* sp. using glyphosate as a source of carbon, phosphorus and nitrogen in an enriched medium, while on day 7 the carbon, phosphorus and nitrogen content in the medium had begun to decrease so that the amount of degradation concentration

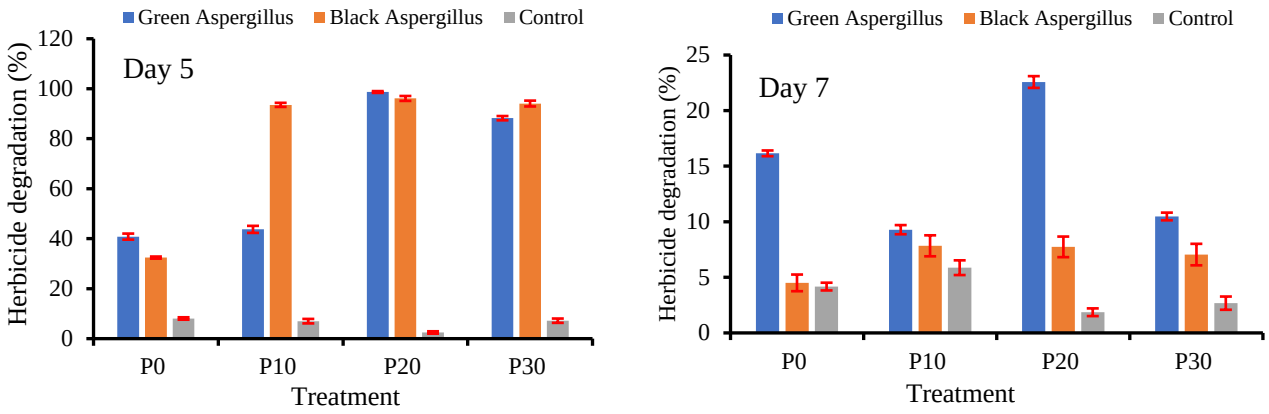


Figure 2. The degradation ability of *Aspergillus* sp. on the 5th day (left) and 7th day (right)

was less. This is supported by research conducted by Cesilia (2017) which stated that when growing *Aspergillus* flavi on media enriched with glyphosate, growth experienced a very significant increase because *Aspergillus* flavi uses glyphosate as a source of carbon, phosphorus and nitrogen for its growth. According to Ristiari *et al*. (2018), *Aspergillus* sp. is the most abundant fungus in the soil and is toxic which is capable of producing proteases that function in the transformation of organic nitrogen in the soil and other organic matter wastes into inorganic N (NH4+). In addition, this fungus is a type of phosphate solubilizing fungus which has been proven to be able to dissolve phosphate from sources that are difficult to dissolve. The type of fungus obtained from this previous study will be tested by degrading the isoprofile of amine glyphosate.

One of the most significant herbicides used in agriculture is glyphosate. This pesticide is degradable by a variety of microbes. Fungi are actually the primary herbicide-degrading microbes and the most resilient to environmental stressors, according to reports. An affordable, practical, and ecologically friendly way to address glyphosate contamination in soils is by fungus bioremediation. In this work, a number of saprotrophic fungi that were isolated from agricultural settings were tested to see if they could withstand and make use of Roundup as a food source under various cultural circumstances. In order to assess Purpureocillium lilacinum's capacity to degrade and employ glyphosate as a P source in a liquid medium, additional screening was conducted (Spinelli *et al*., 2021). Fungi produce large amounts of extracellular enzymes during soil hyphal colonization, resulting in high levels of xenobiotic biodegradation. It should be understood that the degradation of glyphosate in the soil environment is a co-metabolic process and the rate of decomposition must depend on the general activity of the microbial population, soil type and environmental conditions. There are two main paths in the degradation carried out. One pathway leads to the formation of sarcosine and inorganic phosphate via CP lyase, whereas the other type of degradation occurs by glyphosate oxidoreductase (GOX), which cleaves the CN bond of glyphosate to produce aminomethylphosphonic acid (AMPA) and glyoxylate (Carranza *et al*., 2007).

From Figure 2 (left) it can be seen the calculation of the average concentration of *Aspergillus sp.* green, black and control. Observations obtained when reviewed for each treatment, on the 5th day both *Aspergillus sp.* green and black showed relatively higher concentrations when compared to controls. The highest concentration of *Aspergillus* sp. green is indicated by P20 (20 ppm) of 98.69%. As for *Aspergillus* sp. black is also on the P20 (20 ppm) at 96.11%. Figure 2 (right) also shows the calculation of the average concentration of *Aspergillus* sp. green, black, and control. On the 7th day of observation, there was a reduction in concentration which indicated the biodegradation activity carried out by *Aspergillus* sp. neither green nor black. On the 7th day of observation of *Aspergillus* sp. green the highest value is shown by P20 (20 ppm) of 22.57. All treatments on the 7th day with *Aspergillus* sp. green was not significantly different between P10 (10 ppm), P20 (20 ppm), P30 (30 ppm) and control. On the 7th day of observation of *Aspergillus* sp. black the highest value is shown by P10 (10 ppm) of 7.84. The treatments of P10 (10 ppm), P20 (20 ppm), and P30 (30 ppm) were significantly different from the control.

Previous research, the ability to grow *Aspergillus* sp. also affected by the concentration of glyphosate used. Research conducted by Barberis *et al*., (2013), showed that *Aspergillus flavus* and *A. parasiticus* strains were able to grow effectively and produce aflatoxins in high nutrient status media at a range of glyphosate concentrations under different water activity conditions. Another study conducted by Carranza *et al*., (2016), showed the tolerance of non-toxic *A. oryzae* and *A. flavus* strains to high concentrations (100 to 500 mm – 17,000 to 84,500 ppm) of commercial glyphosate formulations. All tested lines were able to develop at the highest concentrations of glyphosate tested regardless of water availability conditions.

4. CONCLUSIONS

Based on the results of the study it was concluded that:

1. There are 15 isolates fungi obtained from exploration the soil to a depth of 10 cm including *Aspergillus* sp. with black, green and yellow colonies, *Cladosporium* sp., *Penicillum* sp., *Rhizopus* sp., *Mucor* sp., *Cephalosporium* sp., *Phymatotrichum* sp., *Pytopthora* sp., *Curvularia* sp., *Microsporum* sp., *Colletothricum* sp., *Acremonium* sp., *Tricophyton* sp. Four isolates are obtained from soil samples with a depth of 20 cm, namely *Chrysosporium* sp., *Geotrichum* sp., *Aspergillus* sp. with black and green colonies. However, after testing for the presence of herbicides with concentrations of 10 ppm, 20 ppm, and 30 ppm, only 2 isolates were able to survive, namely *Aspergillus* sp. with black and green colonies.
2. The optimum biodegradability of *Aspergillus* sp. green and *Aspergillus* sp. black in degrading isopropyl amine glyphosate occurred on the 5th day of incubation. At P20 (20 ppm) optimum biodegradability of *Aspergillus* sp. green is indicated by of 98.69 ppm, and for *Aspergillus* sp. black is 96.11 ppm.
3. The suggestions for further research are to use other means of charging such as GC-MS or HPLC. This research is expected to be continued, because the output of this study is the consortium of fungi as bioremediation agents to overcome environmental pollution problems.

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