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ISOLATION AND IDENTIFICATION OF GLYPHOSATE RESISTANT, AMYLASE AND PROTEASE PRODUCING FUNGI FROM NON-AGRICULTURAL SOIL

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ABSTRACT

Glyphosate herbicide is one of the most used herbicide throughout the world and they are very important to agriculture. Despite the role of herbicides to agriculture, they also posed direct or indirect threats to the health of humans and also to the nature and survival of soil microorganisms. However, there is a need for determination of the effect of herbicide on soil fungi. This research was carried out to determine the effect of glyphosate-base herbicide on the growth of soil fungi of non-agricultural soil of IBBUL Three sites of non-agricultural soil of IBBUL were selected and labelled as site (A, B and C) respectively. A serial dilution method and microscopic method were used for the isolation and identification of the different fungal species in the different soil treatments. A total of 270 isolates were identified and these comprises of thirteen genera namely, *Aspergillus, Fusarium, Penicillium, Microsporum, Candida, Protostoma, Trichoderma, Xenosporella, Stylopage, Meria, Rhizopus, Papulospora*, *Paecilomyces.* The frequency of fungi isolated showed that *Aspergillu*s (33) had the highest occurrence followed by *Fusarium* (28), *Stylopage* (10) and *Papulospora* (12) in all the three sites (A, B and C). *Aspergillus niger, Fusarium spp, Penicillium notatum, Microsporum spp, Candida albicans, Protostroma spp, Trichoderm spp, Xenosporella spp, Stylopage spp*, *Meria laricis*, *Rhizopus spp*, *Papulospora spp,*and *Paecilomyces spp* were all amylase positive while others were amylase negative. *Fusarium spp, Penicillium notatum, Microsporum spp, Candida albicans, Xenosporella spp, Stylopage spp, Rhizopus spp* and *paecilomyces spp* were all protease positive while others were negative. This result indicates that the effect of glyphosate herbicide on the growth of soil fungi is not immediate. The result demonstrates overall negative effect of glyphosate on the growth of soil fungi. However, there is a need for long time studies on effect of glyphosate herbicide on the growth of soil fungi of non-agricultural and agricultural soil of IBBUL.

Keywords: Amylase, Protease, Fungi, Glyphosate, Isolates

INTRODUCTION

Weeds are one of the major threats to the agriculture for ages. Globally, a number of chemicals are tested and used for weed management from time immemorial. However, the major shift in the use of agricultural chemicals was observed after World War II (Choudhury *et al*. 2016). The use of herbicides in agriculture has over the years contributed tremendously to both food and cash crop production all over the world. But one of the challenges undermining the farming business has been the invasion of many common weed species due to favourable environmental conditions in

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Nigeria. As a result of threats posed by weeds, manufacturers have adopted the act of flooding the agrochemical market with all kinds of herbicides that are meant for the elimination of different kinds of weeds at different stages of their growth (Sebiomo *et al*., 2011). Up till 2016, more than 2000 herbicides belonging to 15 different modes of action were introduced in the global market (Choudhury *et al*. 2016). Economic viability and easy application make it one of the most common tools for the weed management in modern-day agriculture. Intensity of utilization was further increased with adoption of conservation agriculture practices and herbicide-resistant genetically (Ntow *et al*., 2006).

Glyphosate [N-(phosphonomethyl) glycine] is a systemic, non-selective, post-emergence herbicide and the most active ingredient worldwide in herbicides formulation (Van Stempvoort *et al*., 2014). It constitutes the active ingredient of more than 750 different broad-spectrum herbicides used worldwide. Recommended glyphosate field doses vary between 0.96 and 2.88 kg a.i./Ha but high doses or repeated applications are common (Zabaloy *et al*., 2016). This herbicide has a moderate persistence in soil and is degraded predominantly by co-metabolic microbial processes (Qin *et al*., 2014). Glyphosate targets a key enzyme in the shikimate pathway (5-enolpyruvyl-shikimate-3-phosphate synthase) involved in the production of aromatic amino acids (phenylalanine, tyrosine and tryptophan) that are essential for the growth of most plants, inhibiting nucleic acid metabolism and protein synthesis (Bortoli *et al.,* 2012). Moreover, the shikimate pathway is present in non-target organisms such as bacteria, fungi and algae.

The relationship between glyphosate and fungi varies from toxicity to biodegradation vitro (Busse *et al.,* 2001). Glyphosate toxicity was demonstrated in vitro (Busse *et al.,* 2001); however, when recommended field doses were tested in a soil microorganism, the changes in soil fungal communities were inconsistent (Qin *et al*., 2014). Several studies have shown that glyphosate may exert at least temporary changes in soil microbial activity (Haney *et al.,* 2010), inhibiting the growth of some fungal species and stimulating others, including plant pathogens (Means *et al.,* 2014).

Fungi are vital in the degradation and decomposition of cellulose, starch, pectin, lignin and hemi-cellulose in the organic matter added to the soil (Firdous *et al.,* 2017). They also serve as food for bacteria. Fungi can be classified into three general functional groups based on how they get their energy. They can either be decomposers, mutualists or pathogens / parasites. Pathogens cause reduced production or death when they colonize the roots and other organisms. Root-pathogenic fungi as *Rhizoctonia* cause major economic losses in agriculture each year. Fungal diseases often reduce crop yield and lower crop quality by producing toxins which are harmful to human health (Placinta *et al.,* 1999). Some of the most commonly observed fungi in the soil are *Alternaria, Aspergillus, Cephalosporium, Mucor, Gliocladium, Penicillium, Trichoderma, Cladosporium, Botrytis, Monilia, Chaetomium, Pythium, Cladosporium,* and so on and so forth (Brady *et al.,* 1999).

Herbicide application has become an integral part of vibrant agricultural productivity in the whole world since its benefit has been overwhelming over the years. However, the excessive use of these non-selective herbicides especially in our agricultural soil has toxic impact on the non-target soil microorganisms which play roles in degrading organic matter, nitrogen and nutrient

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recycling and decomposition. Due to the excessive use of these glyphosates on our farmland there is a need for regular monitoring of these farmlands since, soil microorganisms help in degradation of these herbicides, decomposition of organic matters and also maintenance of soil structure.

Over the years, many research works have been carried out on the effect of selective herbicide on soil microorganisms, microbial population and on agricultural soil. However, there is paucity of information available on the effect of glyphosate (non-selective) herbicide on the growth of soil fungi in Ibrahim Badamasi Babangida University Lapai (IBBUL).

MATERIALS AND METHODS

Description of the Study Area

The study area is non-agricultural soil of Ibrahim Badamasi Babangida University Lapai (IBBUL) situated in Lapai local government of Niger State, Nigeria. Three different sites were identified. These sites were virgin piece of lands where no agricultural activities were said to be carried out and also herbicides were not said to be used. Site A was a piece of land at the back of IBBUL Microfinance Bank, site B was Botanical Garden of Biological Sciences and site C was a piece of land at the back of IBBUL Administrative.

Sample Collection from the Study Area

Soil samples were collected from three different sites in IBBUL with no prior to herbicide treatment. The soil samples were collected from topmost part of the soils (5cm in depth) with sterile hand trowel and transferred inside sterile polyethylene bags (well labelled). The samples were sieved using a 2.0 mm mesh size to remove stones and plant debris.

The Herbicide

The herbicide used was obtained from a local agricultural input dealer in Lapai, Niger state. The selected herbicide that is **"Gobara"** is the most used herbicide brand in the area which contain the following active ingredients 360g Glyphosate per litre in the form of 480g per litre isopropyl amine salt of liquid (SL).

Preparation of Growth Media

Potato dextrose agar (PDA) was used as a media for the growth and maintenance of the fungal isolates all through the experiment. The preparation of the PDA was done according to the manufacturer's recommendation. Exactly 39g of dehydrated PDA (Difco Laboratories, Michigan, USA) was dissolved in 1 litre of distilled water in a 1000 ml conical flask. The solution was autoclaved at 121^0C for 15 minutes and was allowed to cool to 45^0C. The molten PDA was poured into sterile Petri dishes, 9 cm in diameter in such a way that the PDA filled the whole bottom of the Petri dishes. The mouth of the conical flask was flamed every time before and after pouring the PDA into the Petri dishes. The PDA was allowed to solidify and preserved in the refrigerator.

Isolation of Fungi from Soil Samples

A provision of test tubes were made depending on the number of diluents to be obtained. They were then labelled from 10^{-1} – $10^{-11.}$ A known volume of the stock was obtained, usually 1mL (for a solid sample dissolve 1gm or a loop full of the sample inside a separate test tube labelled diluent and collect 1mL). Each test tube was then filled with a known volume of distilled water usually 9mL (peptone water or normal saline can be used as a substitute for distilled water). The 1ml of the sample was then introduced into the 9mL of distilled water in the test tube

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labelled 10^{-1}, this produces 10mL of the dilute solution. The solution was mixed thoroughly. 1mL was then taken from each preceding test tube and added to the next i.e. from 10^{-1} to 10^{-11}.

Identification of Isolated Fungi

The identification of isolated fungi were done by using a microscopic method and biochemical method.

Microscopic Method

A drop of lactophenol cotton blue was placed on a microscopic slide and an inoculum from fungus culture representing all fungal structures were transferred on the slide. The fungal inoculum was separated with teasing needle and mixed with stain. Cover slip was placed on the slide to avoid air entrapment and finally examined under microscope. The structures seen were sketched and morphology of each was described and identified based on the characteristics. Micrographs of Watanabe (2002) was used as atlas for comparison.

Biochemical Method

Screening for Amylase

Production was done by inoculating the fungal isolates on starch agar (containing peptone, 1%; yeast extract, 1%; KH_2PO_4, 0.5%; agar 2% and supplemented with 1% (w/v) starch (HiMedia) as a carbon source and supplemented with antibacterial antibiotic Chloramphenicol) plate with fungal isolates. After incubation, the plates were flooded with iodine solution and clear zone of hydrolysis surrounding the colony was taking as evidence of amylotic (Aneja, 1996; Kathiresan and Manivannan, 2006).

Screening for Protease Production

The fungal isolates were inoculated on Casein agar plates and incubated at 20^0C for 4 days. After inoculation, the plates were observed for possible clear zone surrounding the colony (Aneja, 1996).

RESULTS AND DISCUSSION

Isolation and Identification of Fungi from Soil Samples

The results showed that varying genera and population of fungi were observed in the samples treated with non-selective herbicide (glyphosate) throughout the sample period. A total of two hundred and sixty-eight fungal species were isolated and identified. The identification was done using morphological features and compared with mycological atlas as shown in Table 1.

The total number of fungal species isolated during the studies is Two Hundred and Seventy, which comprises fourteen genera namely; *Aspergillus, Fusarium, Penicillium, Microsporum, Candida, Sporonema, Protostoma, Trichoderma, Xenosporella, Stylopage, Meria, Rhizopus, Papulospora*, and *Paecilomyces.* Of all the genera, *Aspergillus* and *Fusarium* had the highest frequency of occurrence while *Stylopage* and *Papulospora* had the least frequency of occurrence in all the three sites (A, B and C) respectively as shown in Table 2.

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Table 1: Cultural and morphological characteristics of isolated fungi and comparison with mycological atlas.

Samples	Cultural characteristics	Morphological features	Inference
1	Cottony appearance, initially white then turned black after few days	Brown conidial heads splits into columns and hyphae.	*Aspergillus niger*
2	Blue-green in colour with a yellowish pigment	Brightly coloured filamentous hyphae with conidiophores arising from the mycelium.	*Penicillium notatum*
3	Spread flat colonies that are white to creamed coloured with a cottony surface.	Spindle or comb-like in shape with segmented hyphae and microconidia.	*Microsporum spp*
4	Smooth creamy-like colonies	Mycelium septate, conidia hyaline single celled and conidiophores absent.	*Candida albicans*
5	Creamy-greenish in colonies	Conidiophores hyaline upright, much branched with green patches of conidia.	*Trichoderma spp*
6	Transparent or colourless in colonies	Mycelium, slender sparsely branched with conidia hyaline.	*Stylopage spp*
7	Cottony in appearance with some tinge purple to yellow in colonies.	Canoe-shaped, mycelium extensive with conidia hyaline	*Fusarium spp*
8	Rounded, central bulged, thick with orderly margins and radiating ring. Initially white-red wine-dark brown after few days	Hyphae ramose and septate with branched conidiophores.	*Paecilomyces spp*
9	Early stage white then to dark after some days.	Mycelium hyaline branched, conidiophores elongated and septate with conidia hyaline.	*Meria laricis*
10	Brownish yellow then to dark brown	Dark seta-like papulaspores and intercolary hyphal areas.	*Papulaspora spp*
11	Creamy to dark brown in colonies	Conidiophores minute dark single celled conidia	*Protostroma spp*
12	Yellowish brown	Conidiopores dark, comparatively short or stout, branched, septate with dark conidia tightly coiled.	*Xenosporella spp*
13	Early-stage white to dark brown in colonies.	Filamentous branching hyphae which coenocytic	*Rhizopus*

Screening of the Isolated Fungi for Amylase and Protease Production

The Amylase and protease production potentials of the isolated fungi is as shown in Table 3 below. *Aspergillus niger, Fusarium spp, Penicillium notatum, Microsporum spp, Candida albicans, Protostroma spp, Trichoderm spp, Xenosporella spp, Stylopage spp*, *Meria laricis*, *Rhizopus spp*, *Papulospora spp,*and *Paecilomyces spp* were all amylase positive while others were amylase negative. *Fusarium spp, Penicillium notatum, Microsporum spp, Candida albicans, Xenosporella spp, Stylopage spp, Rhizopus spp* and *paecilomyces spp* were all protease positive while others were negative Both negative and positive results of the amylase and protease activities are as display in Figure 1 and 2 respectively.

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Table 2: Frequency of fungal species isolated from soil samples of three different sites of non-agricultural soil of (IBBUL)

Fungal species	Site A	Site B	Site C	Total
Aspergillus niger	11	10	12	33
Fusarium spp	10	9	9	28
Penicillium notatum	9	7	3	19
Microsporum spp	8	5	10	23
Candida albicans	10	2	7	19
Protostroma spp	9	2	8	19
Trichoderma spp	5	4	6	15
Xenosporella spp	9	7	4	20
Stylopage spp	5	3	2	10
Meria laricis	6	2	6	14
Rhizopus spp	7	6	3	16
Papulospora spp	5	4	3	12
Paecilomyces spp	8	8	6	22
Total	**104**	**68**	**78**	**270**

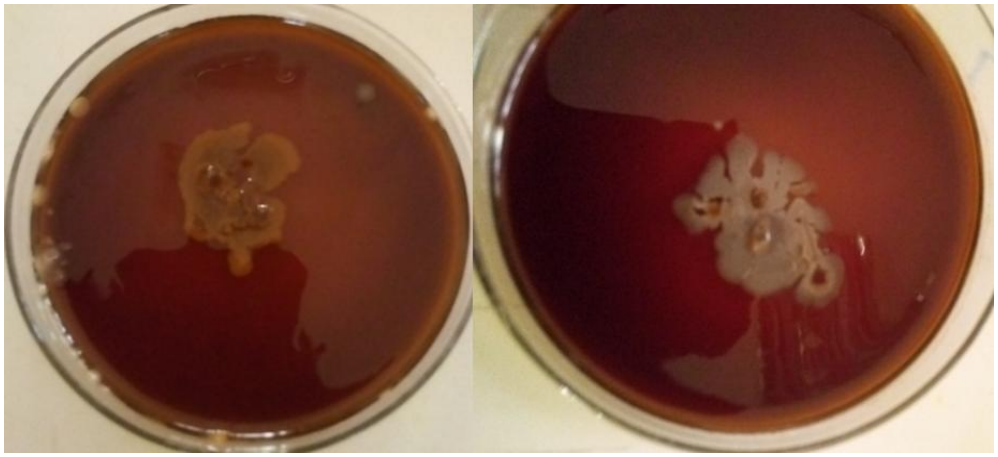

Figure 1: Amylase activity determination: Negative (left), Positive (right)

Figure 2: protease activity determination: Negative (left), Positive (right)

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Table 3: Production potentials of some fungal species isolated from soil samples of non-agricultural soil of IBBUL, Niger State.

Fungal species	Amylase	Protease
Aspergillus niger	+	-
Fusarium spp	+	+
Penicillium notatum	+	+
Microsporum spp	+	+
Candida albicans	+	+
Protostroma spp	+	-
Trichoderma spp	+	-
Xenosporella spp	+	+
Stylopage spp	+	+
Meria laricis	+	-
Rhizopus spp	+	+
Papulospora spp	+	-
Paecilomyces spp	+	+

Present (+) Absent (-)

DISCUSSION

The use of herbicides in agriculture has over the years contributed tremendously to both food and cash crop production all over the world. Thus, it was important to study the effects of these herbicides on the growth of soil fungi. In the present study, *Aspergillus sp* and *Fusarium sp* isolated from non-agricultural soil of Ibrahim Badamasi Babangida University Lapai are the fungal species with highest frequency of occurrence while *Stylopage sp* and *Papulospora sp* were the fungal species with least frequency of occurrence in all the three sites (A, B and C) treated with the glyphosate herbicide. In a related study, Hamza *et al.* (2019) studied molecular and biochemical characterization of some keratinophilic fungi isolated from soil samples from Murtala Amusement park Minna, Niger state, Nigeria and reported that *Aspergillus niger* was the fungus with the highest frequency. This result is also in line with Ashok *et al.* (2014) Olajubu and Folurunso (2014) Similarly, Bashir *et al.* (2018) studied effect of glyphosate herbicide on soil fungi in Yola, Adamawa state and also reported that *Aspergillus niger* and *Aspergillus flavus* had the highest frequency throughout the period of the study. In this study, *Aspergillus sp* and *Fusarium sp* had the highest frequency of occurrence in all the three sites and differ from Sangoyomi *et al.* (2015) who reported that *Lasidiodiplodia theobromae* had the highest frequency of occurrence through all the treatments. Agu *et al.* (2013) also disagrees with this finding who in their study isolated *Aspergillus flavus* with the highest number of occurrence.

The dominance of the *Aspergillus niger* in the soil may be due to their high rate of spore production, dispersal, extreme resistance to environmental conditions and their suitability to adapt and grow in different soil pH concentration. Moreover, the genus *Aspergillus* is known to produce some toxins such as aflatoxins, achrotoxins and these toxins, if secreted may inhibit the growth of other fungi species (Hamza *et al.,* 2019). Several researchers have shown that pathogenic fungi secrete various lytic enzymes, such as proteases and lipases, and these enzymes enhance survival in tissues by digesting host proteins, lipids thus providing a source of energy for the fungi (Ogawa *et al.*, 1992).

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In this current study, accurately 99% of the fungi isolated from non-agricultural soil of Ibrahim Badamasi Babangida University Lapai showed positive for amylase production and these fungi species includes *Aspergillus sp, Fusarium sp, Penicillium sp, Microsporum, Candida sp, Protostoma, Trichoderma sp, Xenospora, Stylopage, Meria sp, Rhizopus sp, Papulospora sp* and *Paecilomyces sp* except for *Sporonema* that showed negative for amylase production and this result is line with Hamza *et al.* (2019) in their study of molecular and biochemical characterization of some keratinophilic fungi isolated from soil samples from Murtala Amusement park Minna, Niger state, Nigeria and reported that exactly 90.90% of the isolates were positive for amylase production. Also in a related sudy, Kathiresan and Manivannan (2006) isolated strains of *Penicillium* species from the coastal soil of a mangrove habitat and later identified *P. fellutonum* and showed positive for amylse production.

In this result 70% of the fungi isolates were positive for protease production and these fungal species are *Fusarium sp, Penicillium sp, Microsporum, Candida sp, Xenospora sp, Stylopage sp, Rhizopus sp,* and *Paecilomyces sp* while *Aspergillus, Protostoma, Trichoderma, Meria* and *Papulospora* were the isolates that showed negative for protease production. Similarly, Hamza *et al.* (2019) studied molecular and biochemical characterization of some keratinophilic fungi isolated from soil samples from Murtala Amusement park Minna, Niger state, Nigeria and reported that exactly 54.50% of isolates were positive for protease production. In related study, Banakar e*t al*. (2012) worked on the isolation and screening of forest soil of Bhadra Wild Life Sanctuary for potent amylotic fungi reported that *Penicillium* species showed positive for protease production.

CONCLUSION

From the data obtained several fungal species were isolated and identified from samples treated with glyphosate (non-selective) herbicide of non-agricultural soil of Ibrahim Badamasi Babangida University Lapai, Niger State. Some of these fungi; *Aspergillus sp, Fusarium sp, Penicillium sp, Microsporum, Candida sp, Protostoma, Trichoderma polysporum, Xenospora, Stylopage, Meria sp, Rhizopus, Papulospora* and *Paecilomyces sp* and *Sporonema* are potential sources of enzymes production such as amylase and protease. The results indicated that the effect of glyphosate is not immediate but demonstrate overall negative effect of glyphosate on the growth soil fungi.

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