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In vitro Evaluation of Growth Inhibition of Some Common Soil Fungi by Selective and Non-selective Herbicides

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Abstract: The indiscriminate and excessive use of herbicides being witnessed in recent times portends possible danger to environmental and human health. To gage the likely impacts of herbicides onfungal ecosystem services, the influence of three narrow [selective (atrazine, butachlor and 2,4-D)] and two broad [non-selective (glyphosate and paraquat)] spectrum herbicides at various concentrations of 0, 0.01, 0.1, 0.5 and 1% v/v on the radial mycelial extension growth, mycelial extension growth rate, percentage mycelial growth inhibition and minimum sporulation time of four common soil fungi, *Aspergillus niger*, *A. flavus*, *Trichoderma viride* and *Penicillium* sp. were investigated on PDA plates for a period of 96 h at 30 ± 2°C (room temperature). The radial mycelial extension growth of fungi decreased with increased concentrations of the herbicides except for *T. viride* where atrazine did not show any significant difference among all the concentrations tested ($P \leq 0.05$). The fastest mycelia extension growth rate for all fungi was recorded in Atrazine at the highest concentration tested, with *T. viride* being the fastest at 0.44 mm h^{-1}. At 1% v/v, save for atrazine, all the herbicides inhibited at least 66% mycelial growth with 2,4-D showing a 100% inhibition. The minimum sporulation time for all fungi in the presence of the herbicides was 48 h. There was no sporulation in *A. flavus* and *T. viride* in presence of paraquat and 2,4-D above 0.1% v/v concentration within the 96 h of investigation. The results of this study suggest that indiscriminate and excessive use of herbicides could negatively affect ecosystem function.

Keywords: Herbicide, Mycelia Growth, Soil Fungi, Sporulation

1. Introduction

Globally, the application of herbicides in weed management and control has become ageneral and acceptable practice because of it success and ease of application as against the traditional manual and mechanical process. In Nigeria, there has been an increasing upsurge in the use of herbicides in recent time by peasant and commercial farmers, home owners, private estate developers etc. to control and kill weeds in farms, residential, official and commercial premises. Herbicides have been consistently linked to serious illnesses such as cancer, reproductive problems and neurological diseases, among others [1]. The indiscriminate and excessive use of these herbicidesportends serious danger not only to human health but also agricultural productivity and environmental wellbeing as various groups of soil microorganisms including fungi could be affected.

Fungi are important members of the soil microbial community in terms of their dominance and ecological function. They play critical role in the degradation of organic waste and pollutants, cycling of nutrients in the biogeochemical cycles, and assisting plants in increased nutrient uptake through root-mychorrizae association. They are also a repository of useful antibiotics in medicine, in addition to being important antagonists to some common soil borne plant pathogens [2].

The growth inhibition of soil fungi by herbicides therefore could drastically limit these enormous ecological functions with grave environmental consequences. It is on the basis of ascertaining the possible impact of herbicides usage on the growth of fungi in soil that this study was carried out.

2. Materials and Methods

2.1. Isolation and Characterization of Test Fungal Isolates

The four test fungi employed in this study (*Aspergillus niger*, *A. flavus*, *Trichoderma viride* and *Penicillium* sp.) were isolated from soil using the soil dilution pour plate method on potato dextrose agar (PDA). Plates were incubated at 30 ± 2°C for five days for the growth of fungi. The pure culture of the test fungal isolates were characterized based on cultural and morphological properties following the fungal identification schemes as described by Rifai, Barnett and Hunter, Humber, and Ellis *et al*. [3-6].

2.2. Preparation of Various Concentrations of Herbicides

In addition to the control, four different concentrations of the herbicides, Atrazine, Butachlor, 2,4-D, Glyphosate and Paraquat were prepared by adding appropriate volume of the respective herbicides (undiluted as constituted and marketed by the manufacturer) to molten PDA at 45°C to obtain the following concentrations of herbicides, 0, 0.01, 0.1, 0.5 and 1% v/v.

2.3. Measurement of Fungal Radial Mycelial Extension Growth on PDA Containing Herbicides

The radial mycelial extension growth of the test fungi on PDA plates containing the different herbicides at various concentrations was determined by obtaining a 5.0 mm agar disc inoculum from the edge of actively growing pure culture of the respective test fungi, using a cork borer. With the aid of an inoculating needle, the agar disc inoculum was placed upside down in the centre of a 12 cm diameter PDA plates containing the various concentrations (0, 0.01, 0.1, 0.5 and 1.0 v/v) of the respective herbicides. Culture plates for each herbicide and concentration were prepared in triplicates.

Measurement of the radial mycelia extension growth (in mm) of each test fungi on PDA plates was carried out using a graduated meter rule after incubation at room temperature (30 ± 2°C) for 96 h. Within the incubation period, test fungi were exposed to alternating 12 h day lighting and 12 h darkness by incubating close to laboratory windows.

2.4. Determination of Percentage Radial Mycelial Extension Growth Inhibition by Herbicides

The percentage radial mycelial extension growth inhibition of the test fungi at the highest concentration tested (1% v/v) was determined by adapting the method of Zain *et al*. [7], using the formula:

% Radial mycelial extension growth inhibition = D_c-D_f/ Dc×100 (1)

Where D_c = Radial mycelial extension growth on PDA without herbicide

D_r = Radial mycelia extension growth on PDA with herbicide

2.5. Determination of Fungal Radial Mycelial Extension Growth Rate

The effect of the various concentrations of herbicides on fungal radial mycelial extension growth rate was determined using the method of Ubogu *et al*. [8]. The radial mycelial extension growth rate (mm h^{-1}) of each of the test fungi growing on PDA plates containing the various concentrations of the herbicides was determined at the endof the investigation period using the formula:

Radial mycelial extension growth rate (mm h^{-1})=Total radial growth (mm) / Total time taken (h) (2)

2.6. Determination of the Effect of Herbicides on the Minimum Sporulation Time of Fungi

The minimum sporulation time of fungi due the presence of the respective herbicides was determined by observing the fungal growth culture at interval of 24 h for 96 h for the presence and appearance of hyphal-bearing spores under low-powered objectives (X 10).

2.7. Statistical Analysis

The various data obtained from replicate samples in this study were analyzed using statistical tools such as measure of central tendency (mean), dispersion (standard deviation), Student's t-test and Analysis of Variance (ANOVA) ($P \leq 0.05$).

3. Results

3.1. Fungal Radial Mycelial Extension Growth on PDA Containing Herbicides

The results of this study showed that for *A. niger*, *A. flavus* and *Penicillium* sp., all the concentrations of herbicides employed in this study inhibited theirradial mycelial extensiongrowth as compared to that of the control (0% v/v). Radial mycelial extension growth decreased with increased herbicides concentrations from 0.01 to 1.0% v/v (Fig. 1, 2 and 4). These decreases were least with atrazine and most profound with 2,4-D. At 96 h, from the lowest concentration of 0.01to the highest of 1.0% v/v, atrazine, butachlor, 2,4-D, glyphosate and paraquat recorded radial mycelia extension growth in the range of 34-23.5, 14.3-5.5, 33.0-0.0,22.5-12.5 and 21- 9.0 mm for *A. niger*; 27.5-17.0, 20.0-7.0, 35.0-0.0, 27.0-11.0 and 25.0-9.0 mm for *A. flavus* and 16.0-5.5, 15.5-3.5, 16.0-0.0, 13.5-4.5 and 15.0-4.0 mm for *Penicillium* sp. as compared to the control with 36.9; 35.7 and 17.3 mm growth respectively. The degree of radial mycelial extension growth of *A. niger*, *A. flavus* and *Penicillium* sp., varied significantly with the type of herbicide irrespective of its selective or non-selectiveness ($P \leq 0.05$).

However in the case of *T. viride*, the radial mycelial extension growth of the fungi also decreased with increased

concentrations of the herbicides from 0.01 to 1.0% v/v with the exception of atrazine where there was no significant difference in the radial mycelialextension growth of the organismin the control as compared to all other concentrations tested (Fig. 3). The lowest radial mycelia extension growth of the fungi occurred with paraquat which was followed by 2,4-D. At concentration as low as 0.1% v/v, the growth of the organism was completely stopped by paraquat. The range of the radial mycelial extension growth of *T. viride* by atrazine, butachlor, 2,4-D, glyphosate and paraquat at 0.01 and1.0% v/v were 42.5-42.5, 34.5-7.0, 42.5-0.0, 42.5-8.5 and 42.5-0.0 mm respectively as compared to the control with 42.5 mm growth at 96 h. Similarly, the degree of radial mycelial extension growth of *T. viride*varied significantly with the type of herbicide irrespective of its selective or non-selectiveness ($P \leq 0.05$).

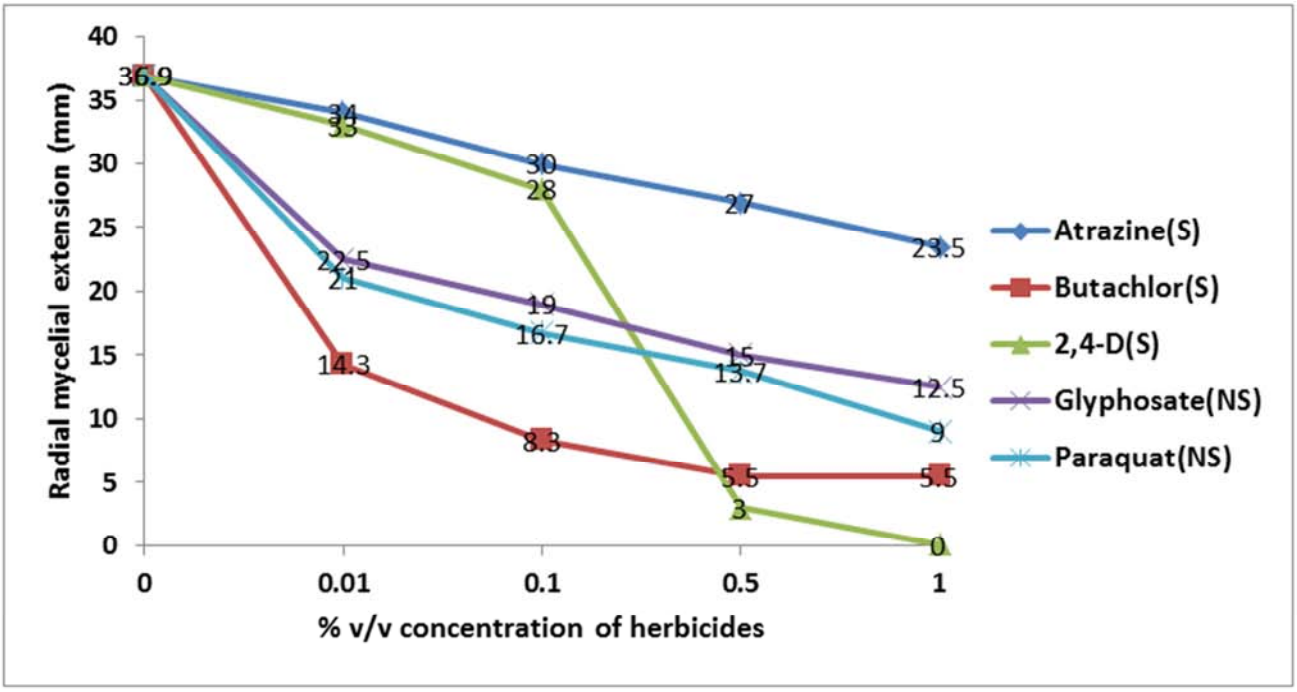

Figure 1. *Radial mycelial extension growth of A. niger at various concentrations of herbicides.*

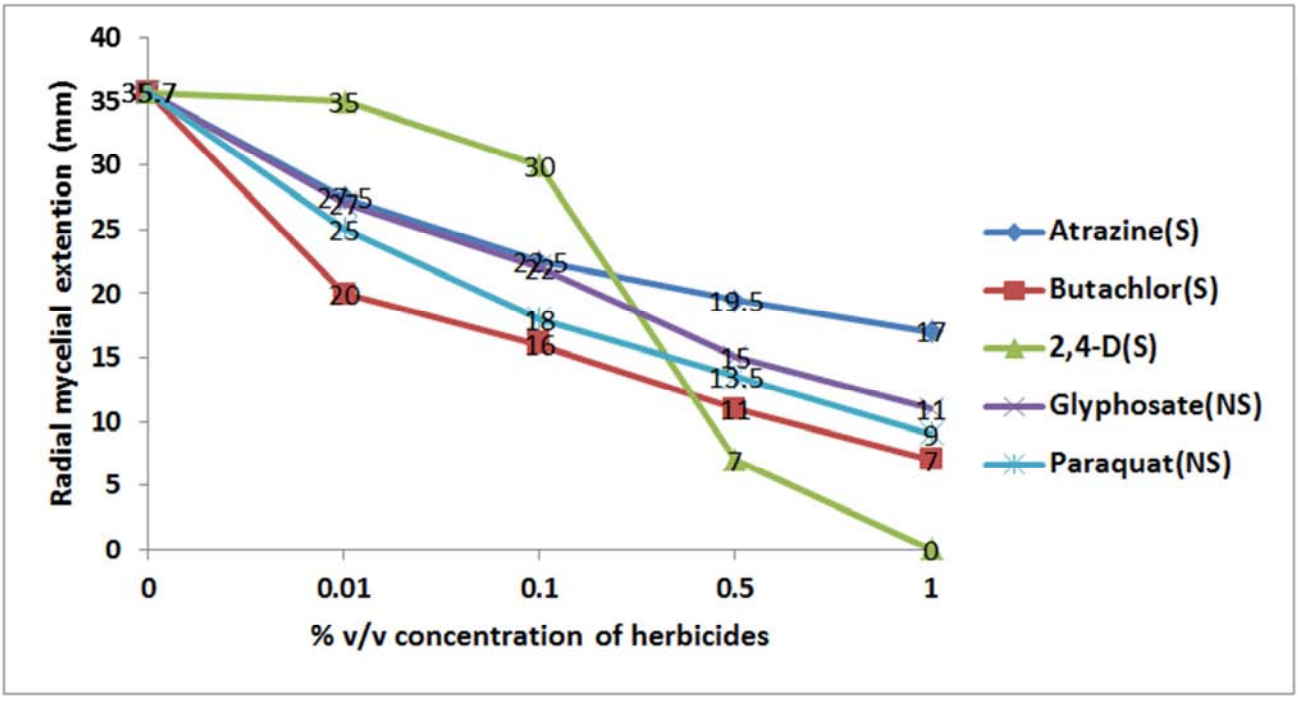

Figure 2. *Radial mycelia extension growth of A. flavus at various concentrations of herbicides.*

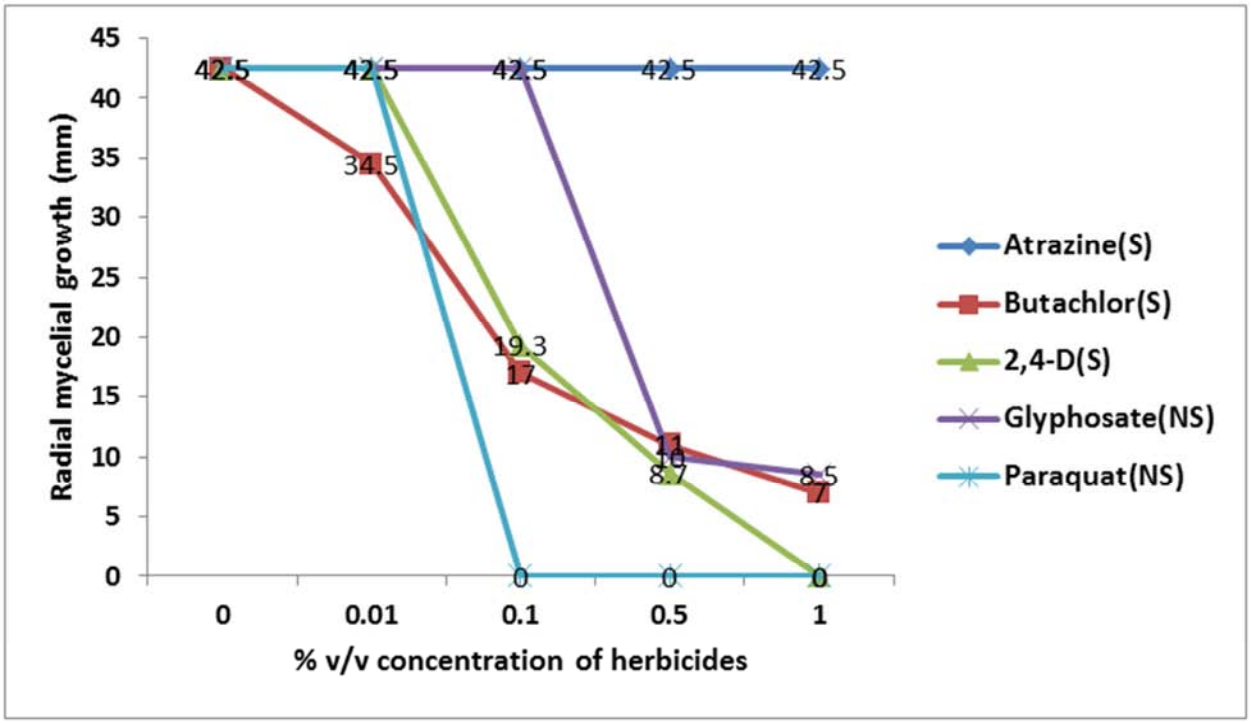

Figure 3. *Radial mycelial extension growth of T. viride at various concentrations of herbicides.*

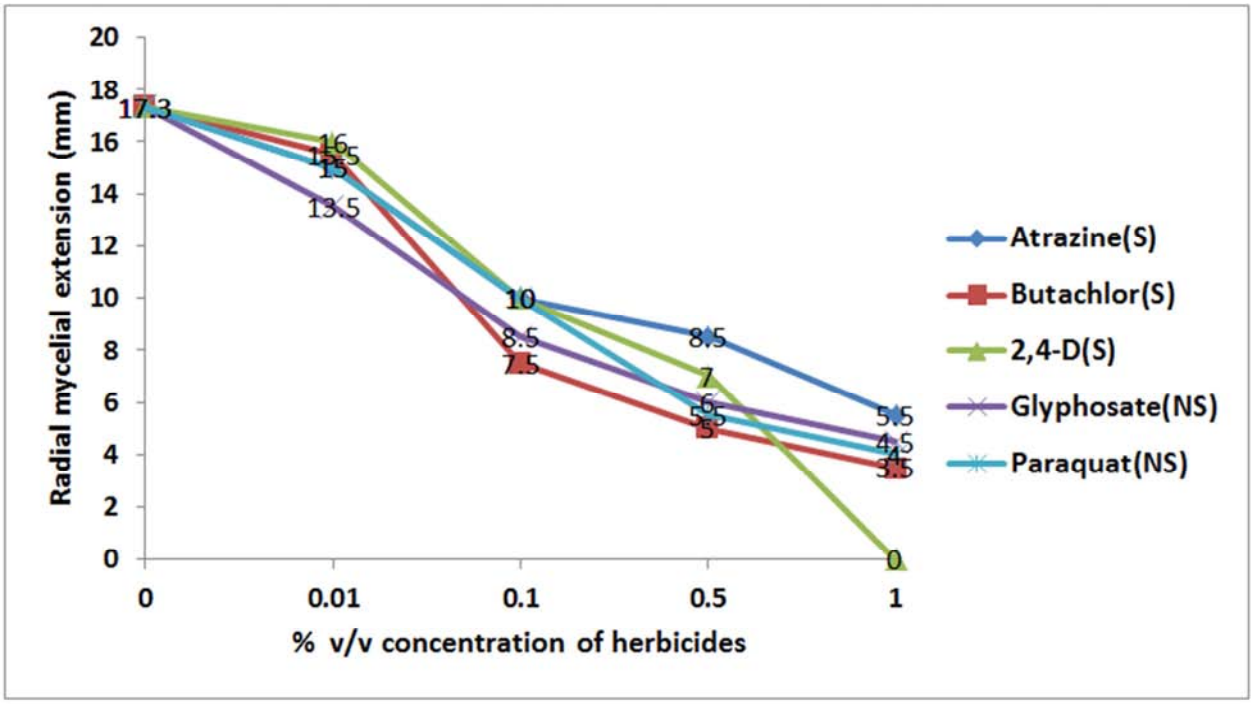

Figure 4. *Radial mycelial extension growth of Penicillium sp. at various concentrations of herbicides.*

3.2. Fungal Radial Mycelial Extension Growth Rate in the Presence of Herbicides

With the exception of atrazine which did show any effect on *T. viride* as compared to the control, the radial mycelial extension growth rate of all the fungi varied significantly with the type of herbicide and the concentration tested in this study($P \leq 0.05$) (Table 1,2, 3 and 4). The radial mycelial extension growth rate decreased with increased concentration. For *A. niger*, *A. flavus* and *Pencillium* sp., with the exception of the control where radial mycelial extension growth rate were 0.38,0.35 and 0.18 mm h^{-1}, atrazine produced the fastest radial mycelial extension growth rate of 0.35, 0.29and 0.17mm h^{-1}at the lowest concentration of 0.01% v/v, while butachlor, 0.06, 0.08 and 0.04mm h^{-1}as the slowest at the highest concentration of 1.0%v/v respectively.

On the contrary, for *T. viride*, with exception of butachlor

with 0.36 mm h^{-1}, the radial mycelial extension growth rate for all the other herbicides were the same with that of the control (42.5 mm $h^{-1)}$ at the lowest concentration of 0.01% v/v, while butachlor produced the slowest growth rate of 0.07 mm h^{-1} at the highest concentration of 1.0%v/v.

However, there was no growth at all among the fungi tested in the presence of 2,4-D. Furthermore, paraquat in addition, also did not produced growth for *T. viride* at 1.0% v/v concentration.

Table 1. *Mycelial extension growth rate (mm h-^{1}) of A. niger in the presence of herbicides.*

Herbicide	Mycelial	Extension	Growth Rate	(mm h^{-1})	
		Concentration	(% v/v)		
	0	0.01	0.1	0.5	1.0
Atrazine (S)	0.38±0.09^{a}	0.35±0.01^{b}	0.31±0.03^{c}	0.28±0.01^{c}	0.24±0.01^{d}
Butachlor (S)	0.38±0.09^{a}	0.15±0.01^{b}	0.09±0.00^{c}	0.06±0.00^{d}	0.06±0.00^{d}
2,4-D (S)	0.38±0.09^{a}	0.34±0.01^{b}	0.29±0.00^{c}	0.03±0.00^{d}	0.0
Glyphosate(NS)	0.38±0.9^{a}	0.23±0.01^{b}	0.20±0.01^{b}	0.16±0.01^{c}	0.13±0.01^{d}
Paraquat (NS)	0.38±0.09^{a}	0.22±0.01^{b}	0.17±0.01^{c}	0.14±0.01^{d}	0.09±0.01^{e}

Key:*Values with the same superscript alphabet in the same row did not differ significantly (P≤0.05)
S- Selective Herbicide
NS-Non-Selective Herbicide

Table 2. *Mycelial extension growth rate (mm h-^{1}) of A. flavus in the presence of herbicides.*

Herbicide	Mycelial	Extension	Growth Rate	(mm h^{-1})	
		Concentration	(% v/v)		
	0	0.01	0.1	0.5	1.0
Atrazine (S)	0.35±0.01^{a}	0.29±0.01^{b}	0.23±0.00^{c}	0.20±0.01^{d}	0.18±0.01^{d}
Butachlor (S)	0.35±0.01^{a}	0.21±0.00^{b}	0.17±0.01^{c}	0.11±0.00^{d}	0.08±0.01^{e}
2,4-D (S)	0.35±0.01^{a}	0.35±0.01^{a}	0.31±0.05^{b}	0.08±0.01^{c}	0.0
Glyphosate(NS)	0.35±0.01^{a}	0.28±0.01^{b}	0.23±0.01^{c}	0.16±0.01^{d}	0.12 ±0.01^{e}
Paraquat (NS)	0.35±0.01^{a}	0.26±0.00^{b}	0.19±0.00^{c}	0.14±0.01^{d}	0.09±0.02^{e}

*Values with the same superscript alphabet in the same row did not differ significantly (P≤0.05)

Table 3. *Mycelial extension growth rate (mm h-^{1}) of T. viride in the presence of herbicides.*

Herbicide	Mycelial	Extension	Growth Rate	(mm h^{-1})	
		Concentration	(% v/v)		
	0	0.01	0.1	0.5	1.0
Atrazine (S)	0.44±0.00^{a}	0.44±0.00^{a}	0.44±0.00^{a}	0.44±0.01^{a}	0.44±0.00^{a}
Butachlor (S)	0.44±0.00^{a}	0.36±0.01^{b}	0.18±0.01^{c}	0.11±0.02^{d}	0.07±0.02^{e}
2,4-D (S)	0.44±0.00^{a}	0.44±0.00^{a}	0.20±0.02^{b}	0.09±0.03^{c}	0.0
Glyphosate(NS)	0.44±0.00^{a}	0.44±0.00^{a}	0.44±0.00^{a}	0.10±0.01^{c}	0.09±0.00^{c}
Paraquat (NS)	0.44±0.00^{a}	0.44±0.00^{a}	0.0	0.0	0.0

*Values with the same superscript alphabet in the same row did not differ significantly (P≤0.05)

Table 4. *Mycelial extension growth rate (mm h-^{1}) of Penicillium sp. in the presence of herbicides.*

Herbicide	Mycelial	Extension	Growth Rate	(mm h^{-1})	
		Concentration	(% v/v)		
	0	0.01	0.1	0.5	1.0
Atrazine (S)	0.18±0.03^{a}	0.17±0.02^{a}	0.11±0.02^{b}	0.09±0.00^{c}	0.06±0.00^{d}
Butachlor (S)	0.18±0.03^{a}	0.16±0.03^{a}	0.08±0.01^{b}	0.05±0.00^{c}	0.04±0.00^{c}
2,4-D (S)	0.18±0.03^{a}	0.17±0.00^{a}	0.11±0.00^{b}	0.07±0.02^{c}	0.0
Glyphosate(NS)	0.18±0.03^{a}	0.14±0.02^{b}	0.09±0.02^{c}	0.06±0.01^{d}	0.05±0.00^{d}
Paraquat (NS)	0.18±0.03^{a}	0.16±0.00^{a}	0.11±0.00^{b}	0.06±0.00c	0.04±0.01^{c}

*Values with the same superscript alphabet in the same row did not differ significantly (P≤0.05)

3.3. Percentage Radial Mycelial Extension Growth Inhibition by Herbicides

The percentage radial mycelial extension growth inhibition of the tested fungi at 1% v/v, varied significantly with the type of herbicide irrespective of its selective or non-selectiveness (P≤0.05) (Fig. 5). While the presence of atrazine did not show any form of radial mycelial extension growth inhibition of *T. viride*, the presence of paraquat resulted in 100% inhibition. In addition to *T. viride*, the presence of 2,4-D also resulted in100% growth inhibition of the three other tested fungi at 1% v/v concentration. Among the herbicides evaluated, Atrazine produced the lowest percentage radial mycelial extension growth inhibition, while 2,4-D produced the highest percentage growth inhibition of the tested fungi. Save for atrazine, the presence of all the other herbicides evaluated resulted in over 66% radial mycelial extension growth inhibition of all the tested fungi.

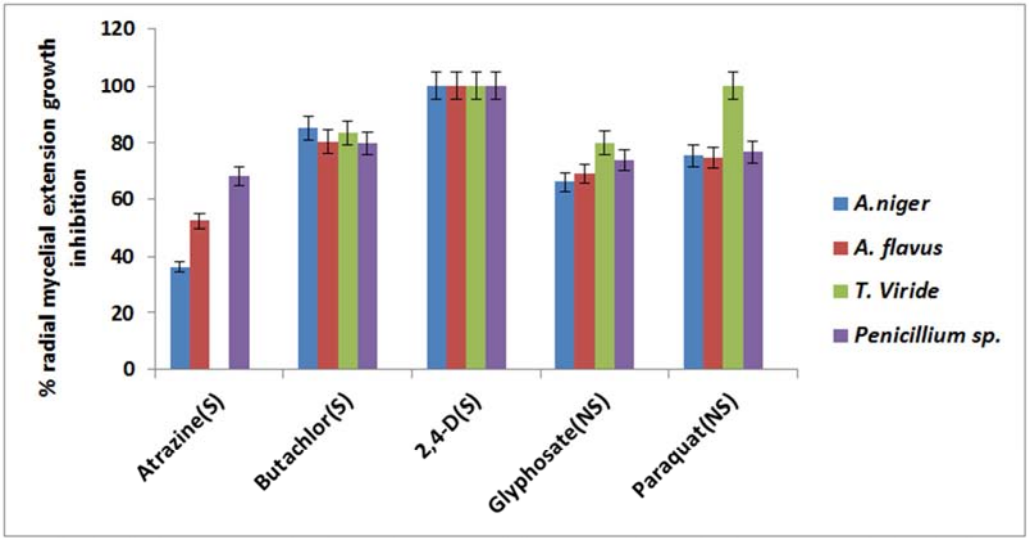

Figure 5. *Percentage radial mycelial extension growth inhibition of test fungi by herbicides at 1%v/v concentrations.*

3.4. Effect of Herbicides at Various Concentrations on Fungal Minimum Sporulation Time

The results of this study showed that the minimum sporulation time for the test fungi in the presence of the various herbicideswas 48 h wherever sporulation occurred including the control (Table 5). However, sporulation did not occur in the presence of 2,4-D at 1.0% v/v for all the fungi, and for *T. viride*in the presence of paraquat beyond 0.01% v/v concentration due to complete absence of any form of growth. On the other hand, in addition to the absence of sporulation in *A. flavus* at 1.0% v/v in the presence of paraquat,2,4-D and paraquat also prevented the sporulation of *A. flavus* and *T. viride* at 0.1 and 0.5% v/v concentrations (where growth occurred) within the 96 h of the investigation.

Table 5. *Effect of herbicides at various concentrations on fungal minimum sporulation time.*

Herbicide Con.(% v/v)	**Minimum**	**Sporulation**	**Time (Hour)**	
	A.niger	***A. flavus***	***T. viride***	***Penicillium* sp.**
Control	++	++	++	++
Atrazine				
0.01	++	++	++	++
0.1	++	++	++	++
0.5	++	++	++	++
1.0	++	++	++	++
Butachlor				
0.01	++	++	++	++
0.1	++	++	++	++
0.5	++	++	++	++
1.0	++	++	++	++
2,4-D				
0.01	++	++	++	++
0.1	++	-	-	++
0.5	++	-	-	++
1.0	++	++	++	++
Glyphosate				
0.01	++	++	++	++
0.1	++	++	++	++
0.5	++	++	++	++
1.0	++	++	++	++
Paraquat				
0.01	++	++	++	++
0.1	++	-	0	++
0.5	++	-	0	++
1.0	++	-	0	++

Key: += 24 h
++= 48 h
+++ = 96 h
-= No sporulation
0= No growth

4. Discussion

With the exception of atrazine which did not inhibit the radial mycelial growth of *T. viride* at all the tested concentrations, all the herbicides investigated in this study showed a progressive reduction of mycelial growth of the tested fungi as the concentrations of the herbicides increased when compared to the control. The extent of radial mycelial growth reduction in the presence of the herbicides varied with fungal species and the type of herbicide irrespective of whether it is a narrow (selective) or broad (non-selective) spectrum herbicide. Previous reports also lend credence to the findings in this work. The effects of herbicides on fungal growth have been shown to be specific and dependent on the type, dose, microbial species and environmental conditions [9, 10]. Pasaribu *et al*. [11], reported differential responses of the mycorrhiza fungus *Glomus mosseae* to alachor and glyphosate with the fungus being more sensitive to alachor. While the herbicides did not affect the external hyphal length, there was increasing reduction ofinternal hyphal growth with increasing rates of alachlor application as against glyphosate which never did. Furthermore, while investigating the effect of the herbicides pretilachlor, 2,4-D, paraquat, glyphosate and anilofos on the mycelia growth of *Fusarium pallidoroseum*, Praveena *et al*. [12], reported that with the exception of pretilachlor which did not affect the mycelia growth of the fungus, all the other herbicides reduced the mycelia growth of the fungus with increased concentrations, and the degree of reduction varied with the type of herbicide. Similarly, Zain *et al*. [7], evaluatedmycelia growth inhibition and influence of exposure periods of paraquat, glufosinate-ammonium, glyphosate and metsulfuron-methyl on*Aspergillus* sp., *Penicillium* sp. and *Mucor* sp. and reported that these varied with fungal species, herbicides and their rate of application.

To understand fungal capacity to colonize and settle on a particular substrate, it is important to study their growth rate [13]. Of all the herbicides employed in this study, 2,4-D appeared to be the most potent as it not only reduced the radial mycelial growth rate with increased concentration like the other herbicides, but resulted in a100% mycelial growth inhibition of all the fungi at 1% v/v. However, on the other extreme, atrazine was the least potent of the herbicides to the point that it completely failed to arrest the mycelial growth of *T. viride* even at the highest concentration tested. This corroborate the findings of Rodriguez-Kahana *et al*. [14], who had earlier reported that all the concentrations of atrazine tested, 8, 20, 40 and 80 ppm enhanced *T. viride* growth; and that of Cupul *et al*. [13], who reported that although, atrazine inhibited the mycelial growth rate of all the tested macrofungi (*Cymatadoderma elegans*, *Daedalea elegans*, *Pleurotus djamor*, *Pleurotus* sp., *Pycnoporus sanguineus* and *Trametes maxima*), non reached 100% under the tested doses(468, 937, 1875 and 3750 mgL^{-1})with some fungi not inhibited at all at lower doses, and concluded that moderate doses of atrazine are not toxicto the fungi. Variation in the degree of mycelial growth inhibition may be attributed to the differential toxicity of the herbicides arising from their chemical composition and the degradative capacity of the respective fungi. Fungi may either degrade herbicides or get affected adversely by their presence depending on the type of herbicide [15, 16].

In this study, the minimum sporulation time of the test fungi in the presence of the herbicides was 48 h wherever growth occurred. While there was mycelialgrowth, there was no sporulation in *A. flavus* at 1.0% v/v in the presence of paraquat. Similarly, sporulation also failed to occur even when there were mycelial extension growth in the presence of paraquat and 2,4-D for*A. flavus* and *T. viride* at 0.1 and 0.5% v/v concentrations within the 96 h of this investigation. Variation in fungal sporulation in the presence of different herbicides and concentrations observed in this work is in agreement with what have been earlier reported. Direct effects of herbicides on fungal sporulation have been found to be variable and often species and dosage dependent [17]. Ray and Pandey [18], reported that while glyphosate stimulated sporulation of *Alternaria alternate*, 2-4-D inhibited same. Sporulation of *F. pallidoroseum* have also been shown to be inhibited by 2,4-D (at 1.0 and 0.25kg ai ha-1) and paraquat (at 0.75 and 0.19 kg ai ha-1) [12]. While investigating the fungistatic effect of lidocaine, a potent local anaesthetic on the growth and sporulation of *A. niger*, Jaiswal *et al*. [19], reported delayed sporulation of the fungi by a day from 0.2 to 1.0% as compared to 0.1% concentration.

Herbicides have been shown to influence fungal competitive colonization of substrates. A change in the patterns of colonization of substrates by microorganisms may affect the rate at which these substrates are subsequently decomposed [20].

5. Conclusion

The results of this study clearly indicate the negative effects of herbicides on the growth of soil fungal community. The growth inhibition of soil fungi is therefore most likely to increase the persistence of herbicides and other organic compounds in the environment.

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