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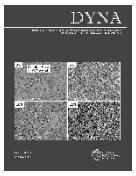

UNIVERSIDAD NACIONAL DE COLOMBIA

In vitro evaluation the toxicity in mixture of glyphosate and methyl metsulfuron against strains of *Trichoderma* spp

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Abstract
Trichoderma is an antagonist fungus used in agriculture and forestry for the diseases control. The use of the herbicides metsulfuton methyl and glyphosate is a common practice for weed control that limits Trichoderma use. This study has evaluated the toxic effects of the herbicides metsulfuron methyl and glyphosate on four strains of fungi of the genus Trichoderma (GRB-HA1, GRB-HA2, GRB-HA8 and GRB-HA9). Poisoned media were prepared with high doses of 25 mL·L-1 of glyphosate + 0.2 g L-1 of metsulfuron methyl (D500) and low doses of 12.5 mL·L-1 of glyphosate + 0.2 g L-1 of methyl metsulfuron (D250) to determine the effects on viability. The effects of the exposure time (30-90 min) on the germination of the conidia were determined using suspensions prepared with the mixtures D500 and D250. The results showed the differential sensitivity of the Trichoderma strains that affected the viability of the conidia and mycelial growth.

Keywords: Trichoderma; herbicides; antagonims; toxicity; glyphosate; metsulfuron-methyl.

Evaluación *in vitro* de la toxicidad de los herbicidas glifosato y metsulfurón metil frente a cepas de *Trichoderma* spp.

Resumen
Trichoderma es un hongo antagonista utilizado en agricultura y silvicultura para el control de enfermedades. El uso de los herbicidas metsulfuton metil y glifosato es una práctica común que limita las aplicaciones de trichoderma. Este estudio evaluó los efectos tóxicos de los herbicidas metsulfuron metil y glifosato sobre cuatro cepas de hongos del género Trichoderma (GRB-HA1, GRB-HA2, GRB-HA8 y GRB-HA9). para determinar los efectos sobre la viabilidad se usaron medios envenenados con dosis altas (25 mL·L-1 de glifosato + 0.2 g L-1 de metsulfuron metil; D500) y dosis bajas (12.5 mL·L-1 de glifosato + 0.2 g L-1 de metsulfuron metsulfuron; D250). Los efectos del tiempo de exposición (30-90 min) sobre la germinación de los conidios se determinaron utilizando suspensiones preparadas con las mezclas D500 y D250. Los resultados evidenciaron la sensibilidad diferencial de las cepas de trichoderma que afectaron la viabilidad de los conidios y el crecimiento micelial.

Palabras clave: trichoderma; antagonismo; herbicidas; glifosato; metsolfuron metil.

1. Introduction

Latin America has a broad productive potential in different crops of commercial interest especially forest species, positioning itself in international markets due to its tropical condition [1]. *Eucaliptus grandis* is one of the most important forest species due to its rapid growth, adaptability and productivity for the production of agglomerates used for the construction and remodeling of spaces [2]. A wide variety of chemicals are used to control pests, diseases and weeds that negatively affect these plantations, especially during the first year of establishment [3]. These products, although efficient, have high costs, cause negative effects on human health and also the environment, generating restrictions on

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marketing [4]. Different investigations have found an alternative that uses entomopathogenic and antagonistic microorganisms for the control of pests and diseases, improving competitiveness and reducing negative effects on health and environment [5,6]. Mixtures of glyphosate (N-(phosphonomethyl) glycine) and metsulfuron-methyl methyl 2- (4-methoxy-6-methyl-1,3,5-triazine-2-ylcarbamoylsulfamoyl) benzoate] [6-7-8] are used for weed management and control as herbicides during the establishment of forest plantations.

There are reports that show that some fungi of the *Trichoderma* genus can metabolize glyphosate and take advantage of it as a source of carbon, nitrogen and / or phosphorus [9], which could represent an advantage for *Trichoderma*-based products. However, this answer is not so clear, some reports indicate that prolonged contact of these herbicides in high concentrations can cause negative effects on the efficacy of microorganisms [10-12]. Despite the agronomic potential of microorganisms for the control of pests and diseases, there are few studies that indicate their limitations against the herbicides used in the usual practices in forest plantations.

This study evaluated, under laboratory conditions, the effects that two mixtures of the herbicides glyphosate and methyl metsulfuron used for the initial control and maintenance of forest plantations in the establishment stage of *Eucalyptus grandis*, have on the viability of the conidia and the mycelial growth of four strains of the genus *Trichoderma*.

2. Materials y methods

2.1. Microorganisms

Four microorganisms were included in the study, two native strains of *Trichoderma asperellum* (GRB-HA1, GRB-HA2) donated by the GRINBIO research group of the University of Medellín isolated from ant colonies and selected for their high agronomic potential and two commercial isolates of commercial *Trichoderma* genus (GRB-HA8 and GRB-HA9). The fungi were maintained in PDA medium (Potato Dextrose Agar), and incubated in dark conditions at 25 ± 2ºC subcultures were done every 15 days.

2.2. Obtaining conidia

The microorganisms were grown separately in solid state fermentations on a rice: water substrate in a 2:1 proportion, prepared inside high-density propylene bags, the bags were manually homogenized, sealed with a cotton stopper and sterilized by humid heat in an autoclave sterilizer (121 ° C, 15 PSI for 15 min). For inoculation, 5 discs of 5 mm diameter of each microorganism were used, finally the inoculated substrates were cultivated under controlled conditions of darkness and temperature (25 ± 2ºC). In order to increase the sporulation and drying of the substrate, after 7 days of cultivation, the growing biomasses of the fungi were extracted and deposited separately in sterile Kraft paper bags and kept in controlled dark conditions at 25 ± 2ºC for 8 days.

The dry biomass resulting from the cultivation was ground until obtaining a fine powder which was sieved (0.25 mm), the powder obtained from each microorganism was stored under cooling conditions at 4 ° C. At the time of the test, the conidia powder concentration was adjusted to values $1x10^9$ conidia mL^{-1}.

2.3. Effect of mixed herbicides on the germination of Trichoderma conidia

The effect of exposure of the microorganisms to mixtures of glyphosate (credit® 747SG, 447 g Kg^{-1}) recommended in doses between 1.87 - 37 mL and metsulfuron methyl (Nufuron 60 WG® 600 g Kg^{-1}) in recommended doses of 100 L ha^{-1} were measured by preparing suspensions of both herbicides in two combinations, one at the initial application concentrations used for weeding of the crops before planting (25 $mL \cdot L^{-1}$ of glyphosate + 0.2 g L^{-1} of metsulfuron methyl, D500) and the other adjusted to the maintenance concentration usually used (12.5 $mL \cdot L^{-1}$ of glyphosate + 0.2 g L^{-1} of metsulfuron methyl, D250), which is applied monthly during the first year of establishment of eucalyptus forest plantations.

To determine the effect of the herbicide mixture (D500, D250) on the germination of conidia of each *Trichoderma* strain evaluated, a solution of herbicide was inoculated separately with the *Trichoderma* strains adjusted to concentration values of $1x10^9$ mL^{-1} by counting in a neubauer chamber [13]. To evaluate the resistance of the conidia to the exposition time of the herbicide mixture in the fumigation pumps during time application, the conidia suspensions contaminated with the herbicide mixtures were shaken for an hour and a half taking samples of 1 mL every 30, 60 and 90 minutes, in this case the controls were performed with uncontaminated conidia suspensions. At the end of the exposure time, serial dilutions were made and sown in water agar medium, the cultures obtained were grown in the dark at room temperature under laboratory conditions. After 48 hours of cultivation, the effects of exposure to the herbicide mixture on the germination of the conidia was determined by microscopy with 40X magnification. The calculation of germination percentage was performed based on the total number of conidia observed [14]. The percentage of inhibition (% I), generated by the treatments of exposure of the fungi to the herbicides, was calculated for each fungus and time of exposure using eq. 1:

$$\%I = 100 - \left(\frac{\%\ germination}{\%\ control\ of\ germination} * 100\right) \quad (1)$$

2.4. Determination of the effect of herbicide mixture on mycelial growth in pure Trichoderma cultures

To evaluate the effect of prolonged exposure of the fungi included in the study to the herbicide mixtures (D500, D250), cultures were performed in culture medium mixed with herbicides poisoned medium on sterile PDA (121 ° C, 15 minutes). They were prepared by adding the herbicide mix in the proposed doses (D500, D250) to the still liquid agar (45 ° C) and shaking it before pouring it into Petri dishes. The

poisoned media were inoculated separately with 1cm diameter discs of the GRB-HA1, GRB-HA2, GRB-HA8 and GRB-HA9 fungi. As an experimental control, the strains were plated on herbicide-free PDA medium. The media were incubated at 25 ± 2ºC for 5 days in dark conditions. The effects of herbicides on fungal growth were determined by comparing radial mycelial growth of fungi in herbicide-poisoned media with growth in non-poisoned control media. In the study, the growth diameters were measured with the help of a Vernier caliper every 24 hours until the mycelium of the control cultures reached the edges of the Petri dishes (6 days). The data was used to calculate growth reduction (PIC RC) using eq. 2 [15].

$$PIC\ RC = 1 - \left(\frac{radio\ del\ medio\ con\ herbicida}{radio\ del\ medio\ control}\right) * 100 \qquad (2)$$

2.5. Statistical analysis

The germination and mycelial growth tests were performed five times, the analysis of the observed data of germination percentage and the percentage of inhibition of the strains were subjected to an analysis of variance (ANOVA) and the multiple range of a Tukey test (P≤0.05) to determine the significant differences between treatments using the R program version 4.0.2 (2020-06-22).

3. Results and discussion

3.1. Effect of the herbicide mixture on the germination of conidia of the fungi of the genus Trichoderma

The results of this study made it possible to demonstrate the toxic effects of the mixture of the herbicides glyphosate and metsulfuron methyl in the germination processes of the conidia of the *Trichoderma* strains evaluated. In the study, toxicity varied between microorganisms and increased with concentration and exposure time (Table 1). Control of hydration time of the conidia for 30 - 90 min for the strains GRB-H1 (% G: 88 -90), GRB-H2, (% G: 78-87), GRB-HA8 (% G: 84 -88) and GRB-HA9 (% G: 97 -99) did not affect its viability, even slightly increased with wetting time (Table 1).

The immersion of the conidia in contaminated suspensions of the herbicide mixture of glyphosate and metsulfuron methyl in the doses D500 and D250 revealed the differences in the sensitivities of the microorganisms. In the tests, the viability of the GRB-HA2 conidia (D250, 30% ± 3; D500. 0% ± 0) was more strongly affected in the contaminated suspensions, causing even the complete loss of viability in D500. In this study GRB-HA8 (D250 26% ± 10; D500, 7% ± 2) and GRB-HA9 (D250, 35% ± 4; D500, 15% ± 3) presented a slightly higher resistance to the toxic effects of herbicides managing to survive even at the highest doses (D500)

The most resistant strain to the toxic effects of herbicide suspension was GRB-HA1 (D250, 79% ± 5; D500, 77% ± 2), with inhibition percentages between 5-12% (D250) and 10-16% (D500). For this strain, the Tukey test did not find significant differences between the effects of the low dose (D250, 90 min, % G: 79 ± 5) and the high dose (D500, 90 min, % G: 77 ± 2) or between the contaminated treatment and the hydration control (90 min, % F: 90 ± 5) considering them a homogeneous group. The low toxicity of herbicides in the germination processes of *Trichoderma* conidia has been previously reported for other species, although the effect of mixtures of different doses of glyphosate and Metsulfuron methyl has not been reported. Meriles et al. [19] evaluated the effect of glyphosate on doses (mL L-1) of 20, 60, 100 and 140 on *Trichoderma viride*, indicating that for this microorganism no evidence of toxicity was observed, finding germinations greater than 90%. Despite the advantages that this low toxicity brings to the possibility of combining these microorganisms with herbicides in the application pumps for up to 90 min, to reduce costs. These results are different for each species and strain and should not be generalized. It is not feasible to know whether the lack of toxicity reported by Meriles et al., [19] is a consequence of the resistance of the specific strain used or, if it was the mixture of the products that increased the toxicity for the others.

Table 1.
Average germination percentages achieved by the *Trichoderma* strains GRB-HA1, GRB-HA2, GRB-HA8 and GRB-HA9 after exposure for 30, 60 and 90 minutes to the mixtures of the herbicides glyphosate and metsulfuron methyl in doses D250 and D500. Lower case letters represent homogeneous groups.

Micro-organisms	Dose	Time (min)	Germination (%)	% de inhibition
GRB-HA1	D500	30	79 ± 8 b	10 ±5
		60	78 ± 3 b	13 ±3
		90	77 ± 2 b	16 ±2
	D250	30	84 ± 5 b	5 ±1
		60	81 ± 2 b	11 ±2
		90	79 ± 5 b	12 ±2
	Control	30	88 ± 4 ab	-
		60	90 ± 3 ab	-
		90	90 ± 5 ab	-
GRB-HA2	D500	30	15 ±5 f	81 ±5
		60	0 f	100 ±0
		90	0 f	100 ±0
	D250	30	30 ± 3 e	66 ±4
		60	28 ± 4 e	67 ±3
		90	25 ± 5 e	68 ±5
	Control	30	78 ± 3 b	-
		60	84 ± 3 b	-
		90	87 ± 2 ab	-
GRB-HA8	D500	30	14 ± 4 f	83 ±4
		60	10 ± 3 f	88 ±6
		90	7 ± 2 f	92 ±1
	D250	30	55 ±13 c	35 ±4
		60	42 ± 6 d	51 ±1
		90	26 ±10 e	67 ±3
	Control	30	84 ±15 b	-
		60	85 ± 8 b	-
		90	88 ± 9 ab	-
GRB-HA9	D500	30	24 ± 4 e	76 ±6
		60	25 ± 9 e	73 ±6
		90	15 ± 3 e	85 ±5
	D250	30	63 ± 5 c	36 ±3
		60	53 ± 6 c	45 ±4
		90	35 ± 14 d	64 ±5
	Control	30	97 ± 2 a	-
		60	97 ± 1 a	-
		90	99 ± 1 a	-

Source: Own elaboration.

The statistical analysis of the separate effects of the dose and the exposure time showed that the concentration of herbicides is more decisive in the processes of inhibition of the germination of conidia, explaining 65% of the inhibition in germination, in this case, the exposure time (30-90 min) was responsible to a lesser extent (35% of the response). This behavior coincides with reports of a decrease in the germination of conidia of the phytopathogenic fungus *Fusarium solani*, which decreased with the increase in glyphosate concentration [21], although their study did not evaluate the combined effect of the immersion of the conidia in contaminated mixture suspensions of the herbicides glyphosate and metsulfuron methyl. The high toxicity of these herbicides found for some of the *Trichoderma* strains evaluated can be compared with the results of other authors who registered a significant decrease in the viability of mycorrhizal fungal conidia after the application of glyphosate in concentrations of 0.8 and 3 L ha^{-1} [20], again highlighting the risks of these applications alongside beneficial soil microorganisms.

The fact that the mixture of the herbicides glyphosate and metsulfuron methyl in the doses D500 and D250 can differentially affect two strains of the same species, such as the *Trichoderma asperellum* GRB-HA1 strains (D250, 79% ± 5; D500, 77% ± 2) and GRB-HA2 (D250, 30% ± 3; D500.0% ± 0) included in this study, allow us to affirm that previous reports do not always serve to validate or predict the effectiveness of a strain in forest plantations managed under schemes that include glyphosate and methyl metsulfuron for weed control. And these results highlight the need to assess the survival of conidia and mycelial growths of each microorganism present in commercial products.

3.2. Herbicide effect on Trichoderma mycelial growth.

After 6 days of culture, the mycelial growths of the evaluated *Trichoderma* strains (GRB-HA1, GRB-HA2, GRB-HA8, GRB-HA9) in the poisoned media were significantly affected with inhibition percentages (% I) that ranged between 68 and 83% (Fig.1). The strain most resistant to the effects of herbicides was GRB-HA1 (% I: 68.2% ± 2.4, D250), followed by GRB-HA8 (% I: 70.1% ± 3.6, D250) and the most drastic effects were found in the highest doses (D500) for the GRB-HA9 (% I: 83.2% ± 2.7, D500) and GRB-HA2 (% I: 82.2% ± 1.2, D500) strains.

The statistical analysis (P≤ 0.05) showed significant differences between the percentages of mycelial growth in the poisoned media both in the D250 and D500 doses (Fig. 1). Tukey's tests showed significant differences, for treatments with D250, 4 homogeneous groups were found that overlapped in the case of fungi (GRB-HA9, 73.2% ± 2.6, D250; GRB-HA1, 68.3% ± 2.4, D250; GRB-HA2, 71.0% ± 3.3, D250; GRB-HA8, 70.1% ± 3.6, D250) and all of them differed from the control (0%, D250). For high doses (D500) all fungus (GRB-HA2, (62.3% ± 1.2, D500; GRB-HA1, 80.6% ± 1.5, D500; GRB-HA8, 82.6% ± 2.7, D500 and GRB-HA9 79.5% ± 2.2, D500) were included in a group, differentiating them from the control (0%, D500).

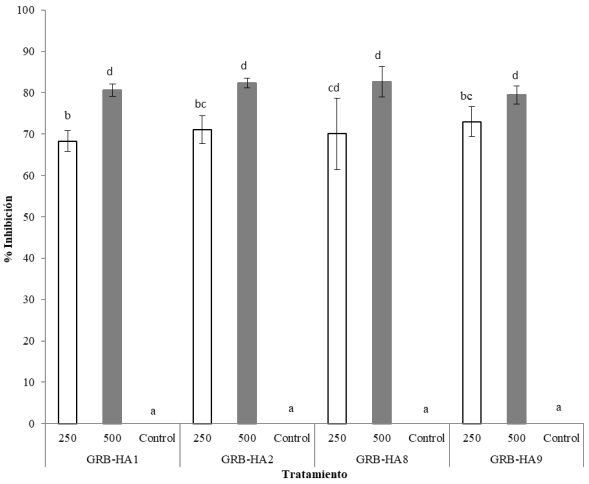

Figure 1. Percentage of inhibition of mycelial growth of the *Trichoderma* strains GRB-HA1, GRB-HA2, GRB-HA8 and GRB-HA9 in culture media poisoned with the initial application glyphosate and methyl metsulfuron mixture 25 mL·L^{-1} glyphosate + 0.2 g L^{-1} of metsulfuron methyl (D500) and maintenance 12.5 ml·L^{-1} of glyphosate + 0.2 g L^{-1} of metsulfuron methyl (D250). The bars indicate the standard error. Different letters present statistically significant difference.
Source: Own elaboration.

No previous studies were found evaluating the combined effect of the herbicides glyphosate and metsulfuron methyl on fungi of the genus *Trichoderma*. Toxicity studies of these herbicides have been carried out individually, Sanato et al., [16], for example, reports low effects of glyphosate on mycelial growth (% C) of *Trichoderma atroviride* in the recommended low doses (D: 0.48 g L^{-1}, %C: 95.7 ± 2.2, D: 0.96 g L^{-1}, %C: 96.3 ± 2.3, D: 1.92 g L^{-1}, %C: 84 ± 1.3). Similar results have been reported by Melo et al., [18] who evaluated the toxicity of glyphosate at different doses (D: 0.6, 6 g L^{-1}) on *Trichoderma* sp. and reported low inhibitions (D0.6, % I: 4; D3, % I: 20; D6, % I: 50). When the low toxicity effects of glyphosate reported in these doses are compared with the high toxicities found in this study (D250, 6g · L^{-1}; D500 12g L^{-1},) it shows that is possible that the toxicity of glyphosate increases with increasing concentration. These results show the risk that the use of glyphosate could have during the establishment of forest plantations when applied in the recommended high doses of 37 g L^{-1}, this product could destabilize soil microbial communities, making them more vulnerable to fungal attack by phytopathogens.

Zain et al., [17] evaluated the effects at different doses of glyphosate (D: 10, 20, 40 g L^{-1}) on the growth of three fungi (*Penicillium* sp., *Aspergillus* sp., *Mucor* sp.), evidencing the differential effect on microorganisms. In this study, glyphosate had a moderate toxicity on *Penicillium* sp., (D10, % I: 20; D20, % I: 57; D40, % I: 60) and *Aspergillus* sp. (D10, % I: 16; D20, % I: 35; D40, % I: 50), while the toxicity was higher for the fungus *Mucor* sp. (D10, % I: 63; D20, % I: 70; D40, % I: 80). Zain et al., [17] also evaluated the effects of methyl metsulfuron (D 0.38, D 0.75, D1.5) on these microorganisms indicating low inhibition for *Penicillium* sp. (D0.38, % I: 0; D0.75, % I: 10; D1.5, % I: 16) and *Aspergillus* sp. (D 0.38, % I: 3; D 0.75, % I: 8; D1.5, % I: 20), and an

increase in the toxicity for *Mucor* sp. These results allow us to think that the increase in toxicity found in this study is generated as a result of the addition of metsulfuron methyl and they show the sensitivity of the *Trichoderma* strains evaluated (GRB-HA1, GRB-HA2, GRB-HA8, GRB-HA9). Highlighting the negative effects that these applications can bring to the soil microflora and the potential effects of reducing the effectiveness of biological products.

4. Conclusions

Suspensions of the herbicides glyphosate and metsulfuron methyl used in combination for weeding of the crops before planting (D500) and in maintenance concentration (D250) have important effects on the germination of conidia and the mycelial growth of *Trichoderma* strains (GRB-HA1, GRB-HA2, GRB-HA8, GRB-HA9).

The toxic effects of the herbicide mixture on the germination of conidia can vary between species and between strains of the same species the strains of the same *Trichoderma* species.

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