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Tolerance of Pisolithus sp. Isolates to Glyphosate in Vitro

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ABSTRACT

Ectomycorrhizae are beneficial to eucalypts plants and can be inhibited by herbicides during crop management. Thus, this study aimed at assessing the tolerance of *Pisolithus* sp. isolates to the herbicide glyphosate. Tolerance was evaluated in liquid and solid mediums through independent testing. Treatments were arranged in a 5x5 factorial scheme using the isolates D10, D17, D51, D106 and D118 of *Pisolithus* sp., which were grown at glyphosate concentrations of 0; 51.7; 103.5; 206.9 and 413.8 mg L^{-1}. Mycelium of the isolates was obtained from collection and cultured on modified Melin-Norkrans solid medium, and let grow in the dark for 29 days at 25ºC. After this period, 5-mm-diameter circular sections were withdrawn from around the edges of each isolate colony. Next, the sections were transferred into Petri dishes containing solid MMN medium and incubated for three more days under the same conditions to reactivate mycelium damaged by cuts, confirm viability and lack of contamination. This procedure was made for both liquid and solid mediums. After 30 days, mycelium growth on solid MMN medium was evaluated by measuring colony diameter, and mycelium dry mass for liquid MMN medium. The highest concentration of glyphosate in solid medium reduced colony diameter compared to control (0 mg L^{-1}), reaching the following ascending order: 5.3% for D106; 19.3% for D17; 21.7% for D118; 22.6% for D51 and 26.8% for D10. The isolated D10 had the highest percentage of diameter reduction at the highest glyphosate concentration as compared to control; however, the same herbicidal dosage promoted major growth to this isolate than to the others. The glyphosate concentration required to inhibit 50% growth of isolates in liquid medium was 70.0 mg L^{-1} for D106, 104.5 mg L^{-1} for D17, 99.0 mg L^{-1} for D118, 111.0 mg L^{-1} for D51 and 129.5 mg L^{-1} for D10. Thus, *Pisolithus* sp. isolates were more sensitive to glyphosate in liquid medium rather than in solid. Isolates of *Pisolithus* sp. had differences regarding glyphosate tolerance and the type of culture medium used. D10 was the most tolerant isolate in both mediums.

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INTRODUCTION

Ectomycorrhizae increase water and nutrient absorption, primarily those with low mobility in soil, besides of increasing tolerance to toxic conditions and pathogens in soil (Marx and Cordell, 1989; Smith and Read, 1997; Grazziotti *et al*., 2003; Gandini *et al*., 2015). Such symbiosis is very common in eucalypts (Ragonezi *et al*., 2013; Rachid *et al*., 2015), which represents 77% of total forest plantation area in Brazil (ABRAF, 2013). Among ectomycorrhizal fungi (EMF) found in commercial

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plantations within Southeastern Brazil, *Pisolithus* sp. is one of high occurrence (Campos *et al*., 2011). It is known by the ability to promote growth of eucalypts inoculated seedlings, being easily isolated and kept in laboratory (Alves *et al*., 2001; Silva *et al*., 2007; Souza *et al*., 2008; Gandini *et al*., 2015).

Even if EMF inoculation has great potential to make the forest industry more sustainable, it has not been used in Brazil yet (Costa *et al*., 2003). On the other hand, countries from northern hemisphere have already placed such inoculants on the market (Plant health care - Mexico, 2014). Moreover, the increasing demand for wood associated with a reduction in planted forest areas due to financial, political and environmental issues (Alvarenga, 1994; Fornari, 2011); therefore, EMF inoculation becomes promising also in Brazil (Alves *et al*., 2001, Gandini *et al*., 2015). However, there has still been missing studies to identify efficient isolates for areas throughout the country, or under conditions in which eucalypts is planted.

Eucalypts has a fast growth rate and is very competitive; however, yields can be impaired quantitatively and qualitatively by weed interference. Cost control, workforce demand, high yield goals as well as herbicide effects on the environment put weeds into the list of worst problems of eucalypts plantations (Tuffi Santos *et al*., 2006; Pereira *et al*., 2012a). Weeds may hinder crop establishment in the field, reducing plant development and growth through allelopathic effects (Toledo *et al*., 2003), besides of competition for water, nutrients and light. Moreover, weed growth can increase fire risk and prevent the use of other forest practices (Tuffi Santos *et al*., 2006). Given the approach, forestry companies have currently increased the use of chemicals as an alternative to reduce production costs (Ribeiro, 1988; Toledo, 2002).

Glyphosate is the most widely used herbicide for weed control in eucalypts (Tuffi Santos *et al*., 2005; Pereira *et al*., 2012b), since it exhibits a broad activity against a wide range of weed species and low environmental impact (Malik *et al*., 1989; Silva *et al*., 2012). This product is classified as non-selective, post-emergent, systemic and with high usage range (Cox, 2000; Çağlar e Kolankaya, 2008).

EMF response to herbicidal activity depends upon product, dosage, plant species, fungal isolate and growth conditions (Trappe *et al*., 1984; Moorman, 1989; Paula Jr *et al*., 1995; Huang *et al*., 2007; Fernandes *et al*., 2014). Evaluating solid medium, Estok *et al*. (1989) observed that *Pisolithus tinctorius* had been affected by concentrations from 1 mg L^{-1} of triclopyr, glyphosate, hexazinone and 2,4-D, being completely inhibited at the dose 5,000 mg L^{-1} of the same products. Likewise, Chakravarty and Sidhu (1987) reported that *Hebeloma crustuliniforme, Laccaria laccata* and *Suillus tomentosum* growth was hindered from 10 mg L^{-1} of glyphosate and hexazinone in solid medium. In addition, Huang *et al*. (2007) found that concentrations of 100 mg L^{-1} DDT completely inhibited *Boletus edulis, Gomphidius viscidus, Laccaria bicolor* and *Leccinum scabrum* growth. The addition of isoxaflutole and glyphosate into liquid and solid mediums reduced growth of four *Pisolithus* sp. isolates, from which Pt24 was underscored as the most tolerant to glyphosate as well as UFVJM04 was to isoxaflutole (Fernandes *et al*., 2014). These authors concluded that glyphosate was more toxic to *Pisolithus* sp. than isoxaflutole was. On the other hand, some studies found a stimulating effect of glyphosate and terbuthylazine on the growth of 64 EMF isolates in solid medium under a dose of 1 mg L^{-1} (Laatikainen and Heinonen-Tanski, 2002). This behavior can be explained by the capacity of some EMFs to degrade certain biocides, e.g. 2,4-D can be degraded by *Paxillus involutus* and *Suillus variegatus* in solid medium as well as in symbiosis with *Pinus sylvestris* (Meharg *et al.*, 1997; Meharg and Cairney, 2000). Likewise, *B. edulis, G. viscidus, L. bicolor* and *L. scabrum* have the ability to degrade DDT in solid medium (Huang *et al.*, 2007). Thus, this study aimed at assessing the tolerance of some *Pisolithus* sp. isolates against glyphosate in liquid and solid mediums.

MATERIALS AND METHODS

Experimental design and trial preparations:

The tolerance of *Pisolithus* sp. isolates were assessed in solid and liquid modified Melin-Norkrans medium (MMN) (Marx, 1969) at independent trials. For both trials, treatments were set in a 5 x 5 factorial scheme, which stands for the isolates D10, D17, D51, D106 and D118, growth in culture medium with glyphosate dosage 0 (control); 51.7; 103.5; 206.9 and 413.8 mg L^{-1}, with nine replications per treatment. The isolates were obtained from the collection of Laboratory of Soil Microbiology of the Universidade Federal dos Vales do Jequitinhonha e Mucuri, in Diamantina - MG, Brazil. Pure isolate cultures were originally obtained from basidiomata sampled in *Eucalyptus* sp. plantations of Alto Jequitinhonha - MG, Brazil. Glyphosate doses were set based on recommended patterns of Roundup Original® (5 L ha^{-1}) for eucalypts (AGROFIT, 2014) extrapolating the area to 0.006362 m^2. These doses were extracted from stock solutions prepared in a laminar flow chamber with sterile distilled water. Roundup Original® is a non-selective, systemic herbicide belonging to N-substituted glycine group and has as active principle the isopropyl-amine salt of glyphosate, and is classified as moderately toxic (MAPA, 2014).

Mycelium of isolates were obtained from collection, subculture onto MMN solid medium, and let grow in the dark for 29 days at 25ºC. After this period, 5-mm-diameter circular sections were withdrawn from around the edges of each isolate

colony. Then, discs were transferred into Petri dishes with MMN medium and incubated for three more days under the same conditions to reactivate mycelium damaged by cuts, in order to confirm their viability and lack of contamination. This procedure was followed for both solid and liquid mediums.

Solid medium trial:

The culture medium was sterilized by autoclaving at 1 atm and 121°C for 15 minutes. After cooling down to 45°C using water bath, glyphosate stock solution aliquots were added into the medium at the concentrations to be evaluated. Subsequently, about 20 mL of this medium was poured into each Petri dish (0.006362 m^2). After solidification, circular sections with a diameter of 5 mm were taken from 3-day grown mycelium and placed in the center of a sterile Petri dish with mycelium turned upwards. Then, these sections were incubated for 30 days at the same conditions described above. At the end of this period, colony radial growth was calculated by the average between colony diameters in two directions using a ruler.

Liquid medium trial:

The MMN medium in an amount of 48 mL was placed in 125 mL Erlenmeyer flasks and sterilized as previously described. After cooling down, aliquots of glyphosate stock solution were added at the established concentrations. Final volume was adjusted to 49 mL at all concentrations, in which 48 mL was liquid medium and 1 mL of herbicide solution. Then, ten 5-mm discs were withdrawn from around the edges of each colony and pre grown for 3 days hereupon they were placed into the flasks and left for incubation at 25ºC in the dark. Flasks were gently hand shaken for 3 seconds every day and, after 30 days, mycelium was sampled using a 0.053-mm mesh sieve, washed with distilled water and dried at 60ºC for 3 days to weigh its dry mass. Comparisons between isolates were made by regression of the concentration that reduced the dry weight of mycelium on 50%.

Statistical Analysis:

The trials were assessed separately and data of diameter and mycelium dry mass underwent variance analysis and, when interaction was significant, regression was set, except D10, for which there was no equation adjustment.

RESULTS AND DISCUSSION

The growth performance of *Pisolithus* sp. isolates in control (0 mg L^{-1}) followed the ascending order of D17 = 5.5 cm; D106 = 5.7 cm; D118 = 6.2 cm; D51 = 6.6 cm and D10 = 8.1 cm. Glyphosate addition decreased colony diameters for all isolates, however, in a distinct way (Figure 1). The D10 isolate was not consistently influenced, having the largest growth at the highest glyphosate concentration (413.8 mg L^{-1}). For D17 and D51, the lowest concentrations of the herbicide increased colony diameters. D106 and D118 colony diameters reduced linearly at the highest concentration, being D106 higher than D118. Overall, growth inhibition was observed up to the concentration of 103.5 mg L^{-1} (Figure 1). Another *Pisolithus* sp. isolate had its growth in solid medium reduced by 59% with the addition of 50 mg L^{-1} glyphosate (Paula Jr *et al*., 1995). Nevertheless, as observed in literature, the isolates of *Cenococcum geophilum*, *Hebeloma longicaudum* and *Pisolithus* sp. can have their growth impaired by adding concentrations of triclopyr, glyphosate, hexazinone and 2,4-D higher than 100 mg L^{-1} (Estok *et al*., 1989).

When compared to control (0 mg L^{-1}), the highest concentration of glyphosate has provided diameter reduction of all colonies by the rates of 5.3% for D106, 19.3% for D17, 21.7% for D118, 22.6% for D51 and 26.8% for D10. In spite of this further reduction of D10, this isolate presented the best performance in growth at the highest herbicide concentration, being followed by D106 > D51 > D118 > D17 (Figure 1).

Comparing the mediums, glyphosate had a greatest inhibitory effect on liquid medium than on solid one. In liquid medium the production of mycelium dry mass varied in the following sequence for the control: D10 = 259 mg, D51 = 222 mg, D17 = 209 mg, D118 = 198 mg and D106 = 140 mg. Therefore, mycelium dry mass of all isolates decreased with increasing doses of glyphosate (Figure 2). This was similar to other findings in the literature for *Pisolithus* sp. isolates grown in liquid medium receiving up to 254 mg L^{-1} glyphosate (Fernandes *et al*., 2014). Contrarily, for D10 and D51, dry mass reduction was lower at the lowest concentrations. Thus, considering intermediate doses, the growing order of tolerance to the herbicide was D106 < D17< D118 < D51 < D10 (Figure 2). The required concentration to restrain 50% mycelium growth was 0 mg L^{-1} for D106, 104.5 mg L^{-1} for D17, 99 mg L^{-1} for D118, 111 mg L^{-1} for D51 and 129.5 mg L^{-1} for D10. Once more, as observed for solid medium, isolates with further growth without herbicide application were also more tolerant, as D10 and D51 in this study. The greater toxicity of glyphosate in liquid medium compared to solid may be due to some changes in herbicidal properties like solubility and diffusivity, or even lower availability thereof to fungi (Paula Jr. *et al*., 1993). This behavior was also observed for glyphosate and isoxaflutole in other study (Fernandes *et al*., 2014).

The largest biomass amount, especially for D10 in both mediums, may contribute to greater herbicide retention within hyphae or adsorption in G-layers (gelatinous layers), melanin and other compounds released by hyphae, as proposed in EMF for atrazine and 2-4D (Donnelly *et al*., 1993), DDT (Huang *et al.*,

2007) and heavy metals (Denny and Ridge, 1995; Grazziotti *et al.*, 2001; Grazziotti *et al*., 2003), including protection to host plant. Furthermore, several studies have been demonstrating that some EMF isolates have the ability to degrade biocide molecules (Meharg *et al*., 1997), as reported for 2,4-D (Meharg *et al.*, 1997; Meharg and Cairney, 2000) and DDT (Huang *et al.*, 2007).

Even under high concentrations of glyphosate, the largest biomass of D10 compared to the other isolates can also foster colonization of host plant roots. Besides of that, a larger mass of isolates facilitates soil exploitation and increases its detoxification capacity thereof. In addition, efficient inoculants have intensive growth as a desirable characteristic as well as its tolerance to potentially harmful substances.

Given the above, it is of great importance to use EMF isolates adapted to herbicide action in large-scale inoculation programs of the forest sector, since this symbiosis can be influenced by herbicide use. Still, more studies should be carried out to evaluate the effect of herbicides on fungus-plant associations and their further benefits. While fungus is growing along with the host plant, it has a wide range of carbohydrates available, which are supplied by the plant. It is known that in symbiosis, the extracellular enzymatic fungal activity increases vastly compared to growth in culture medium. Thus, fungus ability to degrade herbicide molecules can enhance during symbiosis with plants, once this association increases the mass and enzyme activity. Further studies are needed to evaluate the effect of herbicides on EMFs in symbiotic relationship, as well as the environmental factors that may affect herbicide degradation. These fungi can play a major ecological role in degradation processes in addition to retain soil aromatic compounds. The D10 isolate can be potentially used in inoculations; therefore, once it is known the behavior of such mechanism, symbiotic process management will be facilitated in a commercial environment.

Conclusion:

Pisolithus sp. isolates presented different results regarding the tolerance to glyphosate and the type of culture medium used may affect such results.

D10 was the most tolerant isolate in both solid and liquid culture mediums.

The most sensitive isolate to glyphosate on solid medium was D17, and on liquid medium was D106.

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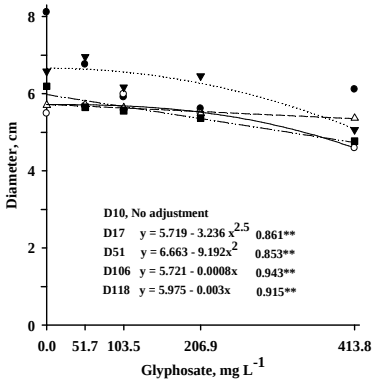

Fig. 1: Colonies diameters of *Pisolithus* isolates sp. grown for 30 days in solid MMN media with glyphosate. ** significant at 1% by F test.

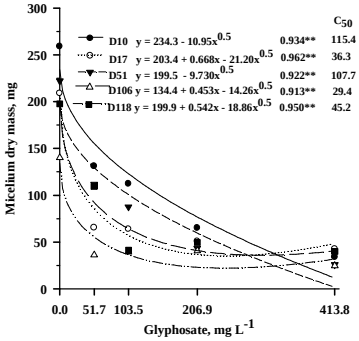

Fig. 2: Mycelium dry mass of *Pisolithus* sp. isolates grown for 30 days in liquid MMN media with glyphosate. ** significant at 1% by F test. C_{50} (mg L^{-1}) is the concentration which reduced isolates growth in 50%.

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