



Revista Árvore

ISSN: 0100-6762

r.arvore@ufv.br

Universidade Federal de Viçosa
Brasil

Silva Fernandes, Mayara Cristina; Soares da Costa, Lidiomar; Graziotti, Paulo Henrique; Fonseca Santos Graziotti, Danielle Cristina; Barbosa dos Santos, José; Rossi, Márcio José
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Revista Árvore, vol. 38, núm. 3, mayo-junio, 2014, pp. 461-468
Universidade Federal de Viçosa
Viçosa, Brasil

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***Pisolithus* sp. TOLERANCE TO GLYPHOSATE AND ISOXAFLUTOLE *In Vitro*¹**

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ABSTRACT – The ectomycorrhizal fungi have different tolerance to herbicides and may promote the survival and growth of the eucalypts tree. This study aimed to evaluate the tolerance of *Pisolithus* sp. isolates to glyphosate and isoxaflutole. The isolates evaluated were D3, D16, D17, Pt24 and UFVJM04. Glyphosate concentrations were: 0, 32, 63, 127 and 254 mg L⁻¹ in liquid medium; 0, 32, 63, 127, 254, 507 and 1014 mg L⁻¹ in solid medium. For isoxaflutole, the concentrations were 0, 295, 589, 1178 and 2355 mg L⁻¹ for both media. Assays were independent for each herbicide and culture medium. The tolerance of isolates depended on the herbicide and its concentration in each type of culture medium. Pt24 was the most tolerant to glyphosate and the UFVJM04 to isoxaflutole. Glyphosate was more toxic to isolates of *Pisolithus* than isoxaflutole.

Keywords: Herbicide; Ectomycorrhiza; *Eucalyptus*.

TOLERÂNCIA DE Pisolithus* sp. A GLYPHOSATE E ISOXAFLUTOLE *In Vitro

RESUMO – Os fungos ectomicorrízicos possuem tolerância diferenciada aos herbicidas e podem promover a sobrevivência e crescimento das plantas de eucalipto. O objetivo deste trabalho foi avaliar a tolerância de isolados do fungo ectomicorrízico *Pisolithus* sp. aos herbicidas glyphosate e isoxaflutole. Os isolados avaliados foram D3, D16, D17, Pt24 e UFVJM04 e as concentrações de glyphosate no meio líquido, de 0, 32, 63, 127 e 254 mg L⁻¹, enquanto no meio sólido, de 0, 32, 63, 127, 254, 507 e 1.014 mg L⁻¹. Para o isoxaflutole nos dois meios de cultura, as concentrações foram de: 0, 295, 589, 1.178 e 2.355 mg L⁻¹. Os ensaios foram independentes para cada herbicida e tipo de meio de cultura. A tolerância dos isolados foi dependente do herbicida e da sua concentração em cada meio. O Pt24 foi o mais tolerante ao glyphosate e o UFVJM04, ao isoxaflutole. O glyphosate foi mais tóxico aos isolados de *Pisolithus* sp. do que o isoxaflutole.

Palavras-chave: Herbicida; Ectomicorriza; *Eucalyptus*.

¹ Recebido em 08.08.2013 aceito para publicação em 29.04.2014.

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1. INTRODUCTION

Sustainability has been an increasing concept in bioenergy production, and ectomycorrhizae can contribute on this, helping in eucalypts establishment in nutrient-poor or degraded soils. They increase volume of exploited soil by roots improving water and nutrients absorption, in special those with low mobility, as phosphorus (MARX; CORDELL, 1989). In addition to this these benefits, ectomycorrhizae can provide great resistance to high temperatures and extreme pH (MARX; CORDELL, 1989) and increase root longevity (ALLEN, 1991), as well as dry matter production stimulation (SOUZA et al., 2004) and provide greater tolerance to soil toxicity conditions and pathogens (ALLEN, 1991; GRAZZIOTTI et al., 2003).

Eucalypts is the forest species most grown in Brazil and, due to increasing demand for wood and limitation of new areas, there is a need to enhance seedling survival and growth in field conditions and, with this, boosting yield of production areas. Thus, ectomycorrhizal fungi (EMF) inoculation in eucalypts seedlings might enhance plant productivity. Among EMF used in commercial plantings in Southeastern Brazil, *Pisolithus* is one with the most occurrence, and several researches have evinced isolate capacity of the genus to promote growth of inoculated seedlings (ALVES et al., 2001; SILVA et al., 2007; SOUZA et al., 2008).

Ectomycorrhizal associations depend on interaction with soil, environment, and host plants and can be influenced by adopted crop management (CAMPOS et al., 2011). In order to use EMF, it is necessary to know about herbicide effect on fungi, since in major planted forests, these products are used to prevent damages by weeds. Invasive species control is necessary once weed impose difficulties in seedling establishment in field, because they harm seedling development and growth by allelopathic effects (TOLEDO et al., 2003), and water, nutrient and light competition. Moreover, weed can increase fire risks and hinder other forestry practices (TUFFI SANTOS et al., 2006).

Herbicide use by forest sector companies has been practiced increasingly and might have, as an indirect consequence, inhibition of ectomycorrhizal formation (ILOBA, 1978; CULDLIN et al., 1983; TRAPPE et al., 1984). Nevertheless, some studies have pointed out that herbicides also may not influence (WARDLE; PARKINSON, 1991) or, even though, stimulate (ROSLYCKY, 1982) EMF growth. This EMF response

to herbicides depend on species, herbicide type and dosage (TRAPPE et al., 1984; MOORMAN, 1989). *Pisolithus tinctorius* had its growth in solid medium reduced from 1 mg L⁻¹ of triclopyr, glyphosate, hexazinone and 2,4-D herbicides, being completely inhibited in 5,000 mg L⁻¹ of same herbicides. Yet *Hebeloma longicaudum* growth was inhibited in 100%, at 1,000 mg L⁻¹ of triclopyr and 2,4-D concentrations, and other herbicides have inhibited growth in 55% at 100 mg L⁻¹ concentration, while *Cenococcum geophilum* growth was stimulated in 53% up to 10 mg L⁻¹ dose for triclopyr and hexazinone (ESTOK et al., 1989). A *P. tinctorius* isolated had growth in solid culture medium reduced by 59% at 50 mg L⁻¹ concentration of glyphosate and completely inhibited in same concentration for oxyfluorfen (PAULA Jr. et al., 1995). However, another *P. tinctorius* isolate did not have growth, in liquid medium, impaired for glyphosate concentrations up to 10 mg L⁻¹ (LAKE et al., 1981). Otherwise, same specie growth was reduced in solid medium with 1 mg L⁻¹ hexazinone (KELLEY; SOUTH, 1980).

Current researches demonstrate that *Pisolithus* sp. isolates have great difference in ability to colonize and promote growth of eucalypts seedlings in nursery gardens (FONSECA, 2013). Nonetheless, searching for terms such as herbicides, ectomycorrhizae, “herbicida”, “ectomicorriza” through Scopus, Scielo and Cab direct bibliographical databases, we have not found any research that had evaluated tolerance of different isolates of a same EMF. Thereby, we aimed in the current research to evaluate *Pisolithus* sp. isolate tolerance to glyphosate and isoxaflutole.

2. MATERIAL AND METHODS

Growth of fungal isolates D3, D16, D17 and UFVJM04 of *Pisolithus* sp., were gotten from *Eucalyptus* spp. plantations in the Alto Vale do Jequitinhonha, and the Pt 24 isolate of *Pisolithus* sp., was gotten from *Pinus* sp. plantations in Santa Catarina. These isolates were evaluated in liquid and solid Melin-Norkrans-modified medium (MNM) (MARX, 1969) added of increasing concentrations of glyphosate (Scout®) and isoxaflutole (Fordor®) herbicides. Pt 24 *Pisolithus* sp. isolate belongs to collection of the Laboratory of Ectomycorrhizae from Microbiology and Parasitology Department in Federal University of Santa Catarina, and other isolates came from the Laboratory of Soil Microbiology collection in Federal University of Vales do Jequitinhonha e Mucuri.

Scout® is a systemic and non-selective herbicide of replaced glycine chemical group, its active principle is glyphosate ammonium salt, and is considered medium toxic (BRASIL, MAPA, 2013). Fordor® is a contact selective herbicide of isoxazol chemical group, its active principle is isoxaflutole that is considered extremely toxic (BRASIL, MAPA, 2013). Both formulas are widely used in eucalypts crop.

For glyphosate, concentrations were established for the two culture mediums, based on preliminary test and at LD₅₀ for trout that is 80 mg L⁻¹, being this the most sensitive test-organism when compared with birds and insects (RODRIGUES; ALMEIDA, 2011). Herbicides concentrations were obtained in a laminar flow chamber from herbicide stock-solution, which were prepared with distilled and sterilized water. Isoxaflutole concentrations were set, also for solid and liquid mediums, based on recommended dosage for eucalypts that is 200 g ha⁻¹, estimated for the Petri dish area (100 mm diameter).

From isolate culture grown by 29 days at 25°C in MNM solid culture medium, 5-mm diameter discs were removed from borders of each isolate colonies. After that, discs were transferred into Petri dishes with same medium and incubated for more three days under same conditions. Then, allowing retrieval of damaged mycelium during subculturing, confirming viability and ensuring contamination absence. This initial procedure was carried out into all performed assays.

2.1. Assay in liquid culture medium

Fifty milliliters of culture medium were placed into 125-mL Erlenmeyer flasks and subsequently sterilized by 15 min at 121°C. After culture medium cooling, in laminar flow hood, stock-solution was added into the medium to produce final concentrations of 0, 32, 63, 127 and 254 mg L⁻¹ of glyphosate and 0, 295, 589, 1178 and 2355 mg L⁻¹ of isoxaflutole. Then, 10 disks of 5 mm diameter from edges of isolate colonies, previously grown per three days, were added into flasks and incubated at 25°C.

Flasks were softly stirred per 3 seconds every day, and, by the end of 30 days, mycelium was collected a sieve with aperture diameter of 0.053 mm, washed with distilled water and dried at 60°C until constant weight, for mycelium dry mass determination (MDM) and calculation of tolerance index (TI). For this calculation, dry mass production of medium containing herbicide

are multiplied by 100, and divided by dry mass production without herbicide. So, this variable was used for isolate comparison since they have a differentiated growth.

2.2. Assay in solid culture medium

Culture medium was sterilized and, after being cooled up to 45°C in water-bath, received aliquots of stock-solution to produce the following concentrations: 0, 32, 63, 127, 254, 507 and 1014 mg L⁻¹ of glyphosate and 0, 295, 589, 1178 and 2355 mg L⁻¹ of isoxaflutole. Immediately after that, 20 mL of the medium were poured into Petri dishes of 100 mm diameter. After medium solidification, a disk, containing a previously grown mycelium per three days, was placed at central part of each Petri dish with mycelium facing upwards and incubated at 25°C per 30 days. At the end of this period, radial growth of isolate colonies was evaluated by two-colony diameter average measured at two different directions. As isolates present different radial growth, we also calculated TI, for tolerance comparison among isolates regarding the herbicide, as described by test in liquid medium.

2.3. Design and statistical analyses

Herbicides and culture medium types were analyzed in independent assays using a totally randomized design with four replications. For glyphosate assays in liquid medium and isoxaflutole in the two medium types (liquid and solid), treatments were organized in a 5x5 factorial scheme, performed by the five EMF isolates and the five herbicide concentrations. In relation of glyphosate test in solid medium, treatments were established by a 5x7 factorial scheme with same isolates and seven herbicide concentrations. Statistical analysis for TI data was performed by variance analysis and, when interaction was significant, we tried to establish regressions.

3. RESULTS

3.1. Tolerance of isolates in liquid medium

In descending order, *Pisolithus* sp. isolates that have presented greater growth in liquid medium without glyphosate and isoxaflutole addition, were: D16 = 232.9 mg; D3 = 203.4 mg; D17 = 177.8 mg; UFVJM04 = 149.6 mg; and Pt24 = 118.4 mg. *Pisolithus* sp. isolates differed ($P > 0.05$) with respect to tolerance to glyphosate and isoxaflutole concentrations (Table 1).

Table 1 – Mean square error and variation coefficient (VC) of tolerance index (TI) of fungal isolates D3, D16, D17, and PT24 UFVJM04 of the *Pisolithus* sp. grown on liquid medium with herbicides.

Tabela 1 – Quadrado médio do resíduo e coeficiente de variação (VC) do índice de tolerância (TI) dos isolados fúngicos D3, D16, D17, UFVJM04 e Pt24 de *Pisolithus* sp. crescidos em meio de cultura sólido com herbicidas.

Variáveis	Fungo (F)	Concentração (C)	F x C	CV%
IT ao glyphosate	6,849.7*	19,008.5*	1,197.9*	28.5
IT ao isoxaflutole	3,225.4*	2,712.7*	2,759.8*	42.6

* = significant at 5% by F test.

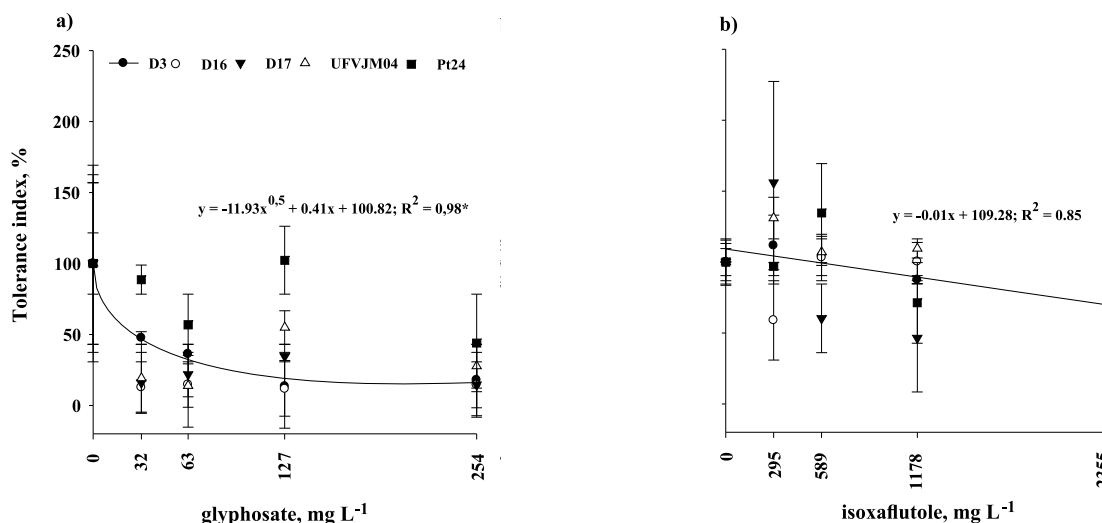
* = significativo a 5% pelo teste F.

For glyphosate, all *Pisolithus* sp. isolates had TI reduced with increasing concentrations, despite no adjustment has been found for regression in majority of isolates, except D3. TI of this isolate showed a behavior explained by an squared root equation, reaching 50% reduction at 33.4 mg L⁻¹ concentration (Figure 1a). In a concentration next to that, 32 mg L⁻¹, isolate tolerance order was: Pt24 = 88.6% > D3 = 47.5% > UFVJM04 = 19.2% > D17 = 16.0% > D16 = 12.8%, being the Pt24 TI at 63 mg L⁻¹ concentration of 58.8%, still bigger than the remaining ones. Pt24 and D3, besides presenting a greater TI than others at 32 mg L⁻¹ concentration, both also presented great MDM production at this glyphosate dosage. Mycelium production at this glyphosate concentration was Pt24 = 104.9 mg; D3 = 96.6 mg; D6 = 29.8 mg; UFVJM04 = 28.7 mg; and D17 = 28.5 mg.

Isoxaflutole promoted a widely varied effect on mycelium production what is confirmed by high value of variation coefficient (Table 1; Figure 1b). D3 TI index presented linear reduction, coming to a maximum of 35% at a concentration of 2,36 mg L⁻¹ (Figure 1b). Even though, we did not find regression curve fit for the other isolates, at this concentration, tolerance order was UFVJM04 = 134.2% > Pt24 = 95.2% > D16 = 71.8% > D3 = 68.3% > D17 = 59.9%.

2.3. Tolerance of isolates in solid medium

In descending order, *Pisolithus* sp. isolates that have presented greater growth in liquid medium without glyphosate and isoxaflutole addition, were: D3 = 74.5 mm; UFVJM04 = 65.0 mm; D16 = 61.3 mm; D17 = 47.1 mm; and Pt24 = 40.3 mm. *Pisolithus* sp.



* = significant at 5% by F test.

* = significativo a 5% pelo teste F.

Figure 1 – Tolerance index (TI) of isolates of the ectomycorrhizal fungus *Pisolithus* sp. grown on liquid culture medium MNM added glyphosate (a) or isoxaflutole (b).

Figura 1 – Índice de tolerância (TI) de isolados do fungo ectomicorrízico *Pisolithus* sp. crescidos em meio de cultura MNM líquido adicionado de glyphosate (a) ou isoxaflutole (b).

isolates have differed ($P > 0.05$) regarding tolerance to glyphosate and were not influenced by isoxaflutole (Table 2).

Glyphosate promoted highly variable effect on colony diameter in solid culture medium (Figure 2a). The TI of D16 reduced linearly with increasing concentrations, reaching a maximum reduction of 50% at 810.7 mg L^{-1} glyphosate concentration (Figure 2a). Despite, there is no adjustment found for regression of major isolates at concentration next to this, 1.014 mg L^{-1} of glyphosate, tolerance order of isolates was: Pt24 = 115.5% > UFVJM04 = 106.6% > D17 = 104.8% > D3 = 82.9% > D16 = 41.6%.

Isolates used in this current research had low tolerance to increasing concentrations of glyphosate, being still smaller than tolerance of *P. tinctorius* to the same herbicide, where fungus had mycelium dry mass reduced in 20%, at a concentration of 250 mg L^{-1} glyphosate in same liquid culture medium, grown per 30 days (PAULA JR. et al., 1995). Nonetheless, all studied isolates were more tolerant than *Scleroderma aurantium* was, which had dry mycelium production completely inhibited at 1 mg L^{-1} glyphosate (LAKE et al., 1981). Although these studies did not specify what commercial product was used, such results indicated importance of knowing EMF tolerance to various herbicides before use in Programs of Eucalypts Seedling Mycorrhization.

Isoxaflutole effect on MDM production have demonstrated variable tolerance of *Pisolithus* sp. studied isolates, since MDM increased for UFVJM04, and it was little influenced by Pt24 or reduced in D16, D3 and D17, confirming a differentiated behavior yet observed. Under bigger concentration of isoxaflutole, TI index of D3, despite being the second smallest, mycelium dry mass was of 153.5 mg, greater than that produced for UFVJM04, isolate with the biggest TI, in medium without herbicide (149.6 mg).

Isolate varied behaviors with regards to presence and herbicide type have demonstrated tolerance variation in accordance with these substances, where TI of *Pisolithus* sp. studied isolates increased for Pt24, had little influence by UFVJM04 and D17 isolates or reduced for D3 and D16, as observed under effect of isoxaflutole in liquid medium (Figure 1b). However, some isolate behaviors was dependent on used herbicide. In the highest concentration, TI of D3, despite being the second smallest, presented a growth in colony diameter of 56.5 mm, which was greater than 46.4 mm of Pt24, that obtained the biggest TI. Thus, as seen in this isolate in liquid medium with addition of isoxaflutole, the great mycelium production can also allow glyphosate use in areas where inoculated seedlings with this isolate were planted.

For isoxaflutole, *Pisolithus* sp. isolates demonstrate tolerance in studied concentrations, presenting colony diameter similar to control. This result differs from the observed, also in solid medium, for triclopyr, glyphosate, hexazinone and 2,4-D herbicides, that at concentrations higher than 100 mg L^{-1} inhibited *C. geophilum*, *H. longicaudum* and *P. tinctorius* growth (ESTOK et al., 1989). As large-scale use of isoxaflutole in eucalypts for direct application in plants, results are interesting, since admit its possible use at lower effect on tested isolates, making them better options of inoculation in plantations, which may use herbicide for weed control.

Isolates of the ectomycorrhizal fungus *Pisolithus* sp. presented differentiated tolerance to studied herbicides, and this tolerance varied according to herbicide type, concentrations and culture medium (liquid and solid). For glyphosate, isolates were sensitive in liquid medium (Figure 1a) and little influenced in solid medium (Figure 2a). Yet for isoxaflutole, isolates had little influence in liquid medium and were tolerant in the solid one. These results disagree with the classification of glyphosate as middle toxicity and

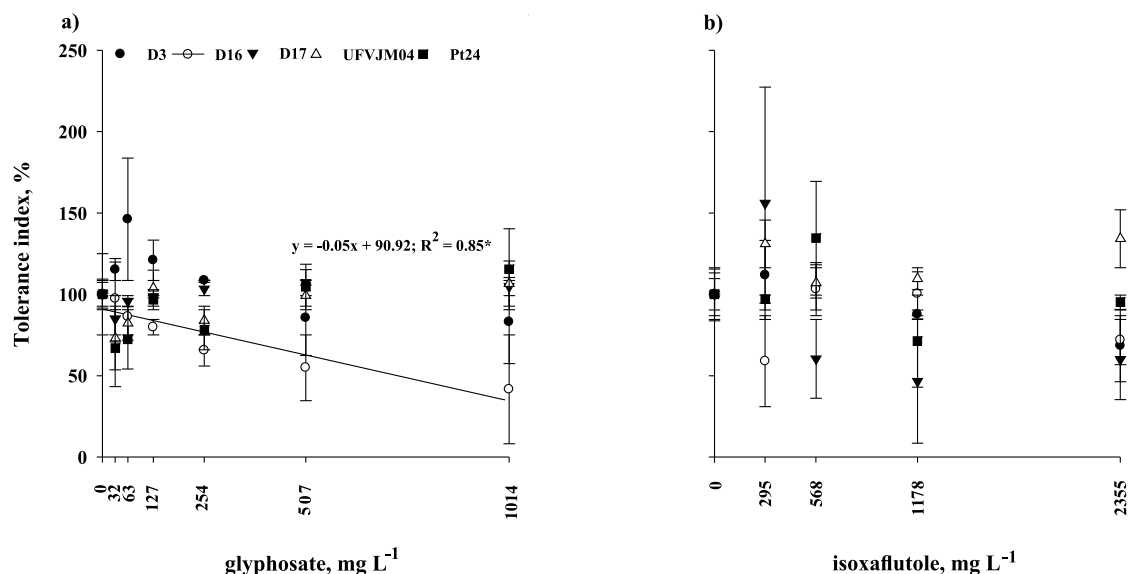
Table 2 – Mean square error and variation coefficient (VC) of tolerance index (TI) of fungal isolates D3, D16, D17, and Pt24 UFVJM04 of the *Pisolithus* sp. grown on solid medium with herbicides.

Tabela 2 – Quadrado médio do resíduo e coeficiente de variação (VC) do índice de tolerância (TI) dos isolados fúngicos D3, D16, D17, UFVJM04 e Pt24 de *Pisolithus* sp. crescidos em meio de cultura sólido com herbicidas.

Variáveis	Fungo (F)	Concentração (C)	F x C	CV%
IT ao glyphosate	4,242.62*	601.10 ^{n.s.}	1,360.36*	19.05
IT ao isoxaflutole	94.47 ^{n.s.}	44.29 ^{n.s.}	83.37 ^{n.s.}	5.89

n.s. = not significant at 5% by F test; * = significant at 5% by F test.

n.s. = não significativo a 5% pelo teste F; * = significativo a 5% pelo teste F.



* = significant at 5% by F test.

* = significativo a 5% pelo teste F.

Figure 2 – Tolerance index (IT) of isolates of the ectomycorrhizal fungus *Pisolithus* sp. grown on solid culture medium MNM added glyphosate (a) or isoxaflutole (b).

Figura 2 – Índice de tolerância (TI) de isolados do fungo ectomicorrízico *Pisolithus* sp. crescidos em meio de cultura MNM sólido adicionado de glyphosate (a) ou isoxaflutole (b).

extremely toxic for isoxaflutole, proposed by the Ministry of Agriculture, Livestock and Food Supply. Therefore, this demonstrates the need for further studies to evaluate the current classification of these herbicides.

The greatest tolerance level of EMF in solid medium than in liquid may be due to agar addition as solidifier agent, which interferes in ion absorption and translocation processes. Meanwhile, they can also represent the best fungus ability to control herbicide ingress into cells, differently of what was observed in liquid medium. This behavior has also been observed in studies that evaluated EMF tolerance to heavy metals in liquid medium (GRAZZIOTTI et al., 2001; GRAZZIOTTI et al., 2003). It is probable that the behavior of herbicide molecules in soil is similar to the observed for solid medium. Thereby, other researches must evaluate herbicide effects on symbiosis formation and effectiveness in their presence.

Stimulation occurrence, effect absence or growth reduction of different *Pisolithus* sp. isolates in culture medium observed in this research was similar to other studies using other species in medium with addition of 2,4-D and glyphosate (ILOBA, 1978).

The lowest toxic effect of isoxaflutole suggests that this herbicide can be used in areas that have been deployed with inoculated seedlings with EMF. Nevertheless, assays under field conditions, in which applications of these products have been frequent, should be conducted to evaluate results in natural reforestation environment because physiological effects on plants can affect mycorrhiza formation.

4. CONCLUSIONS

Isolates of ectomycorrhizal fungus *Pisolithus* sp. differ in tolerance to herbicides, being this result dependent on herbicide type and concentration and culture medium type.

Pt24 was the most tolerant to glyphosate and the UFVJM04 to isoxaflutole. Glyphosate was more toxic to isolates of *Pisolithus* sp. than isoxaflutole was.

5. ACKNOWLEDGEMENTS

We thank the Foundation of Research Support of Minas Gerais State (FAPEMIG) and to the CNPq, for financial support.

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