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Effect of Trichoderma on contamination and seed germination *in vitro* of *S. terebinthifolius* and Characterization of vegetative growth *ex vitro*, on the influence of Trichoderma isolates, in the presence or absence of growth regulator Stimulate®

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ABSTRACT

The production and cultivation of native forest seedling present difficulties, being the interaction between forest species and Trichoderma a promising alternative. *Schinus terebinthifolius* has economic importance because possesses medicinal properties, phytochemical, alimentary and ecological. This study aimed to evaluate the effect of the interaction of *Trichoderma spp.*, in the presence or absence of growth regulator Stimulate® in contamination and germination in vitro and in germination and vegetative growth ex vitro of *S. terebinthifolius*. Through the cellophane *in vitro* technique were evaluated the isolates TSM1 and TSM2 of the *Trichoderma viride*, 2B2 and 2B22 of the *Trichoderma harzianum*. The *ex vitro* experiment was conducted in a greenhouse, where were evaluated the effects of the isolates 2B2 and 2B22, and the commercial product Trichodermil®, all in the presence and absence of the growth regulator Stimulate®. The four tested isolates are efficient on the in vitro controlling of the seeds fungal growth. In ex vitro cultivation, we observed significant differences in leaf area index for the isolates 2B2.

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INTRODUCTION

The regeneration of a degraded environment depends, mainly, on the seed access to the site (REIS *et al*. 2003). To Holl (1999), the low seed contribution rate is the main limiting factor in the restoration of degraded areas. Thereby, the need for conservation of tropical forests and the strengthening of environmental policy promoted an increase in demand for studies on seeds of native species, which constitute a basic input in the recovery programs and ecosystem conservation (CARVALHO *et al*., 2006).

Nevertheless, forest native species are still poorly studied, such as the case of *S. terebinthifolius* popularly known in Brazil as: aroeira-vermelha, aroeira-pimenteira, pimenta-rosa, aroeira-mansa e aroeira-do-sertão. *S. terebinthifolius* is a native tree species of the Anacardiaceae family that has economic importance because it is a plant with medicinal, phytochemical and nutritional properties. Ecologically, this species stands out in environmental reforestation programs and restoration of degraded areas (LORENZI, 1992).

One of the most influential factors on seed germination is the presence of microorganisms, especially fungi, which can cause reduction of its germination, reducing their quality and commercial value (LASCA *et al*., 2004). This practice has been recommended, aiming not only to preserve the quality of seeds, but also to improve the germination performance under adverse conditions (GOULART *et al*., 2000; MACHADO, 2000). In studies with seeds of *S. terebinthifolius*, the fungus with pathogenic potential more often detected in the samples was the *Fusarium sp*. (STRAPASSON *et al*., 2002). Some species of this genus are associated with overturning seedlings in many agronomic and forestry species (CARNEIRO, 1987).

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The biocontrol of plant pathogens and the biggest plant growing can be achieved by management practices to favor native antagonists and, also, through the introduction of selected microorganisms (MELO, 1998). Isolates can be introduced before or at planting preventively and its application can be made to the seeds, on the substrate or at planting. The introduction of the agent may also be made by means of organic matter incorporated before transplanting the seedlings (LUCON, 2009).

The promotion of growth caused by soil microorganisms is due to the action of various factors poorly understood. Several fungal species have been involved in biocontrol of plant pathogens. However, Trichoderma species are, undoubtedly, the biocontrol agents of plant pathogens most studied and used in Brazil and other Latin American countries (MORANDI & BETTIOL, 2009). According to Lucon (2008), this is because these species are not pathogenic and being present in almost all types of soil with organic matter, being easily isolated, cultured and multiplied, effectively colonizing the root system of many plants.

In a study by Fortes *et al*. (2007) it was observed that the survival micropiles of a clone of Eucalyptus sp. increased by treatment with isolates of *Trichoderma spp*. and also promoted an increase in the percentage of rooting. Likewise, studies on the interaction of *Gochnatia polymorpha* (cambará) with *Trichoderma spp*. resulted in increased germination and growth compared with the control treatments without isolates (MACHADO, 2010).

The plant bioregulators or plant growth regulators, organic compounds, natural or synthetic that are not produced by plants, with action similar to that of hormones (auxins, gibberellins, cytokines, ethylene and inhibitors) in plant metabolism, modulating and regulating the growth of various organs of the plant (SANTOS, 2004), in small amounts, inhibit or modify somehow morphological and physiological processes of the plant (CALDAS *et al*., 1990; CASTRO & VIEIRA, 2001). When applied to the seeds or the leaves can interfere with processes such as germination, rooting, flowering, fruiting and senescence (CASTRO & MELOTO, 1989). The use of growth regulators on seed germination improve the seedling performance, accelerate the emergence speed and enhance seed potential in various species. The use of biologically active chemicals such as regulators and growth stimulants may terminate or reduce the impact of adverse factors in the quality and performance of seeds (ARAGÃO *et al*., 2003).

Thus, the objective of this study was to evaluate the effect of different isolates of *Trichoderma spp*. on the contamination and germination *in vitro* of *S. terebinthifolius* and study the influence of Trichoderma isolates, on the presence or absence of the growth regulators Stimulate®, as to the characterization of the emergence of seedlings and vegetative growth *ex vitro* of *S. terebinthifolius* aiming their conservation through propagation of healthy seedlings and the sustainable use of the specie.

MATERIALS AND METHODS

The assays were performed on the Laboratory of Plant-Microorganisms Interaction and in a greenhouse, belonging to the Department of Biology, Center of Natural and Exact Sciences, Federal University of Santa Maria, RS.

The botanical material used consists of seeds from the fruits of *S. terebinthifolius* collected directly from plants growing naturally in the forests of Rio Grande do Sul state. Samples were collected during the months from June to August 2011. The seeds were stored under refrigeration 8 (+/- 2) °C until the early experiments. Four exsiccates (dried and pressed specimens) were incorporated into the herbarium of the Biology Department of the Federal University of Santa Maria, from measurements at different locations, under the registration number SMDB - 13,718; 13,732; 13,742; 13,743.

Effect of Trichoderma on contamination and seed germination in vitro of S. terebinthifolius:

The experiment was carried out using the cellophane *in vitro* technique, modified in Ethur (2002). Were evaluated the effect of Trichoderma isolates in percentage of germination and contamination of the seeds of *S. terebinthifolius.* Were used in the experiments two isolates of *Trichoderma viride*, TSM1 and TSM2, two isolates of *Trichoderma harzianum*, 2B2 and 2B22, which were stored in the Laboratory of Plant-microorganism Interaction - CCNE / UFSM, beyond the control treatment without Trichoderma isolates. These isolates were selected because of the results that both isolates already shown in biological control of plant pathogens research and promotion of growth and seed germination (SILVA, 1991; SILVA, 1997; MACHADO, 2012).

Were evaluated five treatments (Table 1) arranged in a completely randomized design with six replicates per treatment; each experimental plot with 25 seeds, totaling 150 per treatment.

Table 1: Treatments of the experiment conducted by the cellophane *in vitro* technique which were evaluated the effect of Trichoderma on contamination and germination of seeds of *Schinus terebinthifolius.*

Treatmens		Description of thetreatments
T1		TSM1 (*Trichoderma viride*)
T2		TSM2 (*Trichoderma viride*)
T3		2B2 (*Trichoderma harzianum*)
T4		2B22 (*Trichoderma harzianum*)
T5		Control (without isolates)

The culture medium used was a mixture of agar-water and BD culture medium culture, 70% water-agar and 30% BD (potato dextrose - 200g of potato, 20 g of dextrose, and 1000ml of distilled water). It was used 0.7% of agar and pH adjusted to 5.8 ± 0.2 in Petri dishes. The culture medium was covered, aseptically, with a semi-permeable cellophane disk, sterilized (120 °C/40 minutes) and transferred to the center of the plates, discs of culture medium oat-based containing mycelia and spores isolated Trichoderma.

The treatments control plates were covered with a disc of cellophane, however, did not receive the mycelium disc and spore isolated. Plates were sealed with plastic film and the cultures placed in a climatic chamber (BOD) at 20 °C and 16 hours photoperiod for five days. After the incubation period, under aseptic conditions, the Petri dishes were opened and the cellophane was removed along with the discs of mycelia and spores, remaining in the culture medium only the non-volatile metabolites released by the isolates. In these, were spread the seeds that previously were undergone a disinfection process, which consisted of immersion for 15 minutes in sodium hypochlorite (NaClO) 3% and three washes in distilled water and autoclaved.

Cultures were maintained at 20°C with a 16h photoperiod. The evaluations consisted of the determination of seeds contaminated for seven days and the determination of seeds germinated for 24 days, 24 hours after installation of the experiment.

Characterization of vegetative growth ex vitro, on the influence of Trichoderma isolates, in the presence or absence of growth regulator Stimulate®:

This experiment was conducted in a greenhouse, to detect the relationship between the action of Trichoderma isolates and the growth phytoregulator in *ex vitro* conditions. Were used two isolates of Trichoderma, 2B2, 2B22, which showed good results in reducing contamination of *S. terebinthifolius* seeds in vitro, and a commercial product, Trichodermil® (manufacturer: Itaforte Bioproducts). The growth regulator used has the trade name Stimulate® (composition: Kinetin, gibberellic acid, Ácido4-Indol-3-Ilbutírico).

We used the substrate brand Tecnomax®. The application of Trichoderma on the non-sterilized substrate was carried out by means of post-biologicals, prepared with the isolated, with the except of commercial powder (Trichodermil®) which was purchased from the manufacturer Itaforte BioProducts.

The application of the post-biological on the substrates was performed 14 days before the sowing for Trichoderma colonization on the substrate. Were used 2g biological powder per kg of substrate, as recommended by the manufacturer of the commercial product, being this dose adjusted so that all treatments received the equivalent of 109 CFU.g-1 biological powders, except the Trichodermil® that has a concentration of 108 CFU g -1. The isolates 2B2, 2B22 were tested at a concentration of 4 g of powder per kg of substrate, and a control treatment in the absence of Trichoderma (Table 2). It is noteworthy that the three different isolates introduced into the substrate, where the seedlings were produced, were able to colonize and survive in the plants by the end of the culture cycle, under field conditions.

Also, the effects of the interaction of Trichoderma with the growth regulator, Stimulate® were tested. Seeds were soaked using the higher recommended dose, of all the cultures, by the manufacturer for application before planting (1.5 L * 100 kg-1 seed).

Table 2: Description of the treatments constituents of the experiment conducted in the greenhouse.

Treatments	Description of the treatments	Biological powder dosage
T9	2B2	4g
T10	2B22	4g
T11	Control: without isolate	-
T12	Trichodermil®	2g
T13	2B2 + Stimulate®	4g
T14	2B22 + Stimulate®	4g
T15	Control: without isolate + Stimulate®	-
T16	Trichodermil® + Stimulate®	2g

For installation of this experiment were used 12 vases with a capacity of 500 mL, for each treatment, totaling 96 plastic vases. Were sown 10 seeds by repetition at a depth of 0.5 cm. Irrigation was performed daily and consisted of 20 to 80 mL of distilled water per day, varying according to the

need. In this experiment the following assessments of the seedlings were carried out:

Emergence first count: held at 28 days, the obtained data were used to calculate the percentage of emergence first count of seedlings (NAKAGAWA, 1994); Emergency percentage: were counted the emerged seedlings every seven days for 23 weeks. At the end of the 23 weeks it was determined the total percentage of emergency, by the number of emerged plants in the number of placed seeds to germinate (BORGHETTI &FERREIRA, 2004); Emergence speed index (IVE): we calculated the IVE, adding to the number of emerged seedlings every seven days, divided by the number of days elapsed from the sowing, as Maguire (1962) (NAKAGAWA, 1994); Percentage of surviving seedlings: was determined at the end of 23 weeks by the number of surviving seedlings in the number of emerged seedlings; Number of leaves: was counting the number of leaves for seedling and calculated the arithmetic mean (MUNIZ *et al*., 2007); Leaf area: held by the destructive method, with equipment LI3000C model, brand Licor, with the value in mm^2; in cm on the neck of the seedling by the stem apex and calculated the arithmetic mean (NAKAGAWA, 1994); The largest root length: were measured in cm and calculated the arithmetic mean (NAKAGAWA, 1994); Fresh mass of the aerial part (stem and leaves) and root: the seedlings were removed from the substrate, washed in running water to remove the substrate residue retained in the roots and then, left to drain on paper towels. After, there was a cut on the stem base, separating the aerial part of the root. Was held then the weighing, by repetition, in accuracy balance of 0,001g, the aerial part (stems and leaves, separately) and root, and calculated the arithmetic average per seedling (NAKAGAWA, 1994); Dry mass of the aerial part (stems and leaves) and root: after having been obtained the weight of the fresh weight of the aerial part and root, the material was placed in paper bags and placed in an oven, maintained at a temperature of 60- 65 ° C, where it remained until achieve constant weight, when it was weighed and determined the average weight of the dry mass per plant (NAKAGAWA, 1994).

Were evaluated eight treatments, arranged in a randomized design, with 12 replicates per treatment; each plot had 10 seeds totaling 120 seeds per treatment. For each variable evaluated was carried out a statistical analysis. When necessary, the data in percentage were transformed to the arcsine of the square root, using the statistical program Estat. After, they were submitted to analysis of variance (ANOVA) and means compared by Tukey test at 5% probability of error.

RESULTS AND DISCUSSION

Effect of Trichoderma on contamination and seed germination in vitro of S. Terebinthifolius:

According to Baugh and Escobar (2007), Trichoderma action as a stimulator of germination and plant growth is complex and performed by interactions with biochemical factors and production of various enzymes and beneficial compounds. It can be observe that treatment with the highest germination was 2B22 (27.45%) of *T. harzianum*, followed by TSM2 *T. viride* with 21.81%. The control treatment had the lowest germination with 16.49%. However, there were no significant differences in germination between treatments with Trichoderma isolates and control.

For Ozbay *et al*. (2004), the isolates T22 and T95 of *T. harzianum* did not help in the emergence of tomato, but increased the growth of seedlings. Harman (2000) using the application of nitrogen to the soil together with the isolated T-22 of *T. harzianum*, found that initially there were no differences between areas with and without treatment, however, in adult corn plants occurred larger diameter stems and income of grain and silage, on plants in the treated areas.

Harman *et al*. (2004) consider that the increased productivity provided by selected isolates of *Trichoderma spp*. is most evident under stressful conditions for plants, in terms of the presence of pathogens. Under conditions close to ideal, the benefits to plants are less evident.

S. terebinthifolius and other species can benefit the colonization of their roots by *Trichoderma spp*., as a consequence pathogens control and the action of some isolates of this gender, as symbiont species. Likewise, according to Harman *et al*. (2004), the induction of resistance by *Trichoderma spp*. increases the expression of genes related to plant defense and is similar to acquired resistance process.

Thus, it is evident that occurred antagonist action in the biological control of fungi associated with seeds and this interaction with Trichoderma did not affect the germination of *S. terebinthifolius* negatively. Being that, the lower index of initial contamination can generate seedlings with greater future vigor.

Characterization of vegetative growth ex vitro, on the influence of Trichoderma isolates, in the presence or absence of growth regulator Stimulate®:

The effects of Trichoderma, in the presence or absence of growth regulator Stimulate®, on the germination assessments (emergency percentage, first count, seedling survival, emergency speed index) and growth (number of leaves, stem length, root length, fresh weight of leaves, fresh weight of root, fresh weight of stem, dry weight of leaves, dry weight of root, dry weight of stem, leaf area) of *S. terebinthifolius* are shown in Table 3.

Table 3: Emergency (E), first count (FC), seedling survival (SS), emergency speed index(ESI), number of leaves (NL), stem length (SL), root length (RL), fresh weight of leaves (FWL), fresh weight of root (FWR), fresh weight of stem (FWS), dry weight of leaves(DWL), dry weight of root (DWR), dry weight of stem(DWS), leaf area(LA) of aroeira-vermelha (*S. terebinthifolius*) by Trichoderma isolates, in the presence and absence of growth regulator Stimulate®, assessed to 165 days. Santa Maria, RS, 2012.

Trt	E (%)	FC (%)	SS (%)	ESI	NL	SL (g)	RL (g)	FWL (g)	FWR (g)	FWS (g)	DWL (g)	DWR (g)	DWS (g)	LA (cm^2)
T9	21,6[a]	5,0[a]	81,9[a]	0,6[a]	9,4[a]	6,3[a]	6,5[a]	10,1[a]	5,8[a]	3,8[a]	2,7[a]	1,3[a]	1,1[a]	510[a]
T10	26,6[a]	4,2[a]	89,6[a]	0,7[a]	7,7[a]	6,8[a]	7,2[a]	8,6[a]	3,7[a]	3,2[a]	2,4[a]	1,2[a]	1,0[a]	295[b]
T11	33,3[a]	12,5[a]	93,1[a]	1,0[a]	9,3[a]	10,6[a]	9,6[a]	13,1[a]	4,8[a]	5,0[a]	3,8[a]	1,7[a]	1,6[a]	271[b]
T12	25,8[a]	6,7[a]	91,7[a]	0,7[a]	8,5[a]	7,3[a]	7,4[a]	8,5[a]	3,9[a]	3,5[a]	2,6[a]	1,3[a]	1,2[a]	306[b]
T13	30,0[a]	3,3[a]	75,4[a]	0,8[a]	7,9[a]	6,5[a]	6,0[a]	8,7[a]	4,1[a]	3,0[a]	2,5[a]	1,0[a]	0,9[a]	286[b]
T14	26,7[a]	5,0[a]	87,5[a]	0,7[a]	7,9[a]	6,8[a]	6,2[a]	9,1[a]	4,8[a]	3,1[a]	2,7[a]	1,1[a]	1,0[a]	275[b]
T15	20,8[a]	5,8[a]	91,7[a]	0,6[a]	8,3[a]	6,1[a]	5,7[a]	9,3[a]	5,0[a]	3,3[a]	2,8[a]	1,2[a]	1,0[a]	379[b]
T16	26,7[a]	5,8[a]	84,7[a]	0,8[a]	7,7[a]	7,6[a]	6,4[a]	11,3[a]	5,6[a]	4,0[a]	3,5[a]	1,3[a]	1,3[a]	338[b]
Aver	30.1	11.8	77.9	0.7	2.9	2.7	2.6	3.1	2.2	2.0	1.8	1.3	1.3	56.2
C%	35.9	78.9	32.2	3.2	25.5	28.8	30.8	27.8	24.5	24.1	22.8	17.4	17.9	21.7

*Values followed by the same letter are not statistically different from each other. Tukey's test was applied to 5% error probability.

For Melo (1998), the success of plant pathogens control and promoting plant growth by bio-agents depends on the properties and mechanisms of action of the organism. Since, for the *ex vitro* tests, a significant difference was observed only for the variable leaf area, in which the treatment with the isolate of *T. harzianum* 2B2, in the absence of growth regulator Stimulate®, was superior to all other treatments. However, for all other variables were not observed effects, both negative and positive, of Trichoderma isolates studied in the presence or absence of growth regulator Stimulate®, in germination tests and plant growth of *S. terebinthifolius.*

The leaf area can be considered as a productivity index, given the importance of photosynthetic organs in the biological production of the plant (SCALON *et al*., 2003). The light, being the primary source of energy related to photosynthesis (CAMPOS & UCHIDA, 2002) and morphogenetic phenomena (TAIZ & ZEIGER, 2004), is one of the main factors influencing the growth and development of plants, in such a way that, this increase in leaf area promoted by isolated 2B2 of *T. harzianum* can effect on higher growth when analyzed in more advanced stages. The efficiency on growth can be related to the ability of adjustment of the seedling to environmental lighting conditions, being the satisfactory growth of some species in environments with low or high brightness attributed to the ability of the species to quickly adjust its allocation model of biomass and physiological behavior (DIAS-FILHO, 1997, 1999).

For the results of the use of growth regulator Stimulate®, in which was not found positive or negative effects on the germination and growth of *S. terebinthifolius*, it is noteworthy that the manufacturer does not have recommendations for forest species. Such results may be related to the choice of the applied dose, number of applications for this species, among other factors still unclear. Thus, it is shown a clear need for further studies of this growth regulator for forest species. And also, even present satisfactory results of the use in the presence of growth-promoting microorganisms.

The growth promoting capacity of plants caused by microorganisms in the soil occur due to the action of various factors poorly understood. According to Benitez *et al*. (2004) these factors may be such as the composition of soil microbial, nutrient availability, isolated used, soil type and environmental factors. Since the absence of some of these factors may explain the results in Table 4, in which shows no significant effects on the parameters of Trichoderma germination and growth studied.

As for the effect on the development and production of lettuce, it was found that the isolates did not affect the fresh weight of the plants, when compared to controls, even being present in the rhizosphere of plants. These data differ from those observed by Lynch *et al*. (1991) that reported increases 27-54% in fresh weight of lettuce treated with *Trichoderma spp*. On the other hand Ousley *et al*. (1993) demonstrated this variability when they used an isolate of Trichoderma producer of viridiol who interfered negatively in the germination of lettuce (*Lactuca sativa* L.) and other isolate producers of fatty acids and glycerol who acted positively in wheat growth (*Triticum aestivum* L.) .Moreover, Correa (2006), in hydroponic conditions, and Bal & Altintas (2008), in sub-optimal growth conditions in field, not found any effect of *Trichoderma spp*. on the growth of lettuce plants.

The variability among isolates of *Trichoderma spp*., as to the interference in the vegetable growth, consists, mainly, in the production of secondary metabolites and their ability to be competitive in the rhizosphere. Thus, it can be observe that the response of Trichoderma to different cultures may vary, as observed by Chang *et al*. (1986) that, by utilizing soil treatment with conidial suspension of *T. harzianum*, were observed promotion of plant growth through weight of dry mass higher than the control, on bean at 10%, radish at 8%, 37 to tomato, in 42% of pepper and 93% of cucumber.

Although positive effect on the sprouting and little effect on growth of *S. terebinthifolius* by the isolates of Trichoderma were not observed, also adverse effects were not observed (Table 4). The presence of these isolated on the substrate has

potential in advanced stages of development, to provide greater protection against pathogens action, this logically, if proved by detailed studies of biological control for certain pathogens to the studied species. In several studies it has been observed that *Trichoderma harzianum*, introduced into the ground, has reduced disease severity in plants and has induced stimulation of germination and growth of various plant species, however, there are few reports of researches involving the interaction of Trichoderma and forest species (DONOSO *et al*., 2008). The results of this interaction may optimize the production of seedlings and hence, reduce the extraction from natural forests; contribute to reforestation programs in Brazil, accelerating recovery processes of degraded areas; in addition to the genetic conservation of the species and benefits for producers in nurseries. However, the contribution of species of Trichoderma on the increase of the seed germination and plant growth is proved for some Trichoderma isolates in studied on plant species (RESENDE *et al*. 2004; ALMANÇA, 2005; FORTES *et al*., 2007; HOYOS-CARVAJAL *et al*., 2009; MACHADO, 2010; MACHADO, 2012).

Conclusion:

The Trichoderma isolates tested TSM1 and TSM2 of *T. viride* and 2B2 and 2B22 of *T. harzianum*, did not interfere negatively in the germination of *S. terebinthifolius*. These isolates were effective in reducing fungal contamination of seeds of *S. terebinthifolius* by the cellophane *in vitro* technique.

In the *ex vitro* cultivation of *S. terebinthifolius* it was observed that the isolated 2B2 of *T. harzianum* in the absence of growth regulator Stimulate®, did obtain significant increase compared to the vegetative growth of leaf area. For the other variables analyzed was not observed interaction between isolated of Trichoderma, both in the presence and absence of growth regulator Stimulate®, regarding to germination and vegetative growth.

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