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RESEARCH ARTICLE

METHODOLOGY FOR *Trichoderma* sp. MULTIPLICATION IN ORGANIC SUBSTRATES

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ABSTRACT

The current methodologies available for *Trichoderma* sp. multiplication under controlled conditions require long periods to produce the fungal inoculum. This study presents a simple and efficient methodology for multiplication of pure *Trichoderma* sp. cultures within only 13 days of incubation. The differential of the proposed methodology is the multiplication step in commercial organic substrates, which optimizes and reduces the production cost of the fungal inoculum, thus facilitating its use in the production of different species of seedlings of agricultural and forestry interest, as well as in research that aims to evaluate the agricultural potential of different *Trichoderma* sp. isolates.

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INTRODUCTION

The increasing concern with natural resources, potential sources of contamination and food security, has encouraged modern agriculture to use sustainable practices in pest management for a variety of crops. Fungi of the genus *Trichoderma* are among the most important agents of pest biocontrol, due to their competition mechanisms for space and nutrients and the production capacity of inductive acids, lytic enzymes and secondary metabolites with the action of antibiosis, which make it possible to control a wide range of plant pathogens (Harman, 2006; Ethur *et al.,* 2007; Almeida, 2009). Currently, the large number of isolates of the species of *Trichoderma* genus already described are being efficiently used in the control of diseases caused by different species of pathogens *Colletotrichum, Sclerotium, Sclerotinia, Phytophthora, Phythium, Rhizoctonia, Fusarium, Verticillium*and, indirectly, in the suppression of damage triggered by phytonematoids (Srivastava *et al.,* 2016; Bae *et al.,* 2016; Carrero-Carrón *et al.,* 2016; Saravanakumar *et al.,* 2016; Waghunde *et al.,* 2016). Besides pest control, the growth promoting effects attributed to symbiosis with *Trichoderma* sp. establishes a second mechanism of great importance to plant production. According to Kamaruzzaman *et al.* (2016), Fiorini *et al.* (2016) and Shaw *et al.* (2016),the fungus-plant interaction results in the exudation and assimilation of metabolites related to plant biomass production, rooting, nodulation biochemistry and rhizospheric symbioses beneficial to plant development.

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Bisen *et al.* (2016) also highlight the great efficiency that *Trichoderma* sp. present in induced systemic resistance in plants of agricultural interest. Both effects (pest biocontrol and plant growth promotion) confer to *Trichoderma sp*. great technical, economic and environmental importance for global agribusiness in two basic forms: the mass multiplication for commercialization of the bioagent in different formulations and the use of the fungus inoculated in the substrate of seedlings of commercial interest. The latter include mainly vegetable crops and ornamental, bioactive and forest plants. In the last thirty years, different methodologies for multiplication of *Trichoderma* sp. in a controlled environment have been reported. In addition, the use of cereal grains as substrate, or the elaboration of organic substrates can provide a surface for adhesion and energy and nutrition for microbial growth. For mass production, especially for commercial purposes, grains such as rice and sorghum are commonly used in industrial production of *Trichoderma* spp., due to practicality, availability, cost and yield (Fortes *et al.,* 2007). In addition, they are readily biodegradable, facilitating field application (Carvalho Filho *et al.,* 2008). However, other substrates may be used, such as millet grains, wheat grains, wheat straw and rice husk (Rajput and Shahzad, 2015).When the objective is to produce inoculums of *Trichoderma* sp. for commercial seedling production or to evaluate the efficiency of pure strains in *in vivo* tests, some known methodologies present a limiting factor regarding to fungus development time, requiring incubation periods of more than three months. The methodology proposed in this study is an efficient and simple alternative, since it allows the multiplication of the fungal inoculum directly in the organic substrate, which can be used

as support for plant growth, in a reduced period of time. This study presents a simple, fast, efficient and safe methodology for the multiplication of pure *Trichoderma* sp. cultures in organic substrates, aiming toward commercial production of seedlings and research bioassays to evaluate the efficiency of different isolates in the plant production.

MATERIALS AND METHODS

The methodology consists of two stages of multiplication of each *Trichoderma* sp. isolate, the first one in rice grains and the second in an organic substrate for vegetable production (Fig 1).

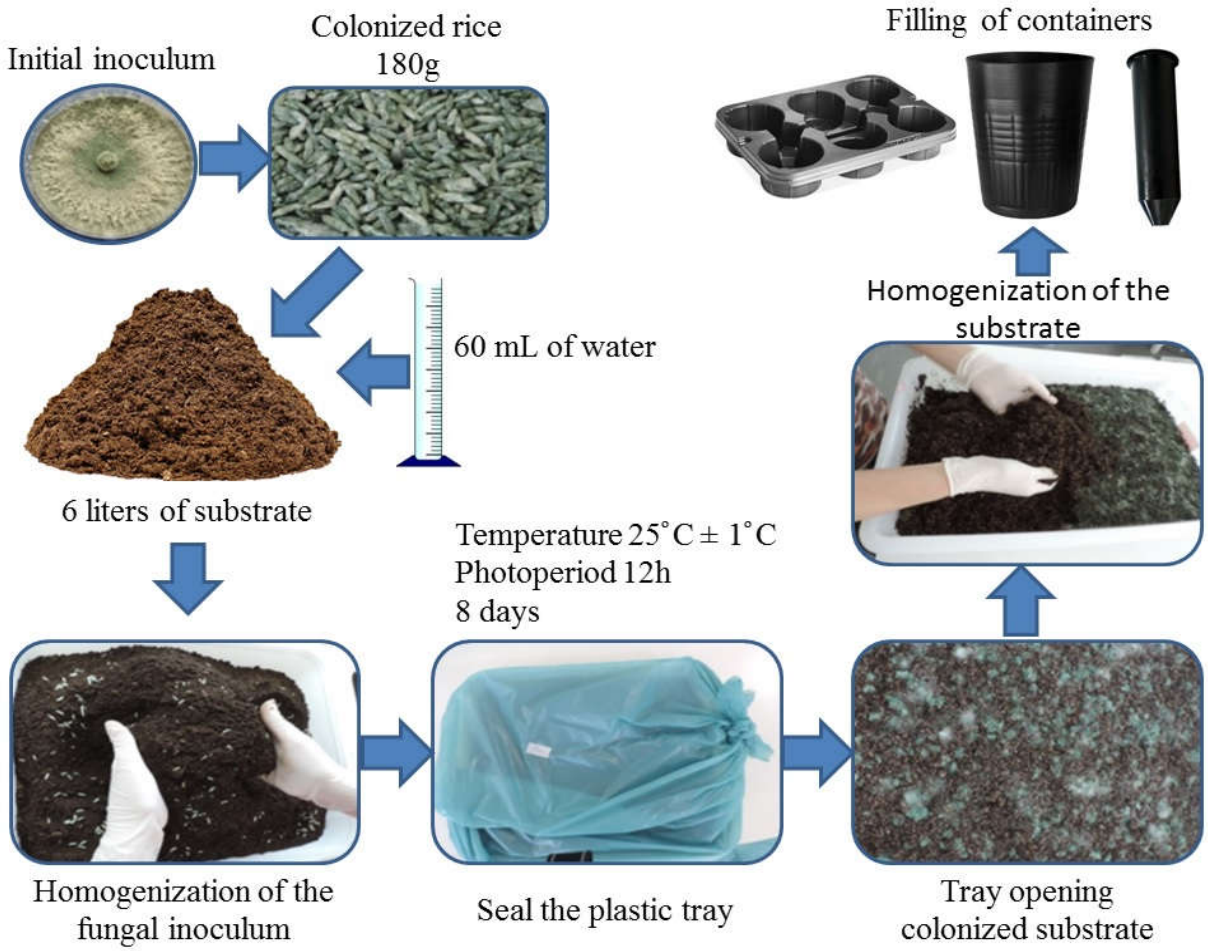

Fig.1. Steps for the production of inoculum of *Trichoderma* sp. in organic substrates

Step 1: Multiplication of the isolate in rice grains

Add 100 grams of parboiled rice and 50 mL of distilled water in polypropylene packs (approximately 20 x 30 cm). Close the packages with crepe tape and sterilize in autoclave for 25 minutes at 121 °C. In a laminar flow chamber, open the packages and transfer five disks (9 mm diameter) of fungal mycelium from pure culture of *Trichoderma* sp. isolate grown in commercial BDA (potato-dextrose-agar) medium. The packaging should be kept in an environment with temperature control (25 ± 1 °C) and brightness, remaining the first 12 hours in the dark and then with a 12-hour photoperiod for five days, when the grains will be completely colonized by the fungus.The packages should be revolved daily to promote aeration of the substrate and the breaking of the mycelium, aiming to increase the contact surface and the sporulation rate of *Trichoderma* sp.

Step 2: Multiplication on organic substrate for plant production

Plastic trays with a capacity of 10 liters should be filled with six liters of commercial organic substrate previously autoclaved (3 cycles of 60 minutes at 121ºC)**.** Use of organic substrates based on peat, expanded vermiculite, carbonized rice husk, among other materials, pH 5.0 (± 0.5), low density and water retention capacity of around 50% is recommended. After autoclaving, add 60 mL of sterilized distilled water and 30 grams of colonized rice per liter of substrate. After complete homogenization of the fungal inoculum in the substrate, seal the trays with plastic packaging that allows passage of light.

The material should be kept under controlled temperature (25 ± 1 ºC) and brightness for eight days, and in the first 24 hours the material should remain in the dark, followed by 12 hour photoperiod. After this period, spores and hyphae of the pure culture of *Trichoderma* sp. have grown on the surface and inside the substrate mass. Remove the plastic packaging and stir manually to completely homogenize the fungal structures in the organic material. You could evaluate the number of spores per cm^3 of substrate with the aid of a Neubauer chamber under an optical microscope, this value will be variable according to the sporulation capacity of each isolate. The organic substrate with inoculum of the pure culture of *Trichoderma* sp. is ready to be used in the seedlings commercial production, or in research for *in vivo* evaluation of the effect of each isolate on growth promotion or plant protection. By dilution of the organic substrate (v:v) in soil or

substrate (commercial or non-commercial formulations), it is possible to evaluate different concentrations of inoculum and efficiency for agricultural and / or forestry use.

DISCUSSION

Currently, the methodologies available for *Trichoderma sp*. multiplication demand a long time and relatively high investment in labor. Shukla *et al.* (2016) point out the logistics and infrastructure needs inherent to the process of multiplying microbial agents. The great differential of the methodology proposed in this study is the possibility of inoculum production in only 13 days of incubation, using as the raw material for multiplication the same substrate that will be used in plant production. Considering that the fungus growth dynamics is directly related to the conditions of space, nutrition, temperature and luminosity, these are the factors that must be optimized for obtaining the final product (colonized substrate) in a shorter time. Research published by Ramanujam *et al.* (2010); Khandelwal *et al.* (2012); Nagur Badu and Pallavi (2013); Emerson and Mikunthan (2015); Rajput and Shahzad (2015) and Tomer *et al.* (2016) have shown the methodological bases inherent in the multiplication of *Trichoderma* sp.at a small or large scale, using different liquid compounds and organic substrates. These publications show as converging points the time required for isolate multiplication in the final substrate (organic substrate), which varies from 40 to 150 days. Depending on the objective, the time required for multiplication of the isolate in the substrate may be a limiting factor. In order to maintain optimal fungal growth conditions, there should be no stress related to the deficiency of carbon, glucose and dispersed oxygen sources, otherwise the physiology of the microorganism will collapse due to the accumulation of volatile, non-volatile and thermo stable secondary metabolites excreted by the microorganism. It is well known that the antagonist potential of *Trichoderma* sp. derives from its ability to produce enzymes, such as cellulase and hemicellulase, capable of degrading lignolytic materials and breaking cell walls of phytopathogenic fungi, as well as glucanases and chitinases. Self-inhibition has been reported by Seidl-Seiboth *et al.* (2005 and 2013), being related to the accumulation of degrading proteins in the medium or substrate already used by the microorganism. Equally limiting to fungal development is the competition for space and nutrients, which present barriers to the growth of filamentous fungi(Samuels, 1996; Boehm and Hoitink, 1999; Druzhinina *et al.,* 2006; Contreras-Cornejo *et al.,* 2016; Karthikeyan, 2016). Therefore, the possibility of efficient multiplication of *Trichoderma* sp. in a short time is an advantage of the methodology presented in this study. However, it is important to consider that there is variation in the sporulation rate among isolates of the genus *Trichoderma* due to differences in nutritional requirements and incubation time for spore production (Carvalho Filho *et al.,* 2008).

Conclusions

The presented methodology allows the multiplication of pure cultures of fungal isolates of the genus *Trichoderma sp*. in organic substrates in only 13 days of incubation in a controlled environment. The inoculum multiplication steps are simple, fast and efficient, with application in seedlings production and in research bioassays to evaluate the potential of different isolates in pest biological control and in plant growth promotion.

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