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Soil sterilization for arbuscular mycorrhizal fungi and the influence of remaining spores on maize plants growth

Camila Sousa dos Santos[1], Tulio Silva Lara[2], Marcos Diones Ferreira Santana[3], Ivan Alves dos Santos Junior[4]

Abstract

Arbuscular Mycorrhizal Fungi (AM) are beneficial to plant growth and development by increasing nutrient and water absorption and tolerance to abiotic stress. However, in controlled studies, a recurrent problem is to define whether the observed benefits come from the inoculated AM fungi or from a native AM fungus from the soil used. The solution is the correct sterilization of the soil before the beginning of the experiment, and, therefore, the objective was to evaluate the efficiency of soil sterilization techniques and to evaluate the responsiveness of corn plants to the spores of AM fungi remaining after sterilization. The experiment was carried out under laboratory conditions, where five soil sterilization techniques were proposed: Soil sterilized in an autoclave at 121 °C for 15 minutes (T2); Soil sterilized in a drying oven at 100 °C for 1 hour (T3); Soil sterilized in a drying oven at 150 °C for 1 hour (T4); Soil watered with 3% Formaldehyde (T5); Soil fumigated with 27% Formaldehyde (T6) and Control (T1) with unsterilized inoculum. After the application of each technique, the percentage of infection (I%) of the AM fungi, originating from the remaining spores, in the roots of maize plants was evaluated and the growth of the plants was analyzed comparing them to the control, in order to evaluate the efficiency of sterilization techniques. The spores of the AM fungi were resistant to all techniques tested, but these reduced the percentage of infection by remaining spores. Treatments T2 and T5 provided the best results, however, the formaldehyde used in T5, still present in the soil, caused damage to plant development. Therefore, soil sterilization by autoclave is the most effective, and repetition of the technique is recommended to achieve the inactivity of the spores of the AM fungi.

Keywords: AMF spores, soil disinfestation and percentage of infection.

Esterilização do solo para fungos Micorrízicos Arbusculares e a influência dos esporos remanescentes em plantas de milho: Fungos Micorrízicos Arbusculares (MA) são benéficos ao crescimento e desenvolvimento das plantas por aumentar a absorção de nutrientes, água e tolerância ao estresse abiótico. Porém, em estudos controlados, um problema recorrente é definir se os benefícios

[1] Bachelor's Degree student in Biological Sciences, ICTA, UFOPA, Santarém- Pará, Brazil, camilasousantos1@gmail.com
[2] PhD in Plant Phisiology, Professor, ICTA, UFOPA, Santarém - Pará, Brazil, tulio.lara@yahoo.com.br
[3] PhD student Biodiversity and Biotechnology (Bionorte), UFPA, Belém- Pará, Brazil, santana.mdf@gmail.com
[4] Master's Degree student in Biosciences (PPGBioc), UFOPA, Santarém- Pará, Brazil, ivanjunioritb@gmail.com

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observados são provenientes dos fungos MA inoculados ou de um fungo MA nativo oriundo do solo utilizado. A solução é a esterilização correta do solo antes do início do experimento, e em virtude disso, objetivou-se avaliar a eficiência de técnicas de esterilização do solo e avaliar a responsividade das plantas de milho aos esporos de fungos MA remanescentes, após a esterilização. O experimento foi executado em condições de laboratório, onde foram propostas cinco técnicas de esterilização do solo: Solo esterilizado em autoclave a 121°C por 15 minutos (T2); Solo esterilizado em estufa de secagem a 100 °C por 1 hora (T3); Solo esterilizado em estufa de secagem a 150 °C por 1 hora (T4); Solo regado com Formaldeído a 3% (T5); Solo fumigado com Formaldeído a 27% (T6) e o Controle (T1) com inóculo não esterilizado. Após a aplicação de cada técnica, avaliou-se o percentual de infecção (I%) dos fungos MA, oriundos dos esporos remanescentes, nas raízes de plantas de milho e analisou-se o crescimento das plantas comparando-as ao controle afim de avaliar a eficiência das técnicas de esterilização. Os esporos dos fungos MA foram resistentes a todas as técnicas testadas, porem estas diminuíram o percentual de infecção por esporos remanescentes. Os tratamentos T2 e T5 proporcionaram os melhores resultados, no entanto, o formaldeído usado em T5, ainda presente no solo, causou prejuízo no desenvolvimento das plantas. Portanto, a esterilização de solo por autoclave é a mais eficaz, sendo ainda recomendado repetição da técnica para alcançar a inatividade dos esporos dos fungos MA.

Palavras-chave: Esporos de FMA, desinfestação do solo e porcentagem de infecção.

1. Introduction

Arbuscular Mycorrhizal fungi (AM) make up the phylum Glomeromycota, with more than 300 species (Oehl et al., 2011; Spatafora et al., 2017). These fungi are found in symbiosis with about 90% of the terrestrial plant species, in practically all ecological niches (Lanfranco, 2016). AM fungi hyphae that emerge from the root system can acquire nutrients and water from soil portions inaccessible to the roots (Smith et al., 2000; Allen, 2011). On the other hand, plants direct carbohydrates and lipids to fungi (Roth & Paszkowski, 2017; Leonieet al., 2017), which are used for hyphae synthesis, spore production and respiratory metabolism (Bago, 2000; Smith & Read, 2008). Thus, if there is a limitation of carbohydrate destined to the AM fungi or an uncontrolled increase of AM fungi in the roots, which can cause a high carbohydrate consumption, both the plant and the fungal development are impaired (Johnson et al., 1982). Despite this, recent studies have shown that inoculation with certain AM fungi is beneficial for the growth and development of maize plants under ideal or stressful conditions (Mathur et al., 2018; Covacevich et al., 2018), mainly for improving nutrient and water acquisition and for increasing tolerance to abiotic stress (Elhindi et al., 2017; Tekaya, et al., 2017).

However, a major problem in working with AM fungi, mainly in controlled environments, is to define whether the benefits come from the AM fungi under study or whether it is a result of the native AM fungi in the

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soil used in the experiment. This situation can result in a false positive, where the benefit to the plant comes from the native fungus rather than the inoculated fungus. In this sense, the correct sterilization of the soil is a critical and little discussed step in scientific studies. Methyl bromide has long been the most used compound for soil sterilization, however, since the Montreal protocol, when its unwanted effects on the environment were presented, alternative products have been sought, with lower risks to man and the environment (Miranda et al., 2007).

Some sterilization alternatives emerged, such as: use of autoclave, fumigation and watering with formaldehyde, in addition to the use of a high temperature drying oven (Runia & Molendijk, 2010; Nouri et al., 2014; Sarabia et al., 2017; Hu et al., 2019). However, most of them are focused on the elimination of bacterial spores (Spry, 2008), phytomatodes or even opportunistic plant seeds (Ritzinger e Rocha, 2010) and few studies have focused on changing the status of the AM fungi after sterilization. However, studies that evaluate efficiency of soil sterilization techniques for AM fungi and study the influence of non-eliminated AM fungi (after sterilization) on plants growth are scarce. Thus, the aim of this work was to evaluate the efficiency of soil sterilization techniques to eliminate AM fungi and to evaluate the responsiveness of maize plants to the remaining AM fungi, after sterilization.

2. Material and method

The experiment was carried out at the Laboratory of Plant Physiology and Plant Growth of the Federal University of Western Pará (UFOPA), Santarem campus, using a concentrate of spores isolated from various native AM fungi species from Santarem region and multiplied in 150 g pots containing the soil-sand mixture (v/v) and sown with ten maize seeds (*Zea mays* L.) to produce the inoculant substrate, and the efficiency of the inoculum used was tested according to Santos et al. (2018). Maize plants were used as hosts, which remained under artificial lighting for 20 days with a photoperiod of 12 hours and daily irrigation, maintaining the field capacity at 65 %. The amount of 1.5 kg of the produced inoculum was homogenized to 4.5 kg of Ferralsols, totaling 6 kg, which was divided between treatments, the remaining 150 g were used for counting spores containing approximately 1200 spores per gram of inoculum.

The soil with the inoculum was submitted to five sterilization techniques: Soil sterilized in autoclave at 121 °C for 15 minutes (T2); Soil sterilized in drying oven at 100 °C for 1 hour (T3); Soil sterilized in drying oven at 150 °C for 1 hour (T4); Soil watered with 3% Formaldehyde (T5); Soil fumigated with 27% Formaldehyde (T6), in addition to Control (T1), unsterilized inoculum used to compare the performed sterilization techniques, totaling six treatments.

At T5, the solution of 10 ml of 3% formaldehyde was watered directly into the soil, in the proportion of 1/100 (v/v). The soil was homogenized, sealed in a plastic bag and exposed to the sun for 72 hours, then, the bag was opened for formaldehyde evaporation. At T6, 10 ml of 27% formaldehyde was used, in the proportion of 1/100 (v/v), formaldehyde was packed in disposable cups on a cotton swab to avoid direct contact with the soil. The cups were placed

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inside the plastic bag that was sealed to occur fumigation only by vapor, then the plastic bag was exposed to the sun for seven days, subsequently it was opened for evaporation.

After sterilization, treatments were schematized in four replicates, and each plot represents a repetition. In the 280 ml plots, 150 g of the sterilized soil was added, thus totaling 24 plots. In each plot, five maize plants were grown for 20 days, under the same conditions mentioned above.

Two plants from each plot were selected, standardized considering the emergency day, to perform the immediate analysis of leaf area, to obtain values of total leaf area (TLA) and, later, aerial part dry mass (APDM), root dry mass (RDM) and total dry mass (TDM). The samples were packed in identified paper bags and were dehydrated in a drying oven at 60 °C for 72 hours. After constant weight, the mass measurement was obtained on a precision scale (0.001 g), the aerial part and the root were measured separately.

The remaining plants in each plot were used to analyze the percentage of mycorrhizal infection according to Phillips and Hayman (1970). The roots were cut in a proportion of 1 cm in length and arranged homogeneously in Petri dish with grid (1 × 1 cm) with a stereoscope microscope observing the intersection points, according to the quadrant intersection technique (Giovanetti & Mosse, 1980). For spores extraction and counting, the wet sieving procedure was used from three repetitions of 50 g of the inoculum (Gerdemann & Nicolson, 1963), followed by centrifugation in 50% sucrose solution (Jenkins, 1964) and the spores were counted in plate with a stereoscope microscope.

The obtained data were tested for normality and an analysis of variance was performed. Scott-Knott test ($p<0.05$) was used to compare the means, in the Software SISVAR version 5.6 (Ferreira, 2014) and the graphs were plot in the R program (version 3.6.2).

3. Results

After applying the sterilization techniques and subsequent cultivation of maize plants, it was observed that, in general, all treatments provided a reduction in the percentage of infection compared to the control, but none was efficient in the total elimination of spores of the AM fungi from the soil. Treatments T2 and T5 showed the lowest rates of I%, with a reduction of about 77% and 68%, respectively, while T3 and T6 were the treatments that contributed least to the efficiency of soil sterilization and inactivity of AM fungal spores, with I% of 16% compared to the control (Figure 1).

Regarding the development of maize plants grown in different sterilization treatments, it was observed that T3 and T5 provided the lowest values compared to the control, on average, about 27%, 17% and 24% for APMD, TDM and TLA, respectively (Figure 2A, C-D), and in RDM, there was no difference in relation to the control (Figure 2B). On the other hand, T2 was the only treatment with outliers above the control, about 32%, 85% and 51% for APMD, RDM and TDM, respectively, except for TLA, when the values were equal to the control, T4 and T6 (Figure 2D)

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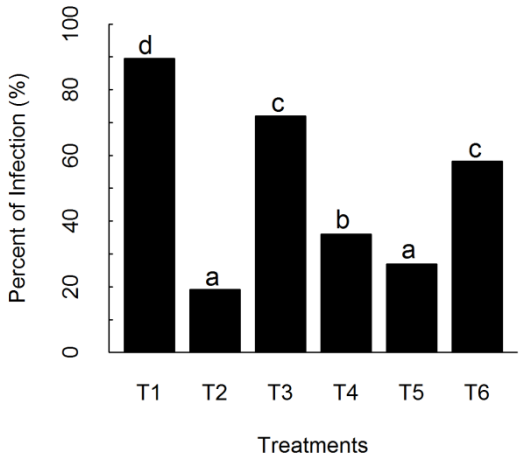

Figure 1: Infection percentage (I%) of the remaining spores of Arbuscular Mycorrhizal Fungi (AM) in roots of maize plants grown in soil subjected to different sterilization techniques. Different letters above the columns indicate significant differences by the Scott-Knott test ($p < 0.05$).

4. Discussion

All the techniques tested reduced the percentage of infection of AM fungi spores in relation to the control, but no treatment completely sterilized the soil. T2 was the treatment that came closest to the efficiency of sterilization, being this one of the most accessed means for soil sterilization because it uses autoclave equipment (Hu et al. 2020). However, Hu et al. (2020) concluded that the time required for total soil cleaning using this technique and, above all, for AM fungal spores inactivity, should be between 0.5 to 1 hour, which is longer than the time the soil used in this study was submitted to, of just 15 minutes, which although short, provided results that tend to corroborate with these authors.

Treatments T3 and T4, although better than the control, showed high values in relation to T2, possibly the short time of exposure to high temperatures did not result in a greater efficiency in the inactivity of AM fungi spores, as in the work by Zhang et al. (2019), where the technique of soil sterilization in a drying oven was used, but with temperature higher than which the soil samples of T3 (100°C) and T4 (150°C) were submitted and for a longer period of exposure of soil, 3 hours. The solarization technique, similar to the T3 and T4 treatments, as it uses heat in the sterilization of the soil to free it from pathogens, indicates an exposure time of more than four weeks (Ritzinger and Rocha, 2010), showing that techniques that use heat need more exposure time for effective sterilization. Treatment T5, using 3% formaldehyde, did not differ from T2 in terms of I%, resulting in low rates of infection by AM fungi spores. However, due to its residual toxicity in the soil, it impaired the development of maize plants used as hosts. Note valid for T6, a treatment that used formaldehyde in a higher concentration (27%) and with a significant reduction in I%. Formaldehyde in the vapor state is most used for air and surface sterilization (Patitucci, 2008) and its inefficiency in soil sterilization possibly occurred because it did not reach all the pores and soil particles. The application of this technique, if necessary, considering the lack of autoclave equipment and its proven efficiency as a germicide, destroying molecules of genetic material and microbial cells (Nour et al., 2014), should be followed by resting the soil for a period longer than 7 days, as formaldehyde is a biodegradable molecule with a tendency to complete degradation after this period..

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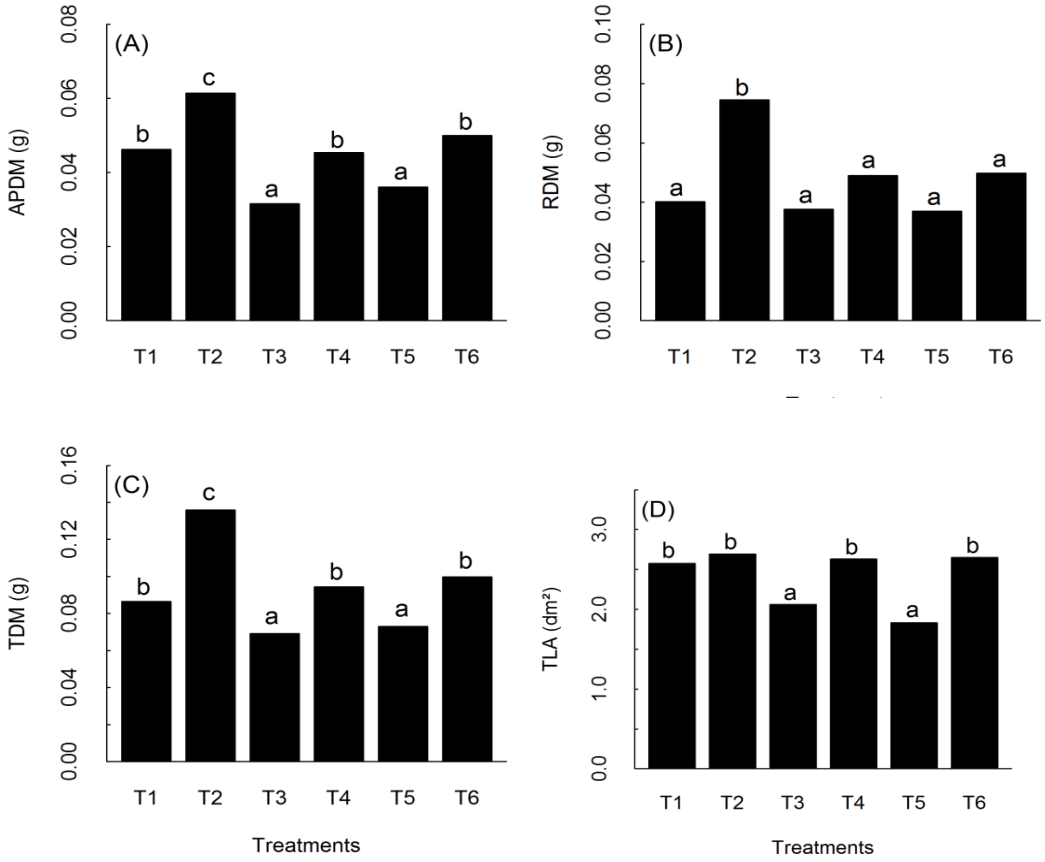

Figure 2: Influence of remnant spores of Arbuscular Mycorrhizal Fungi (AM) on the development of maize plants grown in soil treated under different sterilization techniques. Aerial part dry mass (APDM), Root dry mass (RDM), Total dry mass (TDM), Total leaf area (TLA). Different letters above the columns indicate significant differences by the Scott-Knott test ($p < 0.05$).

AM fungi spores are resistance structures (Siqueira et al 2010), formed by aminopolysaccharides, glycans, proteins, lipids, sporopollenin, mucoran, and others, which protect against lytic enzymes, poisons, high temperatures and other stress factors (Feofilova et al., 2012), a fact confirmed by this study in which, in view of all the tests, part of the spores used remained capable of colonizing plant roots. Thus, the ineffectiveness of soil sterilization in order to inactivate AM fungi spores can harm experiments and generate false results due to the interaction between the remaining spores and the host plant, because although in small quantities, as in the T2 treatment (about 24 spores per gram of soil after applying the technique), the results in plant development can be expressive. This can happen even if the AM fungi propagules are in small concentrations (Salgado et al. 2017), although the effects in situations like this may be irrelevant on plant nutrition and growth (Augé et al. 2015).

Another important aspect that must be considered is that soils sterilized in an autoclave or subjected to high temperatures can change the availability of nutrients (Hu et al.

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2020), making the technique help plants in the absorption of nutrients due to the increase in nutrient availability from the sterilization process (Miransari et al. 2009; Yang et al. 2015; Hu et al. 2020). However, only soil analysis could confirm whether T2, which presented a low density of remnant spores, and consequently low levels of I% after autoclaving, would have improved plant development due to the sterilization technique. This aspect was observed by Zhang et al. (2011) in maize plants grown in sterilized soil, which showed increased height, dry weight and phosphorus absorption when compared to unsterilized soil and by Aguiar et al. (2004) who also observed benefits in the development of plants *Prosopis juliflora* (Sw.) DC. inoculated with AM fungi and cultivated in sterilized soil in relation to plants cultivated in unsterilized soil.

5. Conclusion

Arbuscular Mycorrhizal fungi spores were resistant to the soil sterilization techniques tested in this study and induced an increase in the growth of maize plants. However, all techniques were efficient in the partial sterilization of the soil, with sterilization in an autoclave (T2) being the most efficient. T5, which uses direct soil irrigation with 3% formaldehyde, provided a high rate of sterilization, but the residual formaldehyde in the soil impaired plant development, requiring a greater rest of the soil after the treatment for total evaporation of formaldehyde in the soil. Thus, the sterilization of the soil in autoclave is indicated, however, it is suggested to repeat the process for a better result, inactivating all spores of the AM fungi remaining in the soil.

Disclosure

This article is unpublished and is not being considered for any other publication. The authors and reviewers did not report any conflict of interest during their evaluation. Therefore, the *Scientia Amazonia* owns the copyrights, has the approval and the permission of the authors for disclosure, of this article, by electronic means.

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