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MICROBIOLOGY

Anaerobic soil disinfestation with ethanol or sucrose reduces the viability of *Meloidogyne javanica* and *Stromatinia cepivora*

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ABSTRACT. Anaerobic soil disinfestation (ASD) is an ecological alternative to chemical soil fumigation. However, little is known about the potential of this technique for the management of *Meloidogyne javanica* and *Stromatinia cepivora*. To test the hypothesis that ASD reduces the viability of these two pathogens, we assessed ethanol (5%, v:v) and sucrose (5%, m:v) as carbon sources for ASD, for an incubation period of three weeks. Twenty kilograms of soil naturally infested with *M. javanica* (82 ± 43 J_2 100 cm^{-3} soil) were placed into a plastic container. Polyester traps, each with 15 *S. cepivora* sclerotia, were buried at 10 and 20 cm depth per container. ASD with diluted ethanol or sucrose (5% v:v or m:v) was compared to the untreated control (UTC), chemical fumigant metam sodium (MS), and soil saturation with water. In comparison to the UTC, ASD using ethanol reduced the numbers of J_2 in soil and the galls in tomato roots by more than 93%, a degree of suppression similar to that achieved when using MS. The viability of sclerotia of *S. cepivora* was reduced by ASD using ethanol or sucrose from 38.12 to 58.1% compared to the UTC. ASD for three weeks using ethanol or sucrose (5%) reduces the viability of *M. javanica* and *S. cepivora* in the microcosm.

Keywords: biological soil disinfestation; reductive soil disinfestation; root-knot nematode; *Sclerotium cepivorum*.

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Introduction

Plant-parasitic nematodes and the fungus *Stromatinia cepivora* (syn. *Sclerotium cepivorum*) are widely spread in agricultural areas worldwide. Nematodes may reduce the yield and quality of agricultural products, increase the susceptibility of plants to other pathogens and act as a vector of viruses (Nicol et al., 2011; Lopes & Ferraz, 2016). Root-knot nematodes (*Meloidogyne* spp.) are the most economically important in agriculture, especially the species *Meloidogyne incognita*, *M. javanica,* and *M. arenaria*, which are prevalent in tropical and subtropical regions (Lopes & Ferraz, 2016). In turn, *S. cepivora* is the causal agent of white rot, one of the most devastating garlic and onion diseases worldwide (Fuga, Lopes, & Vieira, 2012; Villalta et al., 2012; Lourenço Júnior, Viera, Lopes, & Villalta, 2018). Attacked plants present symptoms of leaf yellowing and bulb rot, and ultimately, they may die (Lourenço Júnior et al., 2018).

The management of soil-borne pathogens, such as nematodes and sclerotia-producing fungi, requires a combination of various control strategies, including crop rotation, use of biological control agents, application of chemicals, and adoption of physical control methods. Some *Meloidogyne* species have a wide host range, such as *M. incognita* and *M. javanica*, which limits the options for rotation crop systems. The host range of *S. cepivora* is limited to *Allium* species. However, the fungus can survive in the soil for up to 20 years as sclerotia (Coley-Smith, Mitchell, & Ansford, 1990). Thus, the adoption of a sustainable crop rotation system for vegetable growers is restricted, especially where garlic is one of the most profitable during the cold seasons, which is the case of the Cerrado of Minas Gerais, Brazil (Fuga et al., 2012; Lourenço Júnior et al., 2018). Chemical control of root-knot nematodes and *S. cepivora* may be expensive and harmful to the environment and human health. The number of resistant cultivars to root-knot nematodes is limited and, in Brazil, all commercial cultivars of garlic and onion are susceptible to *S. cepivora* (Lourenço Júnior et al., 2018). Solarization, biofumigation, and the application of biocontrol agents may be valuable options for the management of nematodes and *S. cepivora*, especially when used in an integrated approach (Fuga et al., 2012; Elshahawy, Saied, Abd-El-Kareem, & Morsy, 2019; Forghani & Hajihassani, 2020).

Anaerobic soil disinfestation (ASD), also known as biological soil disinfestation, is an ecological alternative for controlling soil-borne pathogens. The technique was developed in the late 1990s and early 2000s (Blok, Lamers, Termorshuizen, & Bollen, 2000; Shinmura, 2000). It has been used for the management of insects, weeds, and various plant pathogens, including *Athelia, Fusarium, Macrophomina, Phytophthora, Pythium, Ralstonia*, *Rhizoctonia, Verticillium* and *Sclerotinia* (Rosskopf et al., 2014; Shennan et al., 2014; Shrestha, Augé, & Butler 2016; Guo et al., 2017; Shrestha, Dee, Ownley, & Butler, 2018; Lopes et al., 2022).

The method consists of incorporating easily decomposable organic material into the soil (carbon source), followed by irrigation to saturation. The soil is maintained covered with oxygen-impermeable plastic for 3 to 10 weeks (Rosskopf et al., 2014; Shennan et al., 2014; Shrestha et al., 2016). The abundance of organic carbon and the anaerobic conditions created by soil saturation and plastic covering stimulate the activity of anaerobic microorganisms (Rosskopf et al., 2014; Shennan et al., 2014; Shrestha et al., 2016). The combination of oxygen depletion, accumulation of toxic byproducts, and antagonism exerted by anaerobic bacteria reduce the viability of plant pathogens in areas under anaerobic soil disinfestation (Runia et al., 2014; Shennan et al., 2014; Strauss & Kluepfel, 2015; Hewavitharana et al., 2019).

The success of ASD depends on the occurrence of anaerobic conditions and the release of a plethora of toxic compounds by microorganisms, including volatile organic acids, methyl sulfides, CO_2, NH_3, H_2S, CH_4, and N_2O (Huang et al., 2016; Hewavitharana et al., 2019). Carbon sources distinctly affect soil microbial populations under ASD (Hewavitharana, Ruddell, & Mazzola, 2014). Cereal bran and cover crop residues have been the most used carbon sources in ASD (Shrestha et al., 2016), especially due to their greater availability in agricultural regions worldwide. However, liquid amendments are generally more effective than solid ones in suppressing plant pathogens, especially ethanol (Momma, Momma, & Kobara, 2010; Momma, Kobara, Uematsu, Kita, & Shinmura, 2013; Shrestha et al., 2016). Liquid sources can be applied via irrigation before soil covering and seep deeper and more uniformly into the soil profile than the solid materials. In addition, highly labile C-sources, such as ethanol and molasses, increase the rate of attainment of anaerobicity in comparison to recalcitrant C-sources, such as some crop residues. For instance, molasses are more efficient than millet as a carbon source in ASD for the management of *Fusarium oxysporum* and *M. incognita*, even under similar anaerobic conditions (Butler et al., 2012).

The efficacy of ASD on the management of *Fusarium*, *Verticillium*, and *Rhizoctonia* species has been widely reported, especially in strawberry (Shrestha et al., 2016, Zavata, Muramoto, Mazzola, & Shennan, 2021). However, little is known about its potential to control other soil-borne pathogens, such as *S. cepivora* and *M. javanica*. Thus, we assessed the effect of ASD for three weeks using diluted ethanol or sucrose as a strategy for reducing the viability of *M. javanica* and *S. cepivora*.

Material and methods

The effect of anaerobic soil disinfestation (ASD) using diluted ethanol (5%, v:v; from ethanol 96%) or sucrose (5%, w:v; from crystal sugar) on the viability of *M. javanica* and *S. cepivora* was assessed in a greenhouse experiment. For the experiment, 600 kg soil was collected in a table beet field (*Beta vulgaris* L.) naturally infested with *M. javanica*. The soil was sieved, homogenized in a cement mixer, and put into 30 plastic containers with a capacity of 20 L. A visual description of the method is illustrated in Figure 1.

Before ASD, 2.5 kg soil was collected from each container: 0.5 kg for extracting second-stage juveniles (J_2) of *M. javanica* from the soil by centrifugal-flotation technique (Jenkins, 1964) and 2 kg for growing tomato cv. Santa Cruz Kada (susceptible indicator plant for *M. javanica*). The number of juveniles of root-knot nematodes was 82 ± 43 J_2 100 cm^{-3} soil. For estimating the viable population of *M. javanica* in soil (i.e. eggs + J_2), tomato plants were cultivated in 2 L plastic pots on a greenhouse bench for 45 days, when the number of root galls per plant was assessed.

Two polyester traps with 15 *S. cepivora* sclerotia each were buried per container: one at 10 cm depth and another at 20 cm depth. To prepare the polyester traps (Figure 1b), the sclerotia were surface disinfected with ethanol 70% (v:v) for 30 seconds, followed by sodium hypochlorite 0.5% for 180 seconds, and finally washed twice with sterile distilled water. Sclerotia were placed on a 0.04-mm^2 polyester mesh cloth (8 x 16 cm), which was on a curtain eyelet component (6 cm of inner diameter). The polyester mesh was folded in half for covering the sclerotia, and the other component of the curtain eyelet was used to close the trap. The sclerotia used in this study were collected from diseased garlic plants in Rio Paranaíba, Minas Gerais State, Brazil, and remained buried for 3 months to overcome constitutive dormancy (Coley-Smith, Parfitt, Taylor, & Reese, 1987).

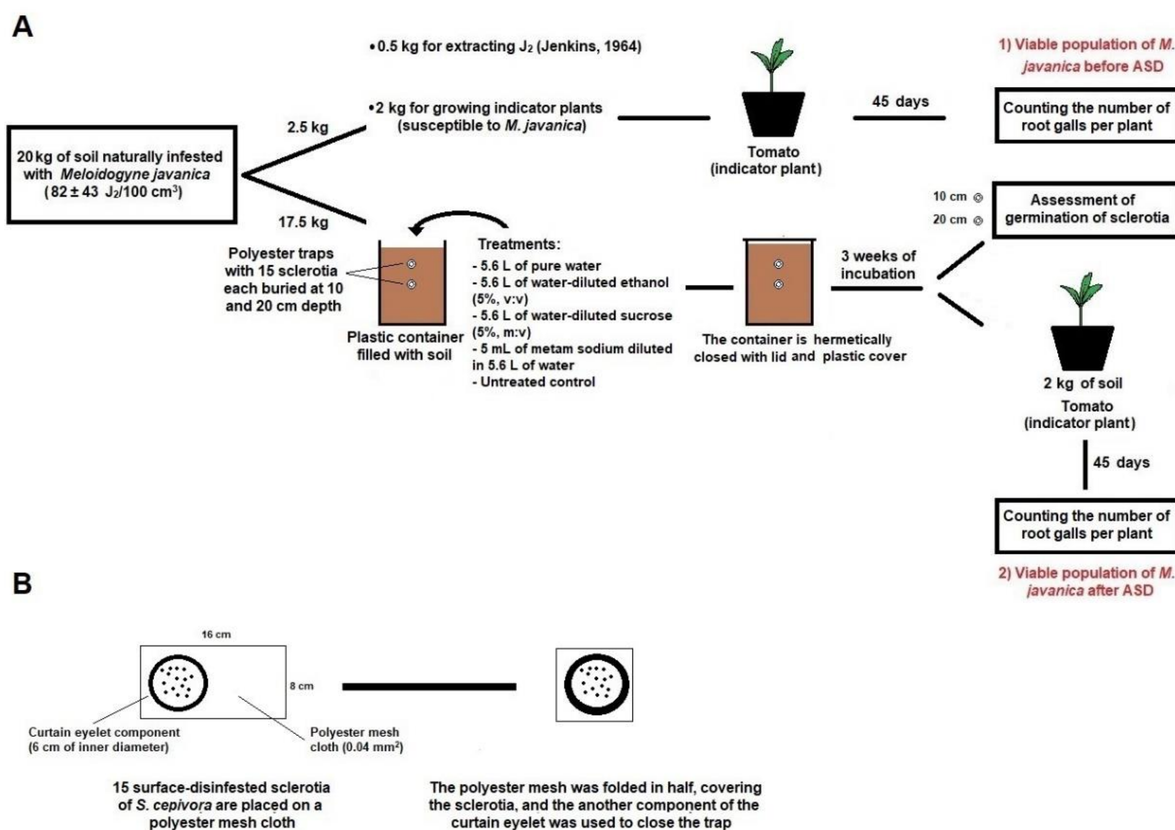

Figure 1. Schematic representation of the methodology used to assess the effect of anaerobic soil disinfestation on the viability of *Meloidogyne javanica* and *Stromatinia cepivora*. A – Overview of the experimental procedures. B – Preparation of polyester traps containing *S. cepivora* sclerotia.

The saturation point of soil moisture was reached with 5.6 L of water. Thus, ethanol and sucrose were diluted in 5.6 L of water at the rate of 5% (v:v or m:v). Untreated plots and 5.6 L of pure water and 5.6 L of metam sodium solution (Bunema® 330 SL, 750 L ha^{-1}) were used as controls. After soil saturation, the containers were hermetically sealed with lids to allow the set-up of anaerobic conditions.

After an incubation period of three weeks, lids were removed and the polyester traps were collected for assessing the germination percentage of sclerotia. In the laboratory, the sclerotia were surface disinfected with ethanol 70% (v:v) for 30 seconds, followed by sodium hypochlorite 0.5% for 180 seconds, and finally washed twice with sterile distilled water. Ten sclerotia per trap were plated on potato dextrose agar (PDA, acidified with 0.6 mL lactic acid) and incubated at 16 ± 2°C for 21 days, with visual assessment of germination every two days. Two and a half kilograms of soil were collected from each container and used for extracting J_2 of *M. javanica* and for growing tomatoes, as described above. The soil temperature was monitored using ibutton thermometers (DS1920-F5, HomeChip, Ltd, United Kingdom), inserted at 15 cm depth, before ASD. The mean temperature was 20.6 ± 1.5°C.

The experiment consisted of 30 plots with five treatments in a completely randomized design and six replications. Each container was used as an experimental plot. All data were tested for error normality (Shapiro-Wilk test), and homogeneity of variances (Bartlett test), and subjected to analysis of variance ($p < 0.05$). The data of numbers of J_2 in soil and galls per plant after ASD were transformed into square roots (x) to achieve a normal distribution before ANOVA. Means were compared by Tukey's test ($p < 0.05$). Data were analyzed using R version 4.0.2 (R Development Core Team, 2020).

Results and discussion

The numbers of J_2 per 100 cm^3 soil and galls per tomato plant (indicator plant) before the application of treatments were statistically equal in all treatments (Table 1). This implies that the nematode inoculum was evenly distributed in the experimental soil, which allows the direct comparison of the effects of treatments at the end of the experiment.

Table 1. Population of second-stage juveniles (J_2) of *Meloidogyne javanica* in soil and the number of galls in tomato (indicator plant) roots before application of soil treatments.

Treatment	J_2 per 100 cm^3 of soil	Number of galls per plant
Anaerobic soil disinfestation using ethanol 5% (v:v)	78.75 ^{ns}	118.17 ^{ns}
Anaerobic soil disinfestation using sucrose 5% (v:v)	87.02	63.67
Soil saturation with water (control)	86.40	76.50
Metam sodium (control)	96.25	92.83
Untreated control	62.63	76.67
CV (%)	54.91	56.15

^{ns} Non-significant by F test ($p > 0.05$). CV = Coefficient of variation.

Anaerobic soil disinfestation for three weeks using ethanol or sucrose (both at 5%, v:v) as carbon sources significantly reduced the viability of *M. javanica* and *S. cepivora* (Table 2). In comparison to the untreated control, ASD using ethanol reduced the numbers of J_2 in soil and the galls in tomato roots by more than 93%, a degree of suppression similar to that achieved using the chemical fumigant metam sodium, which ranged from 89.4 to 95.4% (Table 2). ASD using sucrose reduced from 87.3 to 95.7% the numbers of J_2 and galls of *M. javanica* compared to the control. Soil saturation with water reduced the viability of nematode propagules by 63.0-77.4%, in comparison to the control, although it was less effective than ASD using ethanol or sucrose, except for the effect on $J_{2,}$ in which ASD using sucrose was similar to soil saturation with water (Table 2).

Viability of sclerotia of *S. cepivora* was reduced by ASD with ethanol or sucrose compared to the untreated control, at levels ranging from 40.7 to 58.1% in propagules at 10 cm depth, and from 38.12 to 57.3% in sclerotia at 20 cm depth (Table 2). Fumigation with metam sodium did not suppress the germination of sclerotia of *S. cepivora* in comparison to the untreated control (Table 2). Soil saturation with water for three weeks reduced sclerotia germination by 38.1-42.2% in comparison to the untreated control, with an effect similar to that of ASD using ethanol or sucrose (Table 2).

Table 2. Effect of anaerobic soil disinfestation (ASD) using ethanol or sucrose as carbon sources (5%, v:v), soil saturation with water, and the fumigant metam sodium on the number of second-stage juveniles (J_2) and galls of *Meloidogyne javanica* and the germination (%) of *Stromatinia cepivora* sclerotia at 10 and 20 cm depth.

Treatment	J_2 per 100 cm^3 soil†	Galls per plant†	Germination (%) at 10 cm	Germination (%) at 20 cm
ASD using ethanol	10.5 c	0.5 c	23.3 c	23.3 c
ASD using sucrose	21.3 bc	2.3 c	33.0 bc	33.8 bc
Water (control)	37.3 b	20.0 b	32.2 bc	33.8 bc
Metam sodium (control)	17.8 c	2.5 c	51.0 ab	49.0 ab
Untreated control	168.7 a	54.0 a	55.7 a	54.7 a
CV (%)	18.9	45.4	30.3	28.3

†Data were transformed into root square (x) before analysis of variance. Original means are presented in the table. Means followed by different letters, in the same column, are significantly different by Tukey's test ($p > 0.05$).

Anaerobic soil disinfestation (ASD) for three weeks using ethanol or sucrose, both at a concentration of 5%, reduces the viability of the nematode *M. javanica* and the fungus *S. cepivora* in the microcosm. ASD has been used as an efficient and ecological alternative to chemical soil fumigation, especially for the management of species of *Fusarium*, *Verticillium*, *Rhizoctonia*, *Pythium,* and *Phytophthora* (Rosskopf et al., 2014; Shennan et al., 2014; Shrestha et al., 2016; Guo et al., 2017; Shrestha et al., 2018). However, little was known about the effect of this technique on *M. javanica* and *S. cepivora* (Shrestha et al., 2016). These pathogens, widely spread worldwide, are important threats to vegetable production in Brazil.

The efficacy of ASD depends on various factors, including the carbon sources used to stimulate the activity of the soil microbiota (Lopes et al., 2022). Ethanol and sucrose are amongst the most efficient C-sources for ASD (Momma et al., 2013; Shrestha et al., 2016), which led us to include them in this study. Besides that, they are relatively cheap in Brazil and easy to apply in the soil. The chemical composition of ethanol and sucrose is not variable, which is advantageous for use in ASD, unlike many organic materials with variable composition. For example, Butler et al. (2012) reported inconsistency in the control of pathogens and weeds using cover crops as carbon sources in ASD. In turn, the control was more efficient and consistent across experiments when ethanol was used (Butler et al., 2012).

ASD does not rely on elevated temperatures, using solarization or biosolarization (Lopes et al., 2022), to reduce the viability of soilborne pathogens. However, ASD efficiency decreases under lower temperatures, which requires an increased incubation period (Butler et al., 2014). The ideal temperature for this technique

ranges between 16 and 30°C. Here, the temperature oscillated within the ideal range, although closer to the lower limit (20.6 ± 1.5ºC).

ASD, using both ethanol and sucrose, was so effective in reducing the viability of *M. javanica* as the chemical fumigant metam sodium, especially when ethanol was employed. In the meta-analysis published by Shrestha et al. (2016), ethanol was the carbon source that increased the levels of control of different pathogens and weeds. In many studies, the concentration of ethanol for use in ASD has ranged from 0.5 to 2%. In general, the application of 100 L of 1% ethanol per m^2 in preliminary field experiments is recommended (Momma et al., 2010; Momma et al., 2013; Hewavitharana et al. 2014). Here, we used a concentration greater than 1% since ethanol is easily found in Brazil. Further studies under field conditions may explore the variation in the concentration of this amendment from 1 to 5% and its effect on plant pathogens, for instance.

Molasses have been used as carbon amendment in ASD, especially when combined with cereal brans (Butler et al., 2014; Sanabria-Velazquez, Testen, Enciso, Soilan, & Miller, 2019; Testen & Millen, 2019). Molasses contains approximately 30-35% sucrose, and 10-25% fructose and glucose (Jamir, Kumar, Kaur, Kumar, & Singh, 2021). However, little is known about the effect of pure sucrose on ASD, which was used here. The nematicidal effect of sucrose observed in this study may have been due to the osmotic damage of sugar on the nematode (Santiago, Homechin, Montalvan, & Krzyzanowski, 2005) and, or the increased sulfate-reducing bacteria responsible for the generation of toxic levels of hydrogen sulfides (Rodríguez-Kábana, Jordan, & Hollis, 1965).

In general, the nematicidal effect of ASD was stronger than soil saturation with water. The addition of carbon sources was essential for the proliferation of anaerobic organisms and the accumulation of toxic by-products from anaerobic decomposition (Runia et al., 2014; Shennan et al., 2014; Hewavitharana et al. 2019). However, flooding was similar to ASD in reducing the viability of *S. cepivora* sclerotia in the microcosm. Crowe and Hall (1980) revealed that flooding may be an efficient method to reduce the soil population of *S. cepivora*. In general, ASD is expected to be more efficient in controlling pathogens and weeds than soil saturation with water alone, as observed here for *M. javanica*. Field experiments are required to assess if ASD and flooding on *S. cepivora* have similar effects.

Conclusion

Anaerobic soil disinfestation (ASD) for three weeks using ethanol or sucrose, both at a concentration of 5%, reduces the viability of the nematode *M. javanica* and the fungus *S. cepivora* in the microcosm. The efficiency of this technique was similar to or higher than that obtained with the use of the chemical fumigant metam sodium (Bunema® 330 SL, 750 L ha^{-1}). The effect of soil saturation with water is lower than that of ASD in reducing the viability of *M. javanica,* but they are similar in reducing the viability of *S. cepivora* sclerotia.

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