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Antifungal activity of homeopathic medicines against the white mold causing agent *Sclerotinia sclerotiorum*

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ABSTRACT. White mold disease, caused by fungus *Sclerotinia sclerotiorum* (Lib.) de Bary., is a disease hard to control due to the high amount of sclerotia produced, which guarantees its survival in the soil for years leading to significant yield losses. Alternative techniques to control the pathogen have been researched, including homeopathy. The present work aimed to evaluate the *in vitro* antifungal effect of homeopathic medicines on *S. sclerotiorum* mycelial growth. Homeopathic medicines Sulphur, fungal sclerotium Nosode and Calcarea carbonica, in 30CH, 200CH and 1000CH dynamizations were tested. Assays were carried out in a completely randomized design, with four repetitions. Experiments were performed through the addition of homeopathic medicines on the surface of plates containing culture medium, followed by insertion of a disc containing fungus mycelia and incubation. Control treatment received no homeopathic medicine. The mycelial progression was monitored by seven halo diameter measurements during experiment period. All homeopathic medicines tested and their dynamizations were able to inhibit partially the development of the fungus. Calcarea carbonica at the dynamization of 1000 CH showed the best inhibitory effect on *S. sclerotiorum*, which under its effect produced a mycelial halo 40% smaller than the control treatment.

Keywords: Calcarea carbonica; homeopathy; Nosode; phytopathogen; Sulphur.

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Introduction

The white mold is a disease caused by *Sclerotinia sclerotiorum* (Lib.) de Bary fungus, a pathogen difficult to control due to the production of high amounts of sclerotia, a resistant structure that allows the fungus to survive on soil for long periods (Meyer, Campos, Godoy, & Utiamada, 2014). It is considered a pathogen of great importance and concern, since it occurs in temperate, tropical and subtropical regions, and also due to its ability to infect different plant hosts (Bolton, Thomma, & Nelson, 2006).

Until 2003/2004 crops, the white mold was considered a random soybean disease, however, since that period, the disease has become a worrying pathology (Juliatti, Polizel, & Juliatti, 2004). Dissemination of the disease is increased under favorable weather conditions, such as temperature between 5 and 30 °C and high air humidity (Beruski, Pereira, Jaccoud-Filho, Sartori, & Sentelhas, 2015), due to the central pivot irrigation system (Sediyama, 2009), on susceptible cultures rotation system and by the use of contaminated seeds (Juliatti & Juliatti, 2010).

The control of white mold in soybeans often requires good management procedures, including the use of high quality seeds, ground cover, deposition of plants for good aeration among them, shorter flowering period, fungicide application (Meyer et al., 2014) and, more recently, alternative methods have been used and studied, such as homeopathic medicines (Rissato et al., 2016). In 2008, the homeopathy became official for use in agriculture (Brazil, 2008) and its action on plant diseases is based on the induction of the plant's natural defenses (Bonato, Proença, & Reis, 2009).

As homeopathy therapy offers an alternative of low cost and zero environmental impact for the control of crop diseases and pests, it arises as a promising alternative for agroecological systems (Bonato et al., 2009). The present work aimed to evaluate the *in vitro* antifungal effect of three homeopathic medicines, *i.e.* Nosode,

Sulphur and *Calcareo carbonica*, in the dynamizations of 30CH, 200CH and 1000CH, over the *S. sclerotiorum* growth.

Material and methods

The *S. sclerotiorum* isolate used in this work was provided by Dr. Maria Heloisa de Moraes, from the Seed Pathology Laboratory from *Universidade de São Paulo* – ESALQ/USP (Sisgen register code AF19FF3).

Homeopathic solutions were chosen based on the soybean plant symptoms when attacked by *S. sclerotiorum* and on the recommended treatment for the disease using homeopathy. The Nosode homeopathy controls the disease by the containment of its causative agent, *Calcareo carbonica* was chosen due to ability to treat wilting, one of the symptoms presented by the attack of white mold (Bonato et al., 2007), and Sulphur is indicated for fungal diseases and plant tissue damage (Oliveira et al., 2014).

The experimental design was completely randomized with three homeopathic medicines - Nosode, Sulphur and *Calcareo carbonica*, all in three different dynamizations – 30CH, 200CH and 1000CH, prepared in 70% ethanol solution, plus the control treatment, totaling 10 treatments. Different dynamizations were chosen to assess whether they result in different responses from the fungus development.

Homeopathic treatments in their respective dynamizations were performed in quadruplicate and the control treatment consisted of adding 70% ethanol to the culture medium without the presence of homeopathic medicines.

Medicine Nosode from *S. sclerotiorum* was prepared from fungus sclerotia. One gram of sclerotia was transferred to an amber glass flask previously sterilized containing 29 mL of 70% ethanol. The flask was maintained protected from light for 21 days, in an airy place, away from light and the heat, being vigorously shaken once a day, daily, for preparation of mother tincture (Rissato et al., 2016). After this period, the tincture was taken to a homeopathic pharmacy, for the dynamizations of 30CH, 200CH and 1000CH. Medicines Sulphur and *Calcareo carbonica* were purchased from a ready-to-use pharmacy, in the same dynamizations as Nosode.

Evaluation of the efficiency of the homeopathic medicines as a potential antifungal against *S. sclerotiorum* was performed through the inhibition of mycelia growth, based on the protocols proposed by Damin, Alves, and Bonato (2015) and Rissato et al. (2016), with modifications.

Plates containing 30 mL of Potato Dextrose Agar medium (Himedia, Mumbai, India) were added 150 µL of the homeopathic medicine and, after Drigalsky loop spreading, each plate received a disc of 8 mm in diameter containing *S. sclerotiorum* mycelia, after complete drying of the medicine added. Plates were sealed and incubated under a 12 hours photoperiod at 25 °C, for 20 days. During the incubation period, halo sizes of mycelia growth were evaluated on the fourth, sixth, eighth, eleventh, thirteenth, fifteenth and nineteenth days. The control treatment received the disc containing the fungus mycelia and no homeopathic medicine.

The evaluations of the inhibition test initiated at day four after the experiment was installed and ended when the fungal mycelia reached the edges of the plates. Measurements followed the diametrically opposite method (Stangarlin, Schwan-Estrada, Cruz, & Nozaki, 1999), being the diameters growth of the fungal colonies measured with the aid of a ruler. During the experiment, the mycelial growth was monitored by means of seven diameter measurements on different days, until the maximum mycelium diameter was determined when it reached 9 cm, *i.e.*, the edge of the Petri dishes.

Statistical analyses were performed using software SISVAR (Ferreira, 2011), with analysis of variance by regression at 5% significance level. The analyses were conducted for each evaluated day. The treatments were compared using the Tukey test at 5%. The percentages of mycelial growth inhibition of each day analyzed were transformed into \log_{10} and then compared in relation to the control treatment.

Results and discussion

Each homeopathic medicine tested was analyzed individually, by comparing the respective effects with the control treatment (Table 1).

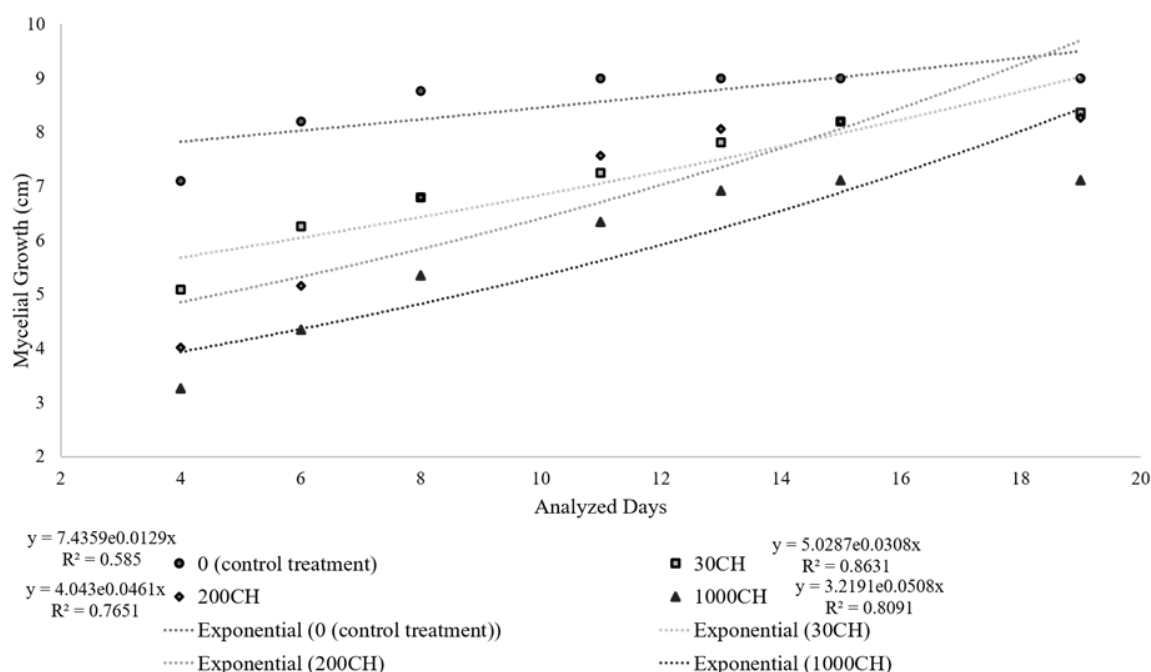
Table 1. Statistical analysis of the inhibition of *Sclerotinia sclerotiorum* mycelial growth in the analyzed days, for each treatment.

Treatment	Mycelial Growth (cm)						
	Analyzed Days						
	4	6	8	11	13	15	19
Sulphur 30CH	5.10 AB	6.28 AB	6.80 AB	7.25 BC	7.83 BC	8.20 BC	8.38 BC
Sulphur 200CH	4.02 A	5.18 AB	6.80 AB	7.58 BC	8.08 BC	8.28 BC	8.28 BC
Sulphur 1000CH	3.28 A	4.35 A	5.35 A	6.35 AB	6.93 B	7.13 B	7.13 B
Nosode 30CH	4.30 AB	5.30 AB	6.53 AB	7.05 B	7.48 BC	8.05 BC	8.08 BC
Nosode 200CH	3.90 A	5.18 AB	6.40 A	7.30 BC	7.75 BC	8.00 BC	8.00 BC
Nosode 1000CH	3.56 A	5.88 AB	6.45 A	6.35 AB	7.75 B	7.38 B	7.38 B
Calcarea Carbonica 30CH	5.05 AB	6.18 AB	6.65 AB	7.10 B	7.38 B	7.68 BC	7.68 BC
Calcarea Carbonica 200CH	3.98 A	5.25 AB	5.78 A	6.25 AB	6.70 AB	6.95 AB	6.95 AB
Calcarea carbonica 1000CH	3.70 A	4.50 A	4.80 A	5.03 A	5.03 A	5.40 A	5.40 A
Control treatment	7.10 B	8.20 B	8.78 B	9.00 C	9.00 C	9.00 C	9.00 C
CV (%)	26.67	22.82	14.49	10.83	9.459	8.55	8.56
Fc (%)	3.64	2.96	5.16	7.39	8.70	9.14	9.33

Averages followed by the same letter do not differ statistically from each other by Tukey's test ($p < 0.05$).

Sulphur

Sulphur homeopathic medicine showed potential to inhibit *S. sclerotiorum* growth in all dynamizations, as shown in Figure 1. In the first evaluation - day 4 - it was possible to verify that the growth of the control treatment was greater than the fungus submitted to treatment with Sulphur, and statistically significant with Sulphur 200CH and 1000CH. The best suppressive effect over the fungus was observed with the dynamization of 1000CH, which has inhibited more efficiently the *S. sclerotiorum* growth during the experiments compared to control treatment.

**Figure 1.** *Sclerotinia sclerotiorum* mycelial growth (cm) from Sulphur treatment at 30CH, 200CH, 1000CH and the control on all analyzed days.

Studies on the inhibitory effects of the homeopathic medicine Sulphur have already shown the potential against the development of phytopathogens. Lorenzetti et al. (2016) researched the potential of Sulphur medicine in the control of *Macrophomina phaseolina*, the causing agent of grey stem in soybean. The authors observed a reduction of 14% and 15% of the area under mycelial growth curve of the fungus with the dynamizations of 12CH and 48CH respectively, when comparing the action of Sulphur to the control treatment. Leonel and Barros (2013) verified the reduction of coffee rust, caused by *Hemileia vastatrix*, for up to three consecutive months after treatment with this medicine with 6CH dynamization. Toledo, Stangarlin, and Bonato (2009) tested the Sulphur homeopathic medicine in the control of *Alternaria solani*, a fungus responsible for tomato early blight, inoculated in tomato plants. Ten days after *A. solani* inoculation, the

authors observed that Sulphur at the dynamizations of 12CH and 60CH reduced the disease severity in 48.82% and 56.47% respectively, when compared to the control treatment. Sinha and Singh (1983) verified that Sulphur, at 200CH, was able to inhibit the growth of the important aflatoxin producer *Aspergillus parasiticus* in 100%.

The results showed that the increase in the dynamization of the Sulphur caused a decrease in the speed of the fungus growth, with the best result being observed with 1000CH dynamization, which showed mycelial growth inhibition statistically different from the control treatment on all evaluated days (as shown in Table 1).

Nosode

Nosode homeopathic medicine was able to inhibit *S. sclerotiorum* mycelial growth, however, the inhibition behavior differed among dynamizations. With Nosode 30CH, the mycelial growth of the fungus was statistically different from the control only on the fourth day, whereas in the 200CH dynamization, the inhibition was statistically significant on the first and third days of analysis. The 1000CH dynamization showed a more consistent inhibition in relation to the mycelial growth of the control treatment, with statistically better results on the first, third, fifth, sixth and last day of evaluation (Table 1, Figure 2).

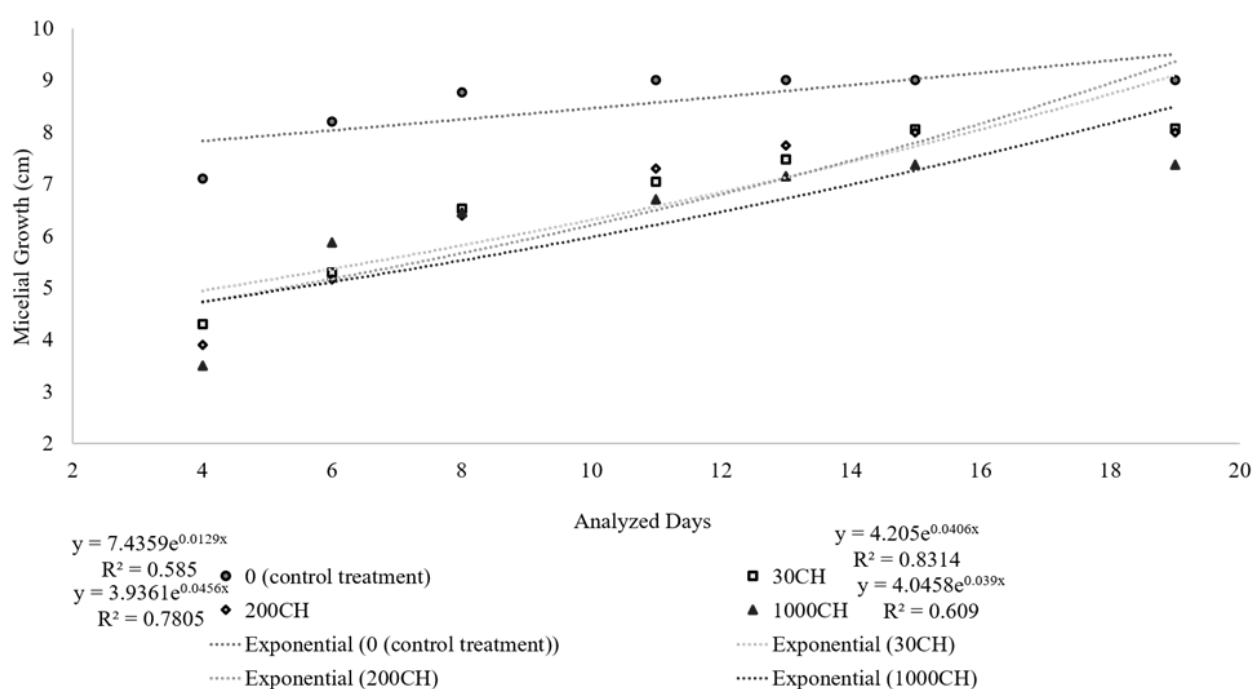


Figure 2. *Sclerotinia sclerotiorum* mycelial growth (cm) from Nosode treatment at 30CH, 200CH, 1000CH and the control on all analyzed days.

These results differ from those found by Rissato et al. (2016), who tested the *Sclerotinia sclerotiorum* sclerotium Nosode for control of this pathogen and obtained, with the dynamization 48CH, an increase of mycelial growth by 10.54%. Diniz et al. (2006) assayed the potential of homeopathic preparation from *Phytophthora infestans*-infected tomato tissue (Nosode) to treat tomato plants with symptoms of late blight and observed no significant reduction of the evaluated inhibition indicators.

Carneiro et al. (2010) evaluated the potential of a biotherapeutic obtained from *Alternaria solani* in the control of early blight of tomato plant and on *in vitro* development of the fungus. They observed that the bioteraphic had no effect on the fungus development *in vitro*, however, the severity of the disease was reduced at the dynamizations 26CH, 27CH and 28CH, suggesting that it might have induced the resistance of the host. Nosode of *Macrophomina phaseolina* was tested *in vitro* and *in vivo* in soybean by Lorenzetti et al. (2016), at the dynamizations of 6, 12, 24, 36 and 48CH, and the treatment showed no effectiveness in reducing the disease or inhibiting the development of the fungus.

Nosode medicine showed variable efficiency in inhibiting mycelial growth in the tests performed and, according to Diniz et al. (2006), each organism reacts differently to this medicine, requiring adjustment of dynamizations to improve performance (Casali, Castro, Andrade, & Lisboa 2006). Agreeing with Casali et al. (2006), Carneiro et al. (2011) stated that the great difficulty of researching vegetables with homeopathy is to find the right dynamization to treat a certain pest or disease.

In this sense, when the correct dynamization of the Nosode has been defined, this medication may represent a potential alternative for the control of phytopathogens, since it is based on isopathy and its matrix is prepared from the agent that causes the organism imbalance (Rezende, 2009).

Calcarea carbonica

With the treatment using homeopathic medicine *Calcarea carbonica*, on all evaluation days, the speed of mycelial growth has slowed. In comparison to the control treatment, *C. carbonica* 30CH significantly inhibited the growth of *S. sclerotiorum* on the fourth and fifth days of evaluation. The suppression of the medicine against the fungus in the 200CH dynamization was significant every day except on the second day of evaluation, and for the *C. carbonica* 1000CH, the medicine significantly inhibited fungal growth every day (Table 1, Figure 3).

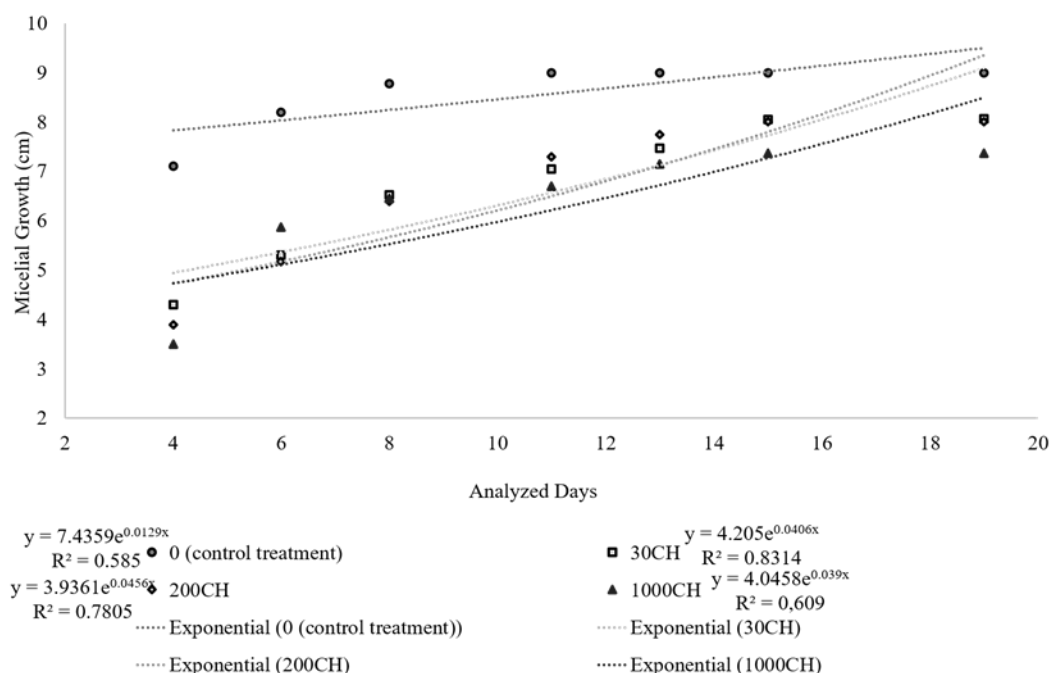


Figure 3. *Sclerotinia sclerotiorum* mycelial growth (cm) from *Calcarea carbonica* treatment at 30CH, 200CH, 1000CH and the control on every analyzed day.

Calcarea carbonica medicine, at 1000CH dynamization, showed the best results on the inhibition of *S. sclerotiorum* mycelial growth. At the end of the experiment, treatment with *Calcarea carbonica* at 200CH showed higher efficiency when compared to 30CH, however, both treatments showed lower inhibitory effect when compared to 1000CH, which reduced the growth rate of the fungus by 40% compared to the control treatment.

Promising results in inhibiting phytopathogenic fungi using *Calcarea carbonica* were also found by Barkund et al. (2018) that tested the use of *Calcarea carbonica* to inhibit *Gibberella fujikuroi* and *in vitro* assays showed inhibition of the fungus with 30CH and 200CH dynamizations. The same medicine was tested *in vitro* by Chinche et al. (2018) for the inhibition of *Eremothecium gossypii* (*Ashbya gossypii*) growth, and the dynamizations 12CH, 30CH and 1M showed inhibitory activity over the fungus.

It is important to note that, although homeopathic treatments were not able to completely inhibit the *in vitro* development of *S. sclerotiorum*, these drugs were applied in a single dose at the beginning of the experiments, and tests with other application dynamics are pertinent.

Controlling *S. sclerotiorum* in soybean is a difficult task due to the rapid progress of the disease and the long-term survival of pathogen sclerotia in the soil, as well as the difficulty of curative control of the disease by chemicals or the limited variety of active substances for rotation, in order to avoid selection of resistant population (Meyer et al., 2014). Therefore, it is necessary to evaluate *Calcarea carbonica* in an *in vivo* test, to determine the activity of the medicine over soybean plants and, if effective, define which is the dynamization to control white mold in a living organism.

Conclusion

The homeopathic medicines Sulphur, Nosode of *S. sclerotiorum* and Calcarea carbonica showed potential to partially inhibit the mycelial growth of *Sclerotinia sclerotiorum*. Calcarea carbonica in the dynamization of 1000CH was the most efficient medication, showing significant inhibition of fungus development compared to the control treatment. The use of homeopathy showed a promising potential for alternative and sustainable management of *Sclerotinia sclerotiorum*.

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