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Fungitoxicity activity of *Phosphorus* and *Calcarea carbonica* against *Sclerotinia sclerotiorum* and control of white mold in common bean (*Phaseolus vulgaris*) with extremely diluted aqueous solutions

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Abstract

Common bean (*Phaseolus vulgaris* L.) is one of the main Brazilian agricultural crops. Numerous diseases have affected such a crop during its life cycle, such as white mold, caused by *Sclerotinia sclerotiorum*. This fungus is quite aggressive and requires an intensive use of pesticides. This study aimed at evaluating white mold control and antimicrobial activity against *S. sclerotiorum* using extremely diluted aqueous solutions of *Phosphorus* and *Calcarea carbonica,* at 6CH, 12CH, 24CH, 36CH and 48CH dynamizations (centesimal Hahnemannian). The tests were carried out in a completely randomized design, with 10 treatments and 5 replicates each, considering water as control. Variables including disease progression, the number of dead plants, the number of sclerotia, and mycelial growth were evaluated by *in vivo* and *in vitro* tests. The treatments *Phosphorus* 12CH, *Phosphorus* 48CH, *Calcarea carbonica* 12CH, and *Calcarea carbonica* 48CH presented resistance-inducing action by slowing down the disease progression up to 83% and decreasing the number of dead plants up to 90%. *In vitro* tests showed that the treatments *Phosphorus* 12CH, *Phosphorus* 48CH and *Calcarea carbonica* 48CH slowed down the mycelial growth. The latter also completely inhibited the production of sclerotia. These results indicate the potential of *Phosphorus* 12CH, *Phosphorus* 48CH, *Calcarea carbonica* 12CH, and *Calcarea carbonica* 48CH for controlling *S. sclerotiorum* in common beans.

Key words: Alternative control, high dilutions, homeopathy, *Phaseolus vulgaris* L., repertorization.

Introduction

Common beans (*Phaseolus vulgaris* L.) are one of the most important edible legumes and Brazilian dietary components, representing the main source of income for a considerable number of farmers (Vieira et al., 2006). For cultivation during the whole year in a large variety of ecosystems, several factors become limiting for common bean production (Vieira et al., 2006). Among these factors, diseases are the main responsible for a low productivity, besides decreasing product quality or even devastating certain areas of cultivation (Vieira et al., 2006). Such diseases include white mold, which is difficult to control and has affected common bean production areas all over Brazil, damages of which can reach 100% (Napoleão et al., 2005). Thus, based on its aggressiveness grade, besides a wide range of hosts (Fancelli and Dourado Neto, 2007), strategies to control white mold in common beans must be integrated in order to adapt management strategies at the least possible degree of ideal conditions for its development (Pereira et al., 2013). In this context, alternative control methods may be useful to maintain the pathogen population below the economic damage threshold, besides reducing damages to the environment (Vieira et al., 2006) due to the indiscriminate use of pesticides. Among the practices allowed to producers, homeopathy has been included in several agriculture sectors using extremely diluted aqueous solutions, which has also been allowed by the Food and Agriculture Organization of the United Nations (FAO) as a technique to be used in certified organic products (Bonato, 2007a). Homeopathy has been recognized as a field of knowledge with great potential within the modern view of food quality and biosafety, since homeopathic medicine does not leave residues in the environment, vegetables or animal foods. It also provides resources and improvements in plant metabolism, activating reactions involved in the production of enzymes related to plant defense mechanisms (Lisboa et al., 2005).

Since no Homeopathic Materia Medica has been specifically dedicated to plants, the choice of drugs used in agriculture has considered analogies between the symptoms

described in the Materia Medica and those presented by diseased plants. Thus, the greater levels of evidence between plant symptoms and those described for a remedy, the greater its influence on organism which will be given, and, then, the better the chances for a cure (Wassenhoven, 2007), since homeopathy acts by the similitude principle.

Thus, the knowledge about the action of homeopathic remedies on metabolism and in inducing the resistance of cultivated plants may develop a potential and viable alternative for controling plant diseases, including white mold.

Taking all these into account, the aim of this work was to verify the fungitoxic activity of extremely diluted aqueous solutions of *Phosphorus* and *Calcarea carbonica* against *S. sclerotiorum*, as well as white mold control on common bean plants.

Results and Discussion

Resistance inducing activity

The first trial (Table 1), for area under the disease progress curve (AUDPC), indicated that the *Phosphorus* treatment differed from the control group in all tested dynamizations, with reductions of 40, 58, 41, 23, and 58%, respectively. Despite a reduction of 23% in the progression of white mold was detected by the use of *Phosphorus* 36CH, it did not reduce the percentage of dead plants (PDP) when compared to the control treatment. However, when tested at 6CH, 12CH, 24CH, and 48CH dynamizations, *Phosphorus* reduced PDP by 60, 90, 70, and 80%, respectively.

Similar results were obtained in the second trial for AUDPC (Table 1), in which all dynamizations tested for the *Phosphorus* treatment differed from the control group. Such reductions were 36, 55, 40, 26, and 52% for 6CH, 12CH, 24CH, 36CH, and 48CH dynamizations, respectively. Likewise, *Phosphorus* 36CH was the least effective for AUDPC and the only treatment that, as well as the control group, resulted in 100% plant mortality by white mold. Regarding 6CH, 12CH, 24CH, and 48CH dynamizations, reductions in plant mortality were 60, 90, 60, and 90%, respectively, in comparison to the control group. These results emphasize the importance of testing several dynamizations for a same remedy, since their responses are not linear.

In the test to evaluate the control of white mold by *Calcarea carbonica*, the two variables were significant (Table 2). For AUDPC, only the dynamization 6CH had a similar action than that of the control treatment, so that the disease progression reduced by about 25% for 24CH and 36CH dynamizations, and by 83% for 12CH and 48CH ones.

The extremely diluted solutions of *Calcarea carbonica* 12CH and *Calcarea carbonica* 48CH reduced white mold progression by about 77% when compared to *Calcarea carbonica* 6CH, *Calcarea carbonica* 24CH and *Calcarea carbonica* 36CH. These data suggest a satisfactory white mold control only for *Calcarea carbonica* 12CH and *Calcarea carbonica* 48CH.

Likewise, in the second test, *Calcarea carbonica* 12CH and *Calcarea carbonica* 48CH stood out, with reductions by 81 and 74%, respectively, in disease progression. Similar results were observed for PDP, with reductions by 85% for *Calcarea carbonica* 12CH and 80% for *Calcarea carbonica* 48CH. The other treatments also decreased plant mortality, but not expressively, so that *Calcarea carbonica* 6CH and *Calcarea carbonica* 36CH did not differ from the control group. A difference was observed for *Calcarea carbonica* 24CH, but the reduction was only by 40%.

These results indicate that the extremely diluted solutions chosen for this study are correct, but the dynamizations 6CH, 24CH and 36CH were not suitable for pathological conditions. Thus, it must be emphasized again the importance of using several dynamizations, since responses may vary depending on drug potency (Bonato, 2007b). Furthermore, by acting on the vital energy, which is a dynamic principle, immaterial, distinct from the body and that integrates the entire body, organizing all physiological phenomena (Bonato, 2007a), the same remedy may be applicable to several bodies and under different situations.

Fonseca et al. (2006) evaluated the effect of a unique application of *Calcarea carbonica* in *Porophyllum ruderale* ("arnica-paulista") plants and observed an increase in leaf polyphenol concentrations, proving the resistance inducing activity of homeopathic remedies. *Calcarea carbonica* has also been mentioned by its inhibitory effect on ethylene production in tomato fruits, by delaying the proportion of fruit sauce and increasing the percentage of salad and colorful fruits (Modolon *et al.* 2012). Since ethylene is a hormone whose growth favors the onset of symptoms of white mold (Al-Masri e Barakat, 2003), a reduction in its synthesis on common bean plants treated with *Phosphorus* and *Calcarea carbonica* could be a hypothesis for the efficiency observed in the control of this disease.

Briefly, at the end of the tests, the replicates of the control treatment presented 100% plant mortality, while those treated with *Phosphorus* and *Calcarea carbonica,* both potentiated at 12CH and 48CH, showed resistance to disease progression, emphasizing their role in inducing resistance. Similarly, Leonel and Barros (2013) observed the control of coffee rust up to three months after application of homeopathic remedies in plants affected by such a disease, confirming their residual effect.

In general, in trials carried out in the present study, plants apparently did not respond linearly to the tested dynamizations. The same substance able to increase the values of AUDPC and PDP also showed suppressive effect under different dynamizations, so that a same substance can be innocuous or efficient for a variable according to changes in its dynamization.

In plants, studies have demonstrated that an increase in dynamizations does not necessarily mean an increase in drug potency (Bonato, 2007b). Andrade et al. (2012) obtained sinusoidal patterns after evaluating the effect of increasing dynamizations of *Arnica montana* on the coumarin content of *Justicia pectoralis* ("chambá") plants.

Likewise, Toledo et al. (2015) evaluated the effects of the homeopathic remedies *Propolis*, *Sulphur* and *Ferrum sulphuricum* at 6, 12, 30, and 60CH dynamizations and obtained a sinuous pattern in response to the severity of early blight in tomato, as well as for plant growth variables. This fact, as proven by several researchers and for different

Table 1. Area under the disease progress curve (AUDPC) and percentage of dead plants (PDP) of common bean treated with extremely diluted aqueous solutions of *Phosphorus* at different dynamizations

Phosphorus				
Dynamization (CH)	Test 1		Test 2	
	AUDPC	PDP	AUDPC	PDP
6	08.79 B	40 AB	10.05 CD	40 B
12	06.15 A	10 A	07.08 A	10 A
24	08.59 B	30 AB	09.41 BC	40 B
36	11.34 C	75 BC	11.70 D	100 C
48	06.22 A	20 A	07.63 AB	10 A
Controle	14.65 D	100 C	15.75 E	100 C
CV (%)	09.59	54.55	10.27	25.86

Averages followed by the same letter in the column. do not differ significantly by Tukey test ($p<0.05$).

Table 2. Area under the disease progress curve (AUDPC) and percentage of dead plants (PDP) of common bean treated with extremely diluted aqueous solutions of *Calcarea carbonica* at different dynamizations

Calcarea carbonica				
Dynamization (CH)	Test 1		Test 2	
	AUDPC	PDP	AUDPC	PDP
6	11.06 C	70 B	10.42 B	80 BC
12	02.63 A	15 A	02.93 A	15 A
24	10.95 B	70 B	12.17 B	60 B
36	10.39 B	90 B	11.45 B	80 BC
48	02.34 A	20 A	04.06 A	20 A
Control	14.65 C	100 B	15.75 C	100 C
CV (%)	11.03	38.26	8.89	33.18

Averages followed by the same letter in the column. do not differ significantly in the Tukey test ($p<0.05$).

Table 3. Area under the curve of mycelial growth (AUCMG) and number of sclerotia (NS) of *Sclerotinia sclerotiorum* treated with extremely diluted aqueous solutions of *Phosphorus* at different dynamizations.

Phosphorus		
Dynamization (CH)	AUCMG	NS
6	09.04 A	41.00 B
12	07.68 A	21.25 A
24	12.56 B	40.50 B
36	13.09 B	22.25 A
48	06.98 A	21.00 A
Control	13.55 B	23.50 A
CV(%)	08.83	08.73

Averages followed by the same letter in the column. do not differ significantly by Tukey test ($p<0.05$).

Table 4. Area under the curve of mycelial growth (AUCMG) and number of sclerotia (NS) of *Sclerotinia sclerotiorum* treated with extremely diluted aqueous solutions of *Calcarea carbonica* at different dynamizations.

Calcarea carbonica		
Dinamization (CH)	AUCMG	NS
6	11.76 C	43.50 CD
12	08.27 B	20.50 B
24	11.94 C	41.50 C
36	11.14 C	47.75 D
48	02.42 A	00.00 A
Control	14.05 D	23.50 B
CV(%)	08.09	08.72

Averages followed by the same letter in the column. do not differ significantly by Tukey test ($p<0.05$).

variables (Bonato et al., 2009; Andrade et al., 2012; Rissato et al., 2016), indicates that the same homeopathic remedy may act differently on a same variable, which can be increased or inhibited depending on the dynamization. Thus, in the present study, regression analysis was not performed for treatments with different dynamizations.

On the above basis, it should also be emphasized that healing with homeopathy is based predominantly on Similar Law and vitalism. Thus, from the homeopathic point of view, healing occurs when the vital force that distinguishes living beings (animals and plants) from inanimate objects is reestablished, leading to the conclusion that a similarity exists between plants presenting white mold and the remedies *Phosphorus* and *Calcarea carbonica*.

Fungitoxic activity

With respect to the development variables of *S. sclerotiorum*, the treatment *Phosphorus* led to differences for mycelial growth, expressed as area under curve of the mycelial growth (AUCMG) (Table 3) at 6CH, 12CH and 48CH dynamizations, when compared to the control group, so that 33, 43 and 48% delays were observed, respectively. The dynamizations 24CH and 36CH had no antimicrobial potential. Regarding the number of sclerotia (NS), *Phosphorus* 6CH and *Phosphorus* 24CH stimulated sclerotial formation by 74 and 72%, respectively, when compared to the control group. For all other dynamizations, NS did not differ from the treatment with hydroalcoholic solution (30%), indicating a probable effect of alcohol on sclerotial formation. The treatment *Calcarea carbonica* (Table 4), at 12CH and 48CH dynamizations, reduced the mycelial growth of *S. sclerotiorum* by 41 and 83%, respectively. The other treatments differed from the control group, but with a low antimicrobial potential, not extending beyond 21% mycelial growth inhibition, which indicates that the antifungal activity of *Calcarea carbonica* is directly related to its energy property. The results obtained for NS (Table 4) demonstrate the importance of using an appropriate dynamization. *Calcarea carbonica* at 6CH, 24CH and 36CH led to differences, with increases in NS by 85, 77 and 103%, respectively, when compared to the control group. Conversely, *Calcarea carbonica* 48CH inhibited sclerotial formation. Thus, *Calcarea carbonica* 48CH emerges as an alternative for integrated control of *S. sclerotiorum* since it slows down the exponential growth of the pathogen over the years, so that NS can be reduced by up to 100%. Although some tested treatments did not result in differences, their potential should not be disregarded. Thus, further studies involving dosage, dynamization, method, and application frequency of extremely diluted solutions are needed.

Materials and Methods

Choice and preparation of treatments

Extremely diluted solutions were selected by repertorization through the software HomeoPro and chosen with the assistance of the Homeopathic Materia Medica (Boerick, 2003). For this purpose, the symptoms of plants infected by *S. sclerotiorum* and environmental conditions favoring its occurrence were considered, i.e. deep epidermal lesions, especially in the lower members, and plant injury and chlorosis favored by high humidity. The Homeopathic Materia Medica (Boericke, 2003) describes the individual to *Calcarea carbonica* as light sensitive, as well as the pathogen *S. sclerotiorum*, which has skin warts and rashes difficult to heal, similarly to the symptoms presented by plants affected by the fungus; and that has difficulty to swallow, as well as the plant to absorb water and nutrients. *Phosphorus* has already been recommended for individuals presenting degeneration of blood vessels, skin lesions and destructive metabolic conditions (Boericke, 2003). Thus, *Phosphorus* and *Calcarea carbonica* were chosen.

Based on previous studies, which reported positive responses regarding induced resistance in plants against pathogens by using extremely diluted solutions (Toledo et al., 2009; Modolon et al., 2012; Rissato et al., 2016), the dynamizations 6CH, 12CH, 24CH, 36CH, and 48CH (centesimal Hahnemannian) were chosen for this work. The hydroalcoholic solution (ethanol 30%) was considered as the control group since it was the diluent used to prepare such remedies.

The remedies were obtained from a homeopathic pharmacy at 6CH dynamization and manipulated to 12, 24, 36, and 48CH in accordance with the Brazilian Homeopathic Pharmacopoeia (1997) by dilution at 1:100 and succussing 100 times. After, the pluralist dilution proposed by Hahnemann was followed, so that a flask was used for each dilution and suction was applied in unidirectional, sequential and vertical movements through a mechanical stirrer. Each remedy was diluted in distilled water at 0.1% ratio at the time of its use, according to Bonato et al. (2009).

Resistance inducing activity evaluation

The experiment was carried out in an acclimatized greenhouse in a completely randomized design, with four tests involving six treatments and five replicates each. The first two tests were carried out simultaneously in June and July 2015, each one of them using *Phosphorus* and *Calcarea carbonica* in their respective dynamizations. The following two tests were carried out similarly to the previous ones, from November to December 2015, in order to impart the results obtained in the first cycle. Common bean seeds (cultivar 'IAC Alvorada') were sanitized in alcohol 70% for 1 min and in sodium hypochlorite solution (3:1) for 2 min, followed by rinsing with distilled water. Seeds were placed into acrylic boxes (Gerbox®) filled with three paper sheets (Germitest®) moistened with distilled water. After, seeds were maintained in a BOD (Bio-Oxygen Demand) incubator at 25°C in a 12h-light/ 12h-dark photoperiod, and aside for the final seeding after three days. Seeds presenting no pathogens and with full and proportional radicles were selected for greenhouse experiments. Sowing was performed in 3l pots filled with a mixture of soil and sand (2:1) previously autoclaved at 120°C for 60 min. Each pot contained six seedlings. At the V1 stage, a thinning was done in order to maintain three seedlings per pot. When plants presented their first fully expanded trifoliate leaf (V2 stage), two segments of common beans (2 mm diameter) replete with *S. sclerotiorum* mycelia were inoculated at the base of each plant. The treatments (0.1%) were administered in the soil three days before inoculation, on the day of inoculation, and at 3, 10 and 17 days after inoculation. Lesions caused by

S. sclerotiorum were measured daily through a caliper as soon as the first symptoms appeared at the base of plants, so that measurements ceased when control plants were dead. For each treatment, the values of the area under the disease progress curve (AUDPC) were obtained through the equation of Shaner and Finney (1977):

$$AUDPC = \left[\left[\frac{(Y_1 + Y_{1+1})}{2} * I\right]\right] + \left[\left[\frac{(Y_2 + Y_{2+1})}{2} * I\right]\right] \ldots \left[\left[\frac{(Y_n + Y_{n+1})}{2} * I\right]\right]$$

Where:
AUDPC = area under the disease progress curve (adimensional);
Y_i and Y_{i+1} = size of lesion of the disease observed in two consecutive evaluations (cm);
I = interval between two consecutive evaluations (days).
The evaluation of plant mortality from white mold disease took place after the tests were completed when the number of dead plants per treatment was counted. The percentage of dead plants (PDP) was obtained through the equation:

$$PDP = \frac{NDP}{\mathrm{TNP}} * 100$$

Where:
PDP = Percentage of dead plants for the treatment in question;
NDP = Number of dead plants in the treatment;
TNP = Total number of plants in the treatment.

Fungitoxic activity evaluation

The experiment was carried out in a completely randomized design, totaling two tests involving six treatments and five replicates each. The first test was carried out with the *Phosphorus* treatment, and the second one with *Calcarea carbonica*, both at 6CH, 12CH, 24CH, 36CH, and 48CH dynamizations. For evaluation of the *in vitro* activity, the treatments (0.1%) under appropriate dynamizations were added to Erlenmeyer flasks containing autoclaved potato dextrose agar (PDA) culture medium. The whole process was occured within a laminar flow chamber. After a complete culture medium solidification, each Petri dish received a disc (7 mm diameter) containing *S. sclerotiorum* mycelia in its center. Then, Petri dishes were sealed with plastic wrap and maintened in BOD incubator at 25 ^{o}C in the absence of light.
For evaluation of mycelial growth, daily measurements were performed by the method of diametrically opposed measures, beginning at 24h after experiment installation and lasting until when fungal colonies eached the edges of Petri dishes. After the end of the experiment, the area under curve of the mycelial growth (AUCMG) was calculated according to the equation of Shaner and Finney (1977). The number of sclerotia (NE) was measured 30 days after the test assembly.

Statistical analysis

To analyze the data, an analysis of variance (ANOVA) was performed and, when relevant, the Tuley test at 5% probability of error was conducted using the statistical program GENES (Cruz, 2006).

Conclusion

The extremely diluted solutions Phosphorus 12CH, *Phosphorus* 48CH, *Calcarea carbonica* 12CH and *Calcarea carbonica* 48CH showed potential to control the white mold bean, as well as antimicrobial activity against *Sclerotinia sclerotiorum*.

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