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USE OF *TRICHODERMA SPP*. AND HIGH-DYNAMIZED DILUTIONS IN THE CONTROL OF *BOTRYTIS CINEREA* AND STRAWBERRY GROWTH

Uso de *trichoderma spp.* e altas diluições dinamizadas no controle de *botrytis cinerea* e crescimento de morangueiros

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ABSTRACT

Agroecological interventions for disease management and plant vigour focus on strategies to enhance crop homeostasis. Biological agents and homeopathic preparations are innovative and efficient agriculture technologies used in the production of pesticide-free food. The objective of this work was to evaluate the effects of the biological control *Trichoderma spp.* and high-dynamized dilutions of *Silicea terra* to manage grey mould (*Botrytis cinerea*) and to promote plant growth in strawberry plants cv. San Andreas. The experiment was carried out in a greenhouse at EPAGRI experimental station, Lages-SC, 2017. The experimental design consisted of six treatments and five repetitions which were completely randomised. Each plot consisted of a strawberry plant cultivated in a 3.6 L plastic pot. Leaf area, number of leaves, root and leaf weights were assessed. The incidence and severity of grey mould on fruits was also evaluated. The statistical analysis was done by environment R®. Plants treated with *Trichoderma spp.* (WP formulation) resulted in a lower fruit incidence of B. cinerea fruits in all evaluated periods. Plants treated with *Silicea*12CH showed wider leaf area, produced more leaves during the cycle and had greater root weight.

KEYWORDS: Agroecology, homeopathy, biological control, grey mould, *Fragaria x ananassa*, pesticide-free food

RESUMO

Intervenções agroecológicas para manejo de doenças e vigor de plantas focam em estratégias para melhorar a homeostase das culturas. Agentes biológicos e preparados homeopáticas são tecnologias agrícolas inovadoras e eficientes utilizadas na produção de alimentos livres de agrotóxicos. O objetivo deste trabalho foi avaliar os efeitos do controle biológico *Trichoderma spp*. e altas diluições dinamizadas de *Silicea terra* para manejar o mofo cinzento (*Botrytis cinerea*) e promover o crescimento de plantas de morangueiro cv. San Andreas. O experimento foi realizado em casa de vegetação na estação experimental da EPAGRI, Lages-SC, 2017. O delineamento experimental constou de seis tratamentos e cinco repetições, inteiramente casualizados. Cada parcela foi composta por uma planta de morangueiro cultivada em vaso plástico de 3,6 L. A área foliar, o número de folhas, o peso das raízes e das folhas foram avaliados. A incidência e severidade do mofo cinzento nos frutos também foram avaliadas. A análise estatística foi realizada em ambiente R®. Plantas tratadas com Trichoderma spp. (formulação WP) resultou em menor incidência de frutos de B. *cinerea* em todos os períodos avaliados. Plantas tratadas com *Silicea Terra* 12CH apresentaram maior área foliar, produziram mais folhas durante o ciclo e tiveram maior peso de raiz.

Palavras Chaves: Agroecologia, homeopatia, controle biológico, mofo cinzento, *Fragaria x ananassa*, alimento livre de agrotóxicos

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INTRODUCTION

Agroecological management is used to support pesticide-free food production in around 43.1 million hectares worldwide, generating about $50 billion per year over the last decade (HATHAWAY et al, 2016). Within this scenario, there is a high potential for organic strawberries (*Fragaria x ananassa*) farmers. Strawberry is the main crop of the so-called red fruits, appealing to the consumer for health, appearance and taste characteristics. In Brazil, 4,500 ha of strawberries yield 105,000 tons annually (approx. 30 t/ha), yet there is a market push to increase yields (FAGHERRAZZI et al.,2017).

The management of the grey mould (*Botrytis cinerea*) is one of the main problems that farmers face in strawberry culture. It is a widespread fungal disease present in more than 200 crops (BARAKAT and AL-MASRI, 2017). It colonizes flowers and fruits, having as its main source of inoculum, dead leaves, and mummified fruits, causing losses either in yield and/or in fruit quality, through reduced shelf life, lower pulp firmness and colour as well as taste alterations (ANTUNES, 2013). The use of pesticides to control plant disease has placed the fruit in the list of highest pesticide-residue foods, and a Brazilian study found that around 72% of the fruit samples contained fungicide residues beyond the legal limit (IBGE 2016).

Research into less harmful agricultural methods is thus required, in order to address society's concerns for safe and fair food production (ABRAHAM et al., 2014). In this respect, studies with high dynamized dilutions applied to crops and livestock has shown promising results (DÖRING et al., 2015; GIESEL et al., 2017). Silica rock-dust is a mineral that can be used to make a homeopathic remedy and the biodynamic formulation P501, which are used in agriculture to promote plant vigour and improve photosynthesis activity, helping plants to build up natural tolerance to diseases and environment stresses (EL-SHETEHY et al., 2021).

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The method of biological control is widely used in agroecology. Biological controls manage the system by increasing the population of naturally occurring biological agents such as types of insects or fungi (BETTIOL, 2011). In conventional farming, strawberry crops are isolated, and this inhibits natural processes of biological control (ANTUNES, 2013). For those conditions, artificially introducing a biological agent is a feasible option. The fungus *Trichoderma spp.* has shown to control a wide range of phytopathogens whilst also stimulating plant growth through its hormonal singling (HERMOSA et al., 2013). According to Barakat and Al-Masri (2017), commercial formulations of *Trichoderma spp.* include: wettable powder (WP); dispersible granules (DG); concentrated suspension (CS); emulsified oil (EO); colonised grains (CG) and dry spores (DS). In fact, in Brazil annually, 550 tons of colonised grains are used exclusively to produce bioagent formulations (FERNANDEZ, 2013).

The objective of this research was to evaluate the effectiveness of spraying leaves with *Trichoderma spp.* from three commercial formulations, concentrated suspension/SL (TSL), emulsified oil/EO (TEO), wettable powder /WP (TWP) and with high-dynamized dilutions of *Silicea terra* in two dynamized dilutions, 12CH and 18CH (CH= hahnemannian centesimal dilution order) to manage *B. cinerea* and to promote strawberry plant growth.

MATERIAL AND METHODS

Experimental design

Greenhouse experiments were carried out at the Experimental Research Station of Santa Catarina Agriculture Research and Rural Extension Service (EPAGRI), Lages/SC – Brazil, in 2017. The experimental plot consisted of one strawberry plant placed in a 3.6 L PVC pot. The treatments were: T1 - concentrated suspension/SL (TSL); T2 - emulsified oil/EO (TEO); T3 - wettable powder /WP (TWP); T4 - *Silicea terra* 12CH; T5 - *Silicea terra* 18CH; T6 - Control (distilled water). The experiment design was completely randomized

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with five repetitions. The study was conducted in duplicate, being named Experiment 1 and Experiment 2. The trial was conducted from June/2017 to December/2017.

The commercial strawberry variety *San Andreas* (remontant) was chosen for its representativeness amongst farmers in the region south region of Brazil. The seedlings were obtained from a certified nursery in the city of Farroupilha-RS. The bare rooted seedlings were washed and separated by similar thinning the shoots and ensuring uniformity of the roots through a cross section, pruning the roots, leaving them 8 cm in length.

The substrate used was, ¼ sheep manure, ¼ local soil – (Humic Cambisoil; EMBRAPA 2006), ¼ commercial expanded vermiculite (medium grain size) and ¼ commercial TECNOMAX® plant compost. All plants received the same management with daily irrigation and ferti-irrigation with SuperMagro® biofertilizer administered at a dose of 50 ml/plant every twenty days. The chemical dry weight content of the biofertilizer is of N (1.43); P (0.26); K (1.01); Ca (0.49); Mg (0.26); S (1.07) g kg^{-1}, and, B (439), Cu (332), Fe (155), Mn (961), Zn (1699) and Na (328) mg kg^{-1}. Its pH was 5.6 and its electrical conductivity was 13.18 dS $m^{-1.}$

Treatment preparation

The *Trichoderma spp.* suspensions were applied weekly using 50 ml/plant of a solution containing $1x10^9$ viable spores per ml. of the commercial formulations: T1 – concentrated suspension/SL (TSL); T2 – emulsified oil/EO (TEO); T3 – wettable powder /WP (TWP).

The homeopathic preparation *Silicea terra* was made by the Laboratory of Homeopathy and Plant Health/EPAGRI according to the recommendations of the Brazilian Homeopathic Pharmacopoeia (Brasil, 2011) at potencies 12CH and 18CH. These potencies were select because they are commonly related to this preparation according to the literature (KAVIRAJ, 2018). 10 ml of homeopathic preparation was added to 490 ml of distilled water and 50 ml of this solution was applied per plant-plot. The graduated hand sprayer containing

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this solution was shaken 10 times before application to activate the solution. The spray was directed at all organs of the aerial parts of the strawberry plant, and this homeopathic preparation was applied weekly, at the same moment of the other treatments, for 7 months (June to December 2017).

To guarantee the infection of *B. cinerea*, a solution with $1x10^6$ viable spores/ml was sprayed on the leaves, flowers, and fruits (50ml / plant). The spore solution was renewed weekly, and it was applied twenty-four hours after *Trichoderma spp.* and *Silicea terra* treatments.

Disease incidence and severity assessments

Incidence and severity of *B. cinerea* on the fruits was evaluated in two moments: a) at the harvest -proportion of fruits with symptoms of *B. cinerea*, b) at the post-harvest - proportion of fruits with *B. cinerea* that showed no symptoms at harvest but five days after harvest. Incidence was assessed as presence (1) and absence (0). Adding the results of these two evaluations we obtained the total incidence - proportion of fruits with *B. cinerea* that had symptoms at harvest and those that were confirmed in post-harvest. Severity on the fruits was assessed in the harvest and post-harvest, using a scale which scores correspond to the affected area by the fungus: 1) 0-20%; 2) 20-40%; 3) 40-60%; 4) 60-80%; and 5) 80-100%. The scores were given with the aid of diagrammatic scales adapted from Orjeda (1998).

Plant growth promotion: leaf area, number of leaves, shoot and root dry weight

Leaf area was measured at the end of the cycle using the IMAGE J® image analytical software at the laboratory of Plant Physiology and Post-Harvest at the Agroveterinary Science Centre (CAV), Santa Catarina State University (UDESC). The number of leaflets were obtained by counting the new completely expanded leaves each fourteen days during the whole season. The dry mass of shoot and root were obtained by weighing the plants using a scale, after they had been placed in an air-forced circulation chamber at 60 ºC until they maintained a constant weight.

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Statistical analysis

To verify the effect of the treatments on the incidence of *B. cinerea*, the binomial model was used, considered as variable response the proportion between the number of fruits with symptoms at the harvest moment and the number of infected fruits after five days (postharvest). For total incidence we considered the proportion of infected fruits at harvest plus postharvest and the total of produced fruits. In cases where high data dispersion was observed in relation to the binomial model, an extra dispersion parameter was used through the quasi-likelihood estimation method – quasibinomial model (Li, 2021).

For severity at harvest and post-harvest, leaf area, number of leaflets, shoot dry weight, and root dry weight were analysed using the classical variance analysis model, and the assumptions of normality and homogeneity of variance were verified by Bartlet and Shapiro Wilk tests, respectively. In cases where at least one of them was not met, the transformation proposed by Box-Cox (VENABLES and RIPLEY, 2002) was used (for total incidence).

For the plant growth, the means of the treatments were compared by the Tukey test (5%). All the statistical analyses were done in free environment R®

RESULTSS AND DISCUSSION

Incidence and severity of disease

The incidence of *B. cinerea* in fruits is shown in Table 1. The biological control *Trichoderma spp.* reduced grey mold incidence on strawberry fruits on harvest moment (25.89%), compared to the control (60.16%), particularly with the treatment wettable powder (TWP). This result suggests that the wettable powder formulation (TWP), which had greater ease of solution homogenization, can ensure better distribution of *Trichoderma spp.* spores

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over the fruits and the plant in leaf spraying. It is known that degradation conditions commonly succeed in tissue colonization at harvest period due to pulp injuries or the permanence of inoculum on the petals that are still attached to the crown tissue (FERNANDEZ et al., 2013). Thus, if the *Trichoderma spp.* spore-solution (WP) was easier to homogenize and consequently better distributed over the tissues, the biofilm layer formed over the fruit would play a significant protection role, which according to Hermoza et al. (2013) would likely ensure lower rates of colonization by *B. cinerea*.

Table 1. Incidence of gray mold in strawberry fruits treated with *Trichoderma spp.* in three commercial formulations: Concentrated suspension (TSL), emulsified oil (TEO), wettable powder (TWP) and the homeopathic preparation *Silicea terra* (Sil) in potencies 12CH and 18CH. Lages, SC, 2017.

Treatment	Harvest incidence (%)	Post-harvest incidence (%)	Total incidence (%)
		Experiment 1	
Control	31.46 ± 8.94 a	69.33 ± 7.10 a	58.16 ± 7.78 a
TEO	24.58 ± 7.15 a	58.16 ± 7.78 a	43.79 ± 4.54 a
TWP	11.40 ± 3.93 a	39.60 ± 6.33 b	31.89 ± 6.25 b
TSL	24.38 ± 7.94 a	60.03 ± 3.38 a	46.48 ± 6.17 a
Sil 12CH	20.38 ± 8.52 a	71.66 ± 6.60 a	58.29 ± 9.87 a
Sil 18CH	22.89 ± 6.74 a	60.35 ± 4.59 a	45.57 ± 7.31 a
		Experiment 2	
Control	60.16 ± 7.18 a	60.33 ± 8.15 a	68.15 ± 8.78 a
TEO	42.25 ± 4.61 a	56.16 ± 8.12 a	53.47 ± 5.72 a
TWP	25.89 ± 6.20 b	33.10 ± 8.33 b	27.69 ± 5.26 b
TSL	48.48 ± 6.57 a	61.09 ± 9.18 a	43.48 ± 8.17 a
Sil 12Ch	41.17 ± 9.80 a	60.66 ± 9.60 a	51.99 ± 6.81 a
Sil 18Ch	55.28 ± 7.36 a	60.51 ± 5.59 a	41.87 ± 6.31 a

Means (± standard error) followed by the same letter in the columns do not differ by Tukey's test (p <0.05).

The incidence of fruits colonized by *B. cinerea* increased in the postharvest period. This was expected because fruit tissues are in a state of natural senescence in this period. So, this state is favourable to the colonization by *B. cinerea* (CALVO and GARRIDO et al., 2013). TWP resulted in the lowest proportion of fruits colonized by *B. cinerea* in the postharvest period, both in Experiment 1 (39.60%) and Experiment 2 (33.10%). The better distribution of the spore solution as observed when using WP could also facilitate spore

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germination and action of *Trichoderma spp.* over *B. cinerea*. It has been seen that the closer the contact surface between *Trichoderma spp.* spores and the antagonist fungi are, the faster the recognition systems and their sets of pattern-recognition-receptor (PPRs) are activated, either on the aerial part or in the roots (HERMOSA et al., 2013). In this way, *Trichoderma spp.* will attack *B. cinerea* rapidly, eliminating its competitor on that ecological spot.

Of the total incidence, the proportion of fruits infected in both moments, TWP again obtained the lowest percentages of fruits colonized in both Experiments 1 (31.89%) and 2 (27.69%) in relation to all treatments. The best performance of TWP is directly related to the spore conditioning medium (WP) that presents greater germination ease due to the manufacturing and storage method. The pure adjuvant fungal colonies favour synchronization between micro parasitism and antibiosis (BARAKAT and AL-MASRI, 2017). By distributing viable spores of *Trichoderma spp.* throughout the plant, secretion of recognition elicitors is facilitated by systematically inducing immune response in plant structures (FERNANDEZ, 2013; HERMOSA et al., 2013; BARAKAT and AL-MASRI, 2017).

The severity of the disease in fruits is shown in Table 2. The *Trichoderma spp.* reduced grey mold severity on strawberry fruits in both periods when compared to the control. At harvest moment, a lowest incidence of disease is directly related to a lower severity at the same moment (FERNANDEZ, 2013).

Consequently, plants treated with TWP had fruits with lower severity indexes in both Experiments, 1 (0.20) and 2 (0.52) for the harvest moment. TWP reduced disease severity on experiment 1 (0.44) and experiment 2 (0.45) at the post-harvest period when compared to the control. Barakat and Al-masri (2017) using the *Trichoderma spp.* biofungicide formula, significantly reduced the development of grey mold lesions on strawberry detached leaves by 19% - 45% and reduced gray mold disease severity on whole plants by 13% - 15% compared to the control.

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Freeman et al. (2001), using 74 isolates of *Trichoderma spp*., *T. harzianum*, *T. atroviride* and *T. longibrachiatum*, showed potential to control grey mold in strawberry. The lowest index severity of disease was found when *T. harzianum* strain T-39 applied at a 2-day interval at 0.4% concentration.

Table 2. Severity Index of grey mold in strawberry fruits treated with *Trichoderma spp.* in three commercial formulations Concentrated suspension (TSL), emulsified oil (TEO), wettable powder (TWP) and the homeopathic preparation *Silicea terra* (Sil) in potencies 12CH and 18CH. Lages, SC, 2017.

Treatments	Experiment 1		Experiment 2	
	Harvest severity	Post-harvest severity	Harvest severity	Post-harvest severity
Control	0.67 ± 0.23 a	1.37 ± 0.14 a	0.71 ± 0.27 b	1.42 ± 0.41 a
TEO	0.41 ± 0.10 a	0.52 ± 0.06 c	0.55 ± 0.16 c	0.87 ± 0.16 a
TWP	0.20 ± 0.07 b	0.44 ± 0.11 c	0.52 ± 0.12 c	0.45 ± 0.09 b
TSL	0.41 ± 0.10 a	0.92 ± 0.13 b	0.69 ± 0.09 b	0.84 ± 3.38 a
Sil 12CH	0.45 ± 0.20 a	1.20 ± 0.21 ab	1.22 ± 0.21 a	1.35 ± 0.27 a
Sil 18CH	0.44 ± 0.12 a	0.70 ± 0.15 bc	0.58 ± 0.27 c	0.99 ± 0.21 a

Means (± standard error) followed by the same letter in the columns do not differ by Tukey's test ($p < 0.05$).

The postharvest time is a critical period for the colonization of *B. cinerea*. The TWP bioagent again obtained the lowest severity index observed with 0.44 in Experiment 1 and 0.45 in Experiment 2 at the post-harvest period. The lowest inoculum potential obtained with TWP has shown that pulverized bioagents can be well suited to the botanical and morphophysiological characteristics of the fungus (TULIPANI et al., 2011). The solution sprayed on the shoot is important within management. This is because the leaves and fruits that are infected at the young stage by *B. cinerea* remain quiescent until they mature and only then does the pathogen resume abundant sporulation growth, a moment that is colonized by *Trichoderma spp.* (BARAKAT and AL-MASRI, 2017). The observed incidence and severity showed a positive effect regarding WP formulation in fruit protection.

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Low potencies of *Silicea terra* used in this study did not interfere directly in the incidence or severity levels, even though *Silicea* 18CH presented positive results regarding the severity. This result aligns with the reported results obtained by other authors working with this homeopathic preparation in other crops (BONFIM et al., 2011; BETTI et al., 2016). The low sensitivity of the plant to the homeopathic preparation is also attributed to the choice of the potentization level and frequency of application (HANIF and DAWAR, 2015). These are the main difficulties in applying high dynamized dilutions to plants, animals and to the environment (BONFIM et al., 2011; BETTI et al., 2016; GIESEL et al., 2017).

Plant growth promotion

The attributes of shoot and root development are presented in Table 3. Plants submitted to high-dynamized dilutions of *Silicea terra* at potency 12CH developed a larger leaf area in both experiments, 139 cm^2/plant in Experiment 1, and 120 cm^2/plant in Experiment 2. Consequently, a higher number of leaflets was observed in plants treated with *Silicea terra* 12CH, 43 leaflets/plant in Experiment 1, and 46 leaflets/plant in Experiment 2. The homeopathic preparation of *Silicea terra* was chosen because of the similarity between the element silica and its indication for induction of stress resistance in the plant, its corresponding description in the homeopathic *materia medica* presenting characteristics of stiffness, hardness and toughness (RESENDE, 2009).

Rationally and timely applied homeopathic preparations, following the law of like-cures-like, promote biomass increases, most likely owing to the response to a physiological adjustment of the plant in relation to the characteristic of mineral Silica, where the expression occurs in the formation of a double layer of silica cuticle and silica cellulose (BETTI et al., 2016).

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Table 3. Strawberry biometrics attributes treated with bio-agent *Trichoderma spp.* in three commercial formulations Concentrated suspension (TSL), emulsified oil (TEO), wettable powder (TWP) and the homeopathic preparation *Silicea terra* (Sil) in two potencies 12CH and 18CH. Lages, SC, 2017.

Treatments	Leaf area (cm^2)	leaflets / plot (nº)	Dry weight aerial part (g)	Dry root weight (g)
		Experiment 1		
Control	94 ± 0,02 b	28.60 ± 0.30 bc	2.08 ± 0.25 a	2.44 ± 1.21 ab
TEO	80 ± 0,02 b	24.80 ± 0.71 c	1.86 ± 0.10 a	1.96 ± 0.38 b
TWP	107 ± 0,04 b	37.40 ± 1.51 b	1.94 ± 0.15 a	2.72 ± 1.15 ab
TSL	89 ± 0,03 b	32.21 ± 0.86 b	1.99 ± 0.24 a	2.57 ± 1.32 ab
Sil 12CH	139 ± 0,05 a	43.40 ± 1.41 a	2.03 ± 0.26 a	3.75 ± 1.99 a
Sil 18CH	81 ± 0,03 b	30.00 ± 1.22 b	1.84 ± 0.30 a	1.90 ± 0.46 b
		Experiment 2		
Control	74 ± 0,03 c	38.80 ± 1.13 b	1.25 ± 0,24 a	0.75 ± 0.16 b
TEO	99 ± 0.03 b	29.20 ± 0.75 c	1.23 ± 0,33 a	0.76 ± 0.32 ab
TWP	113 ± 0.04 ab	36.40 ± 2.26 b	1.44 ± 0,35 a	0.78 ± 1.28 ab
TSL	130 ± 0.05 a	37.60 ± 1.06 b	1.53 ± 0,27 a	0.80 ± 0.21 a
Sil 12CH	120 ± 0.06 ab	46.20 ± 2.82 a	1.57 ± 0,72 a	0.78 ± 1.59 ab
Sil 18CH	95 ± 0.04 b	32.80 ±1.52 bc	1.34 ± 0,35 a	0.75 ± 0.30 ab

Means (± standard error) followed by the same letter in the columns do not differ by Tukey's test ($p < 0.05$).

Such a protective layer has a positive relationship with the reduction of transpiration by the plant (BARBOSA FILHO et al., 2001). The decreasing evapotranspiration of water throughout the cycle, makes the plant less water demanding, with greater drought resistance and greater water use efficiency (BETTI et al., 2013).

This fact highlights the potential of high-dynamized dilutions for increasing the resilience of food production systems, providing healthier plants constituting an efficient management technique (BETTI et al., 2013). These results converge with previous studies which obtained positive increases in leaf area and dry mass of plants treated with high-dynamized dilutions (JÄGER, 2011.; CARNEIRO et al., 2019; ANDRADE et al., 2012).

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Regarding shoot and root dry weight, there was no difference between treatments in both experiments.

Based on our study, it was possible to observe that in plants, the potency response is not linear; that is, the increase in potency did not necessarily affect progressive and / or increasing physiological responses. This behaviour has been reported by other authors (BETTI et al., 2016). This opposes with the data already reported by other authors, who described shorter primary root length and lower seedling height at potencies 6CH, 12CH and 24CH (SILVA et al, 2012).

CONCLUSION

Strawberry plants treated with *Trichoderma spp.* (TWP) foliar spray showed lower incidence of *Botrytis cinerea* on fruits at harvest, postharvest and total incidence. Consequently, the lowest severity indices were also obtained with TWP. It is clear that the application of the antagonist via foliar spray in the TWP (wettable powder) formulation is efficient in the protection of fruits against *B.cinerea*. Regarding plant growth promotion, strawberry plants treated with high-dynamized dilutions of *Silicea terra* 12CH obtained an increase in the leaf area and number of leaflets, evidencing the induction of vigour promoted by the high-dynamized dilutions on plants.

Conflict of Interest Statement

We have no conflict of interest to declare.

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