

Antagonism of *Bacillus subtilis* Against *Sclerotinia sclerotiorum* on *Lactuca sativa*

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Abstract

The objectives of this study were select and test the efficiency of the *Bacillus subtilis* strain and its metabolites against *Sclerotinia sclerotiorum* in all stages of its cycle. The bacterium was selected by performing antibiosis test against this and other pathogens, being selected that one who obtained the best result. For the assessment of radial mycelial growth under the influence of metabolite, this was added to PDA culture medium and the fungus was placed on its surface, being evaluated daily in the both directions. The test with volatiles was performed by using the two surfaces of the Petri dish, each containing culture medium, putting the fungus and the bacteria separately one on each surface without any contact between them, being measured every day the mycelial growth dimensions. For the evaluating the direct effect on the sclerotia was applied 1 mL of bacterial suspension (1.002 absorbance value at wavelength of 550 nm) to observe the possible presence of harmful substances to this structure. The test for the possible protection provided by the strain on lettuce (*Lactuca sativa*) was performed with four varieties grown up to 60 days after sowing, when was sprayed the bacterial suspension following the best incubation time tested. The antibiosis test showed a decreased by 16.33% compared to control. The metabolite, when mixed into the culture medium showed better inhibition results of the radial mycelial growth when concentration and incubation time were greater. The volatile test showed a reduction of 83.33% on mycelial growth. For the test with sclerotia, the mycelial and carpogenic germination were higher than the control. *In vivo* treatments with lettuces were observed differentiated levels of control, and some plants showed extremely sensitive to bacterial cells concentration used. This study show the *B. subtilis* as a biocontrol agent applying different strategies to control the pathogen and also the hipensensitive plant reaction in some cases.

Keywords: metabolites, lettuce, sclerotia, apothecia and phytopathology

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a phytopathogenic fungus and under appropriate conditions can cause a disease called white mold. Its importance as a pathogen is praised for being a necrotrophic fungus and produce in the course of its life cycle, a weather resistant structure, known as sclerotia. The efficient control of this pathogen is still a challenge, and treatment is carried out mainly using biological control agents, fungi and bacteria, as well as targeted fungicides application when it inhabits lower plant parts.

The fungus is the etiological agent of white mold disease in several crops commercially exploited, it is important for the damage caused and destructive potential, when no control measures are employed. Losses of up to 15% due to *Sclerotinia* species commonly happen and losses of up to 60% have been reported in specific conditions (Subbarao, 1998). The control and eradication of this pathogen is difficult due to the existence of a resistance structure called sclerotia that can survive in soil for years without hosts or favorable condition for development (Coley-Smith & Cooke, 1971). Authors such as Huang et al. (2000), and Mueller et al. (1999), pointed out that even using different methods of control were remaining sufficient sclerotia to cause the disease. In attempting to control the pathogen, metabolites from *Bacillus* spp. has been used in different ways against the fungus and distinct results were obtained depending of the hosts (Fernando et al., 2007; Yang et al., 2009).

The metabolites synthesized by bacteria, fungi and actinomycetes are able to prevent the growth of other microorganisms. According Kennedy (1999), the substances that compose metabolites are present in

micro-organisms differ in specific structure and distribution. Some bacteria when placed in culture medium with conducive conditions to development, produces phytotoxic metabolites such as lactic and acetic acids, cyanide and antibiotics (Bakker & Schippers, 1987; Kloepper et al., 1989). The metabolite produced depends of the environment components in which they are, as well as the environmental conditions in which they are inserted (Konings et al., 1992).

Therefore we can say that all secondary metabolites produced depend primarily of the medium in which they are inserted, and subsequently require the presence of specific enzyme complex (Kliebenstein, 2004). Secondary metabolites are products derived from primary metabolites and although not necessarily essential to the organism, perhaps can generate some advantages to ensure their survival (Santos, 1999). According Kutchan (2001), secondary metabolites have its synthesis influenced by environmental conditions. This influence of environmental condition are also affirmed by McCall and Karban (2006), who point out that the metabolites synthesized and accumulated in plants is directly related to environmental factors. All these specifics may change the result obtained in disease control. According Hammami et al. (2009) the effect *in situ* by exposure live *B. subtilis* cells may incite the growth promoting and/or biocontrol. In nature this biocontrol can be promoted directly or indirectly by producing compounds antagonistic or induce resistance in plants after application (Ryu et al., 2004; Ongena et al., 2007).

Bacillus subtilis is an example of antagonistic bacteria that usually act through antibiosis and eventually by parasitism and competition for space and nutrients (Nagórska et al., 2007). Microorganisms acting on antibiosis, generally have a wide action spectrum, and for this reason the fungal inhibition by producing toxic substances is more efficient than any other mechanism of action involved (Kupper et al., 2003; Leelasuphakul et al., 2008). Santos et al. (2006) reported that *Bacillus* sp. is the most numerous rhizobacteria in the soil. This high presence on the ground reveals the great competitive potential, when it is located on the environment with several other microorganisms. According Kupper et al. (2003), some *Bacillus* sp. lineages produce thermostable metabolites, capable of maintaining their activities even after autoclaving, which facilitates large scale production. Marroni and Germani (2011) working with non-volatile metabolites of *Bacillus* sp. inhibited the development of *Macrophomina phaseolina*, a fungus that produce sclerotia, as resistant structure. In the specific case of *S. sclerotiorum*, Hou et al. (2006) reported that among 1,140 isolates of *Bacillus* sp., 8.2% showed antifungal activity to this pathogen.

Leelasuphakul et al. (2008) found strains of *Bacillus* sp. inhibiting the mycelial growth of *Penicillium digitatum*. In the same way Santos et al. (2006) related strains of *Bacillus* sp. *B. subtilis*, *Bacillus megaterium* pv. *cerealis* and *Bacillus pumilus* controlling the bacterial fruit blotch, known as *Acidovorax avenae* subsp. *citrulli*, on melon seedlings due to lipopeptides compounds present of the metabolites produced. Based on these studies the use of *Bacillus* sp. as biological control agents has numerous applications.

Kai et al. (2007) demonstrated that *B. subtilis* is capable of producing volatile elements with antifungal activity. However, many of the volatile substances produced by this microorganism are unknown yet. Chen et al. (2008) identified by gas chromatography mass spectrometry (GC-MS) 14 volatile compounds of *B. subtilis*. The quality of some antifungal compounds such as 2-ethylhexanol, 2,4-bis (2-methylpropyl) phenol, 4-hydroxybenzaldehyde and 2-nonanone was also demonstrated in other pathosystems (Wang et al., 2004, Almenar et al., 2007). Non-volatile metabolites from *B. subtilis*, besides possessing antifungal metabolites are also effective in controlling some phytobacterias (Cunha et al., 2006).

In analyzing the attempts for management of *S. sclerotiorum*, chemical treatment is not recommended as uniquely way to control this pathogen due to its low efficiency (Paula Júnior et al., 2004), so the use of alternative and sustainable practices for the management, has been widely used, and the biological control using metabolites from bacteria assumes importance in combating this pathogenic organism. The objective of this work is to know the fungicidal and/or fungistatic effect of metabolites produced by *B. subtilis* in various phases of the *S. sclerotiorum* life cycle.

2. Materials and Methods

2.1 Antibiosis Test

First of all, one experiment was performed in order to isolate the bacteria that colonize the sclerotia. For this test, 20 sclerotia were placed on the surface of plastic plates containing a mixture of soil and sand in the same ratio, which were kept in an incubator at 20°C for 60 days. After isolating the bacteria, an essay was performed to attest the fungistatic or fungicidal activity of metabolites produced. We used Petri dishes containing PDA culture medium to make the experiment. In testing, disks measuring 9 mm in diameter containing the fungus were inserted on the medium in one pole of the plate. On the opposite pole was inserted 100 µL of bacterial

suspension with absorbance value of 1.002 nm at 550 nm wavelength. After the growth of both organisms were measured the inhibition zones.

2.2 Volatile Test

The presence of volatile substances was observed using both surfaces of the Petri dish, fixing one another with the aid of plastic film. Thus was inserted PDA culture medium inside to two larger parts of the Petri dishes. Caring for there was no contact between the microorganisms, has been inserted a disc with the mycelium in the center of the one those surfaces. In another surface, parallel grooves were made, derived from a bacterial suspension containing 1.002 of absorbance at a wavelength of 550 nm. The plate containing the mycelium and that plate containing the bacteria was fixed one to another. The radial growth measurements were measure daily until take up the entire plate. The treatment was compared with a control without bacteria, generating data to calculate the inhibition percentage.

2.3 Metabolites Production

For this process, a bacterial suspension was prepared with saline solution 0.85% (w/v), which was calibrated according absorbance readings given by a spectrophotometer ($A = 1.002$ at $\lambda = 550$). We transferred 1 mL of the suspension to five flasks containing 75 mL of culture medium MB1 liquid (Kado & Heskett, 1970). The bottles were covered with aluminum foils and placed on a shaker table at 120 rpm for 24, 48, 72, 96 and 120 hours. After this time, we put all flasks into the autoclave for sterilization process at 40 minutes in 120°C.

2.4 Mycelial Growth Test

Once the solution containing the metabolites produced in different incubation times, they were diluted into the PDA medium being adjusted at six different concentrations, 0% (control), 1%, 5%, 10%, 15% and 20%. Then, it was poured in Petri dishes with 9 cm in diameter. Mycelium discs of *S. Sclerotiorum* with 9 mm in diameter were transferred to the center of the plates containing PDA medium and metabolites. The set was sealed with plastic wrap and set aside in cold chamber at 20°C and 12 hours photoperiod. Mycelial growth was assessed with 24 hours apart, marking the two diameters perpendicular to the disk mycelium. The experiment was conducted in three replicates for each concentration.

2.5 Survival of Sclerotia in Soil Test

Into the plastic boxes was placed a mixture of sand and soil (1:1), previously sterilized in autoclave at 120°C for 1 hour. The sclerotia used were previously selected and disinfected. For disinfection, they were immersed for 30 seconds in ethanol 70%, one minute in sodium hypochlorite 2% and three times in distilled water to remove the excess. The arrangement of sclerotia in the substrate occurred uniformly arranged in a standard 5x5, resulting in total of 25 sclerotia per box. Then, was pipetted 500 μ L of bacterial suspension on each sclerotia, proceeding the same for all five incubation times tested (24, 48, 72, 96 and 120 hours). For the control was applied 500 μ L of sterile water on each sclerotia. The set was sealed with plastic film and set aside in a growth chamber at 20°C and 12 hours photoperiod.

2.6 Ascospores Germination Test

Performing this step the metabolite was prepared similar to item 2.3 until 120 hours of incubation. It was tested in four concentrations of bacterial suspension (5%, 10%, 15% and 20%) and a control containing sterile distilled water (0%). Ascospores from apothecia were obtained after incubation of 20 sclerotia for 40 days in the cold chamber at 20°C and 12 hours of light. The bacterial cells were placed in contact with the ascospores for a period of 48 hours, sufficient time for spore germination. The significant results obtained in the F test were used to make a regression analysis.

2.7 In Vivo Test

For the *in vivo* test we used four varieties of lettuce named Pescada, Camurim, Parma and Badejo, being cultivated for 30 days into the growth chamber. Inside the pots was added six sclerotia previously disinfected arranged on the base of the lettuce plants. In this experiment was only tested the incubation period that showed the best results in the previous tests, which is the incubation for 120 hours and the suspension was applied to the point of runoff. After applying all the treatments, the plants were incubated in a growth chamber at 20°C and 12 hours of light. The plants were evaluated for weight and number of healthy leaves.

2.8 Statistical Analysis

Where it was applicable data were subjected to analysis of variance with statistical program SISVAR 4.0 (Ferreira et al., 2000). When it was significant by F test, means were compared by the Tukey test at 5% probability.

3 Results and Discussion

3.1 Antibiosis Test

The metabolite presented antagonism to fungus with a reduction of 17.7% in mycelial growth (Table 1). Even after two weeks of the first measuring, these values have not changed in subsequent evaluations, suggesting the high efficiency of these substances in the control of *S. sclerotiorum*. Zhang et al. (2008) working with *B. subtilis* also achieved zones of inhibition between 10 and 20 mm against *S. sclerotiorum*.

Table 1. Halo inhibition in antibiosis test

Treatment	Mycelial growth (Cm)	Halo inhibition (Cm)
<i>B. subtilis</i>	7.53 b	1.46
Control	9 a	-

* p value = 0; Coefficient of variation (CV) = 0,22.

Favourable results were also found by Yilmaz (2006) who demonstrated the efficiency of *Bacillus* sp. against *Pseudomonas fluorescens* and *Micrococcus flavus*. Han et al. (2005) when working with *Bacillus* sp. also observed inhibition zones against *Streptomyces scabies*, *Pseudomonas syringae* pv. *syringae*, *Fusarium oxysporum* and *Alternaria mali*. This step was used in the initial tests in a practical way to know if the bacterial isolate produces harmful substances to the development of pathogens.

3.2 Volatile Test

The plates containing the bacteria grew less than the control with a reduction of 83.84% on mycelial growth, which attests that our *B. subtilis* has fungistatic volatile substances (Table 2).

Table 2. Mycelial growth index (MGI) and mycelial growth (cm)

Treatment	MGI (cm/day)	Mycelial growth (cm)
<i>B. subtilis</i>	0.24 b	1.5 b
Control	1.5 a	9 a

* p-value for MGI and Mycelial growth, respectively: 0, CV 2.04; 0, CV 3.56.

Table 3. Colonization of the resistance structures and their effects on germination

Incubation time	Colonized sclerotia	Mycelial germination	Carpogenic germination	Surface appearance
24 hours	6.67	1.00	0.00	Discolored - no layer
48 hours	7.33	1.67	0.00	Discolored - no layer
72 hours	16.67	1.00	0.00	Discolored - no layer
96 hours	15.00	1.00	0.00	Discolored - no layer
120 hours	20.67	0.00	0.00	Discolored - no layer
Control	0.00	13.67	2.67	Black

According to Wheatley (2002) volatile organic compounds are molecules which generally have low molecular weight and high vapor pressure. Because of their volatility, these compounds may be scattered over long distances and being a gas can take various forms according to environment. Chaurasia et al. (2005) reported that the production of volatile compounds by *B. subtilis* caused structural deformation in *Cladosporium oxysporum* and *Alternaria alternata*. In our study was not observed any hyphae alteration after the contact with volatile.

The influence of volatile metabolites on mycelial growth was also evaluated by Leelasuphakul (2008) who showed a reduction in the growth of *Penicillium digitatum*. Chen et al. (2008) noted the antagonistic effect of volatiles generated by *B. subtilis* on mycelial growth of *Botrytis cinerea*, a sclerotia producer. The use of volatile substances can be used more efficient, especially, in growing areas protected or small properties, but still in a lettuce commercial-scale production. Fialho et al. (2011) obtained 76.59% of reduction in the *S. sclerotiorum* mycelial growth when working with volatile organic compounds, which supports our result.

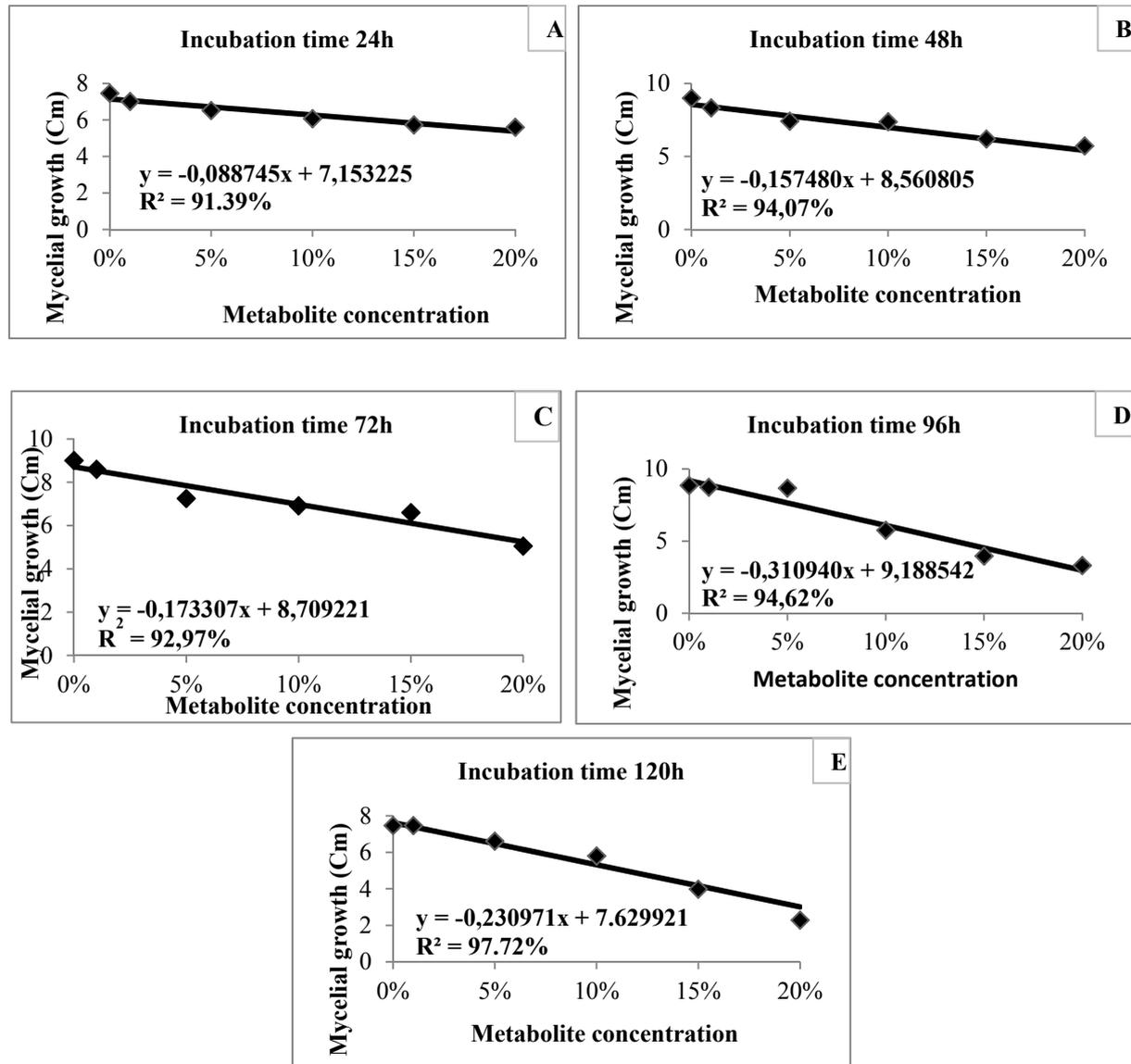


Figure 1. Mycelial growth according to the incubation time and metabolite concentrations. * Significant differences in the F-test at 5% probability. 24 h (p-value 0.003, CV 7.62), 48 h (p-value 0.001, CV 7.99), 72 h (p-value 0.001, CV 3.77), 96 h (p-value 0.000, CV 4.74) e 120 h (p-value 0.000, CV 10.19)

3.3 Survival of Sclerotia in Soil Test

When applying the bacteria on the sclerotia was observed that the incubation time of 120 hours yielded a greater number of sclerotia colonized by bacteria cells, and also prevented the mycelial and carpogenic germination. It was also observed that the sclerotia treated by bacterial cells had changes in color and less resistant with the consistency impaired.

Remuska and Pria (2007) reported that *Bacillus thuringiensis*, besides controlling the *S. sclerotiorum* mycelial growth in 37.44%, additionally did not allow the fungus to form sclerotia. However, this effect is not permanent, since the sclerotia output is delayed by only fungistatic substances. Thus, the search for molecules that decrease the strength and viability of these structures are important for management. Abdullah et al. (2008) worked with culture filtrates of *Bacillus amyloqueliefaciens* cultured for 7 days had an average a reduction of 48.67% in the sclerotia production. Our *B. subtilis* are able to damage the black layer of sclerotia, which protect the mycelium against adverse environmental factors.

3.4 Mycelial Growth Test

Due to the variation in metabolite concentrations and their incubation periods, the best fungistatic results against *S. sclerotiorum* were noted for the treatments which had more concentrated and longer incubation period, reaching up to 69.43% of control (bacterial cells cultivated for 120 hours and metabolites concentration at 20%). However, all treatments had effective inhibition on the mycelial growth of this pathogen (Figure 1).

Zhang and Xue (2010) by cultivating *B. subtilis* for 10 days achieved a reduction in mycelial growth in 75.1%, when treated with a suspension of bacterial cells and approximately 70% of control using the cell-free filtrate, which attesting the great potential on biological control to *S. sclerotiorum*.

3.5 Ascospores Germination Test

It was observed that the filtrate from cells of *B. subtilis* inhibits twinning of ascospore depending on the concentration used (Figure 2). Testing the concentration of 20% was observed a decrease of 39.74% on the spores germination. Yuen et al. (1991) also report the control of *S. sclerotiorum* ascospores germination using a strain of *Bacillus polymyxa* inhibiting approximately 40% of the germination and the growth of germ tubes.

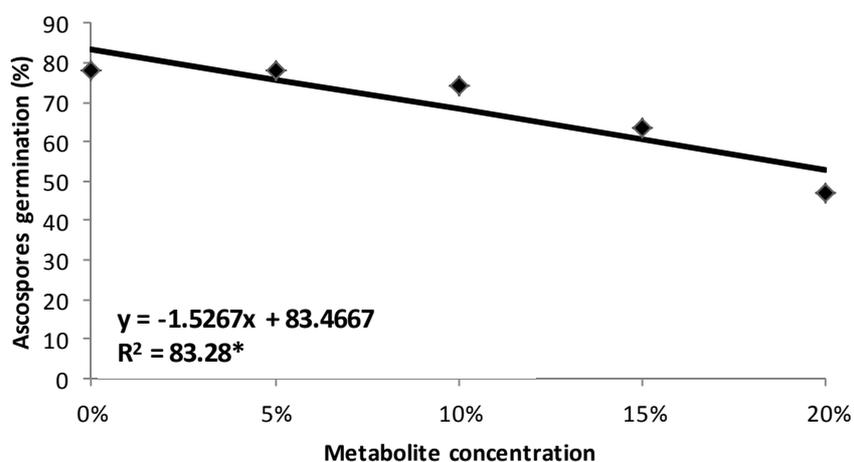


Figure 2. Ascospores germination on the effect of bacterial cells suspension. * Significant differences in the F-test at 5% probability. p – values: 0; CV – 4.53

3.6 In Vivo Test

When analyzing the performance of the bacterial suspension of *B. subtilis*, differences can be observed between the lettuce varieties used (Figure 3). Low values for the number of healthy leaves and weight of shoots of cultivar Pescada can be explained by the hypersensitivity reaction of bacterial suspension considering the concentration used. One day after application of the suspension, the plants were already dead. Even plants that did not die have showed fewer healthy leaves and increased colonization by *S. sclerotiorum*, perhaps by the weakening of the plant tissues once in contact with the bacterial cells. Testing with Badejo cultivar was observed that treatment with *B. subtilis* was efficient in preventing the disease.

This hypersensitivity in lettuce has been observed by others using suspension of *Bacillus* sp. However, others have reported positive effects in treatment with *Bacillus* cells. As Sossai et al. (2011) obtained an increase of 33.33% on the weight of the shoot, when plants were treated with *B. subtilis*. According to Araujo et al. (2005) the promoting growth, occurs through the production of hormones or antibiotics, which promote higher growth and plant vigor that were treated. In our work was not observed the growth promotion of lettuces varieties.

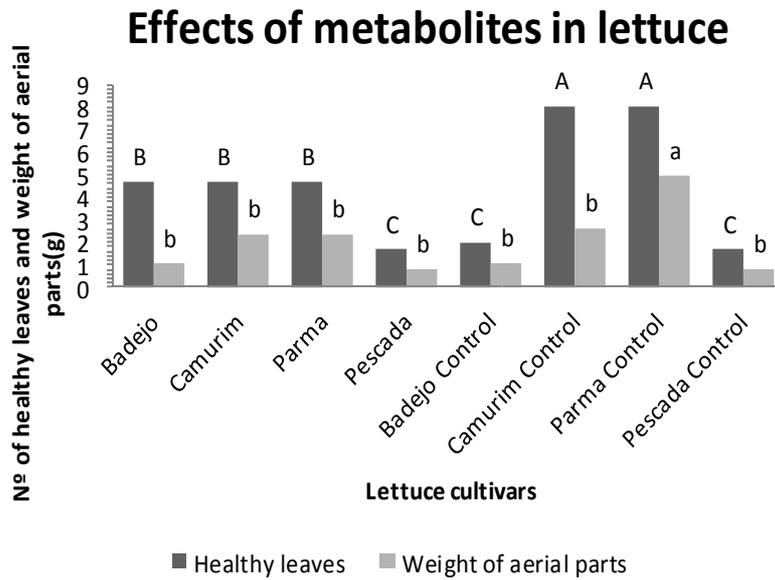


Figure 3. Control of *S. sclerotiorum* by pulverization the bacterial cells in four lettuce cultivars. Different letters in each category represent significant differences at 5% probability. p-value for healthy leaves and weight of aerial parts, respectively: 0.0054, CV: 38.83; 0.0020, CV: 51.53

The bacterium was also revealed by scanning electron microscopy (Figure 2) to prove the isolation of a single bacterium.

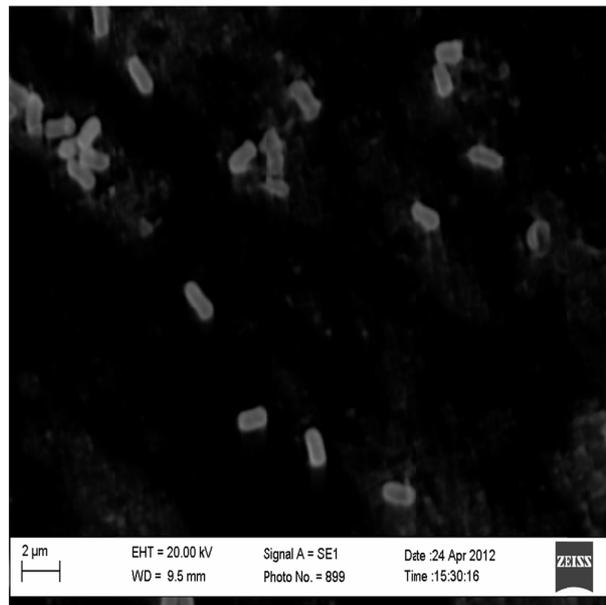


Figure 4. Bacteria antagonist revealed by scanning electron microscopy

4. Conclusion

B. subtilis has an antagonistic effect on all stages of the cycle *S. sclerotiorum*. The use of *B. subtilis* as biocontrol agent is conditioned on lettuce variety and concentration of bacterial cells used in the suspension.

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