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Endophytic Colonization by Brazilian Strains of *Bacillus thuringiensis* on Cabbage Seedlings Grown *in Vitro*

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Bt Research, 2012, Vol.3, No.3 doi: 10.5376/bt.2012.03.0003

Received: 07 May, 2012

Accepted: 14 May, 2012

Published: 22 May, 2012

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Preferred citation for this article:

Praça et al., 2012, Endophytic Colonization by Brazilian Strains of *Bacillus thuringiensis* on Cabbage Seedlings Grown *in Vitro*, Bt Research, Vol.3, No.3 11-19 (doi: 10.5376/bt.2012.03.0003)

Abstract *Bacillus thuringiensis* is a very important bacterium used as a biological control agent of agricultural pests. It may be isolated from soil, water, plants and dead insects. Three Brazilian *B. thuringiensis* strains that present high activity to Lepidoptera pests were inoculated in the seeds of cabbage in order to study the penetration and endophytic colonization of this entomopathogen in cabbage using scanning electron microscopy. The ability of *B. thuringiensis* to colonize endophytic seedlings was verified by the presence of vegetative cells, spores and crystals of the four Brazilian *B. thuringiensis* strains in different parts of cabbage seedlings. The observation of *B. thuringiensis* structure on roots, shoots and leaves of cabbage revealed colonization predominantly on roots. The colonization was shown on the surface, near the stomata and inside stomata pores. The mechanism of penetration of *B. thuringiensis* probably occurs through openings and injuries in roots and then moves through the xylem until reaching the leaves. The endophytic colonization of *B. thuringiensis* did not affect the germination of seeds and initial seedling development. The strains labeled with radioisotopes were inoculated on cabbage and were detected inside the plant. This article demonstrates for the first time the ability of *B. thuringiensis* to colonize cabbage seedlings that are important to control the cryptic pest as *P. xylostella* and others endophytic insect's pests.

Keywords *Bacillus thuringiensis*; Colonization; Cabbage; Strains; Insecticidal activity

Background

Bacteria of the genera *Enterobacter*, *Bacillus*, *Methylobacterium*, *Agrobacterium*, *Serratia*, *Acinetobacter*, *Arthrobacter* and *Pseudomonas* are the microorganisms most frequently found in endophytic associations with plants. The great advantage of endophytic colonization for the microorganism is that the plant's internal tissues provide a good environment that protects the colonizers from adverse conditions, such as ultraviolet radiation, rain, extreme temperature fluctuations and lack of nutrients (Silva et al., 2006); furthermore, these organisms may benefit the host plant by promoting growth and controlling pests by biological means.

Bacillus thuringiensis is a bacterium of cosmopolite occurrence (Krywunczyk and Fast, 1980), which is found worldwide in various substrates such as soil, water, plant surfaces, dead insects, spider webs and stored grain (Bravo et al., 1998). It is a gram-positive and aerobic bacterium, which may present facultative growth in anaerobiosis and is commonly used in

biological control of insects. The latter characteristic is due to the presence of crystalline protein inclusions, produced during sporulation (Bravo et al., 1998; Monnerat and Bravo, 2000). This species is very sensitive to ultraviolet rays, making its systemic use in plants of great agronomic interest.

Although *B. thuringiensis* is found in plants (Maduella et al., 2007; Monnerat et al., 2007), the way in which this bacterium enters them is still unknown. It is thought that it penetrates plants by means of raindrops, but there are few studies that demonstrate the ability of *B. thuringiensis* to colonize plants successfully; in such a way that it reaches insects in the phylloplane (Monnerat et al., 2009). Some studies indicate that the species of *B. thuringiensis* found in the soil and the phylloplane are the same, and that these bacteria, when found on cabbage leaves, are sometimes transported to the leaves from the soil (Bizarri and Bishop, 2007; Maduella et al., 2007). Other studies have demonstrated that colonization by endophytes seems to start with the

migration of bacteria to places where seeds are germinating and roots growing (Hinton and Bacon, 1995). One isolate of *Bacillus* and another of *Pseudomonas* applied to seeds of Norway spruce (*Picea abies*) have been observed to move apoplastically, in the intercellular spaces, being found on branches, leaves and flowers (Shishido and Chanway, 1999), colonizing the plants endophytically. Their penetration and transport mechanisms, however, are not known.

The ecology of *B. thuringiensis* is also little understood. To use it endophytically in the control of insect pests it is important to understand the way that *B. thuringiensis* colonizes cabbage seedlings, so that it may be used in controlling insect pests that live and feed within plant tissues.

The Laboratory of Entomopathogenic Bacteria at Embrapa Genetic Resources and Biotechnology has, since 2003 (Monnerat et al., 2003), been studying a new way of controlling *Plutella xylostella* using *B. thuringiensis* in its endophytic form, with the aim of protecting cabbage crops and other brassicas from this important cryptic pest.

The objective of this work was to evaluate the colonization of Brazilian *B. thuringiensis* strains toxic to *P. xylostella* of cabbage seedlings cultivated *in vitro*, by means of scanning electron microscopy. This study opens up prospects for systemic use of this bacterium in the control of agricultural pests from the Lepidoptera order. In addition, the study evaluated the possible positive effect caused by various strains of *B. thuringiensis* on seed germination and on seedling development, in cabbages cultivated *in vitro*.

1 Results

1.1 Development of cabbage seedlings inoculated by Brazilian *B. thuringiensis* strains

Results of the experiment to evaluation cabbage seed germination and seedling development three days after planting showed that there were no statistically significant differences when Brazilian *B. thuringiensis* strains were compared to the two negative controls (Table 1). This indicated that bacterial strains did not inhibit or stimulate germination (Kruskal-Wallis: $H_5=3.4$; $P=0.639$) or seedling development (Kruskal-Wallis: $H_5=11.679$; $P=0.039$) soon after germination.

Table 1 The effect of *B. thuringiensis* strains in development of cabbage seedlings after germination (average±standard deviation)

Treatment	Length of seedling (cm)	Percentual of germination
Control	3.111±1.079 a	100.00±0.000a
Embrapa medium	3.694±1.742a	100.00±0.000a
S1905	4.688±2.496a	94.44±9.624a
S2122	4.559±1.648a	100.00±0.000a
S2124	3.667±1.590a	94.44±9.624a
S1450–Btk	3.500±1.620a	94.44±9.624a

Note: Measurements followed by the same letter, in the column, do not differ among themselves

As regards development of seedlings after 30 days, stimulatory and inhibitory effects could be observed and varied depending on the bacterial strains and controls used (Table 2).

Thirty days after germination, growth of the aerial part was positively induced by *B. thuringiensis* strain S1905 (±27%) when compared to strain S1450 Btk, but when compared to the controls it did not present a

Table 2 Effect of *B. thuringiensis* strains on development of cabbage seedlings

Treatment	Length of aerial part (cm)	Length of roots (cm)	Weight of fresh material (g)	Weight of dry material (g)	Number of leaves (unit)
Control	8.27±1.041ab	9.88±1.417a	0.500±0.0636a	0.0277±0.003a	2.9±0.316a
Embrapa medium	8.08±1.948ab	7.52±2.004a	0.459±0.0705a	0.0239±0.0041ab	3.0±0.000a
S1905	8.96±1.526a	8.70±1.274a	0.285±0.0671b	0.0193±0.0058b	3.0±0.470a
S2122	7.40±1.518ab	9.07±3.375a	0.310±0.112 b	0.0210±0.0055b	3.1±0.568a
S2124	8.75±0.995ab	9.67±5.147a	0.319±0.0486b	0.0205±0.0042b	2.7±0.483a
S1450–Btk	7.05±0.765b	8.89±1.025a	0.305±0.0795b	0.0195±0.0060b	3.0±0.000a

Note: Measurements followed by the same letter, in the column, do not differ among themselves

statistically significant difference. For root length (Kruskal-Wallis: $H_5=8.226$; $P=0.144$) and number of leaves (Kruskal-Wallis: $H_5=6.326$; $P=0.276$), all the treatments were similar to one another. Furthermore, for mass of fresh matter (ANOVA: $F=14.616$; $P<0.001$) and of dry matter (ANOVA: $F=4.412$; $P=0.002$), the bacterial strains did not present a positive effect.

It can be inferred that after immersing the cabbage seeds in bacterial cultures, the bacteria adhered to the seeds. Then, by natural openings that were formed during the germination and seedling development process, bacteria penetrated and colonized the plant tissues, without causing damage to the cabbage

seedlings (Figure 1).

1.2 Observation of *B. thuringiensis* strains colonizing cabbage seedlings by scanning electron microscope

The colonization results of *B. thuringiensis* in cabbage seedlings were observed by scanning electronic micrographs. The presence of the bacterium was not observed in the blank control sample (Figure 2A) and it was confirmed that cracks were present (Figure 2B), exposing spores and crystals of *B. thuringiensis*. In transverse sections of the roots, visible spores and crystals testified to the existence of a growing population of aggregated bacterial cells (Figure 2C), colonizing tissue cells (Figure 2D).

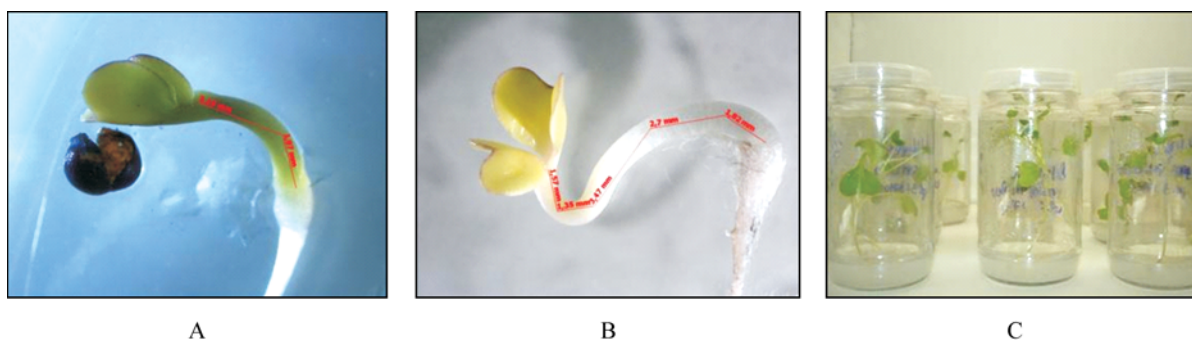


Figure 1 Seedlings of cabbage colonized by *B. thuringiensis* with normal aspect

Note: A: Control seedling after three days; B: Seedling treated with pellet of *B. thuringiensis* and C: Seedlings treated after 30 days

The stems of cabbage seedlings colonized by *B. thuringiensis* were also observed, as can be seen in figure 3A. Spores of strain S2124 of *B. thuringiensis* are visible colonizing stem stomata, a structure that can only be seen in young stems.

Observations of cabbage seedling leaves by scanning electron microscope showed the presence of cells of *B. thuringiensis* in the depressions of the epidermal cells, stomata, guard cells and regions near the stomata (Figure 2B). In most cases the bacterial cells were found aggregated and were rarely isolated, especially in areas protected by cuticular flanges or depressions and around the stomata (Figure 3C). Initial formation of fibrils (Figure 3D) was also observed in the stems of cabbage seedlings.

All the strains of *B. thuringiensis* that were used in this study were successful in colonizing cabbage leaf tissues, but structures of strains S1450 and S1905 of *B. thuringiensis* (Figure 3C; Figure 3D) were seen in more abundance.

1.3 Detection of *B. thuringiensis* marked with methionine ³⁵S on cabbage

To confirm the presence of *B. thuringiensis* strains in cabbage seedlings, these were inoculated with strains of *B. thuringiensis* marked with methionine ³⁵S. Radioactivity could be detected in all parts of the inoculated seedlings. However, radioactivity was not detected in the control seedlings that had been treated only with PBS, demonstrating again the colonization of cabbage by strains of *B. thuringiensis* (Figure 4).

2 Discussion

Bacillus thuringiensis is interesting specie to control insects' pest and were studying about capacity to colonize plants to control cryptic insets and its effect in the plants growth promoter. In this work the seed germination and development of cabbage seedlings were studied and demonstrated a similar results to those presented by Assis et al (1995), who showed that there was no germination promotion in radish (*Raphanus sativus*) seeds that had been inoculated

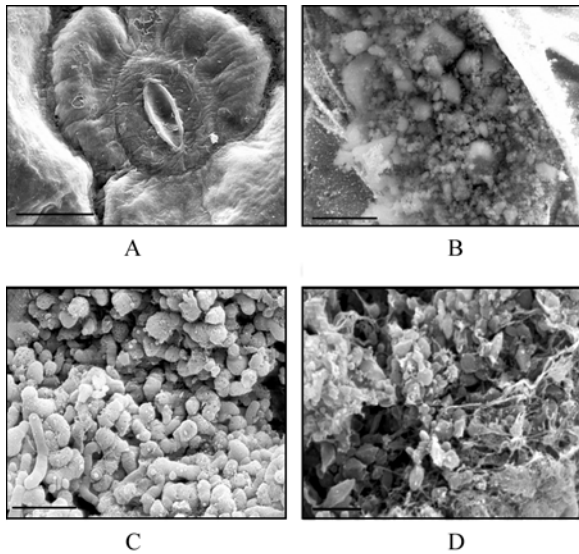


Figure 2 Scanning electronic micrographs showing colonization of different strains of *B. thuringiensis* in different parts of cabbage seedlings

Note: A: Cabbage Seedlings of non-infected control leaves by *B. thuringiensis*; B: Presence of cracks in roots of cabbage seedlings with spore and bipyramidal crystals (cb) of strain S1905 of *B. thuringiensis*; C: Vegetative cells, spore and spherical crystals of *B. thuringiensis* strain S1905 aggregated colonizing leaves of cabbage seedlings; D: Colonization of roots of cabbage seedlings by spores and bipyramidal crystals (cb) of *B. thuringiensis kurstaki* HD-1 S1450; Bars: A: 10 μ m; B: 5 μ m; C: 5 μ m; D: 2 μ m

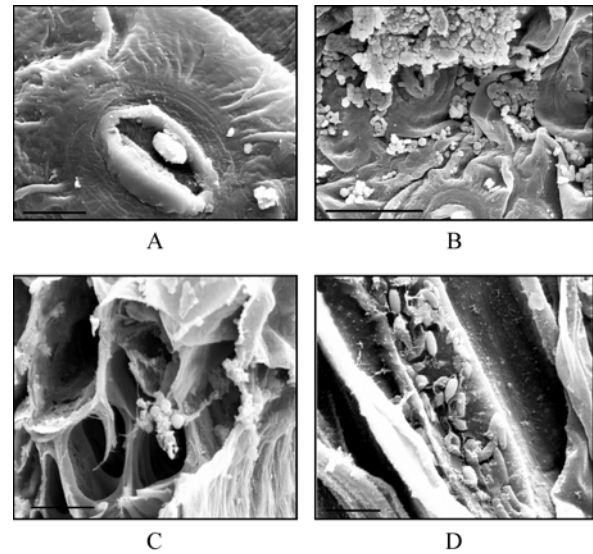


Figure 3 Scanning electronic micrographs showing colonization by *B. thuringiensis* strains in different parts of cabbage seedlings

Note: A: The presence of the spore of *B. thuringiensis* S2124 in stomata of stems of cabbage seedlings; B: The colonization of vegetative cells (cv), spore (ep) and spherical crystals of *B. thuringiensis* S1905 in depressions and stomata of leaves of cabbage seedlings; C: Spherical crystals of *B. thuringiensis* S2122 in xylem of roots of cabbage seedlings; D: Formation of fibrils in spores of *B. thuringiensis* strain S2124 colonizing stems of cabbage seedlings; Bars: A: 5 μ m; B: 20 μ m; C: 5 μ m; D: 2 μ m

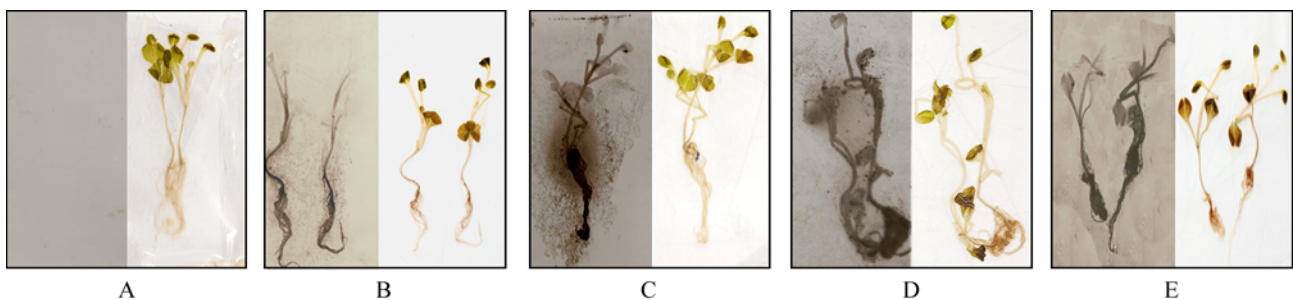


Figure 4 Autoradiography of cabbage seedlings after five days exposed to *B. thuringiensis* strains

Note: A: Control; B: S1450; C: S1905; D: 2122; E: S2124-Btk standard; They are showing the colonization of *B. thuringiensis* marked with methionine ³⁵S in roots, stems and leaves of cabbage seedlings

with *B. subtilis*. However, other studies with *B. subtilis* indicated an increase in *Eucalyptus* seed germination (Campello, 1992). This demonstrates that bacterial strains can have different effects depending on the plant and the bacterium used; the time the seed is exposed to treatment may also have an effect, since mechanisms such as nitrogen fixation, production of phytohormones, competition, and control of plant pathogens, among other aspects, may be involved in the regulatory process of plant growth (Sabino

et al., 2000).

The strains of *B. thuringiensis* studied here did not promote growth of cabbage seedlings under the tested conditions and the bacterial strains did not present a positive effect. Although work carried out with bacteria applied to seeds, using another bacterium of the genus *Bacillus*, *B. amyloliquefasciens*, demonstrated an improvement in the quality of cucumber (*Cucumis sativus*) seedlings (Silveira et al., 2004).

Effective colonization by cells of *B. thuringiensis* on the surface of roots of cabbage seedlings demonstrates that it is possible for this bacterium to exert a physiological effect on this host plant, an event that is essential if this microorganism may act as a growth promoter.

The treatment of seeds with different strains of *B. thuringiensis* for 5 minutes did not present a growth-promoting effect on cabbage seedlings. Nevertheless, further assays should be carried out, with seeds immersed for longer in the bacterial solution, or even direct application on roots. This is because the adherence of bacteria to plants may increase over time and depending on the initial concentration of the inoculums (Reis and Olivares, 2006). Additionally, there may be an interaction between the application method and the concentration, because the substances secreted by *B. thuringiensis* may stimulate plant development in a plant-microorganism interaction (Lee et al., 2009).

Observing the seedlings grown *in vitro* by scanning electron microscope, 30 days after inoculating the *B. thuringiensis* strains in the seeds, allowed vegetative cells, spores and crystals of *B. thuringiensis* to be seen in various parts of the plants. This observation of vegetative cells did not only demonstrate the adherence and penetration of this microorganism in cabbage seeds, but also the infection, multiplication and establishment in the interior of cabbage seedlings. Colonization of the various parts of cabbage seedlings by *B. thuringiensis* was thus proven.

The fact that vegetative cells were viewed by scanning microscope means that the bacteria may have used substances from the plant to develop and keep themselves alive in the seedlings. According to some reports, the isolates that have most ability to use seed exudates have a selective advantage in colonizing roots, because carbohydrates and amino acids are released abundantly in the form of exudates during the seed germination process (Subrahmanyam et al., 1983).

The bacteria may have penetrated by emergence of primary roots (Reis and Olivares, 2006). They may also have entered during the development of lateral roots that grow in the direction of the cortex, tearing the cell layer of the epidermis and emerging to the exterior. Large cavities created during this process

may have formed sites of infection for the strains of *B. thuringiensis*, as have already been observed in bacteria such as *Gluconacetobacter diazotrophicus* (James et al., 1994) and *Azospirillum spp.* (Patriquin et al., 1983). Infection in the roots can also occur due to abrasion with the growth medium, which promotes the formation of injuries during the process of root growth. These injuries are often the gateway for microorganisms to enter plants (Reis and Olivares, 2006), a fact that has been observed in *Gluconacetobacter diazotrophicus*, where a large number of bacteria were noted accumulating in the regions where root epidermis cells had ruptured (James et al., 1994).

The originality of this work with *B. thuringiensis* lies in the fact that cabbage seeds were treated in bacterial culture, rather than using sprayed leaves. It appears that the bacteria adhered to the surface of the seeds, penetrating by means of natural openings during the germination process, such as through roots or injuries, and finally colonizing the cabbage seedlings. Favorable conditions probably led to colonization, as well as the absence of reactions on the part of the plant tissues (Reis and Olivares, 2006). The presence of *B. thuringiensis* spores in the ostiole proves again that the sub-stomata chamber functions as a gateway for entry and egress of the bacteria, a phenomenon reported previously by Oliveira (2006) for the bacterium *H. rubrisubalbicans* in sugarcane (*Saccharum officinarum*).

Although interaction between cabbage plants and *B. thuringiensis* is not completely understood, it is possible that the bacterium reaches a favorable site and is able to resist removal, which constitutes a selective advantage (Reis and Olivares, 2006). This implies that this bacterial species may thus have colonized the roots, stems and leaves through natural openings, arriving on the surface of cabbage leaves via stomata cells by means of evapotranspiration or exudation.

These events could be observed for strains S1905, S2122 and S2124 as well as for the standard strain S1450. Structures from all the *B. thuringiensis* strains tested were visible adhering to the surface of leaves and in their depressions, being concentrated around the stomata. They were also seen in the stomata chamber.

The colonization of leaves seedlings of cabbage could

be observed for strains S1905, S2122 and S2124 as well as for the standard strain S1450. Structures from all the *B. thuringiensis* strains tested were visible adhering to the surface of leaves and in their depressions, being concentrated around the stomata. They were also seen in the stomata chamber. The colonization on stomach and nearby areas of cabbage leaves were observed in this work and similar results were found by Silva-Neto et al (2006) in the penetration of *Acidovorax avenae* subspecies *citrulli* by stomata openings in leaves of yellow melon (*Cucumis melo*).

The bacterial cells were found in areas protected by cuticular flanges or depressions and around the stomata; these events have already been observed for other microorganisms, such as phyto-bacterioses and nitrogen fixers (Silva-Neto et al., 2006; Mattos et al., 2008). The formation of fibrils was also observed by Silva-Neto et al (2006) and Campbell et al (1987) that observed the presence of fibrillar material, favoring adherence to the surface of hosts such as yellow melon (*Cucumis melo*) and canola (*Brassica napus* L. var. *oleifera*). The adherence process is not completely understood, but it is considered essential in the infection process, because it helps bacteria to stick to host surfaces, aiding the process of penetration and colonization.

All the strains of *B. thuringiensis* that were used in this study were successful in colonizing cabbage leaf tissues, but structures of strains S1450 and S1905 of *B. thuringiensis* were seen in more abundance. The latter is a Brazilian strain known to be toxic to larvae of insects from the Lepidoptera order (Monnerat et al., 2006). These strains were visible in the stomata openings, producing cells, spores and crystals, thus demonstrating germination, sporulation and formation of crystals of *B. thuringiensis*. The fact that *B. thuringiensis* was observed in all parts of the seedlings was of great importance for this study, because it shows the possibility of using *B. thuringiensis* in the control of agricultural pests that cause leaf fall, endophagous insects, sucking insects, soil pests and phytopathogenic vectors.

It is likely, then, that strains S1450 and S1905 are good colonizers of cabbage seedlings, standing out for their intense colonization in all parts of the seedlings,

as well as for the presence of bipyramidal, cuboid and spherical crystals, which are essential in toxicity to insect pests. Thirty days after being immersed, bacterial structures such as vegetative cells, spores and crystals of *B. thuringiensis* were found in all parts of the analyzed seedlings, proving that *B. thuringiensis* penetrates and colonizes tissues of cabbage seedlings.

The confirmation of effective colonization of seedlings by *B. thuringiensis* demonstrates that this bacterium may be used endophytically to control *P. xylostella*, a cryptic pest that causes damage to brassica crops in Brazil and worldwide.

Scanning electron microscopy produced proof that *B. thuringiensis* had penetrated the roots, stems and leaves of cabbage seedlings. It was confirmed that the methodology for inoculating *B. thuringiensis* in seeds was appropriate, providing conditions for observing the structures of this bacterium in the cabbage seedling tissues. This is the first demonstration of colonization of cabbage seedling tissues by Brazilian strains of *B. thuringiensis* using scanning electron microscopy.

The strains of *B. thuringiensis* did not promote growth in the cabbage seedlings, but they did present endophytic behavior without inducing disease symptoms.

The *B. thuringiensis* strains marked with methionine ³⁵S were detected in all parts of cabbage seedlings. Similar results were found by Monnerat et al (2009) in kale and cotton plants inoculated with *B. thuringiensis* HD-1 labeled by methionine ³⁵S.

This colonization in cabbage seedlings is of great importance, reinforcing as it does the possibility of using *B. thuringiensis* endophytically in the control of *P. xylostella*, a pest which, when the cabbage head forms, enters it and causes damage to the plant, reducing its value and lowering productivity. This corroborates the results of Monnerat et al (2009), in a study which demonstrated the presence of *B. thuringiensis* in cotton plant tissues for several weeks by means of cell counts after treating them with Btk HD-1.

The seed is one of the main vehicles for spreading microorganisms, and therefore disseminating *B. thuringiensis* via seeds should be more thoroughly investigated, so that this inoculation method can be

improved. It may thus lead to the control of agricultural pests that live within plants of major economic importance to Brazil and other countries worldwide.

3 Materials and Methods

The experiments were carried out in the Laboratories of Entomopathogenic Bacteria, Apomixes and Electronic Microscopy at Embrapa Genetic Resources and Biotechnology, Brasília, Federal District of Brazil.

3.1 Strains of *B. thuringiensis*

Four strains were used: S1450–*B. thuringiensis* subspecies *kurstaki* HD–1 (Btk) a standard obtained from the Collection of *B. thuringiensis* and *Lysinibacillus sphaericus* at the Pasteur Institute, France, and S1905, S2122 and S2124 from the Bank of Bacteria of Invertebrates at Embrapa Genetic Resources and Biotechnology.

3.2 Plant material

Seeds of hybrid cabbage Astro Plus were used in assays to evaluate colonization by the strains of *B. thuringiensis* in cabbage seedlings grown *in vitro*.

3.3 Bacterial growth

The strains were cultivated in Embrapa medium (Monnerat et al., 2007) in a rotating incubator at 28 °C, for 72 hours and at 200 rpm. The lots of each strain were viewed under a phase control optical microscope with 1000× magnification to observe spores and crystals.

3.4 Inoculation of *B. thuringiensis* strains in cabbage seeds

Cabbage seeds were superficially disinfected in ethanol 70% for 5 minutes, and in sodium hypochlorite 2 % with Tween 20 added (0.01%) for 30 minutes. After this period the seeds were washed three times in sterile distilled water and were transferred to autoclaved filter paper to remove the excess of water. All procedures with seeds, bacteria and *in vitro* culture were carried out in a laminar flow chamber. After disinfecting, each lot of 18 seeds was treated with one of the four strains of *B. thuringiensis*. Sterile distilled water and Embrapa medium were applied as negative controls. The seeds were immersed in 10 ml of each of the treatments for 5 minutes, and transferred to dry on Petri dishes with filter paper. Once dry, the seeds were inoculated into Petri dishes containing MS medium

(Murashige and Skoog, 1962) solidified with 0.7% of agar and at pH 5.8. For seed germination, the dishes were incubated in a culture room at (25±2)°C in the dark. The treatments were arranged completely randomly on a shelf in the culture room, in a total of six treatments with 18 repetitions of each. After three days, the seedlings were transferred to glass flasks with MS medium, where they were kept for another 27 days in the conditions described above, with a 12 h photoperiod. The treatments were arranged completely randomly on a shelf in the culture room, keeping the six treatments, but with 10 repetitions of each treatment.

3.5 Evaluation of development of cabbage seedlings

The seedlings were evaluated for percentage of germination and for length of the seedlings three days after they had been inoculated in the medium for germination. At the first evaluation stage, the germinated seedlings were measured with the help of a V8 stereoscopic microscope, using the program AxioVision, both from Zeiss. At the second stage, 27 days after transferring the seedlings to the glass flask, 10 seedlings were individually removed from the medium, taking care not to damage their roots, and were washed in water to remove the culture medium and traces of agar; they were then dried with absorbent paper. After this procedure, each of the seedlings was weighed, the number of leaves was counted and the leaf area and length of roots and aerial part were measured. After drying them in a stove at 50 °C for 16 hours, each seedling was weighed again to determine the weight of dry material. The design was completely random and the above variables were compared by analysis of variance followed by the Student Newman-Keuls test to compare averages; when the data did not fulfill the necessary premises non-parametric analysis of variance (Kruskal-Wallis) was instead used, and the differences between averages were compared by Dunn's test (P<0.05) with the help of the Sigma Stat program (Kuo et al., 1992).

3.6 Scanning Electron Microscopy

Samples of roots, stems and leaves from the plants cultivated *in vitro* were fixed with glutaraldehyde 2.5%, formaldehyde 2.5% and sodium cacodylate buffer 0.1 M pH 7.2 for 24 h at 4 °C. After this stage, the samples were submitted to three washing sessions of 15 min in cacodylate buffer 0.1 M, followed by

immersion in a solution of osmium tetroxide (OsO₄) at 2% in sodium cacodylate buffer for 1 h. The samples post-fixed in OsO₄ were washed twice in cacodylate buffer 0.1 M, at a 10 min interval, and twice in distilled water at a 5 min interval; soon after this, they were submitted to dehydration in a graded ethanol series (30%, 50%, 70%, 90% and 100%), being left for 10 minutes in each of the concentrations. The samples were washed three times for 20 min each time in ethanol at 100%. After this, the samples were dried by the CO₂ critical point method, in a Baltec CPD 030 appliance, and after being mounted on a metal support were sputter-coated with about 25 nm of gold using a MED 010 appliance from Balzers. The samples that had been thus prepared and duly identified were observed in a scanning electron microscope from Zeiss, model DSM 962.

3.7 Detection of *B. thuringiensis* marked with methionine ³⁵S on cabbage

Cabbage seeds were disinfected, as in item 4, and sown in a centrifuge tube of 50 mL, containing 15 mL of MS medium; they were then kept for 15 days in an acclimated room. After 13 days, three bacterial strains were inoculated separately in Petri dishes containing Embrapa Agar with methionine ³⁵S added at a concentration of 10 µCi/µL, and were kept at room temperature for 48 hours. Next, the dishes were scraped with a Pasteur pipette and the bacterial mass of each of the strains was mixed with 5 mL of PBS buffer. Of the 5 mL, 1 mL of each strain was applied to one of the seedlings. One of the seedlings was left as a control. Five days later, the seedlings were placed inside cellophane paper to dry in a gel drier for one hour. Then the seedlings were conditioned in a lead cassette and, in the dark, were exposed to an auto-radiograph film for a period of 30 days. After this, the film was revealed for observation of radio-marking.

Authors' Contribution

LP conducted all the research for this paper and prepared the manuscript. AG participated in seedlings preparation for microscopic analyses. GC participated in the experiments of plant growth promoter. ESM participated in activities with radioactivity. ES participated in data analysis and reviewed the manuscript. RM coordinated the project and was involved in manuscript preparation.

Acknowledgement

This work was financially supported by FAP-DF and Embrapa.

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