Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Translocation of *Bacillus thuringiensis* in *Phaseolus vulgaris* tissues and vertical transmission in *Arabidopsis thaliana*

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Keywords
cabbage looper, *kurstaki*, particle translocation, rhizosphere, vascular tissue.

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2016/2584: received 28 October 2016, revised 17 January 2017 and accepted 17 January 2017

doi:10.1111/jam.13407

Abstract

Aims: To demonstrate the ability of *Bacillus thuringiensis* to penetrate as spore-crystal complex to the internal tissues of bean plants, and keep its insecticidal activity. To test the vertical transmission of the spore-crystal complex in *Arabidopsis thaliana*.
Methods and Results: The experimental strain was transformed with the pMUTIN-*gfp* plasmid which labelled the spores of *B. thuringiensis* HD-73 with the GFP protein. Once the rhizosphere of the bean plants was inoculated with the labelled strain, the bacterium was recovered from leaves, stems, and petioles. Furthermore, toxicity of treated plants was significantly higher than control plants when bio-assayed on cabbage looper larvae. The labelled strain was recovered from the dead insects. When the rhizosphere of *A. thaliana* plants was inoculated with the labelled strain, mature seeds from these plants were surface-sterilized and grown under *in vitro* conditions. The labelled strain was recovered from the seedlings.
Conclusions: We showed that *B. thuringiensis* subsp. *kurstaki* (HD-73) in the rhizosphere can translocate to upper tissues of bean plants, and keep its insecticidal activity. Transmission of the labelled *B. thuringiensis* strain passed to the next generation of *A. thaliana*.
Significance and Impact of the Study: The role of *B. thuringiensis* as a potential facultative endophyte bacterium and the possible biotechnological repercussions are discussed.

Introduction

Bacillus thuringiensis is a Gram-positive bacterium whose main feature is the formation of parasporal crystalline inclusions during its sporulation phase (Bulla *et al.* 1980; Whiteley and Schnepf 1986; Aronson 2002). These inclusions are proteinaceous in nature and are widely studied due to their insecticidal properties (Aronson *et al.* 1986; Höfte and Whiteley 1989; Carlton 1990; Aronson 1993). Commercial products based on *B. thuringiensis* have been continuously used for more than 50 years with great success in agriculture, silviculture, and human health programs. This is mostly due to their specificity towards target pests and a long history of safe use on humans, domestic animals, and plants, as well as their lack of contaminating by-products. However, *B. thuringiensis* products only remain active in the field for short periods of time after their application due mostly to their exposure to several environmental factors (Joung and Côté 2000). Also, sucking insects as well as those with cryptic habitats (i.e. borers) escape to the usual application on the plant surface (Soberón and Bravo 2007).

This problem has been solved by the generation of transgenic plants (known as Bt crops) that systemically express the toxins (Cannon 2000; Ferré *et al.* 2008; Ibarra and Del Rincón-Castro 2015). The toxic activity of Bt crops remains during the whole growing season and significantly reduces the number of synthetic insecticide applications. However, the main concern about the use of Bt crops is the possibility that pests may develop

resistance, due to the continuous selection pressure they are exposed to (Gassmann *et al.* 2011; Tabashnik *et al.* 2013).

On the other hand, in spite of being one of the most studied bacteria, the ecology of *B. thuringiensis* has received little attention. It is known that isolates can be found in a wide and diverse number of habitats (Iriarte and Caballero 2001), but that is only an indication of the presence of viable spores, not of a growing population. Interestingly, some authors have reported the presence of *B. thuringiensis* as an endophytic bacterium in plants such as cotton, soybean, maize, sugarcane, and cabbage (Subrahmanyan *et al.* 1983; Suzuki *et al.* 2008; Praça *et al.* 2012). Observations in cabbage have shown the presence of vegetative cells, spores and crystals, suggesting the colonization of the bacterium from the initial entry through the root to their translocation to the upper parts of the plant (Praça *et al.* 2012). Other authors have suggested that, besides the roots, *B. thuringiensis* may gain entrance to the plants through stomata, wounds, and even by means of hydrolytic enzymes (Hallmann *et al.* 1997; McCully 2001). Monnerat *et al.* (2009) reported the natural presence of *B. thuringiensis* in cotton plants in a field with no previous use of *B. thuringiensis* products, and confirmed the translocation of *B. thuringiensis* from the root to the upper parts of the plant, by expressing the GFP protein in the experimental strain.

The potential endophytic nature of *B. thuringiensis*, which might be improved artificially, may become an alternative pest control strategy, similar to Bt crops, where toxic crystals are protected from the sunlight as well as from leaching by the rain (Griego and Spence 1978; Behle *et al.* 1997; Ruan *et al.* 2004; Raymond *et al.* 2010). For this reason, we tested the ability of the spore-crystal complex of *B. thuringiensis* to translocate from the root to the upper parts of the plant, keeping its viability and, most importantly, its toxicity. Also, we tested the endophytic ability of *B. thuringiensis* to be transferred to the next generation through the seed of treated plants.

Materials and methods

Biological material and culture media

Common bean (*Phaseolus vulgaris*) seeds of the variety 'Cacahuate 72' were kindly provided by Jorge Acosta-Gallegos (INIFAP- Mexico). *Arabidopsis thaliana* ecotype Columbia was provided by The Arabidopsis Information Resource (TAIR). Cabbage looper larvae, *Trichoplusia ni*, were reared in the Cinvestav insectary. The strain HD-73 from *B. thuringiensis* serovariety *kurstaki* was grown on plates with agar for standard methods (Difco, Becton, Dickinson, Mexico) and incubated at 28°C. Recombinant *B. thuringiensis* strains were grown in LB broth complemented with erythromycin (30 μg ml^{-1}). Production of the spore-crystal complex was achieved by culturing the strains in peptonized milk medium (López-Meza *et al.* 1995) or sporulating broth (Lereclus *et al.* 1989) complemented with erythromycin for the culture of the recombinant strains.

Labelling of the *B. thuringiensis* strain

Tracking of *B. thuringiensis* within the plant tissues was achieved by labelling the HD-73 strain with the GFP protein. For that purpose, the vector pMUTIN-*gfp* (Kaltwasser *et al.* 2002) was used to transform competent cells by electroporation, using an electroporator BTX ECM 630 under the following conditions: 2·3 KV, 475 Ω, y 25 μF. Thus, the strain Bt HD73/pMUTIN-*gfp* was obtained. The *gfp* gene was placed under the control of the pspac promoter of *Bacillus subtilis*, which constitutively controls the transcription of the gene during the vegetative growth phase. Once transformed, colonies were selected by their resistance to erythromycin in the medium, and fluorescence of the cells was corroborated under a Zeiss Primo Star fluorescence microscope (Carl-Zeiss GmbH, Oberkochen Germany).

Production and extraction of the spore-crystal complex of Bt HD73/pMUTIN-*gfp*

The recombinant strain was inoculated in peptonized milk broth and incubated at 28°C with 250 rev min^{-1} agitation for 48 h, or until complete autolysis (i.e. after cell walls of sporangia are degraded, spores and crystals are released separately), corroborated under phase contrast microscopy. Cultures were centrifuged (15 590 ***g***, for 12 min at 4°C) to pellet the spore-crystal complex (mixture of spore and crystals released during autolysis), repeating this procedure three more times after re-suspending the complex in cold ddH_2O. Finally, the last pellet was freeze-dried in a Labconco LYPH-Lock 4.5, Markham lyophilizer (Kansas City, MO).

Inoculation of bean plants' rhizosphere with the labelled strain

Common bean seeds of the 'Cacahuate 72' variety were germinated in a sterile substrate of Sunshine mix no. 3 : vermiculite : perlite (3 : 1 : 1), and 2 weeks later (plants showed two complete leaves) inoculation of the recombinant spore-crystal complex was initiated. The roots and the base of each plant's stem were separated from the rest of the plant (main stem and leaves) with a

layer of Parafilm stretched at the top of each pot, to avoid contamination of the upper part of the plant with the inoculum. Furthermore, the Parafilm was covered with several layers of Saran wrap (Fig. 2a). Suspensions of the spore-crystal complexes were prepared with 100 mg freeze-dried powder in 10 ml sterile ddH_2O. Each plant's rhizosphere was inoculated with 3 ml of this suspension (~$1{\cdot}26 \times 10^9$ spores) twice a week during 3 weeks by injecting the suspension through the Parafilm. Rhizosphere of control plants were inoculated with water. Pots were kept at 24°C with a light period of 16 h.

Detection of the labelled strain in the bean's sap

Leaves from plants previously treated with the recombinant spore-crystal complex and from control plants, were processed within a few hours of the last inoculation. These were surface-sterilized in a laminar flow chamber with 2% NaClO for 10 min, then with 70% ethanol for 10 min, and thoroughly washed three times with sterile ddH_2O. They were then kept to dry on sterile filter paper. Pieces of leaves and petioles were cut with a sterile scalpel and placed into a sterile syringe, which was coupled to a sterile Swinnex filter holder fitted with a piece of sterile filter. This device was placed into a 50 ml sterile Falcon tube and centrifuged at 2380 ***g*** for 10 min at 21°C. The sap recovered from the bottom of the Falcon tube was observed under the microscope, and aliquots were inoculated on LB agar plates with erythromycin, which were incubated at 28°C for 48 h. Colonies obtained from the plate were observed under phase contrast and fluorescent microscopy.

Detection of the labelled strain in the bean tissues

In order to detect the recombinant *B. thuringiensis* strain within the bean plant tissues, 7 mm segments of the stem and petiole of the treated plants (as well as the controls) were subjected to cryo-fixation with CryoGel, as described by the manufacturer (Electron Microscopy Sciences, Hatfield, PA). Once samples were assembled, they were frozen in liquid N_2 for 10 s. A LKB Bromma retracting microtome was used to obtain 25–30 μm-thick cuts, which were mounted on microscope slides with Fluoro-Gel (Electron Microscopy Sciences). Finally, cuts were observed under fluorescent microscopy.

Toxicity tests of treated plants

In order to detect toxicity derived from the recombinant *B. thuringiensis* strain inoculated in the rhizosphere of the bean plants, 20 neonate cabbage looper larvae per replicate were transferred to one bean leaf, which was isolated in a notched Petri dish, keeping the petiole connected to the whole plant but avoiding the escape of larvae. The same procedure was used for control plants. Five replicates for each bioassay were performed. Larvae were kept under these conditions for 4 days and then moved to another leaf of the same plant, where they were kept for three more days, for a total of 7 days per bioassay. Mortality was recorded for each replicate and data were statistically analysed by ANOVA and Tukey's analysis.

Detection of the labelled strain in dead larvae

Dead larvae from the previously described bioassay were separately homogenized in 200 μl sterile ddH_2O with a polypropylene pestle, under sterile conditions. Aliquots of the homogenate were inoculated on LB agar plates with erythromycin and incubated at 28°C for 48 h. The generated colonies were observed under phase contrast and fluorescent microscopy.

Inoculation of *A. thaliana*'s rhizosphere with the labelled *B. thuringiensis* strain

Seeds of *A. thaliana* were germinated in a sterile substrate of Sunshine mix no. 3 : vermiculite : perlite (3 : 1 : 1), and 3 weeks later their rhizospheres were inoculated with the recombinant spore-crystal complex. Preparation of the spore-crystal complex suspension, separation of the root system from the shoot system, and rhizosphere inoculation procedures were done as described above, except that three inoculations per week were made during a 2-week period. Rhizosphere of control plants were inoculated with sterile ddH_2O. Plants were grown for 10 weeks until the maturation of seeds (F1).

Detection of the *B. thuringiensis* labelled strain in F1 seedlings of *A. thaliana*

Mature seeds obtained from treated and control *A. thaliana* plants (see above) were surface-sterilized in an airtight gas-chlorine chamber with 30 ml HCl and 50 ml NaClO, overnight (an exposure that was three times longer than the usual procedure). Disinfected seeds were transferred to plates with MS medium (Murashige and Skoog 1962) free of hormones, under sterile conditions. Seeds were incubated at 24°C and a photophase of 16 h. Ten days after germination, F1 seedlings were processed as described above to obtain their sap, which was inoculated in LB agar plates containing erythromycin and incubated at 28°C for 48 h. Resulting colonies were observed under phase contrast and fluorescent microscopy.

Results

Expression of *gfp* in the Bt HD73 strain

In order to be able to track the progress of the HD-73 strain within the plant tissues, strain HD-73 of *B. thuringiensis* was transformed with the pMUTIN-*gfp* vector. Vegetative cells of the HD-73 strain showed the expression of the GFP protein, indicating that the pspac promoter (Kaltwasser *et al.* 2002), originally used to express genes in *B. subtilis*, was successfully recognized by *B. thuringiensis*, as evidenced by observations under fluorescent microscopy of the recombinant strain (Fig. 1a,b). Typical sporulation and crystal formation at the stationary growth phase of the cultured recombinant strain was confirmed, too. Moreover, GFP expression was clearly detected in the sporangia (Fig. 1c,d), but more interestingly, most of the endospores showed the green fluorescence even when released after autolysis (Fig. 1e,f). These results suggest that GFP expressed mainly during the vegetative phase was either expressed during the formation of the spores or that the GFP was 'sequestered' during the formation the spore's layers. This result was very important, as it permitted the subsequent tracking of the recombinant spores in the plant tissues. Crystals showed no fluorescence (Fig. 1c,d), but toxicity of the recombinant strain was corroborated by bioassays performed on *T. ni* larvae, using the wild-type strain as a positive control.

Detection of the labelled strain in the bean sap

To test the ability of the spores of the recombinant strain to penetrate the bean plant after inoculation of its rhizosphere, sap obtained from surface-sterilized leaves and petioles of inoculated and control plants was analysed. The whole sap extraction content of each of the eight replicates was plated on LB agar supplemented with

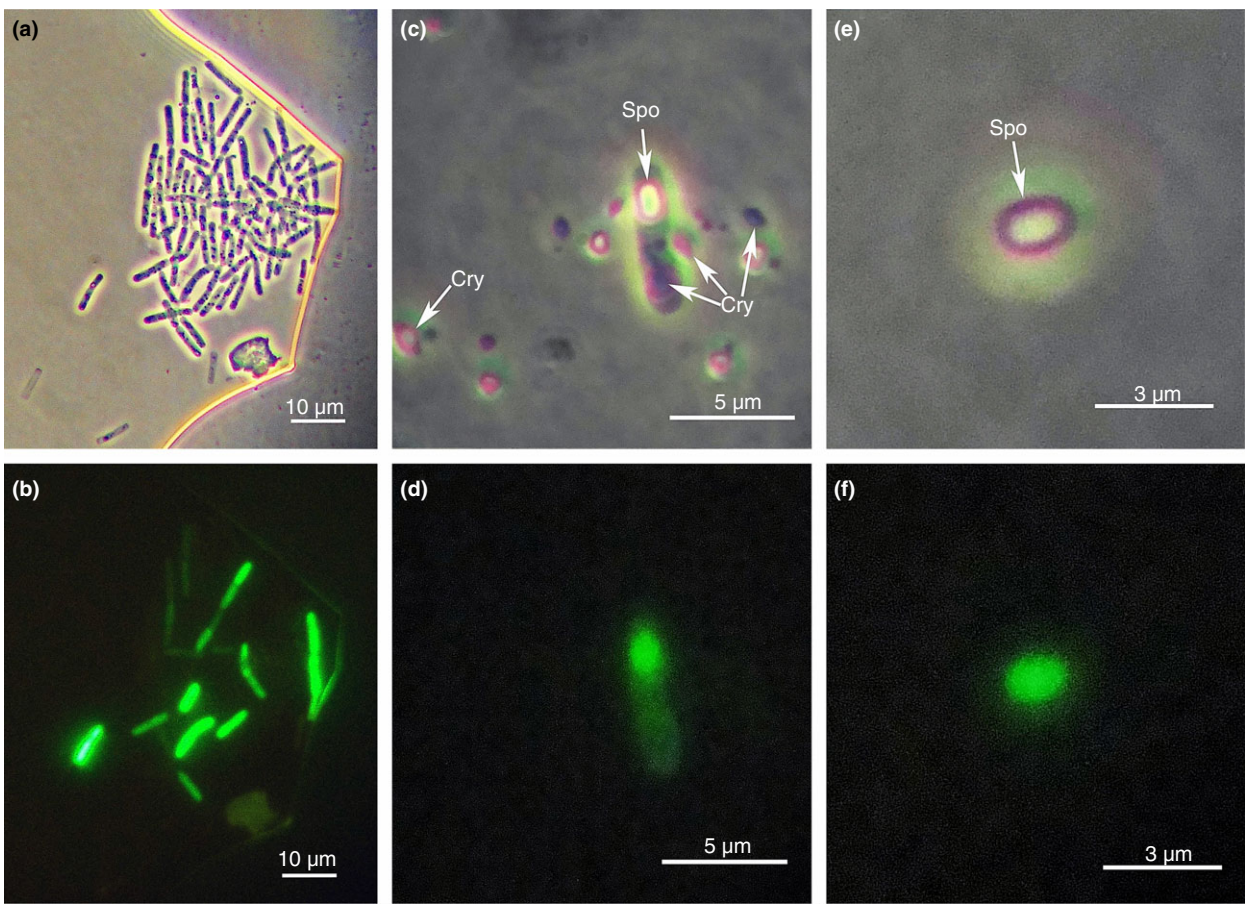

Figure 1 HD-73 strain of *Bacillus thuringiensis* transformed with the pMUTIN-*gfp* vector, containing the *gpf* gene under the control of the pspac promoter (Bt HD73/pMUTIN-*gfp*). (a, c and e) Phase contrast microscopy of vegetative cells (a), sporangium (c), and spore (e). (b, d and f) Fluorescence microscopy of the previous counterparts. Spo: spores; Cry: crystals. [Colour figure can be viewed at wileyonlinelibrary.com]

erythromycin. All plates inoculated with sap obtained from treated plants showed colonies of the labelled HD-73 strain, as corroborated under fluorescent microscopy (Fig. 2b). No colonies developed from sap extracted from the control plants. This result indicates that the labelled strain was able to translocate from the rhizosphere of the bean plant to the upper organs.

Detection of the labelled strain in the bean tissues

Further evidence of the translocation of the spores from the rhizosphere of the bean plants to the upper organs was obtained when cryo-fixed longitudinal cuts of top stems and petioles from treated and control plants were observed under fluorescent microscopy. Although infrequent (cuts were only 25 μm thick), some cuts showed evident fluorescent particles close to the xylem tissue, corresponding to the expected shape and size of a *B. thuringiensis* spore. These observations (not shown) support the previous results showing the recovery of the labelled strain from sap.

Toxicity tests of treated bean plants

Leaves from plants whose rhizosphere was inoculated with the labelled strain and leaves from control bean plants were used to detect any toxicity against first instar larvae of *T. ni*. The results obtained from the five replicates per bio-assay indicated a mean mortality of 48% for the treated plants after 7 days, while 23% mortality was observed in the control plants (Fig. 3). Even if the control mortality was higher than expected, the statistical analysis showed a highly significant difference ($P = 0{\cdot}0008$) between treated and control plants.

Detection of the labelled strain in dead larvae

Further evidence was obtained to demonstrate that larvae from the previously described bio-assays were killed by the action of the labelled HD-73 strain within the leaf tissue. Dead larvae (Fig. 4a) were homogenized independently and the obtained suspension was plated on LB agar supplemented with erythromycin. The labelled strain was recovered from all the samples obtained from larvae fed on treated plants (Fig. 4b). Conversely, none of the dead larvae fed on control plants showed the presence of the labelled strain. These results indicate that, not only the spores, but also the toxic crystals of the labelled strain, are translocated to the upper parts of the plant, considering that spores show no toxicity at all and that strain HD-73 produces neither VIP toxins nor β-exotoxin.

Detection of the labelled strain in F1 seedlings of *A. thaliana*

Due to its short life cycle, *A. thaliana* was used to test the possibility of vertical transmission of translocated *B. thuringiensis* to the next generation. Once plants of the parental generation were inoculated in their rhizosphere with the labelled strain (Bt HD73/pMUTIN-*gfp*), mature seeds were obtained from these plants. Once surface-sterilized, seeds were grown *in vitro*, showing no *B. thuringiensis* growth on the MS medium, in spite of

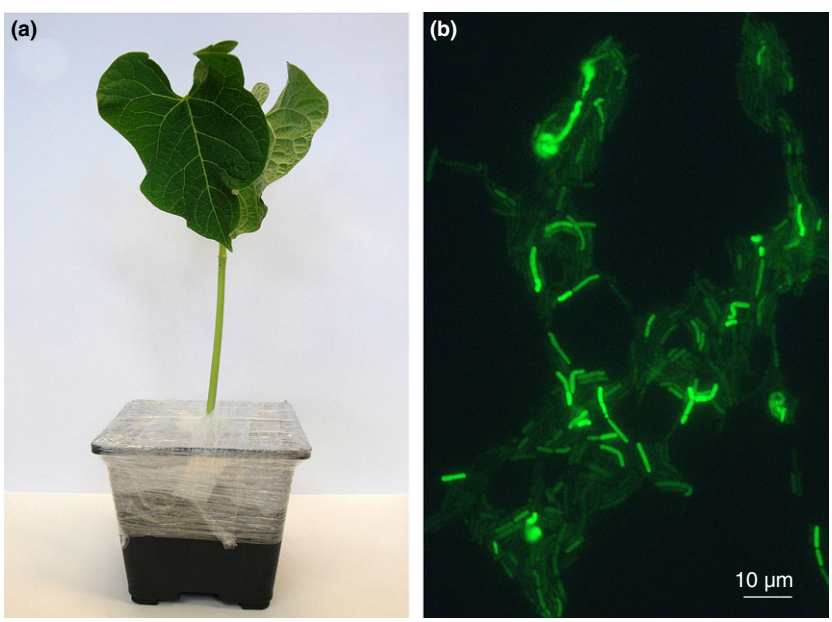

Figure 2 Bt HD73/pMUTIN-*gfp* strain obtained from the sap of bean plants. (a) Experimental unit of a bean plant after its rhizosphere was inoculated with the spore-crystal complex of the Bt HD73/pMUTIN-*gfp* strain. (b) Fluorescence microscopy of the labelled strain obtained from a colony, once the sap from the treated bean plants was inoculated on agar plates. [Colour figure can be viewed at wileyonlinelibrary.com]

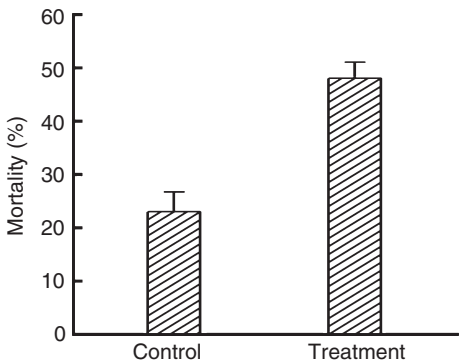

Figure 3 Mortality of *Trichoplusia ni* first instar larvae observed from five replicates (n = 5) of treated (inoculated with the Bt HD73/pMUTIN-*gfp* strain spore-crystal complex), and non-treated (control) bean plants, after 7 days of exposure. Columns indicate the average value and error bars indicate standard error.

B. thuringiensis' ability to grow in this medium. When sap was obtained from the F1 seedlings of the treated plants (Fig. 5a) and subsequently inoculated on LB agar supplemented with erythromycin, it generated colonies of the Bt HD73/pMUITN-*gfp* strain (Fig. 5b), while sap of seedlings coming from control parental plants showed no bacterial growth. These results indicate that spores were able to translocate from the rhizosphere to the flower meristems of the parental plants, where they remained viable within the differentiated seeds until germination, proving the vertical transfer of the bacterium. This was confirmed by the detection of the labelled strain in the resulting F1 seedlings.

Discussion

Results shown in this report indicate that the spore-crystal complex of *B. thuringiensis* is able to penetrate into the bean plant's root from the surrounding rhizosphere and translocate, more plausibly, through the xylem up to the stem, leaves and flowers of the plant. The possibility that the spores inoculated in the plant's highly organic substrate germinate exists, implying that vegetative cell might also be subjected to internalization. This possibility may be important in case that the internalization is an active process, where enzymes are involved in the degradation of root tissues. The GFP labelled strain (Bt HD-73/pMUTIN-*gfp*) was detected in the upper part of the plant, by visual observation of fluorescent spores in the plant tissues, and it was recovered from the plant's sap. Toxicity test of leaves on *T. ni* larvae was demonstrated, and the labelled strain was recovered from the dead larvae. This is an indirect proof that crystals were also internalized in the plant, although, growth of *B. thuringiensis* and production of new crystals within the plant tissues cannot be discarded at this point. Additionally, when the labelled strain was applied to the rhizosphere of *A. thaliana* plants, the labelled strain was recovered from seedlings obtained from the F1 generation, indicating a vertical transmission of this bacterium.

Similar results were reported earlier (Monnerat *et al.* 2009), when a labelled strain of *B. thuringiensis* was detected by microscopic observations in the petiole of cabbage plants, which agree with our results. However, it is noteworthy to comment that the graphical evidence shown in the above-mentioned report was unclear and the particle shown as a *B. thuringiensis* cell is far larger than expected. These authors also reported some degree of toxicity of the treated cabbage plants against the diamond back moth, *Plutella xylostella*, but their toxicity readings were lower than those we obtained in *T. ni*. Nonetheless, toxicity levels in both studies (theirs and ours), were low, compared to those shown by transgenic plants, even if a high concentration of the spore-crystal complex was used to inoculate the rhizosphere. Nevertheless, the higher toxicity of the treated plants was evident in our work, including some additional bioassays where the mortality was recorded daily, clearly showing that mortality started much earlier than the control (data not shown).

Many factors may have interfered on the optimal internalization and translocation of the spore-crystal complex. Among these, the process of internalization might have critically influenced the actual number of spores and

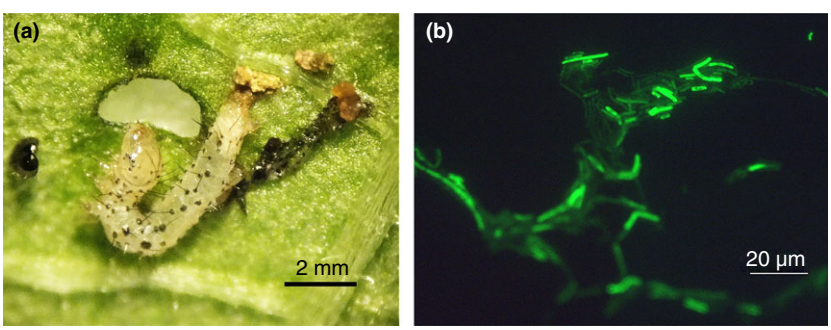

Figure 4 Bioassay of *Trichoplusia ni* first instar larvae fed with treated (inoculated with the Bt HD73/pMUTIN-*gfp* strain spore-crystal complex) bean plants. (a) Dead larva after leaf feeding; (b) fluorescence microscopy of the labelled strain obtained from a colony, once a dead larva homogenate was inoculated on agar plates. [Colour figure can be viewed at wileyonlinelibrary.com]

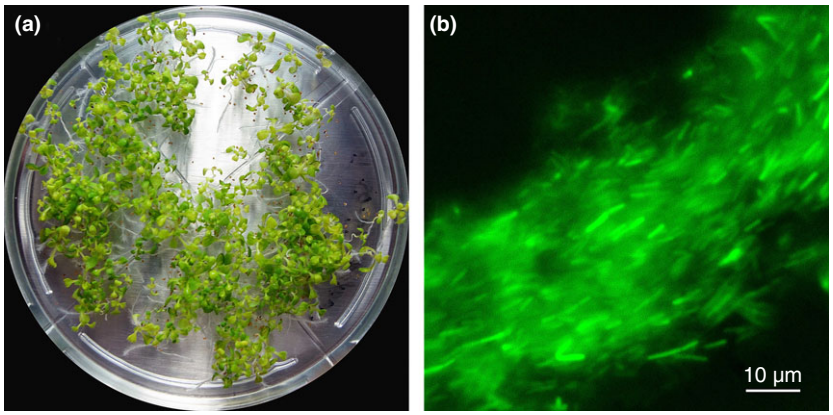

Figure 5 Vertical transmission of the Bt HD73/pMUTIN-*gfp* strain in *Arabidopsis thaliana*. (a) *In vitro* growth of *A. thaliana* seedlings from seeds obtained from plants treated with the Bt HD73/pMUTIN-*gfp* strain spore-crystal complex. Notice that most of the *A. thaliana* seeds were not able to germinate due to the rigorous surface sterilization procedure used. (b) Fluorescence microscopy of the labelled strain obtained from a colony, once the sap from the treated *A. thaliana* plants was inoculated on agar plates. [Colour figure can be viewed at wileyonlinelibrary.com]

crystals that reached the root cells. This process is supposedly controlled by the root cuticle and is highly regulated by the Casparian strip in the root endodermis. Another limiting factor could have been the capacity of the spore and crystals to move in the apoplast system of the root cells (González and Arbo 2013).

Internalization of the spore-crystal complex of *B. thuringiensis* into the root tissues can also be affected by the amount of lignin and suberin in the apoplast. For this reason, Praça *et al.* (2012) suggested that the internalization of *B. thuringiensis* in cabbage plants occurred through lesions in the root, caused either by physical factors, chemical degradation produced by other microorganisms, and/or natural openings due to the formation of secondary roots. In addition, during the differentiation of the root tissues, the Casparian strip is not totally formed, and therefore the so-called 'passing cells' can be found, which allow the entry of small particles into the internal root tissues.

On the other hand, there are reports describing what appears to be a passive internalization of particles into internal root tissues, such as CeO_2 nanoparticles 17 nm to >1 μm long, in squash (*Cucurbita maxima*) (Schwabe *et al.* 2013). Similarly, iron nanoparticles can be absorbed by squash and cucumber plants (Zhu *et al.* 2008; Zhang *et al.* 2011) as well as gold nanoparticles in several plants (Judy *et al.* 2012). However, the same particles could not be absorbed by monocot plants (e.g. wheat and maize) (Birbaum *et al.* 2010; López-Moreno *et al.* 2010). Interestingly, during the first stages of our work using maize plants, no clear results were obtained on the penetration of the spore-crystal complex. Based on the above, it is therefore possible that a mechanism similar to the passive internalization of particles permitted the entry of spores and crystals into the plants we examined. This is in agreement with the findings describing the process in which many natural endophytic bacteria penetrate the root (Hallmann *et al.* 1997). However, other scenarios are possible, such as the speculated utilization by some of these bacteria of an active process based on the degradation of pectin and cellulose (Hallmann *et al.* 1997), which resemble those employed by bacterial plant pathogens. Yet, we are inclined to discard this active process of penetration by *B. thuringiensis* considering that the spores lack any metabolic activity. On the other hand, even if we cannot discard at this point the possibility that the inoculum (spore-crystal complex) may have grown in the soil previous to the translocation or once in the plant tissues, we hypothesize that the inoculum (spore-crystal complex) was translocated directly to the plant, as only fluorescent spores were observed within the plant tissues, and in no case vegetative cells were observed.

Perhaps it is important to comment that an attempt to label the crystal by creating a chimaera with the GFP protein was made in this work, using the acrystalliferous Cry⁻B *B. thuringiensis* strain as transformant. Unfortunately, even when the recombinant crystals showed a distinctive fluorescence at the sporangium stage, this fluorescence was lost after the crystals were released during autolysis. Furthermore, the crystals lost their typical bipyramidal shape and, more interestingly, they were significantly less toxic towards *T. ni* larvae.

One of the most interesting results in this work was the observation of vertical transmission of *B. thuringiensis* into the seeds of treated *A. thaliana* plants. In such manner, *B. thuringiensis* behaved similarly to natural endophytic bacteria which, after entering and colonizing plant tissues, can reach the seeds systemically and be vertically transmitted (Hallmann *et al.* 1997; Coombs and Franco 2003). It is too early to consider that *B. thuringiensis* is a natural endophyte, as more confirming evidence is needed. Even so, it is noteworthy to mention that we have found *B. thuringiensis* in the sap of citrus trees and of bean plants in previous works (JEI, personal observations). Also, Monnerat *et al.* (2009) and Jeong *et al.* (2016) have suggested the possible role of *B. thuringiensis*

as a natural endophyte. In any case, either as a natural or induced endophyte, the important role of *B. thuringiensis* as a biological control agent can be greatly enhanced if the internalization procedure of the spore-crystal complex is improved and/or if the ability of *B. thuringiensis* to grow in the plant's vascular system can be stimulated. Both alternatives may open a novel set of strategies to control insect pests. Also important is the potential ecological relevance of these observations, which merit further research.

Acknowledgements

Authors are in debt with the excellent technical support of Javier Luévano-Borroel, Lino Sánchez-Segura, and Leandro Gabriel Ordóñez-Acevedo.

Funding sources

Cinvestav annual allocation found.

Conflict of Interest

There is no interest or relationship, financial or otherwise, that might be perceived as influencing an author's objectivity that can be considered a potential source of conflict of interest.

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