Encapsulation of Bacterial Metabolic Infiltrates Isolated from Different *Bacillus* Strains in Chitosan Nanoparticles as Potential Green Chemistry-Based Biocontrol Agents against *Radopholus similis*

Hilary Ureña-Saborío[1]*, Sergio Madrigal-Carballo[1], Jorge Sandoval[2], José R. Vega-Baudrit[3] and Alejandro Rodríguez-Morales[2]

[1]*National Center for Biotechnological Innovations (CENIBiot), CeNAT-CONARE, San José, Costa Rica*
[2]*Biocontrol Research Laboratory, National Banana Corporation (CORBANA), Guápiles, Costa Rica*
[3]*National Nanotechnology Laboratory (LANOTEC), CeNAT-CONARE, San José, Costa Rica*

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ABSTRACT: Currently there is a trend towards reducing the use of agrochemicals in developing countries. However, they are still being applied intensively in tropical countries. Thus, there is a trend towards developing new products based on natural chemicals for pest control, leading to second-generation pesticides incorporating nano- and biotechnologies. Costa Rica is one of the largest producers of bananas in the world. One of the most important pests of banana and plantain crops is the burrowing nematode, *Radopholus similis* (Cobb) Thorne. Highly toxic chemical compounds have traditionally been used to control this specific pest in banana plants, which can have dangerous effects on the environment and living beings as well. Biological control agents (BCA) like *Bacillus* isolated from nematode suppressive soils, in combination with nano- and biotechnological approaches, are gaining attention in the National Banana Corporation (CORBANA), as this plague generates great economic losses for the country. In order to perform encapsulation of active banana nematode biocontrol agents, we have been applying biopolymer carrier agents, such as chitosan and alginate, due to their recognized biocompatibility, biodegradability and low toxicity. Therefore, we have developed innovative formulations based on green chemistry approaches for encapsulating bacterial metabolic infiltrates (BMI) from four different *Bacillus* strains in order to improve the persistence and spread of these biocontrol agents in the soil and, consequently, becoming an effective pest control for banana plantations.

KEYWORDS: Banana, *Radopholus similis*, bacterial metabolic infiltrates, chitosan

1 INTRODUCTION

Green chemistry is a set of principles that reduces or eliminates the use or generation of hazardous substances in the design, manufacture and application of chemical products. Green chemistry moves products and processes toward an innovative economy based on protecting and improving soil quality, reducing dependence on nonrenewable resources, such as fuel, synthetic fertilizers and pesticides, and minimizing adverse impacts on safety, wildlife, water quality, and other environmental resources.

Reduction in the use of agrochemicals in developing countries is becoming a trend due to the intensive application of pesticides and environmental pollutants over the years. Different studies have concluded that no more than 0.1% of the agrochemicals applied in the fields can successfully reach the plague they are designed to control; meanwhile, the rest of the product circulates in the environment, polluting the surrounding soil, water and biota [1]. Thus, new trends in agrochemical design are now looking for biocontrol compounds with greater efficiency and less pollution, using natural and biological compounds for pest control [2]. These innovative biocontrol agents (BCA) are based on combined formulations, incorporating key agents to stabilize, protect and activate the natural or biological compounds in the product [3]. Developing of novel agricultural BCA products may involve

*Corresponding author: hilarydic93@hotmail.com

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techniques such as encapsulation, in which the active compounds are covered by a polymeric encapsulating matrix to protect them from environmental degradation or to promote the controlled release of their active compounds, allowing the development of different carrier systems such as microcapsules, nanoparticles and liposomes, among others [4–6]. For several years, the use of encapsulation techniques has been promoted to improve the stability, shelf life and effectiveness of a variety of products in the food, agriculture and medical industries [7, 8].

The process of encapsulation of an active compound is driven by an encapsulating agent, usually consisting of a polymeric matrix that can be either synthetic, artificial or natural [9]. A polymer is suitable to fulfill the role of encapsulation when it meets certain characteristics such as biocompatibility, biodegradability, mechanical strength, chemical stability, and low toxicity. Natural polymers or biopolymers have become very important in biotechnology over the years because they meet all these requirements. Among the wide range of existing biopolymers, chitosan and alginate are generating more interest as encapsulating agents due to their versatility, low cost and good performance as carrying matrix [8].

Costa Rica is one of the leading producers of bananas in the world, with a planting area of over 43000 hectares, for a total production of around 100 million boxes per year [10].

Nematodes are becoming the most important crop pests of bananas and plantains around the world, affecting the growth and development of the plants due to damage in roots and corms. It is pretty common to find large communities of nematodes, mainly *Radopholus similis* (Cobb) Thorne, in banana plantations that have been established for several years [11]. Therefore, control of these organisms using novel encapsulation technologies represents an important innovation for banana producers. CORBANA, the National Banana Corporation of Costa Rica, has been focusing their research on pest biocontrol as an alternative for reducing the use of agrochemicals. Various studies have been developed in this area with promising results; for example, the use of fungi and bacteria from nematode-suppressive soils are shown to be effective for the biocontrol of the nematode *Radopholus similis* [12–14].

Research conducted so far indicates that the use of microorganisms as nematode BCA has been efficient, generating an alternative window for the treatment of banana plantations, and also mitigating the effects on the environment caused by applications of highly toxic nematicides. However, there is a need to improve the stability and control release of the active agents in the field in order for BCA treatment to reach effectiveness [10].

This research is focused on the application of green chemistry approaches for the formulation and evaluation of different encapsulation systems based on biopolymers, such as chitosan nanoparticles, considering the optimal manufacturing conditions, stabilization performance and release from nanostructured encapsulating systems loaded with active BCA and its bacterial metabolic infiltrates (BMI). The application of biopolymer carriers to develop encapsulating systems for BCA will improve the persistence and spread of these microorganisms and, consequently, will produce an improved pest control.

2 MATERIALS AND METHODS

The production of nanoparticle systems and their physical and chemical characterization were performed at the National Center for Advanced Technologies (CeNAT), San José, Costa Rica. The production of BCA and bacterial metabolic infiltrates, as well as the bioassays for nematode control, were performed at the Biocontrol Laboratory in CORBANA's Research Center, Guápiles, Costa Rica.

2.1 Chemicals

All chemicals used in this investigation met the analytical grade or higher. Chitosan from shrimp shells (deacetylation degree of 92% calculated by potenciometric analysis; mean molecular weight of 214 kDa calculated by specific viscosimetry), was provided by CENIBiot. All other chemicals and solvents were obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification.

2.2 Biocontrol Agents (BCA)

Bacillus strains used in the tests belong to a collection of bacteria isolated from nematode-suppressive soils present on banana commercial farms in Costa Rica owned by CORBANA. Selected bacteria species were identified and labeled as *B. cereus* (B-71), *Bacillus* sp (B-72), *B. thuringiensis* (SER-217) and a final strain that was characterized in the genus *Bacillus* sp by PCR (gen 16 S), using specific primers for *Bacillus* and followed by sequencing (B-458).

2.3 Production of Bacterial Metabolic Infiltrates (BMI) from *Bacillus* Strains

Bacterial metabolic infiltrates (BMIs) composed by secondary metabolites were produced in 200 mL liquid culture of Luria-Bertani medium. After incubation for three days, 50 mL of bacterial culture were taken

and centrifuged at 10.000 rpm for 10 minutes, and the supernatant was vacuum filtered using a bioreactor with nitrocellulose filters (0.22 µm pore size). The isolated sterile bacterial metabolic infiltrates were kept at 4 °C for further analysis.

2.4 Characterization of *Bacillus* BMI

Chemical characterization of BMI from *Bacillus* strains was performed using reversed-phase diode-array-detection high-performance liquid chromatography (RP-DAD-HPLC) to separate and identify the phytochemical profile of the main components contained in each sample. An aliquot of 250 µL of concentrated BMI was injected into a HPLC (225 Series, PerkinElmer, USA). The phytochemical profiles of the samples with more heterogeneity were also evaluated by QTRAP mass spectrometry (QTRAP-MS/MS 1200 Series, Agilent Technologies, USA). For analysis by mass spectrometry, an aliquot of 150 µL of each BMI was mixed with 3 mL of methanol and 2 mL of the mixture was taken and placed in the mass spectrometer.

2.5 Preparation and Characterization of Chitosan Nanoparticles Loaded with *Bacillus* BMI

Chitosan nanoparticles were prepared according to a previously described methodology [15]. Briefly, chitosan powder was dissolved in aqueous acetic acid (0.1% w/v) and mixed under continuous stirring with the *Bacillus* BMI dispersed in water at different concentrations (2.5%, 5.0%, 7.5% and 10.0% v/v). Once the samples were mixed for about 10 min, tripolyphosphate (1 mg/mL) was added under vigorous stirring as crosslinking agent to fabricate the nanoparticles. All materials were previously sterilized and all the experimental work was conducted under sterile conditions.

The size of the nanoparticles was measured by dynamic light scattering (NanoZetasizer, Malvern Instruments Ltd, UK). Morphology of the hybrid nanoparticles loaded with BMI from *Bacillus* B-458 10% v/v was characterized by atomic force microscopy (AFM, MFP-3D-SA, Asylum Research, USA), and scanning electron microscopy (SEM, JEM2011, JEOL USA). AFM imaging was performed with a spring contact of k = 0.03 N/m using a nanoprobe cantilever made of silicon nitride (Si3 N4). The SEM samples were immobilized on copper grids. They were dried at room temperature, and then were examined using a SEM without being stained.

The release profiles of *Bacillus* BMI loaded into chitosan nanoparticles were evaluated by UV-vis spectrometry (Lambda 35, PerkinElmer, USA). An aliquot of 2 mL of each encapsulated nanoparticle system was taken and placed into the spectrophotometer. Absorbance at a wavelength of 250 nm was recorded to monitor the release of the BMI from the loaded nanoparticles against empty chitosan nanoparticles as blank control for a total period of 72 hours.

2.6 *In-Vitro* Bioactivity of Chitosan Nanoparticles Loaded with *Bacillus* BMI against *Radopholus similis*

Nematodes from *Radopholus similis* were kindly provided by the Nematology Laboratory of the National Banana Corporation (CORBANA, Guápiles, Costa Rica). *In-vitro* activity of *Bacillus* BMI-loaded nanoparticles was evaluated in a bioassay to determine their antagonistic effect on *Radopholus similis*, according to previously described protocol [16]. Briefly, nematodes were isolated from a carrot disc using sterile ultrapure water to wash the nematodes form the disc and retaining the nematodes on a sterile sieve (28 µm). Nematodes attached to the filter were immersed in a solution of sodium hypochlorite 0.25% for 10 seconds, and then washed three times with sterile ultrapure water. Nematodes (normally composed by up to 95% female) were taken out of the filter and kept in sterile ultrapure water with constant oxygen bubbling previous to standardization at 25 nematodes/100 µL for further examination. An aliquot of 2 mL from each BMI-loaded nanoparticle system was poured into a 24-well plate, and 100 µL of the nematode standardized dispersion was added to each well, for a total count of 25 nematodes per well. Plates were kept under incubation at 27 °C, with no lights, for a total period of 150 hours and nematode mortality was evaluated using an inverted microscope (LIB-305, Leader Precision Instrument Co. Ltd, USA) at 4x magnification. Mortality of nematodes was evaluated according to a stimulation protocol based on nematode mobility *in vitro* [17].

3 RESULTS AND DISCUSSION

3.1 Characterization of BMI Isolated from *Bacillus* Strains

The wavelength absorption spectrum for the BMI from *Bacillus* strains indicated a maximum absorbance between 245 and 260 nm (data not shown), corresponding to the wavelength of absorption of proteins found in a range of 235 and 280 nm [18, 19]. These values may indicate that the most abundant components in the filtrates could be peptides and proteins, which is consistent with previously obtained results reporting

that the BMI showed collagenolytic activity for *Bacillus* strains B-71 and B-72, respectively (data not shown). From these results it was decided to select the wavelength of 250 nm to determine the average absorbance for further stability and release analysis.

3.2 RP-DAD-HPLC Analysis

Analyses of liquid cultures of *Bacillus* have shown that these bacteria are producers of highly active secondary metabolites, which can produce several structurally diverse compounds [20]. For example, *B. thuringiensis* is known for producing highly toxic crystalline proteins, which have been used commercially to control pests [21]. Also, *B. cereus* has been previously reported to produce a mixture of secondary metabolites of high complexity [22]. Reversed-phase high performance liquid chromatography analysis (DAD-RP-HPLC) carried out on the third day after preparation of the *Bacillus* BMI (Figure 1), indicated that the BMI from *Bacillus* strains B-71, B-72 and SER-217 showed a similar phytochemical profile between each other (Figure 1a, 1b and 1c, respectively). According to studies of diversity, *Bacillus cereus* (B-71) is very

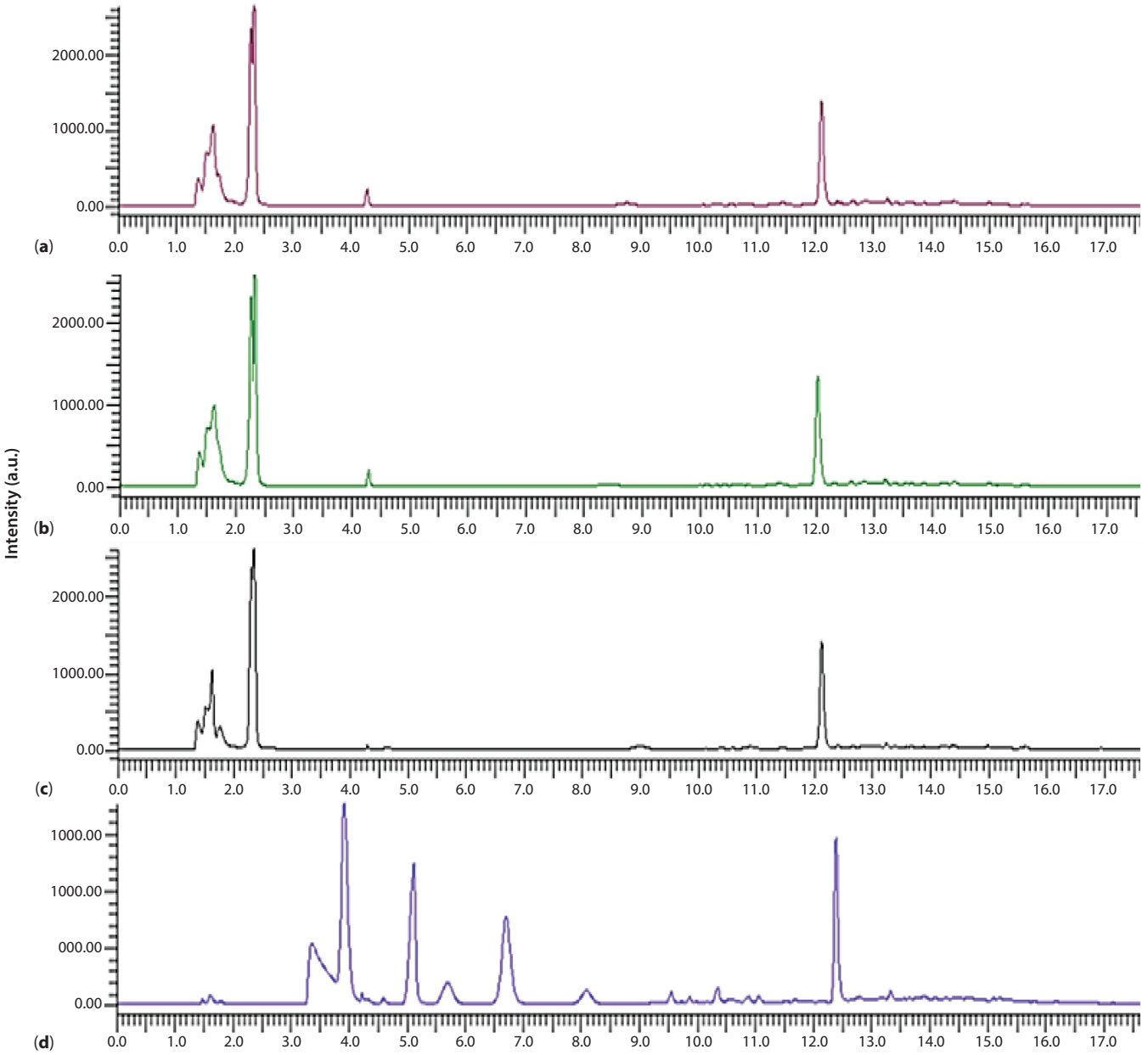

Figure 1 RP-DAD-HPLC chromatograms for BMI isolated from *Bacillus* strains (**a**) B-71, (**b**) B-72, (**c**) SER-217 and (**d**) B-458.

closely related to *Bacillus thuringiensis* (SER-217) [22]. Meanwhile, the BM infiltrate isolated from *Bacillus* strain B-458 showed significant differences in its phytochemical profile (Figure 1d), in which it is possible to identify the presence of a larger number of chemical compounds at retention times between 3 and 11 min from the other *Bacillus* BMI, suggesting a promising distribution of bioactive compounds with potential as BCA. *Bacillus* strain B-458 has only been characterized in the genus *Bacillus* by morphological and sequencing studies, by sequence comparison analysis at available bacterial databases. This strain may correspond to a species with different characteristics and therefore can have a significantly different effect on the mortality of *Radopholus similis*, since studies have shown that different *Bacillus* species have antagonistic capabilities [23, 24].

A second RP-DAD-HPLC analysis of the *Bacillus* BMI was conducted 10 days after preparation (data not shown), showing no significant changes on its phytochemical profiles, indicating that the stability of the chemical components on the BMI were maintained with time when the infiltrates were stored at 5 °C.

3.3 QTRAP-MS/MS

In order to fully characterize the differences observed between the BMI obtained from *Bacillus* strains B-458 against the other strains, we performed a QTRAP mass spectrometry analysis (QTRAP-MS/MS), selecting the BM infiltrate from *Bacillus* strain B-71 as a reference. Figure 2 shows the mass spectrometry spectra of these two *Bacillus* BMIs collected in a QTRAP detector system.

The mass spectrum confirms the presence of multiple chemical compounds in both BMIs, but results clearly confirm that the BMI isolated from *Bacillus* strain B-458 showed a greater variety of compounds in the range of masses below 1000 m/z (Da). Furthermore, in both spectra it is possible to identify the presence of a major compound with mass 570 m/z (Da) that could be a promising candidate for isolation and phytochemical characterization.

3.4 Nanoparticle Characterization by Dynamic Light Scattering (DLS) Analysis

Figure 3 shows the average particle size (calculated as average hydrodynamic diameter) for chitosan nanoparticles loaded with BMI from *Bacillus* strains. Results suggest that BMI may interact with chitosan biopolymer, possible through hydrogen bonding interactions, producing particle agglomeration and flocculation, which is depicted in high particle size values above the nanoscale range (~ 500 nm).

However, chitosan nanoparticles loaded with BM infiltrate from *Bacillus* strain B-458 showed particle size values in the same range as chitosan nanoparticles, thus they have been used for further microscopy characterization.

3.5 Surface Characterization of Nanoparticles by SEM and AFM Microscopy

Figure 4 shows the micrographs obtained by scanning electron microscopy (SEM, Figure 4a) and atomic force microscopy (AFM, Figure 4b) for chitosan nanoparticles loaded with BMI from *Bacillus* strain B-458, which show nanoparticles with uniform spherical shape and an average particle size of around 350 nm.

The observed contraction in size and thickness, compared with the apparent hydrodynamic diameter obtained by dynamic light scattering (DLS), is probably due to the scanning force acting on the surface of the particles [25].

3.6 *Bacillus* BMI Release Profiles from Chitosan Nanoparticles

The release profiles for BMI from different *Bacillus* strains loaded into chitosan nanoparticles are shown in Figure 5. Results confirm the efficiency of the nanoparticle system as controlled release matrix for the *Bacillus* BMI.

The release mechanism from the nanoparticles suggests a slow delivery at the beginning of the process, when chitosan nanoparticles are starting to swell and physically degrade, allowing the loaded BMI to get released into the media at about 5 hours of incubation. After this period of time the rate of degradation of the chitosan matrix and the rate of release of the BMI seem to reach a dynamic equilibrium that is depicted in a sustained release profile as a function of time. According to Agnihotri *et al*. [26], one of the most important factors affecting the release profiles from chitosan nanoparticles is the dispersion media, because the process can occur by diffusion, where water enters the system of nanoparticles, causing swelling of the matrix.

Previous studies have revealed that the chitosan nanoparticles prepared by ionic gelation process with TPP are of great interest because of their capacity for incorporating a large number of active compounds and macromolecules [27, 28]. Studies by Liu *et al*. [29] also indicate that the release of encapsulated

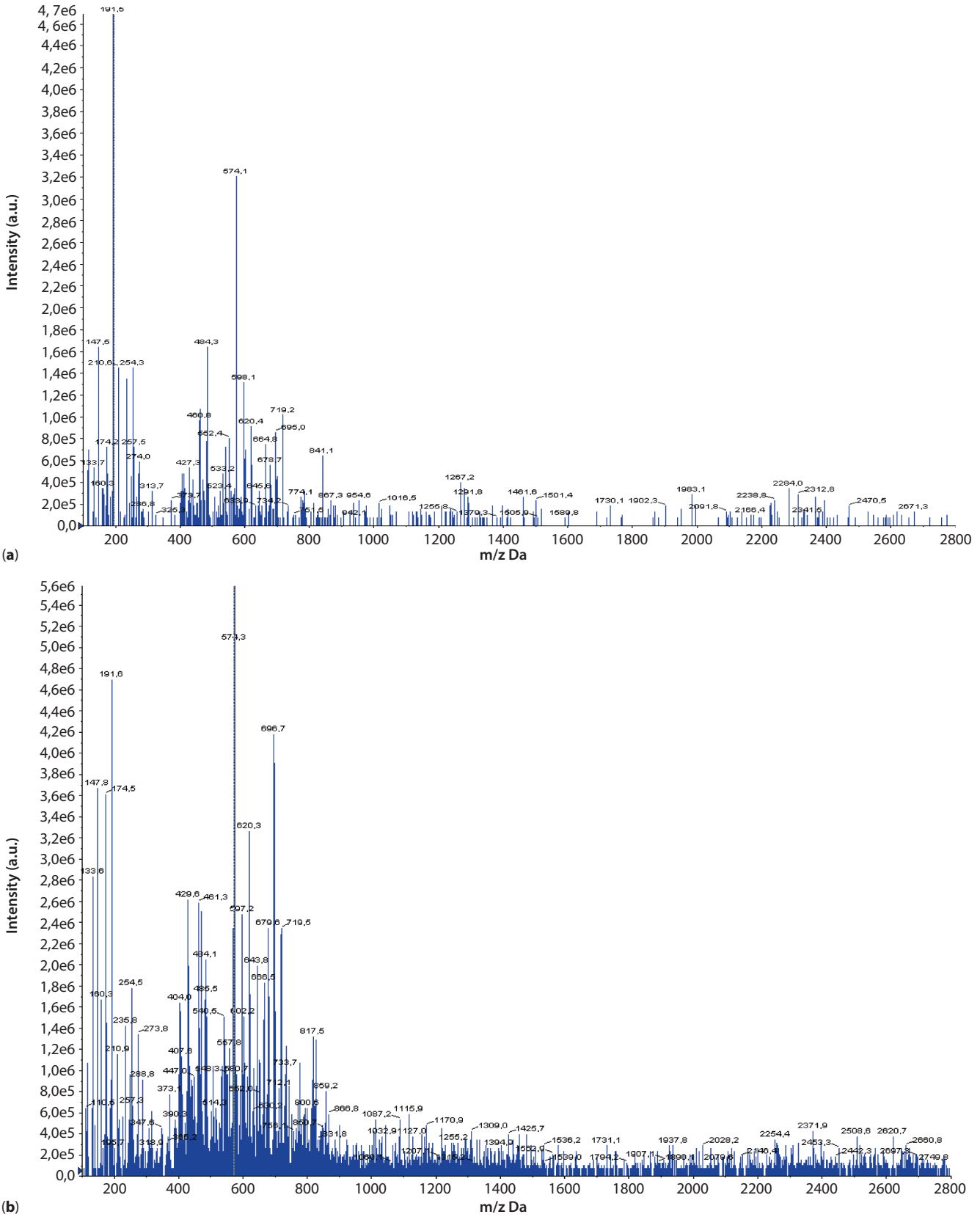

Figure 2 QTRAP-HPLC-MS/MS spectra for bacterial metabolic isolates from *Bacillus* strains (**a**) B-71 and (**b**) B-458.

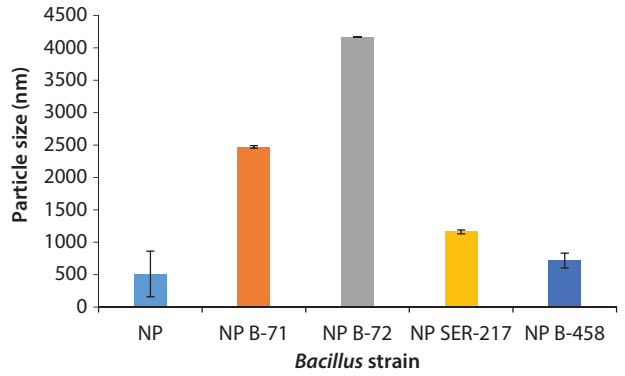

Figure 3 DLS analysis of particle size (reported as average hydrodynamic diameter) for chitosan nanoparticles (NP) loaded with BMI from *Bacillus* strains (10% v/v).

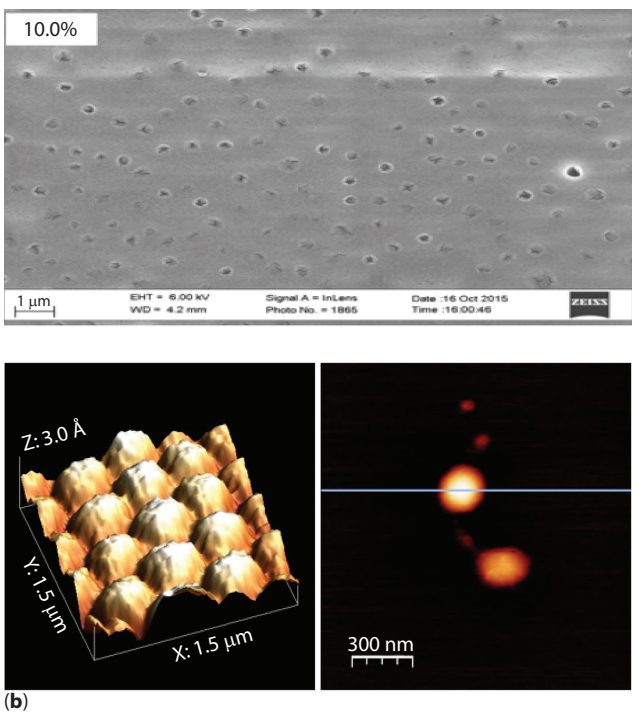

Figure 4 Microscopy analysis of chitosan nanoparticles loaded with BMI isolated from Bacillus strain B-458 (10% v/v). (**a**) SEM micrograph showing uniform spherical shapes and (**b**) AFM showing an average particle size distribution analysis of about 350 nm.

microorganisms occurs efficiently in aqueous medium. Results suggest that the release of BMI from *Bacillus* is driven by a controlled diffusion process, thus promoting the protection and stability of active BMI, and potentially allowing a better distribution and permanence on the soil profile.

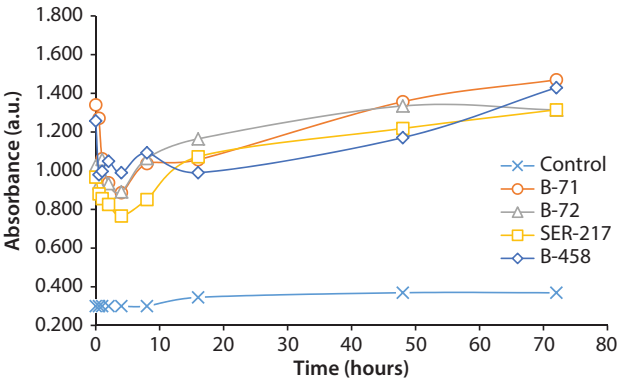

Figure 5 Release profiles for BMI (10% v/v) from different *Bacillus* strains loaded into chitosan nanoparticles.

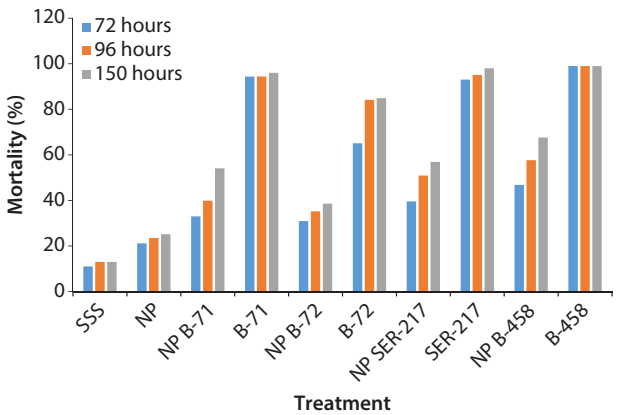

Figure 6 *In-vitro* activity of BMI (10% v/v) against *Radopholus similis* of different *Bacillus* strains encapsulated and non-encapsulated in chitosan. nanoparticles (NP) at three different incubation periods (72, 96 and 150 h). SSS indicates sterile saline solution as Negative control SSS, and NP indicates empty nanoparticles as positive control.

3.7 *In-Vitro* Effect of Nanoparticles against *Radopholus similis*

Non-chitosan-encapsulated BMI (10% v/v) induced higher mortality on *R. similis* at any evaluation period, compared with the chitosan-encapsulated BMI (Figure 6), with all of the samples reaching 100% mortality after 150 hours of incubation.

Results do not show significant differences among the free BMI from different *Bacillus* strains; on the other hand, the differences between the nanoparticle systems can reach values that differ in about 40%. This significant difference can be explain by the release profiles of the nanoparticle systems, where the polymeric matrix needs to be degraded before the BCA is available to

induce mortality of *R. similis*. Results also show that each nanoparticle system loaded with BMI that forms *Bacillus* increases its mortality against *R. similis* as a function of the time of incubation; meanwhile, the free BMI from *Bacillus* strains already reaches 100% mortality at the shortest time of incubation (72 h), suggesting the effectiveness of the nanoparticles as controlled release systems that may lead to a more sustained biological control.

Finally, for illustrative purposes, Figure 7 shows the images obtained from inverted microscopy visualization of the media containing *Radopholus similis* (Fig. 7f) treated with chitosan nanoparticles loaded with BMI from *Bacillus* strains (Figure 7a–e).

Results allow identifying the presence of dispersion of nanoparticles distributed all around the media, for samples in Figure 7b–e, associated with chitosan nanoparticles loaded with BMI from *Bacillus*. Furthermore, Figure 7a shows the system composed by empty chitosan nanoparticles, showing poor light dispersion and a decrease in nanoparticle distribution around the media, possibly depicting its low mortality rate against *R. similis*.

4 CONCLUSIONS

The BMI isolated from different strains of *Bacillus* proved to be sufficiently stable to store under refrigeration at 5 °C. The phytochemical profile analysis of the BMI from *Bacillus* both by RP-DAD-HPLC and QTRAP mass spectroscopy showed that the *Bacillus* strain B-458 has a more complex chemical composition than the other three strains of *Bacillus* analyzed, and this chemical complexity could be associated with a higher rate of mortality against *Radopholus similis*.

Production of chitosan nanoparticles loaded with different BMIs from *Bacillus* was efficient, indicating the ability of these compounds to be efficiently encapsulated in polymeric storing structures, forming active cores with a given amount of BM infiltrate compounds, which are released over time. Results also indicate that the release of encapsulated microorganisms occurs efficiently in aqueous medium, driven by a controlled diffusion process, thus promoting protection and stability of active BMI from *Bacillus* strains, and potentially allowing them a better distribution and permanence in the field.

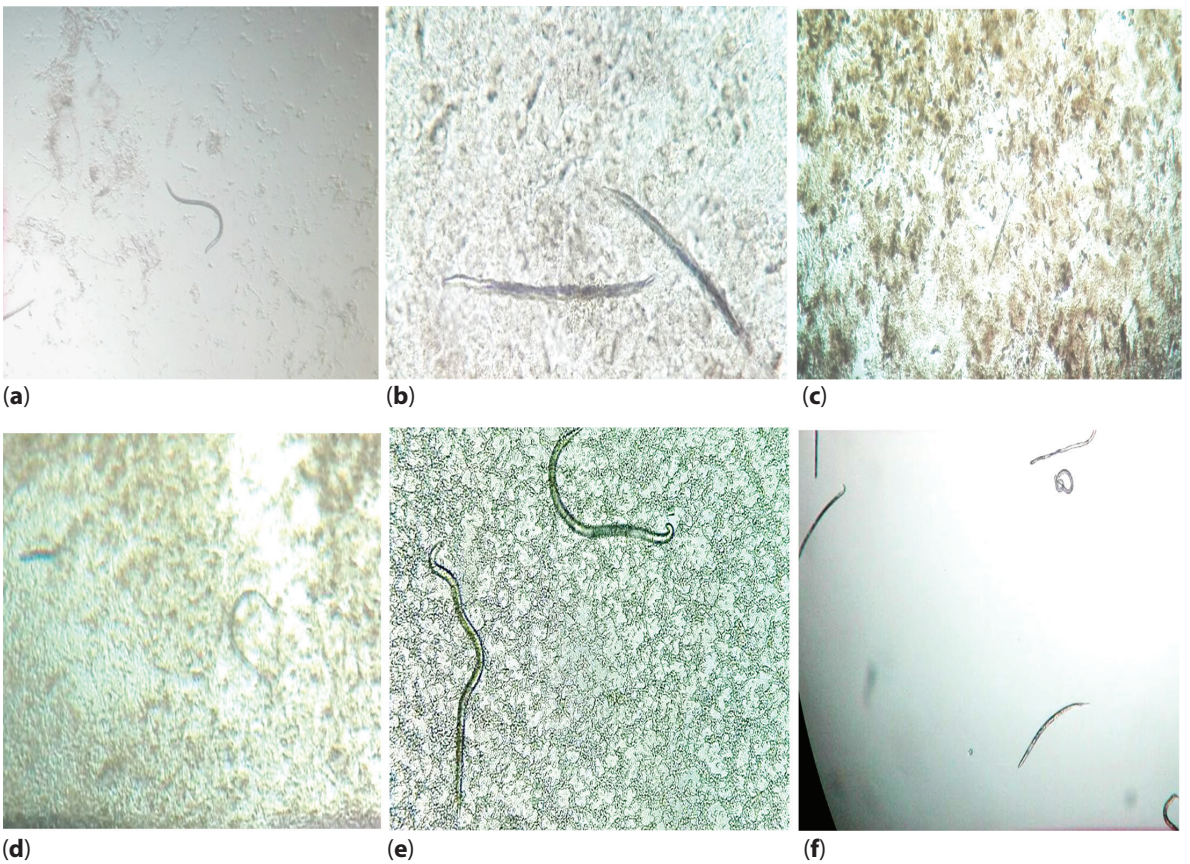

Figure 7 Inverted optical microscopy images showing the bioassays of mortality against *Radopholus similis*. (**a**) Empty chitosan nanoparticles (NP), (**b**) NP B-71, (**c**) NP B-72, (**d**) NP SER-271, (**e**) NP B-458, and (**f**) Negative control SSS (sterile saline solution).

The maximum mortality of *Radopholus similis* achieved with nanoparticles *in vitro* averaged 60% for BMI from *Bacillus* loaded into chitosan nanoparticles at a concentration of 10% v/v. Chitosan nanoparticles loaded with BMI reached lower values of mortality than free BMI by about 40-fold. However, BMI-loaded nanoparticle systems showed an increase in their mortality effect against *R. similis* as a function of time, suggesting a bioactivity driven by the controlled release of BMI from the nanoparticles core.

These bioproducts are currently being evaluated at the nursery level on banana vitro plants (Grande Naine cultivar) at the Experimental Station of CORBANA in La Rita de Guápiles, Costa Rica.

Green chemistry is necessary to generate greener inputs for agricultural production. Thus, green chemistry alternatives involving nano- and biotechnologies are vital to sustainably produce agricultural goods without continued dependence on toxic pesticides and chemicals of concern.

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