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Polymer-based encapsulation of *Bacillus subtilis* and its effect on *Meloidogyne incognita* in tomato

Encapsulación polimérica de *Bacillus subtilis* y su efecto en *Meloidogyne incognita* en tomate

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Abstract. Antagonistic bacteria used as biological control agent may loss effectiveness at the field due to environmental factors such as UV radiation, dryness and high temperature. An inexpensive alternative to protect antagonistic bacteria against such factors is the use of microencapsulating agents. In this work, the effect of microencapsulation of *Bacillus subtilis* with commercial gums on their antagonistic capacity against *Meloidogyne* incognita was evaluated. The efficiency of the microencapsulation was verified by the difference between the initial and final concentrations of protein release. The effectiveness as antagonist was evaluated against *M. incognita* in tomato under greenhouse conditions. The microcapsules based on carboxymethylcellulose (MBC) and xanthan (MBX) were morphologically different. The MBX showed a higher bacterial release efficiency (90.2%) compared to that of MBC (76.6%). Plants inoculated with MBX showed a significant decrease in galls and *M. incognita* eggs in comparison to control plants, but this decrease did not occur on those inoculated with non-microencapsulated *B. subtilis*. The application of MBX to tomato plants at transplanting time provided good protection against *M. incognita* under greenhouse conditions.

Keywords: Rhizobacteria; Plant pathogenic nematode; Ionic gelation; Microencapsulation.

Resumen. Las bacterias antagonistas usadas como agentes de control biológico pueden perder eficacia debido a diversos factores ambientales, tales como la radiación UV, sequedad y alta temperatura. Una alternativa de bajo costo para protegerlas contra estos factores es el uso de agentes microencapsulantes. En este trabajo se evaluó el efecto de la microencapsulación de *Bacillus subtilis* con gomas comerciales sobre la capacidad antagónica contra *Meloidogyne* incognita en plantas de tomate. La eficiencia de la microencapsulación se verificó por la diferencia de las concentraciones inicial y final de la proteína liberada. La efectividad de las microcápsulas como antagonista se evaluó contra *M. incognita* en tomate bajo condiciones de invernadero. Las microcápsulas a base de carboximetilcelulosa (MBC) y xantana (MBX) fueron diferentes morfológicamente. Las MBX tuvieron una eficacia de liberación bacteriana más alta (90,2%) en comparación con las MBC (76,6%). Las raíces de las plantas inoculadas con MBX tuvieron menor número de agallas y huevos de *M. incognita* en comparación con el control y las plantas inoculadas con *B. subtilis* no encapsulado. La aplicación de MBX al momento del transplante proporcionó una buena protección contra *M. incognita* bajo condiciones de invernadero.

Palabras clave: Rizobacteria; Nematodo fitopatógeno; Gelación iónica; Microencapsulación.

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INTRODUCTION

Several hundred species of nematodes are known to nourish themselves from living plants and cause a variety of diseases. These soil-borne pathogens damage a broad range of crops causing a dramatic loss of yield, especially in tropical and sub-tropical regions. Crop losses by nematodes have been estimated to exceed US$ 100 billion globally per year (Mohammed et al., 2008; Raaijmakers et al., 2009).

The nematode *Meloidogyne incognita* attacks the roots of a wide variety of plants. *Meloidogyne incognita* deforms the normal root cells, establishes giant cells, and roots become nodulated. These nodules obstruct absorbent vessels and lead to a deficiency in minerals causing wilting, stunting and chlorosis. Damage can also facilitate the entry of pathogens, such as bacteria and fungi (Agrios, 1999). *Meloidogyne incognita* is extremely polyphagous, having a host range of up to 3000 plant species on *Fabaceae, Cucurbitaceae, Rubiaceae, Brassicaceae, Myrtaceae* and *Solanaceae* (Castagnone, 2002).

The increase on costs and collateral damage caused by chemical pesticides highlights the need for using biological control agents (Killani et al., 2011). Among these agents, the bacterium *Bacillus subtilis* has been widely used as an antagonistic microorganism for soil-borne pathogens. This bacterium produces hydrolytic enzymes (e.g., glucanases, proteases) and antibiotic lipopeptides (e.g., surfactin, fengycin, and/or iturin A) capable of acting against nematodes (Cazorla et al., 2007; Knaak et al., 2007; Snook et al., 2009). Previous works have documented the effects of *B. subtillis* on *M. incognita*. In this context, Siddiqui & Mahmood (1993) showed that *B. subtilis* reduced the multiplication of larvae of *M. incognita* and the number of root galls in chickpea. Similarly, Rahman et al. (2005) observed that the application of *B. subtilis* suppressed the pathogenicity of *M. incognita* on ornamental crops. Munshid et al. (2013) and Khalil et al. (2012) found that *Bacillus subtilis* suppressed *M. incognita* infection by decreasing the nematode population and root galls on green onion and tomato plants.

Antagonistic bacteria may loss effectiveness due to environmental factors such as UV radiation, dryness and high temperature (Myasnik et al., 2001). Several protective methods have been developed to improve the efficiency and performance of antagonistic microorganisms (Saxena et al., 2002). Despite some of them have been successfully tested, their application is still pending because they increment both costs and risks of environmental contamination. An inexpensive method to protect antagonistic bacteria from such factors may include the use of polymeric gums either as carriers or microencapsulating agents (Hernández et al., 2011). This technique not only provides protection from unfavorable environments, but also improves their stability (Bregni et al., 2000; Hernández et al., 2011). Chen et al. (2013) microencapsulated *Bacillus cereus* C1L with natural polymers (maltodextrin and gum arabic), and found that the microencapsulation enhances the activity of this bacterium due to the protection from adverse conditions. Hernández et al. (2011) found that microencapsulated *B. subtilis* strains enhance biocontrol of *Rhizoctonia solani* and *Fusarium oxysporum*. The goal of this study was to evaluate the effects of microencapsulation of *Bacillus subtilis* with commercial gums on their antagonistic capacity against *Meloidogyne incognita* in tomato plants under greenhouse conditions.

MATERIALS AND METHODS

Bacterial strain. Spores of *Bacillus subtilis* strain cbrf24 were obtained from a collection kept at the Plant Pathology Laboratory of the Conkal Technological Institute, in Yucatan, México. This strain has been previously selected for its nematicidal activity against *Meloidogyne incognita* (Ruiz et al., 2014). Bacterial culture was carried out on Potato Dextrose Agar (PDA) and kept in an oven TERLAB (Model TE-E80DM) at 37 °C, until use.

Preparation of microcapsules. For microencapsulation, two different types of commercial gums were evaluated: carboxymethycellulose and xanthan. A basal encapsulation protocol was employed to prepare microcapsules by ionic gelation (Betancur et al., 2011). Briefly, 2% (w/v) gum solution (carboxymethycellulose or xanthan) was prepared by dissolving the polymer in 200 mL of *B. subtilis* spore solution ($1x10^8$ spores/mL), under low stirring at room temperature for 1h. Subsequently, the gum mixture containing *B. subtilis* was added to 250 mL of 0.15 M $FeCl_3$ (Sigma Chemical) and allowed to harden for 30 min. Hardened microcapsules were recovered by decanting, washed with distilled water and dried in a convection oven (FELISA FE-143, Serial Number: 941002) for 36 h at 40 °C.

Characterization of microcapsules. The shape and particle size of microcapsules were measured using a light microscope (Carl Zeiss, Axiostar plus Model 1169-151). Measurements were taken by placing microcapsules individually on the edge of a coverslip to record shape and diameter (Betancur et al., 2011). Ten microcapsules were measured per treatment.

The flow capacity (FC) of microcapsules was calculated by measuring the angle of repose. The microcapsules were passed through a funnel (4 mm internal diameter, 60 mm long) onto a horizontal surface to form a pile. Pile height (h) and cone base radius (r) were measured with a digital caliper, and the angle of repose (φ) was calculated from the following equation (Betancur et al., 2011):

$$\varphi = \tan^{-1}\left(\frac{h}{r}\right) \qquad (1)$$

Efficiency of microencapsulation. The efficiency of the microencapsulating process (E, %) was determined as de-

scribed by García et al. (2011). Briefly, protein concentration was calculated from the difference between the initial protein concentration (IPC) in the microencapsulating process and the final protein concentration (FPC) released from the microcapsules with 55 mM sodium citrate during a 6 h period. The initial and released concentration of protein was analyzed as described by Bradford (1976). The efficiency of the SPA microencapsulating process was calculated from the following equation:

$$E(\%)=\left(\frac{FPC}{IPC}\right) \times 100 \qquad (2)$$

Antagonistic bioassays. Tomato seeds were sown in polystyrene trays of 200 wells using Cosmopeat® as substrate. Seedlings were transplanted in 4-L capacity polystyrene pots when they reached 10 cm height. Substrate for pots was compounded by the autoclaved mixture (%v/v) of 50% soil, 30% commercial substrate (Cosmopeat®) and 20% fine grave. Suspensions of nematode eggs were added to all pots at transplanting (860 eggs/pot) as described by Cristóbal et al. (2006). After nematode inoculation, *B. subtilis* ($1x10^8$ spore/mL) suspensions were applied. Bacterial suspensions were applied as either free cells or non-microencapsulated (NMB: 5 mL), microencapsulated with carboxymethycellulose (MBC: 250 mg) or microencapsulated with xhantan (MBX: 250 mg) bacteria. Control plants had no bacterial inoculation. Treatments were set with twenty replicates (plants). Pots were arranged in a completely randomized design, and maintained in a greenhouse at 35-42 °C. Thirty and sixty days after transplanting, plant growth (plant height, stem width, number of leaves, foliar area, root volume, foliar biomass) and nematode infection (number of galls and number of eggs in the root system) were evaluated (Mohammed et al., 2008).

Statistical analysis. One-way analysis of variance and Tukey mean comparisons were performed using Statgraphics Centurion (Version 15.2.06). Data were expressed as means and standard errors. Means were considered significantly different at $P<0.05$.

RESULTS

Characterization of microcapsules. Carboxymethylcellulose-based microcapsules (MBC) showed a spherical shape, while xanthan gum-based microcapsules (MBX) showed an amorphous shape (Fig. 1). MBX showed an ovoid form with a smooth surface, which became amorphous after drying it. The angle of repose by MBC and MBX was 33.69° and 35.31°, respectively. No significant difference ($P>0.05$) was observed in size between both types of microcapsules (Fig. 1).

The efficiency of release of *B. subtilis* was 76.6 ± 0.7% (13 µg/mL) for microcapsules formed with MBC, and 90.2 ± 1% (15.2 µg/mL) with those formed with MBX (Fig. 2).

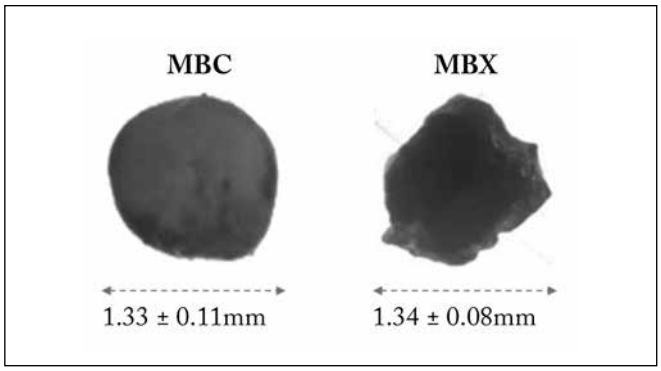

Fig. 1. Morphology and size of *Bacillus subtilis* microcapsules: carboxymethylcellulose-based microcapsules (MBC) and xanthan-based microcapsules (MBX).
Fig. 1. Morfología y tamaño de las microcapsulas de *Bacillus subtilis*: Microcápsulas a base de carboximetilcelulosa (MBC) y a base de xantana (MBX).

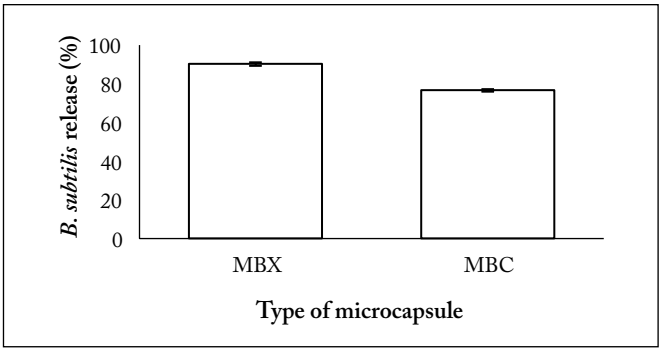

Fig. 2. Release efficiency of *Bacillus subtilis* from microcapsules formed with either carboxymethylcellulose (MBC) or xanthan (MBX).
Fig. 2. Eficiencia de liberación de *Bacillus subtilis* de las microcápsulas formadas con goma carboximetilcelulosa (MBC) y xantana (MBX).

Plant growth. To evaluate the effects of *B. subtillis* containing microcapsules on suppression of *M. incognita* in tomato, stem diameter, plant height, leaf number and area, and dry biomass were determined. In this context, stem diameter, plant height, number of leaves and leaf area showed no significant differences ($P>0.05$) among treatments (Table 1). Effect of inoculation on plant growth was only significant ($P<0.05$) between plants inoculated with non-microencapsulated *B. subtilis* (NMB) and those microcapsulated with MBX, where root volume at 60 days after transplanting was 26.5% higher in plants treated with NMB (Table 1).

Microencapsulation of *B. subtilis* showed no effect ($P>0.05$) on dry biomass of root, stem and leaves (Table 2).

Severity of damage by *Meloidogyne incognita*. Evaluation of the severity of *M. incognita* damage to tomato root (Fig. 3) showed a significant decrease ($P<0.05$) in the number of galls per gram of root in plants inoculated with *B. subtillis* relative

Table 1. Growth characteristics of tomato plants inoculated with *B. subtilis* at 30 and 60 days after transplanting.
Tabla 1. Características de crecimiento de las plantas de tomate inoculadas con *B. subtilis* a los 30 y 60 días después del transplante.

Treatment	Days after transplanting	Stem diameter (mm)	Plant height (cm)	Root volume (cm^3)	Number of Leaves	Total leaf area (cm^2)
Control	30	8.6 ± 0.7 a	39.3 ± 5.3 a	39.3 ± 5.4 a	150.6 ± 14.6 a	732.2 ± 127.3 a
NMB	30	8.1 ± 0.7 a	37.0 ± 2.9 a	37.0 ± 2.9 a	197.0 ± 99.6 a	780.0 ± 155.7 a
MBC	30	7.9 ± 0.7 a	40.2 ± 2.7 a	40.2 ± 2.7 a	151.2 ± 10.8 a	755.9 ± 121.7 a
MBX	30	8.2 ± 0.4 a	39.8 ± 2.1 a	39.8 ± 2.1 a	157.3 ± 24.5 a	813.0 ± 83.8 a
Control	60	12.9 ± 1.6 a	48.5 ± 10.5 a	43.0 ± 5.0 ab	496.4 ± 146.5 a	2173.4 ± 270.7 a
NMB	60	12.9 ± 0.8 a	52.1 ± 8.3 a	58.5 ± 8.1 b	463.9 ± 101.4 a	2552.5 ± 501.8 a
MBC	60	12.3 ± 1.5 a	49.8 ± 8.8 a	36.2 ± 15.8 a	453.4 ± 88.4 a	2116.4 ± 650.7 a
MBX	60	13.1 ± 1.4 a	48.9 ± 10.5 a	44.4 ± 13.5 ab	431.0 ± 114.2 a	1802.0 ± 271.7 a

Means with different letters in the same column after either 30 or 60 days from transplanting are significantly different (P<0.05). MBC: Microencapsulated *B. subtilis* with carboxymethylcellulose gum; MBX: Microencapsulated *B. subtilis* with xanthan gum; NMB: Non-microencapsulated *B. subtilis*.
Las medias con letras diferentes dentro de una misma columna después de 30 ó 60 días del transplante son significativamente diferentes (P<0,05). MBC: *B. subtilis* microencapsulado con goma carboximetilcelulosa; MBX: *B. subtilis* microencapsulado con goma xantana; NMB: *B. subtilis* no microencapsulado.

Table 2. Dry biomass (g) production in tomato plants inoculated with *B. subtilis* at 30 and 60 days after transplanting.
Tabla 2. Producción de biomasa seca (g) de las plantas de tomate inoculadas con *B. subtilis* a los 30 y 60 días después del transplante.

Treatments	Days after transplanting	Roots	Stem	Leaves
Control	30	2.1 ± 0.5 a	3.0 ± 0.8 a	3.4 ± 0.8 a
NMB	30	1.9 ± 0.4 a	2.9 ± 0.7 a	3.3 ± 0.7 a
MBC	30	2.0 ± 0.3 a	3.4 ± 0.8 a	3.6 ± 0.7 a
MBX	30	2.4 ± 0.8 a	3.5 ± 0.8 a	3.5 ± 0.7 a
Control	60	5.5 ± 0.9 a	13.1 ± 3.3 a	10.8 ± 0.7 a
NMB	60	5.8 ± 1.0 a	13.9 ± 3.9 a	13.3 ± 4.7 a
MBC	60	4.0 ± 2.2 a	9.0 ± 5.1 a	10.6 ± 5.6 a
MBX	60	4.5 ± 1.3 a	9.9 ± 3.6 a	10.3 ± 3.2 a

Means with different letters within the same column after either 30 or 60 days from transplanting are significantly different (P<0.05). MBC: Microencapsulated *B. subtilis* with carboxymethylcellulose gum; MBX: Microencapsulated *B. subtilis* with xanthan gum; NMB: Non-microencapsulated *B. subtilis*.
Las medias con letras diferentes dentro de una misma columna después de 30 ó 60 días desde el transplante son significativamente diferentes (P<0,05). MBC: *B. subtilis* microencapsulado con goma carboximetilcelulosa; MBX: *B. subtilis* microencapsulado con goma xantana; NMB: *B. subtilis* no microencapsulado.

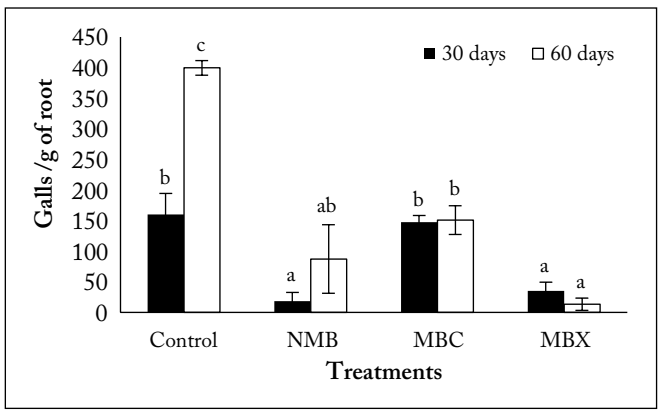

Fig. 3. Effects of *Bacilus subtilis* inoculation on gall formation by *M. incognita* in tomato plants at 30 and 60 days after transplanting. Values followed by the same letters are not statistically different (Tukey, P<0.01). MBC: carboxymethylcellulose-based microcapsules of *B. subtilis*; MBX: xanthan-based microcapsules of *B. subtilis*; NMB: Non-microencapsulated *B. subtilis*.
Fig. 3. Efectos de la inoculación con *B. subtilis* sobre la formación de agallas de *M. incognita* en plantas de tomate a los 30 y 60 días después del transplante. Valores con letras iguales no son estadísticamente diferentes (Tukey, P<0,01). MBC: *B. subtilis* microencapsulado con goma carboximetilcelulosa; MBX: *B. subtilis* microencapsulado con goma xantana; NMB: *B. subtilis* no microencapsulado.

to that on the control plants. Plants treated with either NMB or MBX, but not those with MBC, showed a lower number of galls per gram of root relative to control plants after 30 days from transplanting (Fig. 3). At 60 days from transplanting, the order in the number of galls (g/root) was control > MBC > MBX (P<0.05) (Fig. 3).

After 60 days from transplanting, the number of *M. incognita* eggs per gram of root showed a significant decrease (P<0.05) in plants inoculated with microencapsulated or non-microencapsulated *B. subtilis* relative to that on control plants (Fig. 4). The effect of MBX was significantly higher (P<0.05) than that of MBC and the control on the number of eggs per gram of root.

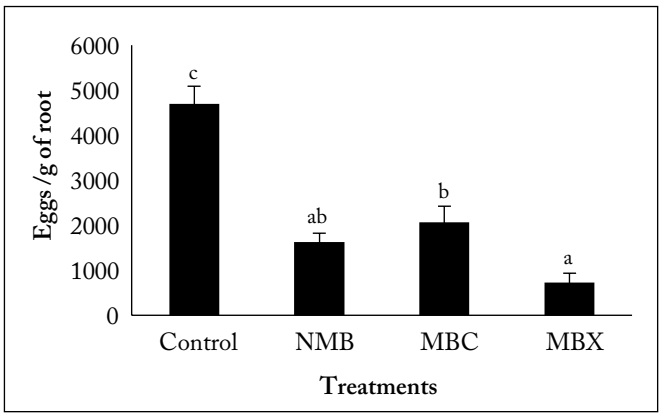

Fig. 4. Effects of inoculation of *B. subtilis* on *M. incognita* eggs in tomato root at 60 days after transplanting. Values followed by the same letters are not statistically different (Tukey, P<0.01). MBC: carboxymethylcellulose-based microcapsules of *B. subtilis*; MBX: xanthan-based microcapsules of *B. subtilis*; NMB: Non-microencapsulated *B. subtilis*.

Fig. 4. Efectos de la inoculación con *B. subtilis* sobre el número de huevos de *M. incognita* en raíz de tomate a los 60 días después del transplante. Valores con letras iguales no son estadísticamente diferentes (Tukey, P<0.01). MBC: *B. subtilis* microencapsulado con goma carboximetilcelulosa; MBX: *B. subtilis* microencapsulado con goma xantana; NMB: *B. subtilis* no microencapsulado.

DISCUSSION

In the present work, polymer-based microencapsulation of *B. subtillis* was carried out in an attempt to protect the bacterial spores and increase effectiveness on suppression of *M. incognita* infection in tomato. Data showed that carboxymethylcellulose-based microcapsules and xanthan-based microcapsules had similar particle diameter, but different particle shape; MBC had spherical form, while MBX was amorphous. The difference in particle shape may be attributed to the type of polymer used (Burkersroda et al., 2002; Kumar et al., 2002). Particle shape is critical for delivering the active ingredient. A previous study showed that irregularly shaped microcapsules have more porous in internal structure, which enhances the microorganism delivery (Jaya et al., 2010). This might have contributed to the higher efficiency in protein release by MBX (90.2%). In this sense, our data agree with those of García et al. (2011), who showed a similar protein release (89%) from sodium alginate-based microcapsules of *Bacillus thuringiensis*.

We observed that inoculation with microencapsulated *Bacillus subtilis* showed no significant effect on most of the plant growth variables. Also, these plants showed slight damage by *M. incognita* (Fig. 3). The slight root damage by nematodes might have caused a compensatory root growth as response to nematode invasion. This response has been well documented in tomato and cotton by Ma et al. (2013). Likewise, Haase et al. (2007) reported that low levels of *M. incognita* infection cause elongation of lateral roots, mainly as a response to wounding and stress by the host plant. The physiological reaction to nematode attack may involve an increase in the production of phytohormones and ethylene, which are critical in the formation and elongation of root hairs (Nagata et al., 2004; Overvoorde et al., 2010).

The severity of root damage by *M. incognita* was reduced when plants were inoculated with MBX. Our results showed that the number of galls per root as well as the number of eggs per gram of root decreased significantly in plants inoculated with MBX. The reduction in numbers of galls and eggs reached a maximum of 96.7% and 84.6%, respectively. The effect of MBX was higher than that reported by other authors that have previously evaluated the effects of *Bacillus* spp on *M. incognita* in tomato. For example, Almaghrabi et al. (2013) found a decrease of 32% in the number of galls, and 52% in that of eggs per gram of root after application of *B. subtilis*. Likewise, Mohammed et al. (2008) reported a reduction of 52% in the number of galls, and 84% in the number of eggs per gram of root after applying *B. thuringiensis* strain Bt7N.

We showed that polymer-based microencapsulation of *B. subtilis*, particularly with xanthan gum, increased the antagonistic effect against *M. incognita* in tomato under greenhouse conditions. The application of this technology may have enormous potential to enhance the effectiveness of *B. subtilis* as a biological control agent for soil-borne pathogens.

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