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Carrier and vector of *Pectobacterium carotovorum* subsp. *carotovorum* and its handling through a base of entomopathogenic fungi in *Agave* sp.

Teodulfo Aquino-Bolaños^{1,*}, José A. Sánchez-García¹, Yolanda D. Ortiz-Hernández¹, Julián Hernández-Cruz², and Carlos I. Cortés-Martínez

Abstract

The presence of the carrier and vector of *Pectobacterium carotovorum* (Jones) (Enterobacteriaceae) was found in *Agave potatorum* Zucc. (Asparagaceae) (larvae and adults of *Scyphophorus acupunctatus* Gyllenhaal [Coleoptera: Curculionidae], larvae of *Drosophila melanogaster* Macquart [Diptera: Drosophilidae] and *Hermetia illucens* [L.] [Diptera: Stratiomyidae], and adults of *Hololepta* sp. [Coleoptera: Histeridae]) in Oaxaca, Mexico. Entomopathogenic fungi have been used as control methods to prevent the development of bacteria under laboratory conditions. After 5 d of inoculation, all the existing insects in the agave plants exhibited 98% of the countable growth of the bacterium, which led to the determination that the 4 species of insects are carriers and vectors of *P. carotovorum*. According to the final growth of the bacterium and the entomopathogenic fungi, the *Beauveria bassiana* (Bals.-Criv.) Vuill. (Cordycipitaceae) (E) strain at a concentration of 1.0×10^4 spores per mL showed a difference of 57.2 mm. For this reason, it was considered the best strain to disable the growth of *P. carotovorum*.

Key Words: agave weevil; *Scyphophorus acupunctatus*; *Beauveria bassiana*; *Metarhizium anisopliae*

Resumen

Se determinaron insectos portadores y vectores de la bacteria *Pectobacterium carotovorum* presentes en plantas de *Agave potatorum* Zucc. (larvas y adultos de *Scyphophorus acupunctatus*, larvas de *Drosophila melanogaster* y *Hermetia illucens*, y adultos de *Hololepta* sp.), y como métodos de control se utilizaron hongos entomopatógenos para inhibir el desarrollo de la bacteria en condiciones de laboratorio. A los 5 días de la inoculación todos los insectos presentes en las plantas de agave presentaron un 98% de crecimiento contable de la bacteria, por lo que se determinó que las cuatro especies de insectos son portadores y vectores de *P. carotovorum*. De acuerdo al crecimiento final de la bacteria y los hongos entomopatógenos, la cepa *Beauveria bassiana* (E) a una concentración de 1.0×10^4 esporas por mL presentó una diferencia de 57.2 mm por lo que se considera como la mejor cepa que inhibió el crecimiento de *P. carotovorum*.

Palabras Claves: picudo del agave; *Scyphophorus acupunctatus*; *Beauveria bassiana*; *Metarhizium anisopliae*

The most serious problem affecting the cultivation of agave in Oaxaca, Mexico, is the putrefaction of the heart of the plants, known as bacterial spot, caused by the *Pectobacterium carotovorum* (Jones) (Enterobacteriaceae) bacterium. The Consejo Regulador de Tequila (CRT 1999) estimated that 22.3% of the 203,000,000 *Agave tequilana* Weber (Asparagaceae) plants cultivated were damaged to different degrees by this bacterium. In Oaxaca, Mexico, *P. carotovorum* is observed principally during rainy seasons; the damage starts with a small round dark brown injury (0.5 to 2.0 cm diam) that grows in an irregular manner (Arredondo & Espinosa 2005). There is little research about the life cycle of the bacteria in agave. Aquino et al. (2009) concluded that the bacterium is not present in the soil or in the young leaves. For this reason, it is important to know which organisms are in direct contact with the agave, and which could be the carriers or vectors of *P. carotovorum*.

Today the unlimited use of chemical products for handling and control of plants and insects has caused illnesses to plants of economic interest such as agave, and the bacteria have become resistant. As

an alternative, it has been proposed that biological control be used rather than of pesticides. Many pathogens are known to be natural enemies and can cause epizootic (high levels) of illness in host populations (Hajek 2004).

Entomopathogenic fungi are an alternative for this practice, because they are microorganisms with alternative strategies which harm neither the agroecosystem nor the beneficial fauna or human beings (Alatorre & Guzmán 1999). The principal entomopathogenic fungi are *Paecilomyces fumosoroseus* (Wize) Br. & Sm. (Cordycipitaceae), *Metarhizium anisopliae* (Metschn.) Sorokin (Clavicipitaceae), *Beauveria bassiana* (Bals.-Criv.) Vuill. (Cordycipitaceae), and *Verticillium lecanii* (Zimm.) Viégas (Cordycipitaceae).

The objective of this research was to determine the carrier and vector insects of *P. carotovorum* bacterium, and their proper handling based on the isolated entomopathogenic fungi of *Scyphophorus acupunctatus* Gyllenhaal (Coleoptera: Curculionidae) adults in plants of *Agave* sp. (Asparagaceae).

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Materials and Methods

DETERMINATION OF CARRIERS AND VECTOR INSECTS OF *PECTOBACTERIUM CAROTOVORUM* PRESENT IN PLANTS OF AGAVE

To determine the vector and carrier insects of *P. carotovorum* present in the agave plants in the community of Sola de Vega, Oaxaca, Mexico, we used agave tobalá (*Agave potatorum* Zucc.; Asparagaceae) plants with a damage level of 4 to 5 (Aquino et al. 2007). At the end, 20 plants of *A. potatorum* and 100 insects were assessed for the study. The collected insects (larvae and adults) were gathered from the stems and leaves of the *A. potatorum* plant with Bio Star entomologic pliers (BONSTAR, Puebla, México). The insects were placed in transparent 300 mL containers (BONSTAR, Puebla, México) for transport to the entomology lab at the Interdisciplinary Center of Research, Integral Regional Development Campus, Oaxaca, Mexico, according to the protocol described by Arredondo & Espinosa (2005).

The tests were done in vitro using the colony growth of bacteria method and the BD Bioxion growth medium MacConkey agar (DIFCO, Madrid, Spain), which was poured into 9 cm diam glass Petri dishes. The bodies of the larvae and adults were scraped with a platinum handle and were cultivated in the BD Bioxion culture medium. Then the larvae and the adults were disinfected with 70% ethyl alcohol, and a longitudinal cut was made in the bodies. A sample was taken from the internal part of the bowel, which also was cultivated in the cultivation medium following the method of Fucikovsky (1999). These were left in place for 6 d at a temperature of 23 ± 2.4 °C, and the corresponding observations were done every d. We conducted 7 repetitions of each assessed insect.

The growth assessment of the colonies of bacteria was done in a qualitative way through the scale proposed by Berta & Loretta (1998). The levels were as follows: +++ = intense growth; ++ = normal growth; + = countable growth; – = no growth.

EFFECT OF THE ENTOMOPATHOGENIC FUNGI FOR THE CONTROL OF *PECTOBACTERIUM CAROTOVORUM* UNDER LABORATORY CONDITIONS

For this assessment, we used 6 strains of native entomopathogenic fungi assessed at a concentration of 1.0×10^4 spores per mL obtained from adults of *S. acupunctatus* in *A. potatorum* plants. To determine the effect of the bacterium and entomopathogenic fungi, *B. bassiana* (A, B, C, D, and E) and *M. anisopliae* (A), the tests were conducted in vitro by cooling method of the colonies. We used MacConkey agar and Dextrose Sabouraud (DIFCO, Madrid, Spain) agar cultivation media mixed at 50% solution. The cultivation medium was poured into 9 cm diam glass Petri dishes. Afterwards, on the sides or ends of the boxes with agar medium, a colony of the bacterium and fungi were cultivated, which were incubated for 13 d at a temperature of 23 ± 2.4 °C (Fig. 1). We used 7 Petri dishes per treatment according to the protocol described by Pozo et al. (2006).

We used a Knova® Vernier (Add To My Toolbox, Company Industrial Vernier, Distrito Federal, México) with 0.1 mm precision to measure the growth of the bacterium and the fungus. Observations were done for 13 d every 24 h. A count of spores was done using a Neubauer camera. (Velaquin, Distrito Federal, México.)

The data obtained in this experiment were subjected to a Variance of analysis (ANOVA) using the Statistical Analysis System (SAS 2017).

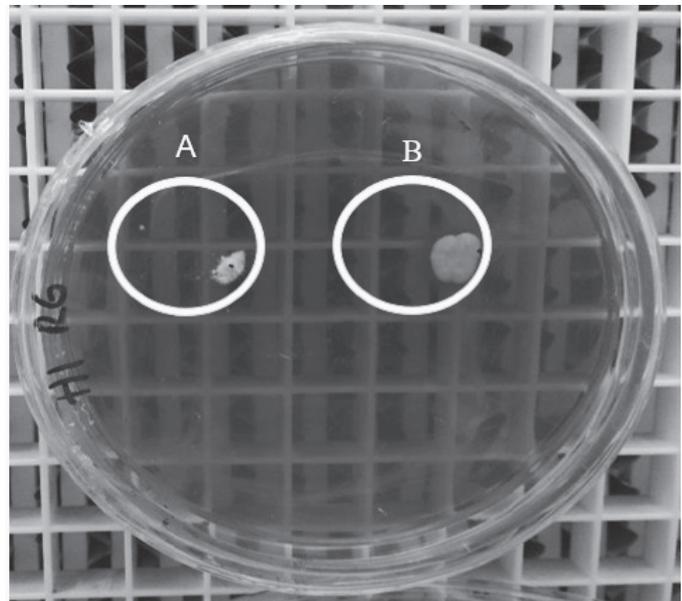


Fig. 1. (A) Growth of the *Beauveria bassiana* bacterium; (B) Growth of the *Pectobacterium carotovorum* bacterium.

Results

VECTOR AND CARRIER INSECTS OF *PECTOBACTERIUM CAROTOVORUM* PRESENT IN AGAVE PLANTS

We found an internal and external countable growth for the insect *Scyphophorus acupunctatus* in larvae and adults after 48 and 72 h (Fig. 2). After 96 and 120 h, we found an intense growth, because this insect was the carrier and vector of the bacterium of the putrefactions (*P. carotovorum*). When the insect feeds from the leaves and the heart of the agave, it ingests the bacterium and transmits it to the healthy plants. These results confirm the results obtained by Fucikovsky (1999), Rubio-Cortés (2007), and Aquino (2011) who determined that when the adults of *S. acupunctatus* tunnel into the tissues of the agave plant, they transmit the bacterium they carry in their bodies, causing severe phytosanitary problems to the plant of *A. potatorum*.

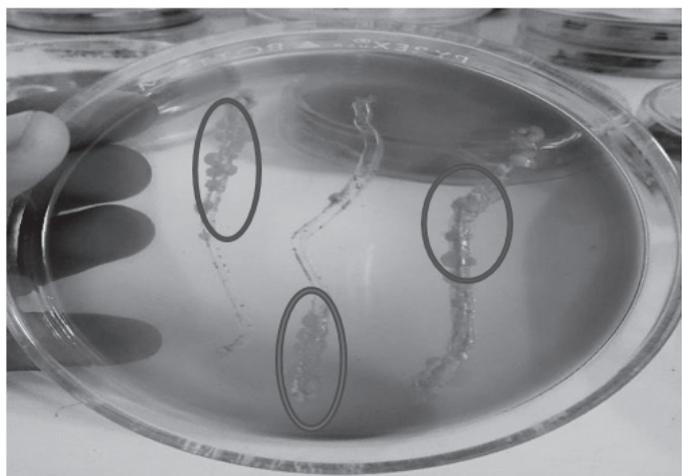


Fig. 2. Countable growth of the *Pectobacterium carotovora* bacterium in a BD Bioxion cultivation medium (MacConkey agar).

Table 1. Growth of *Pectobacterium carotovorum* bacterium in a BD Bioxion cultivation medium in collected insects in agave plants.

Hours (h)	<i>Scyphopphorus acupunctatus</i> larvae		<i>Scyphopphorus acupunctatus</i> adults		<i>Hermetia illucens</i> larvae		<i>Drosophila melanogaster</i> larvae		<i>Hololepta</i> adults	
	External	Internal	External	Internal	External	Internal	External	Internal	External	Internal
24	-	-	-	-	-	-	-	-	-	-
48	+	+	-	+	-	-	-	+	-	-
72	+	+	-	+	-	+	+	+	-	-
96	++	++	+	++	+	++	++	++	+	+
120	+++	+++	++	+++	+	+++	+++	+++	++	++

+++ = intense growth, ++ = normal growth, + = countable growth, - = no growth

Table 1 shows that for the larvae of *Drosophila melanogaster*, the larvae of *Hermetia illucens* and adults of *Hololepta* sp. showed a countable growth of the bacterium *P. carotovora* after 96 h, and after 120 h for these same insects the growth was intense, because these insects are also carriers and vectors of the *Pectobacterium carotovorum*.

EFFECT OF THE ENTOMOPATHOGENIC FUNGI FOR THE HANDLING OF PECTOBACTERIUM CAROTOVORUM UNDER LABORATORY CONDITIONS

It was discovered that the bacterium *P. carotovora* showed a faster growth. After 24 h it reached from 2 to 5 mm in a linear manner. During this same period of time, the *B. bassiana* and *M. anisopliae* entomopathogenic fungi did not show any growth in the cultivation medium. A statistical difference between the growths of the bacterium regarding the 6 strains of the assessed fungi was found. After 96 h, the *P. carotovorum* bacterium reached a growth of 9 to 10 mm. During this same time period, all the assessed strains of the *B. bassiana* fungi (A, B, C, D, and E) reached a similar growth of 7.5 to 10.3 mm, which was different from *M. anisopliae* which showed the highest growth at 16.1 mm. The strains previously mentioned did not have any statistical difference in the presence of the bacterium. After 192 h, there was an exponential growth of the *B. bassiana* (E) fungi at 40.8 mm, and from the *M. anisopliae* fungus at 40.3 mm, exceeding the bacterium by more than 20 mm (Table 2). After 192 h, the bacterium did not show any growth due to the sporulation of all the strains. This proved that the mycelium could spread throughout the cultivation medium. In this way, the entomopathogenic fungi started to take over and stop the growth of the bacterium, because the growth became slower. In most of the samples the growth of the bacterium was totally inhibited. Finally, af-

ter 288 h, all the fungi reached their highest growth, development of the bacterium colonies ceased and, in some cases, the colonies were covered completely (Figs. 3, 4).

Discussion

Basset et al. (2000) say that the *P. carotovorum* bacterium is found in the inner part of the larvae, activating their immune answer system, which is why its role in the dissemination of the bacterium is unknown. Owing to the absence of the bacterium in the soil or in the dry leaves (Aquino et al. 2009), there are insects associated with the spread of the bacterium. Martínez-Ramírez (2011) mentions that *H. illucens* is an insect that helps the dissemination of the *P. carotovorum* bacterium. Finally, Kloepper et al. (1979) reported that the insects of the Diptera order have been associated with the transmission of the illness; the percentage of insects contaminated with the bacterium was 1.0 to 6.1%.

Today there are few works on the growth inhibition of the phytopathogenic bacteria. Aquino et al. (2009), whose research was done under lab conditions, determined that the agrochemicals Agrimicin and sulphur (Bayer CropScience, Whippany, New Jersey, USA) could neither inhibit nor control the *P. carotovorum* colonies more than 90%. They also determined that the *P. carotovorum* bacterium does not show any effect on the populations of entomopathogenic fungi. Rodríguez et al. (2006) say that there is a type of biological control using antagonist microorganisms, which interfere with other pathogenic microorganisms by limiting their development, or by developing themselves on the colonies of the bacteria and blocking their development, and in

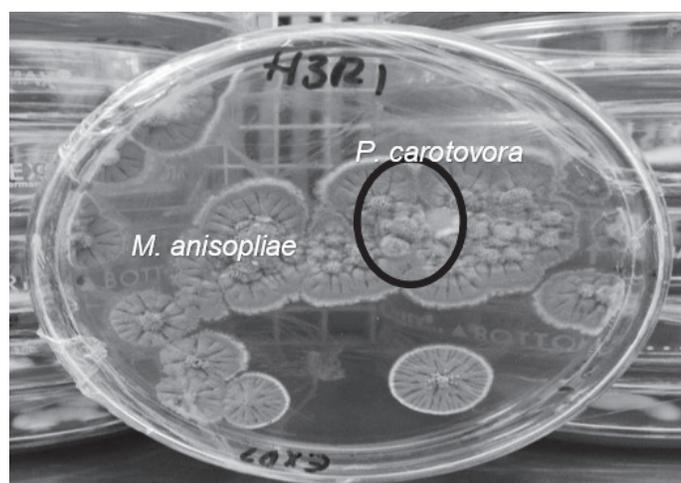


Fig. 3. Massive growth of the *Metarhizium anisopliae* fungus on the *Pectobacterium carotovora* bacterium.



Fig. 4. Massive growth of the *Beauveria bassiana* fungus on the *Pectobacterium carotovora* bacterium.

Table 2. Effect of the entomopathogenic fungi for the handling of the *Pectobacterium carotovorum* bacteria in laboratory conditions.

Hours (h)	Organism	<i>Beauveria bassiana</i> (A) + <i>Pectobacterium carotovorum</i>		<i>Beauveria bassiana</i> (B) + <i>Pectobacterium carotovorum</i>		<i>Metarhizium anisopliae</i> (A) + <i>Pectobacterium carotovora</i>		<i>Beauveria bassiana</i> (C) + <i>Pectobacterium carotovorum</i>		<i>Beauveria bassiana</i> (D) + <i>Pectobacterium carotovorum</i>		<i>Beauveria bassiana</i> (E) + <i>Pectobacterium carotovorum</i>	
		4.3 a	0.0 b	3.4 a	0.0 b	4.7 a	0.0 b	4.1 a	0.0 b	4.0 a	0.0 b	4.3 a	0.0 b
24	Bacterium	4.3 a	0.0 b	3.4 a	0.0 b	4.7 a	0.0 b	4.1 a	0.0 b	4.0 a	0.0 b	4.3 a	0.0 b
	Fungi	9.3 a	8.0 a	9.2 a	9.8 a	10.3 a	16.1 a	9.8 a	9.8 a	9.8 a	9.8 a	9.34 a	9.34 a
96	Bacterium	8.0 a	10.5 b	9.81 a	13.2 b	16.1 a	11.6 b	7.5 a	8.4 a	8.4 a	8.4 a	10.35 a	10.35 a
	Fungi	31.4 a	11.2 b	19.5 a	14.9 b	40.3 a	11.6 b	11.8 b	12.0 b	12.0 b	12.0 b	11.2 b	11.2 b
192	Bacterium	11.2 b	52.3 a	14.9 b	36.4 a	11.6 b	46.6 a	21.1 a	24.1 a	24.1 a	24.1 a	40.8 a	40.8 a
	Fungi	52.3 a		36.4 a		46.6 a		13.6 b	12.8 b	12.8 b	12.8 b	11.5 b	11.5 b
288	Bacterium							45.4 a	37.8 a	37.8 a	37.8 a	62.6 a	62.6 a
	Fungi												

Values in each column followed by different letters indicate difference between treatments; Tukey (0.05).

some cases covering them completely. Some of these microorganisms is the fungi which have been described as biological control agents of illnesses in crops (Ezziyiani et al. 2006).

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