SUSTAINABLE AND INTEGRAL EXPLOITATION OF AGAVE

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BIOCONTROL OF SOFT ROT AGAVE BY BACTERIOPHAGES

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ABSTRACT

Cultivation of several species of the genus *Agave* is used as raw material for the production of beverages such as *A. tequilana* and *A. cupreata*. However, the cultivation of these species is threatened by pests and phytosanitary problems, among them are the weevil (*Scyphophorus acupunctatus*), wilt (*Fusarium oxysporum*) and soft rot (several bacterial species). Especially rot causes great losses in agave fields for the production of tequila and mezcal in the states of Jalisco and Michoacán in Mexico. Results of chemical control of soft rot have not been successful. On the other hand, bacterial viruses (bacteriophages) are employed for efficient fight off bacterial diseases in plants. So the aim of this work was to isolate and characterize bacteriophages associated with the causal agent of soft rot for *A. tequilana* and *A. cupreata*. Samples of crops with agave soft rot were performed on sites of Jalisco and Michoacán. Bacteriophages were isolated from phytopathogenic and virulent bacterial isolates and were characterized by transmission electron microscope and restriction patterns. Three morphotypes of bacteriophages were isolated from four bacterial species involved in soft rot of agave. The bacteriophages lysed differential magnitude the bacterial cultures *in vitro*. These results suggest the possible application for biocontrol of bacteria involved in soft rot of agave, and this would help to the sustainable production of the raw material for tequila and mezcal.

Keywords: Bacterial viruses, *Bacillus* sp, Plant pathogenic bacteria.

INTRODUCTION

Agave tequilana Weber var. Azul is the plant from which tequila is obtained, booze representative of Mexico, where more than 100000 hectares of agave are currently grown in the states of Jalisco, Nayarit, Michoacán, Guanajuato and Tamaulipas.Similarly, *A. cupreata* is an important plant for the production of mezcal in Michoacán (Martínez-Palacios y col. 2011). The propagation of blue agave is mainly through asexual reproduction however, this

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monoculture system has had serious phytosanitary consequences for the tequila industry, increasing the incidence of diseases caused by fungi such as *Fusarium oxysporum* (Vega-Ramos et al. 2013) and bacteria *Pectobacterium*, *Pantoea*, *Pseudomonas*, *Bacillus*, *Arthrobacter* and *Streptomyces* (Rincón-Enríquez et al. 2014; Jiménez-Hidalgo et al. 2004), causing losses estimated in around 40 percent of agave plantations affected by these microorganisms. Presently, the use of copper-based agrochemicals and antibiotics are the main tools used by the producers of agave to combat these pests. However, the use of bacteriophages to control bacterial diseases has been in recent years a field of great interest in the area of plant health with a great potential to replace the use of currently used agrochemicals. For example, in other bacterial pathosystems, bacteriophages have been used with successful results (Frampton y col. 2012). For example in the pathosystem apple (*Malus domestica*) - fire blight (*Erwinia amylovora*), bacteriophages were isolated and assessed successfully decreasing the symptoms of blight in apple by applying the virus (Gill y col. 2003.). Also, in the pathosystem tomato (*Solanum lycopersicum*) - bacterial wilt (*Ralstonia solanacearum*) application of bacteriophages in plants inoculated with *R. solanacearum* showed a significant decrease of the disease (Fujiwara y col. 2011). Bacteriophages can be used effectively as part of integrated management strategies for plant diseases. Also, the use of these presents several advantages over the use of copper compounds and antibiotics, because of the relative low manufacturing cost in addition to the high specificity for the host, which makes them good candidates for use as biocontrol agents (Jones y col. 2007). Therefore, the objective of this work was to isolate and characterize specific bacteriophages of pathogenic bacteria associated with soft rot bud in *A. tequilana* and *A. cupreata.*

METHODOLOGY

Collection of soil samples

183 soil samples (20 cm deep) from the base of healthy agaves and rot symptoms were collected, from different sampling points located in the states of Michoacán (8 sites) and Jalisco (6 sites).

Bacterial host strains

For isolation of bacteriophages 11 bacterial isolates obtained from diseased plants from the same sampling sites (named BV) were used. Bacterial isolates were characterized and analyzed to identify virulence factors such as the production of cellulase enzymes. The genera to which belonged some of these bacterial isolates were: *Pantoea*, *Pseudomonas*, *Bacillus*, *Arthrobacter* y *Streptomyces* (Rincón-Enríquez et al. 2014).

Isolation, propagation and characterization of bacteriophages

The isolation, propagation and characterization of bacteriophages was conducted following the procedure described by Solís et al. (2014). Briefly, this procedure consisted of: isolation which was performed from soil samples by enrichment by using 100 g of soil sample in 100 mL of nutrient broth and 600 µL of each of the bacterial cultures (16 h/ 30°C) and incubated for 18-24 h at 30°C. Subsequently, cultures were centrifuged at 10,000 g × 20 min and supernatants were recovered and filtered through 0.22 µm membrane. The presence of phages was confirmed by a test double-layer soft agar by mixing 100 µL of bacterial culture, 100 µL of filtered supernatant enrichment and 5 mL of nutrient agar (0.7%), incubated at 30°C for 18 h and examined for the presence of lysis areas or plaques. After single plaques were isolated and propagated in liquid medium until high viral titers (>$1x10^8$ plaque forming

units). Pure bacteriophages were used to test the range of infection and with these data a cluster analysis was performed using the Unweighted Pair Group Method algorithm using Arithmetic averages (UPGMA) through the statistical program StatGraphics Centurion XV. Subsequently, purification of the viruses was performed by centrifugation (70-80000Xg 4-6 h) and washing (0.1 M ammonium acetate pH 7) for subsequent analysis of bacteriophages in a transmission electron microscope (TEM) Jeol JEM-1010 (grid Fombar-coal coated copper 300 mesh, stained with 5 µL uranyl acetate).

RESULTS AND DISCUSSION

183 soil samples were collected, of which 77 were tested, and 40 virus isolates were obtained, all clearly showed the formation of plaques which correspond to the replication cycle of lytic bacteriophages, also a high diversity of phages was observed on the size of the plaques formed in double plate assays (Figure 1 A, B, C and D). Cluster analysis of infection-rank test showed two aspects: three viral isolates from 648 and 649 strains were able to lyse up to 8 different bacterial strains, and these were of broad spectrum (Figure 1F); while on the side of the bacterial species, the 648 strain was the one that showed higher susceptibility to infection, as 20 viral isolations lysed this strain (Figure 1G). Moreover, the large number of isolates were obtained in soil samples collected from agave plants with soft rot.

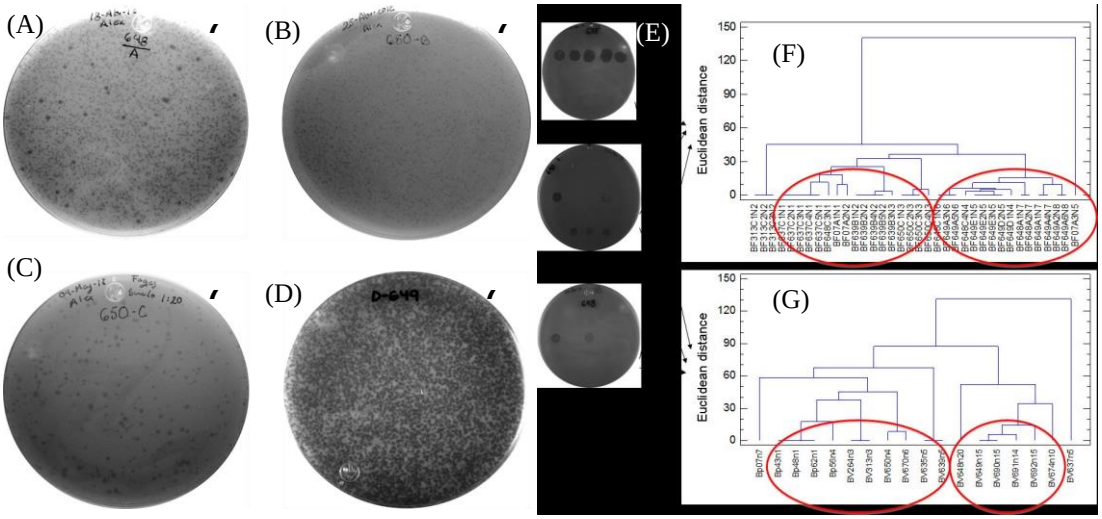

Figure 1. Diversity, density and virulence of bacteriophage associated with bacteria involved in soft rot of agave. Bacteriophages isolated using: (A) 648 strain: two types of lytic plaques are observed. (B) 650 strain: shows a uniform high amount of plaques which suggest a package that is a single virus. (C) 650 strain: 1:20 dilution, different plates with different sizes are observed, suggesting the presence of more than one type of bacteriophage. (D) 649 strain: high number of plates with the same morphology. (E) Infection assay range: in the same bacterial strain different serial dilutions of virus isolation are evaluated. (F) The bacteriophages showed a differential response to infect their bacterial hosts: the last two digits on each label the X axis shows the number of bacteria that phages can to lyse. (G) Some bacterial species were more susceptible to several bacteriophages: the last two digits on each label the X axis show the number of phages that can lyse a strain bacterial.

The characterization of the bacteriophages by TEM revealed that all virus isolates are of the

Siphoviridae family except 637-C-3, which corresponds to the *Podoviridae* family and the 313-C-1 which likely corresponds to *Myoviridae*. These families belong to the order *Caudovirales* or virus with glue, which are exclusive to bacteria and archeas, likewise, this is the group of viruses with more distribution and abundance of all the viruses and represent the majority of the bacteriophages described to date, they are generally lytic, but may present a lysogenic cycle. Bacteriophages belonging to the *Siphoviridae* family have isometric capsids with long noncontractile tails, as λ phage group, while *Myoviridae* has contractile tails. Whereas the *Podoviridae* family bacteriophage has isometric capsids and short tails not contractile (Figure 2).

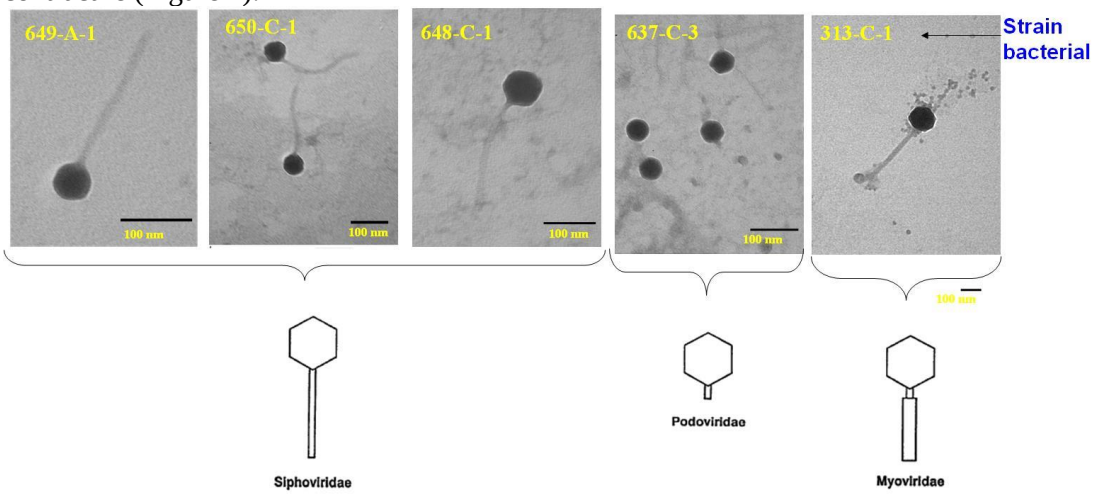

Figure 2. Transmission electron micrographs of bacteriophages from different families involved in the lysis of bacteria associated with soft rot of agave.

The results show the presence of specific bacteriophages for bacteria associated with soft rot of agave in soil samples; these bacteriophages have remained viable in soil samples and suggest alternative biological control for soft rot of agave.

CONCLUSION

Forty viral isolates were characterized and identified in the order Caudovirales of families *Siphoviridae*, *Myoviridae* and *Podoviridae*. All isolates of bacteriophages are lytic for the bacteria associated with soft rot of agave (*A. tequilana* and *A. cupreata*).

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REFERENCES

Frampton RA, AR Pitman, PC Fineran. 2012. Advances in bacteriophage-mediated control of plant pathogens. *International Journal of Microbiology*, article ID 326452, 11 pages (doi:10.1155/2012/326452).

Fujiwara A, Fusisawa A, Hamasaki R, Kawasaki T, Fujie M, Yamada T. 2011. Biocontrol of

Rasoltonia solanacearum by treatment with lytic bacteriophages. *Applied and Enviromental Microbiology* 77: 4155-4162.

Gill JJ, AM Svircev, R Smith, AJ Castle. 2003. Bacteriophages of *Erwinia amylovora*. *Applied and Environmental Microbiology* 69: 2133-2138.

Jiménez-Hidalgo I, G Virgen-Calleros, O Martínez-de la Vega, G Vandemark, V Olalde-Portugal. 2004. Identification and characterization of bacteria causing soft-rot in *Agave tequilana. European Journal of Plant Pathology* 110: 317-331.

Jones JB, Jackson LE, Balogh B, Obradovic A, Iriarte FB, Mogol MT. 2007. Bacteriophages for plant disease control. *Annual Review of Phytopathology* 45: 245-262.

Martínez-Palacios A, JM Gómez-Sierra, C Sáenz-Romero, N Pérez-Nasser, N Sánchez-Vargas. 2011. Diversidad genética de *Agave cupreata* Trel. & Berger consideraciones para su conservación. *Revista Fitotecnia Mexicana* 34: 159-165.

Rincón-Enríquez G, E Quiñones-Aguilar, J Qui-Zapata, K Vega-Ramos, J Uvalle-Bueno. 2014. Selección de bacteriófagos para el control biológico del agente causal de la pudrición del *Agave tequilana. Revista Mexicana de Fitopatología* 32: S112.

Solís-Sánchez GA, EE Quiñones-Aguilar, G Rincón-Enríquez. 2014. Métodos de aislamiento y caracterización de bacteriófagos para el control biológico de bacterias fitopatógenas. *In. Manual básico de microbiología aplicada a la agricultura y al ambiente*. Ferrera-Cerrato R, A Alarcón, JJ Almaraz-Suárez, J Delgadillo-Martínez (eds). Ed. Trillas, México. (*En edición*).

Vega-Ramos KL, JX Uvalle-Bueno, JF Gómez-Leyva. 2013. Molecular variability among isolates of *Fusarium oxysporum* associated with root rot disease of *Agave tequilana*. *Biochemical Genetics* 51: 243-255.