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Diversity of endophytic bacteria in strawberry plants (*Fragaria* sp.) in Sinaloa

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ABSTRACT

Background/Objective. Currently, agricultural systems require management strategies based on crop sustainability. Plant growth-promoting bacteria are an environmentally friendly option to reduce the use of chemicals in agriculture and promote sustainable agricultural practices. The objective of this research was to isolate and identify endophytic bacteria in strawberry plants (*Fragaria x ananassa*) cv Chanler, grown under organic conditions and to evaluate *in vitro* their ability to produce metabolites related to plant growth promotion.

Materials and Methods. Twenty-five healthy-looking strawberry plants of the Chanler variety were collected during the fall-winter 2023-2024 cycle. Genomic DNA was isolated from samples composed of root, stem, and leaf tissue, and the 16S rDNA gene was amplified.

Results. Analysis of genomic sequences identified bacterial genera associated with the phylum Firmicutes, with the *genus Bacillus* predominating, along with the species *B. cereus; B. subtilis, B. thuringiensis, B. xiamenensis,* and *B. zanthoxyli. Shimwellia blattae* and *Burkholderia tropica* were detected in smaller numbers. Of these isolates, *B. cereus, B. subtilis,* and *B. thuringiensis*, in that order, showed the ability to produce siderophores, chitinase, and solubilize phosphorus *in vitro*.

Conclusion. *B. cereus, B. subtilis*, and *B. thuringiensis* exhibited metabolic characteristics related to plant growth promotion *in vitro*. These results in the existence of a diversity of endophytic bacteria in commercial strawberries. This provides information for understanding the endophytic bacteria-plant interaction in the specific environment of strawberry cultivation in the northern state of Sinaloa.

Keywords: Siderophores, Phosphates, Chitinase, *B. cereus.*

INTRODUCTION

At an international level, the production of strawberry (*Fragaria* x *ananassa*) has an important spot due to its excellent profitability and nutritional characteristics (Thakur *et al.,* 2015; Yadav *et al.,* 2017). However, its production has limitations, since it is susceptible to a large number of phytopathogens. This promotes the extensive use of pesticides, causing damage to human health and the environment (Moura *et al.*, 2021).

Due to this, agricultural systems currently require sustainable health and productive management strategies (Kumar *et al.,* 2016). An agroecological strategy to reduce the use of chemical products in agriculture and promote sustainable farming practices is the use of plant growth-promoting bacteria (PGPB) and pathogen resistance-inducing bacteria (Bhattacharyya *et al.,* 2016; Trabelsi and Mhamdi, 2013). The plant-PGPB-environment interaction directly enhances nutrient uptake, promotes plant vigor and reduces susceptibility to phytopathogens.

Several studies have shown that the benefits of PGPB in different fruit species such as cherries, citrus fruits, cranberries apples raspberries and blackberries (Ortiz-Galeana *et al.,* 2018). Esitken *et al.* (2010) studied the effect of three bacterial strains (PGPB), *Pseudomonas BA-8, Bacillus OSU-142* and *Bacillus M-3* individually and in consortia, on raspberry crops. An adequate yield and fruit development was observed, along with an increase in macronutrients, mainly P and Zn in leaf foliar tissue. Likewise, De Melo *et al.* (2012) characterized endophytic bacterial communities in strawberry fruits (*Fragaria x ananassa*). They identified *Curtobacterium citreum, Enterobacter* sp.*, Pseudomonas* sp., *Bacillus subtilis, Bacillus* sp.*, Enterobacter ludwigii, Lactobacillus plantarum,* and *Pantoea punctata*, all with the ability to produce siderophores and IAA (3 indol-acetic acid) equivalents; however, nitrogen fixation capacity was only found in species of the genera *Pseudomonas* and *Enterobacter*. Moura and collaborators (2021) identified five strains of the *Bacillus* sp. genus and one of *Pantoea*, endophytes in strawberry plants, which produced antifungal compounds against *Botrytis cinerea*, the causal agent of the gray mold disease. The taxonomic and functional composition of the microbiome of three commercial strawberry genotypes under field conditions was (Sangiorgio *et al.*, 2022). The plant growth-promoting capacity of native bacteria was confirmed both *in silico* and *in vitro*. The bacteria identified belonged to the classes *Actinobacteria, Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria* and *Bacteroidia*. The current trend to understand the functional mechanisms of PGPB in commercial crops is based on the premise that these bacteria can contribute to reducing the use of fertilizers and active chemical ingredients (e.g., fungicides). This vision aligns with global objectives to promote sustainable agroecological processes (Sangiorgio *et al.,* 2022).

In Mexico, strwawberry is mainly produced in Michoacán, Guanajuato and Baja California (SADER, 2023). The state of Sinaloa managed to increase the strawberry production thanks to the implementation of effective phytosanitary strategies, mostly municipality of Ahome, despite climate limitation for the crop (i.e., extreme weather) (SADER, 2023). Nevertheless, the experience acquired by local farmers has helped strawberry production to become recognized in local and national markets for its high quality, also making it a job-creating alternative, particularly for women. There are currently no known studies related to PGPB in the organic strawberry crop in the region. Consequently, the aim of this investigation was to isolate and identify endophytic bacteria in strawberry plants (*Fragaria* x *ananassa*) cultivated under organic conditions and to

evaluate *in vitro* the ability of bacteria to produce metabolites related to the promotion of plant growth. This would provide a biotechnological resource of PGPB bacteria as a potential sustainable alternative in Sinaloa, Mexico.

The experimental material consisted of Chanler variety strawberry plants, established as an organic crop, at the end of the 2023-2024 autumn-winter production cycle in plots of the Nueve de Diciembre ejido, located in the municipality of Ahome, Sinaloa; Mexico (25°49′50″N 109°46′00″W; HR monthly average 70%; hottest months 36.1 °C and 41.1 °C). Sampling was carried out in random zig zag, using sterile material (gloves, scissors, tweezers). Twenty-five asymptomatic plants were collected, then taken to the Biotechnology Lab of the Universidad Autónoma de Occidente, Guasave, Sinaloa regional unit, for processing. Composite samples of foliar, root, stem and fruit tissues were collected. Samples were washed and surface-sterilized with 70% ethanol and 1% sodium hypochlorite for 10 min and 10% Tween-80 (v/v), and finally rinsed with sterile distilled water. Bacterial isolation was carried out using the direct contact technique, by making transverse and longitudinal cuts on the tissue under aseptic conditions, placing the cut surface in contact with the nutrient agar culture medium/Petri dish. The cultures were incubated at 27 °C for eight days (Yang *et al.,* 2011). The bioassay was performed in triplicate for each tissue type. In order to confirm the efficiency of the sterilization process of the analyzed tissue, aliquots of the sterile distilled water used in the final tissue rinse were placed on Petri dishes with nutrient Agar and incubated under the same conditions as those described above. The absence of microbial growth was confirmed. Colony morphology was examined under a Leica digital stereoscope. Purification was carried out from mother colonies after 48 h, until morphologically similar colonies were obtained. After four purification rounds, a total of 25 isolates were obtained, of which 10 were not cultivable. Population density was determined by direct count on the dish (UFC g^{-1}), selecting the strains distinguished by prevalence, size, shape and color.

For molecular characterization, the genomic DNA was isolated from the bacterial strains, amplifying the 16S rDNA gene was amplified using the specific primers F2C (5'-AGAGTTTGATCATGGCTC-3') and C (5'-ACGGGCGGTGTGTAC-3') (Shi *et al.,* 1997). The PCR products were purified and sequenced at the National Laboratory of Genomics for Biodiversity (LANGEBIO) in CINVESTAV-IPN. The sequences obtained were subjected to GenBank searches of the U.S. National Center for Biotechnology Information (NCBI) using the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic inferences were obtained using the Neighbor-Joining method, based on the Kimura-2-parameter model with a bootstrap test of 1.000 replicates in the MEGA X program.

Evaluations were carried out *in vitro* in triplicate for the identification of potential PGPB bacteria, using the *Bacillus cereus* (B25) strain as a positive control (Figueroa-López *et al.*, 2016). Siderophore production was evaluated in Chrome Azurol S (CAS) agar medium (Schwyn and Neilands, 1987), with colonies forming a yellow/orange halo around them being considered positive. Chitinases were evaluated following Shanmugaiah *et al.* (2008), with the formation of a clear area around the bacterial colony after five days being considered positive. Phosphate-solubilizing activity was evaluated on Pikosvkaya agar plates (Pikosvkaya, 1948), with colonies surrounded by a clear zone considered positive.

Results of this investigation were based on 15 endophytic strains isolated from foliar, root, stem and fruit tissues from strawberry plants. These strains displayed different morphologies regarding edges, shapes, colors and surfaces (Figure 1). Bacterial population

densities presented significant variations between tissues, with high values in roots (3.0 x 10^{10} g^{-1} root), followed by stems (1.0 x 10^{10} g^{-1} stem), fruit (1.0 x 10^{10} g^{-1} fruit) and leaves (1.3 x 10^{9} g^{-1} leaf).

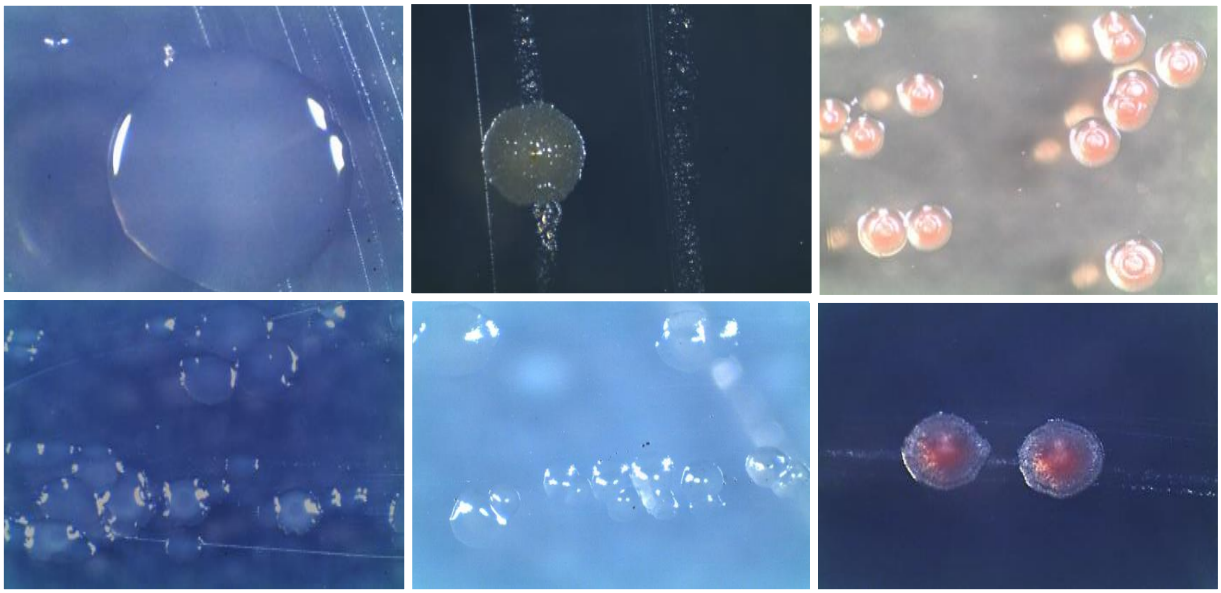

Figure 1. *In vitro* colonial morphology of endophytic bacteria isolated from the roots, stems, leaves, and fruit of strawberry plants (*Fragaria x ananassa*) cv. Chanler, viewed a digital stereoscope after 48 h of growth on nutrient agar. Note: The different morphology: smooth, translucent edges, circular shape, shiny surface, and coloration.

These results confirm the presence of endophytes in different plant tissues, coinciding with reports by Porras-Alfaro and Bayman, (2011) and Sahoo *et al*. (2017), who attribute the highest bacterial density in roots to them being in direct contact with the soil, favoring colonization. The isolates were identified by origin tissue (R: root, T: stem; H: leaf and F: fruit), numbered consecutively. Sequence analysis helped identify bacterial genes with a similarity of 98% with reference sequences in the data base. Most were associated to the phylum Firmicutes, with a predominance of isolations of the genus *Bacillus* (Figure 2). Isolates obtained from root, stem, leaf and fruit were identified as *B. cereus* R5 (PV583387), T6 (PV583388), T7 (PV583389), H8 (PV583390), T9 (PV583391) and F10 (PV583392); three root isolates were identified as *B. subtilis* R1 (PV583383), R2 (PV583384), R3 (PV583385); and one as *B. xiamenensis* R4 (PV583386); *Bacillus thuringiensis* T11 (PV583393), R14 (PV583396); *B. zanthoxyli* R12 (PV583394); *Shimwellia blattae* H13 (PV583395), and *Burkholderia tropica* F15 (PV583397). The *in vitro* bioassays for plant growth-promoting activity showed that *B. cereus, B. subtilis* and *B. thuringiensis* were able to produce siderophores, solubilizing phosphorous and exhibiting chitinase activity (Table 1).

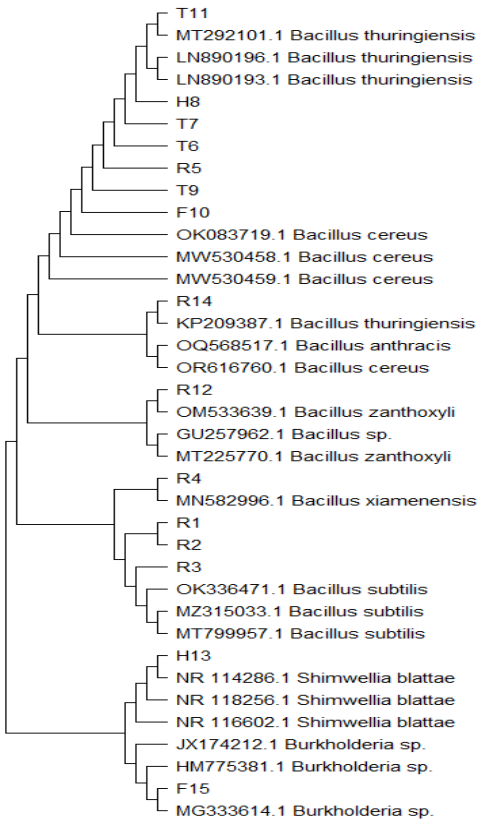

Figure 2. Dendrogram obtained using the Neighbor-Joining algorithm from sequences of the gene encoding the 16S rDNA subunit obtained from endophytic bacteria isolated from strawberry plants (*Fragaria x ananassa*), cv. Chanler, from organic commercial crops. Municipality of Ahome, Sinaloa, Mexico. The isolates obtained in this research are identified with acronyms and numbers: R-n: root; T-n: stem; H-n: leaf; F-n: fruit.

The results contrast with studies performed on cranberries in which *Pantoea, Pseudomonas, Burkholderia* and *Bacillus* are reported to be the most abundant genera. Similar to this study, some strains of *Bacillus* were proven to have a greater production of phytohormones and siderophores than their positive control, rhizobacteria *Pseudomonas fluorescens* UM270 (Ortiz-Galeana *et al.*, 2018).

Table 1. Qualitative *in vitro* siderophore, chitinase, and phosphate production activity of six species of endophytic bacteria isolated from Chanler strawberry plants grown under organic production. Municipality of Ahome, Sinaloa, Mexico.

Isolate	Siderophores	Chitinases	Phosphates
***Bacillus subtilis* (R1, R2, R3)**	+++	++	++
B. xiamenensis (R4)	-	-	-
***B. cereus* (R5, T6, T7, H8, T9, F10)**	+++	++	++
***B. thuringiensis* (T11, R14)**	++	+	++
B. zanthoxyli (R12)	++	-	+
Shimwellia blattae (H13)	-	-	-
Burkholderia tropica (F15)	+	+	-
*****B. cereus* (25)	+	+	+

Positive control (*); Absence of activity (-); + < 3mm; ++ > 3 < 4 mm; +++ > 4 mm. The letters indicate the plant tissue: **R** root, **T** stem, **H** leaf, **F** fruit. The number corresponds to the isolate.

The *Bacillus* genus presents high adaptability to different environmental conditions due to its broad genetic diversity, making it a reference to understanding metabolic processes. The results showed that the *B. subtilis* strains (R1, R2, R3) had a high phosphate-solubilizing capacity, and siderophore and chitinase production (Table 1), coinciding with other studies in which it is considered an endophyte of great interest, since the colonization of roots promotes the development of biocontrol mechanisms, which are important against biotic and abiotic diseases (Corrales-Ramírez *et al.,* 2017; Gond *et al.,* 2015). However, in this study, *B. cereus* was isolated from the diverse organs analyzed (root, stem, leaf and fruit), suggesting a greater adaptability and showing metabolic characteristics similar to those of *B. subtilis* (Cuadro1). This is consistent with reports in rice, where it was isolated as an endophyte, showing a high capacity to promote plant growth, as well as to mobilize phosphate, allowing the uptake of insoluble forms and releasing them, thereby enhancing soil fertility (Chamorro-Anaya *et al*., 2020). Nevertheless, some *B. subtilis* and *B. cereus* strains have been associated to risks to human health, and the former has been linked to soft rot in garlic (*Allium* sp.), making it a factor worth considering in future biotechnological developments (https://microbenotes.com/bacillus-subtilis/).

The *B. zanthoxyli* strain, isolated from roots, exhibited a phosphate-solubilizing ability and siderophore production, consistent with studies in tomato and pepper crops, where it confers resistance to infections caused by *Ralstonia solanacearum* and *Phytophthora capsici,* as well as promoting growth in cucumber, tomato and cabbage plants by increasing shoot and root development (Usmonov *et al.,* 2021). In this study, *Burkholderia tropica* was only isolated from fruit with a low siderophore and chitinase production capacity. Bolívar-Anillo *et al.* (2016), who mention that *B. tropica* produces volatile compounds and siderophores, exerting suppressive effects on phytopathogens and promoting growth in tomato, sugarcane and maize due to its nitrogen-fixing abilities and phosphate solubilizing capacity.

In conclusion, the Chanler variety strawberry (*F*. x *ananassa*), grown under commercial organic conditions, have an endophytic bacterial community composed of three genera. The most prevalent was *B. subtilis*, although *B. cereus*, followed by *B. thuringiensis*, displayed a wider distribution across plant organs, suggesting a greater endophytic evolutionary adaptation. These species also stood out for their plant growth-promoting properties across all three metabolic traits analyzed. This study contributes to the understanding of endophyte-planta interactions in the specific environment of strawberry farming in Sinaloa, which may help improve sustainable phytosanitary and productive management strategies.

Limitations: The sample size (i.e., number of plants and varieties, conventional production) could have influenced the number of genera and species found in this study.

Conflict of interest. N/A

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Contribution of authors

Conceptualization and design of the study, data analysis, contextualization of results and revision of manuscript: RM Longoria-Espinoza, R Félix-Gastélum. Experimental execution and revision of literature: DR. Hernández-Luna.

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