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Alejandro Quispe , Mayra Contreras , Diego Meza , Jossimar Vicente *

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Article

Molecular Characterization of Native Strains of *Bacillus* spp. in rhizospheric Soil in Three Regions of Peru

Alejandro Quispe [1], Mayra Contreras [2], Diego Meza [3] and Jossimar Vicente [4,*]

[1,2,3,4] Agricultural Molecular Biotechnology Laboratory - National University of Cañete; Cañete - Peru

* Correspondence: rvicente@undc.edu.pe; Tel.: +511927059291

Abrstract: *Bacillus* spp. it is a genus that is used to biocontrol post-harvest diseases in various vegetables and fruits during transportation and storage. It suppresses the development of pathogens such as gray mold (*Botrytis cinerea*) in strawberry cultivation. In this work, the molecular characterization of *Bacillus* spp. species was carried out. in three cities in Peru: Huaura, Chanchamayo and Cañete. The samples were collected from rhizospheric soil from crop areas, subsequently processed for microbiological isolation, molecular analysis using the 16S rRNA gene and bioinformatic analysis. In total, 14 strains were obtained where they were identified with a minimum of 92.05% and a maximum of 100% similarity: 8 strains of *Bacillus subtilis*, 4 strains of *Bacillus licheniformis*, 1 strain of *Bacillus paralicheniformis* and 1 strain of *Bacillus cereus*, all the strains were related by constructing a phylogenetic tree.

Keywords: *Bacillus*; 16S rRNA; molecular characterization; rhizosphere; biocontroller

1. Introduction

Crops such as vegetables and fruits become diseased between harvesting and consumption, resulting in significant food waste and economic losses. About 45% of harvested fruits, vegetables, roots and tubers are lost. Most of this loss occurs during storage due to the development of pests and pathogens (bacteria, fungi and insects), unfavorable environmental conditions (rain, humidity, frost and heat), water loss, saccharification and sprouting. [1]

Bacteria of the genus *Bacillus* spp. they are recognized as safe microorganisms for application in the food industry. They occupy the same niche as many pathogens and have the ability to produce a wide range of bioactive substances with antibiotic activity. These substances induce various physiological characteristics in the metabolism of the host plant without causing adverse effects on the environment and human health [2]. Furthermore, *Bacillus* spp. (i.e., *Bacillus subtilis*), produce endospores resistant to dynamic physical and chemical treatments, such as heat, desiccation, organic solvents, and ultraviolet radiation, which therefore maintain their ability to trigger defense responses in plants guest, even in unfavorable conditions.[3]; this makes it capable of easily formulating and storing Bacillus-based biological products and serves as a potent bioactive component against pathogens [4].

Currently, the protective effect of *Bacillus* strains is used in several plant species against a wide variety of biotic (pathogens, pests) [5] and abiotic (drought, salinity, extreme temperatures, metals, stresses toxic) [6].

The purpose of the study is to carry out the molecular identification of *Bacillus* spp. samples. collected in rhizospheric soil from 3 regions of Peru and the relationship that exists between these species through a phylogenetic tree.

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2. Materials and Methods

2.1. Sample collection and isolation

The collection of rhizospheric soil samples was carried out in the areas of 1) Canín Annex of the Checras district of the province of Huaura, 2) Kimiri Sector of the Chanchamayo district of the province of Chanchamayo and 3) Catapalla Annex of the district from Lunahuaná in the province of Cañete. Subsequently, the samples were processed for the isolation of *Bacillus* spp. strains: 10 g were weighed. of the rhizospheric soil sample and dissolved in physiological saline, then dilutions were carried out in 3 series, subsequently the diluted sample was seeded in the culture medium Glucose Tryptone Meat Extract (TGE) and incubated at 37°C for 24 hours, finally Colonies were selected and purified by streaks [7].

2.2. DNA extraction, quantification and quality

The isolated strains were seeded in the Luria Broth (LB) culture medium and incubated for 18 hours at 37 °C [8]. Subsequently, genomic DNA extraction was carried out according to the instructions of the commercial Genomic DNA Extraction Kit – Gram (+) Bacteria/Yeast/Fungi (Cepham Life Sciences). The samples were then quantified using the Eppendorf BioSpectrometer kinetic spectrophotometer [9] and to verify the quality and integrity of the DNA, a 1% agarose electrophoresis run was performed in a 1X TAE buffer solution [10].

2.3. PCR amplification with the 16S rRNA gene and DNA sequencing

Amplification was performed by polymerase chain reaction (PCR) [11] using 2 µl of the DNA sample with the DreamTaq PCR Master Mix kit (2X) and the 16S rRNA 27 F primers (5 ' AGA GTT TGA TCM TGG CTC AG 3') and 1525 R (5' AAG GAG GTG WTC CAR CC 3') [12]. For a negative control, a mix without sample was used. The mixtures were processed in the Eppendorf Mastercycler minutes, elongation at 72°C for 1 minute and a final extension cycle at 72°C for 15 minutes [13]. The PCR products were visualized by electrophoresis in a 1.5% agarose gel in a 1X TAE buffer solution and were subsequently sent for Sanger sequencing [14] to the University of Minnesota - USA.

2.4. Bioinformatic analysis

The sequences obtained were edited with the Chromas v2.66 software (https://technelysium.com.au/wp/chromas/) [15], then they were analyzed with the online BLAST platform [16], subsequently these were aligned using the ClustalW tool and finally the construction of a phylogenetic tree from the 16S rRNA gene was carried out using the software MEGA 11.0.13 [17] using the following parameters: Analysis (phylogenetic reconstruction), statistical method (Neighbor-Joining), Phylogeny test (Bootstrap method, number of bootstrap replications: 1000), model (Maximun composite likelihood) [18].

3. Results

3.1. Sample collection and isolation

A total of 14 strains of Bacillus spp. were isolated and analyzed: 7 in the Canín annex of the Checras district of the province of Huaura, 5 in the Kimiri sector of the Chanchamayo district of the province of Chanchamayo and 2 in the Catapalla annex of the Lunahuaná district of the province of Cañete.

3.2. DNA quantification and quality

For the DNA quantification of the samples, an average result of 30.35 ng/µl was obtained, with a minimum of 21.4 ng/µl for samples UNDC_06, UNDC_09 and a maximum of 39.5 ng/µl for sample UNDC_05. While in the quality of the extracted genomic DNA, defined bands with a large molecular

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weight exceeding 10,000 bp were evident, which indicate an acceptable quality for PCR amplification and corroborates the quantification previously carried out. (Figure 1)

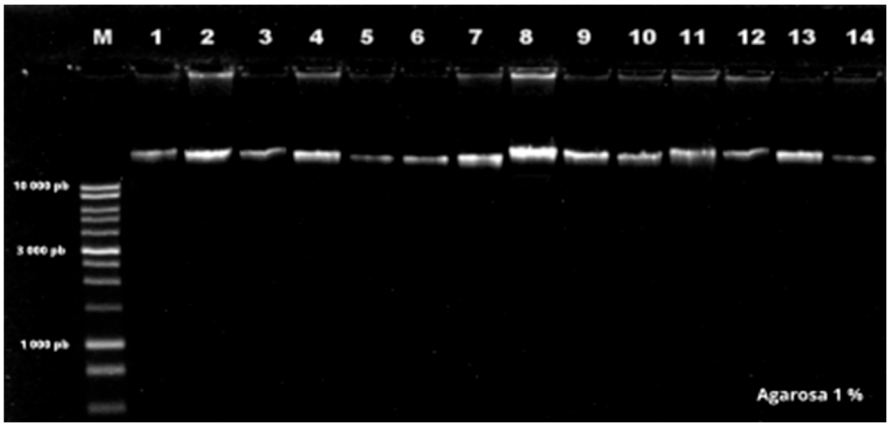

Figure 1. 1% agarose gel of genomic DNA extracted from Bacillus spp.

3.3. PCR amplification with the 16S rRNA gene and DNA sequencing

The PCR amplification products of the 14 samples were analyzed by 1.5% agarose electrophoresis and visualized amplicons with an expected weight of 1500 bp. (Figure 2). As a result of the sequencing, 14 electropherograms were obtained which were subsequently analyzed.

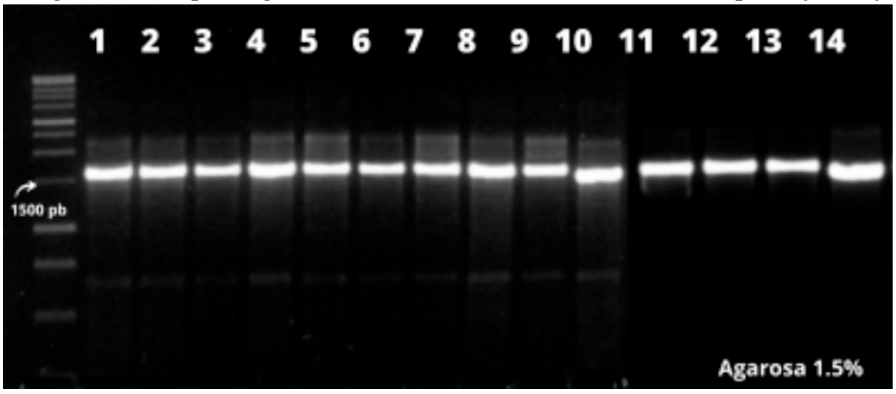

Figure 2. 1.5% agarose gel of PCR products from *Bacillus* spp. samples.

3.4. Bioinformatic analysis

According to the molecular identification analysis with the BLAST platform, the species of Bacillus subtilis, Bacillus licheniformis, Bacillus paralicheniformis and Bacillus cereus were identified, with a minimum of 92.05% and maximum of 100% similarity (Table 1).

Table 1. This is a table. Tables should be placed in the main text near to the first time they are cited.

Code	Species	Location	Identify
UNDC_01	*Bacillus subtilis*	Huaura	98,86%
UNDC_02	*Bacillus subtilis*	Huaura	95,79%
UNDC_03	*Bacillus subtilis*	Huaura	98,09%
UNDC_04	*Bacillus subtilis*	Huaura	100,00%
UNDC_05	*Bacillus licheniformis*	Huaura	98,18%
UNDC_06	*Bacillus paralicheniformis*	Huaura	95,38%
UNDC_07	*Bacillus cereus*	Huaura	99,79%
UNDC_08	*Bacillus licheniformis*	Chanchamayo	92,05%
UNDC_09	*Bacillus subtilis*	Chanchamayo	99,56%
UNDC_10	*Bacillus subtilis*	Chanchamayo	98,28%

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UNDC_11	*Bacillus subtilis*	Chanchamayo	97,91%
UNDC_12	*Bacillus licheniformis*	Chanchamayo	97,15%
UNDC_13	*Bacillus subtilis*	Lunahuaná	94,05%
UNDC_14	*Bacillus licheniformis*	Lunahuaná	98,93%

The construction of the phylogenetic tree reveals the evolutionary relationship and homology between the 14 strains worked on. (Figure 3)

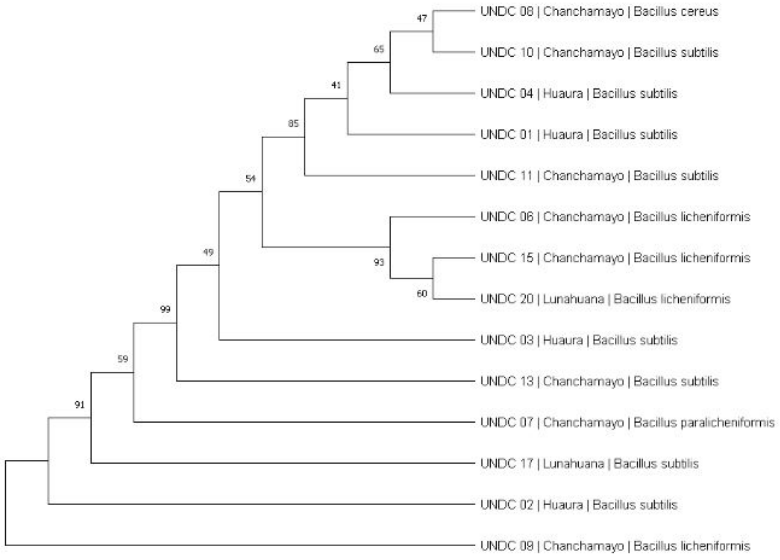

Figure 3. 1.5% agarose gel of PCR products from *Bacillus* spp. samples.

4. Discussion

In the collection stage, the strawberry rhizosphere soil was used as a sample to obtain the Bacillus spp strains, similar to a work reported in the rhizosphere of cacti [19]. On the contrary, studies have been carried out where they obtain samples of mites [20], plants [21], fish [22], birds, bovines [23], fungi [24] among others. In this work, rhizospheric soil is used due to the high diversity of microbiota and the environmental conditions that favor the development of the genus *Bacillus* spp due to its close relationship with the roots of vegetable crops [25]. In the PCR amplification stage, the universal primers of the 16S rRNA gene were used [26], however other authors used different primers such as Bc-Rep-1, Bc-Rep-2 [27] and M-B1 [28].

The sanger sequencing used in this work was efficient for molecular identification, however, the results can be improved using third generation sequencing such as Oxford Nanopore Technologies technology [29], its greatest advantage is the reading length and the speed of the process [30]. This will allow us to expand the database, having more sampling points and repetitions of them. The molecular characterization of species was *Bacillus subtilis* [31], *Bacillus licheniformis* [32], *Bacillus paralicheniformis* [33], *Bacillus cereus* [34], unlike other authors who found other types of species such as *Bacillus amyloliquefaciens*, *Bacillus vallismortis*, *Bacillus halotolerans* [35], *Bacillus thuringiensis* [36].

Finally, the phylogenetic tree was built using the Neighbor Joining method, aligned with the Clustal can be improved by increasing the number of primers for the 16S rRNA gene and the number of replications using a specialized server for bioinformatic analysis.

5. Conclusions

The molecular characterization of the genus Bacillus spp. Through the analysis of the 16S rRNA gene, it allowed the identification of 8 strains of *Bacillus subtilis*, 4 strains of *Bacillus licheniformis*, 1 strain of *Bacillus paralicheniformis*, 1 strain of *Bacillus cereus*; with a minimum of 92.05% and maximum of 100% similarity in the regions of Huaura, Chanchamayo and Cañete.

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Conflicts of Interest: The authors declare no conflict of interest.

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