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RESEARCH NOTE / NOTA CIENTÍFICA

BACTERIAL IDENTIFICATION USING 16S RRNA GENE SEQUENCING FROM BANANA PSEUDOSTEM WET ROT SAMPLES

IDENTIFICACIÓN BACTERIANA MEDIANTE SECUENCIACIÓN DEL GEN 16S RRNA A PARTIR DE MUESTRAS DE PUDRICIÓN BLANDA DEL PSEUDOTALLO DEL BANANO

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ABSTRACT

Wet rot affects organic banana crops in the northern tropical region of Peru. Several bacteria have been reported to cause wet rot on different crops, leading to severe problems, particularly in humid climates. Bacteria infect the banana pseudostem through wounds caused during plant pruning and insect bites. Knowledge about bacteria identification that causes wet rot may provide a valuable tool for controlling this issue. The present study explored the primary bacterial component of banana pseudostem wet rot samples at three localities

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in the Sullana province of Piura, using 16S rRNA gene sequencing. A total of one hundred and thirty-six sequence data were obtained from the three Sullana localities: forty sequences of bacterial isolates from the Querecotillo locality sample, forty-eight from Mambre, and Salitral each. The genus Klebsiella of the Enterobacteriaceae family was identified as the main bacterial component in organic banana pseudostem wet rot. This finding reinforces the need to continue with studies that provide evidence of the bacterial role of the genus *Klebsiella*.

Keyword: Wet rot – 16S rRNA – Banana organic – *Klebsiella*

RESUMEN

La pudrición blanda afecta los cultivos de banano orgánico en la región tropical del norte de Perú. Se ha informado que varias bacterias causan pudrición blanda en diferentes cultivos, lo que lleva a problemas graves, particularmente en climas húmedos. Las bacterias infectan el pseudotallo del plátano a través de heridas causadas durante la poda de plantas y picaduras de insectos. El conocimiento sobre la identificación de bacterias que causan la pudrición blanda puede proporcionar una herramienta valiosa para controlar este problema. El presente estudio exploró el componente bacteriano primario de muestras de pudrición blanda del pseudotallo del banano en tres localidades de la provincia Sullana de Piura, utilizando la secuenciación del gen 16S rRNA. Se obtuvieron un total de ciento treinta y seis datos de secuencia de las tres localidades de Sullana: cuarenta secuencias de aislados bacterianos de la muestra de la localidad de Querecotillo y cuarenta y ocho de Mambre y Salitral, cada una. El género *Klebsiella* de la familia Enterobacteriaceae fue identificado como el principal componente bacteriano en la pudrición blanda del pseudotallo del plátano orgánico. Este hallazgo refuerza la necesidad de continuar con estudios que aporten evidencia del papel bacteriano del género *Klebsiella*.

Palabra clave: Pudrición blanda – ARNr 16S – Plátano orgánico – *Klebsiella*

INTRODUCTION

Banana and plantain (*Musa* sp.) are the fourth most important food crop with a fastest-growing in agricultural production and world trade (Food and Agricultural Organization of the United Nations (FAO), 2020). Over the past decade, the organic banana of Cavendish triploid variety crops promoted economic growth in Sullana province on the tropical coast of Peru, categorize the country among the main producers in Latin America and the Caribbean (MINAGRI, 2014).

However, several phytopathogens affect banana crops with a negative impact on performance and productivity. These are influenced by alterations in climatic conditions that increase the level of aggressiveness in phytosanitary problems like bacterial rot (Blomme *et al.*, 2017; Martínez-Solórzano *et al.*, 2020).

The bacterial soft and wet rot affect the pseudostem and rhizome of bananas and is widespread in tropical regions worldwide. The rhizome rot and pseudostem wet rot is due to the secretion of bacterial pectinases that degrade the pectin at the primary cell walls and middle lamella (Hugouvieux-Cotte-Pattat *et al.*, 2014), causing tissue maceration, and a wet and foul smell of rotting (Loganathan *et al.*, 2019). The pseudostem wet rot is characterized by external brown or dark brown coloration and rotting of the internal pseudostem tissue with amber-colored liquid (Loganathan *et al.*, 2019).

The bacterial pathogens spread to newly planted suckers and young plants transported by insects and during pruning or crop management using tools, soil, and irrigation water contaminated with the bacterial agents (Charkowski, 2018).

The banana pseudostem wet rot development is associated with several bacterial causal agents such as *Dickeya chrysanthemi* Samson *et al. 2005* [earlier *Pectobacterium chysanthemi* (Burkholder *et al. 1953)* Brenner *et al. 1973* (Approved Lists 1980) emend. Hauben *et al.* 1998)], *D. paradisiaca* (Fernandez-Borrero and Lopez-Duque 1970) Samson *et al.* 2005, *Pectobacterium carotovorum subsp. carotovorum* (Jones 1901) Hauben *et al.* 1999 emend. Gardan *et al. 2003, and Klebsiella variicola* Rosenblueth *et al.* 2004 (Lin *et al.*, 2010; Basim *et al.*, 2019; Rajamanickam *et al.*, 2018; Loganathan *et al.*, 2019).

Since these bacterial pathogens could be found together or individually in decaying vegetables, detecting, and identifying the principal bacterial component is essential to understand its contribution to banana pseudostem wet rot. This study assesses the primary bacterial component of three different banana pseudostem wet rot samples from the organic banana crop Cavendish triploid variety in Sullana, Piura.

The current project aims to differentiate the isolated bacteria from samples with soft rot symptoms and classify them based on their 16S rRNA sequence.

MATERIALS AND METHODS

Samples and bacterial isolation

The banana pseudostem samples with wet rot symptoms (Figure 1) were collected from three localities of Sullana: Vichayal Querocotillo (Latitude -4,85; Longitude -80,65), Salitral (Latitude-4,84, Longitude -80,67) and Mambre (Latitude -4,88, Longitude -80,69). The samples were transported to the Universidad Nacional Federico Villarreal in Lima – Peru and the bacterial isolation was carried out at "Laboratorio de Investigación de Bioquímica y Biología Molecular" (LIBBM-SL10LA105).

Figure 1. Banana pseudostem samples with wet rot symptoms. To the left, Organic banana with pseudostem soft wet rot symptoms. To the right, pseudostem sample collected.

The samples were washed with plenty of water, and 2 cm^2 of inner section was taken in sterile conditions and homogenized with 2 mL of Tryptic Soy Broth (TSB). Serial tenfold dilutions were made for each of the cultures from the different locations and were cultured in plates with Tryptic Soy Agar (TSA). After 24 hours of incubation at 25 °C, the fourth dilution for Querecotillo and Salitral, and the sixth dilution for Mambre, was used for individual and aleatory colony selection. A total of 150 colonies were selected; 48 from Querocotillo, 51 from Mambre, and 51 from Salitral.

Molecular characterization using 16S rRNA gene

The genomic DNA of each bacteria colony was extracted by cell rupture through a thermal shock, each colony was put in a test tube containing 300 µl of molecular grade water and 5 cycles of 10 minutes at -20 °C and 3 minutes at 65 °C were carried out

with a final centrifugation step for five minutes at 10 000 rpm. Five µl of the supernatant were used for the PCR.

Amplifications of the 16S rRNA gene region were carried out using universal primers: 27F, 5'-AGAGTTTGATCMTGGCTC-3 and 1492R, 5' TACGGYTACCTTGTTACGACTT-3' (Lane, 1991; Liu *et al.*, 2009; Turner *et al.*, 1999). The PCR reactions were performed using the SuperFiTaq DNA polymerase kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The amplification cycles were performed under the following conditions: an initial pre-denaturation step at 98°C for 5 min, followed by 35 cycles at 94°C for 30 s, 58°C for 45 s, 72°C for 90 s, and a final elongation step of 72°C for 6 min.

The amplicon products were verified by 1,0% agarose gel electrophoresis, and 20 µl of each amplified product were sequenced at MACROGEN laboratories (Korea), using the Sanger sequencing method. Three sequence reads for PCR product were made to achieve the maximum sequence length (approx. 1492 bp of the 16S rRNA gene), the sequences were aligned and assembled in MEGA X bioinformatic software (https://www. megasoftware. net/), and finally the sequences were compared with the public sequences using Nucleotide Basic Local Alignment Search Tool (BLAST) at www.ncbi.nlm.nih.gov/ BLAST, and available from the National Center for Biotechnology Information (NCBI).

Ethic aspects

Ethical aspects were followed in this study to contribute positively to both scientific knowledge and the well-being of the ecosystems by using Good Laboratory Practices (GLPs).

RESULTS AND DISCUSSION

A total of 136 isolates were successfully obtained from the three banana pseudostem samples with wet rot symptoms. The number of isolates selected and recovered from Querocotillo was forty isolates, while for Mambre, and Salitral were forty-eight isolates.

The principal bacteria found was *Klebsiella* genus, in Querecotillo 36 of 40 isolates were classified into the *Klebsiella* genus, (29 *Klebsiella michiganensis* Saha *et al.* 2013 and 7 *Klebsiella variicola* Rosenblueth *et al.* 2004). For Mambre 47 of 48 isolates were *Klebsiella oxytoca* (Flugge 1886) Lautrop 1956; while for Salitral 41 of 48 isolates were classified as *K. variicola* (41 isolates) (See Table 1).

This high prevalence of the genus *Klebsiella* was previously reported. *K. variicola* was found in banana pseudostems and rhizome in tropical regions of Haiti, India, and was reported recently at Salitral province of Sullana-Piura (Fulton *et al.*, 2020; Loganathan *et al.*, 2021; Rafael-Rutte *et al.*, 2022). Likewise *K. variicola* was involved with bacterial soft rot of carrot (Chandrashekar *et al.*, 2018), and with wet wood disease of ironwood trees in a complex association with *Ralstonia solanacearum* (Smith 1896) Yabuuchi *et al.* 1996 emend. Safni *et al. 2014 and K. oxytoca* (Ayin *et al.*, 2019)

Table 1. Principal bacterial isolates by Pseudostem sample.

Location	Specie	Sequence with highest blastn scores (Accession number)	Numbers of bacterial isolates
Querecotillo	*Klebsiella michiganensis*	*CP054159.1*	29
	Klebsiella variicola	MF144432.1	7
	Pseudomonas entomophila	MN493076.1	2
	Stenotrophomonas sp.	KX817899.1	2
Mambre	*Klebsiella oxytoca*	LR133932.1	47
	Kosakonia radicincitans	CP040392.1	1
Salitral	*Klebsiella variicola*	MF144432.1	41
	Kosakonia radicincitans	CP040392.1	3
	Pseudomonas entomophila	MN493076.1	2
	Pseudomonas plecoglossicida	MT367715.1	2

In addition, genomic analyzes of *Klebsiella michiganensis* Saha *et al.* 2013, obtained from diseased blackberry roots have revealed the ability of these bacteria to degrade the cell wall of plants as they have the necessary genes for the biosynthesis of type I and type II bacterial secretion systems, which would allow secreting enzymes to degrade the cell wall of plants (Luo *et al.*, 2022; Yu *et al.*, 2018). The potential capacity of *K. variicola* to be involved in or produce diseases is not limited to plants, in animals was found in Bovine mastitis disease and rare human pulmonary and urinary tract infections clinical cases as well as *K. oxytoca* and *K. Michiganensis* (Barrios-Camacho *et al.*, 2019; Rodríguez-Medina *et al.*, 2019; Seiffert *et al.,* 2019; Duran-Bedolla *et al.*, 2021; Neog *et al.*, 2021).

Nevertheless, the *Klebsiella* genus was also categorized by other studies as benefic bacteria due to the endophytic, nitrogen-fixing, and growth-promoting properties (Duran-Bedolla *et al.*, 2021), so the bacterial role involving the production of enzymes that degrade the cell wall in banana pseudostem wet rot is still uncertain.

The study determines the principal bacterial component while the less representative bacteria could have been excluded due to population reduction due to transport and sample dilution factors.

The genus *Klebsiella* of the Enterobacteriaceae family were found as the main bacterial component in organic banana pseudostem wet rot.

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