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Posted Date: 30 September 2025

doi: 10.20944/preprints202509.2539.v1

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Bacteria Causing Pith Necrosis and Tissue Discoloration in Tomato

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Abstract

Tomato is one of the most widely cultivated and consumed vegetables in Costa Rica, where favorable environmental conditions enabled year-round production. However, these same conditions also promote the development of plant diseases. Diverse bacterial infections causing pith necrosis in tomato crops have become of frequent observation in recent years. Nevertheless, the causal agents in Costa Rica remain unidentified, and management strategies are poorly documented. This study evaluated the pathogenicity of bacterial isolates obtained from tomato plants collected in Costa Rica's Central Valley. Isolates of *Pseudomonas alliivorans*, *P. flavescens*, and *P. capsici* were identified as causal agents of pith necrosis in tomatoes. Additionally, *P. straminea* and *Cedecea* sp. were associated with tissue discoloration. These findings provide valuable information to the tomato production sector in Costa Rica by identifying previously unreported pathogenic bacteria and expanding their known distribution and host ranges. These findings also demonstrate the ability of these isolates to cause pith necrosis.

Keywords: plant pathogenicity; plant pathogenic bacteria; MLSA; whole-genome sequencing

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1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely produced and consumed vegetables worldwide [1,2]. In Costa Rica, tomato cultivation primarily occurs in the Central Valley regions, with a production of 55,307 metric tons on 1,510 hectares reported in 2018 [2]. The high demand for tomatoes in most countries makes it an intensive production crop in which techniques must be used to maximize yield per unit of land [3]. Also, producing countries must have climatic conditions such as temperatures around 18-32°C and relative humidity around 55-90% that favor year-round cultivation [4,5], and measures to control the development and spread of fungal and bacterial diseases.

Bacterial infections affect crop yields, causing significant economic losses [6,7] which can reach up to 91% in yield losses in some cases, such as bacterial wilt caused by *Ralstonia solanacearum* [8]. As a result, bacteria pose a significant global threat to agricultural production, and their control is crucial for ensuring food security [9,10].

Among the most frequently reported bacterial diseases in Costa Rica, bacterial wilt caused by the *Ralstonia* species complex is the most important and destructive. In recent years, the damage caused by other bacteria has focused on soft rot caused by *Pectobacterium* spp., bacterial speck caused by *Pseudomonas* spp., and bacterial spot caused by *Xanthomonas* spp. [11–13]. In addition to these diseases, others, such as pith necrosis, are either infrequently reported in crops or have not yet been officially recorded in the country. One such case is tomato pith necrosis, a disease whose characteristic symptom has been repeatedly observed in samples collected between 2021 and 2023 in various locations in the Central Valley of Costa Rica.

Tomato pith necrosis was first described in the 1970s, caused by *Pseudomonas corrugata* [14], since then, other causal agents have been identified, including various *Pseudomonas* species such as *P. mediterranea* [15], *P. cichorii* [16], *P. viridiflava* [17], *P. marginalis* [18], *P. fluorescens* and *P. putida* [19], as well as species from other genera such as *Xanthomonas perforans* [20] and *Pectobacterium carotovorum* [21].

Pseudomonas corrugata has been the principal species associated with the disease since its initial description and is now widely distributed across most tomato-producing countries worldwide [22]. In the Americas, *P. corrugata* has been reported in Brazil [23], Argentina [24], Uruguay [25], Mexico [26], and the United States [27–31]. In Costa Rica, the presence of *P. corrugata* is presumed; however, no official reports or targeted studies have been made regarding the identification of the species responsible for tomato pith necrosis in the country.

Incidence of pith necrosis within plantations can range from 10% to 100%, depending on factors such as the bacterial species, environment, cropping system, and management practices [14,18,29,32]. Additionally, up to 20% losses in fruit weight and size have been reported and related to the presence of pathogens such as *P. corrugata* and *P. mediterranea* [33]. In severe infections, the wilting caused by pith necrosis becomes widespread and leads to plant death [25].

These considerations highlight the importance of evaluating pathogenic bacteria present in crops. The objective of this study was to identify the bacteria present in tomato plants exhibiting symptoms of pith necrosis using last-generation techniques and to evaluate the pathogenicity of bacterial isolates collected from different locations in the Central Valley of Costa Rica.

2. Materials and Methods

2.1. Bacterial Isolates and Plant Material

We evaluated 13 Gram-negative bacterial isolates obtained from tomato plants showing stem necrosis symptoms as the most recurrent symptom. Isolates were collected in Paraíso (2) in Cartago province, Alajuela (5) and Zarcero (1) in Alajuela province, and Santa Bárbara (5) in Heredia province, all located in the Central Valley of Costa Rica. Bacterial isolates were classified to the genus level by partial sequencing of the *16S rRNA* gene and were selected from a broader set of isolates based on previous reports of plant pathogenic genera and species.

Five-week-old seedlings of the commercial tomato variety “JR”, which is susceptible to bacterial diseases, and the “Acorazado” variety, developed by the Tomato Breeding Program at the Fabio Baudrit Moreno Agricultural Experiment Station (EEAFBM-UCR) and carrying resistance genes to *Ralstonia solanacearum* phylotype I and Tomato Yellow Leaf Curl Virus (TYLCV) strain Israel [34], were used as plant material. Seedlings were transplanted into 12.8 × 10 cm pots filled with sterile soil and maintained under greenhouse conditions at 26.3°C ± 2.9°C and 58.8% ± 9.1% relative humidity.

2.2. Inoculation and Evaluation

The inoculum was prepared from fresh cultures on tryptic soy agar (TSA); colonies were suspended in phosphate-buffered saline (PBS) to a concentration of 1×10^8 CFU/ml. Inoculation was performed by directly injecting 100 µl of bacterial suspension into the stem at the height of the first true leaf. Six plants per bacterial isolate and per variety were inoculated, along with six plants per variety as PBS-inoculated controls. In total, 168 plants were evaluated over a 21-day period (21 days after inoculation [dai]). Plant growth, defined as the difference between final height and initial height ($Height_{21\ dai} - Height_{0\ dai}$) was measured, and the presence of external and internal symptoms was assessed. Internal symptoms were evaluated by making a longitudinal cut of the stem from the inoculation point towards both ends. The extent of pith necrosis or tissue discoloration (cm) was measured in plants that exhibited the symptoms. The severity scale described by Aysan [35] was used to classify the isolates. These were classified as “non-virulent” if no symptoms were observed; “low-virulent” if necrosis extended 0.1–2.0 cm; “virulent” if necrosis extended 2.1–4.0 cm; and “highly virulent” if necrosis extended ≥ 4.1 cm. To fulfill Koch’s postulates, the bacteria were reisolated from the symptom on nutrient agar (NA), and their identity was confirmed through partial *16S rRNA* gene sequencing.

2.3. Identification of Bacterial Isolates

Pseudomonas isolates causing pith necrosis were identified by Multilocus Sequence Analysis (MLSA) using concatenated partial sequences of the *16S rRNA* [36], *gyrB* [37], *rpoD* [38], and *rpoB* [39] genes. Genomic DNA was extracted from fresh bacterial colonies (< 24 h) using the CTAB protocol [40] with modifications described by Trout [41]. PCR reactions were carried out in a final volume of 25 µl containing 2.5 µl of 10X Dream Taq buffer (Thermo Scientific™), 2.5 µl of 2 mM dNTP mix (Thermo Scientific™), 1.25 µl of each 100 µM primer (Table S1), 0.25 µl of Dream Taq DNA polymerase 5 U/µl (Thermo Scientific™), 1 µl of DNA template (60.6 ± 37.5 ng/µl), and ultrapure water to complete the volume. The thermal profile for each reaction was programmed using amplification parameters specific to each primer pair. PCR product amplification was confirmed by 1% agarose gel electrophoresis.

PCR products were purified with Exonuclease I and sequenced at Macrogen, Inc. (Seoul, South Korea) using the Sanger method [42]. Sequences were edited and assembled in BioEdit v.7.0.5.3 [43], along with type strain sequences downloaded from the NCBI GenBank database (Table S2). Sequence alignment for each locus was performed using MAFFT v.7 [44] with default parameters. Aligned and trimmed sequences of the genes *16S rRNA* (1,266 bp), *gyrB* (443 bp), *rpoD* (409 bp), and *rpoB* (669 bp) were concatenated into a single matrix using Mesquite v.3.61 [45], totaling 2,787 bp. JModelTest v.2.1.10 [46] was used to determine the best nucleotide substitution model for each locus.

Bayesian phylogenetic analysis was performed in MrBayes v.3.2.6 [47]; four chains were run under the GTR+I+G model for the *16S rRNA*, *gyrB*, and *rpoD* genes, and the SYM+I+G model for *rpoB*, with 1,000,000 Markov chain Monte Carlo (MCMC) generations. *Pseudomonas aeruginosa* strain DSM 50071^T was used as the outgroup. A maximum likelihood phylogenetic analysis was also conducted using RAxML v.8.2.12 [48] through the raxmlGUI v.2.0.9 interface [49], with the GTR+I+G model, 1,000 replicates, and bootstrap support values. The consensus phylogenetic tree was edited in iTOL v.6.9.1 [50]. Partial housekeeping gene sequences (*gyrB*, *rpoB*, and *rpoD*) of pathogenic *Pseudomonas* isolates were deposited in the GenBank database under accession numbers PQ883186–PQ883197.

The remaining bacterial isolates were identified by partial sequencing of the *16S rRNA* gene and compared with type sequences from the GenBank database (Table S3), using the same procedure as the multilocus analysis, except for the concatenation step. The GTR+I+G substitution model was used, with a total alignment length of 1,346 bp, and *Bacillus subtilis* strain IAM 12118^T was used as the outgroup. Partial *16S rRNA* gene sequences of the bacterial isolates from this study were deposited in GenBank within the accession numbers PQ857593–PQ857653.

2.4. Genome Sequencing and Analysis

A single colony of virulent isolates *Pseudomonas alliivorans* LTM 13.1.2 and *Pseudomonas capsici* LTM 78.3.2 picked from the NA media plates was used for genomic DNA extraction using the CTAB 2% method [40] with some modifications described by Trout [41]. DNA concentration and quality were assessed using a NanoDrop™ One (ThermoScientific™, USA). The DNA libraries were prepared using the Illumina DNA Prep Kit, and the whole genome sequencing was performed using the Illumina NovaSeq Plus X (2 x 151) paired end method at SeqCenter in Pittsburgh, Pennsylvania. The raw reads were trimmed using Trimmomatic 0.36 [51] and assembled with Unicylcer v0.4.8 [52]. The quality of the genomes was assessed with Quast [53]. The taxonomic classification of the genomes was conducted using GTDB-Tk [54], and the functional annotation was conducted using EggNOGmapper 2.0 [55]. Other genomic features such as secondary metabolites and virulence factors were also annotated using Antismash 8.0 [56] and MmetaVF toolkit [57], respectively.

doi:10.20944/preprints202509.2539.v1

Genomes data can be found in the NCBI BioProject accession number PRJNA1314254. For details on the draft genomes, see [58].

2.5. Statistical Analysis

A linear model was fitted to evaluate the mean plant growth $\Delta Height$ as a function of bacterial isolate, tomato variety, and their interaction. Model selection based on the Akaike Information Criterion (AIC) supported the inclusion of the interaction term:

$$\mu_{\Delta Height_{ijk}} = \beta_0 + \beta_1 isolate_j + \beta_2 var_k + \beta_3 (isolate_j \times var_k) + \epsilon_{ijk} \tag{1}$$

Where $\Delta Height_{ijk}$ represents the height increment of plant i exposed to bacterial isolate j and tomato variety k; β_0 is the intercept corresponding to the reference isolate and variety; β_1 and β_2 represent the main effects of specific bacterial isolate and tomato variety, respectively; β_3 captures the interaction between bacterial isolate and variety; and ϵ_{ijk} is the residual error term, assumed to follow a normal distribution with mean zero and constant variance.

The same linear model structure was used to evaluate the effect of bacterial isolates, tomato variety, and their interaction on internal stem lesions, with lesion length (Nec) as the response variable. The model with interaction was selected based on AIC as the best fit for the data:

$$\mu_{Nec_{ijk}} = \beta_0 + \beta_1 isolate_j + \beta_2 var_k + \beta_3 (isolate_j \times var_k) + \epsilon_{ijk} \tag{2}$$

Where Nec_{ijk} represents the extent of internal lesion in each plant i for each bacterial isolate j and tomato variety k; β_0 is the intercept; β_1 and β_2 represent the main effects of specific bacterial isolate and tomato variety, respectively; β_3 captures the interaction between isolate and variety; and ϵ_{ijk} is the residual error term, assumed to follow a normal distribution with mean zero and constant variance.

A Fisher's LSD test for pairwise comparisons was applied to the fitted linear models to determine statistically significant differences among each bacterial isolate-tomato variety combination.

To assess the effect of internal lesion length (pith necrosis) on plant growth (height increment), two multiple linear regression models were fitted: one including an interaction term between necrosis and tomato variety, and another without interaction. Model selection based on AIC favored the model without interaction:

$$\mu_{\Delta Height_i} = \beta_0 + \beta_1 Nec_i + \beta_2 var_i + \epsilon_i \tag{3}$$

Where $\Delta Height_i$ represents the expected increase in height of plant i; β_0 is the intercept; $\beta_1 Nec_i$ is the coefficient for the extent of pith necrosis; and $\beta_2 var_i$ is the coefficient for the tomato variety; and ϵ_i is the residual error term, assumed to follow a normal distribution with mean zero and constant variance.

All analyses were performed in the R environment [59]. Model selection based on AIC was done using the 'performance' package [60], mean contrast tests were conducted using the 'agricolae' package [61], and models were plotted using the 'ggeffects' [62], 'sjPlot' [63] and 'ggplot2' [64] packages.

3. Results

3.1. Pathogenicity

A total of 61 bacterial isolates were obtained from symptomatic tomato plants and taxonomically classified by *16S rRNA* sequencing. Based on previous reports of plant pathogenic genera/species, 13 isolates were selected for pathogenicity assays to confirm their ability to cause pith necrosis or related stem tissue symptoms in tomato plants, the most recurrent symptom observed in the collected samples. Five of the 13 bacterial isolates assessed in this study caused internal symptoms in both tomato varieties, three of which caused pith necrosis and two caused tissue discoloration. Isolates that only caused slight oxidation at the inoculation site without affecting adjacent tissue were considered non-pathogenic. The most virulent isolate was *Pseudomonas alliivorans* LTM 13.1.2, with a pith necrosis extension of 11.55 ± 5.25 cm in var. “JR” and 4.57 ± 1.37 cm in var. “Acorazado” (Table 1, Figure 1). The necrosis caused by this isolate represented 27.3% of the final plant height (42.35 ± 2.23 cm) at 21-dai in var. “JR.” This was the only isolate that showed significant differences in necrosis extension between varieties, being 39.57% greater in var. “JR” ($p < 0.05$) (Figure 1a). The second most virulent isolate was *P. capsici* LTM 78.3.2, with pith necrosis of 4.63 ± 1.03 cm in var. “JR” and 4.12 ± 1.86 cm in var. “Acorazado” (Table 1, Figure 1). Both isolates were classified as “Highly virulent” according to the scale by Aysan [35]. *P. flavescens* LTM 14.2.2 was classified as “Virulent” for causing 3.10 ± 2.49 cm of necrosis in var. “JR” and 2.45 ± 2.37 cm in var. “Acorazado” (Table 1, Figure 1).

Table 1. Extent of pith necrosis and tissue discoloration (cm) in tomato plants of the varieties “Acorazado” and “JR” inoculated with bacterial isolates from different locations of the Central Valley of Costa Rica at 21 days after inoculation (dai) under greenhouse conditions. Mean ± 95% confidence interval (95% CI) are shown.

Isolate	Species/Genus[a]	Locality	Var[b]	Symp[c]	Plant growth[de] (cm)		Lesion length[e] (cm)		Scale[f]	Virulence category
LTM 13.1.2	*Pseudomonas alliivorans*	Paraíso, Cartago	Ac	PN	19.95 ± 4.18	bcdefg	4.57 ± 1.37	b	3	Highly virulent
			JR	PN	18.85 ± 3.24	cdefg	11.55 ± 5.25	a	3	Highly virulent
LTM 14.2.2	*Pseudomonas flavescens*	Paraíso, Cartago	Ac	PN	20.67 ± 3.05	bcdefg	2.45 ± 2.37	cde	2	Virulent
			JR	PN	23.32 ± 2.91	abc	3.10 ± 2.49	bcd	2	Virulent
LTM 32.2.1	*Pseudomonas* sp.	Alajuela, Alajuela	Ac	O	19.37 ± 4.55	cdefg	0.38 ± 0.28	ef	0	Non-virulent
			JR	O	21.92 ± 3.30	abcde	0.33 ± 0.22	f	0	Non-virulent
LTM 40.2.3	*Pseudomonas* sp.	Alajuela, Alajuela	Ac	O	17.10 ± 5.95	fgh	0.32 ± 0.20	f	0	Non-virulent
			JR	O	21.15 ± 2.51	abcdef	0.32 ± 0.08	f	0	Non-virulent
LTM 49.1.1	*Stenotrophomonas* sp.	Zarcero, Alajuela	Ac	O	19.75 ± 1.66	cdefg	0.35 ± 0.26	ef	0	Non-virulent
			JR	O	21.25 ± 3.80	abcdef	0.47 ± 0.26	ef	0	Non-virulent
LTM 71.3.2	*Serratia* sp.	Alajuela, Alajuela	Ac	O	19.58 ± 5.73	cdefg	0.28 ± 0.18	f	0	Non-virulent
			JR	O	22.50 ± 3.15	abc	0.20 ± 0.13	f	0	Non-virulent
LTM 72.2.1	*Cedecea* sp.	Alajuela, Alajuela	Ac	TD	12.38 ± 2.01	i	1.70 ± 2.31	def	1	Low-virulent
			JR	TD	20.00 ± 3.29	bcdefg	0.74 ± 1.11	ef	1	Low-virulent
LTM 74.3.1	*Serratia* sp.	Alajuela, Alajuela	Ac	O	17.52 ± 1.42	efgh	0.32 ± 0.14	f	0	Non-virulent
			JR	O	20.85 ± 4.01	abcdefg	0.35 ± 0.24	ef	0	Non-virulent
LTM 78.1.1	*Pseudomonas* sp.	Santa Bárbara, Heredia	Ac	O	17.60 ± 2.79	defgh	0.75 ± 0.77	ef	0	Non-virulent
			JR	O	20.97 ± 1.59	abcdef	0.72 ± 0.55	ef	0	Non-virulent
LTM 78.2.1	*Pseudomonas straminea*		Ac	TD	16.93 ± 2.90	fghi	1.32 ± 1.92	def	1	Low-virulent

		Santa Bárbara, Heredia	JR	TD	24.47 ± 1.60	ab	2.03 ± 1.83	cdef	2	Virulent
LTM 78.2.3	*Stenotrophomonas* sp.	Santa Bárbara, Heredia	Ac	O	22.20 ± 1.59	abcd	0.50 ± 0.40	ef	0	Non-virulent
			JR	O	21.38 ± 2.75	abcdef	0.45 ± 0.15	ef	0	Non-virulent
LTM 78.3.1	*Pantoea* sp.	Santa Bárbara, Heredia	Ac	O	13.62 ± 2.48	hi	0.47 ± 0.21	ef	0	Non-virulent
			JR	O	21.85 ± 3.28	abcde	0.58 ± 0.35	ef	0	Non-virulent
LTM 78.3.2	*Pseudomonas capsici*	Santa Bárbara, Heredia	Ac	PN	16.32 ± 2.64	ghi	4.12 ± 1.86	bc	3	Highly virulent
			JR	PN	19.20 ± 3.40	cdefg	4.63 ± 1.03	b	3	Highly virulent
Control[g]	-	-	Ac	-	25.33 ± 2.89	a	0.00 ± 0.00	f	-	-
			JR	-	21.50 ± 2.87	abcdef	0.00 ± 0.00	f	-	-

[a] Isolates identified at the genus level by *16S rRNA* gene analysis; isolates identified at the species level by Multilocus Sequence Analysis (MLSA) using the genes *16S rRNA*, *gyrB*, *rpoD*, and *rpoB*. [b] Var (Tomato variety): Ac = "Acorazado"; JR = "JR.". [c] Symp (Symptom): PN = pith necrosis; TD = tissue discoloration; O = Oxidation at the inoculation site. [d] Plant growth (cm) = $Height_{21\,dai} - Height_{0\,dai}$. [e] Means ± 95% confidence interval (CI) followed by different letters in the same column indicate statistically significant differences according to Fisher's LSD test (alpha = 0.05). [f] Severity scale [35]: 0 = no visible internal symptoms; 1 = 0.1–2.0 cm pith necrosis; 2 = 2.1–4.0 cm pith necrosis; 3 = ≥ 4.1 cm pith necrosis. [g] Control = plants inoculated with 100 µl phosphate-buffered saline (PBS) injected into the stem.

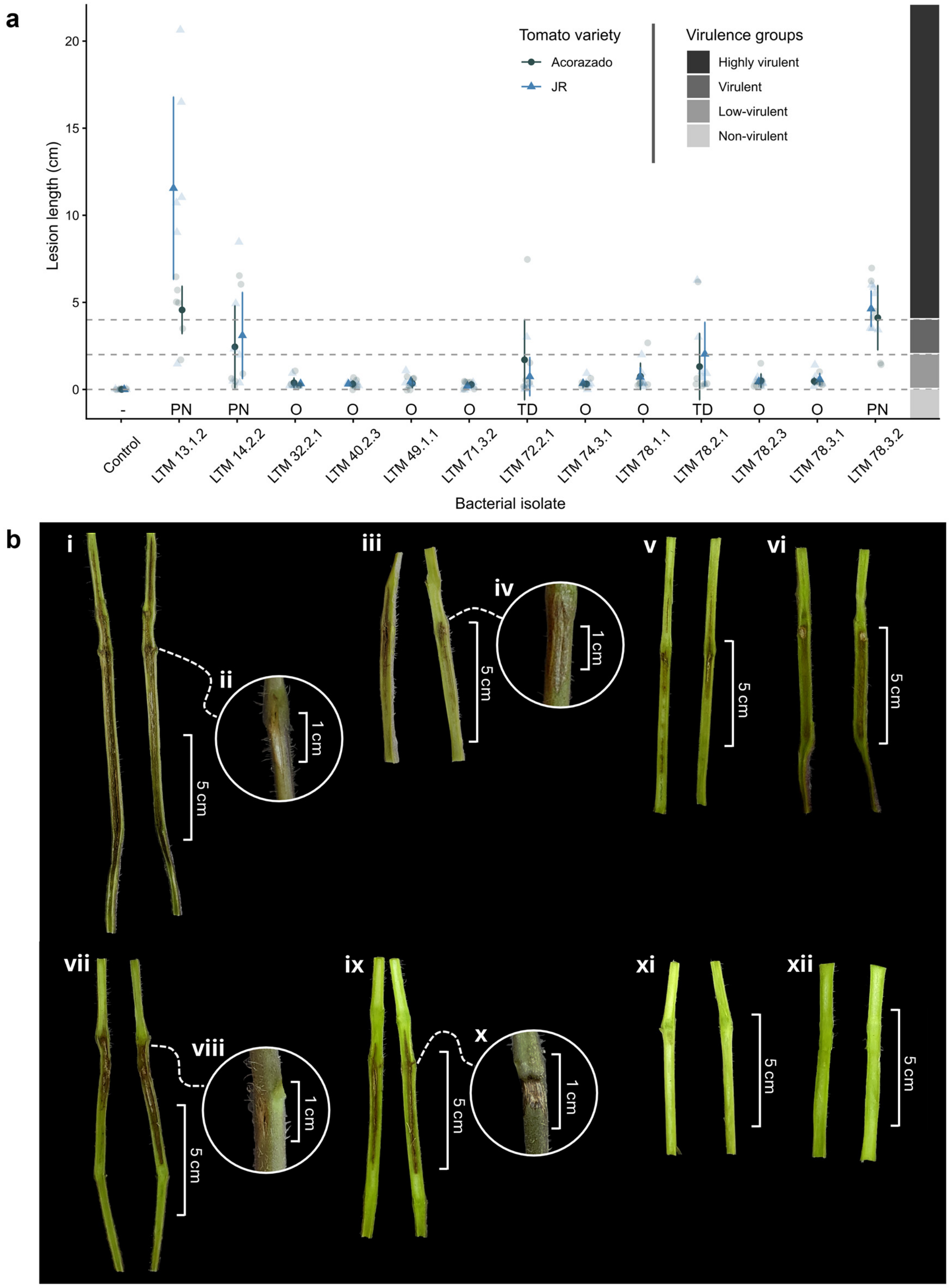

Figure 1. (**a**) Extent (cm) of pith necrosis (PN) or tissue discoloration (TD) in tomato plants of the varieties "Acorazado" and "JR" inoculated with bacterial isolates and control plants inoculated with PBS 21-dai under greenhouse conditions. Oxidation at the inoculation site (O). Virulence categories according to the severity scale [35]. Dark symbols indicate the mean lesion extent and error bars represent the 95% CI. (**b**) Representative internal and external symptoms of pith necrosis observed in tomato plants inoculated with bacterial isolates: *Pseudomonas alliivorans* LTM 13.1.2 in var. "JR" (i, ii) and var. "Acorazado" (iii, iv); *Pseudomonas flavescens* LTM 14.2.2 in var. "JR" (v) and var. "Acorazado" (vi); *Pseudomonas capsici* LTM 78.3.2 in var. "JR" (vii, viii) and var. "Acorazado" (ix, x); Control in var. "JR" (xi) and var. "Acorazado" (xii).

P. straminea LTM 78.2.1 was classified as "Virulent" for causing 2.03 ± 1.83 cm of tissue discoloration in var. "JR", and *Cedecea* sp. LTM 72.2.1 as "Low-virulent" with 1.70 ± 2.31 cm of tissue discoloration in var. "Acorazado" (Table 1, Figure S1). These last two isolates showed greater variability than other isolates classified as "Low-virulent", which only caused mild discoloration at the inoculation site without spreading through the tissue. A trend of greater necrosis or discoloration extension was recorded in the "JR" variety, except for isolate LTM 72.2.1, which caused greater discoloration in the var. "Acorazado." External symptoms such as discoloration and tissue depression at the inoculation site were observed in both tomato varieties with isolates that caused pith necrosis, mainly in isolates LTM 13.1.2 and LTM 78.3.2 (Figure 1b).

Plant growth ($Height_{21\ dai} - Height_{0\ dai}$) was variable, ranging from 4.1 to 32.4 cm, with a mean of 19.91 ± 0.36 cm. Plants of the "Acorazado" variety inoculated with *Cedecea* sp. LTM 72.2.1 showed the lowest average growth (12.38 ± 2.01 cm), followed by those inoculated with *Pantoea* sp. LTM 78.3.1 (13.62 ± 2.48 cm) of the same variety (Table 1). The highest growth was recorded in the control plants of the "Acorazado" variety (25.33 ± 2.89 cm) and plants of the "JR" variety inoculated with *Pseudomonas straminea* LTM 78.2.1 (24.47 ± 1.60 cm). A general trend of lower growth was observed in most plants of the "Acorazado" variety compared to those of the "JR" variety when inoculated with the same isolate, with significant differences ($p < 0.05$) for isolates LTM 72.2.1, LTM 78.2.1, and LTM 78.3.1 (Table 1, Figure 2a).

The effect of internal lesion on plant growth was analyzed, and although an average reduction of -0.20 ± 0.12 cm (mean ± standard error) was observed for each cm increase in internal lesion, it was not statistically significant ($p > 0.05$). However, a significant difference in growth between varieties was found ($p < 0.05$) (Table S4, Figure 2b).

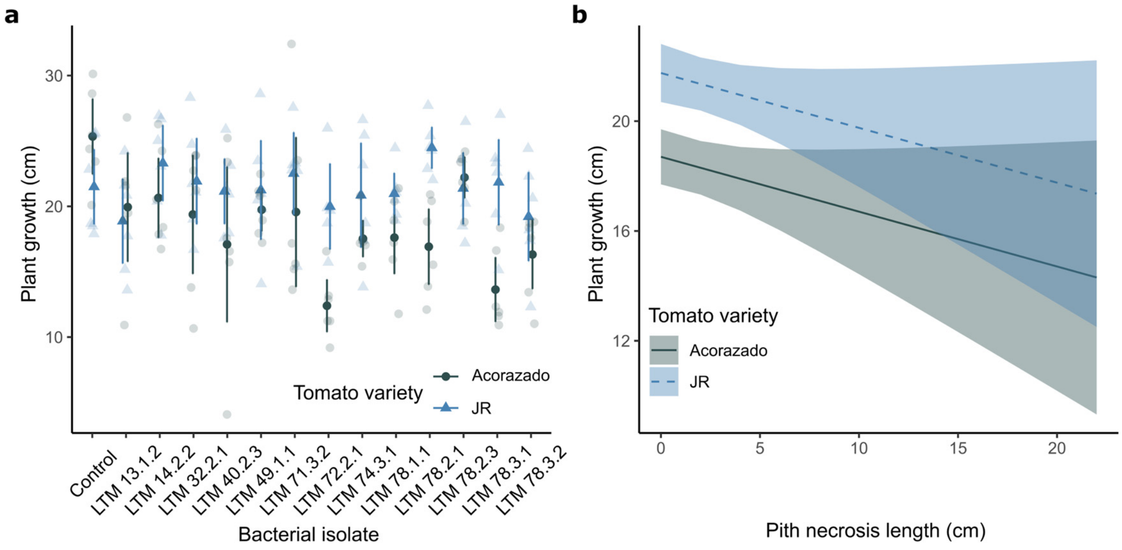

Figure 2. (**a**) Growth ($Height_{21\ dai} - Height_{0\ dai}$; [cm]) of tomato plants of the varieties "JR" and "Acorazado" inoculated with bacterial isolates and control plants inoculated with phosphate-buffered saline (PBS) at 21 days after inoculation (dai) under greenhouse conditions. Dark symbols indicate the mean plant growth for each isolate and variety; error bars represent the 95% confidence interval. Light symbols show the actual values to illustrate variability. (**b**) Effect of pith necrosis length (cm) on plant growth (cm) in tomato plants of the "JR" and "Acorazado" varieties.

3.2. Taxonomic Classification and Phylogenetics

The taxonomic classification of pathogenic bacteria using the Multilocus Sequence Analysis (MLSA) method yielded the following results: the isolate LTM 13.1.2 was identified as *Pseudomonas* *alliivorans*, isolate LTM 78.3.2 as *Pseudomonas capsici*, isolate LTM 14.2.2 as *P. flavescens*, and isolate LTM 78.2.1 as *P. straminea* (Figure 3); isolate LTM 72.2.1 was identified as *Cedecea* sp. (Figure S2).

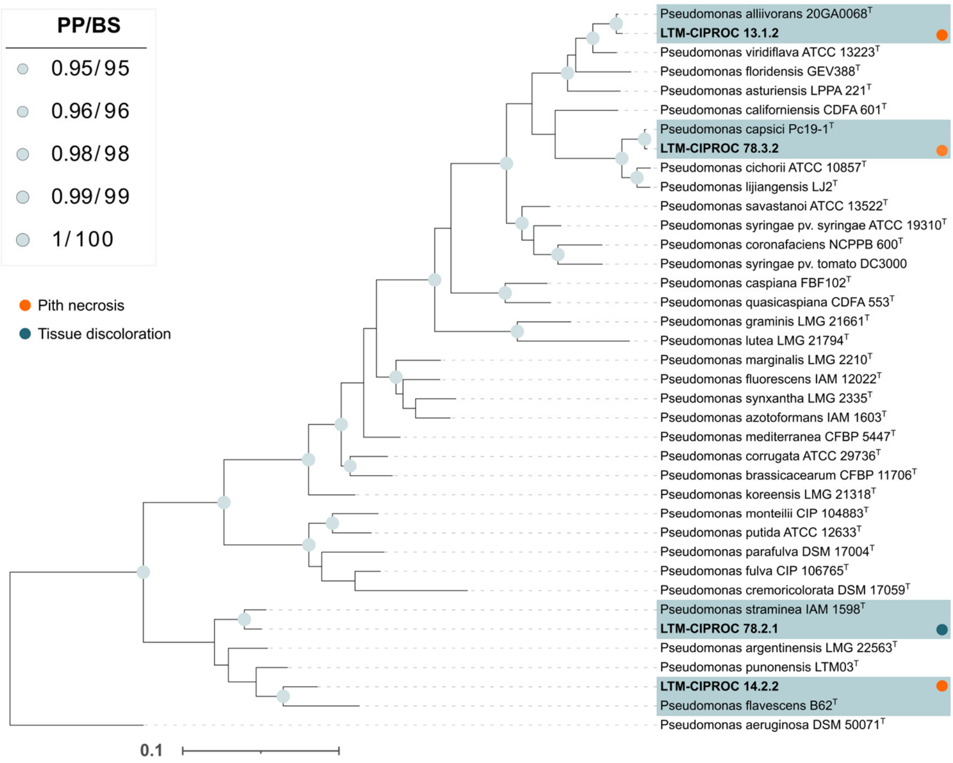

Figure 3. Consensus phylogenetic tree of *Pseudomonas* spp. based on the concatenated partial sequences of the *16S rRNA*, *gyrB*, *rpoD*, and *rpoB* genes. The isolates analyzed in this study are shown in bold with the acronym 'LTM-CIPROC' (Molecular Techniques Laboratory, CIPROC-UCR) and are shaded along with the closest type strain. The tree shows the topology of the Bayesian phylogenetic analysis. Circles at the nodes indicate the combination of support values (PP = Bayesian posterior probability ≥ 0.95; BS = Maximum Likelihood bootstrap support ≥ 95%). ^{T} = Type strain. The scale bar represents the estimated number of substitutions per site.

3.3. Genome Sequencing and Analysis

Genome sequencing of the two most virulent isolates using Illumina technology results in a genome assembly size of 6.15 Mb for isolate LTM 13.1.2 with a coverage of 143X, and 5.87 Mb for isolate LTM 78.3.2 with a coverage of 146X. The number of contigs in each genome assembly were 52 and 54. The N50 of the assemblies were 249,463 bp and 182,980 bp. The total number of predicted genes in each genome were 5,527 and 5,000. The GC content of the genomes was 59.1% and 58.37%, respectively (Table S5). Taxonomic classification of the isolates was confirmed with GTDB-Tk tool and database, the Average Nucleotide Identity (ANI) shows that isolate LTM 13.1.2 has an ANI value of 98.86% when compared to *P. alliivorans* 20GA0068^T, and isolate LTM 78.3.2 has an ANI value of 99.53% when compared to *P. capsici* Pc19-1^T.

Virulence factors and the gene clusters associated with secondary metabolites were annotated for each genome assembly. Twenty-eight genes related to virulence factors such as Type IV pili (T4P), alginate biosynthesis (mucoid exopolysaccharide), pyoverdine (siderophore) and flagella were detected in the genome of *P. alliivorans* LTM 13.1.2. For *P. capsici LTM 78.3.2* genome assembly, thirty genes associated with virulence factors including T4P, Tap T4P, alginate, pyoverdine and flagella were detected (Table S6).

Regarding biosynthetic gene clusters, eleven clusters associated with secondary metabolites were detected in *P. alliivorans* LTM 13.1.2 genome, including arylpolyene, hydrogen cyanide, Non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) products, siderophores, N-acetylglutaminylglutamine amide (NAGGN), a redox-cofactor, a terpene, and several NRPS-derived compounds. In *P. capsici* LTM 78.3.2 genome, fourteen clusters were identified, comprising NRPS, PKS, siderophores, NAGGN, arylpolyene, hydrogen cyanide, a redox-cofactor, and diverse NRPS-derived products (Table S7).

4. Discussion

This study provides the first evidence of several bacterial species affecting tomato plants in Costa Rica. *Pseudomonas alliivorans*, recently described associated with foliar symptoms in onion and cucurbits [65,66], is reported here for the first time as a causal agent of pith necrosis in tomato. Likewise, *P. capsici*, a species associated with foliar diseases in a wide range of hosts [65,67–69], is also reported for the first time causing tomato pith necrosis, as well as its presence in Costa Rica, with the additional relevance that it can induce symptoms at higher temperatures compared to other species like *Pseudomonas syringae* [65]. Given the symptoms induced by these isolates at early stages of tomato cultivation under experimental conditions, further evaluation of their effect on crop yield is necessary, despite the fact that some studies have reported that such symptomatology caused by related species like *P. viridiflava* does not always result in significant yield losses [70]. Furthermore, the ability to colonize both foliar and vascular tissues underscores the potential phytosanitary risk these bacteria pose not only to tomato but also to other solanaceous crops in tropical environments. Consequently, it is imperative to implement monitoring strategies to prevent their spread, as well as effective management strategies.

The phylogenetic proximity between *P. alliivorans* with *P. viridiflava* [66] and *P. capsici* with *P. cichorii* [68], well-known tomato pathogens [16,17,24,25], raises the possibility of historical misidentifications in different regions of the world, as has occurred with several *Pseudomonas* species [71]. Moreover, recent taxonomic revisions showed that some strains previously identified as *Pseudomonas cichorii* are in fact *P. capsici* [69], reinforcing the need for accurate molecular identification and ongoing epidemiological surveillance to correctly map pathogen distributions in tomato-producing regions.

The recovery of *P. flavescens* from symptomatic tomato plants further illustrates the growing diversity of *Pseudomonas* taxa capable of colonizing and damaging tomato vascular tissues. As with the previous isolates, this is the first report of *P. flavescens* causing pith necrosis in tomato. Although this species has been only sporadically reported in association with other hosts [72,73], their association here with necrosis suggests it may contribute to a broader disease complex in tomato.

Although some isolates exhibited low virulence under greenhouse conditions, their detection in symptomatic tissue should not be dismissed as incidental. Opportunistic bacteria detected in this study causing only tissue discoloration, such as *Pseudomonas straminea* and *Cedecea* sp., may act as secondary colonizers or potentially emerge as pathogens under specific environmental or host conditions [20,74]. The presence of pathogens of different taxonomic groups and that its pathogenicity in vegetables has not been reported, such as *Cedecea* sp. [75,76], underscores the importance of genomic and functional characterization to determine pathogenic potential and epidemiological relevance in vegetable crops.

Importantly, previously reported synergistic interactions among *Pseudomonas* spp. such as *P. straminea* with other pathogenic *Pseudomonas* species and with *Xanthomonas perforans* [20,77] suggest that field outbreaks may frequently involve multispecies infections that act together to exacerbate symptoms. This factor may complicate diagnosis and reduce the effectiveness of single-target control measures.

The bacterial isolates that caused pith necrosis also triggered external symptoms such as tissue discoloration and sunken lesions at the inoculation site, similar to those described for other causal agents of pith necrosis [16,32]. Although the formation of adventitious roots on the stem was not observed, this symptom may emerge in later stages of the crop cycle [16,32,78]; therefore, evaluations throughout the entire production cycle are suggested to capture the complete symptomatology and its temporal dynamics.

The variability in plant growth observed across treatments suggests that the response to bacterial inoculation is not uniform and may be influenced by multiple factors, including the intrinsic physiological variability between plants [79]. Although a general trend of reduced growth was observed in the “Acorazado” variety compared to the “JR”, this pattern was not evident in the control plants, indicating that the variety itself may not be inherently less vigorous but rather may be more sensitive or susceptible to specific microbial interactions [80]. Notably, inoculation with highly virulent isolates did not result in measurable reductions in plant growth, suggesting that internal damage is not always associated with immediate loss in vegetative development, at least within the evaluation period.

The genome analysis of the two most virulent isolates revealed the presence of several virulence factors and biosynthetic gene clusters that may confer a competitive advantage as plant pathogens. Some of those genomic traits have been reported in well-known plant pathogenic bacteria and contribute to their pathogenicity and virulence [81–84]. Phytotoxin syringomycin detected in one isolate has been associated with necrosis promotion in the host and enhancement of bacterial growth in a wide variety of plants [85]. This toxin along with virulence factors may be involved in the highly virulence expressed. Furthermore, genomic traits implicated in protection against plant defense mechanisms [86–88], microbial competition [89,90], and environmental stress tolerance [91–94] may benefit these isolates in microbe-microbe interactions and provide them efficient ways for epiphytic and environmental survival. Despite the damage observed in plants, the present study does not provide evidence that these virulence genes are expressed under in vitro or in vivo conditions.

This study represents the first systematic effort in Costa Rica to identify bacteria associated with tomato pith necrosis. The findings of this study contribute to the existing body of knowledge by including new species of pathogenic bacteria that have been observed to cause pith necrosis in tomato plants. This suggests that the range of causal agents may be broader than previously recognized, and that there are potentially additional unidentified species present. Furthermore, the presence of bacteria that cause tissue discoloration could weaken the plant and make it more susceptible to attack by more aggressive pathogens. All of the above highlights the importance of considering aspects such as the identity and virulence of pathogenic organisms when diagnosing and implementing plant health management strategies.

5. Conclusions

Five bacterial isolates were identified as causal agents of internal symptoms in tomato: *Pseudomonas alliivorans* LTM 13.1.2, *P. flavescens* LTM 14.2.2, and *P. capsici* LTM 78.3.2 caused pith necrosis, while *Cedecea* sp. LTM 72.2.1 and *P. straminea* LTM 78.2.1 caused tissue discoloration. *P. alliivorans* LTM 13.1.2 was the most virulent isolate, followed by *P. capsici* LTM 78.3.2. Genome sequences of the most virulent isolates revealed several virulence factors and gene clusters associated with secondary metabolites that may contribute to the pathogenicity of these isolates, while also providing a competitive advantage over other microbes and enhancing environmental survival.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1. Primers used for the amplification and partial sequencing of the *16S rRNA*, *gyrB*, *rpoD*, and *rpoB* genes; Table S2. Reference strains used in the Multilocus Sequence Analysis (MLSA). Partial sequences of the *16S rRNA*, *gyrB*, *rpoD*, and *rpoB* genes from type strains were retrieved from the GenBank database of the National Center for Biotechnology Information (NCBI); Table S3. Reference strains used in the phylogenetic analysis for the identification of isolate LTM 72.2.1 and the non-pathogenic isolates. Partial sequences of the *16S rRNA* gene from type strains were retrieved from the GenBank database of the National Center for Biotechnology Information (NCBI); Table S4. Effect of pith necrosis length (cm) on plant growth ($Height_{21\ dai} - Height_{0\ dai}$; [cm]) in the tomato varieties “Acorazado” and “JR” at 21 days after inoculation (dai) under greenhouse conditions.; [cm]) in the tomato varieties "Acorazado" and "JR" at 21 days after inoculation (dai) under greenhouse conditions; Table S5. Assembly statistics, genome features and genome annotation summary for *Pseudomonas alliivorans* LTM 13.1.2 and *Pseudomonas capsici* LTM 78.3.2; Table S6. Virulence genes detected in *Pseudomonas alliivorans* LTM 13.1.2 and *Pseudomonas capsici* LTM 78.3.2 genomes; Table S7. Gene clusters associated with secondary metabolites annotated for the *Pseudomonas alliivorans* LTM 13.1.2 and *Pseudomonas capsici* LTM 78.3.2 genome assemblies; Figure S1. Vascular tissue discoloration in tomato plants inoculated with bacterial isolates, evaluated 21 days after inoculation (dai). *Pseudomonas straminea* LTM 78.2.1 in var. "JR" (i) and var. "Acorazado" (ii); *Cedecea* sp. LTM 72.2.1 in var. "JR" (iii) and var. "Acorazado" (iv); Control in var. "JR" (v) and var. "Acorazado" (vi); Figure S2. Consensus phylogenetic tree constructed from partial *16S rRNA* gene sequences of isolate LTM 72.2.1, which caused tissue discoloration, and non-pathogenic isolates. Isolates analyzed in this study are shown in bold with the acronym 'LTM-CIPROC' (Molecular Techniques Laboratory, CIPROC-UCR) and shaded. The topology of the Bayesian phylogenetic analysis is shown. Circles at the nodes indicate combined support values (PP = Bayesian posterior probability ≥ 0.80; BS = Maximum Likelihood bootstrap support ≥ 80%). ^{T} = Type strain. The scale bar represents the estimated number of substitutions per site.

Author Contributions: Conceptualization, D.A-R., M.B-M., F.G-S. and W.B-S.; methodology, D.A-R., M.B-M., F.G-S. and G.VA.; software, D.A-R. and G.VA.; validation, D.A-R., M.B-M., F.G-S and G.VA.; formal analysis, D.A-R. and G.VA.; investigation, D.A-R. and G.VA.; resources, D.A-R., M.B-M., F.G-S., W.B-S. and G.VA.; data curation, D.A-R. and G.VA.; writing—original draft preparation, D.A-R.; writing—review and editing, D.A-R., M.B-M., G.VA., F.G-S. and W.B-S.; visualization, D.A-R.; supervision, M.B-M.; project administration, M.B-M.; funding acquisition, M.B-M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Vicerrectoría de Investigación de la Universidad de Costa Rica, grant number 813-C0-466. The APC was funded by Vicerrectoría de Investigación de la Universidad de Costa Rica.

Data Availability Statement: All data analyzed during this study are available from the corresponding author upon reasonable request.

Acknowledgments: We want to express our acknowledgments to the producers of the Cartago, Alajuela, and Heredia areas for facilitating tomato samples. To Anny Calderón-Abarca, Gabriela Chinchilla-Salazar, Alejandro Sebiani-Calvo, Edgar Vargas, Carlos Chacón and Yeimy Ramírez for their technical support in laboratory procedures.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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