Abstract: Jalapeño peppers (Capsicum annuum var. Jalapeño) represent one of the most important crops in Mexico. However, many plant diseases, such as wilt caused by strains of the genus Fusarium, reduce its yield. A sustainable alternative to control diseases is the use of biological control agents (BCAs), for example, beneficial microorganisms such as strains of the genus Bacillus. This study aims to analyze the potential use of B. cabrialesii subsp. tritici TSO2T as a BCA and elucidate its potential modes of action against Fusarium strains causing wilt in Jalapeño peppers. For this, symptomatic samples were collected in a commercial field in the Yaqui Valley, Mexico. Six Fusarium isolates were morphologically and molecularly characterized. After pathogenicity tests, F. languescens CE2 was found to be pathogenic. In screening assays for biocontrol bacteria, strain TSO2T was isolated from soil in a wheat commercial field under an organic production system and preserved in the Culture Collection of Native Soil and Endophytic Microorganisms (COLMENA), had the best biocontrol effect against CE2, and its cell-free filtrate reduced mycelial growth by 30.95%. Genome mining (antiSMASH) of strain TSO2T allows us to identify gene clusters associated with biocontrol, such as fengycin, surfactin, bacillibactin, bacilysin, bacillaene, subtilosin A, and sporulation killing factor, which codify to antimicrobial metabolites and are associated with swarming motility of the studied beneficial strain. These results demonstrate the effectiveness of B. cabrialesii subsp. tritici TSO2T as a potential BCA for the control of Fusarium wilt, through competition and a complex of antifungal metabolites, which still need to be validated through metabolomic analysis. This study highlights the importance of the use of integrative genomic and bioactivity-guided methodologies in understanding biocontrol pathosystems.

Keywords: B. cabrialesii; biological control agent; wilt; Fusarium; plant disease; sustainable agriculture; secondary metabolites
which harvested 22,132.5 tons in 2022 [5]. However, its yield has been affected by the incidence of plant diseases, such as wilt, mostly caused by different members of the genus *Fusarium* [1,6,7], mainly by the *Fusarium oxysporum* species complex (FOSC) [8]. This fungus is one of the most common wilt-inducing pathogens and belongs to one of the main groups of plant-pathogenic fungi known to agriculture and horticulture. It causes a variety of diseases affecting over 100 plant species, and is responsible for up to 50% yield losses, even up to 80% have been reported in favorable environmental conditions for its development, and ranks as the fifth most lethal plant pathogen [9–13]. Members of this species complex have been found in virtually all environments and agroecosystems and can be dispersed through the air, soil, and water, distributed with infected host plants, and anthropogenic dispersal [14,15]. Plant-colonizing *Fusarium* species commonly are dependent on the presence of a host and on the appropriate climate for the host that they colonize [14], but some strains are also capable of colonizing crop residue and quickly reoccupying chemically fumigated soils [15,16].

In recent years, the excessive use of insecticides, pesticides, and inorganic fertilizers has had detrimental effects on soil fertility and health [17]. Scientific evidence has confirmed that employing agrochemicals to enhance crop yield and manage pests and diseases has led to negative effects on ecosystems and human health [17,18]. Additionally, this practice has resulted in the development of pathogen resistance, further complicating its control [18]. Therefore, there is an urgent global demand to embrace sustainable agricultural approaches that prioritize the environment and reduce dependency on agricultural chemicals [19]. Recently, the use of beneficial microorganisms has gained significant importance in the agricultural area, providing a sustainable option focused on increasing crop yield, soil fertility, and salinity management [19,20]. These microorganisms present in agro-ecosystems contribute to the sustainability of food security, and their use as biological control agents (BCA) represents a successful strategy for disease management, for they not only inhibit phytopathogen proliferation but also promote plant growth [20,21]. Promising results have been obtained with the application of several BCA, among them, strains of the genus *Bacillus*, which can replicate rapidly and resist adverse environmental conditions, as well as having a broad spectrum of biocontrol abilities [22]. As an example of disease management, *B. thuringiensis* is a soil bacterium that produces spores, which contain crystals that possess strong and targeted insect-killing capabilities [23]. Likewise, strains of *B. cabrialesii*, a bacterial species recently discovered which is widely reported as a BCA and plant growth promoter [24]. In this sense, the genus *Bacillus* inhibits the proliferation of phytopathogens through the synthesis of growth-suppression molecules, such as cell wall-destructive enzymes, antibiotics, lipopeptide biosurfactants, or by triggering defense signals in plants [25–27].

Recently, a novel subspecies of *Bacillus cabrialesii*, named *Bacillus cabrialesii* subsp. *tritici*, has been described with its type strain being TSO2T [28]. This strain has shown promising results as a BCA against the responsible agent of spot blotch disease in wheat, *Bipolaris sorokiniana* TPO3, by the production of diffusible extracellular metabolites with potent antifungal activity. Additionally, the inoculation of strain TSO2T to wheat plants positively regulated the synthesis of soluble carbohydrate and fructan concentrations and increases root length, stem height, dry weight, and total nitrogen and chlorophyll content [29,30]. Because of its prospective capacity for the biological management of phytopathogens and promoting plant growth, further studies have been conducted on various important crops and plant diseases. Thus, this study aims to describe *B. cabrialesii* subsp. *tritici* TSO2T as a BCA and elucidate its potential modes of action against *Fusarium* species causing wilt of Jalapeño peppers in the Yaqui Valley, Mexico.

2. Materials and Methods

2.1. Collecting Sites and Sampling Methodology

In February of 2022, surveys were conducted in a Jalapeño pepper commercial field in the Yaqui Valley, Sonora, Mexico (N 27° 17′ 40.308″, W 109° 51′ 50.5″) for wilting and rot.
symptoms. Wilt symptoms were observed on *Capsicum annuum* var. Jalapeño (Figure 1). Representative samples of wilting plants were taken whole, transported to the laboratory in plastic bags, and stored at 4 °C until processing (24 h). A total of 12 Jalapeño pepper plants exhibiting wilting were assayed for *Fusarium* detection.

**Figure 1.** Wilt symptoms on Jalapeño peppers on a commercial field in the Yaqui Valley, Mexico. Wilt symptoms are indicated with yellow arrows.

### 2.2. Fungal Isolation

The isolation of *Fusarium* from wilted plants was performed following the protocol described by Santillán–Mendoza et al. [31]. Briefly, to separate the soil from the roots, small subsamples, which consisted of radical tissue, were rinsed with tap water, and then disinfested with 0.6% sodium hypochlorite for 1 min, rinsed three times in sterile water, and blotted dry on sterile paper. Small explants of 10 to 12.5 mm² were excised and transferred to 90 mm × 15 mm Petri dishes with peptone agar (peptone 15 g L⁻¹, KH₂PO₄ 1 g L⁻¹, MgSO₄·7H₂O 0.5 g L⁻¹, agar 20 g L⁻¹) [32], supplemented with 0.15 g L⁻¹ of ciprofloxacin. After 24 to 48 h at 30 °C, to purify *Fusarium* isolates, hyphal tips were transferred directly to synthetic low-nutrient agar (SNA; KH₂PO₄ 1 g L⁻¹, KCl 0.5 g L⁻¹, KNO₃ 1 g L⁻¹, MgSO₄·7H₂O 0.5 g L⁻¹, glucose 0.2 g L⁻¹, sucrose 0.2 g L⁻¹, agar 20 g L⁻¹) from colonies that emerged from the tissue explants [33]. Isolates were incubated at 30 °C for 7 days. Purified isolates were stored at −80 °C in 15% glycerol, in the Culture Collection of Native Soil and Endophytic Microorganisms (COLMENA, for its Spanish acronym; www.itson.mx/colmena accessed on 27 July 2023) in the Instituto Tecnológico de Sonora (ITSON), Mexico, where they are available upon request [20,34].

### 2.3. Fusarium Morphological Characterization, DNA Sequence-Based Identification, and Phylogenetic Analysis

A total of six *Fusarium* isolates were obtained from symptomatic Jalapeño pepper samples. *Fusarium* isolates were grown on plates of potato dextrose agar (PDA; potato extract 4 g L⁻¹, dextrose 20 g L⁻¹, agar 15 g L⁻¹) at 30 °C in darkness. Morphological characters were identified using 7 to 10 days old cultures. For each isolate, colony characteristics and the presence of chlamydospores were recorded, as well as macroconidia and microconidia characteristics [32].

The six isolates obtained from symptomatic Jalapeño peppers were processed by extracting genomic DNA. For this, mycelial mats from 7-day-old static cultures grown in potato dextrose broth (PDB; potato extract 4 g L⁻¹, dextrose 20 g L⁻¹) (30 °C) were pelleted by centrifuging for 3 min and then rinsed with sterile distilled water. DNA was extracted using a procedure described by Raeder and Broda [35], with modifications: ground mycelium was incubated in a lysis buffer at 65 °C for 30 min and no RNAse A was used.
A spectrophotometer (Nanodrop; Thermo Scientific, Waltham, MA, USA) was used to determine the DNA concentration. The high-quality genomic DNA of *Fusarium* isolates was amplified by PCR. For this, a fragment of the translation elongation factor 1-α (TEF1) was amplified as previously described by O’Donnell et al. [36], using primer pairs EF1 (5′-ATGGGTAAGGARGACAAGAC-3′) and EF2 (5′-GGARGTACCAGTSATCATG-3′). All PCR amplifications employed MyTaq™ DNA Polymerase (Meridian Bioscience, Cincinnati, OH, USA) in an Analytik Jena Biometra TAdvanced thermocycler (Jena, Germany). The following program was used: 1 cycle of 90 s at 94 °C; 35 cycles of 30 s at 94 °C, 90 s at 55 °C, and 1 min at 72 °C; followed by 1 cycle of 5 min at 72 °C and a 4 °C soak. All BLASTn searches on the National Center for Biotechnology Information (NCBI) and Pairwise ID of FUSARIOID-ID (https://www.fusarium.org/ accessed on 20 June 2023) were conducted by using TEF1 partial sequences as queries. After a pathogenicity test (described below) and to further the identification of the pathogenic strain, a phylogenetic analysis of two-locus DNA sequence data from the pathogenic *Fusarium* isolate CE2 belonging to the *Fusarium oxysporum* species complex (FOSC) and 28 previously characterized isolates of FOSC, was carried out [9], including fragments of the TEF1 and the second largest RNA polymerase subunits (RPB2). RPB2 amplifications used the primer pair 5f2 (5′-GGGGWGAYCAGAAGAAGGC-3′) and 11ar (5′-GGRTGGATCTTRCTCRTCSACC-3′) and the same cycling programs described before but with an annealing temperature of 60 °C [36]. Amplicons were sequenced at the Laboratorio de Servicios Genómicos (LABSERGEN-CINVESTAV), Mexico, by Sanger sequencing. Sequence chromatograms were edited with MEGA v. 10.2, exported as FASTA files, aligned with MUSCLE in SeaView v. 5.0.5 [37,38], concatenated in SequenceMatrix v. 1.7.8 [39], and then subjected to partitioned maximum likelihood (ML) phylogenetic analyses with IQ-TREE 1.6.10 [40] with ultrafast bootstrap [41]. Model-Finder [42] was employed to identify the optimal molecular evolution models for each partition based on the Bayesian information criterion scores [43]. DNA sequences were deposited in the NCBI GenBank under the following accession numbers: TEF1 pdaOR168964 to OR168969, and RPB2 OR168973.

2.4. *Fusarium* Pathogenicity Tests in Jalapeño Peppers

Healthy seedlings of Jalapeño peppers were kindly donated by the Yaqui and Mayo Vegetable Producers Association (APHYM, for its Spanish acronym). Three representative *Fusarium* isolates obtained in this study (CE2 (FOSC), CE3 (*Fusarium incarnatum-equiseti* species complex, FIESC), and CE6 (*Fusarium solani* species complex, FSSC)), were grown on PDA plates at 30 °C in darkness for 10 days. Spores were recovered from the culture plates with sterile distilled water. Following procedures previously described with modifications [31,44], ten Jalapeño peppers seedlings per treatment were submerged for 30 min in 200 mL of a 1 × 10⁶ spores mL⁻¹ suspension of each studied isolate. Control plants were immersed in sterile distilled water. After this, the treated Jalapeño pepper seedlings were individually transferred into 100 mL pots filled with garden soil previously sterilized (121 °C for 2 h, for three consecutive days). The pots with the seedlings were kept in greenhouse conditions (24.2 ± 2.26 °C) with natural lighting for 20 days with irrigation every third day. At the end of the pathogenicity test, fungal isolates recovered from the treatments were identified by Sanger sequencing of a fragment of the TEF1 gene. The experiment was conducted twice.

2.5. Biocontrol Screening Assay by Confrontation In Vitro

To find a bacterial strain capable of controlling the growth of *Fusarium* causing wilt in Jalapeño peppers, six bacterial strains belonging to COLMENA, previously reported with biological control and/or plant growth promotion activity [25,29,30,45,46], were tested in confrontations in vitro. These strains were: (i) *Bacillus cabrialesii* subsp. *cabrialesii* TE3¹ [28,47], (ii) *B. cabrialesii* subsp. *tritici* TSO2¹ [28], (iii) *B. paralicheniformis* TRQ65 [48], (iv) *Bacillus* sp. FSQ1 [49], (v) *Bacillus* sp. TE3¹-UV25, and (vi) *Bacillus* sp. TSO22. These bacterial strains were grown in nutritive broth (NB; peptone 5 g L⁻¹, beef extract 3 g L⁻¹)
for 24 h at 30 °C and 180 rpm for agitation. After this, the biomass of the bacterial strains was pelleted by centrifugation at 5000 rpm for 10 min and then resuspended in sterile distilled water to adjust to OD_{630nm} = 0.3. After the pathogenicity assay (described above, Section 2.4), the Fusarium strain CE2 was selected for further analysis and its spore suspension was obtained from PDA plate cultures and adjusted to 1 × 10^6 spores mL^{-1}. Then, in the center of 90 mm × 15 mm PDA plates, 20 µL of Fusarium strain CE2 spore suspension was inoculated and 20 µL of each biocontrol bacterial suspension was inoculated, at three equidistant points from the fungal strain (10 mm from the edge). Control plates consisted of just the inoculation of Fusarium sp. CE2 in the center of the plate. Three replicates were made of each treatment. Plates were incubated at 30 °C, in darkness for seven days. After this time, the growth of the Fusarium sp. CE2 was determined by measuring the radius of the colony in the direction of the biocontrol bacteria. The bacterial strain B. cabrialesii subsp. tritici TSO2\textsuperscript{1} was selected from the dual confrontation assay since it showed the best biocontrol. Strain TSO2\textsuperscript{1} was isolated from soil in an organically fertilized wheat commercial field in the Yaqui Valley, using a serial dilution method in nutrient agar at 28 °C for 2 days, this strain was cryopreserved using nutrient broth supplemented with 30% glycerol, in COLMENA [28]. For this assay, cellular biomass of TSO2\textsuperscript{1} (grown for 72 h in NB at 30 °C with 120 rpm agitation) and Fusarium CE2 (grown in PDA at 30 °C for 7 days) spore suspension was obtained as previously described, and adjusted to OD_{630nm} = 0.5 and 1 × 10^6 spores mL^{-1}, respectively. The dual confrontation assay was carried out on PDA plates, placing 20 µL of spore suspension and bacterial biomass at opposite sides of the plate (10 mm from the edge); control plates consisted of 20 µL of the CE2 spore suspension and sterile distilled water. The plates were incubated at 30 °C, in darkness, for 10 days, measuring the fungal colony’s growth daily.

2.6. Bacillus cabrialesii subsp. tritici TSO2\textsuperscript{1} Hybrid Genome Assembly and Annotation

High-quality genomic DNA from a fresh culture of Bacillus cabrialesii subsp. tritici TSO2\textsuperscript{1} was extracted, which was cultivated in NB for 24 h at 32 °C with an orbital shaker at 121 rpm, resulting in a concentration of 1 × 10^6 CFU mL^{-1}. The DNA extraction followed the phenol-chloroform method described by Valenzuela–Aragon et al. [29]. Subsequently, the bacterial DNA underwent sequencing using both the Illumina MiSeq platform and the MinION sequencing technology by Oxford Nanopore Technology (ONT).

For the assembly process, Unicycler version 0.4.8 [50] was used for de novo assembly, employing short reads as a foundation and long reads to bridge gaps. Furthermore, the genome hybrid assembly through SPAdes version 3.13.1 [51] with default parameters in bold mode. To assess the quality of the assembly, Quast v.4.4 [52] and CheckM v.1.0.18 [53] through the KBase online platform [54] were used. The genome annotation was executed using the Rapid Prokaryotic Genome Annotation (Prokka) version 1.0.0 [55] on the annotation platform Proksee (https://proksee.ca/ accessed on 27 July 2023) [56].

2.7. Bacillus cabrialesii subsp. tritici TSO2\textsuperscript{1} Genome Mining for Secondary Metabolite Biosynthetic Gene Clusters (BGCs) and Antagonistic Activity against Fusarium sp. CE2 by Diffusible Extracellular Metabolites

To evaluate the biosynthetic potential of B. cabrialesii subsp. tritici TSO2\textsuperscript{1} and identify putative genes involved in the biological management of phytopathogens, the whole genome of the strain (accession number JAHBMK000000000.2) was submitted to Antibiotics & Secondary Metabolite Analysis Shell (antiSMASH) web-server (https://antismash.secondarymetabolites.org accessed on 27 July 2023) under detection strictness parameter = strict [57].

In addition, the antifungal action of those putative extracellular metabolites produced by B. cabrialesii subsp. tritici TSO2\textsuperscript{1} was tested against Fusarium sp. CE2, as follows: liquid bacterial culture was prepared by inoculating a full loop of strain TSO2\textsuperscript{1} in 20 mL of NB and incubated at 28 °C, under constant stirring at 120 rpm. After 24 h, the bacterial culture was centrifuged at 5000 rpm for 10 min, to obtain a biomass pellet that was resuspended in sterile distilled water to OD_{630nm} = 0.5. In 50 mL of NB, 250 µL of the resuspended bacterial
biomass was inoculated and incubated with the previously mentioned conditions for 72 h. After this, the bacterial culture was centrifuged at 5000 rpm for 10 min and the supernatant was recovered and passed through a hydrophilic syringe filter (0.22 µm). The obtained CF was evaluated against *Fusarium* sp. CE2 in a micro-bioassay method, in sterile 24-well plates [25,58]. To do this, 100 µL of fungal conidia suspension (1 × 10⁵ spores mL⁻¹) was inoculated into each sterile well, containing: 1.5 mL of PDB as a culture medium, for control treatment; for CF treatment, 750 µL were replaced with CF (to reach a concentration of 50% v/v). The plates were sealed and incubated at 28 °C for 3 days under constant stirring at 120 rpm. The dry weight of fungal biomass was recorded, and the inhibitory activity of CF against *Fusarium* sp. CE2 was determined by applying the formula: inhibition (%) = \[\frac{\text{FBControl} - \text{FBCF}}{\text{FBControl}}\] × 100, where FBControl = fungal biomass in the control treatment, and FBCF = fungal biomass under CF. The assay was conducted by using four independent replicates.

2.8. Statistical Analysis

Statistical analyses were carried out using the STATGRAPHICS Plus ver. 5.1. Data were analyzed by one-way ANOVA and Tukey (HSD) multiple comparisons test (p ≤ 0.05). The values shown express the mean between replicates or independent experiments, and the bars represent the standard deviation (SD).

3. Results

3.1. *Fusarium* Isolates Recovered from Jalapeño Peppers with Wilt Symptoms in the Commercial Fields

From the sampled Jalapeño peppers plants with wilt symptoms in the Yaqui Valley, México, fungal isolates that exhibited aerial conidiophores and produced abundant conidia in the typical canoe shape were considered as *Fusarium* spp., and transferred to new plates for further examination. Thus, six *Fusarium* isolates were recovered from the radical tissue of peppers. In general, isolates grown on PDA had abundant aerial mycelia and were white and pink to pale salmon. Pigmentation on the undersides of colonies was white, pale orange, and pinkish white to salmon (Figure A1). Microscope slide preparations were made for detailed examinations, staining them with crystal violet (Figure A1). Conidia were arranged in false heads; microconidia were mostly ovoid, ellipsoidal, or reniform (kidney-shaped) with 0 to 1 sept; macroconidia were fusiform, falcate to straight, with 1 to 5 septs (mostly 3 septs). Two isolates had the presence of chlamydospores (CE2 and CE6). All of these characteristics are typical of the genus *Fusarium*.

3.2. DNA Sequence-Based Identification of *Fusarium* Isolates and Molecular Phylogenetic Analysis

BLASTn searches and Pairwise ID of FUSARIOID-ID, using TEF1 partial sequences as queries, identified that these six isolates belong to three *Fusarium* species complexes: four isolates in FOSC, one in FIESC and one in FSSC, with similarity percentages above 98% (Table 1).

**Table 1.** The pairwise ID of FUSARIOID-ID and BLASTn results of partial TEF1 sequences of the six studied isolates obtained from Jalapeño pepper from the Yaqui Valley, Mexico.

<table>
<thead>
<tr>
<th><em>Fusarium</em> Isolates</th>
<th>Pairwise ID FUSARIOID-ID</th>
<th>Similarity</th>
<th>BLASTn NCBI</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE1</td>
<td><em>Fusarium curvatum</em> (FOSC), LC13739 = F155</td>
<td>99.08%</td>
<td><em>Fusarium oxysporum</em> (FOSC), CPO 3.09</td>
<td>100%</td>
</tr>
<tr>
<td>CE2</td>
<td><em>Fusarium curvatum</em> (FOSC), KG003</td>
<td>99.07%</td>
<td><em>Fusarium oxysporum</em> (FOSC), CPO 3.09</td>
<td>100%</td>
</tr>
<tr>
<td>CE3</td>
<td><em>Fusarium nanum</em> (FIESC), LLC3032</td>
<td>100.00%</td>
<td><em>Fusarium nanum</em> (FIESC), LLC3032</td>
<td>100%</td>
</tr>
<tr>
<td>CE4</td>
<td><em>Fusarium curvatum</em> (FOSC), LC13739 = F155</td>
<td>99.08%</td>
<td><em>Fusarium oxysporum</em> (FOSC), CPO 3.09</td>
<td>99.85%</td>
</tr>
</tbody>
</table>
To further the identification of the only pathogenic strain CE2 (results of pathogenicity test in Section 3.3), a phylogenetic analysis of isolate CE2 and 28 previously characterized isolates of FOSC, including twelve ex-type strains and the \textit{F. oxysporum} CBS 144134 epitype, using fragments of TEF1 (650 bp, 21 informative, TNe model) and RPB2 (877 bp, 36 informative, TNe model), was carried out (Figure 2). The pathogenic isolate CE2 was nested closely to strains of the species \textit{F. languescens} (Figure A2).
3.3. Fusarium Pathogenicity Tests in Jalapeño Peppers

Three *Fusarium* isolates (one of each isolated *Fusarium* species complex) were assayed for their potential to cause wilt symptoms in Jalapeño peppers seedlings. By day ten after the inoculation, some plants of the CE2 isolate started to show wilt symptoms; by day 20 of the experiment, 100% of the plants of this treatment were wilted. The treatments with the isolate CE3 and CE6 showed only 10% of plants with symptoms, while the control treatment did not present wilt symptoms (Figure 3). In general, the observed symptoms were wilting and drooping of leaves that faded to yellow, then to brown, and finally died (Figure 3). The partial TEF1 sequence of the causal agents recovered from these symptomatic plants confirmed that they were the same as those used to inoculate the healthy plants (accession numbers OR168970 to OR168972).

Figure 3. *Fusarium* pathogenicity test in Jalapeño pepper seedlings. (a) Un-inoculated control; (b) CE2 (FOSC); (c) CE3 (FIESC); (d) CE6 (FSSC). The scale bar represents 1 cm.

3.4. Biocontrol Assay by Confrontation In Vitro

To determine the best bacterial strain to control the pathogenic *F. languescens* CE2, a confrontation assay in vitro was carried out. A statistical analysis ($p \leq 0.05$) was used to determine the differences between the radial growth of the *F. languescens* CE2 colonies in relation to the biocontrol agent. It was observed that the *Bacillus* strains that possessed swarming motility were the most efficient to inhibit the growth of strain CE2 with a higher rate (Figure 4). Strains TSO2T, TE3T-UV25, TSO22, and TE3T presented the best biocontrol over strain CE2, when compared against controls, with inhibition percentages of 61.8%, 60.94%, 58.07%, and 56.35%, respectively (Figure 5). Among these strains, *Bacillus cabrialesii* subsp. *tritici* TSO2T has the best biocontrol effect over the *F. languescens* CE2.
After testing the biocontrol of several Bacillus strains against *Fusarium languescens* CE2, *B. cabrialesii* subsp. *tritici* TSO2T was selected as BCA for additional in vitro assays, due to its previously reported potential as a biocontrol bacterium and its swarming motility [24,28,30]. Thus, the radial growth of the fungal colonies was recorded every 24 h for ten days (Figure 6). Statistical analysis resulted in significant differences ($p \leq 0.05$) on the last day of the assay (240 h), between the control treatment (50.3 ± 5.34 mm) and treatment with bacterial cells of strain TSO2T (27.68 ± 1.38 mm), for fungal growth inhibition of 44.9%, by competition for nutrient and space and secretion of antifungal molecules as shown in Figure 4d.

**Figure 4.** Confrontations between (a) *Fusarium languescens* CE2, against biocontrol strains: (b) *Bacillus cabrialesii* subsp. *cabrialesii* TES; (c) *B. paralicheniformis* TRQ65; (d) *B. cabrialesii* subsp. *tritici* TSO2T; (e) *Bacillus* sp. FSQ1; (f) *Bacillus* sp. TSO22; (g) *Bacillus* sp. TE3T-UV25.

**Figure 5.** *Fusarium languescens* CE2 growth inhibition percentage when confronted with biological control agents of the genus *Bacillus*, compared with unconfronted control, seven days after the inoculation. The formula used: inhibition (%) = $$\frac{\text{FG}_{\text{Control}} - \text{FG}_{\text{xB}}}{\text{FG}_{\text{Control}}} \times 100$$, where FGControl = mean of fungal colony radial growth of control treatment, and FGxB = fungal colony radial growth confronted with *Bacillus*. Different letters represent significant differences among treatments ($p \leq 0.05$).
3.5. Bacillus cabrialesii subsp. tritici TSO2T Hybrid Genome Assembly

Illumina MiSeq platform was employed for bacterial DNA sequencing, generating 3,584,209 paired-end reads (2 × 300 bp), and the MinION sequencing technology by Oxford Nanopore Technology (ONT), obtaining 13,764 reads. During the hybrid assembly Unicycler polishes its final assembly with Illumina reads and Pilon to decrease the rate of small base-level errors, getting a total of four polishing rounds. Thus, this assembly resulted in two contigs, improving the previous Illumina draft genome (47 contigs) reported by Valenzuela Ruiz et al. and de los Santos–Villalobos et al. [28,30], with a total of 4,297,452 bp and 44.0% G+C content (Figure 7). No contamination was detected using Quast and CheckM.

Figure 6. Radial growth of Fusarium languescens CE2 when confronted with B. cabrialesii subsp. tritici TSO2T during the assay at ten days after inoculation.

Figure 7. Circular chromosome of Bacillus cabrialesii subsp. tritici TSO2T. Created through Proksee (https://proksee.ca accessed on 27 July 2023), displaying the results generated through the genome annotation from PROKKA and the identified biosynthetic gene clusters associated with biocontrol by AntiSMASH.
Thus, genome annotation was carried out through PROKKA [55], resulting in the creation of a circular chromosome map of *B. cabrialesii* subsp. *tritici* TSO2\[^T\] including the biosynthetic clusters that were identified using antiSMASH V 7.0 [57]. The PROKKA annotation predicted a total of 4374 CDS (coding DNA sequences), with a total of 123 RNAs (90 tRNAs, 32 rRNAs, and 1 tmRNA) (Figure 7).

### 3.6. *Bacillus cabrialesii* subsp. *tritici* TSO2\[^T\] Genome Mining for Secondary Metabolite BGCs and Antagonistic Activity against *Fusarium languescens* CE2 by Extracellular Metabolites

Based on genome mining and in addition to the observed nutrient and space competition of strain TSO2\[^T\] against *Fusarium languescens* CE2, the role of bioactive secondary metabolites potentially produced by *B. cabrialesii* subsp. *tritici* TSO2\[^T\] was explored. Thus, the strain TSO2\[^T\] genome mining predicted seven regions (Figure 7; Table 2), with a similarity above 76% (percentage of genes in the current region that exhibit a significant BLAST match to genes within the nearest known compound) [57] and belong to BGCs that are involved in the synthesis of secondary metabolites with relevant bioactive characteristics. The BGCs found in the genome of strain TSO2\[^T\] were biosurfactant lipopeptides (region 1.2 = surfactin (86%) and region 1.5 = fengycin (100%)), dipeptides (region 1.11 = bacilysin (100%)), siderophores (region 1.8 = bacillibactin (100%)), bacteriocins (region 1.10 = subtilosin A (100%)), polynene antibiotics (region 1.4 = bacillaene (100%)), and sactipeptides (region 1.1 = sporulation killing factor (100%)).

**Table 2.** Biosynthetic gene clusters potentially produced by *B. cabrialesii* subsp. *tritici* TSO2\[^T\] obtained from genome mining in the antiSMASH web server.

<table>
<thead>
<tr>
<th>Region</th>
<th>From</th>
<th>To</th>
<th>BGCs Type</th>
<th>Most Similar Known Cluster</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>206,190</td>
<td>228,364</td>
<td>Sactipeptide, ranthipeptide</td>
<td>Sporulation killing factor</td>
<td>100%</td>
</tr>
<tr>
<td>1.2</td>
<td>387,653</td>
<td>451,968</td>
<td>NRPS</td>
<td>Surfactin</td>
<td>86%</td>
</tr>
<tr>
<td>1.4</td>
<td>1,808,165</td>
<td>1,913,030</td>
<td>TransAT-PKS, T3PKS, NRPS</td>
<td>Bacillaene</td>
<td>100%</td>
</tr>
<tr>
<td>1.5</td>
<td>1,980,438</td>
<td>2,108,149</td>
<td>NRPS, transAT-PKS, betalactone</td>
<td>Fengycin</td>
<td>100%</td>
</tr>
<tr>
<td>1.8</td>
<td>3,303,808</td>
<td>3,355,586</td>
<td>NRP-metallophore, NRPS</td>
<td>Bacillibactin</td>
<td>100%</td>
</tr>
<tr>
<td>1.10</td>
<td>3,895,261</td>
<td>3,916,872</td>
<td>Sactipeptide</td>
<td>Subtilosin A</td>
<td>100%</td>
</tr>
<tr>
<td>1.11</td>
<td>3,919,824</td>
<td>3,961,242</td>
<td>Other</td>
<td>Bacilysin</td>
<td>100%</td>
</tr>
</tbody>
</table>

Given the previously mentioned biocontrol assay results and predicted BGCs, the antagonistic activity of the CF of strain TSO2\[^T\] against *F. languescens* CE2 was tested in a micro-bioassay. After 72 h, *B. cabrialesii* subsp. *tritici* TSO2\[^T\] diffusible extracellular metabolites contained in the CF significantly reduced *F. languescens* CE2 biomass growth compared with the control treatment (3.98 mg ± 0.86 vs. 5.77 mg ± 0.65) (Figure 8). The inhibition percentage resulted in a reduction of 30.95% of fungal biomass when treated with strain TSO2\[^T\] CF compared with untreated control. Microscopic observations of the micro-bioassay showed that when treated with TSO2\[^T\] CF, the spores of *F. languescens* CE2 germinated but the hyphae did not grow as much as in controls, where mycelial growth was abundant (Figure 8).
Given the previously mentioned biocontrol assay results and predicted BGCs, the phylogenetic analysis of the TSO2 CF strain showed a significant inhibition zone (8.0 ± 0.1 mm) in assays with F. languescens CE2, which belongs to the FOSC, was identified as pathogenic, being the responsible of this disease under the used experimental conditions. 

In this sense, we tested six Bacillus strains preserved in COLMENA as BCA against F. languescens CE2, and found that B. cabrialesii subsp. tritici TSO2T was one of the best strains controlling this pathogen’s growth in vitro. The strain TSO2T was recently reported as a novel subspecies of B. cabrialesii [28], named B. cabrialesii subsp. tritici. This strain has been proven to have plant growth promotion abilities such as phosphate solubilization (40.0 ± 1.2%), and indole production (5.4 ± 0.1 ppm) [29]. In previous works, the strain TSO2T was assayed in dual confrontations for biocontrol activity against the wheat pathogen Bipolaris sorokiniana TPQ3, and it was found that it produced a significant inhibition zone (8.0 ± 0.2 mm) when compared to other potential biocontrol rhizobacteria [38]. In this work, the radial growth of the phytopathogen F. languescens CE2 was reduced significantly compared with the control treatment (Figures 4–6). It is also worth noticing that the strain TSO2T possesses swarming.

4. Discussion

Peppers have great economic and cultural importance in Mexico and around the world, and as the population grows, so does the demand for food, which has led to extensive and intensive use of agrochemicals to increase agricultural production and control plant diseases [19,20]. In this sense, wilt in peppers can be caused by Rhizoctonia solani, Phytophthora capsici, F. oxysporum complex, and F. solani complex [6,7,59,60]. In this study, six Fusarium isolates were obtained from symptomatic Jalapeño pepper plants in commercial fields located in the Yaqui Valley, Mexico; however, based on Koch’s postulates, only strain F. languescens CE2, which belongs to FOSC, was identified as pathogenic, being the responsible of this disease under the used experimental conditions.

Currently, the use of microorganisms for the biological control of diseases of agricultural importance has proven an effective and ecologically sustainable alternative to the use of pesticides. Various species of the genus Bacillus have been reported as effective BCA, due to their potential to produce a broad range of antimicrobial metabolites [22,26]. In this sense, we tested six Bacillus strains preserved in COLMENA as BCA against F. languescens CE2, and found that B. cabrialesii subsp. tritici TSO2T was one of the best strains controlling this pathogen’s growth in vitro. The strain TSO2T was recently reported as a novel subspecies of B. cabrialesii [28], named B. cabrialesii subsp. tritici. This strain has been proven to have plant growth promotion abilities such as phosphate solubilization (40.0 ± 1.2%), and indole production (5.4 ± 0.1 ppm) [29]. In previous works, the strain TSO2T was assayed in dual confrontations for biocontrol activity against the wheat pathogen Bipolaris sorokiniana TPQ3, and it was found that it produced a significant inhibition zone (8.0 ± 0.2 mm) when compared to other potential biocontrol rhizobacteria [38]. In this work, the radial growth of the phytopathogen F. languescens CE2 was reduced significantly compared with the control treatment (Figures 4–6). It is also worth noticing that the strain TSO2T possesses swarming.

Figure 8. Mycelial growth inhibition of F. languescens CE2 through extracellular metabolites produced by Bacillus cabrialesii subsp. tritici TSO2T. Representative photographs of F. languescens CE2 mycelial growth during the micro-bioassay. Microscopic photographs at 100× show the difference in mycelial growth and spore germination. Different letters show significant differences among treatments (p ≤ 0.05).
motility associated with its biocontrol ability (Figure 4), which is related to surfactant synthesis and secretion, reducing the tension among the substrate and the bacterial cell, allowing it to spread on the surface, and competing for space and nutrient [61].

In addition, BGCs were mined from the genome of *B. cabrialesii* subsp. *tritici* TSO2T to elucidate the potential secondary metabolites produced by this strain. At the same time, the CF of strain TSO2T—potentially containing these putative metabolites—was used in a biocontrol micro-bioassay against *F. languescens* CE2. Thus, seven BGCs related to the production of metabolites with biocontrol properties were found in the genome of this strain (Figure 7), and the CF of strain TSO2T reduced the mycelial growth (dry weight) of strain CE2 by 30.95%. The BGCs for lipopeptides surfactin and fengycin were identified in the genome of *B. cabrialesii* subsp. *tritici* TSO2T, which are surfactants involved in swarming motility for competition for nutrients and space [61,62]. Surfactins and fengycines per se also have antimicrobial properties and could be involved in biocontrol functions in the rhizosphere [26]. For example, Luo et al. [63], tested the toxicities of lipopeptides produced by *B. subtilis* 916 against *F. oxysporum*, and found that the fungus showed high sensitivity to fengycines, with a 50% inhibitory concentration (IC50) for hyphal growth, below 2 µg mL−1; but had a low sensitivity to surfactins, with an IC50 for hyphal growth over 50.0 µg mL−1 [63]. Thus, these lipopeptides could be involved in the biocontrol ability of strain TSO2T both in competition for nutrients and space and also in inhibiting the mycelial growth by toxicity, degrading the cell wall of the studied phytopathogen [64,65]. Other BGC coding for antimicrobial metabolites were found in strain TSO2T genome, such as bacillaene, whose antifungal activities have been validated previously; for example, in an antifungal assay on agar plates against *Coriolopsis* sp., *Umbelopsis* sp., *Fusarium* sp., *Trichoderma* sp. and *Pseudoxylaria* sp., where the crude extract and purified bacillaene A of *B. subtilis*, suppressed the growth of the fungi in a dose-dependent mode [66]. Bacillibactin and bacilysin, based on genomic, transcriptomic, and chromatography/mass spectrometry analyses, were found in the genome and CF of *B. amyloliquefaciens* [67], and these genes were upregulated in response to *F. oxysporum* f. sp. *cubense* race 4 (FOC4) while showing a significant power to suppress the growth of FOC4 when both microorganisms were co-cultivated in vitro [68].

Another BGC of interest found in strain TSO2T genome was subtilosin A, which is one of many antibiotics produced by *Bacillus* strains and it works against a diversity of Gram-positive bacteria [69]; thus, this metabolite could not be involved in the observed biocontrol against *F. languescens* CE2. Finally, the BGC for the sporulation killing factor (SKF) was present in strain TSO2T genome; during the initial phase of sporulation, sporulating cells of *Bacillus* secrete extracellular killing factors to eliminate the non-sporulating cells whose immunity to these toxins was not developed. Consequently, the nutrients released from the dead cells become available for utilization by the sporulating cells, facilitating their growth resurgence [70,71].

This study provides new insight into possible modes of action of *B. cabrialesii* subsp. *tritici* TSO2T for controlling *Fusarium* wilt in Jalapeño peppers. After identifying BGCs with antimicrobial activity in *B. cabrialesii* subsp. *tritici* TSO2T, it was assumed antibiosis and competition for nutrients and space (swarming) as the potential action modes against the studied phytopathogen. For example, it has long been known that *Bacillus* strains produce lipopeptides that have strong antifungal activity [64,65]. In addition, the presence of BGC for the siderophore bacillibactin suggests that iron competition could also contribute to strain TSO2T biocontrol effect against strain CE2, and its swarming motility allows it to grow and expand in the substrate, competing for space and bringing the excreted antimicrobial metabolites closer to the phytopathogen. Thus, additional approaches to confirm the biosynthesis of molecules found through BGCs genome mining are still needed, as are currently being carried out by our research team.
5. Conclusions

*B. cabrialesii* subsp. *tritici TSO2* is capable of inhibiting *F. languescens* CE2 in a 61.8% by competition for nutrients and space (swarming), and secretion of antifungal molecules. In addition, its CF reduced the mycelial growth of this fungus by 30.95%. Biocontrol of this plant pathogen was achieved in vitro through direct dual confrontations and the metabolites present in the CF of strain TSO2. We have identified, through genome mining the *B. cabrialesii* subsp. *tritici TSO2* genome, the potential biosynthesis of seven antimicrobial secondary metabolites involved in the biocontrol, which suggests that the mode of action is antibiosis and swarming. This was confirmed by the micro-bioassay using only the CF of strain TSO2, and dual confrontation against *F. languescens* CE2. Nevertheless, it is not common for an exclusive mechanism to be used for the suppression of phytopathogens. In conclusion, *B. cabrialesii* subsp. *tritici TSO2* has a high potential for sustainable control of *F. languescens* CE2, the causal agent of Jalapeño pepper wilt.

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**Data Availability Statement:** The genetic sequence data generated in this study are openly available in NCBI accession numbers OR168964 to OR168973. The complete genome sequence has been deposited in DDBJ/ENA/GenBank under accession number JAHBMK000000000, under BioProject number PRJNA728132, and BioSample number SAMN19070894.

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Appendix A

Figure A1. *Fusarium* colonies in PDA (reverse and surface) and microscopic characterization (dyed with crystal violet), after 7–10 days at 30 °C. (a) *Fusarium oxysporum* CE1; (b) *F. languescens* CE2; (c) *F. nanum* CE3; (d) *F. oxysporum* CE4; (e) *F. oxysporum* CE5; (f) *F. falciforme* CE6.
Appendix B

Figure A2. *Fusarium languescens* CE2: (a) macroconidia; (b) microconidia; (c) chlamydoospore; (d) hypha.

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