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Cladophialophora guangxiense sp. nov., a New Species of Dark Septate Endophyte, Mitigates Tomato Bacterial Wilt and Growth Promotion Activities

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Abstract: Bacterial wilt of tomatoes, caused by *Ralstonia solanacearum*, is a significant soilborne disease that often causes significant reductions in the yield of tomatoes. Dark septate endophytic fungi (DSE) represent potential biocontrol agents against plant pathogens that can also enhance plant growth. To collect DSE fungi with potential for biocontrol, the fungus *Cladophialophora guangxiense* HX2 was isolated from the rhizosphere soil of sugarcane in Hengzhou Guangxi Province, China, and a novel species of *Cladophialophora* was identified based on morphological properties and DNA sequence analysis. *C. guangxiense* HX2 demonstrated a controlling effect of 76.7% on tomato bacterial wilt and promoted a 0.5-fold increase in tomato seedling height. It colonized tomato seedling roots, enhancing the activity of antioxidant and defensive enzyme systems. Transcriptomic and qPCR approaches were used to study the induction response of the strain HX2 infection by comparing the gene expression profiles. Gene ontology (GO) and Kyoto Encyclopedia of Gene and Genome (KEGG) pathway enrichment revealed that tomatoes can produce salicylic acid metabolism, ethylene-activated signaling, photosynthesis, and phenylpropanoid biosynthesis to the strain HX2 infection. The expression of *IAA4* (3.5-fold change), *ERF1* (3.5-fold change), and *Hqt* (1.5-fold change) was substantially enhanced and *Hsc* 70 (0.5-fold change) was significantly reduced in the treatment group. This study provides a theoretical foundation for further investigation into the potential of *C. guangxiense* HX2 as a biological agent for the prevention and control of tomato bacterial wilt.

Keywords: *Cladophialophora guangxiense*; new species; *Ralstonia solanacearum*; biological control; plant hormone transduction; phenylpropanoid biosynthesis; MAPK pathways

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1. Introduction

Tomatoes (*Solanum lycopersicum* L.) have significant economic value due to their rich nutrients and bioactive compounds such as lycopenes, ascorbic acid, and polyphenols. China has emerged as the global leader in tomato production [1–4]. However, tomato cultivation faces challenges from diseases caused by various pathogens, including viruses, bacteria, fungi, and root-knot nematodes (*Meloidogyne* spp.) [5]. Bacterial wilt of tomatoes is a typical vascular disease caused by *Ralstonia solanacearum*, which has the characteristics of sudden onset, rapid spread, and high mortality rate [6,7]. *R. solanacearum* was a soilborne bacterium that can be widely transmitted through soil and water, making it difficult to control and causing serious impact on tomato production [8,9]. Control strategies such

as chemical bactericides, field sanitation, resistant variety breeding, and cropping system improvements have been employed [10–12]. However, the pathogen's high variability, limited chemical control options, resilience in various environments, and extensive host range make it difficult to manage [13]. Therefore, sustainable, cost-effective, and environmentally friendly biological control methods have been proposed as viable alternatives in recent decades.

Recent studies have demonstrated the efficacy of various antagonistic microorganisms in controlling *R. solanacearum*. For instance, Sun et al. [14] reported that *Bacillus subtilis* R31 effectively colonizes the root tissues of tomatoes, thereby reducing the population of *R. solanacearum*. Similarly, *B. velezensis* RC116 produces extracellular enzymes and possesses genes involved in antibiotic biosynthesis, which contribute to its ability to manage *R. solanacearum* infections [15]. *Pseudomonas fluorescens* VSMKU3054 has been shown to combat *R. solanacearum* by producing the phytopathogen inhibitor 2,4-diacetylphloroglucinol (DAPG) [9]. While these findings are promising, the majority of research has been conducted on bacterial agents for controlling tomato bacterial wilt, with a notable lack of investigation into the potential role of endophytic fungi in managing this disease. Endophytic fungi, which colonize the internal tissues of plants without causing disease, have garnered significant interest [16]. These fungi are residents within the host plant and play a vital role in bolstering plant immunity. Moreover, they can enhance the host plant's antioxidant defenses, thereby managing the detrimental effects of excessive reactive oxygen species (ROS) generated and accumulated during periods of stress [17,18].

Dark septate endophytes (DSEs) are a group of endophytic fungi characterized by their melanized hyphae, which colonize the root epidermis and cortex both intracellularly and intercellularly. These fungi are also known to form microsclerotia [19,20]. Extensive research has underscored the pivotal role played by DSEs in enhancing plant resilience to stress and improving metal tolerance [21,22]. The potential of DSE fungi to combat crop diseases has garnered significant attention from the plant protection scientific community. These fungi have demonstrated remarkable efficacy as biocontrol agents against a variety of pathogens. Notably, DSE fungi have been successfully utilized to manage Fusarium wilt in crops such as strawberries, asparagus, banana, and Chinese cabbage, caused by *Fusarium oxysporum*, and Verticillium wilt in cabbage, caused by *Verticillium longisporum* [23–27]. Yakti et al. [28] have highlighted that DSEs can extract nutrients from regions inaccessible to plant roots, thereby making these nutrients available to the host plants. However, there is a notable absence of research focusing on the interaction between DSE fungi and tomato bacterial wilt disease to date.

The research focuses on the following aspects: (I) isolate and characterize DSEs from the rhizosphere soil; (II) identified as a new species of *Cladophialophora,* confirmed through a combination of morphological examinations and molecular sequencing methods; (III) to evaluate the effectiveness of DSE fungi in combating tomato bacterial wilt disease and promoting growth activities, pot experiments were conducted. The research primarily; (IV) investigated the mechanisms by which the fungi colonize tomato roots and elicit changes in the tomato transcriptome, thereby enhancing the plant's resistance to the disease. Thus, this study aims to evaluate the potential of *C. guangxiense* HX2 as a biocontrol agent.

2. Material and Methods

2.1. Fungal Isolation and Endophyte Screening

The rhizosphere soil was collected from a sugarcane field in Baihe Town, Heng County, Nanning City, Guangxi Province (22°41′23.24″ N, 109°27′37.36″ E, altitude 60.74 m) using a five-point sampling method on 17 January 2016. The 5 soil samples were taken back to the laboratory as soon as possible and stored at 4 °C.

The tomato seedings were used as bait plants to capture endophytic fungi from soil samples [29]. The tomato seeds were surface disinfected with 75% alcohol, 1% sodium hypochlorite, and sterile water for 30 s, respectively, and then placed on water agar medium (WA) for germination. The rhizosphere soil was mixed with sterilized seedling substrate

in a ratio of 2:1 and placed in culture cups. Germinated tomatoes were transplanted into the cups for baiting. After two months, the roots of the tomato seedlings were collected for endophytic fungi isolation after washing by running tap water and surface sterilizing with 75% alcohol, 1% sodium hypochlorite, and sterile water 3 times for 1 min, respectively. Subsequently, sterilized roots were inoculated onto half-strength cornmeal agar medium (8.5 g corn meal agar, 7.5 g bacto agar, for 1 L distilled water) at 25 °C. Hyphae emerging from the root segments were transferred to cornmeal malt yeast agar medium (CMMY, 10 g malt extract, 2 g yeast extract, 8.5 g corn meal agar, 7.5 g bacto agar, for 1 L distilled water). Finally, a total of 142 fungal isolates were obtained and preserved at the Institute of Plant Protection, Guangxi Academy of Agricultural Sciences.

To eliminate saprotrophic and pathogenic fungal isolates, eighteen isolates were selected based on their morphology as representatives from different species or groups of fungi. The endophyte screening on selected isolates was carried out after growing the fungus in 6 cm Petri dishes filled with oatmeal medium (10 g oatmeal, 18 g bacto agar, 1 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 1 g $NaNO_3$, for 1 L distilled water). After the plates were largely covered by fungal colonies, disinfected 2-day-old seedlings of tomatoes were transplanted onto the growing fungal colonies, placed into a sterile plastic pot, and incubated for two weeks at 23 °C, with 18 h:6 h (L:D) and 180 mol m^{-2} s^{-2} light intensity. A control group was concurrently maintained without endophytic fungal exposure. Assessed plants were harvested and oven-dried at 40 °C for 48 h. The dry weight of treated plants per plate was taken and compared with control plants. The single best candidate of fungal isolate was defined and selected for the subsequent assay.

2.2. Fungus Identification

2.2.1. Morphological Observation

The endophytic fungi was inoculated onto CMMY to examine its growth and colony morphological characteristics. The observation of the fungal sporulation structure was conducted as follows: A 0.5 cm × 0.5 cm cube of oatmeal agar medium (OMA, 10 g oatmeal, 10 g bacto agar, 1 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 1 g $NaNO_3$, for 1 L distilled water) inoculated with endophytic fungi was placed on a sterile coverslip over a WA plate and incubated at 28 °C for 14 days. Following this, the plates were moved to a refrigerator set at 4 °C to promote sporulation [30]. Thereafter, the coverslips were removed and observed under an Olympus BX53 microscope.

2.2.2. Molecular Sequencing and Phylogenetic Analysis

The DNA of endophytic fungi was extracted using the Solebol Fungal Genomic DNA Extraction Kit (Solarbio, China). Polymerase chain reaction (PCR) was employed to amplify the internal transcribed spacer (ITS), the large subunit ribosomal RNA gene (LSU), and the small subunit ribosomal RNA gene (SSU). Amplification was conducted with specific primers: ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) for ITS, LROR (ACCCGCTGAACTTAAGC) and LR5 (TCCTGAGGGAAACTTCG) for LSU, and NS1 (GTAGTCATATGCTTGTCTC) and NS4 (CTTCCGTCAATTCCTTTAAG) for SSU [31–34]. The PCR reaction mixture consisted of 25 μL 2×Easy Taq PCR SuperMix, 1 μL of each forward and reverse primer, 1 μL DNA template, and ddH_2O to a final volume of 50 μL. The PCR reaction parameters were as follows: predenaturation at 94 °C for 5 min, followed by 35 cycles with denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min.

The PCR-amplified products were purified and subsequently sequenced by the Beijing Genomics Institute (BGI). Subsequent sequence alignment and the construction of a neighbor-joining phylogenetic tree were carried out using MEGA 6.0, with all available SSU, ITS, and LSU sequences downloaded from the representative *Cladophialophora* genus in GenBank (Table S1). The Kimura two-parameter model was used for distance estimation, and bootstrap analysis was conducted with 1000 replicates.

2.3. Pot Experiment

2.3.1. Tomato Culture

Tomato seeds (Guihong II: susceptible tomato bacterial wilt) were treated with a 3% sodium hypochlorite solution for 5 min for disinfection. Following disinfection, the seeds were rinsed repeatedly with sterile distilled water [35,36]. Subsequently, the seeds were cultivated in individual pots with a diameter of 10 cm in a greenhouse environment maintained at a temperature of 30 °C, a humidity of 90%, and an illumination schedule of 16 h light to 8 h darkness (L:D ratio).

2.3.2. Effect of Endophytic Fungi on Tomato Growth

The strain HX2 was cultured on PDA medium at 37 °C for 14 d. Then, the colonies were washed with sterile water to collect the spores, and the spore concentration was adjusted to 1×10^8 spores/mL, 1×10^6 spores /mL, and 1×10^4 spores /mL. Tomato plants with similar growth vigor were selected and treated with endophytic fungal fermentation liquid at three concentrations (1×10^8 spores/mL, 1×10^6 spores /mL, 1×10^4 spores /mL) [37], with PDA treatment as the control. Each treatment consisted of three tomato plants, and the experiment was replicated three times for reliability. The height and stem diameter of the tomato plants were measured at 10, 20, and 30 d post-treatment, and root length was measured at 30 d [38].

2.3.3. Effect of Endophytic Fungal Against *R. solanacearum* Infection on Tomato

The experiment was carried out when the tomato grew to a 4-leaf stage, with an experimental design as shown in Table 1. Each treatment consisted of three tomato plants, and the experiment was replicated three times for reliability. Disease severity was assessed on the 14th day post-treatment, utilizing a 0–4 scale [39]. Subsequently, the disease index and control efficacy were computed using the following formulas:

$$\text{Disease index} = [\sum (\text{The number of diseased plants in this grade} \times \text{Disease grade}) / (\text{Total number of plants investigated} \times \text{the highest disease grade})] \times 100$$

$$\text{Control efficacy (\%)} = [(\text{Disease index of control} - \text{Disease index of treated group}) / \text{Disease index of control}] \times 100$$

Table 1. Determination and treatment of the effect of biocontrol strain on tomato bacterial wilt.

Treatment	Inoculation	Inoculation After 7 d
CK	--	--
T1	HX2 (1×10^6 spores /mL)	--
T2	HX2 (1×10^6 spores /mL)	*R. solanacearum* (1×10^8 CFU /mL)
T3	Thiediazole copper (Jiangsu Huifeng Bio-agriculture Co., Ltd., Jiangsu, China, 20 mg/L)	*R. solanacearum* (1×10^8 CFU /mL)
T4	--	*R. solanacearum* (1×10^8 CFU /mL)

2.4. Root Colonization

Endophytic fungi was were cultured on OMA for 10 days. DesinfestedDisinfested tomato seeds were then transplanted into endophytic fungal colonies and placed within tissue culture bottles, accompanying OMA Petri dishes. Subsequently, these were incubated in an illumination incubator at 25 °C, with a light intensity of 180 μmol/(m^2·s) and a photoperiod of 16 h/d. A control group was concurrently maintained without endophytic fungal exposure. Following a 14-day co-cultivation period with tomato seedlings, their growth was assessed, subsequent to which the tomatoes were dried and weighed. The tomato seedling roots were then re-isolated to verify the identity of the initial inoculated strain. Moreover, the colonization of endophytic fungial within the tomato seedling roots was observed using an Olympus BX53 microscope, following staining with lactic acid cotton blue.

2.5. Determination of Antioxidant Enzymes Activities

Consistent with Section 2.3.3., leaf samples were obtained from the same plant parts at 0-, 2-, 4-, 6-, 8-, and 10-day intervals post-experimental treatment and assessed for their tomato peroxidase (POD) [40], phenylalanine ammonia-lyase (PAL) [41], polyphenol oxidase (PPO) [42], catalase (CAT) [43], and superoxide dismutase (SOD) [22] activities. Enzyme extractions and activity measurements were performed using a kit from Solarbio: Phenylalanine Ammonialyase (PAL) Assay Kit (Cat#BC0210); Catalase (CAT) Activity Assay Kit (Cat#BC0200); Peroxidase (POD) Activity Assay Kit (Cat#BC0090); Superoxide Dismutase (SOD) Activity Assay Kit (Cat#BC0170); and Polyphenol Oxidase (PPO) Activity Assay Kit (Cat#BC0190).

2.6. Transcriptome Analysis

Transcriptome analyses of tomato roots was conducted by infecting with endophytic fungi. The experiment was initiated 8 d after inoculation of the endophytic fungi and included a non-inoculated control group. Three biological replicates were executed for each treatment, with each replicate consisting of a pooled sample from at least three distinct plants. Following sample collection, each sample was rapidly frozen in liquid nitrogen and then stored at a low temperature in solid carbon dioxide prior to sending it to Shanghai Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China, for transcriptomic sequencing.

Subsequently, the Illumina platform sequencing was used to obtain the raw reads, which were subjected to quality control to determine the suitability of the sequencing data for subsequent analysis. After quality control, clean reads were obtained by filtration and compared to the reference sequence. In this study, high-quality sequencing data (clean reads) obtained by sequencing were compared and analyzed using the reference genomic sequence of tomatoes. The false discovery rate (FDR) and fold change (FC) are important indices for screening differentially expressed genes (DEGs). The larger the fold change, the smaller the FDR value, indicating that the expression difference is more significant. Meanwhile, FC $\geq$ 2 and FDR $\leq$ 0.05 were defined as DEGs with biological duplication. FC $\geq$ 2 and FDR $\leq$ 0.005 were defined as DEGs without biological duplication. Volcano, Venn, and bubble diagrams were used to analyze DEGs among samples. Pathway enrichment analysis and functional annotation of DEGs were performed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Gene Ontology (GO) to identify the significantly enriched pathways of tomatoes in response to endophytic fungi and screen out the key candidate genes involved in the main enrichment pathways.

2.7. Quantitative Real-Time PCR

To ensure the reliability of the transcriptome data, primers targeting three disease-resistant genes and one pathogenic gene were designed using data from NCBI (Table 2). Subsequently, qRT-PCR analysis was performed, with reverse transcription conducted using 1 µg of each RNA sample and the Toloscript ALL-in-one RT EasyMix for qPCR (Tolo Biotech Co., Ltd., Nanjing, China). The relative transcript levels of target genes were determined in triplicate using a 7500 Real-Time PCR System. The *GAPDH* gene was used as the internal control [44], and the data were analyzed employing the $2^{-\Delta\Delta CT}$ method [45,46].

Table 2. Sequences of primer sets used in this study.

Primers	Sequence (5′->3′)	Length	Tm	GC	Product Length
*ERF*1	GAAAGAGGCCATGGGGTAAA	20	57.47	50.00	150
	GTAGAGACCAAGGACCCCTCA	21	60.27	57.14	
*IAA*4	AACAAGAGGGCTTTGCCTGA	20	59.81	50.00	160
	TCCCACATTCAGCTTCTGCTT	21	59.93	47.62	
Hqt	AATCGCGAGTCCAGTACCAC	20	59.83	55.00	226
	CTAGGTAACCCGGTGGCAAG	20	60.11	60.00	

Table 2. *Cont.*

Primers	Sequence (5′->3′)	Length	Tm	GC	Product Length
Hsc70	ATTCTTGTGTCGGCGTTTGG	20	59.41	50.00	203
	AGACGCATCACTGAACCTCC	20	59.75	55.00	
GAPDH	ACCACAAATTGCCTTGCTCCCTTG	24	--	--	--
	ATCAACGGTCTTCTGAGTGGCTGT	24	--	--	

2.8. Statistical Analyses

The data were subjected to a one-way analysis of variance (ANOVA) with SPSS 26.0 software (IBM, New York, NY, USA), and Duncan's test was used to determine significant differences. Results were presented as the mean ± standard deviation.

3. Results

3.1. Fungal Isolation and Endophyte Screening

The dominant isolated fungi are species of *Pestalotiopsis*, *Colletotrichum*, *Fusarium*, *Thrichoderma*, *Penicillium*, *Phythium*, *Caldosporium*, and *Phomopsis*. Most ineffective isolates, once re-inoculated in axenically-grown tomato seedlings, caused extreme yellowing of leaves and suppression of plant growth. Only one of the isolates (strain HX2) did not cause typical external symptoms on leaves, including etiolation. Compared to non-treated control plants, HX2 could stimulate seedling growth, resulting in taller plants and larger leaves (Figure 1a,b). Thus, strain HX2 was selected for further practical usage.

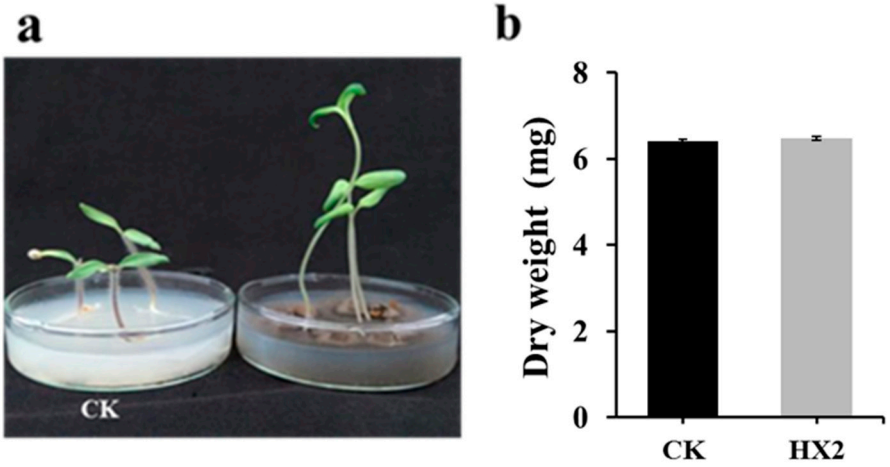

Figure 1. Tomato–HX2 (*C. guangxiense*) symbiont. (**a**): Co-culture results of strain HX2 and tomato seedlings. (**b**): Tomato seedling dry weight. CK: Tomato cultured on PDA; HX2: Tomato treated with strain HX2. Bars indicate the standard error of the mean. Columns marked with the same letter are not significantly different according to Duncan's Multiple Range Test at $p \leq 0.05$.

3.2. Taxonomy and Phylogeny

3.2.1. Taxonomy

Cladophialophora guangxiense Y.Y. Long, Yanlu Chen and L. Xie, sp. nov. Figure 2 MycoBank 855619.

Etym.: The species is named for Guangxi Province, the locality of the type of strain. Asexual morph: The growth of strain HX2 on CMMY was sluggish, with colonies reaching a diameter of 18–20 mm after 14 d culture at 28 °C. Colonies were approximately circular with a color range from gray to dark gray, and exhibited a black border. The colony texture was slightly firm, and the surface was velutinous (Figure 2a). The mycelia of HX2 on OMA were septate and displayed an olivaceous to yellowish-brown color, measuring 1.3–4.4 µm (av. 3.0 ± 0.7 µm, $n = 30$) in diameter. Conidiogenous cells were terminal or lateral, olivaceous to

light yellowish-brown, oblong, cylindrical to somewhat fusiform, (6.0–17.8) × (3.3–6.4) μm [av. (10.7 ± 2.6) × (4.7 ± 0.8) μm, *n* = 44] (Figure 2b–h). Conidia one-celled, unbranched, occasionally branched, produced in short coherent chains of 2–17, oval, cylindrical to sub-cylindrical in shape, or curled string of sausage-shaped, olivaceous to yellow-brown in color, measuring (4.9–10.5) × (4.0–6.4) μm [av. (6.7 ± 1.2) × (5.1 ± 0.6) μm, *n* = 37] in diameter. Phialide-like budding cells were occasionally observed, while chlamydospores were absent and the sexual morph remained unknown.

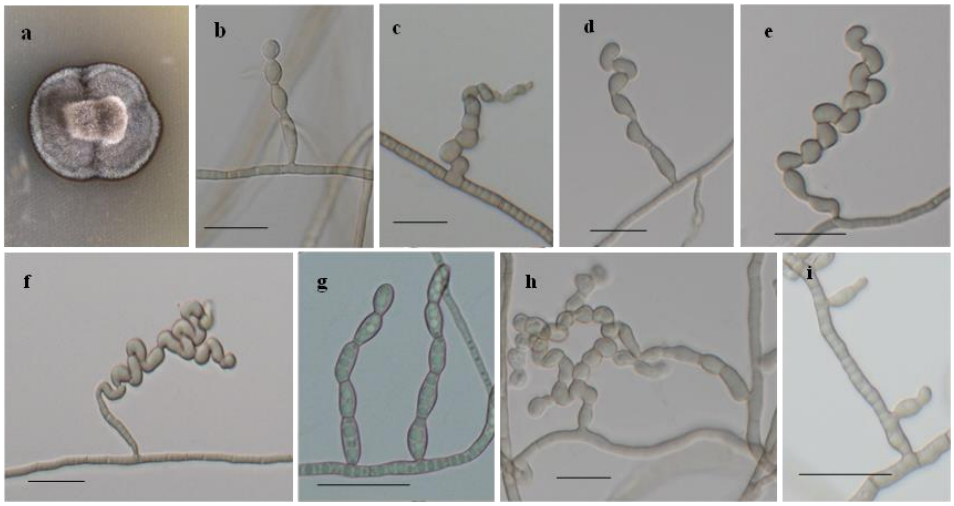

Figure 2. Morphology of endophytic fungi *Cladophialophora guangxiense* HX2. (**a**): Colony on CMMY after 2 weeks at 28 °C. (**b**,**c**): Lateral conidiogenous cells and oval conidial chains on OMA after 3 weeks at 28 °C. (**d**–**f**): Curled string of sausage-shaped conidial chains on OMA after 3 weeks at 28 °C. (**g**): Cylindrical to sub-cylindrical conidial chains on OMA after 3 weeks at 28 °C. (**h**): Solitary conidiophores and oval conidial chains on OMA after 3 weeks at 28 °C. (**i**): Budding cells on OMA after 3 weeks at 28 °C. Scale bars 20 μm.

Type: China, Guangxi Province, Hengzhou City, in the sugarcane rhizosphere, 22°41′23.24″ N, 109°27′37.36″E, altitude 60.74 m, January 2016 (holotype, HMAS 353147, ex-type culture CGMCC 41498).

Notes: The strain HX2 is similar to *Cladophialophora* based on morphological analysis. However, the strain HX2 exhibits a unique species form characterized by non-septate, melanized, curled conidia arranged in coherent and unbranched chains. Therefore, the strain HX2 is a new species of *Cladophialophora*, and was named as *C. guangxiense.*

3.2.2. Phylogenetic Analyses

The *ITS*, *LSU*, and *SSU* regions were sequenced for strain HX2 using the NCBI database (http://www.ncbi.nlm.nih.gov/, accessed on 3 August 2023.) and BLAST analysis. Phylogenetic analysis revealed that strain HX2 and *C. inabaensis* (EUCL1) constituted a monophyletic group, albeit with a statistical support value of only 87%. The similarity of ITS and LSU sequences between HX2 and *C. inabaensis* (EUCL1) was 92.07% and 98.91%, respectively (Figure 3). This provided further evidence to support that strain HX2 is a new species of *Cladophialophora*.

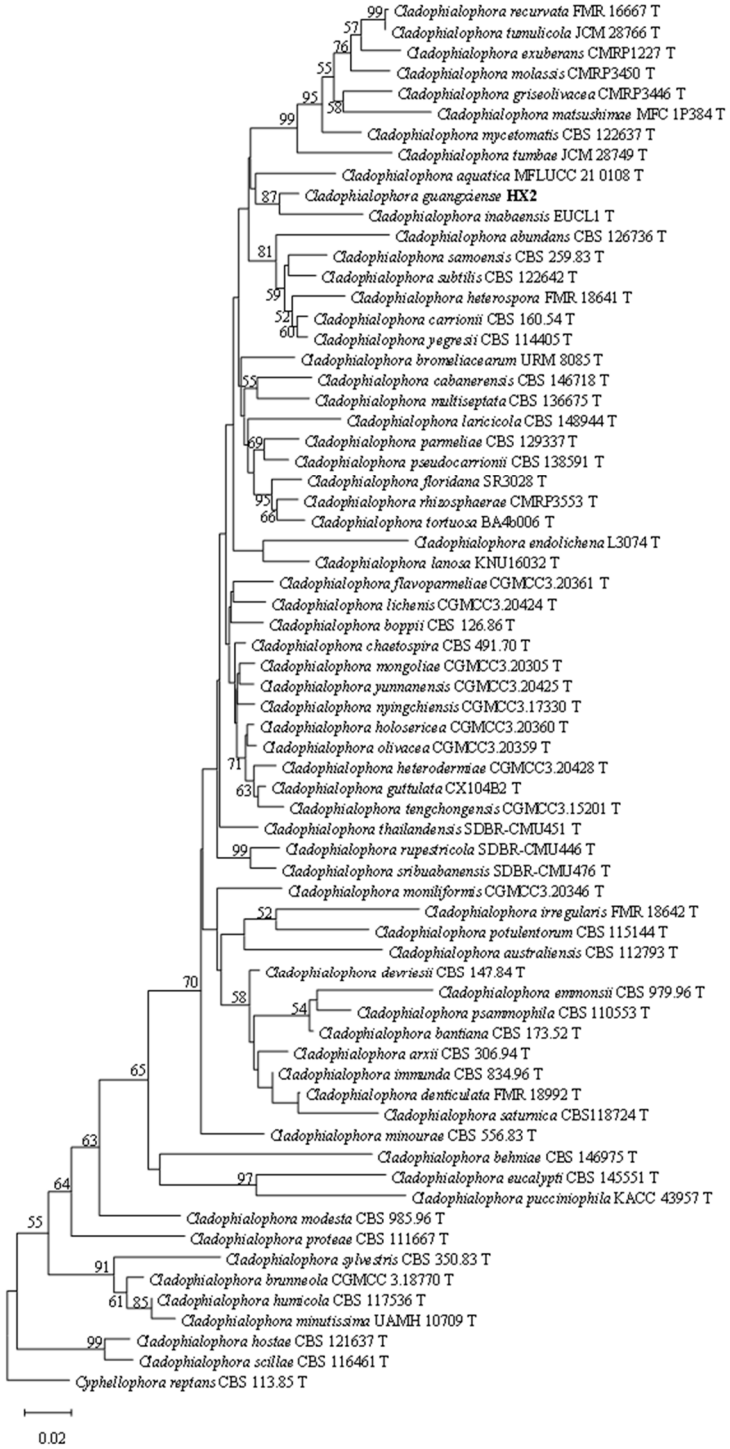

Figure 3. NJ phylogenetic tree based on the combined sequences ITS+LSU+SSU of *Cladophialophora* species. Bootstrap values > 50% are shown at nodes. *Cyphellophora reptans* CBS 113.85 was used as an outgroup. T: type strain. The isolated strain of this study is indicated in bold. The bar indicates 0.02 nucleotide substitutions per site.

3.3. Effect of Strain HX2 on Tomato Growth

To assess the impact of strain HX2 on tomato growth, we measured various parameters, including shoot height, stem diameter at 10, 20, and 30 d post-treatment, and root length at 30 d. As shown in Figure 4, strain HX2 treatments (1×10^8 spores/ mL, 1×10^6 spores/mL, 1×10^4 spores/mL) significantly enhanced tomato root length (Figure 4A), plant height (Figure 4B), and stem diameter (Figure 4C). Notably, the highest effects were observed with

the 1×10^6 spores/mL concentration of strain HX2. Consequently, this concentration was selected for subsequent experiments.

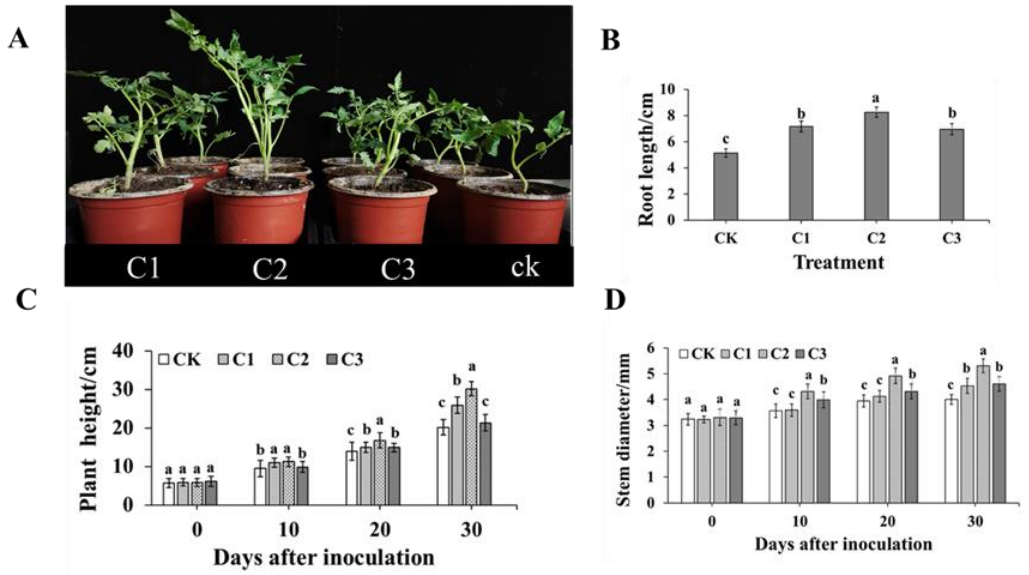

Figure 4. The effect of spore suspension from strain HX2 at different concentrations on tomato growth parameters. This figure illustrates the differences in root length plant height and stem diameter between the treated and control groups. C1: 1×10^8 spores/ mL spore suspension of strain HX2 C2: 1×10^6 spores/mL spore suspension of strain HX2 C3: 1×10^4 spores/mL spore suspension of strain HX2 ck: H_2O. (**A**): Potted Plant Experiment. (**B**): Root length. (**C**): Plant height. (**D**): Stem diameter. Bars indicate the standard error of the mean. Columns marked with the same letter are not significantly different according to Duncan's Multiple Range Test at $p \leq 0.05$.

3.4. Effect of HX2 Against R. solanacearum Infection on Tomatoes

The control effect of strain HX2 against *R. solanacearum* infection on tomatoes was displayed in Figure 5. Compared with the control treatment (T4), both strain HX2 (T2) and Thiourea copper (T3) mitigated the disease index, with control effects of 76.7% and 83.3%, respectively (Table 3). Furthermore, there were negligible differences in disease index between treatment T2 and T3. Additionally, tomato plants treated solely with strain HX2 or distilled water (H_2O) exhibited no detrimental effects. These findings indicate that strain HX2 enhances the suppression of 70 *R. solanacearum* in tomato plants.

CK indicates inoculation with ddH_2O (30 mL) as a control; T1 indicates inoculation only with the spore suspension of HX2 (1×10^6 spores /mL, 30 mL); T2 indicates simultaneous inoculation with the spore suspension of HX2 (1×10^6 spores /mL, 30 mL) and the bacterial solution of *R. solanacearum* (1×10^8 CFU /mL, 30 mL); T3 indicates simultaneous inoculation with the Thiediazole copper (20 mg/L, 30 mL) and the bacterial solution of *R. solanacearum* (1×10^8 CFU /mL, 30 mL); T4 indicates inoculation only with the bacterial solution of *R. solanacearum* (1×10^8 CFU /mL, 30 mL).

Table 3. Control effect of HX2 against tomato bacterial wilt.

Number	Treatment	Disease Index	Control Effect/%
CK	H_2O	5.0 ± 1.2 c	--
T1	HX2	5.5 ± 1.3 c	--
T2	HX2+ *R. solanacearum*	12.5 ± 2.1 b	76.7 ± 9.9% b
T3	Thiediazole copper + *R. solanacearum*	17.5 ± 1.9 b	83.3 ± 9.7% a
T4	*R. solanacearum*	75.0 ± 8.9 a	--

Numerical values were mean ± SD of triplicates; Means were tested with Duncan's Multiple Range Test. Means followed by the same letter are not significantly different ($p \leq 0.05$) within the same column.

Figure 5. Effect of HX2 on the control of *R. solanacearum* in tomatoes.

3.5. Strain HX2 Colonization in Roots of Tomatoes

Microscopy examination revealed that the hyphae of HX2 were capable of colonizing the epidermis and cortex cells of the tomato roots (Figure 6a,b). No typical DSE microsclerotia were detected in this study. The successful establishment of the tomato—HX2 symbiosis was confirmed. These findings suggested that the hyphae of strain HX2 could penetrate the cells of tomato roots and establish colonization.

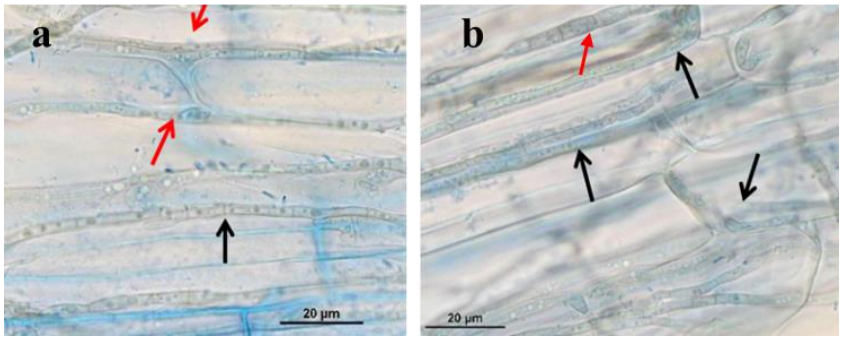

Figure 6. *C. guangxiense* HX2 colonized in the roots of tomato seedlings. (**a**,**b**): the colonization of endophytic fungal within the tomato root tissue was observed using an Olympus BX53 microscope, following staining with lactic acid cotton blue. Intracellular (black arrows) and intercellular (red arrows) colonization of hyphae.

3.6. Determination Antioxidant Enzymes Activities

To determine whether the tomato plant's defense system is activated in response to strain HX2, activities of five defense enzymes (PAL, PPO, POD, CAT, and SOD) were selected and detected. Compared with the control, the treatment group exhibited significantly elevated activities of these enzymes. Compared to the CK, the T1 group achieved its peak activity on the 8th day, with increases of 2.75-fold, 1.47-fold, 1.80-fold, 1.72-fold, and 1.40-fold, respectively. In the T2 group, the maximum activities were recorded on the 6th day, which were 1.49-fold, 1.13-fold, 1.36-fold, 1.18-fold, and 1.25-fold greater than those in the T4 group on the 8th day. The T1, T3, and T4 groups all reached their peak activities on the 8th day (Figure 7). These findings indicate that inoculation with strain HX2 significantly enhances the enzymatic defense response in tomato plants, thereby enhancing their resistance against *R. solanacearum*.

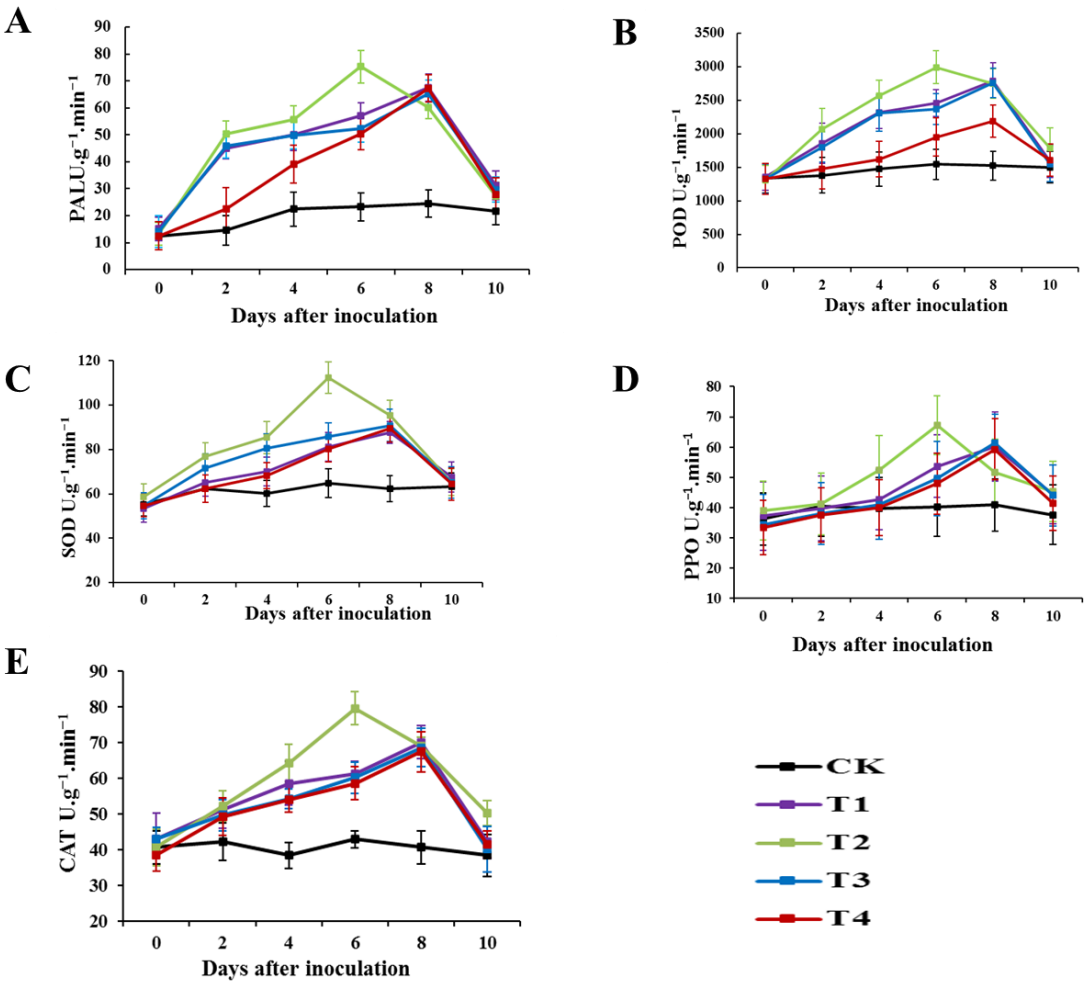

Figure 7. Effect of strain HX2 on activities of antioxidant and defense−related enzymes in leaves of tomato plants. Antioxidant enzymes including phenylalanine ammonia−lyase (PAL) (**A**), peroxidase (POD) (**B**), and superoxide dismutase (SOD) (**C**). Defense−related enzymes including polyphenol oxidase (PPO) (**D**) and catalase (CAT) (**E**). CK: sterile water. T1: HX2; T2: HX2+ *R. solanacearum*; T3: Thiediazole copper + *R. solanacearum*; T4: *R. solanacearum*. Bars indicate the standard error of the mean.

3.7. The Results of Transcriptome Analysis

A total of six cDNA libraries were constructed, each with approximately 44 million reads, and the average error rate was found to be 0.025% (Table 4). The correlation coefficients between biological replicate samples treated identically averaged above 0.95, suggesting high-quality RNA-Seq data (Figure 8a). Upon treatment with HX2, plants exhibited differential expression of 18,553 genes, with 1012 upregulated and 1386 downregulated genes (Figure 8b). GO annotation revealed that these DEGs were predominantly categorized into three domains: biological processes (BP), cellular components (CC), and molecular functions (MF). Notably, a significant number of gene products associated with BP, particularly in cellular and metabolic processes, were identified in the HX2-treated group compared to the control (Figure 8c). KEGG pathway analysis further indicated that the DEGs were most enriched within metabolic pathways (Figure 8d). In terms of significant differentially expressed genes between the control and treatment (CK vs. T) groups, enrichment was observed in both GO and KEGG pathways. The top 20 GO terms included processes such as salicylic acid metabolism, ethylene-activated signaling, photosynthesis, and defense response (Figure 9a). In the KEGG pathway, plant hormone signal transduction ranked second, followed by the MAPK signaling pathway and phenylpropanoid biosynthesis in third and fourth place, respectively (Figure 9b). In conclusion, HX2 strain

enhances plant resistance to pathogen attacks by regulating intracellular metabolism and activating the plant's immune response.

Table 4. Description of RNA sequences from CK or HX2-treated tomato roots.

Sample	Raw Reads	Clean Reads	Error Rate (%)	Q30 (%)	GC Content (%)
CK-1	47,982,218	47,518,134	0.0257	94.93	42.96
CK-2	53,646,110	53,108,916	0.0256	95.02	42.95
CK-3	57,158,118	56,584,476	0.0256	95.05	43.13
T-1	49,639,454	49,147,642	0.0258	94.89	43.37
T-2	44,060,606	43,574,568	0.0257	94.89	43.31
T-3	46,153,870	45,699,214	0.0252	95.3	43.29

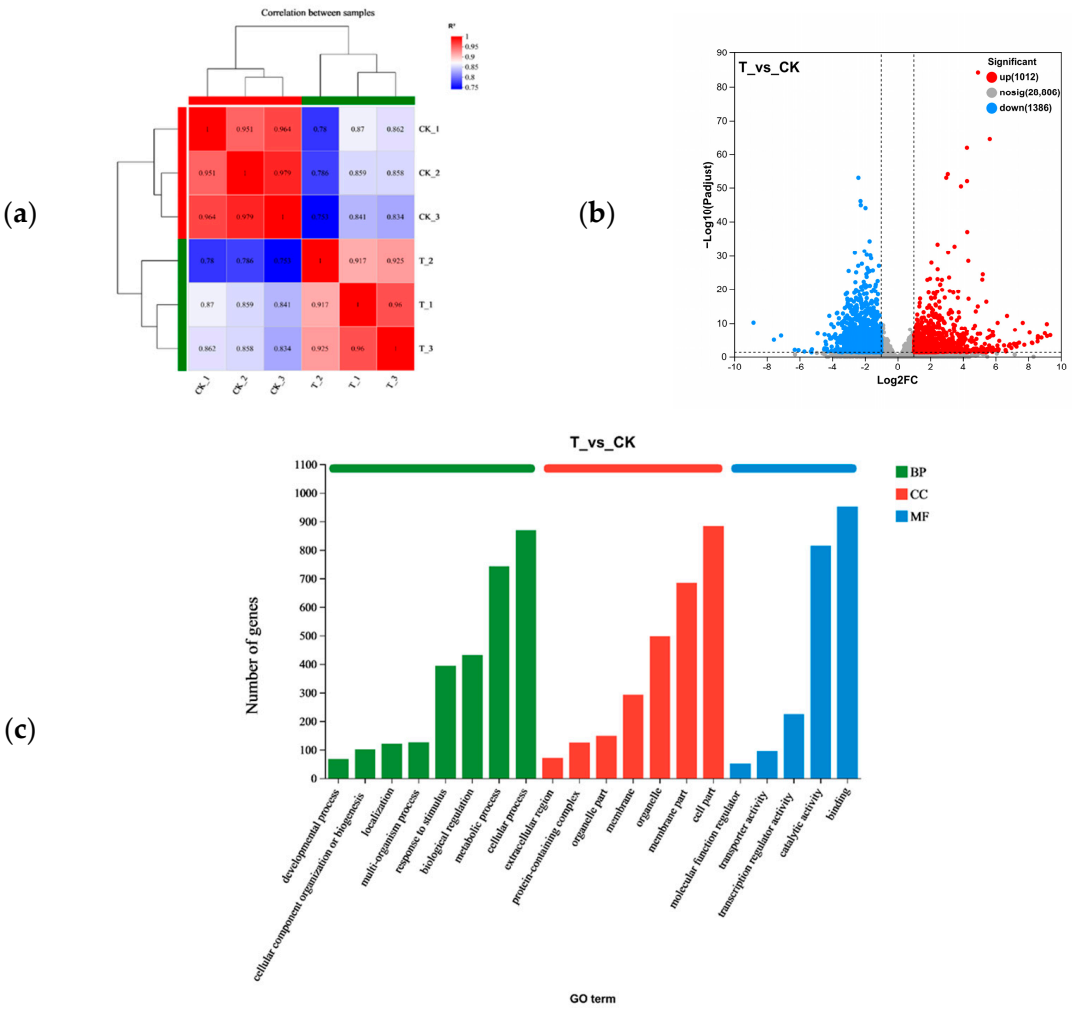

Figure 8. *Cont.*

(**d**)

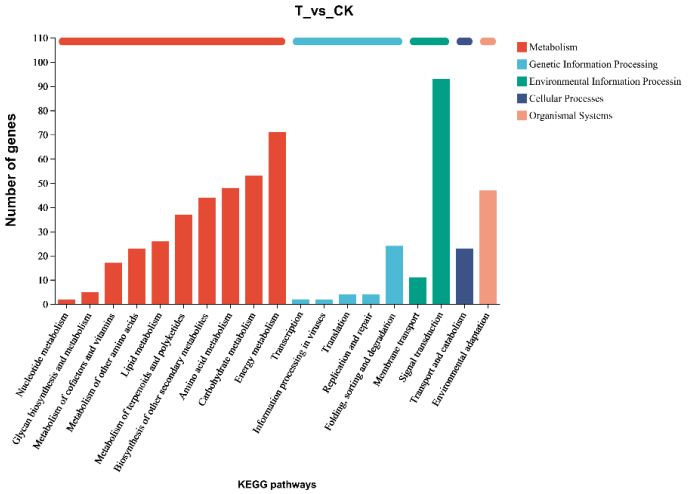

Figure 8. DEG analyses of tomatoes with HX2 vs. PDA inoculation. (**a**): The module clusters and their relationships. (**b**): Volcano plots (the abscissa indicates the multiple changes of gene expression in different samples (log2FoldChange), and the ordinate indicates the significant level of expression difference (−log10padj); upregulated genes are represented by red dots and downregulated genes by blue dots). (**c**): Gene Ontology (GO) annotation category statistics. (**d**): Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification statistic. ((**c**,**d**): The abscissa is the Term, and the ordinate is the number of genes annotated to the Term).

a

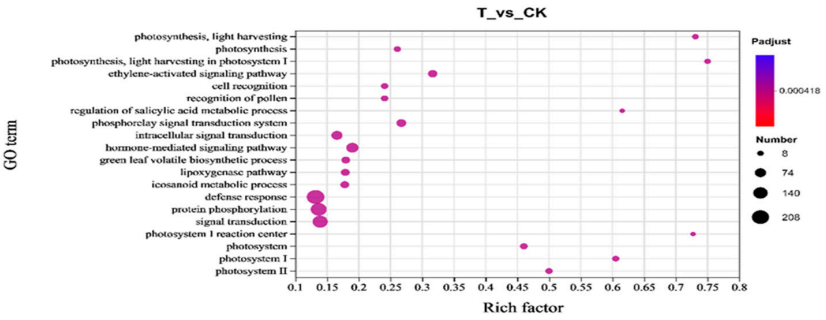

Figure 9. *Cont.*

b

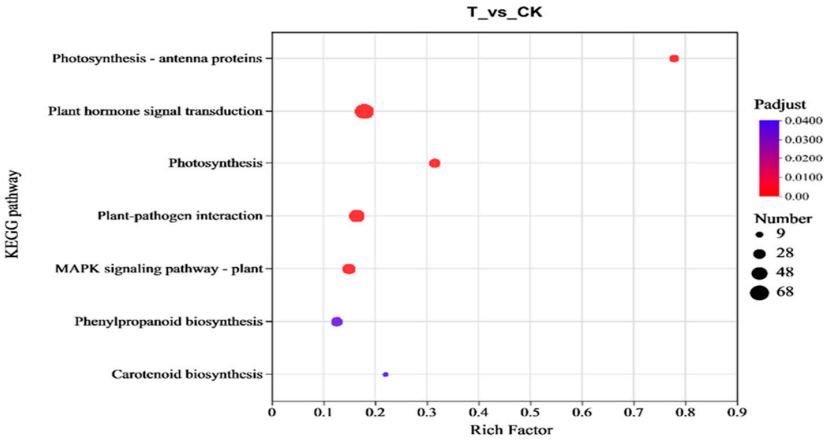

Figure 9. Enrichment analysis of tomatoes with HX2 vs. PDA inoculation. (**a**): Gene Ontology (GO) enrichment analysis. (**b**): Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. Circle size represents the number of enriched genes. The X axis displays the enrichment factor.

3.8. The Results of Quantitative Real-Time PCR

To assess the expression levels of disease-resistant and pathogenic genes across various treatment groups, samples were subsequently subjected to quantitative real-time PCR analysis. The results indicated that the relative expression levels of *IAA*4, *ERF*1, and *Hqt* were significantly upregulated in the HX2 group compared to the control (CK) group, while the expression level of *Hsc*70 was markedly downregulated in the HX2 group (Figure 10). Notably, the expression of *IAA*4 (3.5-fold change), *ERF*1 (3.5-fold change), and *Hqt* (1.5-fold change) was substantially enhanced in the treatment group. Conversely, the expression of the pathogenic *Hsc*70 was significantly reduced (0.5-fold change), suggesting a pronounced inhibitory effect of the HX2 conditions on this gene.

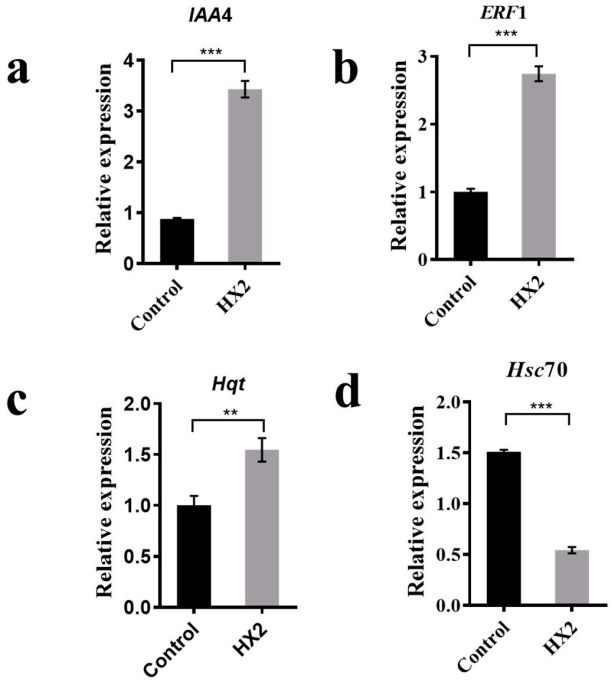

Figure 10. The effect of strain HX2 on the expression level of disease-resistant and pathogenic genes by quantitative reverse-transcription PCR analysis. Relative expression levels of (**a**) *IAA*4, (**b**) *ERF*1, (**c**) *Hqt*, and (**d**) *Hsc*70 in the root of tomatoes through treatment with strain HX2 and sterile water (CK). Error bars represent mean standard deviation of triplicate experiments. ** $p < 0.05$ *** $p < 0.001$.

4. Discussion

The genus *Cladophialophora* was first established by Tanapol with *C. ajelloi* (currently known as *C. carrionii*) as the type species [47]. At present, a total of 75 known species of *Cladophialophora* have been validated, accepted, and recorded in this genus in the Index Fungorum, of which 22 taxa were first introduced in 2023 (Available at: http://www.indexfungorum.org, accessed on 7 September 2024). *Cladophialophora* is a genus of asexual dematiaceous fungi which were characterized by the presence of branched or unbranched conidial chains with hyaline conidial scars produced through blastic conidiogenesis [48,49]. The conidiophores were typically reduced into oblong or cylindrical conidiogenous cells in the majority of *Cladophialophora* species. However, our collection is morphologically distinct in having non-septate, melanized, curled strings of sausage-shaped conidia produced in coherent and unbranched chains that often arise from oblong, cylindrical to somewhat fusiform conidiogenous cells, and could not be ascribed to any known species, hence *C. guangxiense* as new taxon is introduced. Molecular phylogeny showed that *C. guangxiense* strain HX2 formed a distinct clade with *C. inabaensis* (EUCL1) based on a combined dataset, however, the ITS and LSU sequence similarities between the two types of strains were only 92.07% (476/517) and 98.91% (818/827). Furthermore, *C. guangxiense* and *C. inabaensis* were morphologically easy to distinguish from each other by the shape of conidiogenous

cells and conidia [oblong, cylindrical to fusiform conidiogenous cells and oval, cylindrical, sub-cylindrical and curled strings of sausage-shaped conidia in *C. guangxiense* vs. semi macronematous differentiated conidiophores and subglobose (length/breadth ratio <1 to 2:1) conidia in *C. inabaensis*] [50], which also provides further evidence to support placement of the fungus as a new species within the genus *Cladophialophora*. *C. guangxiense* strain HX2 was morphologically similar to *C. tortuosa* in possession of conidial chains consisting of ellipsoid to ovoid conidia which were often bent [51], however, *C. guangxiense* strain HX2 and *C. tortuosa* BA4b006 were in two distinct clades far from each other in the NJ phylogenetic tree in this research, and the ITS and LSU sequence similarities between the two types of strains were only 92.19% (425/461) and 99.03% (819/827), which indicated they were two different species.

Extant research indicates that DSE fungi potentiate plant defenses by mechanisms including intracellular colonization, modulation of metabolic output, and the triggering of systemic acquired resistance [48,52]. *Cladophialophora* is the dominant species of DSEs, which can colonize in a wider variety of host plants and is largely distributed in various habitats around the world [53]. This research showed that some *Cladophialophora* species have the ability to promote plant growth. Our study reported that *C. guangxiense* as a new species plays an active role in tomato growth promotion and disease prevention for the first time in this paper.

Defense enzymes are crucial for plant physiological responses to disease stress [54], including key enzymes such as PAL, PPO, POD, SOD, and CAT. Recent studies have shown that PAL significantly contributes to the reduction of pathogen infection [55]; PPO and POD are involved in the polymerization of polyphenols into lignin and the production of hydroxyproline-containing glycoproteins (HRGP), which aid plants in resisting penetration by pathogens [40]. Additionally, SOD, POD, and CAT participate in the elimination of reactive oxygen species (ROS), initiating downstream processes that involve the ET, jasmonic acid (JA), and SA pathways, and safeguarding rice crops from rice sheath blight damage [56,57]. Our findings demonstrate that pre-colonization of plants with *C. guangxiense* HX2 enhanced the activities of SOD, POD, and CAT against the pathogen *R. solanacearum*. Thus, the augmented antioxidant enzyme activities mediated by *C. guangxiense* HX2 may represent one of the mechanisms by which tomato resistance to bacterial wilt disease is improved.

The strain HX2 has shown potential to colonize tomato roots. The colonization by endophytic fungi can elicit host defenses, which can establish long-term, systemic resistance against a diverse array of pathogens, thereby proving particularly effective in the control of plant diseases [58]. In this study, we employed Illumina sequencing to delve into the molecular underpinnings of tomato resistance induced by strain HX2. Differentially expressed genes between the control (CK) and treatment (T) groups were significantly enriched in GO and KEGG pathways, primarily involving the SA metabolic process, ethylene-activated signaling, plant hormone signal transduction, MAPK signaling, and phenylpropanoid biosynthesis—processes integral to plant defense mechanisms [59].

Hormone-regulated genes are crucial for plant growth, development, and responses to biotic stresses [60]. Key phytohormones in this context include SA, JA, and ethylene [61]. Recently, Kawagoe [62] et al., revealed that the cyclic lipopeptide iturin A activates the SA signaling pathway to induce the defense response in Arabidopsis. Ethylene, upon pathogenic bacterial infestation, upregulates disease resistance-related genes, thereby enhancing plant disease resistance [31,63]. The *ERF* (ethylene response factor) gene family constitutes plant-specific transcription factors that bind to ethylene-responsive elements and regulate gene expression subsequent to ethylene signaling [64]. MAPK pathways are among the conserved defense-related signaling pathways [65], and studies have shown that MAPK pathways are interconnected with ethylene signaling in plants [63]. Phenylpropanoid metabolism is vital for the production of typical antibacterial compounds, such as phenols, flavonoids, and lignans [66]. PAL, as the rate-limiting enzyme and the initial step in the phenylpropanoid metabolic pathway, is essential for disease defense [40,67].

In this study, the expression of *ERF*1 and *Hqt* were substantially enhanced. Zou [68] et al. revealed that *ERF*1 acts in a positive feedback loop and regulates autophagy activity by transcriptionally activating ATG expression in response to root-knot nematode (RKN) infection. Therefore, to strengthen the plant's immune response, enabling it to resist damage from root-knot nematodes. *Hqt* transferases play a crucial role in the synthesis of chlorogenic acid (CGA). CGA possesses antioxidant and antibacterial properties, which can help plants resist pathogen invasion [69,70].

In conclusion, *C. guangxiense* HX2 colonizes the tomato root system and induces resistance by activating metabolic processes related to salicylic acid, ethylene-mediated signaling, plant hormone transduction, the MAPK pathways, and phenylpropanoid biosynthesis, triggering a comprehensive defense response against pathogen invasion. This research provides a foundational understanding of the molecular underpinnings of *C. guangxiense*-induced resistance in tomatoes. However, the underlying mechanism remains elusive. This study demonstrates that strain HX2 has the commercialization potential to be used as a biocontrol agent. Future studies may employ additional omics technologies to dissect the intricate molecular mechanisms by which *C. guangxiense* confers resistance to tomatoes.

5. Conclusions

The fungus *C. guangxiense* HX2, isolated from sugarcane rhizosphere soil, was a new species of DSE fungus in the genus *Cladophialophora*. *C. guangxiense* HX2 could establish a symbiotic relationship with the roots of tomatoes as an endophytic fungus, significantly enhancing the tomatoes' resistance to bacterial wilt and promoting tomato growth. *C. guangxiense* HX2 enhanced the activity of antioxidant enzymes (SOD, POD, CAT, PPO, and PAL). Inoculation with HX2 upregulated genes involved in SA metabolism, ethylene-activated signaling, plant hormone signal transduction, MAPK signaling, and phenylpropanoid biosynthesis, while downregulating pathogenic gene expression. Therefore, *C. guangxiense* HX2 can effectively enhance the resistance of tomatoes to bacterial wilt disease. Consequently, the antagonistic effect of *C. guangxiense* HX2 against bacterial wilt of tomatoes was heightened. This research sheds light on the mechanisms associated with the improved antagonistic efficacy of microorganisms used for disease control of tomatoes, offering an innovative solution for the prevention of bacterial wilt of tomatoes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14122771/s1, Table S1: List of Cladophialophora species used for phylogenetic study.

Author Contributions: Conceptualization, Y.Z. and Z.S.; Methodology, X.W., Y.L., Y.C. and X.J.; Software, X.W., Y.L. and X.J.; Validation, S.N.M. and Y.Z.; Formal analysis, X.W. and Y.C.; Investigation, X.J. and L.X.; Resources, Z.S.; Data curation, X.W., Y.L. and S.N.M.; Writing—original draft, X.W., Y.L., Y.C. and X.J.; Writing—review & editing, X.W., S.N.M., Z.S. and L.X.; Visualization, Y.C. and S.N.M.; Supervision, Y.Z.; Project administration, Y.Z., Z.S. and L.X.; Funding acquisition, Z.S. and L.X. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declared that they have no conflicts of interest in this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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