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Research Article

Molecular Identification of *Juglans Regia* Endophyte LTL-G3, Its Antifungal Potential and Bioactive Substances

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Background: Endophyte is one of the potential biocontrol agents for inhibiting plant pathogens. However, the mechanisms and characteristics involved in the inhibition of different phytopathogenic fungi by endophytes, especially walnut endophytes, are still largely unknown.

Objectives: The present study aimed to identify the walnut endophytic fungus LTL-G3 from a genetic point of view, assess the strain's antifungal activity, and determine the bioactivities of the substances it produces against plant pathogens.

Materials and Methods: The homologous sequence of strain LTL-G3 was examined, and typical strains of the *Trichoderma virens* group were used to build NJ phylogenetic trees and analyze the taxonomic position of the strain. The biocontrol agent's antagonistic potential for many plant pathogenic fungi. By using silica gel G chromatography, the active components of the strain were separated and purified. The active components were identified using GC-MS and NMR.

Results: The strain LTL-G3 was identified as *Trichoderma virens*. Its fermentation and secondary metabolite extracts had a broad spectrum and strong inhibitory effect on the spread of six plant pathogens (*Botrytis cinerea*, *Fusarium graminearum*, *Gloeosporium fructigenum*, *Phytophthora capsici*, *Rhizoctonia solani*, and *Valsa mali*) evaluated, of which, its inhibition rate against *Valsa mali* reached 76.6% (fermentation extract) and 100% (ethyl acetate and n-butanol extracts). On silica gel G chromatography, bioactive compounds were divided into 6 fractions and 7 sub-fractions. Fr.2-2 was the sub-fraction that showed the greatest inhibitory against *V. mali*, as an inhibition percentage of 89.36% in 1 mg. mL^{-1}. Fifteen key inhibitory chemicals identified using GC-MS. By examining the NMR data, the chemical make-up of the precipitated white solid was identified. The inhibition rate against V. mali increased by over 95% at a dosage of 1 mg. mL^{-1}, indicating a significant linear association between compound A and that rate.

Conclusions: The strain LTL-G3 can be applied as an efficient biological control agent against *V. mali*, and its highly inhibitive secondary metabolites provide the mechanism for this action.

Keywords: Antifungal activity, Bioactive substances, Phytopathogens, *Valsa mali*

1. Background

Endophytic fungus is called to any fungus that lives inside its host plant at some point during its life-cycle and does not infect or harm the host, and has a complicated connection with its host plant(s) (1). There are over thirty thousand species of unidentified plants

in the globe that could serve as the host for a variety of endophytes, and endophytic fungi are extensively distributed (2). As a potential biocontrol resource, many endophytic fungi have the function of controlling plant diseases (3) and insect pests (4), promoting the growth of their host plants (5), enhancing plant resistance (6), and promoting the degradation of organic pollutants (7). Among all biological impacts, endophytic fungi have been extensively studied recently for their antifungal activities. In the endophytic fungi of Cupressaceae, *Trichoderma koningii* CSE32 and *Trichoderma atroviride* JCE33 exhibited complete growth inhibition of both *Aspergillus fumigatus* and *Aspergillus niger* within 3 to 7 days (8). The *Lilium davidii* endophyte *Acremonium* sp. Ld-03 had the highest antifungal activity against *Botryosphaeria dothidea*, which was 78.39±4.21% (9).

In the fruit planting industry, spraying chemical pesticides helps trees prevent diseases. But it may lead to environmental pollution, pesticide residues in agricultural products, and pathogen resistance (10). The effectiveness of disease control is decreased and the risk of disease breakout is increased by the overuse of chemical fungicides. More critically, chemical residues that leak into groundwater or flow into water streams might have negative effects on the ecosystem and people's health (11).

In order to deal with ecological and environmental concerns and meet consumers' demands for pesticide-free food, the development of biological pesticides is imminent. Biological insecticides might reduce environmental harm and possible health risks while defending plants against pathogenic infections as compared to chemical pesticides (12-13). Therefore, using bio-control techniques to manage plant pathogenic fungus can be a sustainable option, and using biopesticides instead of conventional pesticides may be preferable.

Beneficial biological activity of biopesticides may be connected to the metabolites that are secondary (14). The secondary metabolites might play a role in antimicrobial action or in the cooperative inhibition of harmful microbial growth and development. Secondary metabolites generated by endophytes include alkaloids, flavonoids, quinones, phenols and other compounds, which have anticancer, antibacterial and antioxidant activities (15). Malhadas *et al*. (16) showed that the ethyl acetate extract of *Olea europaea* L. endophyte *Alternaria alternata* (Fr.) Keissl revealed the broadest spectrum of anti-microbial activity at a minimum inhibitory concentration $\leq$0.095 mg. mL^{-1}. The volatile chemicals were discovered using GC-MS, and the most prevalent ones with antibacterial potentials were 3-methyl-1-butanol and phenethyl alcohol. The findings highlighted *O. europaea* endophytes' antibacterial potential for the first time and the potential for their use because of their antimicrobial compounds.

Walnut (*Juglans regia* L) is considered as the oldest and the most widely distributed nut in the world (17). The pharmaceutical and cosmetic industries have made substantial use of green walnut, shell, husk, kernel, bark, roots, and leaves (18). Walnuts are rich in fatty acids, proteins, minerals and polyphenols (19) exhibiting a range of antimicrobial (20), antioxidant (21), anti-inflammatory (22), cholesterol reducing (23), blood pressure reducing, cardiovascular disease risk reducing (24) and many other properties that positively affect human health. However, the majority of earlier investigations on walnut endophytes concentrate on how the substrates and nutritional requirements of the species affect the effectiveness of their biocontrol. Currently, there are relatively few studies on walnut endophytes. For the first time, *Bacillus subtilis* HB1310, an oleaginous endophyte, was isolated by Zhang *et al*. (25) from the walnut with a thin shell. This strain uses cotton stalk hydrolysate for a substrate to efficiently and quickly acquire cellular lipids. The fatty acid composition of the bacterial lipids generated by this strain suggests that it might be ideal for producing biodiesel.

2. Objectives

Based on the information above, the present study aimed to identify the walnut endophytic fungus LTL-G3 from a genetic point of view, assess the antimicrobial capacity of fermentation and secondary metabolic extracts isolated from the LTL-G3 against six pathogenic fungi, identify the principal antibiotic fractions engaged in LTL-G3's biological control activity, and determine the biological effects of the substances generated by LTL-G3 against plant pathogens.

3. Materials and Methods

3.1. Endophytic Fungus and Plant Pathogenic Fungi

The endophytic fungus LTL-G3 was isolated from walnut

roots according to the method described by Wang, with a few adjustments (The culture temperature was 28 °C) (26). Pathogenic fungi used in the test—*Botrytis cinerea* (ascomycetous fungus), *Fusarium graminearum* (ascomycetous fungus), *Gloeosporium fructigenum* (ascomycetous fungus), *Phytophthora capsici* (an oomycetous microorganism), *Rhizoctonia solani* (a famous basidiomycetous fungus), and *Valsa mali* (ascomycetous fungus)—were provided by Pesticide Research Service Center, Northwest A&F University, Yangling, China, and stored in our laboratory. Before usage, pure culture isolates were cultivated at 28 °C for up to 7 days on potato dextrose agar (PDA) medium.

3.2. Molecular Identification of Strain LTL-G3

The isolated endophytic fungus LTL-G3 was cultivated on 300 mL of potato glucose broth (PDB) medium for 7 to 10 days at 28 °C and 180 rpm. According to the producer's guidelines, the genomic DNA was obtained using a fungal genomic DNA extraction kit (D2300, Beijing Solarbio Science & Technology Co.,Ltd., PR China). Employing primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-CCTCCGCTTATTGATATGC-3') as described by Le *et al*. (27), a rDNA region was amplified by PCR. PCR products were sequenced at Xi'an Thermo Biotechnology Corporation (http://www.wolsen.net).

The nucleotide sequence information was then run through the online BLAST program (ncbi.nlm.nih.gov/BLAST) to identify related sequences with known taxonomic information in NCBI (https://www.ncbi.nlm.nih.gov/), properly confirming the strain LTL-G3. The 16S rDNA nucleotide sequences were aligned using the MEGA7.0 program (Mega Limited, Auckland, New Zealand) (28), and neighbor-joining (NJ) was used to generate a phylogenetic tree (29).

3.3. Preparation of Endophytic Fungal Extracts

The purified endophytic fungal strain was inoculated into a 300 mL PDB medium and it was then grown at 28 °C for 7 to 10 days at 180 rpm on a rotary shaker. To separate the mycelium from the filtrate, the endophytic fungus fermentation products were filtered. The fermentation extract was then extracted from the filtrate using a rotary evaporator.

3.4. Extraction of Secondary Metabolites

The solvent partition method was used to extract the LTL-G3 strain's secondary metabolites (30). To make seed fermentation broth, the strain was cultured in PDB broth at 28 °C and 180 rpm shaking for 7 days. A 500 mL conical flask with solid culture medium was infected with 20 mL of seed fermentation broth (31). To prepare this medium, 80 g polished round-grained rice (COFCO Group Co., Ltd, Beijing, China) was mixed with distilled water (120 mL), placed in a 500 mL conical flask, sterilized for 20 min at 121 °C, then let to cool at room temperature overnight. The solid fermentation product was produced after 45 days of room temperature incubation of the culture. Then, the fermentation product was extracted with twice the volume of 95% ethanol solution for 5 times. The crude extract was produced by condensing the filtrates after filtering the fermentation broth. The organic phase was concentrated in a water bath at 75 °C to get rid of the organic solvents after the crude extract was subjected to three separate extractions using petroleum ether, ethyl acetate, and n-butanol.

3.5. Antifungal Activity

Under *in vitro* testing circumstances, the isolated endophyte LTL-G3 was tested to have antifungal activity against six strains of pathogenic fungus, including *B. cinerea*, *F. graminearum*, *G. fructigenum*, *P. capsici*, *R. solani*, and *V. mali*. The growth rate approach was used to conduct the antifungal bioassays for the fermentation extract and secondary metabolite extracts. Distilled water was added to the extracts to prepare solution of certain concentrations (The concentration of the fermentation extract used was 100 mg. mL^{-1} and the concentration of the secondary metabolite extracts were 50 mg. mL^{-1}). The solution and the PDA culture medium were combined to produce a 1:9 culture medium. Finally, the mixture was distributed into the Petri dishes. The fungal cake of pathogenic fungi was transferred to the center of the medium and incubated in an incubator at 28 °C. Each treatment was replicated three times. As controls, PDA plates with fungal plugs (but no extract solution) were employed. The development of the fungal pathogen strains on the plates was routinely monitored. After the fungus mycelium in the control plate approached the edge of the plate, the fungus growth area was calculated. Inhibition rate (%) is calculated as [(Dc - Dp) / Dc]×100, where Dc and Dp are the colony diameters of the test pathogens in the control and test plates (30), respectively.

3.6. Purification and Evaluation of Endophytic Strain LTL-G3 Active Compounds Antifungal Activity

The extract was put to a silica gel G chromatography column and purified by petroleum ether, petroleum ether/ethyl acetate (2:1, 1:1, 1:2, v/v), ethyl acetate, and ethyl acetate/acetone (1:1, v/v), yielding six fractions, Fr. 1~Fr. 6. Each fraction was prepared as a 20 mg. mL^{-1} test solution using sterile water as the solvent after drying at 60 °C for fungicidal test. The approach used to calculate the antagonistic effect was the same as that outlined in section 3.5. Following further separation using a silica gel G chromatography column and elution by petroleum ether/ethyl acetate (10:0~0:10, v/v), the fraction with the greater fungicidal activity was determined. The highly efficient fraction Fr.2 yielded seven sub-fractions. The seven sub-fractions were given the following names, in order: Fr.2-1~Fr.2-7. Each sub-fraction was dried at 60 °C and then prepared as a 10 mg. mL^{-1} solution by sterile water for fungicidal test.

3.7. Identification and Antifungal Testing of the Effective Antifungal Substances

Analyses using gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) were conducted to ascertain the chemical structures of the purified active ingredients. For fungicidal testing, the substance was dissolved in sterile water to concentrations of 1.00, 0.50, 0.10, 0.05, and 0.01 mg. mL^{-1}. Each treatment was replicated three times.

3.8. Statistical Analysis

The study's data are reported as means standard deviations (SD), which are the means of three replicated measurements. One-way analysis of variance (ANOVA) and Duncan's multiple range test were employed to establish the statistical significance using the statistical program SPSS 17.0. Using Origin software (OriginLab Corp., USA) to draw the graph. The IC_{50} was subjected using the SPSS 17.0.

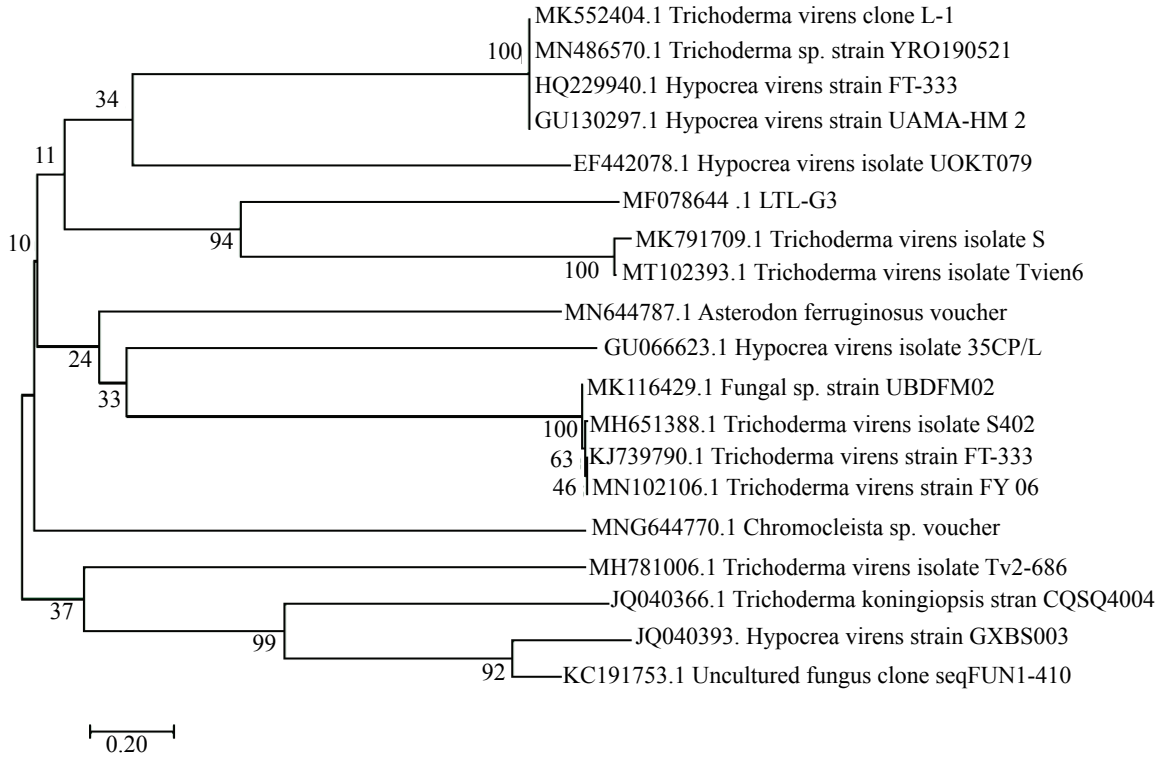

Figure 1. The location of the endophytic fungus LTL-G3 in the phylogenetic tree based on ITS sequences. The BLAST program in the GenBank database on the NCBI website (http://www.ncbi.nlm.nih.gov/genbank/) was used for strain proximate sequence queries. The phylogenetic tree was then constructed based on neighbor-joining clustering analysis.

4. Results

4.1. Molecular Identification of Strain LTL-G3

The DNA of endophytic fungus was amplified by rDNA-ITS methodology, the PCR amplicons for ITS domain of purified strain was sequenced and the similarity was compared. The walnut endophytic fungus LTL-G3 phylogenetic tree was created and is displayed in **Figure1**.

The DNA of strain LTL-G3 was extracted and 619 bp fragment was obtained after amplification. The GenBank database received the sequencing results and assigned the accession number MF078644. The rDNA sequence of our isolate and eighteen sequences of related species isolated from plants were used to build a phylogenetic tree. Phylogenetic analysis of the ITS gene sequences revealed that the endophytic fungus LTL-G3 from walnut joined to other isolates of *Trichoderma virens* strain S (MK791709) and *T. virens* isolate Tvien 6 (MT102393) showing a bootstrap value of 94%. Thus, the endophytic strain LTL-G3 was known to be a member of the fungal genus *Trichoderma* identified as *T. virens*.

Table 1. Inhibition rate of strain LTL-G3 fermentation extracts

Pathogenic fungus	Fungal colony diameter (mm)		Inhibition rate (%)
	Control	Treatment	
Thanatephorus cucumeris	84.67±0.58	36.30±2.86	57.13±3.23b
Valsa mali	65.00±6.06	15.19±1.10	76.60±0.50a
Fusarium graminearum	67.17±3.69	34.77±0.93	48.15±2.63b
Gloeosporium fructigenum	57.50±2.18	35.89±5.72	37.76±7.62c
Botrytis cinerea	60.50±0.50	40.68±0.77	32.46±0.95c
Phytophora capsici	62.50±1.73	59.36±5.81	31.36±9.68c

* Data are presented as means±standard deviation

Table 2. Inhibitory effect of secondary metabolite extracts

Extracts	Fungal colony growth Inhibition rate (%)					
	Valsa mali	*Fusarium graminearum*	*Gloeosporium fructigenum*	*Thanatephorus cucumeris*	*Phytophthora capsici*	*Botrytis cinerea*
Petroleum ether extract	56.65±2.33	32.41±3.65	42.16±2.98	36.53±3.12	23.30±4.02	33.73±2.62
Ethyl acetate extract	100.0±0.00	54.58±4.21	72.25±3.45	94.52±0.86	65.38±3.45	47.39±2.47
N-butanol extract	100.0±0.00	46.38±2.54	67.53±1.32	88.24±1.99	44.32±2.83	32.16±0.45
Residue extract	43.81±1.66	39.52±1.44	21.96±1.88	56.93±3.01	27.94±3.17	26.14±1.23

* The antifungal activity of the secondary metabolite extracts was tested with PDA medium. The test concentration was 50 mg. mL^{-1}. Incubated in a constant temperature incubator at 28 °C for 7 days. Each treatment was replicated three times.

4.2. Inhibitory Effect of LTL-G3 Fermentation on Plant Pathogens

The inhibitory capacity of the fermentation extract of walnut endophytic fungus LTL-G3 against *B. cinerea*, *F. graminearum*, *G. fructigenum*, *P. capsici*, *R. solani*, and *V. mali* was calculated (**Table 1)**. All six pathogenic fungi that were evaluated for growth were capable of being inhibited. Among them, the fermentation extract of strain LTL-G3 had significant antifungal activity against *V. mali* (76.6% inhibition rate). The inhibition rates against *G. fructigenum*, *F. graminearum* and *R. solani* between 37% and 58%. The oomycetous pathogen P. capsici had the lowest inhibitory impact, with an inhibition rate of less than 32%.

4.3. Inhibitory Effect of Secondary Metabolism Extracts Against Plant Pathogens

The secondary metabolic extracts were prepared as 50 mg. mL^{-1} solutions and their antifungal effects on the pathogens were determined. Petroleum ether, ethyl acetate and n-butanol extracts showed different inhibitory effects against plant pathogens, respectively (**Table 2**). Ethyl acetate and n-butanol extracts exhibited significant antifungal activity against the pathogen *V. mali*, completely inhibited the growth of the pathogen hypha, and the inhibition rates were 100%. Meanwhile, the pathogen *R. solani* was highly inhibited by the ethyl acetate and n-butanol extracts, with inhibition rates exceeding 88%. The petroleum ether and residue extract of secondary metabolites had less inhibitory effects on all pathogens, with the inhibition rates less than 57%.

4.4. Inhibitory Effect of Strain LTL-G3 Fractions Against Plant Pathogens

Using a bioassay-guided fractionation technique, six fractions were produced of the ethyl acetate extract from the LTL-G3 fermentation product. The results of the bioassay testing showed that the anti-fungal efficiency of the fractions against specific plant diseases altered with the fractions (**Table 3**). The Fr.2 fraction had the strongest inhibitory effect as a dosage of 10 mg. mL^{-1}, with inhibition ratios of 100%, 88.79%, and 87.21% for *V. mali*, *Th. cucumeris* and *G. fructigenum*, respectively. With an inhibition rate of 90.51%, the Fr.1 fraction was found to have the second-highest inhibitory effects against *V. mali*. Fr.6 did, however, have a very weak inhibitory impact on the pathogens, with inhibition rates not exceeding 25%. Therefore, the percentage Fr.2 fungicidal activity was selected for additional research on the three pathogens.

4.5. Critical Chemicals with Antifungal Effects Inhibiting the Pathogen

Using a silica gel G chromatography column for purifing by petroleum ether/ethyl acetate, the fraction with the highest level of fungicidal activity (Fr.2 in **Table 3**) was further divided into seven sub-fractions (Fr.2-1~Fr.2-7) (**Table 4**).

Table 3. **The antifungal activity of the fractions against three plant pathogens**

Fractions	Fungal colony growth inhibition rate (%)		
	Valsa mali	*Thanatephorus cucumeris*	*Gloeosporium fructigenum*
Fr.1	90.51±3.11	65.42±1.87	35.42±2.64
Fr.2	100.0±0.00	88.79±2.33	87.21±2.73
Fr.3	56.50±1.58	67.44±2.86	75.00±1.61
Fr.4	68.14±1.89	50.42±0.41	65.15±0.31
Fr.5	23.73±0.46	38.19±0.84	48.25±0.43
Fr.6	14.41±0.66	22.36±0.78	15.18±0.27

* The antifungal activity of the fractions was tested with PDA medium. The test concentration was 20 mg. mL^{-1}. Incubated in a constant temperature incubator at 28 °C for 7 days. Each treatment was replicated three times.

Table 4. The antifungal activity of fraction 2 sub-fractions against three plant pathogens.

Fractions	Fungal colony growth Inhibition rate (%)		
	Valsa mali	*Thanatephorus cucumeris*	*Gloeosporium fructigenum*
Fr.2-1	36.18±0.58	45.33±0.89	15.28±1.05
Fr.2-2	89.36±2.53	78.45±1.73	54.23±1.36
Fr.2-3	55.36±1.51	12.15±0.49	46.58±0.68
Fr.2-4	17.50±0.54	26.38±0.69	37.48±1.03
Fr.2-5	10.73±0.56	47.36±1.08	59.18±2.38
Fr.2-6	17.51±0.21	22.18±0.54	48.98±1.56
Fr.2-7	16.38±0.48	18.55±0.99	20.41±1.06

* The antifungal activity of the sub-fractions was tested with PDA medium. The test concentration was 10 mg. mL^{-1}. Incubated in a constant temperature incubator at 28 °C for 7 days. Each treatment was replicated three times.

Table 5. The key chemical compounds in the effective sub-fraction Fr.2-2 identified through GC-MS

No.	Retention time	Formula	Compound name	molecular weight	Relative content (%)
1	5.03	$C_6H_{12}O_2$	Butyl acetate	116	0.16
2	8.08	$C_8H_9NO_2$	Methoxyphenoxime	151	0.10
3	19.32	$C_6H_{11}NO$	Caprolactam	113	0.43
4	26.42	$C_{14}H_{22}O$	2,4-Di-tert-butylphenol	206	0.06
5	26.48	$C_{15}H_{24}O$	2,6-Di-tert-butyl-4-methylphenol	220	0.42
6	28.47	$C_{16}H_{34}$	N-hexadecane	226	4.27
7	30.72	$C_{17}H_{36}$	Heptadecane	240	6.08
8	30.87	$C_{15}H_{32}$	2,6,10-Trimethyldodecane	212	4.18
9	31.30	$C_{15}H_{26}O_2$	Oxidized disaccharide alcohol B	238	0.49
10	36.93	$C_{15}H_{22}O_2$	Curcumenol	234	6.19
11	38.51	$C_{26}H_{44}O_2$	Ethyl iso-5α-cholate	436	0.13
12	40.66	$C_{18}H_{36}O_2$	Cetyl acetate	184	8.45
13	41.45	$C_{14}H_{29}NO$	N,N-Dimethyllauramide	227	0.95
14	41.78	$C_{35}H_{70}$	17-Pentatriacontene	490	10.39
15	44.23	$C_{23}H_{32}O_2$	2,2’-Methylenebis[6-(1,1-dimethylethyl)]-4-methyl-phenol	340	46.37

With respective inhibition percentages of 89.36% and 78.45%, against the pathogens *V. mali* and *R. solani*, Fr.2-2 showed the highest inhibition among the seven sub-fractions examined at a dosage of 1 mg. mL^{-1}. The pathogen *G. fructigenum* was more effectively inhibited by Fr.2-2, with an inhibitory rate of 54.23%.

The most efficient sub-fraction Fr.2-2 (**Table 5**) contained fifteen important chemical components that together accounted for 88.67% of the overall peak area. These chemicals were identified using GC-MS techniques. Among them, 2,2'-methylenebis [6-(1,1-dimethyleth yl)]-4-methyl-phenol was the most abundant (46.37%), followed by 17-Pentatriacontene (10.39%). The rest mainly included lipids, amines, alcohols and alkanes such as cetyl acetate (8.45%), caprolactam (0.43%), and heptadecane (6.08%).

4.6. Structure Identification and Antifungal Activity of Compound A

The highly active component Fr.2-2 was selected for further column chromatography, and a white solid was precipitated in Fr.2-2-2. The precipitated solid was determined to be pure substance of compound A by thin layer chromatography(TLC) test. Analytical TLC was employed on thin layer silica gel plates (GF254 Ocean, 0.25mm, 50100 mm). For elution, petroleum ether/ethyl acetate (1:1) was utilized. UV254/365 light was initially used to view the compound. Then, it was given further visualization using sprayers that included a sulfuric acid-ethanol (1:1) solution. Their plates were equally sprayed with the chemicals until they became totally damp. The plates were then heated for 2 minutes at a temperature of roughly 105 °C. The silicone plate showed a single spot. Thus, it was identified as a single compound.

The structure of compound A was confirmed through the use of ^{1}H-NMR and ^{13}C-NMR and related literature. Compound A: white amorphous powder, easily soluble in ethyl acetate, chloroform and other organic solvents. Molecular formula: $C_{23}H_{32}O_2$, ESI-MS m/z: 340. ^{1}H-NMR (500 MHz, Chloroform-d) δ (ppm): 7.17 (s, 1H), 4.94 (s, 1H), 4.86 (s, 1H), 4.15-4.08 (m, 1H), 2.94

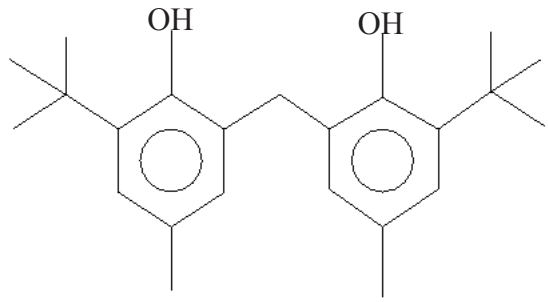

Figure 2. Structure of compound A. (Compound structure information was referenced from NMR data. Compound structure drawing through Integle ChemistryDraw [http://www.integle.com])

Table 6. The toxic regression equation of compound against Valsa mail.

Concentration (mg mL^{-1})	Inhibitory rate (%)	Regression equation	Coefficient (r^2)	IC_{50} (mg.mL^{-1})
0.01	7.94±1.61			
0.05	11.53±1.85			
0.10	20.17±1.42	y=89.389x+8.84	0.9963	0.254
0.50	56.17±1.02			
1.00	96.79±1.28			

(dd, J =17.7, 5.9 Hz, 1H), 2.24-2.14 (m, 2H), 2.04 (s, 1H), 1.99 (d, J=8.1 Hz, 2H), 1.89 (d, J=13.2 Hz, 1H), 1.46 (t, J=10.6 Hz, 1H), 1.29-1.16 (m, 2H), 0.96 (d, J=6.8 Hz, 3H), 0.80 (d, J=6.8 Hz, 3H). ^{13}C-NMR (126 MHz, Chloroform-d) δ (ppm) 171.84, 104.78, 66.66, 51.36, 46.49, 45.99, 36.91, 32.31, 27.34, 26.86, 21.34, 15.14, 49.59, 141.19, 127.61.

Compared with the GC-MS results, compound A was determined to be 2,2'-methylenebis [6-(1,1-Dimethylethyl)]-4-methyl-phenol. The structure is shown in **Figure 2**.

The inhibition rates were evaluated using analysis of regression to determine the toxicity of the various amounts of drug A that were used to suppress *V. mali* (**Table 6**). The concentration of compound A and the rate of inhibition of *V. mali* had a substantial and direct linear relationship. The application of compound A at a dosage of 1 mg. mL^{-1} increased the inhibition rate to 96.79%.

5. Discussion

There is increasing evidence for the beneficial effects of endophytic fungi on host plants, as they are thought to inhibit plant pathogens through the production of secondary metabolites that indirectly protect the healthy growth of the host (32). Endophytic fungi are still poorly studied despite these characteristics, especially in walnuts. We thus looked into the potential of the walnut endophyte LTL-G3 strain as a biological control agent and its capacity to suppress the growth of six phytopathogenic fungi.

In this study, we identified the biological taxonomy of the walnut endophyte LTL-G3 and found it to be *T. virens*. *Trichoderma* has been utilized successfully as a biological control agent to treat a variety of plant diseases, according to numerous publications and studies (33-36). According to Zhang *et al*. (30), who identified and assessed the antifungal activity of *T. longiformis* TL-6, multiple types of plant diseases could be significantly inhibited by the TL-6 strain's fermentation and crude extract. According to the research, the strain of TL-6 might be utilized for an agent of biological control. Trilongins BI-BIV isolated from the *Begonia venosa* endophytic fungus *Trichoderma* sp. P8BDA1F1 may be able to successfully block the activities of *G. fructigenum* and the proteasome targets, according to Grigoletto *et al*. (37), validating these substances as potential medicines and agrochemicals. The discovery of trilongins BI-BIV's antifungal activity against *C. gloeosporioides* and the proteasome target was made here for the first time, and it showed great promise for the investigation of a novel category of inhibiting agents. The mechanism by which the walnut endophytic fungus *T. virens* LTL-G3 combats plant diseases, however, is not well understood. The results of the current study shown that a variety of plant diseases were effectively stopped from growing by the LTL-G3 strain. In example, the apple tree canker pathogen, *V. mali*, was significantly inhibited by the LTL-G3. The isolation and characterisation of the secondary metabolites produced by the walnut endophyte fungus *T. virens*, which is antagonistic to the plant pathogenic fungus *V. mali*, are described in this article for the first time. Thus, the endophytic fungal strain LTL-G3 has great value in the control of plant diseases.

In the current work, we discovered that the LTL-G3 strain's ethyl acetate extract had a potent inhibitory impact on plant pathogens. There are also some similar studies in other plants. *Dillenia indica* L.endophytic fungi were assessed for their antibacterial capability by Kumar *et al*. (38), who also discovered the bioactive substances that were responsible for their antimicrobial activity. With an inhibition range of 15-29 mm, the *Fomitopsis meliae* ethyl acetate extract demonstrated the best inhibitory activity on a number of human pathogenic microorganisms. Three fungal endophytes from the leaves of *Olea europaea* L. were tested for their antibacterial capacity by Malhadas *et al*. (16), who also looked at how the host plant extract affected the antimicrobial activity. At 0.095 mg. mL^{-1}, the least inhibitory concentration, the ethyl acetate extracts exhibited the broadest breadth of antibacterial activity. The ability of the bacteria isolated from strawberry leaves and roots to indirectly promote plant growth was assessed in vitro and *in vivo* by Moural *et al*. (39). The bioactive substances were produced by liquid culture of these strains and extraction with ethyl acetate. Overall, our findings demonstrate that the endophytic fungus ethyl acetate extract has superior antimicrobial activity compared to other polar extracts and is a superior solvent for the isolation and purification of antimicrobial active ingredients.

In this investigation, silica gel column chromatography and GC-MS were used to separate and purify the ethyl acetate extracts. Key bioactive substance was obtained, and its structure and biocontrol effect were further

analyzed. At present, chromatographic separation and GC/HPLC analysis are common means applied in the extraction and separation of key compounds from endophytic fungi crude extracts and bioactive metabolites. Through silica gel column chromatography, the garlic endophyte *T. brevicompactum* 0248 strain yielded the potent antifungal chemical T2. Through spectral analysis, mass spectrometry data, and a comparison of earlier literature, it was determined that the substance was 4-acetoxy-12,13- epoxy-9-trichothecene (*trichodermin*) (40). The *Orychophragmus violaceus* endophyte *Aplosporella javeedii* was isolated from six novel polyketides using semi-preparative HPLC and silica gel column chromatography. Analysis of the NMR and MS data of the novel metabolites revealed their structures (41). So, the application of these separation and purification methods provides convenient technical support for the utilization of endophytic fungi and further development.

Overall, we investigated the antimicrobial activity of the walnut endophyte *Trichoderma* LTL-G3 against six common phytopathogenic fungi, evaluated the biocontrol effect of ethyl acetate extracts, and eluted the active components through silica gel column chromatography. After separation and purification, the key active compounds were identified through GC-MS and NMR as 2,2'-methylenebis [6-(1,1-Dimethylethyl)]-4-methyl-phenol. This is the first instance of the crucial antifungal chemical that we are aware of, which is produced by the walnut endophytic fungus *T. virens*, has been identified. It was indicated that the endophytic fungus from *Juglans regia* may be potentially useful for screening bio-control agents and finding new bioactive substances. At same time, currently reported compound is promising candidate for bio-pesticide development. Future research should look at other diseases as we only investigated antifungal activity against 6 plant pathogenic fungi. Only one compound was isolated in this study, and the detailed isolation of the active antifungal compounds and the multi-faceted activities of the highly active compounds deserve further study. Future research into the practical field efficacy of the biological agent 2,2'-methylenebis [6-(1,1- Dimethylethyl)]-4-methyl-phenol, which has the ability to control the plant disease, is warranted.

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Authors' contributions

Conceptualization: (YRH, JCH, MZZ), Methodology: (YRH, JCH), Formal analysis and investigation: (YRH, JCH), Writing - original draft preparation: (YRH); Writing - review and editing: (YRH, JCH, TYD, XRG), Funding acquisition: (MZZ).

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