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Full Length Article

Trichoderma viride Controls *Macrophomina phaseolina* through its DNA disintegration and Production of Antifungal Compounds

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Abstract

Macrophomina phaseolina is a highly destructive pathogen of more than 500 plant species. It is difficult to eradicate it through chemical means as no patented fungicide is available against this pathogen. Biological control is the possible alternative method for its suitable management. The present study was carried out to evaluate the biocontrol potential of five *Trichoderma* spp. against *M. phaseolina* and the possible mechanisms of action. Identifications of all the *Trichoderma* spp. *viz. T. hamatum*, *T. harzianum*, *T. koningii*, *T. longipile* and *T. viride* were confirmed on molecular basis by using two universal primer pairs namely ITS and EF1. Their biocontrol potential was evaluated in dual culture plate method where *T. viride* showed the highest inhibitory efficacy (63%) against *M. phaseolina*. *T. koningii*, *T. hamatum* and *T. longipile* showed akin effects by arresting growth of the pathogen by 46–47% followed by *T. harzianum* (28%). To find out the mechanisms of action, secondary extrolites of the best biocontrol fungus *T. viride* were tested against the pathogenic genomic DNA where all the concentrations partially degraded DNA bands after 24 h of incubation and a complete DNA band disappearance was noted after 48 h incubation. In addition, *T. viride* culture filtrates were partitioned with chloroform and ethyl acetate and subjected to GC-MS analysis for identification of potential antifungal constituents. The most abundant identified volatile compounds in the two organic solvent fractions were 9,12-octadecadienoic acid (Z,Z)- (44.54%), *n*-hexadecanoic acid (24.02%), hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (14.25%), 9-tricosene, (Z)- (10.43%) and [1,1'-bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester (10.43%). To conclude, *T. viride* was the best biocontrol agent against *M. phaseolina* and acts against the pathogen by DNA disintegration and production of antifungal secondary metabolites. © 2021 Friends Science Publishers

Keywords: Biocontrol; DNA cleavage; GC-MS analysis; *Macrophomina phaseolina;* Secondary extrolites; *Trichoderma viride*

Introduction

The fungus *Macrophomina phaseolina* is a noxious pathogen that infects more than 500 host plant species belonging to about 100 families globally (Schroeder *et al.* 2019). The pathogen has a wide geographical distribution, and is commonly found in Europe, Africa, Asia, South and North America (Zimudzi *et al.* 2017). It causes stem rot, charcoal rot and root rot in major crops including cotton, sorghum, maize, sunflower, soybean, sesame, jute, green gram and common bean with severe yield losses (Khan *et al.* 2017; Degani *et al.* 2020). The pathogen rapidly propagates under high temperature and drought conditions (Muchero *et al.* 2011). Due to its persistent nature, it can remain viable in the form of microsclerotia as resistant structures in soil or infected plant debris for up to 3 years (Short *et al.* 1980; Vasebi *et al.* 2013). At initial stage of infection, its hyphae invade the plant cortical tissues which turn into grey-black sclerotia in infected areas (Chowdhury *et al.* 2014). As the disease progresses, the infected plant turns yellow and ultimately dies (Farnaz *et al.* 2018). As the pathogen is soil-borne and has a wide host range, it is very difficult to manage it through traditional methods that have been largely based on the use of agronomic and cultural practices (Khalili *et al.* 2016). Chemical fungicides used to control fungal diseases are often applied in large quantities with repeated use in agricultural production, which pose drastic effects on the consumers and environment by toxins production (Chamorro *et al.* 2016). Therefore, high risk fungicides have increased the awareness to eliminate the use of synthetic products and encouraged the farmers to increase dependency on biocontrol agents for disease control (Zhang *et al.* 2018).

Biological control is a potential alternate to the synthetic chemical fungicides. It is considered to be an eco-friendly and low-cost strategy for the management of soil-borne pathogens (Muller-Scharer *et al.* 2020). To date, a number of registered biocontrol agents are commercially available belonging to *Trichoderma*, *Candida*, *Gliocladium*, *Coniothyrium*, *Streptomyces*, *Bacillus*, *Pseudomonas* and *Agrobacterium* genera (Bayoumy *et al.* 2017; Deng *et al.*

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2018; Zhao *et al.* 2018). Among them, the genus *Trichoderma* has a wide biotechnological interest and hence comprising on mycoparasitic species, particularly *T. pseudokoningii, T. hamatum, T. harzianum, T. koningii* and *T. viride* that have received a great attention in reducing the populations of soil-borne pathogens including *Sclerotium rolfsii, Rhizoctonia solani, Fusarium oxysporum* and *M. phaseolina* (Bastakoti *et al.* 2017). The antagonistic mechanism of *Trichoderma* species is a combination of diverse mechanisms including direct confrontation with fungal pathogens, competition for nutrients and the production of cell-wall degrading enzymes (Anjum *et al.* 2019). So far, *Trichoderma* spp. are the most studied biocontrol agents and are commercially marketed as biofertilizers, biopesticides and for soil amendments (Kumar *et al.* 2017). These serve as important antibiotic, fast growing, strong spore producer, secondary opportunistic invaders and a source of chitinases, glucanases and cellulases cell wall degrading enzymes (Hewavitharana *et al.* 2018). Secondary metabolites produced by *Trichoderma* spp. are antifungal strain dependent substances belonging to different classes of volatile compounds (Pascale *et al.* 2017). The direct application of anti-microbial compounds of antagonistic fungi instead of living organisms is more advantageous in agriculture and industry because of their inability to reproduce and spread (Soesanto *et al.* 2019). Therefore, the objective of the present study was to assess the comparative antagonistic effect of five *Trichoderma* species against *M. phaseolina,* and to investigate the antagonism mechanism as well as to identify the secondary extrolites produced by *T. viride.*

Materials and Methods

Molecular characterization of selected isolates

Five *Trichoderma* species *viz. T. hamatum*, *T. harzianum*, *T. koningii*, *T. longipile* and *T. viride* were procured from the First Culture Bank of Pakistan (FCBP). Genomic DNA of all the selected fungi were extracted by using CTAB method (Doyle and Doyle 1990). For molecular identification, the contiguous Internal Transcribed Spacer (ITS) and elongation factor 1-alpha (EF1) regions were amplified with primer sets given in Table 1. ITS and EF1 amplicons were loaded to electrophoresis on 1% agarose gel, purified and sequenced at the Molecular and Cellular Imaging Center (MCIC) of the Ohio Agricultural Research and Development Center, Wooster, OH, USA on the Illumina MiSeq platform. The obtained sequences were subjected to BLAST analysis and deposited in NCBI.

In vitro antagonistic activity of *Trichoderma* species

Dual culture experiments were conducted to determine the *in vitro* biocontrol potential of *Trichoderma* spp. against *M. phaseolina* where both the species were inoculated on peripheries of the same 90-mm diameter malt extract agar plates. Mycelial agar plugs (5 mm in diameter) of each filamentous *Trichoderma* culture were placed at opposite end of the tested pathogen (5 mm in diameter) for the establishment of dual culture and a control was also prepared by placing *M. phaseolina* plugs only. Each treatment contained six replicates and the plates were incubated at 28°C for 5 days. The experiment was carried out in a completely randomized design. The antagonistic potential of each *Trichoderma* species was assessed by measuring the pathogen radial growth in the direction of *Trichoderma* isolates and calculated by using the following formula (Rini and Sulochana 2008):

$$\text{Inhibition (\%)} = \frac{\text{Colony diameter in control} - \text{Colony diameter in dual culture}}{\text{Colony diameter in control}} \times 100$$

DNA degradation study

T. viride secondary metabolites were used to explore their potential antagonistic mechanism against *M. phaseolina* in a DNA degradation experiment. For the preparation of secondary metabolites, mycelial agar plugs (5 mm in diameter) of *T. viride* were inoculated in malt extract broth flasks which were agitated for two weeks on an orbital shaker (150 rpm) at 30°C. Subsequently, the broth was passed through two layers of filter paper and the resultant liquid was kept in an electric oven at 40°C to concentrate it for the preparation of higher concentrations *viz.* 100, 200, 300, 400 and 500%. Next, 5 μL of each concentration were mixed in equal volume of *M. phaseolina* DNA in separate vials and incubated at 37°C for 24 and 48 h. A control was also set for comparison. Thereafter, all the treatments were loaded on 1% agarose gel and run for 45 min at 100 volts to examine the extent of DNA degradation (Katrahalli *et al.* 2019).

GC-MS analysis

Mycelial discs were taken from the margins of an actively growing culture of *T. viride* and inoculated in 100 mL autoclaved malt extract broth in Erlenmeyer flasks (250 mL) under aseptic conditions. Inoculated flasks were agitated on an orbital shaker (150 rpm) at 30°C for 15 days. The broth was filtered through Whatman filter paper to remove mycelia. The broth was then partitioned with chloroform followed by ethyl acetate in a glass separating funnel. The obtained fractions were subjected to GC-MS analysis for identification of compounds.

Analysis was done on GC-2010 plus, Shimadzu attached with DB-5MS. The capillary column was 0.25 μm × 0.25 mm × 30 m with temperature capacity of 350°C. A mass spectral library, Version 2.70, Shimadzu Co., was used. Helium as a carrier gas was used in pure form in accordance with split-less injection system (1.0 μL volume), developed for operating the chromatograph with 1 cm^3 min^{-1} spill count at 250°C. The total sample running time was set for 11 min.

Statistical analysis

The dual culture experiment was conducted in a completely randomized design with six replicates. For each treatment, standard errors of the means were calculated. Data regarding percentage inhibition in growth of *M. phaseolina* due to various *Trichoderma* spp. in dual culture plates were analyzed statistically by applying one-way ANOVA followed by LSD test at P≤0.05, using computer software Statistix 8.1.

Results

Molecular identification of antagonistic fungi

In the present study, molecular characterization of *T. hamatum*, *T. harzianum*, *T. koningii*, *T. longipile* and *T. viride* rDNA was carried out with ITS and EF1 universally accepted primers (Fig. 1). The resultant PCR product sequences were subjected to BLAST analysis where all the isolated strains showed 99 to 100% similarities with the already submitted sequences and deposited to Genebank for respective accession numbers (Table 2).

Interactions of *Trichoderma* spp. with *M. phaseolina*

Among the five tested *Trichoderma* spp., *T. viride* showed the best antagonistic potential against *M. phaseolina* by arresting its growth up to 64% over control. Rest of the *Trichoderma* species were less effective than *T. viride* where about akin reduction (46–47%) in the pathogen growth was observed due to *T. koningii*, *T. hamatum* and *T. longipile*. *T. harzianum* showed the least suppressive ability and reduced growth of the pathogen only by 28% (Fig. 2–3).

DNA degradation study

Interaction of *M. phaseolina* ribosomal DNA with secondary metabolites of *T. viride* is illustrated in Fig. 4. All the concentrations except 500% partially cleaved the pathogenic fungus DNA and form smears after 24 h of incubation whereas the 500% concentration was found to be more effective than lower concentrations and completely disintegrated DNA of the fungal pathogen. After 48 h of incubation, it was noted that all the concentrations completely damaged *M. phaseolina* DNA.

GC-MS analysis

The GC-MS chromatogram of *T. viride* chloroform and ethyl acetate fractions showed the presence of 4 and 9 peaks of volatile compounds, respectively (Fig. 5). The compounds in chloroform fraction with their details of percent peak areas and retention time are reported in Table 3. The compound present in the highest concentration was

Table 1: List of oligonucleotide primers used for the characterization of *T. viride* at molecular level

Primer name	5´ to 3´ sequence	Annealing temperature
ITS 1 Forward	TCCGTAGGTGAACCTGCGG	60°C
ITS 4 Reverse	TCCTCCGCTTATTGATATGC	
EF1-728 Forward	CATCGAGAAGTTCGAGAAGG	60°C
EF1-986 Reverse	TACTTGAAGGAACCCTTACC	

Table 2: *Trichoderma* spp. with their respective accession numbers and products amplicon size

Penicillium spp.	ITS		EF-1	
	Amplicon size (bp)	Accession numbers	Amplicon size (bp)	Accession numbers
T. hamatum	~ 535	MT573507	~ 354	MN736405
T. harzianum	~ 556	MN721820	~ 333	MN736406
T. koningii	~ 538	MT573514	~ 363	MN736407
T. longipile	~ 547	MT573512	~ 435	MN736408
T. viride	~ 554	MT573511	~ 368	MN736410

Table 3: Compounds identified from chloroform fraction of culture filtrates of *T. viride* through GC-MS analysis

Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak area (%)
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	7.067	24.02
9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	7.809	44.54
[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-,methyl ester	$C_{21}H_{38}O_2$	322	9.197	10.43
Octadecanoic acid, 9,10-dihydroxy-, methyl ester	$C_{19}H_{38}O_4$	330	9.415	20.89

Table 4: Compounds identified from ethyl acetate fraction of culture filtrate of *T. viride* through GC-MS analysis

Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak area (%)
Benzene, nitro-	$C_6H_5NO_2$	123	2.857	15.05
Naphthalene	$C_{10}H_8$	128	3.395	4.99
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	7.073	6.55
1-Nonadecene	$C_{19}H_{38}$	266	7.203	5.70
9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	7.796	40.09
9-Tricosene, (Z)-	$C_{23}H_{46}$	322	8.015	10.43
Cis-9-Hexadecenal	$C_{16}H_{30}O$	238	8.621	2.84
Hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	330	9.628	14.25
3-*n*-Butylthiophene-1,1-dioxide	$C_8H_{12}O_2S$	172	10.544	4.20

9,12-octadecadienoic acid (Z,Z)- (44.54%). The moderately abundant compounds in this fraction were *n*-hexadecanoic acid (24.02%), octadecanoic acid, 9,10-dihydroxy-, methyl ester (20.89%) and [1,1'-bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester (10.43%).

Details of ethyl acetate fraction spectrum profile are given in Table 4. Ethyl acetate fraction showed the highest abundunce of 9,12-octadecadienoic acid (Z,Z)- (40.09%) whereas, the compounds present in moderate concentrations were benzene, nitro- (15.05%), hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (14.25%) and 9-tricosene, (Z)- (10.43%). On the other hand, *n*-hexadecanoic acid (6.55%), 1-nonadecene (5.70%), naphthalene (4.99%), 3-*n*-butylthiophene-1,1-dioxide (4.20%) and *cis*-9-hexadecenal (2.84%) were ranked as less abundant compounds.

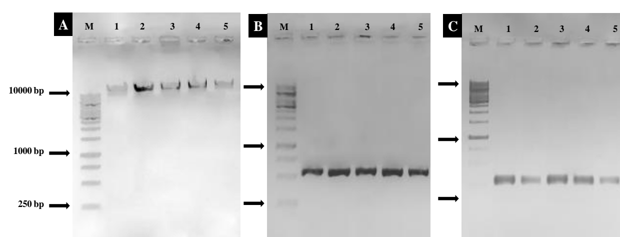

Fig. 1: Molecular characterization of *Trichoderma* species
A)- Genomic DNA of *Trichoderma* species. B)- ITS1/ITS4 amplified PCR product of *Trichoderma* species. C)- $EF1_f/EF1_r$ amplified PCR product of *Trichoderma* species (M): 1 kb DNA standard marker, (1): *T. hamatum,* (2): *T. harzianum,* (3): *T. koningii,* (4): *T. longipile,* (5): *T. viride*

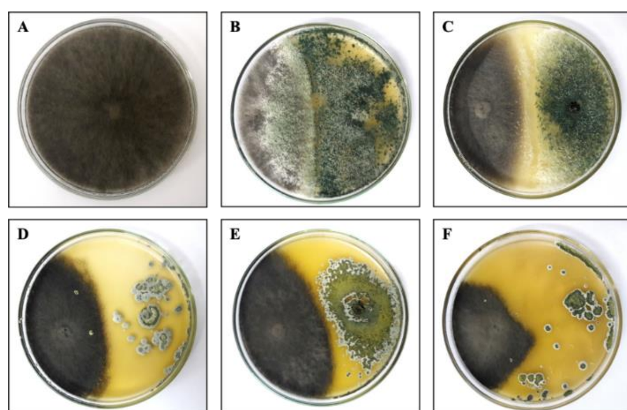

Fig. 2: Interaction of *M. phaseolina* with *Trichoderma* species
A)- Pure culture of *M. phaseolina* (MP); B)- MP co-culture with *T. viride;* C)- MP co-culture with *T. hamatum;* D)- MP co-culture with *T. longipile;* E)- MP co-culture with *T. harzianum;* and F)- MP co-culture with *T. koningii*

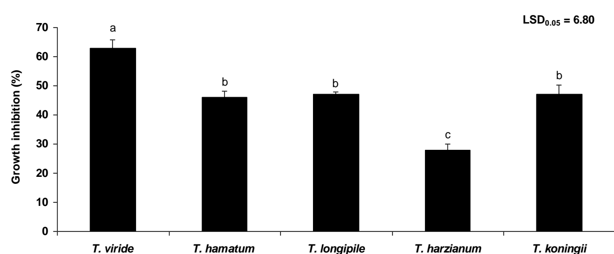

Fig. 3: Inhibition in radial growth of *M. phaseolina* due to interaction with different *Trichoderma* species Vertical bars show standard errors of means of six replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD test

Discussion

Accurate identification of *Trichoderma* spp. is necessary to study their detailed mechanism of antagonism and for the preparation of effective management strategies against the soil-borne fungal pathogens (Mokhtari *et al.* 2017). Genomic DNA sequence and amplification through universal set of primer pairs is the most authentic tool for identification on molecular basis (Sawant *et al.* 2019). In the present investigation, *Trichoderma* spp., especially *T. viride,*

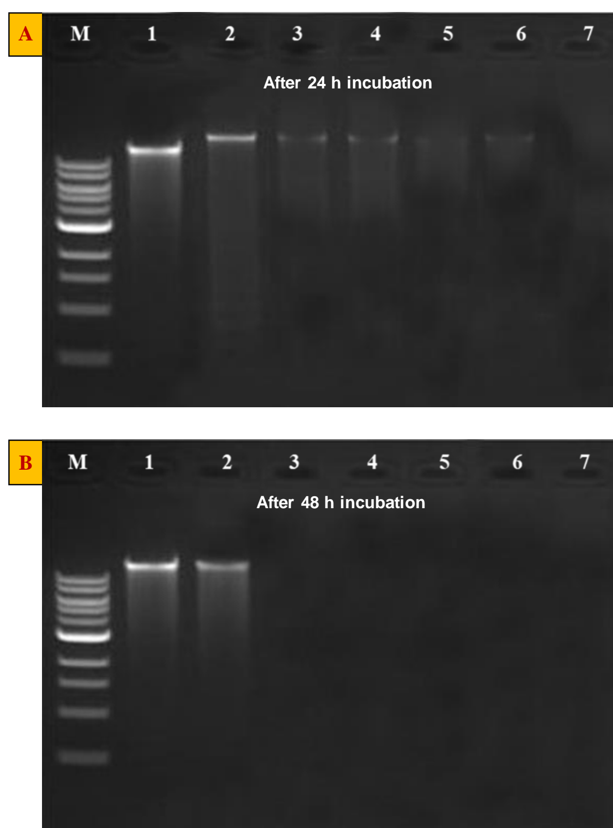

Fig. 4: Gel electrophoresis showing effect of different concentrations of secondary metabolites of *T. viride* on cleavage of *M. phaseolina* DNA samples incubated for 24 h (A) and 48 h (B)
(M): 1 kb DNA standard marker, (1): Genomic DNA of *M. phaseolina,* (2): Negative control (genomic DNA of *M. phaseolina* + malt extract broth), (3): original or 100% metabolites, (4): 200% metabolites, (5): 300% metabolites, (6): 400% metabolites, and (7): 500% metabolites. Arrows indicate the presence or absence of DNA

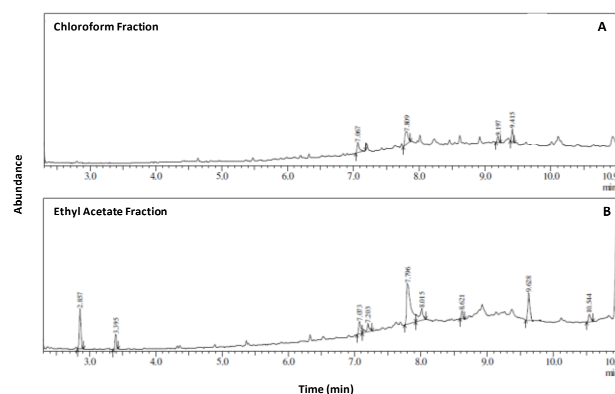

Fig. 5: GC-MS chromatograms of chloroform and ethyl acetate fractions of culture filtrate of *T. viride*

remarkably inhibited the growth of *M. phaseolina*. Previously, Gaur (2016) worked on *T. atroviride*, *T. viride* and *T. harzianum* under *in vitro* conditions to assess their antagonistic potential towards the *M. phaseolina* by direct co-culturing on Czapek's dox medium where all the isolates showed promising inhibitory effects on growth of the

Table 5: Potential antimicrobial constituents in chloroform and ethyl acetate fraction of *T. viride*

Names of compounds	Property	Reference
9,12-Octadecadienoic acid (Z,Z)-	Antifugal, antibacterial, nematicidal, anti-coronary and anti-inflammatory	Tahir *et al.* (2019); Arora and Kumar (2018); Prajapati *et al.* (2017)
n-Hexadecanoic acid	Antifungal, nematicide, pesticide and antioxidant	Pavithra *et al.* (2018); Vats and Gupta (2017); Elaiyaraja and Chandramohan (2016); Pohl *et al.* (2011)
Hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester	Antibacterial, anti-inflammatory and antioxidant	Al-Marzoqi *et al.* (2015); Pandey *et al.* (2014)
9-Tricosene, (Z)-	Pesticidal	Verma *et al.* (2015)
[1,1'-Bicyclopropyl]-2-octanoic acid,2'-hexyl-,methyl ester	Pesticide, anticancer and antidiabetic	Banakar and Jayaraj (2017)
1-Nonadecene	Antifungal and Anticancer	Premathilaka and Silva (2016)
Naphthalene	Antioxidant and antibacterial	Shareef *et al.* (2016)
cis-9-Hexadecenal	Antimicrobial	Juliet *et al.* (2018); Arora and Meena (2017)

pathogen. Similarly, a clear inhibition zone formation was observed against *M. phaseolina* by *T. viride* in dual culture assay performed by Piperkova *et al.* (2016). Likewise, Mishra and Dantre (2017) treated soybean seeds with secondary metabolite formulations of *T. viride* to manage the charcoal rot disease caused by *M. phaseolina* under field conditions. *Trichoderma* spp. are known to have a number of mechanisms through which these control the growth of pathogenic fungi. *Trichoderma* spp. may control the growth of pathogenic fungi through mycoparasitism (Mukhopadhyay and Kumar 2020). A complex system of various extracellular enzymes such as chitinase (Hoell *et al.* 2005), proteolytic enzymes (Pozo *et al.* 2004) and β-1,3-glucanolytic system (Kubicek *et al.* 2001), results in lysis of cell wall of the fungal pathogens (Verma *et al.* 2007). In addition, *Trichoderma* spp. also inhibit hyphal growth of the fungal pathogens through antibiosis by producing antimicrobial compounds in the culture (Gajera *et al.* 2020).

Considering the significant antifungal activity of *T. viride* in dual culture assay, its extrolites were selected for evaluation of their effect on *in vitro* degradation of DNA of the pathogenic fungus. In this study, secondary extrolites showed great potential in degrading the genomic DNA after 48 h incubation. Earlier, this mechanism of action of *T. viride* against fungal pathogens has not been reported. Instead, generally this methodology was adopted to assess the antibacterial mechanism of action of nanoparticles (Dong *et al.* 2017; Dashamiri *et al.* 2018; Jadhav *et al.* 2018). A variety of lytic enzymes and antifungal compounds are produced by *T. viride,* which might be responsible of DNA degradation in the present study (Calistru *et al.* 1997; Parizi *et al.* 2012).

The GC-MS analysis showed many compounds from chloroform and ethyl acetate fractions that were previously reported to have antimicrobial properties. Among the major identified constituents, 9,12-octadecadienoic acid (Z,Z)- was previously isolated from the methanolic extract of *Cenchrus biflorus* with potent antibacterial, nematicidal and fungicidal activities as given in Table 5 (Arora and Kumar 2018; Tahir *et al.* 2019). Similarly, Pavithra *et al.* (2018) worked on bioactivity of *n*-hexadecanoic acid (also known as palmitic acid) and reported that it possesses strong pesticidal and antioxidant properties. This compound is known to exhibit antifungal activity against a number of fungal species including *Aspergillus terreus, A. niger, A. nidulans* (*Emericella nidulans*) *Alernaria solani, Fusarium oxysporum* and *Cucumerinum lagenarium* (Pohl *et al.* 2011). Likewise, hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester was isolated from the methanolic extract of *Limonia acidissima* and tested against pathogenic bacterial strains. The compound showed excellent antibacterial potential against *S. aureus*, *S. epidermidis* and *B. subtilis* (Pandey *et al.* 2014). Verma *et al.* (2015) reported pesticidal potential of 9-tricosene, (Z)- and [1,1'-bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester. Similarly, 1-nonadecene; naphthalene and *cis*-9-hexadecenal are potent antifungal and antioxidant compounds (Premathilaka and Silva 2016; Shareef *et al.* 2016; Juliet *et al.* 2018).

Conclusion

There was antagonistic potential of *T. viride* against a highly problematic fungal pathogen *M. phaseolina*. *T. viride* possibly controlled the pathogen by degrading its DNA through its secondary metabolites released in the surroundings. Moreover, the GC-MS analysis of secondary metabolites showed the presence of compounds such as 9,12-octadecadienoic acid (Z,Z)- and *n*-hexadecanoic acid, which are known for their antifungal effects.

Author Contributions

IHK did experimental work and wrote the paper. AJ supervised the work and contributed in writing and finalizing the paper. DA provided GC-MS facility.

Conflict of Interest

There is no conflict of interest among the authors and institutions where the work has been done

Data Availability Declaration

All data reported in this article are available with the corresponding authors and can be produced on demand

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