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Enhancing the plant growth promoting activity of *Trichoderma viride* biopesticide using substrate supplementation and co-cultivation

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Substrate supplementation and co-cultivation approaches were used to enhance activity of biofungicide *Trichoderma viride*. Biopesticides were produced using corn cobs (T), corn cobs + 1% keratin (K), corn cobs + 5% lignin (L), and corn cobs + 1% keratin + 5% lignin (K+L). Also, biopesticides were produced through co-culturing with *Bacillus subtilis MTCC8142*, by sequential inoculation (B+T) and simultaneous inoculation (T3+B2) (B2+T3). The highest spore count was produced by (T3+B2) of 6.25×10^8 spores/g of substrate, followed by (T) of 6.20×10^8 spores/g of substrate. GC-MS characterization of ethyl acetate extract of biopesticides revealed different metabolites produced under different conditions. The zone of inhibition of extract was greatest for (T) of 21 mm, followed by (K) of 20.7 mm and for (B2+T3) of 18 mm at 10 mg/mL concentration. (K) and (B2+T3) did not affect antifungal properties; however, other substrate-supplemented and co-cultivated biopesticides decreased them. Competition between two microbes prevented (T3+B2) from producing antimicrobial metabolites; hence, no zone of inhibition was observed. The biopesticides (K), (L) and (B2+T3) significantly enhanced growth of chili plants (*Capsicum frutescens* L.), outperforming other biopesticides. The production of phthalates, also known as phytotoxins, by B+T resulted in a decrease in plant growth. Adding 1% keratin to corn cobs (K) and (B2+T3) sequential inoculation are best ways to boost plant growth promoting activity of *T. viride* without affecting its biocontrol activity.

Keywords: Biocontrol, Biofungicide, Biopesticides, *Capsicum frutescens*, Corn cobs, Plant-growth promotion, Solid-state fermentation

Organic farming in India and globally is becoming more prominent as consumer awareness of the importance of adopting healthy dietary habits rises. A growing number of farmers are opting for biopesticides over chemical pesticides, the latter of which have been linked to environmental and health hazards. *Trichoderma* spp. is widely recognised and utilised in the commercial sector as biopesticides and plant growth promoters. Narwade *et al*.[1] have recently published a simple and robust method to produce *Trichoderma viride* spore-biopesticide using corn cobs in earthen vessel. The farmers can easily adopt this method for captive consumption on their farms. The present paper reports further improvement in the productivity of the biopesticide by supplementation of substrate, and co-culturing with other microbes. One of the factors contributing to the widespread utilisation of *T. viride* is its broad spectrum of crop application, which can be ascribed to the inclusion of numerous metabolites, growth hormones, and enzymes[2-4]. Pathogen growth is reportedly inhibited through the secretion of cell wall-degrading enzymes and antibiotics[5]. Moreover, antibiosis-related secondary metabolites demonstrate direct antagonistic activity against phytopathogens[6]. Unexpected versatility is displayed by the emissions of specific metabolites towards a wide range of substrates[7,8]. Furthermore, their production is dependent on the presence of other microbes[9]. Subsequently, their activity can be augmented via different approaches such as co-cultivation and substrate supplementation through solid-state fermentation (SSF).

Various agricultural wastes are commonly utilized as substrates in SSF[10-13]. *Trichoderma* spores are predominantly produced from cellulose-rich wastes due to their high biodegradability[1,12]. Conversely, lignin and keratin-rich recalcitrant wastes are difficult to decompose[14-16]. These are good carbon and nitrogen sources for fungal growth. Additionally, humic substances and amino acids derived from lignin and keratin promote plant growth[17-19]. Although studies have demonstrated that *Trichoderma* can break down lignin and keratin-containing materials,

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their recalcitrant nature makes them challenging to employ as the only substrate[20,21]. Therefore, supplementing them to other cellulosic waste, such as corn cobs could be helpful approach to enhance their activity. Another method of activity enhancement could be co-cultivation of *Trichoderma* with other microorganisms. *Trichoderma* and bacteria co-culture produces results comparable to those of chemical pesticides[22]. Co-cultivation improves plant growth, disease resistance, and soil quality by combining the complimentary properties of *Trichoderma* and *Bacillus*. The metabolites produced through co-cultivation exhibit synergistic effects on both plant growth and disease control[23]. Previous studies have shown that simultaneous inoculation resulted in interference between fast-growing and slow-growing microorganisms[24,25]. Karuppiah *et al*.[24] found that inoculating *T. asperellum* and *B. amyloliquefaciens* sequentially proved successful than co-inoculation. Although co-cultures are less prevalent in SSF than in submerged fermentation, their benefits in terms of solid substrate colonization, permeation, and degradation may be more substantial[26]. Thus, sequential inoculation of *Trichoderma* and *Bacillus* on agricultural waste could help to boost their activity.

The present study was undertaken for activity enhancement of *Trichoderma viride* spore-based biopesticide. Substrate supplementation and co-cultivation methods were explored in a polyethylene autoclavable disposable bags (PADB). The study used corn cobs as a substrate and supplemented with lignin and keratin. Co-cultures have been produced by utilizing simultaneous and sequential inoculation methods. One of the main aspects of the research was the production of *T. viride* spores using different approaches. We carried out further extraction, characterization, and evaluation of the antifungal activity of metabolites against *R. solani* MTCC 4633. Finally, studies were conducted to assess the impact of biopesticide application on the growth of chilli plants.

Materials and Methods

Microorganisms

Trichoderma viride, a biopesticide, was given by the POABS Group Kerala, India. *Rhizoctonia solani* MTCC 4633, a phytopathogen, was provided by Ramnarain Ruia, Mumbai, India. *Bacillus subtilis* MTCC 8142 was procured from MTCC India showing antifungal activity against several plant pathogens and produces humic acid to increase crop growth[23,24]. Fungal strains were cultured on PDA plates at 30°C for 5 days in an incubator. Spores were collected in a sterile solution (0.01% Tween 80). *Bacillus subtilis* MTCC 8142 was grown on nutrient agar plates at 30°C for 24 h. All cultures were stored as 50% glycerol stocks at –80°C for future use.

Substrate

Corn cobs were obtained from the local market and processed as per method described by Narwade *et al.*[1]. Keratin and lignin were prepared as shown in Fig. 1 and used for corn cobs supplementation.

Preparation of keratin and lignin for substrate supplementation

Hair alkaline hydrolysate was given by the Department of Chemical Engineering, ICT Mumbai. It was acidified with hydrochloric acid (pH 3). Keratin in the form of yellowish supernatant was lyophilized with a lyophilizer from Labconco[25]. Alkaline supernatant of sorghum straw was obtained from ICT-DBT centre for lignin extraction. It was acidified with concentrated sulfuric acid until the pH 3 and filtered through a muslin cloth[26]. Lignin was precipitated, washed 2-3 times, and dried at 70°C in an oven. Keratin and lignin powder was used for corn cobs supplementation. In the previous research, keratin and lignin were found to be suitable substrate supplements for microbial growth[13,15,27]. Consequently, those were used in present study for corn cobs supplementation.

Solid-state fermentation

SSF was carried out in PADB, purchased from HiMedia HiDispo Bag-7. The substrates were prepared with 1% keratin, 5% lignin, and 1% keratin + 5% lignin supplementations with corn cobs. *T. viride* spore-based biopesticides produced using corn cobs (T), corn cobs + 1% keratin (K), corn cobs

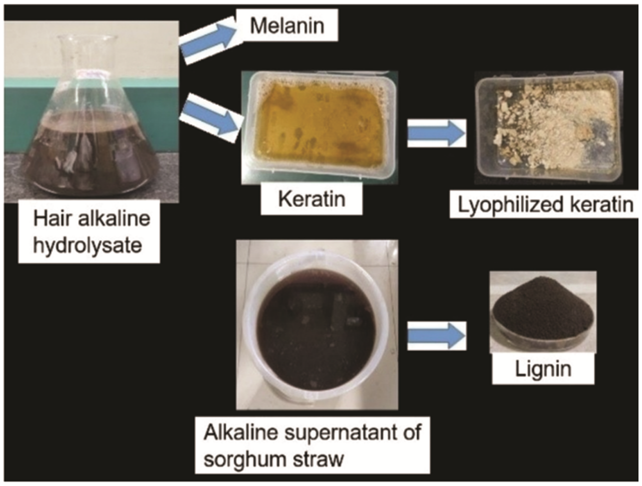

Fig. 1 — Preparation of keratin and lignin samples.

+ 5% lignin (L), and corn cobs + 1% keratin + 5% lignin (K+L). Another approach used was simultaneous and sequential inoculation with *B. subtilis* MTCC 8142. *T. viride* and *B. subtilis* MTCC 8142 monoculture and co-cultures inoculated on processed corn cobs. These were inoculated with 1×10^7 cells/mL of *B. subtilis* MTCC 8142 and 1×10^7 spores/mL of *T. viride* in a laminar airflow cabinet. *T. viride* monoculture (T), as well as co-cultures with *B. subtilis* MTCC 8142 after simultaneous inoculation (B+T) and sequential inoculation (T3+B2), (B2+T3) were produced. Those were incubated at 30°C, and spores were counted after five days of incubation.

Extraction and characterization of metabolites

Biopesticides were extracted with ethyl acetate as described by Narwade *et al.*[1]. GC-MS was carried out using a Shimadzu QP2020 instrument coupled to a capillary column (SH-Rxi-5Sil MS, 30 m × 0.25 mm × 0.25 m).

Antifungal activity

The extracts dissolved in methanol shown highest zone of inhibition against *R. solani* MTCC 4633 and retained their effect for the longest duration[1]. Hence, the extracts were dissolved in methanol and used for agar diffusion test. The diameter of the inhibition zone was measured after 3 days of incubation.

Soil application of biopesticides for chili plant growth

For plant growth study, keratin and lignin-supplemented biopesticides and co-cultivated biopesticides were produced as discussed previously. Bavistin fungicide and *T. viride* from Banana Research Station, Kerala Agricultural University were used as a control. Pot experiments were performed at Bejo Sheetal Seeds Pvt. Ltd. Jalna, India. The chili plant (*Capsicum frutescens* L.) growth was assessed by applying formulations to the potting soil monthly before and weekly after flowering.

Analytical methods

A hemocytometer was used to count the number of *T. viride* spores. *B. subtilis* MTCC 8142 cells were counted with the grid method. Spore-based biopesticide production in PADB was carried out in triplicates. For analysis of multiple treatment means, one- and two-way analysis of variance (ANOVA) was used, followed by Tukey multiple comparison tests (using GraphPad Prism Software; Inc., San Diego, CA, USA). The origin Pro 8 was used to plot the graphs. The presented results were the means of triplicate values with standard error.

Results and Discussion

Spore production

Modifying culture conditions is regarded as a moderate strategy[31]. Therefore, different approaches were used to enhance sporulation, biocontrol and plant growth promoting properties.

First method involved supplementing corn cobs with lignin and keratin. Biopesticides (T), (K), (L) and (K+L) were produced using corn cobs by supplementing with 5% lignin and 1% keratin (Fig. 1). Highest spore count of 6.2×10^8 spores/g of substrate obtained with (T), followed by 5.97×10^8 spores/g of substrate using (K), and 5.95×10^8 spores/g of substrate with (L).The lowest spore count of 5.2×10^8 spores/g of substrate was obtained with (K+L) due to complex polymers such as lignin and keratin as shown in Fig. 2A. Previous studies shown that substrate can be supplemented with lignin and keratin for microbial growth[18,20,28]. The essential elements and amino acids in the keratin make it an ideal medium for fungal growth[19,29]. Lignin is a good source of organic carbon found in nature[30]. In case of (K+L), simultaneous supplementation of 1% keratin and 5% lignin reduced spore production because those were difficult to decompose and could had produced toxic byproducts.

In the second method, co-cultures were produced by simultaneously and sequentially inoculating *T. viride* and *B. subtilis* MTCC 8142 (Fig. 1). The

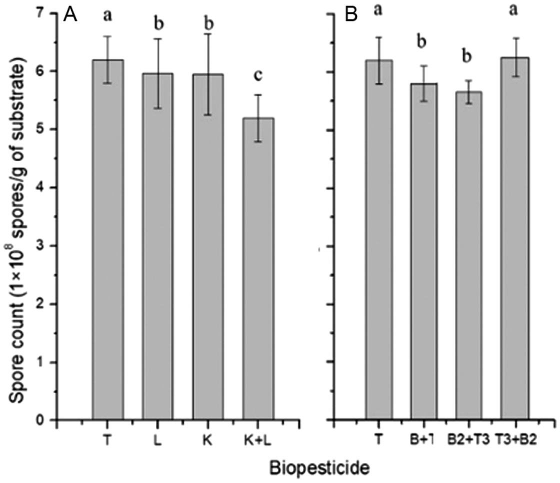

Fig. 2 — Spore count of *Trichoderma viride* biopesticides produced by (A) substrate supplementation; and (B) co-cultivation with *B. subtilis* MTCC 8142 using corn cobs. Results are displayed as means ± standard error. Spore count means followed by different letters differ significantly according to Tukey's multiple comparisons test ($P \leq 0.05$)]

biopesticides (B2+T3) and (T3+B2) were produced through sequential inoculation, whereas (B+T) was produced via simultaneous inoculation. Highest spore count of 6.25 × 10^8 spores/g of substrate obtained with (T3+B2), followed by 6.20 × 10^8 spores/g of substrate with (T), and 5.8 × 10^8 spores/g of substrate with (B+T). Lowest spore count of 5.65 × 10^8 spores/g of substrate was obtained with (B2+T3) as presented in Fig. 2B. There was no complete inhibition of either microorganism in (B+T). In case of (B2+T3), *B. subtilis* MTCC 8142 could have overgrown the *T. viride* and competed with it. Another similar study shown overgrowth of *B. velezensis* than *T. harzianum*[31]. Karuppiah *et al.*[24] observed competition between *T. asperellum* and *B. amyloliquefaciens* under co-cultivation. They found that inoculation of *Bacillus* in the 48 h grown *Trichoderma* pre-culture promoted the growth of both microbes. However, they had not examined the possibility of reverse sequential inoculation. Similar studies of simultaneous and sequential inoculation were carried out to recognize one that provides the best interaction among the microorganisms[24,32]. In the present study, *T. viride* spores were produced by both sequential inoculation methods; however, the highest spore count of (T3+B2) was due to colonization's "first-come, first-served" nature[33].

With substrate supplementation approach, recalcitrant nature of keratin and lignin reduced spore production. Sequential co-cultivation of (T3+B2) yielded the highest spore count due to the availability of nutrients for *T. viride* growth. Consequently, sequential co-cultivation of (T3+B2) can be utilized for spore production. Although other biopesticides not able to increase spore count, they might be responsible for alterations in the bioactive metabolites and their activities. Therefore, further studies focused on characterization of biopesticides.

Characterization of biopesticides

Trichoderma viride monocultures and co-cultures were extracted with ethyl acetate and the extracts were characterized by GC-MS. The previous studies were used to attribute activities to the metabolites because no studies had been conducted.

The extracts of biopesticides produced by substrate supplementation were characterized and peaks of the metabolites are shown in Fig. 2. Table 1 details the

Table 1 — GC-MS identification of *Trichoderma viride* metabolites produced on corn cobs with keratin and lignin supplementation

Metabolites	Molecular Formula	Retention time (min.)	Area (%)
Biopesticides produced on only corn cobs			
n-Propyl acetate	$C_5H_{10}O_2$	3.83	7.41
2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	23.40	1.29
3-Hydroxy-4-methoxybenzoic acid	$C_8H_8O_4$	30.54	52.76
Undecanal, 2-methyl-	$C_{12}H_{24}O$	34.04	2.17
N-Hexadecanoic acid	$C_{16}H_{32}O_2$	40.12	4.86
10(E),12(Z)-Conjugated linoleic acid	$C_{18}H_{32}O_2$	43.85	4.55
Oleic Acid	$C_{18}H_{34}O_2$	43.97	4.78
Octadecanoic acid	$C_{18}H_{36}O_2$	44.47	2.21
Benzenesulfonyl isocyanate	$C_7H_5NO_3S$	48.73	3.76
1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-,[S-(E,Z,E,E)]-	$C_{20}H_{32}$	50.73	14.95
Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	51	1.12
Methanesulfonic acid, (benzylsulfonyl)phenyl-, 4-bromophenyl ester	$C_{20}H_{17}BrO_5S_2$	54.96	6.12
Biopesticides produced on corn cobs supplemented with 1% keratin			
N-Propyl acetate	$C_5H_{10}O_2$	3.83	6.38
Ethane, 1,1-diethoxy-	C6H14O2	4.13	0.77
2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	28.51	1.41
3-Hydroxy-4-methoxybenzoic acid	$C_8H_8O_4$	30.56	48.65
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	C17H24O3	38.76	0.94
N-Hexadecanoic acid	$C_{16}H_{32}O_2$	40.09	4.89
9(E),11(E)-Conjugated linoleic acid	C18H32O2	43.82	4.29
Oleic Acid	$C_{18}H_{34}O_2$	43.93	2.99
Octadecanoic acid	$C_{18}H_{36}O_2$	44.43	1.76
2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	$C_{18}H_{26}O_3$	47.83	2.25
Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	51	5.76
Octocrylene	$C_{24}H_{27}NO_2$	52.94	1.60

(*Contd.*)

Table 1 — GC-MS identification of *Trichoderma viride* metabolites produced on corn cobs with keratin and lignin supplementation — (*Contd.*)

Metabolites	Molecular Formula	Retention time (min.)	Area (%)
N-Propyl acetate	$C_5H_{10}O_2$	3.83	6.38
Biopesticides produced on corn cobs supplemented with 5% lignin			
n-Propyl acetate	$C_5H_{10}O_2$	3.845	5.82
Ethane, 1,1-diethoxy-	C6H14O2	4.15	0.65
3-Hydroxy-4-methoxybenzoic acid	$C_8H_8O_4$	30.55	18.38
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	38.79	1.08
Dibutyl phthalate	$C_{16}H_{22}O_4$	39.97	0.92
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	40.16	21.07
10(E),12(Z)-Conjugated linoleic acid	$C_{18}H_{32}O_2$	43.87	6.9
Oleic Acid	$C_{18}H_{34}O_2$	43.99	5.1
Octadecanoic acid	$C_{18}H_{36}O_2$	44.49	5.71
2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	$C_{18}H_{26}O_3$	47.86	2.1
Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	51.01	2.59
Octocrylene	$C_{24}H_{27}NO_2$	52.99	1.92
Biopesticides produced on corn cobs supplemented with 1% keratin and 5% lignin			
n-Propyl acetate	$C_5H_{10}O_2$	3.84	5.65
2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	28.52	1.07
3-Hydroxy-4-methoxybenzoic acid	$C_8H_8O_4$	30.54	24.76
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	40.11	15.97
10€,12(Z)-Conjugated linoleic acid	$C_{18}H_{32}O_2$	43.82	6.62
Octadecanoic acid	$C_{18}H_{36}O_2$	44.44	3.17
2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	$C_{18}H_{26}O_3$	47.83	2.00
Benzenesulfonyl isocyanate	$C_7H_5NO_3S$	48.72	0.74
Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	50.99	3.12
Octocrylene	$C_{24}H_{27}NO_2$	52.96	1.35
Cyclononasiloxane, octadecamethyl	$C_{18}H_{54}O_9Si_9$	53.86	0.79

metabolites obtained by varying one parameter for corn cob supplementation. As shown in Table 2, these compounds were identified by GC-MS and based on their activities from previous studies. The mass spectra of (T) revealed that the extract contained 12 compounds, of which 10 were antimicrobial. The most abundant compound was 3-Hydroxy-4-methoxybenzoic acid (antimicrobial compound) with Peak Area (PA) of 52.76% and Retention Time (RT) of 30.54 min. This was followed by 1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]- with a PA of 14.95% and RT of 50.73 min. The smallest percentage compound was Bis(2-ethylhexyl) phthalate with a peak of 1.12% and a RT of 51.00 min. The mass spectra of (K) indicated that the extract contained 12 compounds, from which 11 were known to be antimicrobial. The most abundant compound was 3-Hydroxy-4-methoxybenzoic acid (antimicrobial compound), with PA of 48.65% and RT of 30.56 min. This is followed by n-Propyl acetate with a PA of 6.38% and RT of 3.83 min. Ethane, 1,1-diethoxy-is the smallest percentage compound with PA of 0.77% and a RT of 4.13 min. The mass spectra of (L) confirmed that the extract contained 12 compounds, from which 11 were known to be antimicrobial. The most abundant compound was n-Hexadecanoic acid (antimicrobial compound), with PA of 21.07% and RT of 40.16. This is followed by 3-Hydroxy-4-methoxybenzoic acid, with a PA of 18.38% and RT of 30.55. Ethane, 1,1-diethoxy-is the smallest percentage compound with a peak of 0.65% and a RT of 4.15 min. The mass spectra of (K+L) disclosed that the extract contained 11 antimicrobial compounds. The most abundant compound was 3-Hydroxy-4-methoxybenzoic acid (antimicrobial compound), with PA of 24.76% and RT of 30.54 min. This is followed by n-Hexadecanoic acid, with a PA of 15.97% and RT of 40.11 min. The smallest percentage compound was Benzenesulfonyl isocyanate with PA of 0.74% and a RT of 48.72 min. Common antimicrobial compounds produced by (T), (K), (L) and (K+L) were included n-propyl acetate, 3-hydroxy-4-methoxybenzoic acid, n-hexadecanoic acid, octadecanoic acid, bis(2-ethylhexyl) phthalate.

Bioactive metabolites were characterized in monoculture and co-culture extracts. The peaks of compounds are in Fig. 3. The mass spectra of (T) obtained as discussed in previous section. Table 3 reveal the metabolites obtained from *T. viride*

Table 2 — Activities of *T. viride* metabolites identified in the biopesticides produced using corn cobs (T) supplemented with 1% Keratin (K), 5% Lignin (L), 1% Keratin and 5% Lignin (K+L)

Name	Present in	Activity
n-Propyl acetate[45]	(T), (K), (L), (K+L)	Antifungal
2-Methoxy-4-vinylphenol[46]	(T)	Antimicrobial
3-Hydroxy-4-methoxybenzoic acid[47]	(T), (K), (L), (K+L)	Antimicrobial
Undecanal, 2-methyl-[48]	(T)	Antimicrobial
n-Hexadecanoic acid[49]	(T), (K), (L), (K+L)	Antioxidant, antimicrobial, nematicidal
10(E),12(Z)-Conjugated linoleic acid[49,50]	(T), (L), (K+L)	Antibacterial
Oleic Acid[51]	(T), (K), (L)	Antimicrobial
Octadecanoic acid[50]	(T), (K), (L), (K+L)	Antioxidant and antimicrobial
Benzenesulfonyl isocyanate[52]	(T), (K+L)	Antimicrobial
1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]-[53]	(T)	Cytotoxic, insect deterrent, antimicrobial
Bis(2-ethylhexyl) phthalate[50]	(T), (K), (L), (K+L)	Antibacterial and antifungal
Methanesulfonic acid, (benzylsulfonyl)phenyl-,4-bromophenyl ester[54]	(T)	Antimicrobial
Ethane, 1,1-diethoxy-[55]	(K), (L)	Anti-inflammatory, antipyretic, anti-thrombotic and analgesic
2,4-Di-tert-butylphenol[56]	(K), (K+L)	Antifungal
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione[57]	(K), (L)	Antimicrobial
2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester[58]	(K), (L), (K+L)	Antimicrobial
Octocrylene[59]	(K), (L), (K+L)	Antimicrobial
Cyclononasiloxane, octadecamethyl[60]	(K+L)	Antifungal

Table 3 — GC-MS identification of *Trichoderma viride* metabolites produced on corn cobs by co-cultivation with *Bacillus subtilis* MTCC 8142

Metabolites	Molecular Formula	Retention time (min.)	Area (%)
The co-cultures obtained by simultaneous inoculation of *T. viride* and *B. subtilis* MTCC 8142 (B+T)			
n-propyl acetate	$C_5H_{10}O_2$	3.84	6.85
Diisobutyl phthalate	$C_{16}H_{22}O_4$	37.61	2.34
Butyl decyl phthalate	$C_{22}H_{34}O_4$	38.77	2.18
Dibutyl phthalate	$C_{16}H_{22}O_4$	39.94	0.93
n-hexadecanoic acid	$C_{16}H_{32}O_2$	40.14	10.07
10(E),12(Z)-Conjugated linoleic acid	$C_{18}H_{32}O_2$	43.82	6.62
Oleic acid	$C_{18}H_{34}O_2$	43.97	4.01
Octadecanoic acid	$C_{18}H_{36}O_2$	44.48	4.46
Benzenesulfonyl isocyanate	$C_7H_5NO_3S$	48.72	0.74
Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	51.01	39.53
The co-cultures obtained by sequential inoculation of *B. subtilis* MTCC 8142 for 2 days and *T. viride* 3 days incubation (B2+T3)			
Undecane	$C_{11}H_{24}$	13.03	2.76
Dibutyl phthalate	$C_{16}H_{22}O_4$	39.89	3.19
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	40.17	6.28
1,8,11-Heptadecatriene, (Z,Z)-	$C_{17}H_{30}$	43.89	12.54
1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-	$C_{20}H_{32}$	50.67	54.35
Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	50.94	4.34

co-cultures. As shown in Table 4, these compounds were identified by GC-MS and based on their activities from previous research. The mass spectra of (B+T) indicated that the extract contained 10 compounds, from which 9 were known to be antimicrobial. Four of the compounds were phthalate, which are phytotoxins. The most abundant compound was bis(2-ethylhexyl) phthalate with PA of 39.53% and RT of 51.01 min. This is followed by n-hexadecanoic acid with a PA of 10.07% and RT of 40.14 min. Benzenesulfonyl isocyanate was the smallest percentage compound with a peak of 0.74% and a RT of 4.14 min. The mass spectra of (B2+T3) shown that the extract contained 6 compounds from which 5 were antimicrobial compounds. The most abundant compound was 1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, with PA of 54.35% and RT of 50.67 min. This is followed by 1,8,11-heptadecatriene, (Z, Z)-

with a PA of 12.54% and RT of 43.89 min. Undecane is the smallest percentage compound with a peak of 2.76 % and a RT of 13.03 min. The mass spectrum of (T3+B2) showed that the extract consisted of unknown compounds. Antimicrobial compounds such as n-hexadecanoic acid and bis(2-ethylhexyl) phthalate were produced by (T), (B+T) and (B2+T3).

The (T) biopesticides produced the highest concentration of antimicrobial compounds without substrate supplementation and co-cultivation. Also, it was important to note that some metabolites they produced were common while others were different. Therefore, further studies focused on analyzing their biocontrol activity.

Examination of antifungal activity of extracts against *R. solani* MTCC 4633

The extracts were dissolved in methanol and evaluated for antifungal activity on *R. solani* MTCC 4633 using PDA plates. Methanol was used as a control. The inhibition was visible against pathogen at 10 mg/mL concentration, which was selected based on previous study[1].

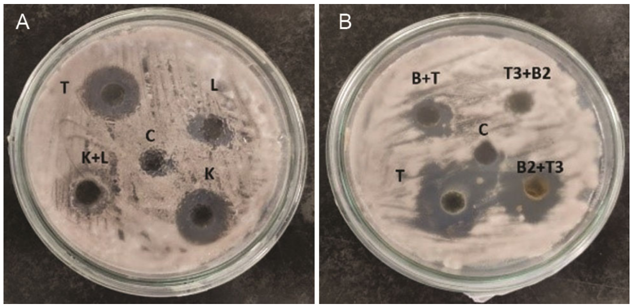

Fig. 3 — Antifungal activity of extracts of *Trichoderma viride* biopesticides produced using (A) corn cobs supplementation; and (B) co-cultivation with *B. subtilis* MTCC 8142. [Each well contained 100 µL of extract with 10 mg/mL concentration]

Biopesticide (T) showed the greatest inhibition with 21 mm, while (K+L) exhibited the least with 10 mm (Fig. 3A & 4A. The biopesticide (K) gave a comparable inhibition to that of (T). Addition of 1% keratin did not affect antifungal activity. However, simultaneous supplementation of keratin and lignin decreased it. This is due to less concentration of produced antimicrobial compounds. Andreo-jimenez *et al.*[19] found that keratin play important role in *R. solani* MTCC 4633 disease suppression. Our studies shown that supplementation of keratin not affecting antifungal activity of *T. viride*, however, lignin reduced it.

In the co-cultivation methods, the highest inhibition was shown by (T) with an area of inhibition of 21 mm followed by (B2+T3) of 18 mm at 10 mg/mL. The biopesticide (B+T) showed the lowest zone of inhibition of 15 mm. However, sequential co-culture of (T3+B2) did not show any antifungal activity due to no antimicrobial compound production (Fig. 3B & 4B. The success of co-cultivation depends on their abilities and cooperation[34]. According to Karuppiah *et al.*[35], *Trichoderma* pre-culture enhanced antagonistic and biocontrol activity by promoting the growth of both microorganisms and inducing secondary metabolites. The order in which the

Table 4 — Activities of *T. viride* metabolites identified in the biopesticides produced using corn cobs (T) co-cultivated with *B. subtilis* MTCC 8142 by simultaneous inoculation (B+T) and sequential inoculation (B2+T3)

Name	Present in	Activity
n-Propyl acetate[45]	(T), (B+T)	Antifungal
2-Methoxy-4-vinylphenol[46]	(T)	Antimicrobial
3-Hydroxy-4-methoxybenzoic acid[47]	(T)	Antimicrobial
Undecanal, 2-methyl-[48]	(T)	Antimicrobial
n-Hexadecanoic acid[49]	(T), (B+T), (B2+T3)	Antioxidant, antimicrobial, nematicidal
10(E),12(Z)-Conjugated linoleic acid[49,50]	(T), (B+T)	Antibacterial
Oleic Acid[51]	(T), (B+T)	Antimicrobial
Octadecanoic acid[50]	(T), (B+T)	Antioxidant and antimicrobial
Benzenesulfonyl isocyanate[52]	(B2+T3)	Antimicrobial
1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]-[53]	(T), (B+T), (B2+T3)	Cytotoxic, insect deterrent, antimicrobial
Bis(2-ethylhexyl) phthalate[50]	(T)	Antibacterial and antifungal
Methanesulfonic acid, (benzylsulfonyl)phenyl-,4-bromophenyl ester[54]	(T), (B+T)	Antimicrobial
Undecane[61]	(B2+T3)	Antimicrobial
Diisobutyl phthalate[62]	(B+T)	Phytotoxic
Butyl decyl phthalate[63]	(B+T)	Phytotoxic
1,8,11-Heptadecatriene, (Z,Z)- [64]	(B2+T3)	Antimicrobial

microorganisms inoculated for co-culturing is also one factor in obtaining a higher yield[32]. Castilo *et al.*[36] stated that inoculation order must be carefully customized due to variations among different growth rates, metabolites production rates, and the possibility of one microbe dominating the others. *Trichoderma* spp. colonise nutrient-deficient conditions, and starvation is required for expression of crucial characteristics in biocontrol mechanism[37]. In sequential inoculation, preculture of *B. subtilis* MTCC 8142 could have consumed maximum nutrients and created nutritional stress. After 2 days when *T. viride* inoculated they could have expressed important traits. Hence, co-coulture (B2+T3) showed higher zone of inhibition than (T3+B2).

The antifungal activity of *T. viride* was not affected by the keratin-supplemented substrate, but lignin diminished it. The biopesticide (B2+T3) contained more antimicrobial compounds and antifungal activity, even though (T3+B2) produced a more significant number of spores. Further research is essential to determine whether substrate supplementation and co-cultivation affect the plant growth-promoting activity.

Effect of biopesticides on chili plant growth

Biopesticides were applied to the potting soil monthly before and weekly after flowering. After a period of three months, the growth of the chili plants was evaluated in terms of root length, stem height, leaf count, and number of stems. Substrate supplemented (Fig. 4) and co-cultivated (Fig. 5) biopesticides shown different effects on plant development.

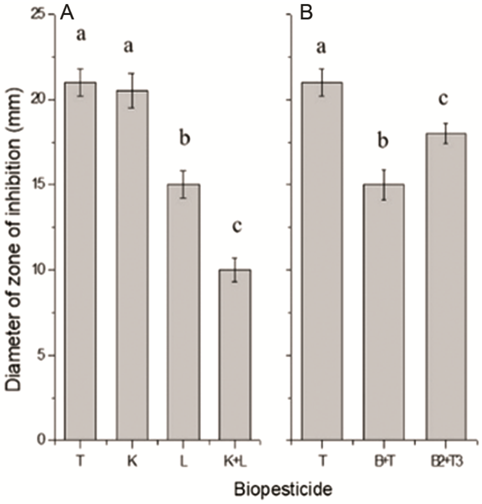

Fig. 4 — Diameter of zone of inhibition of extracts obtained from *Trichoderma viride* biopesticides produced using (A) corn cobs supplementation; and (B) co-cultivation with *B. subtilis* MTCC 8142. [Results are displayed as means ± standard error. Means of diameter of zone of inhibition followed by different letters differ significantly according to Tukey's multiple comparisons test ($P \leq 0.05$)]

The (K) and (L) biopesticides proved significantly superior to the others as shown in Fig. 5A. Keratin contains free amino acids, peptides, and ammonium ions as a nitrogenous fertilizer and promotes plant growth[38,39]. According to Calin *et al.*[28], the metabolites produced by keratin hydrolysis by *T. asperellum* enhanced crop growth and yield. Lignin-degrading microorganisms promoted the development of biologically active humus[40]. Savy *et al.*[41] suggested that amending soils with lignin can help to stabilize humified molecules in soils, resulting in total Carbon buildup. They found that lignin acted as a plant biostimulator and soil conditioner. Therefore, (K) and (L) biopesticides enhanced chili plant growth.

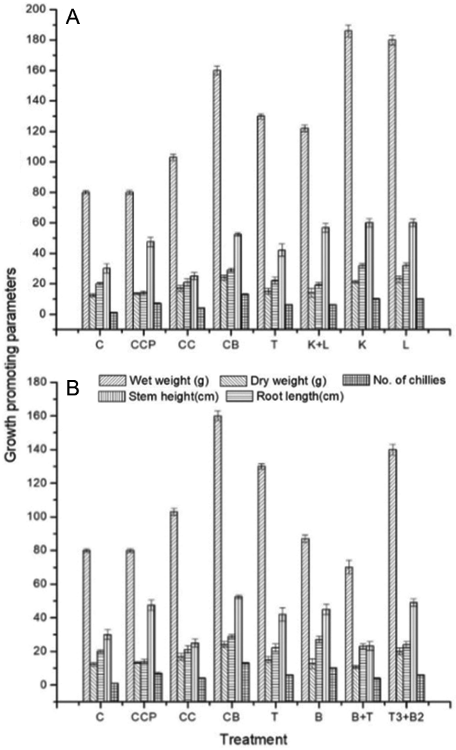

Fig. 5 — Effect of *Trichoderma viride* biopesticides produced using (A) substrate supplementation; and (B) co-cultivation on chili plant growth. [Biopesticides produced using corn cobs (T) supplemented with 1% Keratin (K), 5% Lignin (L), 1% Keratin and 5% Lignin (K+L). C: Control, CCP: Commercial chemical pesticide, CC: Corn cobs, CB: Commercial biopesticide, B: *B. subtilis* MTCC 8142, B+T: *B. subtilis* MTCC 8142 and *T. viride* co-culture, B2+T3: sequential co-culture of *B. subtilis* MTCC 8142 for 2 days and *T. viride* 3 days incubation, T3+B2: sequential co-culture of *T. viride* 3 days and *B. subtilis* MTCC 8142 for 2 days of incubation. Results are displayed as means ± standard error]

Soil application of biopesticide (B2+T3) resulted in higher growth-promoting parameters than other biopesticides as shown in Fig. 5B. Phthalates produced from (B+T) reduced chili plant growth. According to Martinuz *et al*.[33], co-cultures of microorganisms treated with seed or planting material rarely showed a synergistic effect. Plants growth was reduced with (B+T) due to competition between two agents and production of phytotoxins such as phthalates. Karuppiah *et al*.[24] found that sequential co-cultures improved maize plant growth and defence potential under normal and biotic stress. Similarly, the present study also demonstrated enhanced growth using (B2+T3) co-culture.

The soil application of biopesticides (K), (L) and (B2+T3) promoted the growth of chili plants more effectively than the others. Hence, those can be used to enhance plant growth. Plant growth promoting effects of *Trichoderma* are majorly due to production of secondary metabolites, siderophores, hydrogen cyanide, plant growth hormones and phosphate solubilization[42-44]. The amino acids from keratin and humus from lignin contributes to plant development. In addition, both microorganisms in a co-culture can produce plant growth promoters, such as indole-3-acetic acid IAA), zeatin and gibberellin[25]. Further research should be focused on in vitro studies to support plant growth promotion.

Conclusion

Agricultural waste residues are an essential substrate for *Trichoderma* production, and supplementation with them can enhance its efficacy. Keratin from hair waste helps *Trichoderma* make more of its chemicals, which protect plants better against plant pathogens and promote better plant growth. Co-culturing *Trichoderma* spp. with synergistic *Bacillus* spp. can significantly enhance *Trichoderma's* activity. Sequentially inoculated co-cultures enhanced chili plant growth than simultaneously inoculated. (K) and (B2+T3) enhanced plant growth without disturbing their antifungal activity. However, other biopesticides decreased antifungal activity, while some decreased both antifungal and plant growth promoting activity. However, the antifungal activity of other biopesticides was diminished, and (B+T) exhibited a reduction in both antifungal and plant growth promoting activity. Thus, sequential inoculation along with substrate supplementation can make *Trichoderma-based* plant management systems work better and more efficiently.

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Conflict of interest

Authors declare no competing interests.

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