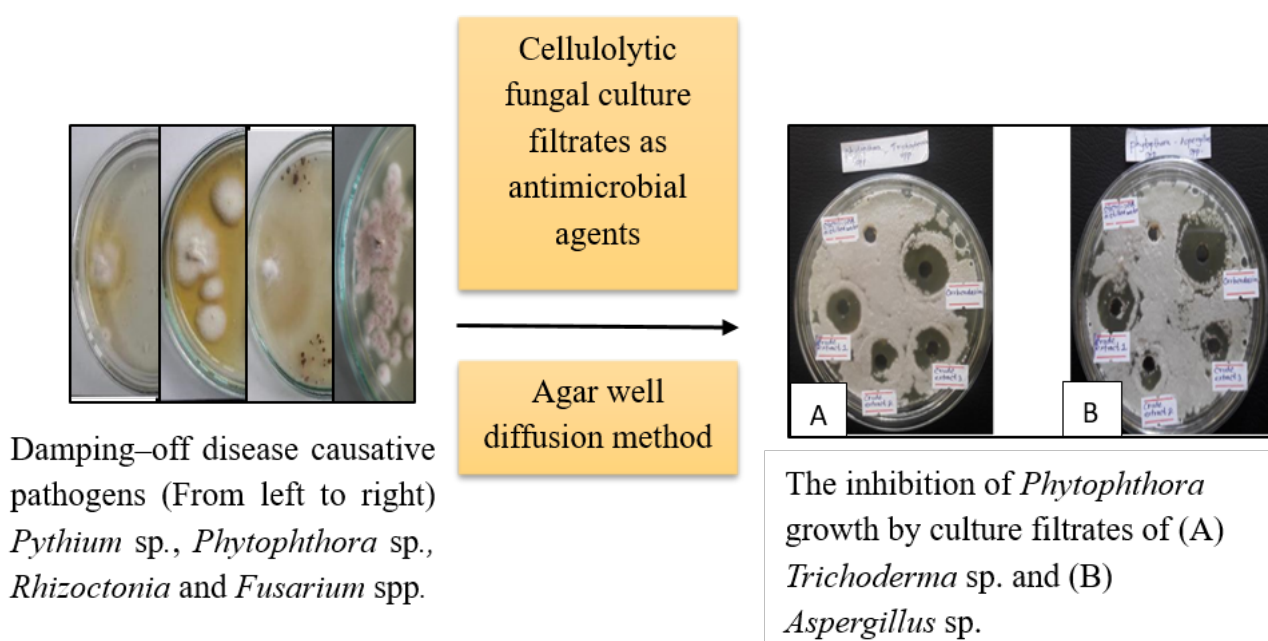


Suppression of damping-off disease causative agents using cellulase enzyme extracts of locally isolated cellulolytic fungi and their co-cultures

S.K. Jayasekara, K.B.M.D.K. Karunarathna, K.L.W. Kumara and R.R. Ratnayake*



Highlights

- Damping-off, is caused by soil-borne microbes viz; *Phytophthora*, *Fusarium*, *Rhizoctonia* and *Pythium*.
- Crude cellulase enzyme extracts of *Trichoderma* sp. and *Aspergillus* sp. inhibited *Phytophthora* sp.
- *Talaromyces* + *Aspergillus* + *Penicillium oxalicum* co-culture crude cellulase extract is an effective seed treatment agent against *Phytophthora*.
- Use of cellulolytic fungi for inhibiting pathogens with cellulose containing cellular structures is a novel concept.

RESEARCH ARTICLE

Suppression of damping-off disease causative agents using cellulase enzyme extracts of locally isolated cellulolytic fungi and their co-cultures

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Received: 26/12/2020; Accepted: 30/10/2021

Abstract: Damping-off disease is caused by *Pythium* sp., *Phytophthora* sp., *Rhizoctonia* sp., and *Fusarium* sp., and affects seedlings at the nursery stage. *Phytophthora* and *Pythium* cell walls contain cellulose. The current study aimed to determine potential use of cellulase extracts of selected fungal species *i.e.* *Talaromyces* sp., *Trichoderma* sp., *Aspergillus* sp., *A. niger*, and *Penicillium oxalicum* to suppress these pathogens and to investigate the potential use of fungal co-culture extracts with the highest cellulase activity for seed treatment to control damping-off. The impact of cellulase extracts on pathogen growth was determined by agar well diffusion method. For seed treatment, tomato seeds were soaked in cellulase extracts from the selected co-culture for 24 h and were sown in pathogens inoculated soil samples. Cellulase extracts of *Trichoderma* and *Aspergillus* suppressed *Phytophthora* growth with 63% and 57% of inhibition percentages respectively. When seeds were treated with *Talaromyces* sp.+*Aspergillus* sp.+*P. oxalicum* co-culture extracts, the highest seedling emergence percentage was observed in *Phytophthora* sp. *Fusarium* sp., and four-pathogen mixture-inoculated soils respectively. Lowest disease incidence was observed in *Phytophthora* sp. inoculated soil. Results show the potential of cellulolytic fungal species; *Trichoderma* sp., *Aspergillus* sp., and co-culture of *Talaromyces* sp.+*Aspergillus* sp.+*P. oxalicum* in suppression of *Phytophthora* sp. a common causative agent of damping-off.

Keywords: Anti-pathogenic fungi; cellulolytic fungi; damping-off; *Phytophthora*; seed treatment agents.

INTRODUCTION

Damping-off is considered as one of the most destructive diseases both in nurseries and fields during crop cultivation and it is a major constraint responsible for significant reduction of yields and severe economic losses (Lamichhane *et al.*, 2017). The disease is mainly recognized by poor seed germination, prevention of seedling emergence above soil level after germination or decay and death of young seedlings at the soil level (Lamichhane *et al.*, 2017).

The incidence of damping-off is governed by various abiotic factors *viz*; excessive soil moisture, excessive overhead misting, low soil temperatures (below 20 °C)

before germination of seeds, high soil temperatures (above 25 °C) after seedlings emergence and overcrowded seed beds. Many fungi and fungi-like species are considered as the most predominant biotic factor which gives rise to this disease (Kraft *et al.*, 2000). Soil-borne fungi such as *Fusarium* spp., *Rhizoctonia* spp., and soil-inhabiting Oomycetes including *Pythium* spp. and *Phytophthora* spp. are the most important and frequent causal agents of damping-off (Starkey and Enebak, 2012).

An effective management of damping-off is achievable by deploying several control strategies that include chemical control methods such as application of fungicides (e.g. captan, metalaxyl, or thiram), use of proper cultivation practices (*i.e.* application of optimum dose of nitrogenous fertilizers, providing sufficient drainage, bio-fumigation, removal and replacement of top soil with fresh healthy soil every five years, soil solarization), and biological control using bacteria and fungi (Lumsden and Locke, 1989; Deadman *et al.*, 2006; Al-Sadi *et al.*, 2008; Pagoch *et al.*, 2015; Hyder *et al.*, 2021). The use of chemical fungicides has certain disadvantages. They are costly, have negative environmental impacts, and may also induce fungicide-resistance in pathogens.

On the contrary, biological control, which uses microorganisms or their cellular extracts for pathogen suppression, offers an attractive ecofriendly alternative to chemical control strategies of plant disease management (Wang *et al.*, 1999). Many fungal species have already been reported as effective bio control agents against damping-off disease (Jayaraj *et al.*, 2006; Kamala and Indira, 2011; Loliam *et al.*, 2012; Boat *et al.*, 2019; Soheiliara *et al.*, 2020). These studies have reported the potential use of different individual fungal isolates in controlling damping-off disease. Among them, *Trichoderma harzianum*, *T. viride*, and *T. hamatum* are reported to be effective for the control of damping-off disease together with strains of *Gliocladium* (Kevan and Shipp, 2017). Moreover, there are number of innovative fungal-based bio control products currently available commercially to support the implementation of damping-off management

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strategies. For example, many manufacturers use several *Trichoderma* species (e.g. *T. viridae*, *T. harzianum*) in their liquid or powder form formulations.

Laboratory-scale experiments have proven microbial co-culturing to be a promising approach for the activation of frequently silent biochemical pathways in microorganisms. Accordingly, fungi could be co-cultured with other bacteria or fungi to trigger their potential production of novel antifungal compounds for controlling plant diseases (Bertrand *et al.*, 2013; Netzker *et al.*, 2015; Serrano *et al.*, 2017). Wu *et al.* (2018) demonstrated the potential of a *B. amyloliquefaciens* ACCC11060 and *T. asperellum* GDFS1009 co-culture to produce more specific biocontrol substances on controlling *Botrytis cinerea*. Karuppiyah *et al.* (2019) recently developed and demonstrated the effectiveness of a co-culture, *Trichoderma asperellum* GDFS1009 and *Bacillus amyloliquefaciens* 1841 in biocontrol of *Fusarium graminearum* (wheat head blight and root rot pathogen).

One objective of the current study was to assess the impact of crude cellulase enzyme filtrates of selected locally-isolated cellulolytic fungi and their co-cultures in suppression of the causal agents of damping-off disease. Especially, it is found that *Pythium* and *Phytophthora* have cell walls that contain cellulose as a major constituent (Cooper and Aronson, 1967; Blaschek *et al.*, 1992; Grenville-Briggs *et al.*, 2008; Jones and Ospina-Giraldo, 2011). Therefore, it was hypothesized that the cellulase extracts could suppress the growth of these pathogens by damaging their cell walls through cellulolysis. Moreover, it would be a novel concept to study whether cellulolytic potential of fungi could carry out control of pathogenic microorganisms that have cellulose in their cellular structures including cell walls. Additionally, there are various studies that have been conducted to prove the potential of fungal isolates to produce and subsequently secrete other extracellular secondary metabolites during their growth that could exhibit antimicrobial properties (e.g. Svahn *et al.*, 2012; Jayatilake and Munasinghe, 2020). Correspondingly, although they do not have cellulose in their cell walls, the growth of *Fusarium* sp. and *Rhizoctonia* sp. could be inhibited by these extracellular metabolites present in the crude cellulase enzyme extracts.

Pre-sowing seed treatment is an agricultural practice that intended to protect the crops against various seed- and soil-borne diseases (Ayesha *et al.*, 2021). Furthermore, seed treatment using fungal biomass or fungal cellular extracts for the control of damping-off causative agents has been previously reported based on the various antimicrobial compounds present (Mao *et al.*, 1998; Pfeiffer *et al.*, 2021). In the present study, it was evaluated whether it is possible to use the cellulase enzyme extracts from fungal co-cultures as a seed treatment agent against soil-borne damping-off causative microorganisms. The use of cellulolytic potential of fungal co-cultures to control disease causative fungi will be a novel approach.

MATERIALS AND METHODS

Determination of total cellulase activities of cellulolytic fungal co-cultures

Five locally-isolated cellulolytic fungal species reported by us previously (Jayasekara *et al.*, 2020), were employed in the present study. They were *Trichoderma* sp., *Aspergillus niger*, *Penicillium oxallicum*, *Aspergillus* sp., and *Talaromyces* sp. According to the previous study by Jayasekara *et al.* (2020), the highest total cellulase activity was observed in *P. oxallicum* as 0.37 FPU mL⁻¹ while *Talaromyces* sp showed 0.31 FPU mL⁻¹. Total cellulase activity of *Trichoderma* sp. was recorded as 0.28 FPU mL⁻¹. *Aspergillus* sp. showed 0.25 FPU mL⁻¹ while *Aspergillus niger* showed 0.23 FPU mL⁻¹ (Jayasekara *et al.*, 2020). These five cellulolytic fungal isolates were co-cultured with each other in a random manner in two, three, and four and five possible combinations in the cellulase production broth described by Mandels and Weber (1969).

The co-culturing was done in triplicate for each fungal combination and a control sample was maintained without inoculating the fungi. The broth was initially inoculated with 100 µL of spore suspensions with a spore concentration of 1 × 10⁷ Spores mL⁻¹ of each selected component fungus and incubated at 28 °C for 7 d with shaking at 120 rpm. At the end of incubation, co-cultures were centrifuged at 10,000 g for 10 min to obtain a cell free supernatant as the crude extract. Total cellulase assay described by Ghose (1987) was conducted to determine the total cellulase activity of each co-culture filtrate.

Isolation and identification of damping-off disease causing pathogens

For the current study, *Rhizoctonia solani* was obtained from the Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya, Sri Lanka while *Phytophthora* sp. was provided by the Horticultural Crop Research and Development Institute (HORDI), Gannoruwa, Peradeniya, Sri Lanka. The fungal cultures were stored in agar slants at room temperature.

Isolation of *Fusarium* sp.

A soil dilution series with concentrations varying from 10⁻² to 10⁻⁷ was prepared with soil taken near a diseased tomato plant. Roots of a diseased tomato plant were washed with flowing tap water and surface sterilized using 70% ethanol. Subsequent wash with sterilized distilled water removed residual ethanol from the plant tissue. One gram of cleansed roots was ground using a sterilized mortar and pestle and the ground sample was dissolved in 10 mL of sterile distilled water to prepare the root extract. Komada medium which is a selective medium for *Fusarium* (Komada, 1975) was used for the isolation. Hundred microliters of 10⁻³ to 10⁻⁶ concentrations of the prepared soil dilution series and root extract were inoculated into Komada medium using the spread plate technique. The cultures were incubated at 25 °C for a week and cultures having morphological characteristics similar

to *Fusarium* sp. were purified and transferred to PDA. Microscopic slides were prepared using sticky tape method and purified fungal cultures were observed under the microscope for identification based on their morphological characteristics using fungal identification keys (Barnett and Hunter, 1998; Dugan, 2006).

Isolation of *Pythium* sp.

Vigna radiata; Mungbean is previously reported in various studies to be infected by *Pythium* spp. causing number of diseases including damping-off (Pandey et al., 2018). Accordingly, in order to isolate *Pythium* sp., mung beans were soaked in water overnight. They were planted in sterilized petri dishes containing soil collected from the dry zone areas such as Jaffna, Ampara, Kurunegala, and Kuliyaipitiya where a warm climate prevails which favors the growth of *Pythium* sp. A portion of mung beans were also grown on a layer of cotton wool. The dishes were placed in a moisture chamber and were observed for damping-off disease symptoms. With the initial observation of the symptoms, roots and infected stem parts were cut into small pieces and ground using a mortar and pestle, and extract was prepared using sterilized distilled water. Tomato agar, water agar (Atlas, 2010), and PDA media were prepared and root extract (100 µL) and infected root and stem parts were placed in each medium. Culture plates were incubated for 24 h and the hyphae that emerged were examined under the light microscope.

Determination of the effect of cellulolytic fungal culture filtrates on damping-off causative agents by well diffusion method

The effects of cellulolytic fungal culture filtrate on the damping-off pathogens were detected by well diffusion method (Cole, 1994). Mueller Hinton agar plates were prepared by adding methylene blue to a final concentration of 5 µg mL⁻¹ to enhance zone edge definition.

The inocula of fungal pathogens were prepared as spore suspensions in sterile Tween 80 (0.1% v/v) in water using seven day-old cultures in agar slants; *Phytophthora* in water agar slant, *Rhizoctonia*, *Pythium*, and *Fusarium* in PDA slants. The spore concentrations were adjusted to 10⁷- 10⁸ spores mL⁻¹ using a Neubauer chamber and 100 µL of each spore suspension was inoculated into solidified Mueller Hinton agar plates by spread plate method. The agar plate contained five wells with 5 mm diameter. Crude extracts were prepared by centrifuging seven-day old previously mentioned five cellulolytic fungal isolates grown in Mandels and Weber broth, at 4200 rpm for 15 min. Three wells were filled with 100 µL of respective crude extract and the other two were filled with a positive control (Carbendazim solution with a concentration of 7 µL mL⁻¹ of sterilized distilled water) and a control solution (fresh culture broth). They were incubated at 30 °C and observed for the occurrence of growth inhibition zones after 16 to 18 h. The diameters of inhibition zones were measured using a ruler graduated to 0.5 mm.

Using culture filtrates from cellulolytic fungal co-cultures as a seed treatment agent

The culture filtrates (crude cellulase mixture) from the cellulolytic fungal co-culture with the highest cellulase activity was evaluated for seed treatment. As it is reported in various studies that damping-off caused by *Pythium* sp, *Phytophthora* sp, *Rhizoctonia* sp, and *Fusarium* sp. is one of the worst diseases of tomato, which is occurring in the nursery that can kill both germinating seeds and young seedlings (Moussa, 2002; Agrios, 2005; Smith, 2007), in the current study tomato seeds were used. Initially, 100 seeds from tomato variety “Thilina” were placed on a sterilized cotton wool pad moistened with sterilized distilled water in a sterilized Petri dish and incubated at 30 °C for two weeks to observe the healthy germination of seeds without any hints of fungal infection because it was necessary to confirm that there were no seed surface-borne pathogen spores. The experiment was conducted in triplicate. The germination percentage was above 95% without any fungal growth or damping off symptoms. Five hundred seeds were dipped in the crude enzyme mixture for 24 h. Another set of 150 seeds were soaked in fresh culture broth for 24 h to be used as controls.

Soil (20 kg) collected from a forest area was sterilized at 121 °C for 20 min and divided into six parts. Pure broth cultures of *Pythium* sp., *Fusarium* sp. *R. solani*, and *Phytophthora* sp. and the mixture of four pathogens were inoculated to the previously prepared five soil fractions separately, while the sixth fraction was maintained without any inoculum. After 24 h, six soil samples (500 g each) were transferred into clean, aluminium foil containers (5×6×2 inches of width×length×height dimensions). Total numbers of 40 soil containers were maintained under greenhouse conditions. Twenty-five treated seeds were sown per container in each treatment consisting of three replicates. Emergence percentage was calculated 7 d after seed sowing which refers to the number of plants emerged out of total seeds sown as a percentage (Gummert, 2010). Damping-off incidence was recorded 7 d after emergence as the percentage of the number of diseased plants out of the total number of plants observed (Cardoso et al., 2004).

RESULTS AND DISCUSSION

Determination of total cellulase activities of cellulolytic fungal co-cultures

The analysis of variance (culture versus mean enzyme activity) indicated that the differences in mean enzyme activity of screened co-culture combinations (alpha = 0.05) were significant (F = 19.23, p-value = 0.000). As given in table 1, twenty-five different co-culture combinations were tested for their cellulase activity. The highest three mean enzyme activities were shown by *Talaromyces* sp.+*Aspergillus* sp.+*P. oxallicum* (0.4725 FPU mL⁻¹), *Talaromyces* sp.+*A. niger*+*P. oxallicum* (0.4720 FPU mL⁻¹), and *Talaromyces* sp.+*A. niger*+*Aspergillus* sp.+*P. oxallicum* (0.4694 FPU mL⁻¹), respectively. However, those three values were not significantly different from each other although they showed enhanced total cellulase

Table 1: A comparison of cellulolytic activities of monocultures and co-cultures.

Monoculture/ Co-culture	Mean enzyme activity (FPU mL ⁻¹)	Tukey's grouping
<i>Talaromyces</i> sp.+ <i>Aspergillus</i> sp.+ <i>P. oxallicum</i>	0.4725	a
<i>Talaromyces</i> sp.+ <i>A. niger</i> + <i>P. oxallicum</i>	0.4720	a
<i>Talaromyces</i> sp.+ <i>A. niger</i> + <i>Aspergillus</i> sp.+ <i>P. oxallicum</i>	0.4694	a
<i>A. niger</i> + <i>Aspergillus</i> sp.+ <i>P. oxallicum</i>	0.4261	ab
<i>A. niger</i> + <i>P. oxallicum</i>	0.4189	abc
<i>Talaromyces</i> sp.+ <i>Trichoderma</i> sp.+ <i>A. niger</i> + <i>Aspergillus</i> sp.+ <i>P. oxallicum</i>	0.3906	abcd
<i>Trichoderma</i> sp.+ <i>P. oxallicum</i>	0.3891	abcd
<i>Aspergillus</i> sp.+ <i>P. oxallicum</i>	0.3684	abcd
<i>P. oxallicum</i>	0.3659	abcd
<i>Trichoderma</i> sp.+ <i>A. niger</i> + <i>P. oxallicum</i>	0.3448	abcd
<i>Talaromyces</i> sp.	0.3100	abcde
<i>Trichoderma</i> sp.	0.2811	bcdef
<i>Talaromyces</i> sp.+ <i>Trichoderma</i> sp.+ <i>A. niger</i> + <i>P. oxallicum</i>	0.2732	bcdef
<i>Talaromyces</i> sp.+ <i>Trichoderma</i> sp.+ <i>Aspergillus</i> sp.+ <i>P. oxallicum</i>	0.2600	bcdef
<i>Talaromyces</i> sp.+ <i>Trichoderma</i> sp.+ <i>P. oxallicum</i>	0.2575	bcdef
<i>Aspergillus niger</i>	0.2509	bcdefg
<i>Aspergillus</i> sp.	0.2322	cdefgh
<i>Talaromyces</i> sp.+ <i>Trichoderma</i> sp.+ <i>A. niger</i>	0.2174	defghi
<i>Trichoderma</i> sp.+ <i>Aspergillus</i> sp.+ <i>P. oxallicum</i>	0.2035	defghi
<i>Trichoderma</i> sp.+ <i>A. niger</i> + <i>Aspergillus</i> sp.	0.1280	efghi
<i>Talaromyces</i> sp.+ <i>Trichoderma</i> sp.+ <i>A. niger</i> + <i>Aspergillus</i> sp.	0.1125	fghi
<i>A. niger</i> + <i>Aspergillus</i> sp.	0.0692	ghi
<i>Trichoderma</i> sp.+ <i>Aspergillus</i> sp.	0.0640	ghi
<i>Talaromyces</i> sp.+ <i>A. niger</i>	0.0597	hi
<i>Talaromyces</i> sp.+ <i>Aspergillus</i> sp.	0.0595	hi
<i>Talaromyces</i> sp.+ <i>P. oxallicum</i>	0.0595	hi
<i>Trichoderma</i> sp.+ <i>A. niger</i>	0.0595	hi
<i>Talaromyces</i> sp.+ <i>Trichoderma</i> sp.	0.0592	hi
<i>Talaromyces</i> sp.+ <i>Trichoderma</i> sp.+ <i>Aspergillus</i> sp.	0.0588	hi
<i>Talaromyces</i> sp.+ <i>A. niger</i> + <i>Aspergillus</i> sp.	0.0375	i

activities than in the monocultures considered (Jayasekara *et al.*, 2020). The co-culture with the highest cellulase activity of 0.4725 FPU mL⁻¹ was applied in the seed treatment experiment.

The lowest mean enzyme activity shown by a co-culture; *Talaromyces* sp.+*A. niger*+*Aspergillus* sp., was 0.0375 FPU mL⁻¹. Some co-cultures showed significantly enhanced total cellulase activities than their respective monocultures while some of them exhibited significantly decreased total cellulase activities. This can be mainly due to either positive (growth induction, enzyme production induction) or negative interactions (competition, antibiosis)

present between the fungal isolates in these co-culture combinations (Serrano *et al.*, 2017).

Determination of the impact of cellulolytic fungal culture filtrates on damping-off causative agents by well diffusion method

According to agar-well diffusion assay, growth of *Phytophthora* sp. was inhibited by crude enzyme extracts from *Trichoderma* sp. and *Aspergillus* sp. (Table 2). The inhibition zone diameter, given by *Trichoderma* extract (Figure 2a), and its respective percentage inhibition (Table 2) is larger than that formed by crude extracts of

Aspergillus sp. and its percentage inhibition (Figure 2b, Table 2). A study conducted by Grenville-Briggs *et al.* (2008), using *Phytophthora infestans* suggests that the interferences that occurs to the microbe during cellulose biosynthesis could adversely affect their pathogenesis. Accordingly, it is possible to conclude that when cellulase enzyme molecules are available in the growth medium, the ability of *Phytophthora* to become pathogenic would be diminished and subsequently their growth would be inhibited because of the degradation of biosynthesized cellulose in their cell walls. Especially, there are also β -glucans in *Phytophthora* cell wall (Tokunaga and Bartnicki-Garcia, 1971). Cellulase is an enzyme complex which is usually composed of exoglucanases, endoglucanases, and β -glucosidase (Jayasekara and Ratnayake, 2019). Therefore, the β -glucosidase component could take part in breaking down *Phytophthora* cell wall by breaking down β -glucans.

However, the cellulase extracts from *Penicillium* and *Talaromyces*, the fungal isolates that exhibited the

highest initial cellulolytic activity in the current study did not inhibit the growth of *Phytophthora*, as was done by *Trichoderma* and *Aspergillus* that showed lower cellulolytic activity. It would be possible to overcome these unexpected results by using a protein purification technique such as gel permeation chromatography to separately obtain the crude protein molecules from the culture filtrates (Coskun, 2016). Then the cellulase assay could be performed to the relevant crude protein extract to improve the reliability of cellulase activity assay results and repeat well- diffusion assay with the purified protein with higher cellulase activity.

In addition to cellulase molecules, there may be other secondary metabolites in the crude culture filtrate that may exhibit inhibitory effect against *Phytophthora* growth. Therefore, it is also possible that the inhibitory effect of *Trichoderma* against *Phytophthora* is a combined effect of both cellulase and other secondary metabolites. The current study must be further modified to clarify these phenomena.

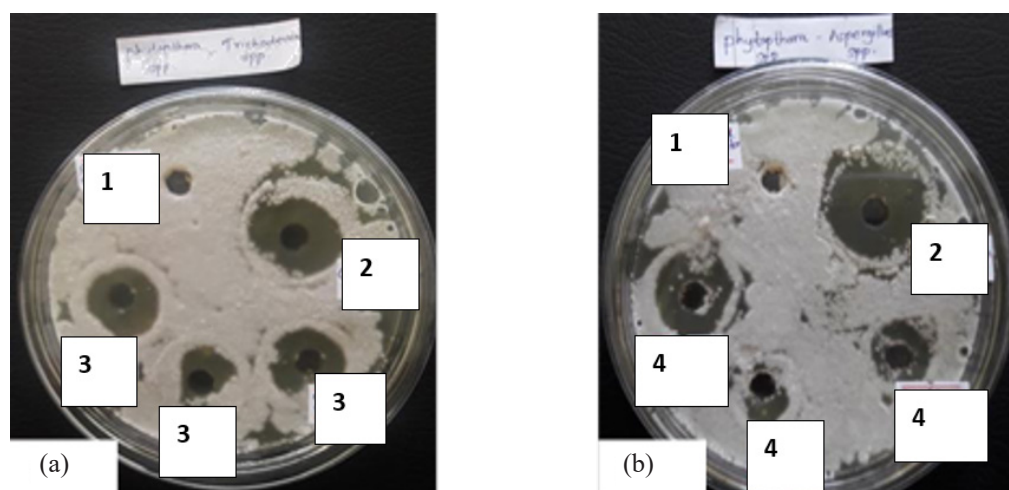


Figure 2: Inhibition of *Phytophthora* growth by crude cellulase enzyme extracts. (a) Inhibition zones caused by *Trichoderma* sp. (b) Inhibition zones caused by *Aspergillus* sp. (1) negative control (fresh culture broth); (2) positive control (Carbendazim); (3) clear zones formed by *Trichoderma* and (4) clear zones formed by *Aspergillus*.

Table 2: Mean diameter of inhibition zones observed in the well diffusion assays for *Phytophthora* sp. with crude enzyme extracts of cellulolytic fungi.

Pathogen-Cellulolytic fungus	Mean diameter of inhibition zone (mm) (A)	*Diameter of inhibition zone of negative control (mm) (B)	*Diameter of inhibition zone of positive control (mm) (C)	Percentage Inhibition $X = (A-B/C-B)100$
<i>Phytophthora</i> sp.- <i>Talaromyces</i> sp.	0	0	24	0
<i>Phytophthora</i> sp.- <i>Trichoderma</i> sp.	17.5	0	26	67%
<i>Phytophthora</i> sp.- <i>A. niger</i>	0	0	27	0
<i>Phytophthora</i> sp.- <i>Aspergillus</i> sp.	15	0	26	58%
<i>Phytophthora</i> sp.- <i>P. oxalicum</i>	0	0	23	0

*Negative control-fresh culture broth, positive control-Carbendazim

Various studies have been conducted to determine the antimicrobial secondary metabolites production of both *Trichoderma* spp. and *Aspergillus* spp. In a recent study, metabolites extracted from cultures of *Trichoderma atroviride*, *T. petersenii*, and *T. virens* isolates, grown in a minimal salt broth, showed strong inhibitory activities against *Phytophthora* isolates (Bae *et al.*, 2016). However, they did not perform purification and identification of these chemical compounds with strong biocontrol activities against *Phytophthora*.

Aspergillus species also generally produce wide range of secondary metabolites which exhibit inhibitory effects pathogenic microorganisms including *Phytophthora* sp. (Siddiqui *et al.*, 2004; Abdallah *et al.*, 2015). They use several mechanisms *viz*; mycoparasitism, competition, mycelial lysis, and antibiosis *via* synthesis of volatile and/or non-volatile metabolites in controlling fungal pathogens (Daami-Remadi *et al.*, 2006; Kaewchai and Soyong, 2010; Bhattacharyya and Jha, 2011).

As previously mentioned, cellulose is a component in the cell wall of *Pythium* sp. (Blaschek *et al.*, 1992; Besnard and Davet, 1993; Chérif *et al.*, 1993), it was expected that the crude cellulase enzyme extracts of cellulolytic fungi employed in this assay would inhibit the growth of *Pythium* by cell wall lysis. However, *Pythium* was not inhibited by cellulase extracts as expected. Several factors, including inadequacy of cellulase concentration, and its composition in crude extract may have resulted these results. In order to clarify further on this, methodology of cellulase production, and extraction could be further optimized followed by enzyme purification.

Besides, none of the crude cellulase extracts of the five selected cellulolytic fungal isolates inhibited *Fusarium* sp., and *R. solani* which confirms the absence of any antifungal compounds, enzymes and mycoparasitic structures in the cellulase extracts that could inhibit their growth. According to previous studies, *Trichoderma* sp., *Aspergillus* sp., *Penicillium* sp., and *Talaromyces* sp. produce inhibitory effects against *Fusarium* and *R. solani* growth by producing different bioactive compounds (Penttila *et al.*, 1988; Harkki *et al.*, 1989; Howell *et al.*, 2003; Singh *et al.*, 2008; Manoch and Dethoup, 2011; Junaid *et al.*, 2013).

There were no such inhibitory effects observed in the current study against the selected pathogens. The major difference between the current study and previous studies is the culture medium used for growing the isolates. Some studies used dual culture technique in PDA (e.g. Manoch and Dethoup, 2011) while others used different salt solutions supplemented with nutrients for their microbial growth. In the current study, fungi were grown in a cellulose broth in order to facilitate cellulase production. The inability to produce bioactive compounds by these fungal isolates against the damping-off pathogens may be partly due to the impact of cellulase fermentation medium on the biosynthetic pathways of producing bioactive compounds by the cellulolytic fungi.

Using culture filtrates from cellulolytic fungal co-cultures as a seed treatment agent

Mean seedling emergence percentage

The highest mean seedlings emergence percentage (87%) was displayed by soil containers inoculated with *Phytophthora* sp. (Table 3). The comparison of emergence percentages of *Phytophthora* sp. treatment and its control revealed that the effect of seed treatment was significantly higher relative to its control ($t = 0.0280$). The lowest significant mean emergence percentage (41%) was exhibited by seeds grown on soil inoculated with a mixture of all four damping-off pathogens, while its corresponding control gave significantly lower mean seedling emergence percentage (24%, Table 3). Moreover, when a two sample *t*-test was carried out for samples treated with *Fusarium* sp, it revealed that there was a significant higher difference between the treatments and their controls (t value = 0.0056). Further, the analysis of variance (Mean emergence % *vs* inoculated pathogens indicated that the differences in mean emergence % of treatments with different pathogens ($\alpha = 0.05$) were highly significant corresponding to their control samples ($F = 21.10$, p -value = 0.000).

These results indicate that the seed treatment has a positive effect on emergence of tomato seedlings. Especially, crude cellulase extract may have suppressed the pathogenesis of *Phytophthora* in the soil by cell wall inhibition and allowed consequent seedling emergence while reducing the damping off occurrence. It is also possible that the microbial cellulase softened the seed coat and thereby enhanced seedling emergence.

Table 3: Tomato seed treatment using crude cellulase enzyme extract against damping-off causative agents.

Pathogen	Seedling emergence percentage			Disease incidence percentage		
	Test	Control	t-value	Test	control	t-value
<i>Phytophthora</i>	87	52	0.0280	15	69	0.0001
<i>Pythium</i>	65	48	0.0858	35	58	0.0514
<i>Fusarium</i>	77	44	0.0056	14	73	0.1024
<i>R. solani</i>	60	52	0.3566	40	46	0.9926
Mixture of pathogens	41	24	0.0201	46	100	0.1039

Moreover, the solution may also contain seed germination enhancers because seedling emergence was significantly higher in the test samples than in the control samples for all the pathogens considered in the study. *Penicillium* sp. which is a component fungus in this seed treatment agent has known to enhance crop growth by facilitating enhanced germination of seeds and enhanced rooting (Bailey and Lumsden, 1998; Harman and Bjorkman, 1998). For instance, fungal crude enzyme extracts may contain plant growth promoting hormones produced which could enhance seed germination and rooting (Yedidia et al., 2001).

Mean disease incidence percentage

The analysis of variance (Mean disease incidence % vs inoculated pathogenic fungi) indicated that the differences in mean disease incidence percentage of seedlings emerged from seeds treated with culture filtrates from different pathogenic microorganisms ($\alpha = 0.05$) were significantly higher ($F = 8.99$, p -value = 0.002). Table 3 shows that the seed treatment has been more effective against *Phytophthora* and *Fusarium* because they showed the lowest significant disease incidence. Highest disease incidences were shown by *R. solani*, *Pythium* sp. and mixture of pathogens inoculated soil samples respectively. However, when the disease incidences of pathogens were compared with their controls using two sample t tests, it was revealed that the effect of seed treatment against disease incidence of *Phytophthora* sp. was significantly higher relative to its control with a t value of 0.0001. The mean disease incidence percentage of *Phytophthora* was 15% while the control sample showed 69% disease incidence. According to these results, it is possible to conclude that the crude cellulase extract from the co-culture (*Talaromyces* sp.+*Aspergillus* sp.+*P. oxallicum*) has the potential of controlling damping off disease incidence induced by *Phytophthora* sp.

The study further revealed the possibility of harnessing underutilized fungal diversity in tropical Sri Lanka for industrial process by isolating and forming their co-cultures. However, in order to make the study more impactful further assays must be conducted to determine the other enzymes available in these crude enzyme mixtures in addition to cellulase that could perform inhibitory effects on the pathogens or could enhance plant growth. Especially, it would be beneficial profiling the plant growth promoting hormones and other active metabolites available in the crude enzyme extracts from both individual cellulolytic fungal isolates as well as in the co-culture extract considered in the current study. If so it would be possible to understand other active compounds that could be produced, extracted and purified from a cellulase enzyme mixture except cellulase.

CONCLUSION

Crude cellulase enzyme extracts of locally isolated *Trichoderma* sp. and *Aspergillus* sp. could individually suppress the growth of *Phytophthora* sp.; a common causative agent of plant diseases including damping-off. The novelty of the study is the use of *Talaromyces*

sp.+*Aspergillus* sp.+*P. oxallicum* co-culture crude cellulase enzyme filtrate as a successful seed treatment agent against damping-off disease causative agent *Phytophthora*. By being effective in reducing damping-off disease incidence and enhancing seedling emergence in *Phytophthora* sp. inoculated soil, the crude enzyme extract indicated its potential use as a seed treatment agent to control soil-borne pathogen. Furthermore, the study emphasizes the importance of characterizing locally isolated fungal species for their potential multiple applications in industrial process (e.g. cellulase production, production of plant growth promoters, production of plant disease control agents).

ACKNOWLEDGEMENT

Our sincere gratitude goes to Prof. (Mrs.) Devika De Costa, Department of Agriculture Biology, Faculty of Agriculture, University of Peradeniya, Sri Lanka and Dr. (Mrs.) M.G.D.L. Priyantha, Department of Agriculture, Seed Certification Service, Gannoruwa, Peradeniya for providing the authors with *Rhizoctonia* and *Phytophthora* cultures. The authors thank Miss. G.D.I.S Karunarathna of the Department of Agricultural Biology, Faculty of Agriculture, University of Ruhuna, Sri Lanka and Dr. Mohanan Kathirgamanathan, Scientist, Genetech Molecular Diagnostic & School for Gene Technology, Sri Lanka for their enormous support rendered to the authors during the study.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no competing interests.

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