Biocontrol activity of *Trichoderma viride* and *Pseudomonas fluorescens* against *Phytophthora infestans* under greenhouse conditions

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The biocontrol potential of *Trichoderma viride*-ES1 and *Pseudomonas fluorescens*-Bak150 against potato late blight pathogen, *Phytophthora infestans*, were studied *in vitro* and under greenhouse conditions. *In vitro* antagonism test carried out between *T. viride* and *P. infestans* showed a radial growth inhibition of the pathogen by 36.7% and a complete overgrowth of *T. viride* on *P. infestans* later, whereas *P. fluorescens* inhibited the radial growth of the pathogen by 88%. Foliar spray method used in the greenhouse trials involved foliar spray of healthy potato plants with suspensions of (i) *T. viride* only, (ii) *P. fluorescens* only, (iii) mixed culture of *T. viride* and *P. fluorescens*, and (iv) Mancozeb, followed by spraying the pathogen inoculum three days later. The infected leaf area was measured weekly and area under the disease progress curve (AUDPC) was calculated and compared among the treatments. The result showed that *T. viride* (AUDPC=260) and *P. fluorescens* (AUDPC=765.1) significantly ($P<0.05$) reduced the disease compared to the untreated check (AUDPC=1045). *T. viride* was found to be more efficient than *P. fluorescens* and mixed culture. No significant difference was observed between the mixed culture and the inoculated/untreated check. This study revealed that the foliar application of *T. viride*-ES1 has good potential in controlling the late blight disease of potato.

Key Words: Biological control, Potato late blight, *Phytophthora infestans*, *Trichoderma viride*, *Pseudomonas fluorescens*

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Introduction

Late blight disease caused by the pathogen *Phytophthora infestans,* is probably the single most important disease of potatoes and tomatoes worldwide (Son *et al*., 2008). Worldwide losses due to late blight are estimated to exceed $5 billion annually and thus the pathogen is regarded as a threat to global food security (Latijnhouwers *et al.,* 2004). In the past few decades, the frequency and severity of the disease have increased in many parts of the world including Ethiopia and have been a serious threat to potato production (Bakonyi *et al.,* 2002). Despite the fact that much of the success in controlling the disease has been due to the application of large quantities of chemical fungicides, their extensive use is causing a serious pollution problem in the environment (Ragunathan and Divakar, 1996). Further, the chemical control of late blight is becoming more difficult due to the appearance of new and more aggressive *P. infestans* strains (Fernandez-Northcote *et al.,* 2000). Thus, an alternative control strategy such as biological control should be sought (Ellis *et al.,* 1999). Biological control of crop disease is receiving increased attention as an environmentally sound alternative to chemical pesticides. Some species of *Trichoderma* and *Pseudomonas* are among the major microorganisms that have shown great potential for biological control of several plant pathogens. *Trichoderma* species have shown biocontrol potential against many plant pathogens including diseases caused by *Sclerotinia minor* (Jones and Stewart, 1997; Dolatabadi *et al*., 2011), *Botryosphaeria berengeriana* f.spp. *piricola, Cladosporium herbarum* (Barbosa *et al.,* 2001), *Dioscorea* spp. (Okigbo and Ikediugwu, 2000) and *Pythium ultimum* (Naseby *et al.,* 2000). Besides, *Trichoderma* species have also shown efficacy against diseases caused by *Rhizoctonia solani*, *Pythium aphanidermatium*, *Fusarium oxysporum, Fusarium culmorum, Gaeumannomyces graminis* var.*tritici, Sclerotium rolfsii, Phytophthora cactorum, Botrytis cinerea* and by *Alternaria* spp. (Kucuk and Kivanc, 2003). Similarly, specific strains of *Pseudomonas* species have shown efficacy in controlling a number of fungal diseases, including *Pythium* root and seed rot of many crops (Mazzola, 1998; Ellis *et al.,* 1999), *Fusarium* wilt in cotton and tomato (Gamliel and Katan, 1992), *Verticillium* wilt of potato (Leben *et al.,* 1987), *Rhizoctonia* stem and root rot of peanut (Savithiry and Gnanamanickam,1987), banana wilt caused by *Fusarium oxysporum* (Sivamani and Gnanamanickam, 1988), bean disease caused by *Sclerotium rolfsii* (Gamliel and Katan, 1992), *Rhizoctonia solani* root infection in tomato (Siddiqui and Shaukat, 2002) and late blight of tomato (Tran *et al.,* 2007). However, significant studies on biological control of late blight of potato are scarce and hence the main aim of this study was to evaluate the efficacy of

local isolates, *Trichoderma viride*-ES1 and *Pseudomonas fluorescens*-Bak150, against late blight of potato *in vitro* and under greenhouse conditions.

Materials and methods

Media used for the culture cultivation and bioassay of the strains were King's B broth for *P. fluorescens* (20 g l^{-1} proteose peptone No.3, 1.5 g l^{-1} K$_2$HPO$_4$, 1.5 g l^{-1} MgSO$_4$. 7H$_2$O and 15 ml glycerol) and for agar plates 15 g l^{-1} agar was added. For *T. viride*, Potato dextrose agar (PDA, Oxoid) was used. Rye agar (60 g l^{-1} rye extract, 20 g l^{-1} sucrose, 0.05 g l^{-1} β- sitosterol and 15 g l^{-1} agar) was employed for antagonistic tests. To isolate *P. infestans*, vegetable juice (V8) (100 ml V8 juice, 1.5 g l^{-1} CaCO$_3$, 0.05 g l^{-1} β-sitosterol and 15 g l^{-1} agar) supplemented with dimethylsulfoxide mix of 20 mg l^{-1} Griseofulvin, 19 mg l^{-1} Nystatin, 10 mg l^{-1} Benlate (50 % w/v, 5 mg l^{-1} Methoxypurine, 30 mg l^{-1} Rifamycin, 5 mg l^{-1} Nalidixic acid, 40 mg l^{-1} 8-azaguanine and 30 mg l^{-1} Neomycin was used.

Microorganisms

T. viride-ES1 was isolated and maintained in PDA slant and *P. fluorescen*s-Bak150 was isolated from a potato field in Bako district, Ethiopia, and maintained in King's B agar slants. *P. infestans* was isolated from infected potato leaves at Holetta Agricultural Research Center (HARC) potato field, Ethiopia.

In vitro antagonistic tests

Antagonistic test between T. viride and P. infestans

Dual culture method was employed to analyze whether *T. viride* inhibits the growth of *P. infestans* as described in Sivakumar *et al.* (2000). Briefly, 1 cm diameter mycelial plug of *P. infestans* (9 days old) was placed on one side of a petri-dish (9 cm diameter) containing rye agar and pre-incubated at 18°C for 2 days to initiate growth. Later, 1 cm diameter disc of *T. viride* (9 days old) was placed 6 cm away from the pathogen on the dual plates; whereas sterile PDA disc was placed in the control plates. The assay was done in triplicates and the radial growth of the pathogen was measured 4 days after incubation at 18°C. The percent radial mycelial growth inhibition (I) was calculated as
I= [(C–T)/C] X100 (Sivakumar *et al.,* 2000); where C is radial growth measurement of the pathogen in the control plates and T is radial growth of the pathogen in the dual plates.

Antagonistic test between P. fluorescens and P. infestans

The antagonism test was carried out on rye agar as described in Georgakopoulos *et al.* (2002). A 20 μl of an overnight culture of *P. fluorescens* grown in King's B broth was spotted at the center of a petri-dish and pre-incubated for 2 days at 18°C followed by placing 1 cm disc of *P. infestans* (9 days old) at either side of the bacterial culture. In the control plates, 20 μl of sterile King's B broth was spotted at the center. This test was done in triplicates. Radial growth inhibition of *P. infestans* was assessed ten days later by measuring the radial growth of the pathogen in the dual and control plates.

Antagonist compatibility test

In vitro compatibility test between *P. fluorescens*-Bak150 and *T. viride*-ES1 was conducted in order to determine whether they can be used in combination. Dual culture plate method described by Siddiqui and Shaukat (2003) was employed. Accordingly, an overnight culture of *P. fluorescens* which was grown in King's B broth was streaked on one side of a petri-dish (9 cm diameter) containing King's B agar (KBA). The other side of the petri-dish was inoculated with 1 cm disc of *T. viride* (9 days old). The plates were then incubated at 25°C and zone of inhibition (if any) was measured. The test was performed in triplicates.

Observation of mycoparasitism by T. viride

Slide culture method described in Sivakumar *et al.* (2000) was employed to determine whether *T. viride*-ES1 parasitizes *P. infestans*. Clean slides were placed on a Z shaped glass rod in 9 cm petri-dishes and sterilized. Small amount of molten rye agar was then poured evenly on the slides. A few drops of sterile water were added to the petri-dish to prevent drying. Mycelial disc of *P. infestans* was inoculated on one side of the slide and pre-incubated at 18° C. *T. viride* was inoculated two days later at a distance of 2.5 cm from *P. infestans*. The presence or absence of coiling and other hyphal interactions were studied under compound microscope after four days of incubation at 18°C.

Greenhouse Trials

Experimental design

Chambers were made in greenhouse using transparent 0.3 mm thick polythene sheet. The temperature in the chambers was adjusted to range

between 14 and 24°C while conducting the experiment. The relative humidity of the chamber was maintained at above 90% by humidifier. Foliar spray method was employed to evaluate the efficiency of spraying the antagonists in controlling airborne inocula of the pathogen. The treatments were (i) *T. viride* only, (ii) *P. fluorescens* only (iii) mixed culture of *T. viride* and *P. fluorescens*, and (iv) Mancozeb (a chemical fungicide included as a standard check). Negative and positive controls were included. Nine replicates were used for each treatment and the pots were arranged in a completely randomized block design method.

Soil, pots and fertilizers

Three kilograms of clay soil (pH=5.7) was added to each of surface sterilized plastic pots of 5 L volume (diameter and depth of 20 cm each) after being autoclaved at 121°C for 30 min. Appropriate doses of fertilizers (i.e., 0.6 g di-ammonium phosphate and 0.5 g urea) were added to each pot.

Selection and preparation of potato seed tubers

The potato cultivar Awash (CIP-378501.3) was used in the experiment. Both the foliage and the tubers are known to be highly susceptible to *P. infestans* strains in Holetta region, Ethiopia. The seed tubers were harvested from HARC and had been stored for 6 months. Disease free and uniform tubers (6-8 cm long) were selected and surface sterilized with 3.5% sodium hypochlorite for one minute and rinsed with sterile distilled water three times.

Preparations of microbial suspensions for foliar spray

A 250 ml suspension of *T. viride* spores was prepared from 9 days old culture plates which were grown on PDA at 25°C. The plates were rinsed with sterile distilled water and the mycelia were carefully scraped off the agar with a bent glass rod. The suspension was then filtered through four layered gauze bandage to separate the spores from the mycelia. The concentration was adjusted to 3.7×10^8 spores/ml (Dubos, 1987) with the help of haemocytometer. A 250 ml of *P. fluorescens* cell suspension was prepared by inoculating the strain into King's B broth followed by shaking for 48 hr (150 rpm) at room temperature. The bacterial suspension was roughly adjusted optically at $ca.1 \times 10^9$ cfu/ml (O.D$_{600}$= 1) (Mulya *et al.,* 1996). A mixed culture of *T. viride* and *P. fluorescens* was prepared by mixing 125 ml of each of *T. viride* (3.7×10^8

spores/ml) and *P. fluorescens* ($1x10^9$ cfu/ml). Mancozeb (Dithane M-45) at a concentration of 5 g l^{-1} was employed.

P. infestans was grown on V8 agar for 9 days in dark at 18°C. Sporangia were harvested from the plates by rinsing the sporangial/mycelial mat with sterile distilled water and scraping the mat using bent glass rod. The suspension was filtered through four folds of sterile gauze bandage to separate the sporangia from the mycelia. The concentration of the sporangia was adjusted to $1x10^3$ sporangia per ml with the help of haemocytometer.

Foliar spraying of antagonists and the pathogen

Potato tubers were planted in pots containing the sterilized soil. After the seedlings reached the rapid expansion phase (18 days after emergence), the control agents were sprayed to run-off onto the plants. For the negative and the positive controls, the plants were sprayed with sterile distilled water. Three days following the application of the control agents, 300 ml suspension ($1x10^3$ sporangia/ml) of *P. infestans* was sprayed to all plants of the different treatments except to those of the positive controls. Prior to spray of the pathogen, the plants were exposed to a high relative humidity (R.H=100%) to moisten their leaves. Following spray of the pathogen, the individual plants were covered with polythene bags to prevent cross infection among adjacent plants.

Data collection and Statistical analysis

The individual plants were rated visually on weekly intervals for percentage of leaf area with symptoms of late blight over the disease progress period based on the assessment key of James, (1971). The average amount of disease developed over the disease progress period was expressed as the area under the disease progress curve (AUDPC) and estimated using the midpoint rule described in Campbell and Madden (1990).

The ratio of infected to total leaves at each leaf position on the main stem of the replicates were also calculated at the 14th day after the foliar inoculation of the pathogen (Kirk *et al.,* 1999). The first leaf above the soil on the main stem was counted as leaf one. Statistical analysis was conducted using the general linear models procedures of the SPSS (version 12). Analysis of variance of differences in the treatments and least significance tests were carried out. Significance was evaluated at *P<0.05* for the tests.

Results

The results of antagonistic activity of *T. viride* against *P. infestans* indicated a radial growth inhibition of 36.7%. After the 4th day, the mycelia of *T. viride* started to overgrow the mycelia of *P. infestans* and by 9th day, *T. viride* had completely overgrown and sporulated over *P. infestans*. The average radii of *P. infestans* in the dual culture plate and the control plate were 0.3 cm and 2.5 cm respectively, ten days after the inoculation of the pathogen. The radial growth inhibition was calculated to be 88%. Figure 1 shows the inhibition of *P. infestans* by *P. fluorescens.* A clear zone of inhibition of *P. infestans* was observed in the dual plate.
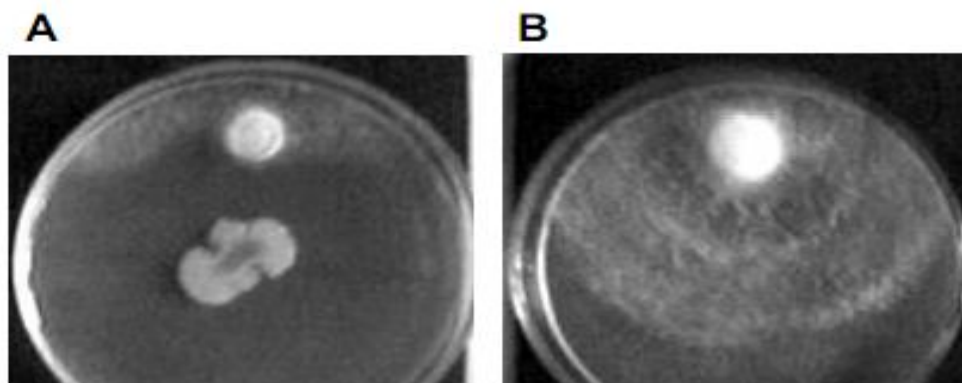

Figure 1. Antagonistic test between *P. fluorescens* and *P. infestans*. Plate A: Dual plate culture of *P. fluorescens* at the centre and *P. infestans* near the periphery and Plate B: control, *P. infestans* alone

Antagonist compatibility test

The average radii of *T. viride* on the 4th day in the dual plate cultures were 2.6 cm whereas 3.9 cm in the control plates. By the 9th day, the colonies of *T. viride* and *P. fluorescens* met and neither organism grew any further (Figure 2). No clear inhibition zone was observed between the bacterial and the fungal colonies.
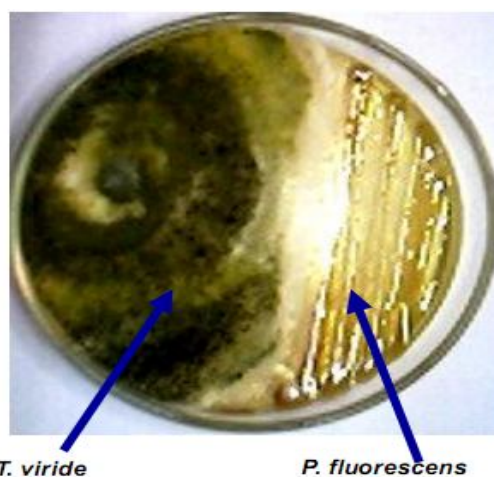
Figure 2. Compatibility test between *T. viride* and *P. fluorescens*. The picture was taken on the 9th day after dual inoculation.

Observation of mycoparasitism by T. viride

Microscopic examination of the hyphal interactions between *T. viride* and the *P. infestans* revealed that *T. viride* hyphae coiled around and/or grew along the hyphae of *P. infestans* (data not shown).

Greenhouse trials

The average AUDPC values of the treatments are presented in Table 1 Each AUDPC value was calculated using the midpoint rule from five disease severity assessments taken weekly. Plants sprayed with *T. viride*, *P. fluorescens* and the chemical fungicide mancozeb had significantly ($P<0.05$) reduced the disease severity compared to the negative control (inoculated/untreated checks) and the mixed culture. The smallest AUDPC values (less disease severity) was recorded in mancozeb (AUDPC=85.9) followed by *T. viride* treated plants. Mancozeb and *T. viride* treated ones were not significantly different while they were different from *P. fluorescens*. *P. fluorescens* also significantly reduced the disease but to a lesser extent compared to the performance of *T. viride* and mancozeb. On the contrary, the mixed culture (AUDPC=999) gave the least control of all the treatments and it was not significantly different ($P>0.05$) from the negative control. The result of the ratio of infected to total leaves at each leaf position on the main stems at the 14th day after foliar spray of the pathogen is presented in Figure 3. Mancozeb and *T. viride* treated plants had the lowest ratio. The highest ratio (0.88) was observed at the 9th leaf of the mixed culture treated plants followed by the 11th leaf of the negative control (0.77). The

overall result showed a relatively higher infection between the 5th and the 11th leaves especially in those plants treated with mixed culture, *P. fluorescens* and the negative control. In most of the treatments, a relatively lower infection was observed in the first three lower leaves. The rate of residual variation (absolute error) for *T. viride* and *P. fluorescens* treatments were found to be 0.07 and 0.7, respectively.

Table 1. Effects of foliar application of *T. viride* and *P. fluorescens* on the progress of late blight disease of potato

Treatments	Mean AUDPC
T. viride	260.0 ± 190.0 c
P. fluorescens	765.1 ± 218.6 b
Mixed culture	999.0 ± 274.5 a
Mancozeb	85.9 ± 77.8 cd
Negative control (inoculated/untreated)	1045.1 ± 227.2 a
Positive control (non-inoculated/untreated)	0.00 ± 0.00 d

Means followed by the same letter are not significantly different.
The area under progress curve (AUDPC) was calculated from five consecutive weekly assessments of percentage of leaf area with symptoms of late blight. The nine replicates were arranged in a completely randomized block design and the midpoint rule was used to calculate AUDPC values.
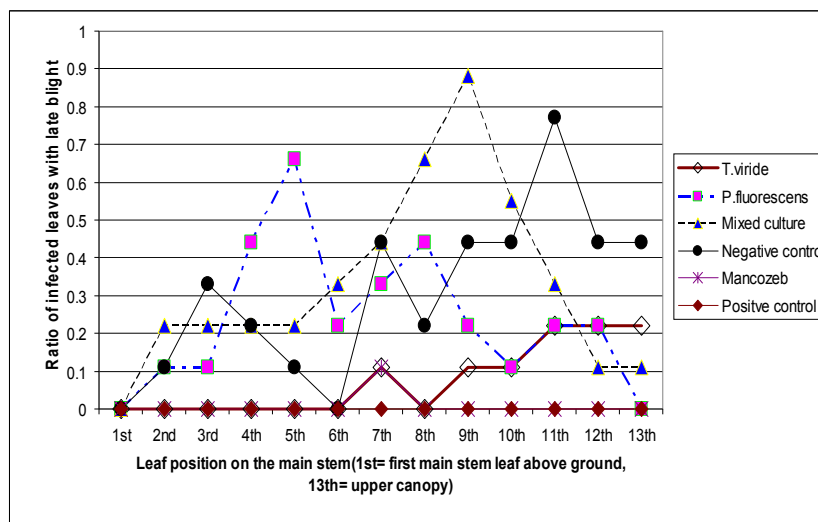

Figure 3. Variation of the ratio of late blight infected leaves with respect to the leaf position on the main stem. The data were taken at the 14th day after the foliar inoculation of *P. infestans*. The values are calculated as ratio of infected to total leaves on the main stem.

Discussion

Late blight, caused by the oomycete pathogen *Phytophthora infestans*, is probably the single most important disease of potatoes worldwide. It is destructive wherever potatoes are grown without the use of fungicides, except in hot, dry, and irrigated areas (Thurston and Schultz, 1981). Biological control is a good alternative for sustainable agriculture to overcome the problems of public concern associated with pesticides and pathogens resistant to chemical pesticides and to become eco-friendly (Akhtar and Siddiqui, 2008). In this study *T. viride* has been found to retard the radial growth of *P. infestans*. This antagonistic mode of action of *Trichoderma* could be attributed to the production of antibiotics and fungal cell wall degrading enzymes (Chutrakul *et al.,* 2008; Sharma *et al.,* 2009). A similar inhibitory action of *Trichoderma* strains (TH1, N47 and T12) against a related oomycete pathogen, *Pythium ultimum,* was reported earlier (Naseby *et al.,* 2000). The observed mycoparasitic action of *Trichoderma* in this study suggests that it has good potential in controlling *P. infestans*. Similar mycoparasitic action of *Trichoderma* strains were also reported against related pathogens, *Phytophthora cinnamomi* (Pugeg and Ian, 2006) and *Phytophthora capsici* (Ezziyyani *et al.,* 2007). Similarly, the pathogen was also strongly (88%) inhibited by *P. fluorescens*. This is corroborated by the study of Torres-Rubio *et al.* (2000) in which *P. fluorescens* inhibited *P. infestans* to an extent of 74%. The clear inhibition zone that was observed in the dual plate is suggestive of production of antagonistic metabolites by *P. fluorescens*. In the compatibility test between *T. viride* and *P. fluorescens*, the colonies of the fungus and the bacterium met on the 9th day and no inhibition zone was formed between the two. In addition, no overgrowth of either organism occurred. The absence of inhibition zone may indicate that their antagonistic metabolites were not inhibitory to each other. This observation was the basis for testing a combination of the two antagonists as "mixed culture" in the greenhouse trial. Similar *in vitro* compatibility was reported between *Pseudomonas aeruginosa* and *Pochonia chlamydosporia* (Siddiqui and Shaukat, 2003) and between *Trichoderma harzianum* and *Streptomyces rochei* (Ezziyyani *et al.,* 2007). The result obtained from the foliar spray method under greenhouse indicated that both of the individual antagonists significantly reduced the severity of foliar phase of late blight infection. The smallest area of leaf infection was observed in mancozeb treated plants and the highest in the untreated checks (negative control). Even though mancozeb treated plants showed the least infection, the difference with that of *T. viride* treated plants was not statistically significant. This indicated that the performance of *T. viride* was comparable to that of the chemical fungicide. It is possible that more than one antagonistic mechanism

1598

could have been involved in the reduction of the disease. However, it needs to be further examined as the relative importance of the mechanisms is dependent on the particular isolate, the target organism and also the ambient environmental conditions (Tronsmo, 1996). The rationale behind the use of mixed culture is that multiple strains allow the deployment of several different biocontrol mechanisms simultaneously. Besides, effective control of the target pathogen over diverse set of environmental conditions could be expected if strains with same ecological requirements are included in the inoculant (Mazzola, 1998). The performance of the mixed culture, however, was the least and it was not significantly different from the inoculated/untreated check. Perhaps competition for iron between the two antagonists could be one possible reason; as such incompatibility was reported between *Trichoderma hamatum* and *Pseudomonas spp* in iron deficient soil (Hubbard *et al.,* 1983). However, evidence for the role of siderophores in competition for iron in the phyllosphere is needed if this argument is to be true. The agar test showed compatibility as neither inhibition zone nor overgrowth between the antagonists was observed. Hence, this implies that *in vitro* compatibility does not always indicate *in vivo* compatibility. Therefore, at this point it would be worth mentioning that sometimes mixtures of bioagents do not ensure improved biological control (Mazzola, 1998). In the greenhouse trial, the control agents were applied three days ahead of the pathogen. The reason is antagonists should occupy the site earlier than the pathogen's arrival (Baker and Cook, 1974). In a study by Kexiang *et al.* (2002), *Trichoderma* species were found to give better control of *Botryosphaeria berengeriana* when inoculated three days in advance than when the two are co-inoculated. The ratio of infected leaves on the main stem was significantly lower in those treated with mancozeb and *T. viride* than the rest of treatments. This reflected the strong performance of *T. viride*. The reason for a lower infection that was observed in the lower leaves of most of the treatment is because potato leaves at the base of the stem are less susceptible to late blight infection than leaves lying closer to the flower (Carnegie and Colhoun, 1982). This result is in agreement with Kirk *et al.* (1999).

Germination of fungal spores on the leaf surface is a critical stage in the development of the host-pathogen interface, and one in which the pathogen is often vulnerable (Campbell, 1989). The motile zoospores of *P. infestans* have no cell wall and hence are probably extremely vulnerable to adverse conditions, and are also the main infective propagules (Erwin and Ribeiro, 1996). Thus, zoospores can be a potential target in biocontrol of *P. infestans*. In conclusion, *T. viride*-ES1 showed good potential in controlling potato late blight under greenhouse conditions. In addition to that, the *T. viride* used in this study was

isolated from local climatic conditions and effectively proved that it has potential to be used as a biocontrol agent in the future. Further research is needed to determine its field performance. In general, as any other biocontrol systems, biological control of late blight should be regarded as one facet of the integrated control program rather than a method to be used alone.

Acknowledgements

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