Characterisation of *Trichoderma* isolates as agents for engineering disease suppressive composted growing media

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Abstract

Six isolates of *Trichoderma* were assessed for suitable attributes for potential inoculation into composted material to develop disease suppressive growing media. Each isolate had previously been shown to possess *in vitro* suppressive properties against root-rot diseases via the production of inhibitory secondary metabolites. In the present study, isolates were further investigated for *in vitro* mycoparasitic properties and *in vivo* suppression of the pathogens *Pythium ultimum* and *Fusarium oxysporum*. Mycelial growth of each isolate was also tested *in vitro* on potato dextrose agar (PDA) against ranges of abiotic properties found in composted materials, such as electrical conductivity(EC), pH and temperature. Four of the six isolates displayed no reduced growth on PDA media with EC values of 9.45 mS cm^{-1}, with two isolates showing slightly reduced growth. pH was found to have a much more influential effect on mycelia growth, with only two of six isolates displaying uninterrupted *in vitro* growth across a range of pH6 to pH10. The *Trichoderma* isolate CS30-01 was found to be the sole isolate with identifiable mycoparasitic properties *in vitro*. Isolate CS30-01 also displayed no reduced growth at the EC and pH ranges tested. Temperature tests found isolate CS30-01 to possess the highest tolerance at 37C°, while also displaying good growth across a temperature range of 15-35°C. Inoculation of a peat based growing media with a suspension of CS30-01 spores had no negative effects on plant germination or growth, and indicted co-incubation of CS30-01 and *P. ultimum* significantly reduced disease severity (P=0.005) on *Brassica rapa* seedlings. Evidence of suppression of *F. oxysporum* disease severity on *Allium cepa* was observed, but not found to be statistically significant. The results from this study indicate that the isolate CS30-01, identified as a strain of *T. harzianum*, possesses suitable biocontrol and growth properties for developing disease suppressive growing media.

INTRODUCTION

Over the years numerous composted organic materials (COMs) have been shown to possess inhibitory properties against soil borne fungal and oomycete root rot pathogens, when used as components of plant growing media (Fuchs *et al.*, 2017). However, disease suppressive properties of COMs have been shown to be highly inconsistent and levels of actual disease suppression are often variable (Termorshuizen *et al.*, 2006). Much research has focused on linking the components of specific COMs and their microbial ecology to disease suppressive properties in order to predict/produce consistently suppressive COMs (Mehta *et al.*, 2014). However, the key to producing large scale batches of consistently suppressive composts still remains elusive.

Current research suggests that disease suppressive properties can be attributed mainly to biotic properties of COMs such as the presence of specific microbial groups/species (Blaya *et al*., 2016). Many studies have isolated and characterised microbial species with inhibitory properties against the growth of microbial pathogens from suppressive COMs (Mohamed *et al*., 2016). Suppressive *Trichoderma* species have been widely isolated from suppressive COMs and have been shown to control growth of pathogens through the production of inhibitory secondary metabolites (antibiosis) and mycoparasiticism (Harman *et al*., 2004). Mycoparasitic properties of *Trichoderma* species have been attributed to the combined action of antibiosis and production of fungal cell wall degrading enzymes (CWDEs), such as chitinases and cellulases (Harman *et al*., 2004). *Trichoderma* chitinases have been shown to be key CWDEs in mycoparasiticism, inducing lysis of target fungi by attacking the chitin present in the cell wall, but not affecting the *Trichoderma's* own cell wall (Gruber and Seidl-Seiboth, 2012). Oomycetes, such as *Pythium* and *Phytophthora* species, have cellulose based cell walls which lack chitin (Fauke *et al*., 2015). Cellulase enzymes have been shown to be important CWDEs in the control of *Pythium* and *Phytophthora* pathogens (Picard *et al*., 2000).

Trichoderma species have been successfully used as seed coatings in plant growth systems as biostimulants and biocontrol agents (BCAs) in soil systems (Xiong *et al*., 2017). However, suppressive effects of *Trichoderma* species have been shown to be more potent and have longer lasting effects in COM substrates, rather than in growing media solely composed of soil or peat (Mehta *et al*., 2014). Inoculation of small and large scale batches of COMs with *Trichoderma* species has been shown to successfully control a range of root rot pathogens in specific waste streams (Lopez-Lopez *et al*., 2016; Pascual *et al*., 2017). However, selection of *Trichoderma* strains suitable for inoculation into a wide range of COMs has received less attention.

In the present study, suppressive *Trichoderma* isolates obtained from COMs were assessed for their potential for inducing disease suppressive properties in various composts. Mycelial growth of *Trichoderma* isolates were assessed *in vitro* for their ability to tolerate the ranges of abiotic parameters that were identified in COMs in a previous study (McGee *et al*., 2014). *Trichoderma* isolates were isolated from various COMs and identified as producing inhibitory secondary metabolites against fungal and oomycete pathogens *in vitro* previously (McGee *et al*., 2016). In the present study the *Trichoderma* isolates were assessed *in vitro* for evidence of mycoparasiticism of the pathogens *Pythium ultimum, Phytophthora erythroseptica* and *Fusarium oxysporum*. An isolate of *Trichoderma harzianum* "CS30-01" was found to be the most suppressive tested, and displayed the most tolerant mycelial growth against abiotic properties *in vitro*. Plant based pot trials were conducted to determine the ability of CS30-01 to suppress *P. ultimum* and *F. oxysporum* in a peat based growing medium. The purpose of this study was to identify an isolate with characteristics suitable for inoculated into various COMs producing value added benefits of disease suppression.

MATERIALS AND METHODS

Isolates

The six isolates of *Trichoderma*: CS07-01, CS08-01, CS21-02, CS21-08, CS30-01 and CS31-08 assessed in the present study were obtained from various COMs and have been characterised in a previous study (McGee *et al*., 2016). The pathogens *P. ultimum* and *P. erythroseptica* were obtained from Mr. Brain Fagan, Plant Pathology Laboratory, University College Dublin, Dublin. The *F. oxysporum f. sp. cepa* isolate was isolated from a diseased onion in Kinsealy Teagasc Horticultural Centre, Malahide, Co. Dublin. A full characterisation of the isolates was performed in a previous study (McGee *et al*., 2014).

In vitro growth trials

In vitro growth trials were conducted on plates of potato dextrose agar (PDA). Trials on the effect of pH, EC and temperature on mycelial growth were conducted in triplicate and in dark conditions, trials were terminated once growth of colonies in one treatment reached

the periphery of the PDA plate. EC and pH trials were conducted at 25°C. For the pH trials pre-autoclaved media pH was adjusted with 1M NaOH and HCI to pH 5, 6, 7, 8, 9 or 10. The EC level in PDA was adjusted with 1M NaCl in pre-autoclaved media. Unadjusted PDA (2.46 mS cm^{-1}) was used as a control in the EC trial; tests were terminated when mycelial growth in colonies in the control plates reached the periphery. Upper temperature tolerance tests conducted on all isolates were conducted between 30-40°C using 1°C increments. Temperature trials on CS30-01 were conducted simultaneously in separate incubators set to 25, 30, 35 and 40°C. Mycelial growth was recorded as surface area (πr^2) of colonies.

Carbon Utilisation Tests

Carbon utilisation tests were conducted *in vitro* on the 6 *Trichoderma* isolates to determine degradation characteristics associated with mycoparasiticism of fungal cell walls. Isolates were grown on a media composed of 0.67 $g.l^{-1}$ of yeast nitrogen base, 15 $g.l^{-1}$ bacteriological agar, and 10 $g.l^{-1}$ of either cellulose or chitin. As a control, carbon source free media was used. Plates of media were inoculated with spores of *Trichoderma* cultures and incubated in dark conditions at 25°C for 5 days before being assessed for growth and sporulation. Spore inoculation was chosen over plug inoculation of plates to reduce the residual effect of nutrients present in bored culture plugs influencing mycelial growth.

Investigation of Mycoparasiticism

Evidence of parasitic properties was investigated *in vitro* in the 6 *Trichoderma* isolates using dual culture techniques against the pathogens *P. ultimum, P. erythroseptica* and *F. oxysporum*. Bored plugs (5 mm) of the *Trichoderma* isolate and the pathogen were inoculated on opposite sides of a PDA plate and incubated at 25°C in dark conditions until mycelial growth was roughly 5 mm apart. A glass cover slide, sterilised on both sides with UV light in a laminar hood, was then placed over the mycelial contact region, and dual cultures were then further incubated to allow contact between the isolates to occur. Once contact was observed, mycelial interactions beneath the cover slide region were observed daily using a compound light microscope (Olympus, BX41).

Plant Growth Trials

The ability of the *T. harzianum* isolate CS30-01 to suppress *P. ultimum* and *F. oxysporum in vivo* was investigated with inoculated peat based growing media and tested using plant growth trials with the *Brassica rapa - P. ultimum* and *Allium cepa - F. oxysporum* pathosystems. A homogenous growing medium was produced using Irish moss peat, fertilised with 4 $g.kg^{-1}$ compound fertilizer (NPK: 12-12-24) and limed to pH 6.37, with a measured EC of 0.61 $mS.cm^{-1}$. 100g batches of the growing medium were added to sterile 500 ml conical flasks. Four flasks were aseptically inoculated with *P. ultimum* (PU) or *F. oxysporum* (FO) using three bored 5 mm plugs. Flasks were sealed with sterile cotton wool and thin foil caps and incubated at 25°C in dark conditions overnight. After 24 hours three bored 5 mm plugs of CS30-01 were added to two *P. ultimum* inoculated flasks (TRIC-PU) and two *F. oxysporum* inoculated flasks (TRIC-FO) and incubated for 14 days to induce colonisation, producing two replicates of each treatment. As a control four inoculated batches of CS30-01 inoculated peat (Tric) were produced, two for each pathogen growing trial. After the 14 day incubation period peat batch replicates were tested for inoculation by adding peat particles to PDA plates.

Once peat batches were identified as successfully inoculated, duplicate batches were homogenised. The treatments of the pure pathogen inoculum, the CS30-01 inoculum and the co-inoculated media and were randomly added to seed modules. Two seeds were added to each module with seven replicates for each treatment in the *B. rapa - P. ultimum* pathosystem

and nine replicates for each treatment in the *A. cepa* - *F. oxysporum* pathosystem. Plant growth trials were conducted in glasshouse units for a 14 day period, after which germination rates and plant fresh weights (FW) were recorded. Non germination of seeds was recorded as 0.

Statistical analysis of plant FW was carried out using ANOVA with the software package SAS 9.1.3.

RESULTS

Growth Trials

Initially, growth of the six *Trichoderma* isolates were tested against a range of temperatures, pHs and the upper limit of EC that were identified in a survey of COMs. Only, two isolates, CS30-01 and CS21-08, displayed uninterrupted growth across the pH ranges tested. A general tendency of mycelial growth to diminish as pH levels increased was observed for the 4 other isolates (Figure 1a). High EC of a growth medium was found to have little effect on mycelial growth of *Trichoderma* isolates, with growth only slightly reduced for two isolates: CS08-01 and CS31-08 (Figure 1b). Temperature trials indicated growth tolerances of between 31-37°C for the isolates, with the isolate CS30-01 having the highest tolerance of 37°C (Figure 1c). A temperature dependent growth tests was conducted on the isolate CS30-01, indicating optimum growth range between 20-30°C (Figure 1d).

Mycoparasiticism and Carbon Utilisation

Carbon utilisation tests indicated that all *Trichoderma* isolates were capable of utilising cellulose efficiently as a carbon source, with colonies reaching the periphery of the plate and displaying extensive sporulation (Figure 2a). Growth of the *Trichoderma* isolates on the carbon free medium was severely limited. Mycelial growth and sporulation on the chitin medium was observed for all isolates, however limited growth was observed for CS07-01 (Figure 2b). Microscopic investigation of mycoparasiticism indicated that only one isolate, CS30-01, displayed clear mycoparasitic properties. Observation of mycelial growth indicated extensive growth of CS30-01 mycelia towards pathogens *in vitro*. Mycelia of CS30-01 were observed growing parallel to, and curling around mycelia of the pathogens *P. ultimum* and *P. erythroseptica*, indicating mycoparasitic characteristics (Figure 3a & 3b). Evidence of mycoparasiticism of *F. oxysporum* was less obvious, though *T. harzianum* mycelia was observed towards mycelia of *F. oxysporum* and appeared to interact.

Plant Growth Trials

Plant germination and growth trials were carried out in inoculated growing media. Growth trials on *B. rapa* were performed in growing media inoculated with CS30-01 (TRIC), *P. ultimum* (PU) and co-inoculated media (TRIC-PU). Average FW of seedlings planted in PU was significantly reduced compared to media inoculated with TRIC (P=0.005) and TRIC-PU (P=0.0001), indicating pathogenicity of the treatment (Figure 4a). No significant difference was observed between FW in TRIC and TRIC+PU inoculated growing media (P=0.09), indicating suppression of pathogenicity of the *P. ultimum* inoculum. 100% seed germination was observed in TRIC and TRIC-PU, while germination in PU was 29%. The lack of evidence of disease symptoms in *B. rapa* seedlings grown in TRIC-PU indicates that CS30-01 completely inhibited growth of *P. ultimum in vivo*.

Growth trials on *A. cepa* were performed in growing media inoculated with CS30-01 (TRIC), *F. oxysporum* (FO) and co-inoculated media (TRIC-FO). Average FW of *A. cepa* seedlings was significantly reduced in FO compared to TRIC (P=0.002) indicating the pathogenicity of the treatment (Figure 4b). However, though average FW in TRIC-FO was

higher than FW in FO, the difference was not found to be significant. Germination of seedlings in TRIC was 100%, however, germination of seeds in TRIC-FO and FO was found to be 56% and 22% respectively, indicating the presence of pathogenicity in both treatments. FW in TRIC-FO was not found to be significantly different to either TRIC or FO. The higher level of germination in the TRIC-FO treatment indicates that some evidence of suppression of *F. oxysporum*.

DISCUSSION

Trichoderma species are widely distributed in the natural environment and have been isolated from various habitats such as decaying woody materials (Bharathiraja *et al*., 2017), soil (Gill *et al*., 2009) and compost (McGee *et al*., 2016). There are numerous studies detailing the isolation of *Trichoderma* species from environmental samples and subsequent selection of the most efficient suppressive strains based on *in vitro* suppressive characteristics, usually secondary metabolites, for use in growing media trials (Aleandri *et al*., 2015; El Komy *et al*., 2015; Widmer, 2014). However, for many microbial isolates, suppressive characteristics identified *in vitro* have often lacked efficacy when tested in *in vivo* plant trials, with little investigation into the underlying reasons. In particular, few studies have reported the influence of *Trichoderma* growth characteristics on their ability to colonise growing materials and suppress plant pathogens. Given how growing media have substantially differing physicochemical characteristics, the ability of *Trichoderma* biocontrol agents to tolerate wide ranges of abiotic properties and grow efficiently is critical for establishment of suppressive properties.

This study has highlighted the varying growth characteristics of a selection of six *Trichoderma* isolates with plant pathogen suppressing properties. The isolates were obtained from screening a range of commercial COMs for cultivatable fungal species with potential biocontrol properties (McGee *et al*., 2016). Characterisation of the COMs in a previous study identified broad ranges of physicochemical properties, such as pH, EC and temperature (McGee *et al*., 2014). In this study, the growth of two *Trichoderma* isolates; CS21-08 and CS30-01, was found to be highly tolerant to the ranges of pH and EC found in COMs. The isolate CS30-01 was also found to tolerate the highest temperatures and displayed good growth across a broad temperature range.

All of the *Trichoderma* isolates investigated in the present study, had been previously shown to suppress a selection of plant pathogens *in vitro* through a combination of over-growth and inhibitory secondary metabolites (McGee *et al*., 2016). In the present study, isolate CS30-01 was the sole isolate where mycoparasitic properties against the selection of plant pathogens tested, were identified. Mycoparasitic properties have been linked to the production of suppressive secondary metabolites as well as CWDEs (Harman *et al*., 2004). The isolate CS30-01 had been previously shown to produce the most efficacious secondary metabolites of the six isolates tested (McGee *et al*., 2016). However, all six of the *Trichoderma* isolates were identified to be capable of utilising cellulose and chitin enzymes, indicating the ubiquitous presence of CWDEs. This indicates that the high level of antibiosis of CS30-01 may play a key role in its mycoparasiticism.

The combination of suppressive mechanisms identified in CS30-01 and its broad tolerance for abiotic properties in COMs indicated that this isolate had suitable properties for developing disease suppressive growing media. Growth trials on the two pathosystems investigated in this study indicated high efficacy of the pathogen treatments which induced stunted growth and reduced germination. The growth trial on *B. rapa* seedlings in the growing media co-incubated with CS30-01 and *P. ultimum* indicated complete suppression of disease symptoms. Similarly evidence of reduced disease severity of *F. oxysporum* on *A.*

cepa seedlings was observed. This study demonstrated that the isolate CS30-01 could efficiently establish itself in non-sterile growing media and out complete high levels of pre-inoculated pathogens. This particular isolate will receive further research to investigate its commercial potential.

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FIGURES

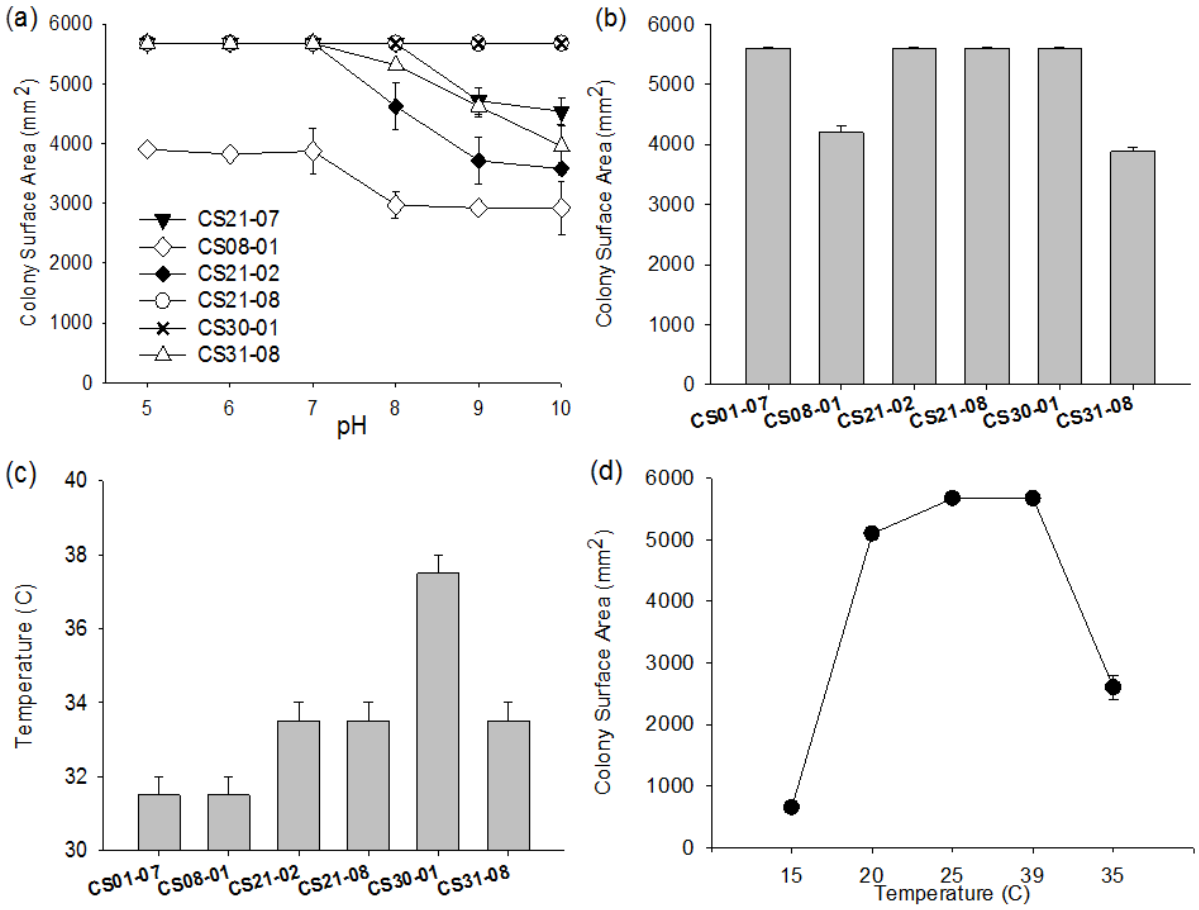

Figure 1. Mycelial growth of *Trichoderma* isolates in response to a pH gradient (a), high EC (b) and highest temperature tolerance (c). Mycelial growth of the isolate CS30-01 in across a temperature gradient (d). Bars = standard deviation.

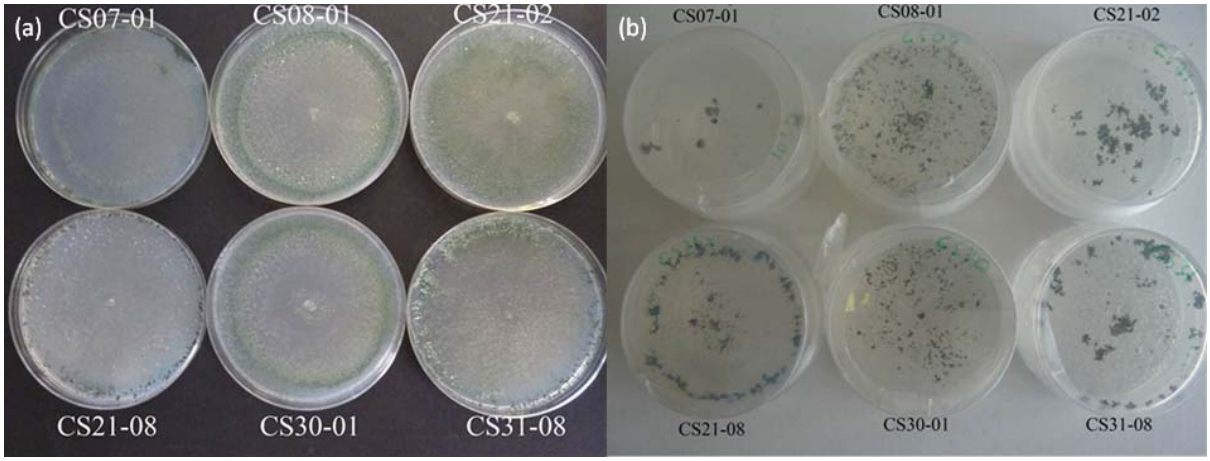

Figure 2. Growth and sporulation of *Trichoderma* isolates in carbon utilisation trials on cellulose (a) and chitin (b).

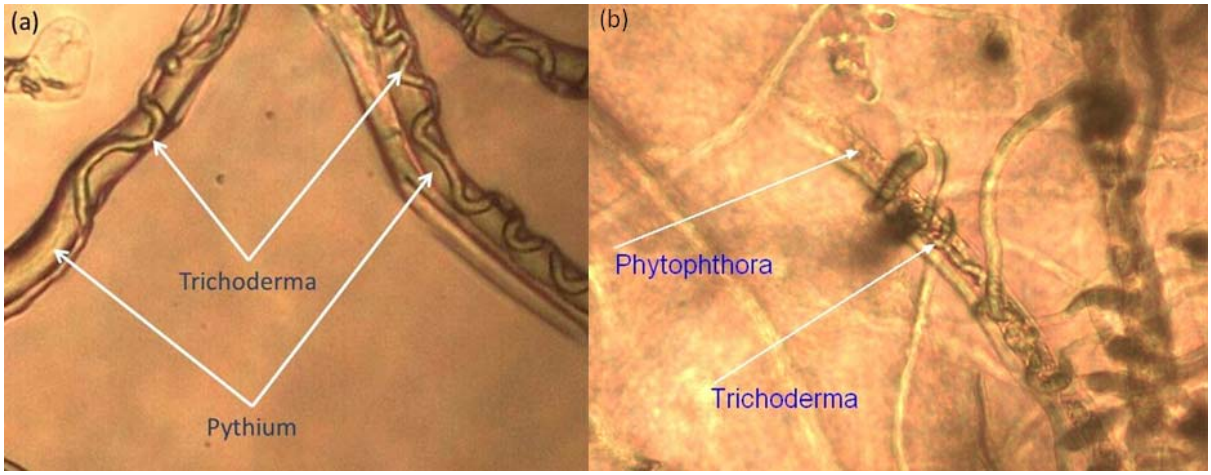

Figure 3. Coiling of *T. harzianum* isolate CS30-01 mycelia on and around mycelia of *P. ultimum* (a) and *P. erythroseptica* (b).

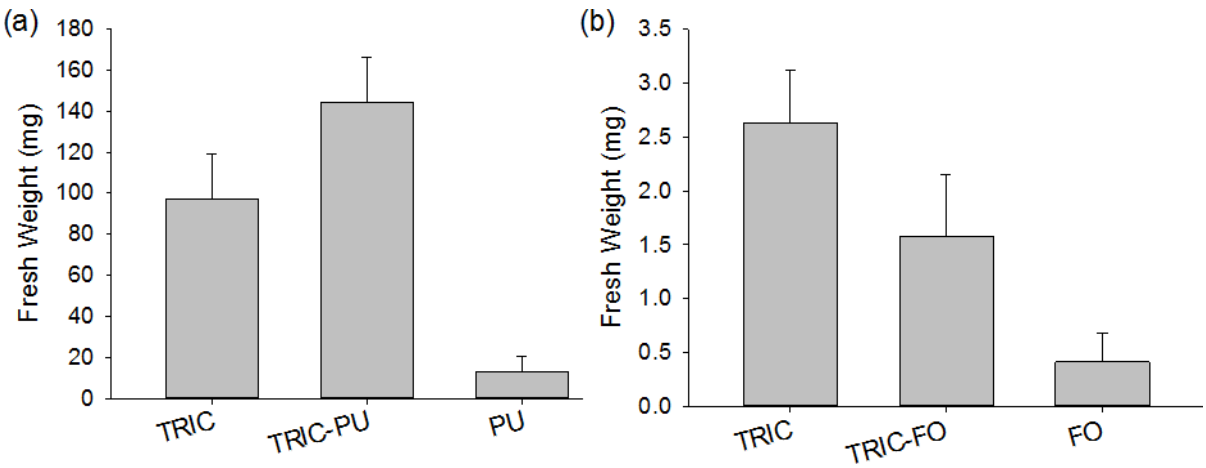

Figure 4. Growth trials testing the suppressive ability of the isolate *T. harzianum* CS30-01 on the disease severity *of P. ultimum* on germination and growth of seedlings of *B. rapa* (a) and *F. oxysporum* on germination and growth of seedlings *A. cepa* (b). Bars = Standard Error.