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Mass production of *Trichoderma asperellum* SQU7 using *Phragmites australis* as a substrate carrier for managing damping-off disease in cucumber

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Trichoderma species are commonly used as biocontrol agents to manage various soilborne fungal and oomycete pathogens affecting crop plants. Various organic and inorganic carriers for mass production of *Trichoderma* intended for field application have been documented in the literature. In this study, *Trichoderma asperellum* strain SQU7, isolated from Omani soil, was screened for antagonistic activity against *Pythium aphanidermatum*, the causal agent of damping-off disease in cucumber. In addition, the possibility of using common reed (*Phragmites australis*) for mass production of *T. asperellum* was explored and its efficacy in controlling *P. aphanidermatum*-induced damping-off in cucumber was evaluated under controlled glasshouse conditions.

The findings showed that *T. asperellum* provided a strong inhibitory effect on the growth of *P. aphanidermatum* in the confrontation assay and caused morphological abnormalities in the pathogen's hyphae as determined by means of scanning electron microscopy analysis. *Trichoderma asperellum* proliferated well on common reed, and significantly suppressed *P. aphanidermatum* damping-off incidence in cucumber seedlings grown in pots. This study presents an efficient and affordable approach for the large scale production of *T. asperellum*, which can be utilised to effectively control damping-off of cucumber in an environmentally sustainable way.

Key words: biofungicide, biological control, common reed, *Cucumis sativus*, disease management, soilborne pathogen.

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Houby rodu *Trichoderma* jsou běžně využívány pro biokontrolu půdou přenášených patogenních hub a oomycetů, napadajících pěstované rostliny. V literatuře jsou doloženy různé možnosti produkce druhů rodu *Trichoderma* na anorganické i organické hmotě pro jejich potenciální využití v kulturách plodin. Kmen *Trichoderma asperellum* SQU7, izolovaný z ománské půdy, byl v této studii testován coby antagonista *Pythium aphanidermatum*, původce vadnutí okurek. Mimoto byla prověřena možnost využití rákosu (*Phragmites australis*) pro masovou produkci *T. asperellum* a ve skleníkových podmínkách sledována účinnost této houby pro potlačení pythiového vadnutí okurek.

Výsledky konfrontačních testů ukazují, že *T. asperellum* má silný inhibiční efekt na růst *P. aphanidermatum* a působí abnormální formování hyf tohoto patogena, jak je vidno na snímcích z elektronového mikroskopu. Dále je zřejmé, že *T. asperellum* se dobře rozrůstá na rákosovém substrátu a její aplikace v květináčích prokazatelně omezuje vadnutí okurkových sazenic. Studie tak představuje efektivní a cenově dostupnou možnost produkce *T. asperellum* ve větším měřítku a její využití v účinné kontrole vadnutí okurek environmentálně udržitelným způsobem.

INTRODUCTION

Damping-off, incited by *Pythium aphanidermatum* (Edson) Fitzpatrick, is a major disease problem in cucumber (*Cucumis sativus*, *Cucurbitaceae*) production in both glasshouses and fields. Synthetic chemical fungicides are frequently employed to manage this oomycete disease when disease-resistant cultivars are unavailable. This practice causes pollution of the environment and raises the risk of emergence of pathogenic strains resistant or tolerant to fungicides. The shift in agricultural practices from conventional to organic farming prompts the utilisation of naturally existing antagonistic soil microorganisms performing functions similar to chemicals. In agricultural practice, biocontrol agents (BCAs) are safe and sustainable substitutes for pesticides. In the 'augmentative biological control' strategy, effective antagonistic microorganisms are identified, mass-produced on suitable substrates, and applied to plants or soil to control plant diseases (Kohl et al. 2019).

Trichoderma species are employed extensively to manage different soilborne fungal diseases which affect crop plants, such as damping-off, wilts and root rots. *Trichoderma* species are asexually reproducing filamentous fungi commonly found in agricultural soils. The main mechanisms by means of which *Trichoderma* spp. combat soilborne fungal diseases take place through mycoparasitism, competition for vital nutrients, lytic enzyme production and antibiosis (Sood et al. 2020, Yao et al. 2023). In addition, *Trichoderma* application enhances seed germination rates, promotes plant growth and optimises nutrient uptake efficiency (Yao et al. 2023). Certain strains of *Trichoderma* also synthesise plant hormones such as

auxins, cytokinins, ethylene, indole acetic acids, and gibberellins (Sinha et al. 2021). Furthermore, *Trichoderma* has the potential to break down complex substances like hemicellulose and cellulose (Sinha et al. 2021). Several species of *Trichoderma* including *T. viride* Pers., *T. harzianum* Rifai, *T. hamatum* (Bonord.) Bainier, *T. atroviride* P. Karst., *T. longibrachiatum* Rifai, *T. virens* (J.H. Miller, Giddens & A.A. Foster) Arx, *T. koningii* Oudem., *T. ghanense* Yoshim. Doi, Y. Abe & Sugiy., *T. asperellum* Samuels, Lieckf. & Nirenberg, and *T. citrinoviride* Bissett have been reported as BCAs (Mukherjee et al. 2014, Ghazanfar et al. 2018, Kumar et al. 2023, Yao et al. 2023, Al-Shuaibi et al. 2024). A wide range of *Trichoderma*-based biocontrol products including Tenet, Sentinel, Trianum-G, BINAB-T, Remedier, Xedavir, Tusal, Esquive, Tri-Soil, TrichoFlow WP, Trichodex, RootShield, and Supervivit are available on the market (McLean et al. 2005, Stewart & McLean 2007, Altintas & Bal 2008, Martinez et al. 2023)

The successful implementation of biological control using microbial antagonists requires the development of suitable formulations and delivery systems. The formulation of biocontrol agents depends on the efficiency of biomass production and maintenance of viability throughout the process (Adekunle et al. 2001). Growing *Trichoderma* spp. on readily biodegradable substrates with an extended shelf life can be advantageous for field uses. Several organic and inorganic substrates have been tested for multiplication of *Trichoderma* spp. based on their local availability (Kumar et al. 2023). In Oman, common reed (*Phragmites australis*, *Poaceae*) is a perennial plant species commonly found in 'wadi' ecosystems. It is a tall grass used as a source of biofuel, roof thatching, coarse fodder, and bedding material for suckler cows (Durant et al. 2020), and has found application in bioremediation of petroleum production water, which contains high concentrations of petroleum hydrocarbons, trace levels of phenols, emulsifiers, and a variety of metals in various concentrations (Denney 2006).

A reedbed produces approximately 40 tonnes of dry biomass/ha/year. We hypothesised that common reed could serve as an excellent organic substrate for mass production of *Trichoderma* spp. and that the inoculum on the carrier might be effective in managing soilborne diseases including damping-off of vegetables. During the isolation of native fungal and bacterial antagonists from Oman agricultural soils, we obtained the native isolate *T. asperellum* SQU7 from a soil sample collected in an organic greenhouse. This study aims to (i) determine the antagonistic activity of *T. asperellum* strain SQU7 against *P. aphanidermatum*, (ii) explore the possibility of using common reed as a medium for large scale production of *Trichoderma* spp. biomass, and (iii) evaluate the efficacy of *T. asperellum* SQU7 inoculum on common reed to control *P. aphanidermatum* damping-off disease of cucumber.

MATERIAL AND METHODS

Trichoderma asperellum. *T. asperellum* strain SQU7 (GenBank accession number PV052721) was originally isolated from a soil sample collected in an organic greenhouse. The strain was obtained from the Plant Pathology Research Laboratory, Sultan Qaboos University and maintained on potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, UK) slants at 4 °C.

Oomycete pathogen. Axenic culture of *Pythium aphanidermatum* (Edson) Fitzpatrick strain Sala5 isolated from a diseased cucumber plant (Al-Mahmooli et al. 2024) was obtained from the Plant Pathology Research Laboratory, Sultan Qaboos University and maintained on PDA at 4 °C.

Assessing *T. asperellum* for its antagonistic potential. The antagonistic potential of *T. asperellum* SQU7 against *P. aphanidermatum* was tested in vitro using a confrontation assay (Pandian et al. 2016). Briefly, one mycelial disc of both *T. asperellum* and *P. aphanidermatum* were placed on the same PDA plate (9 cm diameter) approximately 1 cm from the edge and incubated at 27 °C for 5–7 days. After the incubation period, the radial growth of *P. aphanidermatum* towards the antagonist was assessed and the percentage of inhibition in relation to unchallenged control colonies was determined in triplicate plates.

Scanning electron microscopy (SEM) analysis of *P. aphanidermatum* hyphae. The hyphal morphology alterations in *P. aphanidermatum* when co-cultivated with *T. asperellum* SQU7 were examined using scanning electron microscopy. Agar plugs (6 mm) containing mycelium of *P. aphanidermatum* from the interaction zone of the confrontation assay plates were collected and prepared for SEM following the protocol by Bozzola & Russell (1999). The samples were coated with gold using a BioRad SEM coating system and analysed with a JEOL JSM 4500LV SEM (JEOL, Akishima, Japan) at 20 K accelerating voltage. Agar plugs from *P. aphanidermatum* colonies grown without *T. asperellum* SQU7 and prepared using the same procedure served as controls.

Mass production of *T. asperellum* using common reed. Shade-dried reed biomass (1 kg) was chopped into small (approx. 5 cm-long) pieces and soaked in 1% glucose (w/v) for 30 min. The excess glucose solution was drained, plant biomass was packed in autoclavable polythene bags and autoclaved. The sterilised reed biomass was inoculated with three 8-mm mycelial discs of *T. asperellum* taken from an active (7-day-old) PDA culture and incubated at 28 ± 2 °C. The population of *Trichoderma* on the substrate was estimated 14 and 30 days after inoculation with the serial dilution technique using Rose Bengal Agar (RBA) medium as described by Pandya & Sabalpara (2012).

Glasshouse experiment. A pot culture experiment was conducted under controlled conditions to evaluate the efficacy of reed-grown *T. asperellum* SQU7 in controlling *P. aphanidermatum* damping-off in cucumber. Reed biomass (25 g or 50 g) containing *T. asperellum* was mixed with 400 g of sterilised multi-purpose compost (Bulrush Horticulture Ltd, Magherafelt, UK), poured into sterile pots (14 cm in diameter, 12.5 cm in depth), and incubated for 48 h. *Pythium aphanidermatum* grown on a 90 mm-diameter PDA plate was cut into eight parts and one part of the pathogen inoculum was added to each pot and mixed thoroughly with soil; following, 10 cucumber seeds (Hybrid Ezz cucumber F1; Agrimax Group S.L.U., Barcelona, Spain) per pot were sown. As a positive control, 50 ml of Metalaxyl (0.1%; RidomilGold; Syngenta, Basel, Switzerland) was applied to the pots to compare its efficacy with that of the *T. asperellum* strain. Five replications were maintained. Damping-off disease incidence was recorded 17 days after sowing by counting the number of infected plants. The experiment was repeated twice.

Statistical analysis. The data were analysed using analysis of variance (ANOVA) with the general linear model, and treatment means were compared using Tukey's test at $P = 0.05$. Statistical analysis was performed using SAS v8 software (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Antagonism of *Trichoderma asperellum* against *Pythium aphanidermatum*

The in vitro confrontation assay showed that *T. asperellum* SQU7 exhibited antagonistic activity against *P. aphanidermatum* with 55% mycelial growth inhibition compared to the control (Figs 1 and 2). SEM analysis of *P. aphanidermatum* hyphae from the confrontation assay plates at the interaction zone of the pathogen with *T. asperellum* revealed marked abnormalities, including deformation, shrinkage, distortion, and hole formation, while hyphae from the control plates appeared to be normal, with a smooth surface (Fig. 3).

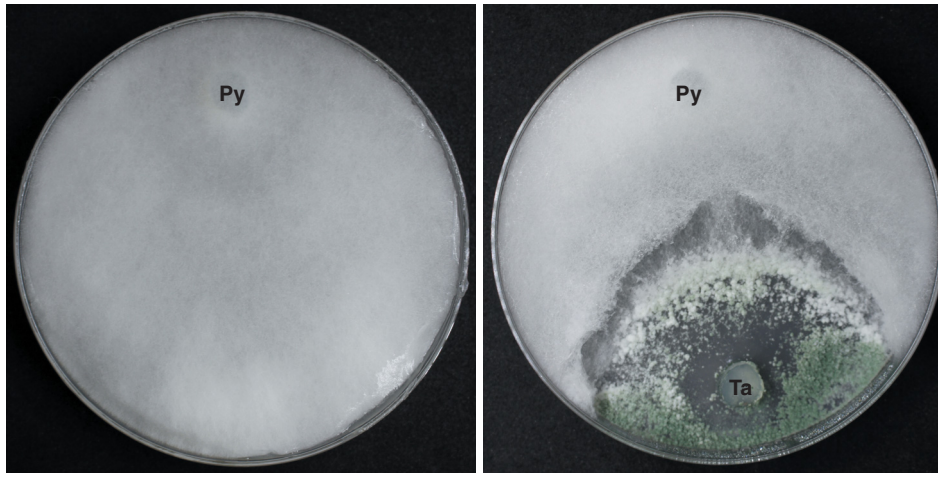
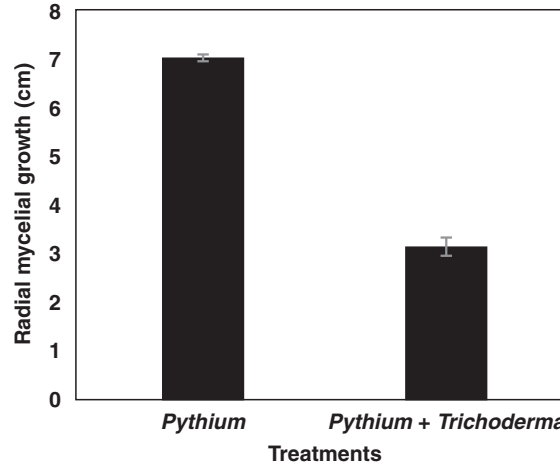


Fig. 1. Confrontation assay showing inhibition of *Pythium aphanidermatum* (Py) by *Trichoderma asperellum* SQU7 (Ta). Photos A.I. Al-Kharusi.

Fig. 2. Inhibition of *Pythium aphanidermatum* by *Trichoderma asperellum* SQU7 as assessed by in vitro confrontation assay. Data are visualised as mean ± standard deviation of three replications.



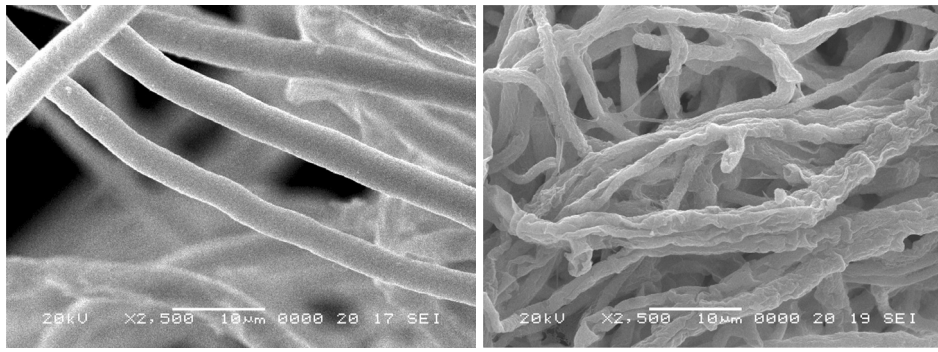


Fig. 3. Scanning electron micrographs showing abnormalities in the hyphae of *Pythium aphanidermatum* upon co-cultivation with *Trichoderma asperellum* SQU7. Bars = 10 µm. Photos M.A. Al-Kindi.

Similar findings were obtained by Rajani et al. (2021), when *Trichoderma* species were co-cultivated with phytopathogens *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*. The authors reported extensive deformation and lysis of *S. sclerotiorum* and *S. rolfsii* hyphae due to the antagonistic effects of *Trichoderma* spp. Conceivably, the deformation of fungal hyphae might be due to the action of antibiotic metabolites of *Trichoderma*, including lytic enzymes such as chitinases, β-1,3-glucanases, β-1,4-glucanases, and proteases, on the cell membrane of the host fungi (Kubicek et al. 2011, Bae et al. 2016). Elad et al. (1983) observed coiling of *Trichoderma* hyphae around the hyphae of *Rhizoctonia solani*, attachment of *Trichoderma* to the host mycelium by newly-formed hooks, and penetration into the host by degradation of its cell walls. Inbar et al. (1996) observed coiling of *T. harzianum* hyphae around *S. sclerotiorum* hyphae in dual culture and partial degradation of the host cell wall at later stages of the parasitism. Manzali et al. (1993), while studying the antagonism of *Trichoderma* sp. against *R. solani* through SEM, observed production of short branches by hyphae of *Trichoderma* growing towards *R. solani* hyphae, and also penetration of pathogen hyphae, coiling of pathogen hyphae, and degradation of cell walls of the pathogen at the penetration sites. As part of its antagonistic activity against *Fusarium sudanense*, which causes seedling blight and seed rot on wheat, *T. harzianum* hyphae attached to *F. sudanense* hyphae, penetrated with or without appressorium-like structures, coiled, caused plasmolysis, and formed a veil (Larran et al. 2020). The shrinkage of *P. aphanidermatum* hyphae could result from the leakage of cellular contents caused by the effect of *T. asperellum* on the pathogen's cell membrane (Garg et al. 2010).

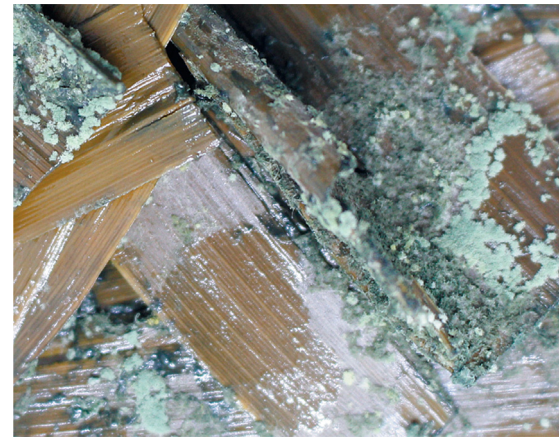


Fig. 4. Growth of *Trichoderma asperellum* SQU7 on common reed. Photo I.H. Al-Mahmooli

Common reed as efficient substrate

Common reed effectively supported the growth of *T. asperellum* (Fig. 4), yielding 3.7×10^7 spores/g and 6.2×10^7 spores/g at 14 and 30 days after inoculation, respectively.

Several organic carriers including coffee husk (Sawant & Sawant 1996), sawdust (Kolet 2014), fruit, vegetable, and crop wastes, poultry manure and farm yard manure (Simon & Anamika 2011), neem cake, coir pith (Saju et al. 2002), wheat bran (Mustafa et al. 2009), and cassava peels (Zhang et al. 2022) have been tested for large-scale production of *Trichoderma* species. Thangavelu et al. (2004) reported that dried banana leaves supported the growth of *T. harzianum* Th-10, which quickly multiplied and covered the entire leaf surface within 4 days, while the development of *T. harzianum* was significantly enhanced when 1% jaggery (unrefined sugar) was added to the leaves. Sachdev et al. (2018), while evaluating different agro-wastes for mass production of *T. lixii*, reported that sugarcane bagasse, spent tea leaves, vegetable waste, vermicompost, and cow dung supported the growth of *T. lixii* and recorded a spore density of 2.01×10^8 spores/g, 3.3×10^7 spores/g, 5.2×10^7 spores/g, 3.2×10^7 spores/g, and 2.7×10^7 spores/g, respectively, after 20 days of incubation at 27 °C. Yadav (2012) reported that maize husk supported a maximum spore production of 2×10^8 spores/g substrate in *T. viride* and 1.2×10^8 spores/g substrate in *T. harzianum* as compared with other substrates such as wheat bran and rice husk. Prathibha et al. (2015) reported that the combination of neem seed kernel powder and coir pith (1:1) showed the highest growth rate of *T. harzianum* (3.2×10^{10} colony forming units/g). In the present study, the production of *T. asperellum* reaching a density of 6.2×10^7 spores/g after 30 days of inoculation suggests the suitability of common reed for mass production of *T. asperellum*.

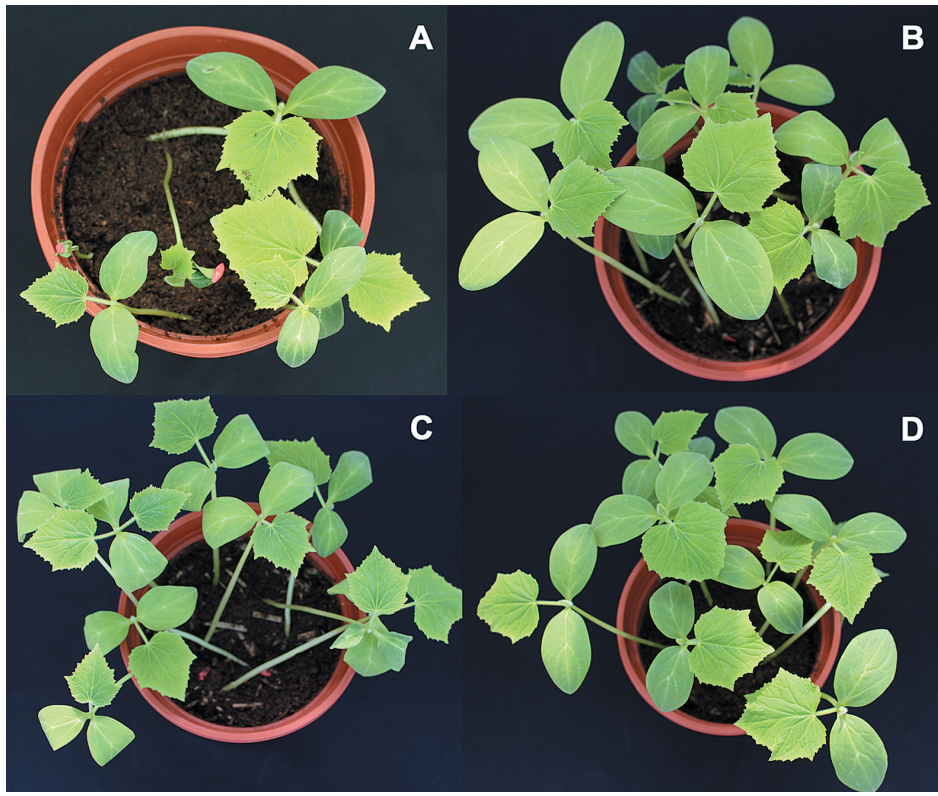


Fig. 5. Efficacy of common reed-based *Trichoderma asperellum* SQU7 inoculum on the suppression of damping-off of cucumber caused by *Pythium aphanidermatum*. **A** – *P. aphanidermatum*-infected control; **B** – soil treatment with 25 g of common reed-based *T. asperellum* inoculum; **C** – soil treatment with 50 g of common reed-based *T. asperellum* inoculum; **D** – soil treated with Metalaxyl (0.1%). Photos A.I. Al-Kharusi.

Suppression of damping-off in pot experiment

The pot experiment revealed that application of 25 g and 50 g common reed biomass with *T. asperellum* inoculum reduced the damping-off incidence by 88% and 91%, respectively, compared to the control, which recorded 68% disease incidence (Tab. 1; Fig. 5). The application of Metalaxyl exhibited 91% disease reduction, which is not significantly different from *T. asperellum* application.

Elshahawy & El-Mohamedy (2019) reported that *Trichoderma* isolates belonging to the species *T. harzianum*, *T. asperellum* and *T. virens* combined, suppressed *P. aphanidermatum* damping-off and resulted in a 74.5% improvement in tomato plant survival. According to research by Jayaraj et al. (2006), tomato damping-off was reduced by up to 74%, and plant biomass increased when seeds were treated with *T. harzianum* strain M1. *Pythium ultimum*-induced damping-off

Tab. 1. Biocontrol efficacy of common reed-based *Trichoderma asperellum* inoculum in controlling *Pythium aphanidermatum* damping-off in cucumber.

Treatments	Damping-off incidence (%)	% reduction over control
Common reed-based *T. asperellum* inoculum 25 g	8.0 ± 3.7 b	88.2
Common reed-based *T. asperellum* inoculum 50 g	6.0 ± 2.4 b	91.2
Metalaxyl (0.1%; 50 ml/pot)	6.0 ± 2.4 b	91.2
Pythium aphanidermatum-infected control	68.0 ± 8.6 a	–

Data are means of five replications (± standard deviation).
Means followed by the same letter in the column are not significantly different from each other at $P < 0.05$ according to Tukey's test. The data come from an experiment which was repeated twice with similar results.

was dramatically reduced in cucumbers when two biocontrol agents, *Pythium nunn* and *T. harzianum* isolate T-95, were applied together, as shown by Paulitz et al. (1990). The effectiveness of the reed-based *T. asperellum* inoculum in managing *Pythium* damping-off in cucumber, as shown here, may be related to its high efficiency and optimal sporulation on the substrate.

CONCLUSIONS

In conclusion, this research demonstrates that common reed is a feasible substrate for *Trichoderma* multiplication. The resulting inoculum reduced damping-off disease in cucumber caused by *P. aphanidermatum* effectively. This study also confirms the possible use of common reed as a carrier for *Trichoderma* mass production, thus offering a sustainable approach to the management of cucumber damping-off. More research is needed to evaluate the modes of action of *T. asperellum* strain SQU7 against phytopathogenic fungi and the effectiveness of common reed-based *Trichoderma* inoculum in controlling other soilborne diseases affecting various crop plants, including cucumber.

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