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MASTER'S DEGREE IN PLANT HEALTH IN SUSTAINABLE CROPPING SYSTEMS

EXPLORING THE POTENTIAL OF *TRICHODERMA* FOR BROAD-SPECTRUM PROTECTION AGAINST PESTS AND DISEASES

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MASTER THESIS

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EXPLORANDO EL POTENCIAL DE *TRICHODERMA* PARA UNA PROTECCIÓN DE AMPLIO ESPECTRO CONTRA PLAGAS Y ENFERMEDADES DE PLANTAS

RESUMEN

Los patógenos y las plagas de las plantas causan graves pérdidas en cultivos en todo el mundo. Aunque el control químico sigue siendo la herramienta más ampliamente utilizada, la demanda por métodos de control más sostenibles ha aumentado significativamente. El control biológico es una alternativa ecológica y eficiente al control químico. Idealmente, los agentes de control biológico multifuncionales, que podrían actuar contra los patógenos y plagas de las plantas, son de gran interés. Los microbios beneficiosos, como *Trichoderma*, pueden ser útiles contra tales amenazas bióticas. En este trabajo, hemos realizado varios experimentos en invernadero para evaluar el potencial de *Trichoderma asperellum* BB005 para actuar contra i) la enfermedad *Pythium ultimum* y ii) contra los áfidos. Para los experimentos usamos plantas de pepino cultivadas en lana de roca. Las interacciones *Trichoderma*-patógeno se estudiaron en cuatro ensayos. Los síntomas de la enfermedad fueron decoloraciones amarillo-marrón y pudrición en la base del tallo. En infecciones graves el desarrollo de la raíz se redujo, sin embargo, no se observó putrefacción de la raíz. Se probaron dos concentraciones de inóculo del patógeno y solo la dosis alta de una mezcla de micelio y oosporas fue efectiva para causar enfermedad. Es importante destacar que el desarrollo del brote de las plantas fue un poco mayor para las plantas inoculadas con la dosis baja que para las plantas control. Una aplicación de *Trichoderma* tuvo como resultado que el 60% de las plantas tuviera lesiones más leves y raíces más desarrolladas en comparación con las plantas sin *Trichoderma*. La adición del regulador osmótico glicina betaína (GB) no tuvo efecto sobre la gravedad de los síntomas causados por Pythium, sin embargo, el 75% de las plantas tratadas con *Pythium* en combinación con GB presentaron las peores puntuaciones respecto al desarrollo de la raíz. De las seis variedades de pepino evaluadas respecto a su susceptibilidad a *Pythium*, Dee Zire fue la más susceptible y Sargon, la más tolerante. La aplicación de *Trichoderma* disminuyó la gravedad de los síntomas de la enfermedad en Dee Zire. Las interacciones *Trichoderma*-herbívoro se estudiaron en un ensayo con plantas de pepino infestadas con *Aphis gossypii* y en dos ensayos con habas y *Acyrtosiphon pisum*. Para eso, se realizaron dos métodos de aplicación de *Trichoderma*: mezclarlo en el sustrato (suelo) o aplicado directamente a las semillas. En todos los ensayos, el crecimiento poblacional de áfidos fue menor en las plantas tratadas con *T. asperellum* BB005 que en las plantas control. En conclusión, nuestros resultados demostraron el potencial de *T. asperellum* BB005 para reducir de la severidad los síntomas causados por *P. ultimum* y favorecer el desarrollo de la raíz en pepino hidropónico. Además, la aplicación de *T. asperellum* BB005 afectó negativamente el crecimiento poblacional de dos especies de áfidos en dos cultivos diferentes. Estos resultados sugieren que *T. asperellum* BB005 es un candidato prometedor para ser explorado como una herramienta multifuncional contra patógenos y plagas en el pepino y otros cultivos.

PALABRAS-CLAVE: *Trichoderma asperellum*, *Pythium ultimum*, control biológico, áfidos, pepino

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EXPLORING THE POTENTIAL OF *TRICHODERMA* FOR BROAD-SPECTRUM PROTECTION AGAINST PESTS AND DISEASES

ABSTRACT

Plant pathogens and arthropod pests cause severe yield losses in crop production worldwide. Even though chemical control is still a widely used tool, the demand for more sustainable crop protection methods has increased significantly. Biological control is an environmentally friendly and efficient alternative. Ideally, multifunctional biological control agents, that could act against both pathogens and arthropod pests, show great promise. Beneficial microbes, such as *Trichoderma* can be useful against such biotic threats. Here, we conducted several greenhouse experiments to test the potential of *Trichoderma asperellum* BB005 to act i) against the disease *Pythium ultimum* and ii) against aphids. We conducted our trials using cucumber plants grown on rockwool. The experiments were carried out at the GreenLab facilities of Biobest Group N.V. in Belgium. The *Trichoderma*-pathogen interactions were studied in four trials. The disease symptoms were yellow-brown discolorations and rot in the stem base. The root development also decreased in severe infections, however, no root rot was observed. Two inoculum concentrations of the pathogen were tested and only the high dose of a mix of mycelium and oospores was effective. Interestingly, the shoot development was higher for the plants inoculated with the low dose than for the control plants. An application of *Trichoderma* resulted in 60% of the plants with smaller lesions and enhanced root system compared to plants without *Trichoderma*. Addition of the osmotic regulator glycine betaine (GB) resulted in no differences in disease severity between treatments, however, 75% of the plants treated with *Pythium* combined with GB presented the worst scores for root development. When comparing six cucumber cultivars regarding susceptibility to *Pythium*, Dee Zire was the most susceptible and Sargon, the most tolerant. Importantly, application of *Trichoderma* decreased the disease severity in Dee Zire. The *Trichoderma*-herbivore interactions were studied in one trial on cucumber infested with *Aphis gossypii* and in two trials on fava beans with *Acyrtosiphon pisum*. For that, two application methods of *Trichoderma* were performed: mixing it in the substrate (peat) and by seed treatment. In all trials, the aphid population development was lower in plants treated with *Trichoderma* than in control plants. In conclusion, our results showed the potential of *T. asperellum* BB005 in reducing disease severity of crown rot caused by *P. ultimu*m on hydroponic cucumber, but not in preventing plant death in severe infections Additionally, root development was consistently enhanced. Finally, application of *T. asperellum* BB005 affected negatively the population growth of two aphid species in two different crops. These results suggest that *T. asperellum* BB005 is a promising candidate to be further explored as a multifunctional tool against plant pathogens and insect pests in cucumber and other crops.

KEY WORDS: *Trichoderma asperellum*, *Pythium ultimum*, biological control, aphids, cucumber

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BCA	Biological control agent
CFU	Colony forming units
DAMPs	Damage-associated molecular patterns
ET	Ethylene
GB	Glycine betaine
IPM	Integrated Pest Management
GLM	Generalized Linear Model
HIR	Herbivore induced resistance
ISR	Induced systemic resistance
JA	Jasmonic acid
MAMPs	Microbe-associated molecular patterns
PAMPs	Pathogen-associated molecular patterns
PDA	Potato dextrose agar
PDAS	Potato dextrose agar with streptomycin
PRRs	Pattern-recognition receptors
PTI	Pattern Triggered immunity
rpm	Rotations per minute
SA	Salicylic acid
SAR	Systemic acquired resistance
UPV	Universitat Politecnica de Valencia

INTRODUCTION

Integrated pest management and biological control

Plant pathogens and insect pests cause severe yield losses in crop production worldwide. Even though chemical control is still a widely used tool for crop protection, the demand for more sustainable crop protection methods has increased significantly. It has been driven by consumer demand for products free from residues, the hazards to human health and concerns with environmental contamination. Biological control alone or as part of an integrated pest management (IPM) program, represents a solid alternative to chemical control. IPM has been recognized as a sustainable and environmentally friendly approach for crop protection. There are several definitions in the literature, but all involve the combination of available actions to manage pests in order to keep damage below an economic threshold while maintaining the applications of chemical pesticides to a minimal level (Stenberg, 2017).

The use of natural enemies to combat arthropod pests has been extensively explored while the research against plant diseases is now advancing. Also, there is an increasing interest in finding multifunctional biological control agents that could act against both pathogens and arthropod pests.

Biological control is an important non-chemical method of IPM strategies for protecting the crops against pests and pathogens. Biocontrol is usually based on the use of beneficial macroorganisms and biopesticides against pest and diseases. Biopesticide is the term used to refer to natural materials (e.g. plant extracts, semiochemicals, minerals) and beneficial microorganisms (Ravensberg, 2017). Species and strains of beneficial microorganisms have been proved to be successful in controlling several plant diseases, particularly against root and soil-borne pathogens on main agricultural crops and different vegetables (Belete et al., 2015; Heydari & Pessarakli, 2010). The existing commercial formulations of microbial biopesticides used in plant disease contain, mostly, soil-borne bacteria or fungi that are beneficial root colonizers. These include species and strains from *Agrobacterium*, *Aspergillus*, *Bacillus, Pseudomonas, Trichoderma* among others (Sutton et al., 2006; Van Lenteren et al., 2018; Vinale et al., 2008).

There are several ways in which biological control agents (BCAs) and pathogens/pests interact. Some of these interactions may happen with a direct action over the biotic threats, characterized by a physical contact between the BCA and the threat (i.e. parasitism, antibiosis, competition for space and nutrients), while others act indirectly through the plant immune system, by improving and stimulating the plant host defence, triggering local and/or systemic resistance (Fernández et al., 2017; Harman et al., 2004). In many cases, a BCA has multiple modes of action.

Plants respond to external stresses, either biotic or abiotic, by activating mechanisms of resistance. The responses upon herbivory or pathogen attack are different, depending on the feeding behaviour of the herbivore and the trophic strategy of the pathogen. The expression of such resistance occurs via a signalling cascade and pathways which phytohormones, such as Salicylic acid (SA), Jasmonic acid (JA) and Ethylene (ET) are biosynthesized and regulate the responses (Lazebnik et al., 2014; Pieterse et al., 2014; Pineda et al., 2013).

Upon a local infection by a pathogen or phloem feeding insects the systemic acquired resistance (SAR) pathway is activated, involving the upregulation of the phytohormone SA (Thaler et al., 2012). In contrast, upon herbivory by chewing insects, JA and ET are crucial for the immune response trigger herbivore-induced resistance (HIR) (Lazebnik et al., 2014; Pieterse et al., 2014). Beneficial microbes, used as biological control agents, stimulate the synthesis of JA, activating induced systemic resistance (ISR) (Bruce & Pickett, 2007; Pieterse et al., 2014; Rahman et al., 2018). An important remark about ISR is priming. The defence capacity of the plants is enhanced. Primed plants are characterized by a faster and stronger activation of defence upon biotic attack resulting in a better protection (Hermosa et al., 2012; Pieterse et al., 2014).

Crop production under protected conditions

Growing vegetable crops in greenhouses is advantageous due to the continuous production throughout the year, high yields and quality of agricultural products. Belgium, Netherlands, Canada and other northern countries usually adopt glasshouses, soil or soilless substrate and modern hi-tech systems to control the microclimate and irrigation (Martin & Loper, 1999; Sutton et al., 2006). Nevertheless, the conditions that favour the growth of the plants and yields, at the same time, may promote the growth of plant pests and pathogens (Rur, 2016).

Cucumber (*Cucumis sativus* L.) belongs to the Cucurbitaceae family and is among the most important horticultural crops in the greenhouse industry (Mohammadi & Omid, 2010). The cultivars can be monoecious (male and female separately but in the same plant), gynoecious (mostly female flowers) or parthenocarpic (no need for fertilization and seedless). Nowadays, most of the greenhouse cucumber cultivars for fresh consumption are parthenocarpic and are grown hydroponically in northern countries (Seminis, 2017; Singh et al., 2017; Staub & Delannay, 2011). The optimum temperature for its growth ranges from 16 °C to 32 °C degrees and it a fast-growing vegetable. Moreover, cucumbers cultivated in hydroponics are among the highest yielding plants grown in greenhouses (Jett, 2011).

In protected production using hydroponic systems, the choice of the soilless substrate is an important factor. The substrate needs to meet some physical and biological requirements. Most importantly, it must be pathogen free in its initial use (Schnitzler, 2004). Furthermore, it must be suitable for optimal water and nutrient holding capacity, as well as for the exchange of oxygen. Several substrates may be used such as coconut fibre, perlite, rockwool and others. In greenhouses in northern countries, rockwool is the most popular one in hydroponic vegetable cropping systems (Van Der Gaag & Wever, 2005).

Pythium spp.

Some *Pythium* species are serious pathogens in greenhouses and have a wide host range. Taxonomically, the genus *Pythium* belongs to the family Pythiaceae, order Pythiales, class Oomycetes, group Stramenopiles, eukaryotic supergroup SAR (Stramenopiles-Alveolata-Rhizaria) (Mcgowan & Fitzpatrick, 2017). The genus possesses a large amount of described species in which many are plant pathogens. The range of hosts and the level of virulence varies depending on the species (Martin & Loper, 1999; Postma et al., 2001; Sutton et al., 2006).

Previously, *Pythium* and other oomycetes were considered as fungi due to similarities in morphology, absorption of nutrients and production of filamentous mycelium. However, morphological, biochemical and molecular analyses have demonstrated several characteristics that place them as fungi-like organisms and not as true fungi. Some important distinctions between oomycetes and true fungi are the reproduction and cell wall composition. Sexual reproduction of oomycetes forms oospores whereas true fungi do not. Oospores are thick-walled spores which are the survival structures and possess constitutive dormancy (Martin & Loper, 1999; Deacon, 2006). Additionally, their asexual reproduction occurs through sporangia and some species can form zoospores (that are adapted for rapid dispersal under aquatic conditions) (Martin & Loper, 1999; Deacon, 2006). The cell wall of oomycetes is composed mainly of β-glucans and cellulose, whereas in true fungi is composed mainly by chitin. Oomycetes are also called water molds. Different environmental conditions influence the disease severity and symptom expression caused by *Pythium* spp. The main ones are water content, temperature and presence of other microorganisms in the root environment (Sutton et al., 2006).

The management of *Pythium* spp. includes preventive use of chemicals (e.g. propamocarb and metalaxyl) (Balk, 2014; Rai et al., 2018), cultural methods based on diseased free substrate, seed treatments, crop rotation, use of suppressive soils/substrates, sterilization of tools and hydroponic systems, other sanitation methods (Paulitz & Bélanger, 2001; Rai et al., 2018) and biological control (Djonović et al., 2006; Harman et al., 2004; Heydari & Pessarakli, 2010; Martin & Loper, 1999; Sutton et al., 2006; Vinale et al., 2008)

Aphids

Aphids (Hemiptera: Aphididae) are significant pests of several crops (Van Emden & Harrington, 2017). They feed on plant-sap (phloem) and depending on the species the damages can range from reduced growth and leaf deformation to transmission of toxic substances or viruses. The most important species found in greenhouses are *Myzus persicae* (Sulzer) (the green peach aphid), *Myzus persicae subsp. nicotianae* (the tobacco aphid), *Aphis gossypii* (Glover) (the cotton aphid), *Macrosiphum euphorbiae* (Thomas) (the potato aphid) and *Aulacorthum solani* (Kaltenbach) (the foxglove aphid) (Malais & Ravensberg, 2003).

In many aphid species, reproduction occurs asexually, giving rise to offspring genetically identical to the mother. Under crowded conditions, winged forms are produced that migrate to exploit new resources. Furthermore, some species complete their entire life cycle in just one host plant, whereas other species alternate between a host in the summer to reproduce asexually and in another (primary) plant host during winter, where they reproduce sexually, and lay eggs to overwinter. Aphids are particularly threats in greenhouses, since, under favourable conditions, the asexual reproduction occurs continuously, and the population increases exponentially in a very short period of time (Malais & Ravensberg, 2003; Resh, 2013).

The management of aphids in greenhouses relies on insecticide applications and biological control. The most common natural enemies used in glasshouses include the predatory larvae of *Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomydae) (gall midge) and *Chrysopa carnea* (Stephens) (Neuroptera: Chrysopidae) (green lacewing), predatory adults and larvae of *Adalia bipunctata* (L.) (Coleoptera: Coccinelidae) (two-spotted ladybird) and the parasitic wasps *Aphidius*

colemani (Viereck)*, Aphidius ervi* (Haliday) (Hymenoptera: Braconidae) and *Aphelinus abdominalis* (Dalman) (Hymenoptera: Aphelinidae) (Beltrà et al., 2018). There are also entomopathogenic fungi that may appear naturally under certain conditions (Malais & Ravensberg, 2003).

Trichoderma spp.

Beneficial root-colonizing fungi, such as some isolates of *Trichoderma* spp. have been investigated extensively as biocontrol agent of plant pathogens. Moreover, *Trichoderma* represents around 60% of fungal based BCAs used nowadays (Fraceto et al., 2007; Harman, 2000; Muckherjee et al., 2012; Verma et al., 2007; Waghunde et al., 2016).

Depending on the strain, *Trichoderma* spp. presents several advantages for crop plants. The root colonization ability of some *Trichoderma* strains stimulates plant growth and improve plant health in abiotic stress by enhancing nutrient mobilization and uptake (Harman, 2006; Waghunde et al., 2016). In fact, studies performed by Qi & Zhao (2013) and Zhao & Zhang (2015) demonstrated that *Trichoderma asperellum* (Samuels, Lieckf. and Nirenberg) strain Q1 possess abilities to suppress salt stress effects in cucumber plants as well to solubilize phosphate. In addition, it can be considered 'rhizosphere competent' with a rapid establishment (Harman et al., 2004).

Also, supresses pathogens using different mechanisms, including competition for nutrients and space, mycoparasitism and antibiosis (Belete et al, 2015; Harman, 2006). *Trichoderma* spp. are very efficient competitors for carbon and iron. Hence, they may control disease development by inhibiting the growth of other microorganisms, such *Pythium,* a weak competitor. Disease suppression by mycoparasitism involves a direct attack of *Trichoderma* on another fungal species. It is a complex process with several steps from recognition to penetration and killing. Also, antibiotics produced by *Trichoderma* spp. inhibit the growth of other microorganisms (Harman, 2006).

Several *Trichoderma* species were found to be very effective biocontrol agents. Diseases caused by *Rhizoctonia solani* (J.G.Kühn) in cucumber, *Fusarium oxysporum* f.sp. *lycopersici* (Fol) in tomato and *Phythophotora capsici* (Leonian) in sweet-pepper were found to be effectively controlled by *T. asperellum* strain T34 by Trillas et al (2006), Segarra et al (2010) and Segarra et al (2013), respectively. The already commercial *Trichoderma harzianum* (Rifai) strain T22 as well a natural isolate, *Trichoderma hamantum* (Bonord.) strain T17, were efficient in controlling Fusarium wilt and promoting growth on melon plants (Martínez-Medina et al., 2014). Furthermore, Harman et al (2004) found that *T. harzianum* T22 efficiently protected maize plants from *Pythium ultimum* (Trow) whereas Djonović et al (2006) found the involvement of *Trichoderma virens* (J.H.Mill., Giddens and A.A.Foster) in controlling *P. ultimum* in bioassays and cotton seedlings.

Several strains of *Trichoderma* spp. can supress plant diseases by inducing systemic resistance (Fernández et al., 2017; Pieterse et al., 2014; Van Wees et al., 2008). Nowadays this is one of the major focus on research about *Trichoderma* spp. (Harman et al., 2008). Several strains are already known to stimulate biochemical changes to enhance plant defences against different pathogens in numerous crops.

Yedidia et al (2003) and Shoresh et al (2005) demonstrated the potential of *T. asperellum* strain T203 (reclassified as *T. asperelloides*) (Samuels & Petrini, 2010) in inducing systemic resistance in cucumber plants. Moreover, Martínez-Medina et al (2013) studied the mechanisms behind the induced systemic resistance triggered by *T. harzianum* strain T78 in tomato plants against *Botrytis cinerea* (Pers.) Also, Salas-Marina et al (2011) investigated the colonization of *Trichoderma atroviride* (Bisset) in Arabidopsis and demonstrated enhanced development of plants as well a systemic induced defence response activation.

There are very few studies exploring the effects of *Trichoderma* spp. towards insect pests. A strain of *T. asperellum* was reported to reduce the offspring of *Aphis fabae* (Scopoli) and *Acyrthosiphon pisum* (Harris) in fava beans (Akello & Sikora, 2012). Coppola et al (2017) assessed the effects of *T. harzianum* T22 towards the aphid *Macrosiphum euphorbiae* (Thomas) and the parasitoid *A. ervi*. They concluded that priming stimulated by the beneficial microbe released volatiles that are attractive to the parasitoids resulting in improved aphid control. In another study, plants inoculated with *T. atroviride* strains MT-20 and S-2 were found to negatively affect the development and reproduction of whiteflies and aphids (Menjivar, 2010).

OBJECTIVES

This study intended to optimize the application of a strain of *Trichoderma asperellum* strain BB005 on cucumber under hydroponic system.

The specific goals were:

(i) Optimize the application of this *T. asperellum* BB005 to control diseases caused by *Pythium ultimum* in hydroponic cucumber crop.
(ii) Explore the potential of *T. asperellum* BB005 to act against aphids.

The following questions are intended to be answered:

1. Can *T. asperellum* BB005 control diseases caused by *P. ultimum* in hydroponically grown cucumber plants?
2. Can *T. asperellum* BB005 enhance cucumber shoot and root growth?
3. Does *T. asperellum* BB005 plant colonization affect the population growth of aphids?

MATERIAL AND METHODS

Greenhouse experiments were carried out from February to August 2018 at the Green Lab facilities of Biobest Group N.V., in Westerlo, Belgium.

The daily average and the range of minimum and maximum for the air temperature and the relative air humidity was recorded during the experimental periods with a data logger (EL– USB 2).

TRICHODERMA - PATHOGEN INTERACTION

Inoculum preparation

The *P. ultimum* isolate was obtained from spinach (*Spinacea oleracea* L.) at the Plant Pathology Laboratory of the Polytechnic University of Valencia (UPV), Valencia, Spain. The inoculum was prepared using the method described in Hultberg et al (2011), with some modifications. The pathogen was grown in the dark for 3-5 days on potato dextrose agar (PDA). Afterwards, 3 mm-discs of mycelium were taken from the edge of the colonies and placed into water agar plates. These plates were kept in the dark for 2 days, at room temperature. Then, pieces of 1 cm x 1cm were cut from the periphery of the colony and placed into petri dishes containing 15 ml of cleared V8 broth. The broth was prepared by first mixing 200 ml of a commercial V8 vegetable juice with 800 ml of reverse osmosis water. The suspension was centrifuged for 20 min at 3500 rpm (Hermle z 400). The supernatant was diluted with a 1:1 ratio and 3 gL^{-1} $CaCO_3$ was added and autoclaved. The petri plates were kept in the dark for 3 days at 25 °C. The content of the petri plates was transferred to a flask, diluted in water and with a hand blender, homogenised for 1 minute. The number of oospores were counted with the aid of a Neubauer haemocytometer and a microscope. The inoculum was a mixture of mycelium and oospores.

Greenhouse experiments

The greenhouse experiments consisted of four trials and were performed in a randomized design. The repetitions (number of plants/treatment) are mentioned for each trial (Table 1). The cucumber plants (*Cucumis sativus* L.) were hydroponically cultivated. The rockwool blocks Plantop® delta, Grodan (10cm x 10cm x 6.5cm) were soaked for 24 hours prior to sowing. The nutrient solution was applied once a week and consisted of 3 gL^{-1} Peters fertilizer 15-11-29+TE (Scotts - Pot Plant Special).

Trichoderma asperellum BB005 was applied as a spore suspension (6.5 x 10^4 spores/ml), using a formulated product containing 10^9 CFU/g, stored at 4 °C. The osmotic regulator Greenstim®, containing more than 96% of glycine betaine (GB), was applied directly on the rockwool blocks (2 gL^{-1}). For each treatment, plants received 100 ml of the suspensions.

Table 1. Summary of trials carried out in hydroponic cucumber at the Green Lab facilities at Biobest Group N.V.

Trial	Cultivar	N° Plants/ treatment	Sowing date	*Trichoderma* inoculation	Glycine betaine application	Pathogen inoculation
A	Marketer	6	16-Feb	-	-	01-Mar
B	Marketer	15	06-Mar	08-Mar	-	16-Mar
C	Marketer	12	15-Mar	19-Mar	19-Mar	28-Mar
D	Six cultivars	6	08-May	14-May	-	25-May

Trial A

A preliminary trial (trial A) was designed to evaluate the inoculation method and concentration of *P. ultimum.* In this initial test, six plants (cultivar Marketer) were used per treatment. The content of two petri plates with a mix of oospores and mycelium was used to inoculate the plants. The concentration of the high dose was 2 x 10^4 oospores/ml. The low dose was approximately 1.4 x 10^4 oospores/ml. For each treatment, the plants received 15 ml of the respective concentration, or water, for the control.

Trial B

Trial B intended to evaluate the interaction between *T. asperellum* BB005 and the plant pathogen *P. ultimum* in cucumber grown in rockwool. The pathogen was inoculated with a mix of oospores and mycelium, using five petri plates and the concentration was approximately 2 x 10^4 oospores/ml.

Trial C

Trial C was performed to investigate if an osmotic regulator (glycine betaine) would enhance the performance of *Trichoderma*. GB is used as osmotic regulator of plants in practice. However, GB can also be used by microorganisms in the rhizosphere (Moe, 2013). Its effect on beneficial microorganisms has not been explored. The pathogen was inoculated using 12 petri plates and the concentration was 2.25 x 10^4 oospores/ml. Additionally, the leftover from the suspension of *Trichoderma* diluted in the glycine betaine was plated to evaluate the effects on the germination of *Trichoderma* spores. A serial dilution was performed and 500 µl was plated in a semi-selective medium.

Trial D

The *Trichoderma* – pathogen interaction was also evaluated in other six cucumber cultivars provided by Enza Zaden (Sumapol, Dee Zire, Kurios, Dee Lite, Oribi and Sargon). This experiment was set in a randomized design with 6 plants of each cultivar per treatment. For the pathogen inoculation, 10 petri plates were used with a mix of oospores and mycelium in a concentration of 2.25 x 10^4 oospores/ml.

For all trials, plants were approximately a month old when they were collected, evaluated and colonization assessments were performed.

Shoot and root development assessment

Shoot fresh weight (g) and number of leaves and flowers per plant were determined. The root development was scored visually. It was based on the retrieved roots from the rockwool blocks (Figure 1).

Figure 1. Scores used to assess the root development of cucumber plants grown under hydroponic system.

Disease severity assessment

The assessment was done by scoring the size of the lesion on the stem of the cucumber plants (Figure 2).

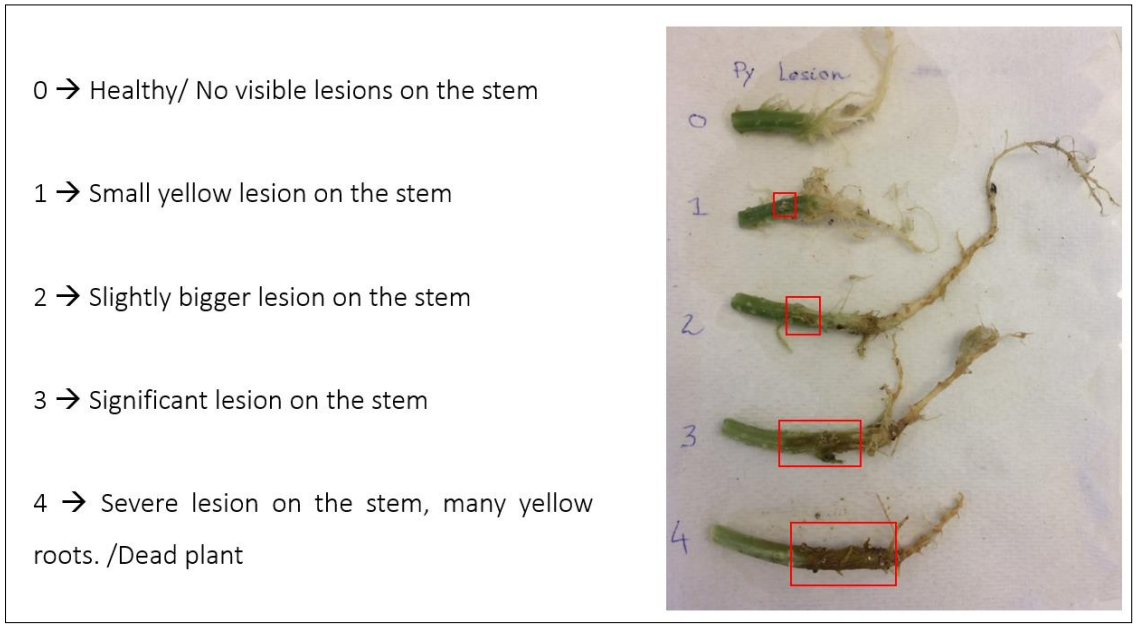

Figure 2. Scores used to assess stem lesions caused by *Pythium ultimum* on cucumber plants grown under hydroponic system.

Colonization by Trichoderma

In trials B and C, root colonization was checked to assess the presence of *Trichoderma.* Root pieces of approximately 1 cm were collected from plants of each treatment and placed into petri

plates containing a semi - selective medium described in (Chung & Hoitink, 1990). The medium was prepared with $MgSO_47H_2O$ (0.2 gL^{-1}); K_2HPO_4 (0.9 gL^{-1}); NH_4NO_3 1 gL^{-1}); KCL (0.15 gL^{-1}); glucose (3 gL^{-1}); $FeSO_47H_2O$ (0.02 gL^{-1}); $MnSO_47H_2O$ (0.02 gL^{-1}); $ZnSO_47H_2O$ (0.02 gL^{-1}); agar (15 gL^{-1}); After autoclaving, Rose Bengal (0.03 gL^{-1}); chloramphenicol (0.01 gL^{-1}); streptomycin (0.05 gL^{-1}); pentachloronitrobenzene (0.02 gL^{-1}) and tergitol (1 ml L^{-1}) were added. Fungi grown on roots were identified as *Trichoderma*, by morphology of the colony. In addition, the presence of *Trichoderma* was confirmed by observing the colonies with the aid of a stereomicroscope.

For trial D, 5 to 10 g of rockwool were collected from the blocks, transferred into an Erlenmeyer, completed to 100 ml of sterile water and was kept shaking at 140 rpm for 30 minutes. Afterwards, a serial dilution was performed and 500 µl was plated on *Trichoderma* semi - selective medium, with two plates per dilution, to quantify the colonies of the *Trichoderma* per dry weight of rockwool.

To confirm the presence of *P. ultimum*, root pieces of about 1 cm were collected and plated on PDA with streptomycin (PDAS) (500 µl L^{-1}).

In addition, discs of 3 mm diameter from 3-5-day-old colonies of *Trichoderma* and *P. ultimum* were placed on PDA plates with different concentrations of glycine betaine: 2 gL^{-1}; 1 gL^{-1} and 0.2 gL^{-1} and growth was measured.

TRICHODERMA - HERBIVORE INTERACTION

CUCUMBER

Greenhouse experiments

The experiment in cucumber (cv. Marketer) consisted in a randomized design. The seeds were sown on rockwool blocks and inoculated with *T. asperellum* BB005 or water (control).

The aphid species used was the cotton aphid (*Aphis gossypii*), reared originally in sweet pepper then in cucumber plants at Biobest N.V. facilities. The infestation occurred introducing 5 individuals/plant. The plants were at least 5 cm of shoot length and 1 true leaf. The number of aphids/plants was counted two weeks after infestation.

The trial was sown on 11th July and 6 plants per treatment were used. The aphid infestation occurred on the 30th of July.

Shoot and root development

The method was similar to the one already described in the *Trichoderma*-pathogen interaction part.

Colonization by Trichoderma

The colonization of *Trichoderma* was determined using the same protocol described for the trial D in the *Trichoderma*-pathogen interaction part.

FAVA BEANS

Greenhouse experiments

The experiments on fava beans (*Vicia faba* cv. Hiverna) consisted of two trials. The experiments included three treatments, in a randomized design. The treatments were:

1. Seeds soaked in water for 24 h (control);

2. Seed treatment - seeds soaked in *Trichoderma* suspension (1 x 10^4 spores/ml) for 24 h;

3. Seeds soaked in water for 24 h and the *Trichoderma* suspension (1 x 10^4 spores/ml) was mixed in the substrate (10% of the substrate volume).

The aphid species used was the pea aphid (*Acyrtosiphon pisum)* reared on fava beans at Biobest N.V facilities. The infestation occurred introducing 5 individuals/plant. The plants had at least 5 cm of shoot length and 1 true leaf at the moment of the infestation. The number of aphids/plants was counted one week after infestation.

The first trial was sown on the 9th of February and consisted on sixteen plants per treatment. The aphid infestation occurred on the 20th of February. The second one contained twelve plants per treatment. The trial was sown on 1st of March and infestation occurred on the 13th of March.

Shoot and root development assessment

Shoot fresh weight (g) and height (cm), root weight (g), number of leaves and extra floral nectar glands per plants were determined. The root development was scored visually based on the root growth on the outside of potting soil (Figure 3).

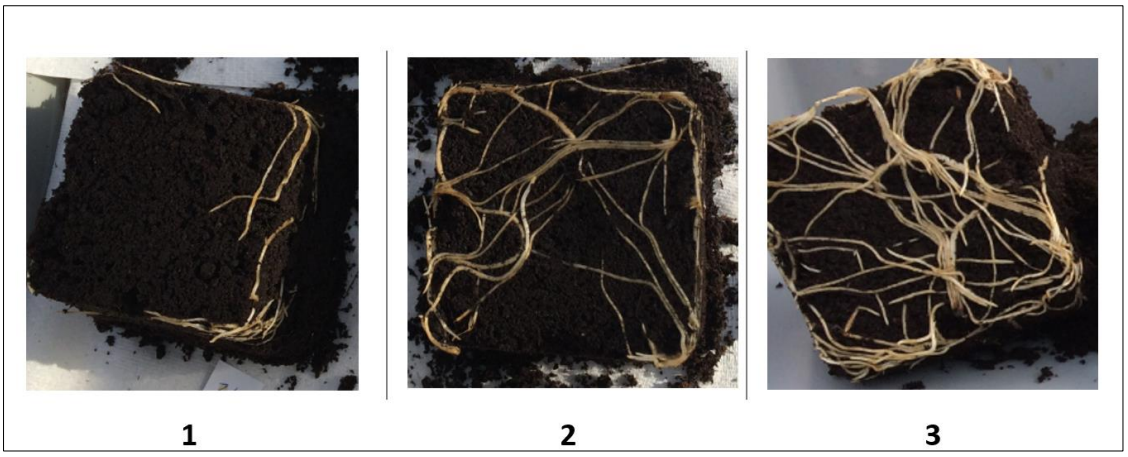

Figure 3. Root development scores for fava beans grown on potting soil. (1=poor development; 2=intermediate development; 3=good development).

Colonization by Trichoderma

The presence of *Trichoderma* on the fava beans plants was determined using the same protocol described on the *Trichoderma*-pathogen interaction part.

DATA ANALYSIS

For *Trichoderma*-pathogen experiments, data were analysed with SPSS STATISTICS v.24 (IBM). The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests and the Spearman's rank correlation was calculated. The root development and stem lesion scores were numerical. A factorial analysis of variance and an interaction model were performed. The plant development traits were the dependent variables and the treatments (microorganisms and control) were the independent variables. For the *Trichoderma*-herbivore experiment, a generalized linear modelling (GLM) technique was applied assuming Poisson error variance to build models considering the plant growth, number of aphids, leaves and extra floral nectar glands (per plant) as the dependent variables and the treatments as the explanatory variables. If over or under dispersion was detected, the significance of the explanatory variable was re-evaluated using an F-test after re-scaling the statistical model by a Pearson's chi-square divided by the residual degrees of freedom (Crawley 2007). Moreover, Tukey's post hoc tests were performed using the function glht from the MULTCOMP package for R. The data analyses were performed with the R freeware statistical package (http://www.R-project.org/).

RESULTS

TRICHODERMA - PATHOGEN INTERACTION

Trial A

Greenhouse experiments

The disease symptoms were characterized by tissue rot and yellow-brown discoloration in the plant stem. The symptoms initially appeared on stems base, then, in some plants, enlarged. Also, some plants had their lower leaves turned yellow, and then the entire plant wilted and died. The volume of the roots also decreased in severe infections, however, no root rot symptoms were found.

Average temperature was 23.93 °C (range: 21.53 °C - 26.38 °C) and average relative humidity was 37.24% (range: 22.26% - 54.84%).

The shoot fresh weight had a strong positive correlation with the number of leaves ($r = 0.941$, $p < 0.0001$) and flowers ($r = 0.782$, $p < 0.0001$). In addition, the number of leaves and flowers were also positively correlated ($r = 0.729$, $p = 0.001$). On the other hand, the stem lesion score was negatively correlated with all traits, being significant for shoot fresh weight ($r = -0.625$, $p = 0.006$), number of leaves ($r = -0.670$, $p = 0.002$) and number of flowers ($r = -0.484$, $p = 0.042$), but not significant for root score ($r = -0.411$, $p = 0.090$). In fact, only root score was not significantly correlated to any of the traits. Significant differences among the treatments were found for all evaluated traits, except for the root development (Annex Figure 1).

Shoot development assessment

The shoot fresh weight and the number of leaves and flowers/plants were, on average, higher for the plants inoculated with the low dose of the pathogen (Annex Figure 1 a, b and c). The

treatments were statistically significant the number of leaves/plants (X_i^2 = 6.505, df = 2, P = 0.038) and the number of flowers/plant (X_i^2 = 7.993, df = 2, P = 0.018) but not for the shoot fresh weight (X_i^2 = 5.460, df = 2, P = 0.065). These traits were, on average, higher for the plants inoculated with the low dose of the pathogen compared to the control plants.

The low dose did not differ statistically from control for leaves (X_i^2 = 2.152, df = 1, P = 0.142) and shoot fresh weight (X_i^2 = 1.652; df = 1; P = 0.198). On the other hand, it was different from control for the number of flowers/plant (X_i^2 = 5.710, df = 1, P = 0.016) (Annex Figure 1 c).

The high dose also did not show statistical differences from control for the number of leaves/plant (X_i^2 = 1.999, df = 1, P = 0.157), for the shoot fresh weight (X_i^2 = 5.460, df = 1; P = 0.292) and the number of flowers/plant (X_i^2 = 0.118, df = 1, P = 0.730).

Comparing the high and low dose, statistical differences were found for the number of leaves/ plant (X_i^2 = 5.487, df = 1, P = 0.019), the shoot fresh weight (X_i^2 = 5.460, df = 1; P= 0.030) and flowers/plants (X_i^2 = 5.710, df = 1, P = 0.016).

Root development assessment

There were no statistical differences among treatments regarding root development (X_i^2 = 3.618, df = 2, P = 0.163). However, some variability was observed among plants. In the high dose treatment, two plants died, thus, no developed root system (score 3). Despite the presence of stem lesions caused by the pathogen, the other plants seemed to develop well and were scored either with 0 or 1. For the low dose, two plants presented a small root development (scored 2) whereas the remaining plants were scored with 0 or 1 (Annex Figure 1 e).

Disease severity assessment

Concerning the stem lesions caused by *P. ultimum*, there was a significant difference among treatments (X_i^2 = 13.203, df = 2, P = 0.0013) (Annex Figure 1 d).The low dose and control were not statistically different (X_i^2 = 1, df = 1, P = 0.317) while the high dose differed from both the control (X_i^2 = 9.658, df = 1, P = 0.002) and the low dose (X_i^2 = 6.909, df = 1, P = 0.0008). Only one plant inoculated with the low dose presented a considerable lesion on the stem and received a score of 2. Although the rest of the plants were also positive for the presence of the pathogen, they did not display any visible symptoms of the disease. For the high dose, one plant presented a small lesion being scored with 1, whereas three other plants had a bigger lesion and were scored with 2. The two remaining plants died.

Colonization assessment

After plating five pieces from roots of each plant in PDAS, the presence of *P. ultimum* was confirmed to be positive in all plated roots, from both treatments (low and high dose), even from the plants that were symptomless.

TRIAL B

Greenhouse experiments

The trial B evaluated plants inoculated by *P. ultimum* and *T. asperellum* BB005. The symptoms caused by the pathogen were already described on trial A. Dead plants were found for both treatments where the pathogen was inoculated (T+ P+ and T- P+).

Average temperature was 24.62 °C (range: 22.74 °C - 28.56 °C) and average relative humidity was 43.84% (range: 29.52% - 57.39%).

A factorial analysis of variance was performed (Table 2). There were no significant interactions among *Trichoderma* and *P. ultimum* for any evaluated trait (leaves: F = 0.638, df = 1, *P* = 0.427; flowers: F = 1.160, df = 1, *P* = 0.286; weight: F = 1.397, df = 1, *P* = 0.242; root scores: F = 3.321, df = 1, *P* = 0.073; lesion scores: F = 1.624, df = 1, *P* = 0.207). The effect of *Trichoderma* was statistically significant only for root scores (F = 7.622, df = 1; *P* = 0.007).The effect of the pathogen was significant for leaves (F = 5.784, df = 1, *P* = 0.019), weight (F = 7.858, df = 1, *P* = 0.006) and root scores (F = 9.522, df = 1, *P* = 0.003), but not for the number of flowers (F = 2.61; df = 1; *P* = 0.111).

Table 2. Factorial analysis of variance. Interaction model and main effects of shoot fresh weight, number of flowers/plants, number of leaves/plants, plants per score for root development and stem lesions caused by *Pythium ultimum* of the hydroponic cucumber plants inoculated with *Trichoderma asperellum* BB005 and *P. ultimum* (trial B).

	Shoot fresh weight	Number of leaves	Number of flowers	Root scores	Stem lesions
Trichoderma	ns	ns	ns	**	ns
Pythium	**	**	ns	**	***
Trichoderma: Pythium	ns	ns	ns	ns	ns

Significance codes: 0.001 '***'; 0.01 '**'; 0.05 '*'; 'ns' Not significant.

The Spearman's rank correlations were calculated. The shoot fresh weight was significantly correlated with the number of leaves/plants (r = 0.636, p < 0.0001) and flowers/plants (r = 0.272, p = 0.043). The stem lesion score was negatively and significantly correlated with all traits, except for shoot fresh weight (r = -0.191, p = 0.159). The root score was significantly correlated with both the number of flowers and leaves (r = 0.316, p = 0.042 and r = 0.420, p = 0.001, respectively) but not for the shoot fresh weight (r = 0.068, p = 0.617).

Significant differences among treatments were found for stem lesions scores (X_i^2 = 32.5, df = 3, *P* < 0.0001) and root development scores (X_i^2 = 18.127, df = 3, *P* = 0.0004) (Figure 4).

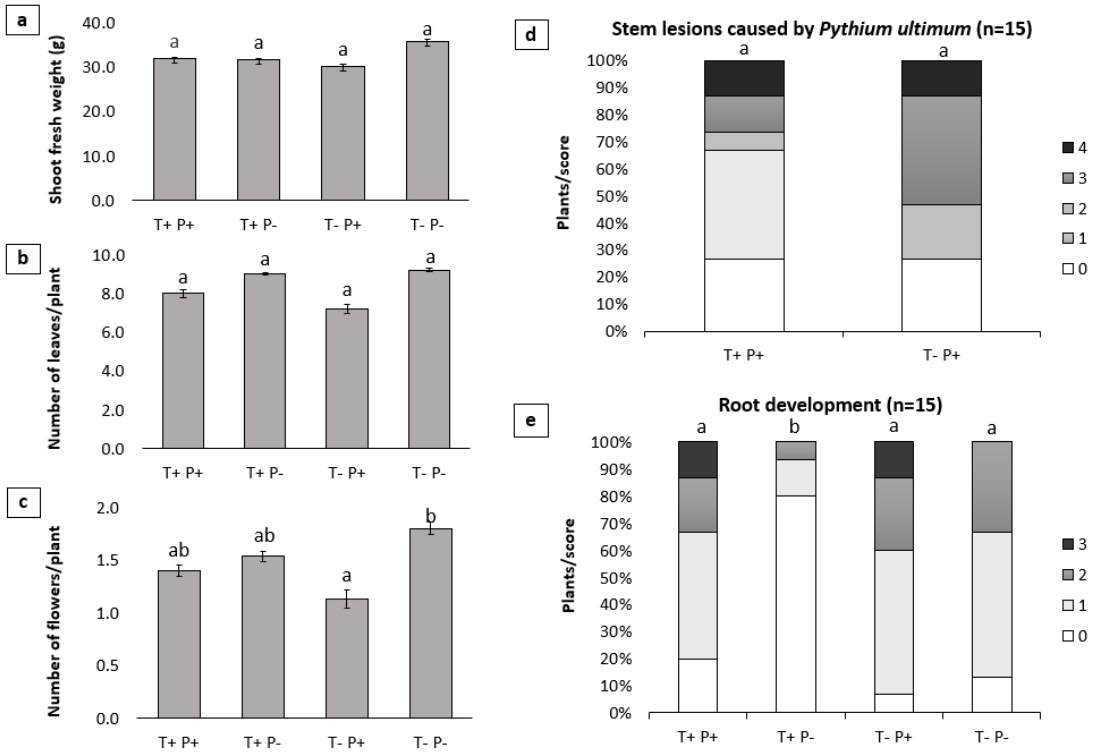

Figure 4. Plant development and disease severity of cucumber plants under a hydroponic system inoculated with *Trichoderma asperellum* (T+) and *Pythium ultimum* (P+) or not inoculated (T- and P-) (trial B). **(a)** Averages of shoot fresh weight, **(b)** number of leaves/plant, **(c)** number of flowers/plant, **(d)** number of plants per score for the stem lesions caused by *P. ultimum* (0= healthy, 1= small lesion on the stem, 2= intermediate size of lesion on the stem, 3= big lesion on the stem, 4=severe lesion/dead plant) and **(e)** root development (0= very well developed root system, 1= intermediate root system, 2= small root system, 3= root system of dead plants). The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests (Mean ± SE). Different letters indicate significant difference between the treatments at a significance level of 0.05. (n=15 for stem lesions caused by *P. ultimum* and root development; n vary for the other traits since dead plants were removed from the calculations).

Shoot development assessment

No statistical differences among treatments were found for shoot fresh weight (X_i^2 = 4.518, df = 3, P = 0.210) and the number of leaves/plants (X_i^2 = 4.462, df = 3, P = 0.215) (Figure 4 a and b). Regarding the number of flowers/plants the treatment, T- P+ was statistically different from the control (X_i^2 = 4.998, df = 1, P = 0.025), however it did not differ statistically from T+ P+ (X_i^2 = 1.668, df = 1, P = 0.196) and T+ P- (X_i^2 = 2.818, df = 1, P = 0.093). Furthermore, T+ P+ and T+ P- did not differ from control (X_i^2 = 1.159, df = 1, P = 0.281; X_i^2 = 0.875, df = 1, P = 0.349, respectively) (Figure 4 c).

Root development assessment

For the root development scores, there was a significant difference among T+ P- and control (X_i^2 = 11.632, df = 1, P < 0.0001); T+P- and T- P+ (X_i^2 = 14.33, df = 1, P < 0.0001) and T+ P- and T+P+ (X_i^2 = 9.961, df = 1, P = 0.001). The plants treated with *T. asperellum* (T+ P-) presented and enhanced growth with 80% of the plants with well-developed roots (score 0) (Figure 4 e).

Disease severity assessment

There was a statistical difference among treatments regarding stem lesions caused by the pathogen (X_i^2 = 32.5, df = 3, P < 0.0001). The treatment with *Trichoderma* (T+ P+) did not avoid death caused by *P. ultimum*. However, 60% of the plants in this treatment presented smaller lesions (scores 0 and 1) in comparison with plants without *Trichoderma* (T- P+) (Figure 4 d).

Colonization assessment

Root pieces of approximately 1 cm were used to confirm the presence of the inoculated microorganisms on the evaluated plants. To check the presence of *Trichoderma,* for the treatments T+ P+ and T+ P-, four root pieces of each plant were plated in the semi-selective growth medium. Positive presence of *Trichoderma* from the treatment T+ P- occurred, on an average, in 3.6 root pieces (90%), whereas for the T+ P+ on average, in 3 root pieces (75%).

To confirm the presence of the pathogen, for T+ P+ and T- P+, the PDAS medium was used. Four root pieces from each plant from T+ P+ were used. On average 2.4 roots pieces (60%) were positive for *P. ultimum*, while for T- P+, ten pieces were used and 7.5 were positive (75%), on average.

TRIAL C

Greenhouse experiments

The trial C assessed plants inoculated with the microorganisms *T. asperellum and P. ultimum* with the addition of glycine betaine (GB). The symptoms of disease caused by *P. ultimum* on the plants were already described in the previous trials. Dead plants were found for the treatments T+ P+ and T+ GB+ P+.

The execution of this trial overlapped with trial B, therefore the values for both temperature and relative air humidity are the same as described before.

A factorial analysis was performed. A significant interaction among *Trichoderma* and *P. ultimum* occurred only for the stem lesions (F = 18.209, df = 1, P < 0.0001). Additionally, there was an interaction among *Pythium* and glycine betaine for shoot fresh weight (F = 15.597, df = 1, P < 0.0001), root scores (F = 9.456, df = 1, P = 0.002), stem lesions (F = 24.593, df = 1, P < 0.0001) and number of leaves/plant (F = 6.173, df = 1, P = 0.014). No statistical significance was found for the interaction among *Trichoderma* and glycine betaine for any trait (leaves: F = 1.414, df = 1, P = 0.237; weight: F = 1.201 , df = 1, P = 0.276; roots: F = 0.065, df = 1, P = 0.798; lesions: F = 0.332, df = 1, P = 0.565), neither for *Trichoderma*, *Pythium* and GB for any trait (leaves: F = 0.535, df = 1, P = 0.466; weight: F = 0.415 , df = 1, P = 0.520; roots: F = 0.262, df = 1, P = 0.609; lesions: F = 0.119, df = 1, P = 0.730). An effect of *Trichoderma* was observed only for the root development (F = 12.871, df =1, P = 0.0005) and stem lesions scores (F = 16.293, df = 1, P < 0.0001), whereas the pathogen was significant for all traits except stem lesions (F = 0.119, df = 1, P = 0.727). Glycine betaine was significant for shoot fresh weight (F = 11.703, df = 1, P = 0.00093) and stem lesions (F = 16.578, df = 1, P < 0.0001) (Table 3).

Table 3. Factorial analysis of variance. Interaction model and main effects or shoot fresh weight, number of leaves/plants, number of plants per score for root development and stem lesions caused by *Pythium ultimum* of the hydroponic cucumber plants inoculated with *Trichoderma asperellum* BB005, *P. ultimum* and glycine betaine (trial C).

	Shoot fresh weight	Number of leaves	Root scores	Stem lesions
Trichoderma	ns	ns	***	***
Pythium	***	***	ns	****
Glycine betaine	**	ns	ns	***
Trichoderma: Pythium	ns	*	ns	***
Trichoderma: Glycine betaine	ns	ns	ns	ns
Pythium: Glycine betaine	***	ns	**	***
Trichoderma: Pythium: Glycine betaine	ns	ns	ns	ns

Significance codes: 0.001 '***'; 0.01 '**'; 0.05 '*'; 'ns' Not significant.

According to the results from the Spearman's rank correlations, the stem lesion scores were negative correlated with all characters. Shoot fresh weight was positively correlated with the number of leaves/plant ($r = 0.857$, $p < 0.0001$) and the root development scores ($r = 0.613$, $p < 0.0001$). Furthermore, the number of leaves/plants was also positively correlated with the root development scores ($r = 0.538$, $p < 0.0001$).

Shoot development assessment

Significant differences among treatments were found shoot fresh weight ($X_i^2 = 35.871$, df = 7, $P < 0.0001$) and the number of leaves/plants ($X_i^2 = 28.328$, df = 7, $P = 0.00019$). The treatment combining *Trichoderma* and glycine betaine (T+ GB+) did not differ statistically from the control for shoot fresh weight ($X_i^2 = 0.404$, df = 1, $P = 0.524$) and for the number of leaves/plant ($X_i^2 = 2.571$, df = 1, $P = 0.108$), while the other treatments decreased shoot fresh weight. For shoot fresh weight, the other treatments did not differ statistically among each other (Figure 5 a). While for number of leaves/plants, GB+ and T+ GB + P+ were not statically different from the control ($X_i^2 = 2.884$, df =1, $P = 0.08$; $X_i^2 = 0.508$, df = 1, $P = 0.475$, respectively) (Figure 5 b).

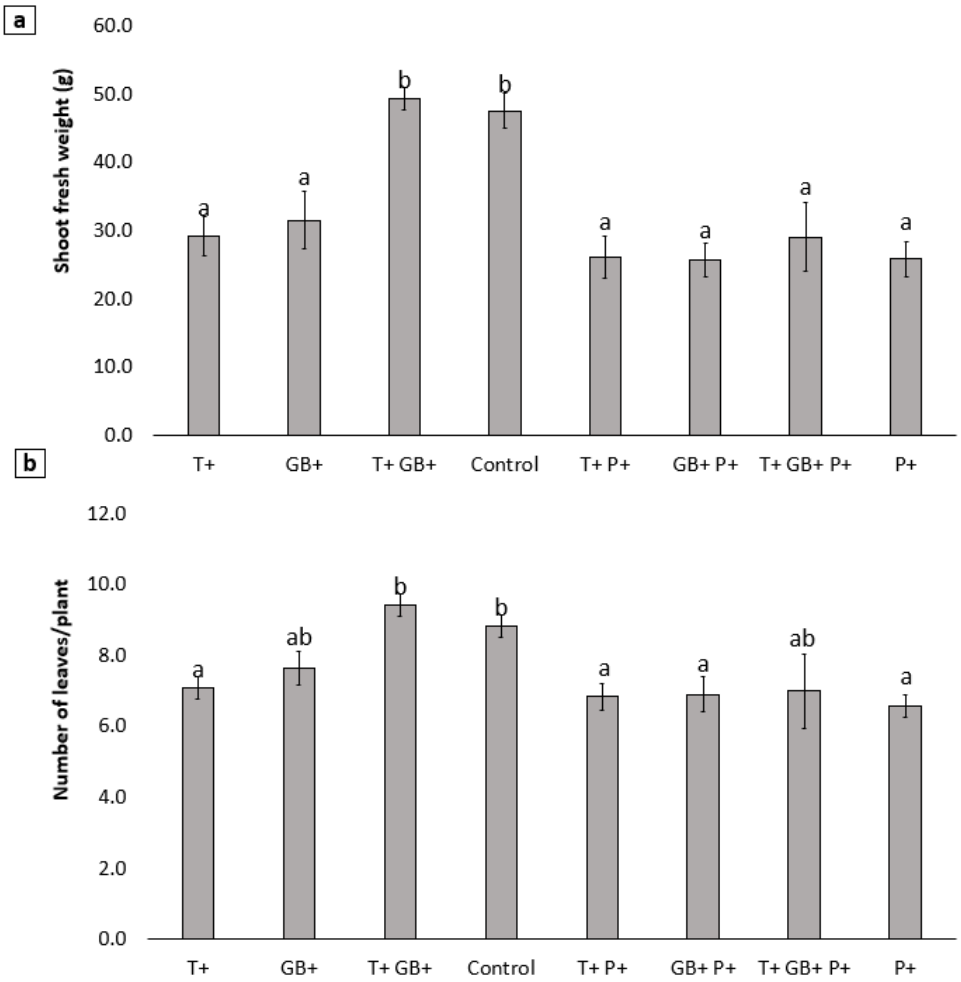

Figure 5. Shoot development of cucumber plants under a hydroponic system inoculated with *Trichoderma asperellum* (T+), *Pythium ultimum* (P+) and glycine betaine (GB+), alone or in combination (trial C). **(a)** Averages of shoot fresh weight and **(b)** number of leaves/plants. The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests (Mean ± SE). Different letters indicate significant difference between the treatments at a significance level of 0.05.

Root development assessment

With regards to the root development, significant differences were found among treatments (X_i^2 = 24.045, df = 7, P = 0.001). The treatment GB+ P+ was statistically different from control (X_i^2 = 6.879, df = 1, P = 0.008), P+ (X_i^2 = 8.086, df = 1, P = 0.004), T+ (X_i^2 = 13.729, df = 1, P = 0.0002) and T + GB+ (X_i^2 = 11.997, df = 1, P = 0.0005). All other treatments did not differed statically from the control (T+ GB: X_i^2 = 1.321, df =1, P = 0.250; T+ GB + P+: X_i^2 = 1.131, df =1, P = 0.287; P+ : X_i^2 = 1.15, df =1, P = 0.283; T+ P+ : X_i^2 = 2.145, df =1, P = 0.143; GB+: X_i^2 = 0.045, df =1, P = 0.831; T+ : X_i^2 = 1.194, df =1, P = 0.274) (Figure 6).

An important highlight was the treat T+ GB+. Sixty percent of the plants presented the best growth, followed by the T+ with 50% of the plants. While in the control, only 30% showed such behaviour. In contrast, 75% of the plants from the treatment GB+ P+ presented the worst scores for root development.

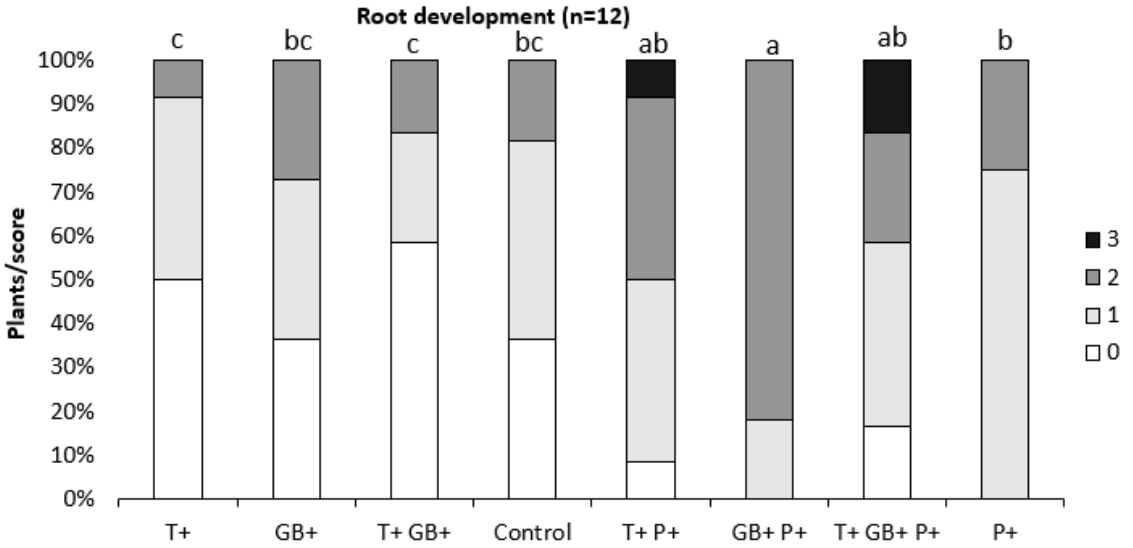

Figure 6. Root development of cucumber plants under a hydroponic system inoculated with *Trichoderma asperellum* (T+), *Pythium ultimum* (P+) and glycine betaine (GB+), alone or in combination (trial C). Number of plants per score for root development (0 = very well-developed root system, 1 = intermediate root system, 2 = small root system, 3 = root system of dead plants). The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests (Mean ± SE). Different letters indicate significant difference between the treatments at a significance level of 0.05.

Disease severity assessment

Considering the stem lesions, no significant differences were found among treatments (when *Pythium* was present) (X_i^2 = 0.615, df = 3, *P* = 0.892). In addition, at least one plant died on all treatments inoculated by the pathogen (Annex Figure 2).

Colonization assessment

On the petri plates with the *Trichoderma* semi-selective medium, two root pieces of each plant were plated. For the plants treated only with *Trichoderma* (T+), the colonization was positive in 100% of the roots; The roots treated with both the glycine betaine and *Trichoderma* (GB+ T+), on average 95.5% of roots returned as positive. The plates containing roots from the treatment GB+, presented a contamination or a different strain of *Trichoderma*, since some roots were positive for the fungi.

On PDAS, *P. ultimum* was found to be positive, on average, in 1.3 roots (65%) from T+ P+, 1.41 roots (70%) from P+ and GB+ P+ and 1.5 roots (75%) from T+ GB+ P+.

In addition to that, was observed that when *T. asperellum* was diluted on the osmotic regulator, on average, 1.95 x 10^4 spores/L were found in the *Trichoderma* suspension whereas when diluted on the osmotic regulator 1.64 x 10^4 spores/L.

Regarding the effects on the growth of *T. asperellum* and *P. ultimum* under different concentrations of glycine betaine no significant differences were found among treatments for fungi growth (F = 0.915, df = 2, *P* = 0.346).

TRIAL D

Greenhouse experiments

The trial D evaluated the response of plants from different commercial cultivars inoculated with *Trichoderma and P. ultimum*. The symptoms on the plants were already described in the previous trials. Only one dead plant was found, in the treatment T+ P+ (cultivar KURIOS).

Average temperature was 26.76 °C (range: 21.31 °C - 31.42 °C) and average relative humidity was 49.16 % (range: 32.79% - 71.12%).

A factorial analysis of variance was performed. No significant interaction effects among *T. asperellum* and *P. ultimum* occurred for the shoot fresh weight (F = 1.476, df = 1, $P = 0.226$). However, it did occur for the average number of leaves/plant (F = 19.951, df = 1, $P < 0.0001$), and for the scores of root development (F =6.969, df = 1, $P = 0.009$) and stem lesions (F = 4.823, df = 1, $P = 0.029$). A significant interaction among *Pythium* and cultivars was found only for stem lesions (F = 2.957, df = 5, $P = 0.014$). Furthermore, the cultivars were significant for number of leaves /plant (F = 5.416, df = 5, $P = 0.00015$) and stem lesions (F = 2.957, df = 5, $P = 0.014$). *T. asperellum* main effect was found for shoot fresh weight (F = 5.416, df = 1, $P = 0.021$), root development (F = 18.552, df = 1, $P < 0.0001$) and stem lesion scores (F = 4.23, df = 1, $P = 0.029$). Additionally, the effects from the pathogen occurred for all traits (leaves: F =19.951, df =1, $P < 0.0001$; weight: F =7.698, df= 1, $P = 0.006$; lesions: 39.678, df = 1, $P < 0.0001$; roots: F = 46.351, df = 1, $P < 0.0001$) (Table 4).

Table 4. Factorial analysis of variance. Interaction model and main effects or shoot fresh weight, number of flowers/plants, number of leaves/plants, number of plants per score for root development and stem lesions caused by *Pythium ultimum* of the hydroponic cucumber plants inoculated with *Trichoderma asperellum* BB005 and *P. ultimum* (trial D).

	Shoot fresh weight	Number of leaves	Root scores	Stem lesions
Cultivar	ns	***	ns	*
Trichoderma	*	ns	***	*
Pythium	**	***	***	***
Trichoderma: *Pythium*	ns	**	**	*
Trichoderma: cultivar	ns	ns	ns	ns
Pythium: cultivar	ns	ns	ns	*
Trichoderma: Pythium: cultivar	ns	ns	ns	ns

Significance codes: 0.001 '***'; 0.01 '**'; 0.05 '*'; 'ns' Not significant.

Spearman's rank correlations were calculated. All characters were significantly correlated. Lesion scores were negatively correlated to root scores (r = -0.436, p < 0.0001) and to fresh weight (r = -0.181, p = 0.030).

Significant differences among treatments were found for shoot fresh weight: $X_i^2 = 19.373$, df = 3, $P = 0.0002$; number of leaves/plants: $X_i^2 = 30.516$, df =3, $P < 0.0001$; root development: $X_i^2 = 50.855$, df = 3, $P < 0.0001$; and stem lesions: $X_i^2 = 45.89$, df = 3, $P < 0.0001$.

Shoot development assessment

In the absence of *Trichoderma*, the cultivars DEE ZIRE, DEE LITE and SUMAPOL were more affected by the inoculation of *P. ultimum*. On the other hand, in the presence of *Trichoderma*, there was no effect of *Pythium* on shoot development. However, inoculation of *Trichoderma*, in the absence of *Pythium*, reduced, significantly, the shoot fresh weight in ORIBI, DEE LITE and SUMAPOL compared to non-inoculated plants.

For shoot fresh weight, there were no statistic differences among treatments for the cultivars KURIOS (X_i^2 = 1.056, df = 3, P = 0.7875), ORIBI (X_i^2 = 3.7212, df = 3, P = 0.2932) and SARGON (X_i^2 = 2.455, df = 3, P = 0.48). In contrast, the cultivars DEE LITE (X_i^2 = 9.177, df = 3, P = 0.027), DEE ZIRE (X_i^2 = 11.916, df = 3, P = 0.007) and SUMAPOL (X_i^2 = 10.677, df = 3, P = 0.013) showed significant differences among treatments (Figure 7).

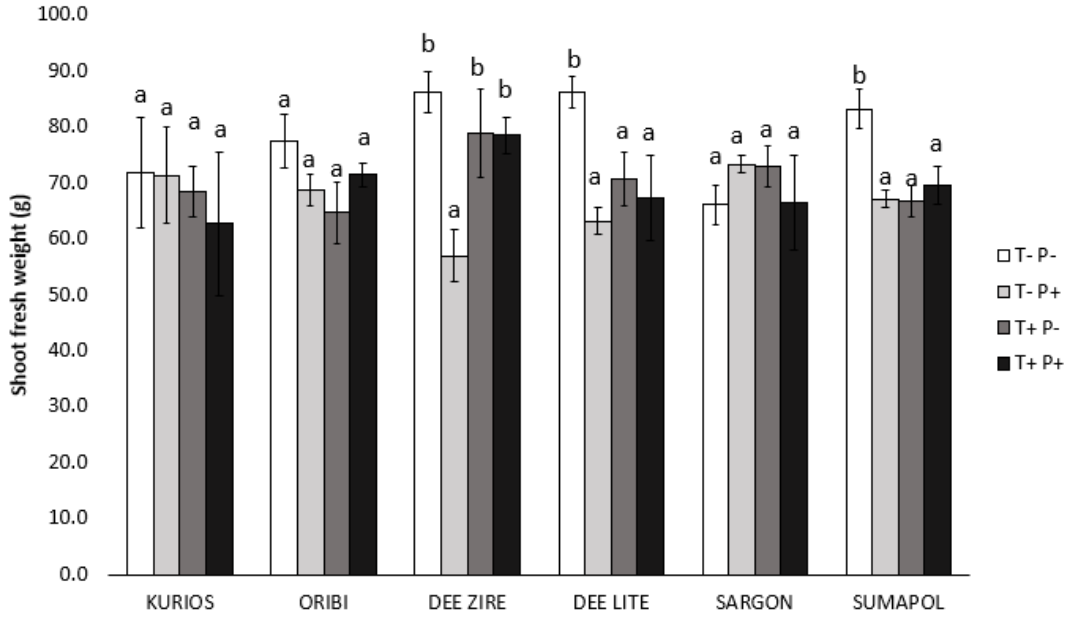

Figure 7. Shoot development of different commercial cucumber cultivars under a hydroponic system inoculated with *Trichoderma asperellum* (T+) and *Pythium ultimum* (P+) or not inoculated (T- and P-) (trial D). Averages of shoot fresh weight. The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests (Mean ± SE). Different letters indicate significant difference among treatments within each cultivar at a significance level of 0.05.

Regarding the number of leaves/plants, the cultivars DEE LITE (X_i^2 = 10.315, df = 3, P = 0.016), DEE ZIRE (X_i^2 = 13.671, df = 3, P = 0.003), SARGON (X_i^2 = 12.091, df = 3, P = 0.007) and SUMAPOL (X_i^2 = 7.292, df = 3, P = 0.027) showed significant differences among treatments while KURIOS (X_i^2 = 0.744, df = 3, P = 0.862) and ORIBI (X_i^2 = 6.5833, df = 3, P = 0.086) did not (Figure 8).

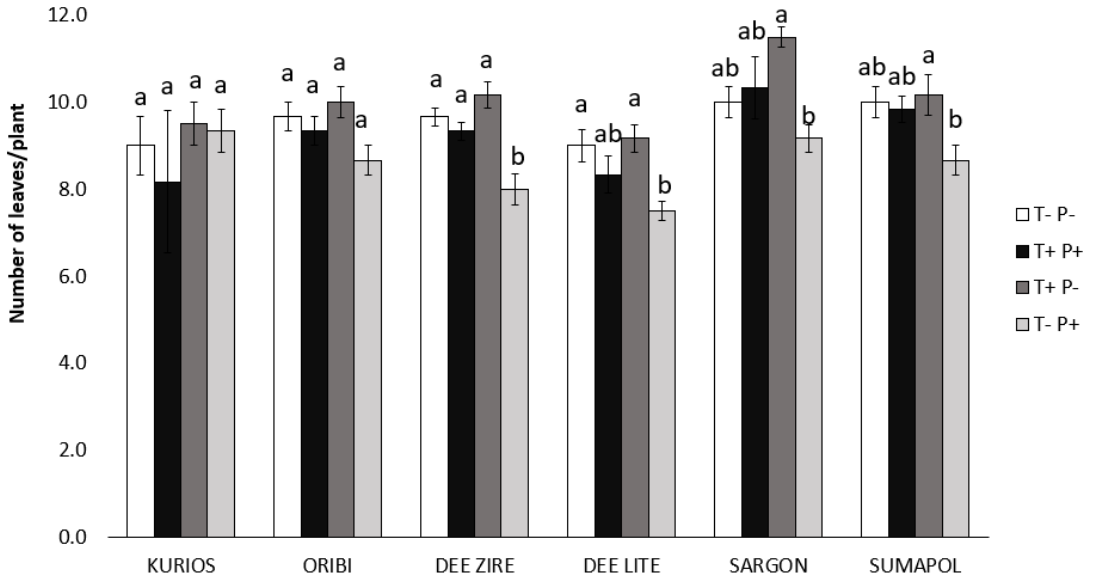

Figure 8. Shoot development of different commercial cucumber cultivars under a hydroponic system inoculated with *Trichoderma asperellum* (T+) and *Pythium ultimum* (P+) or not inoculated (T- and P-) (trial D). Average of number of leaves/plants. The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests (Mean ± SE). Different letters indicate significant difference among treatments within each cultivar at a significance level of 0.05.

Root development assessment

The root development showed significant differences among treatments (X_i^2 = 50.855, df = 3, P < 0.0001) (Figure 9). In the absence of *Trichoderma*, all cultivars seemed to be more affected by the inoculation of *P. ultimum*. On the other hand, in the presence of *Trichoderma*, there was a positive effect for the cultivars DEE ZIRE, DEE LITE, ORIBI and SUMAPOL for the root development in which 50% of plants were well developed. The inoculation of *Trichoderma*, in the absence of *Pythium*, did not show any effects in root development compared to non-inoculated plants.

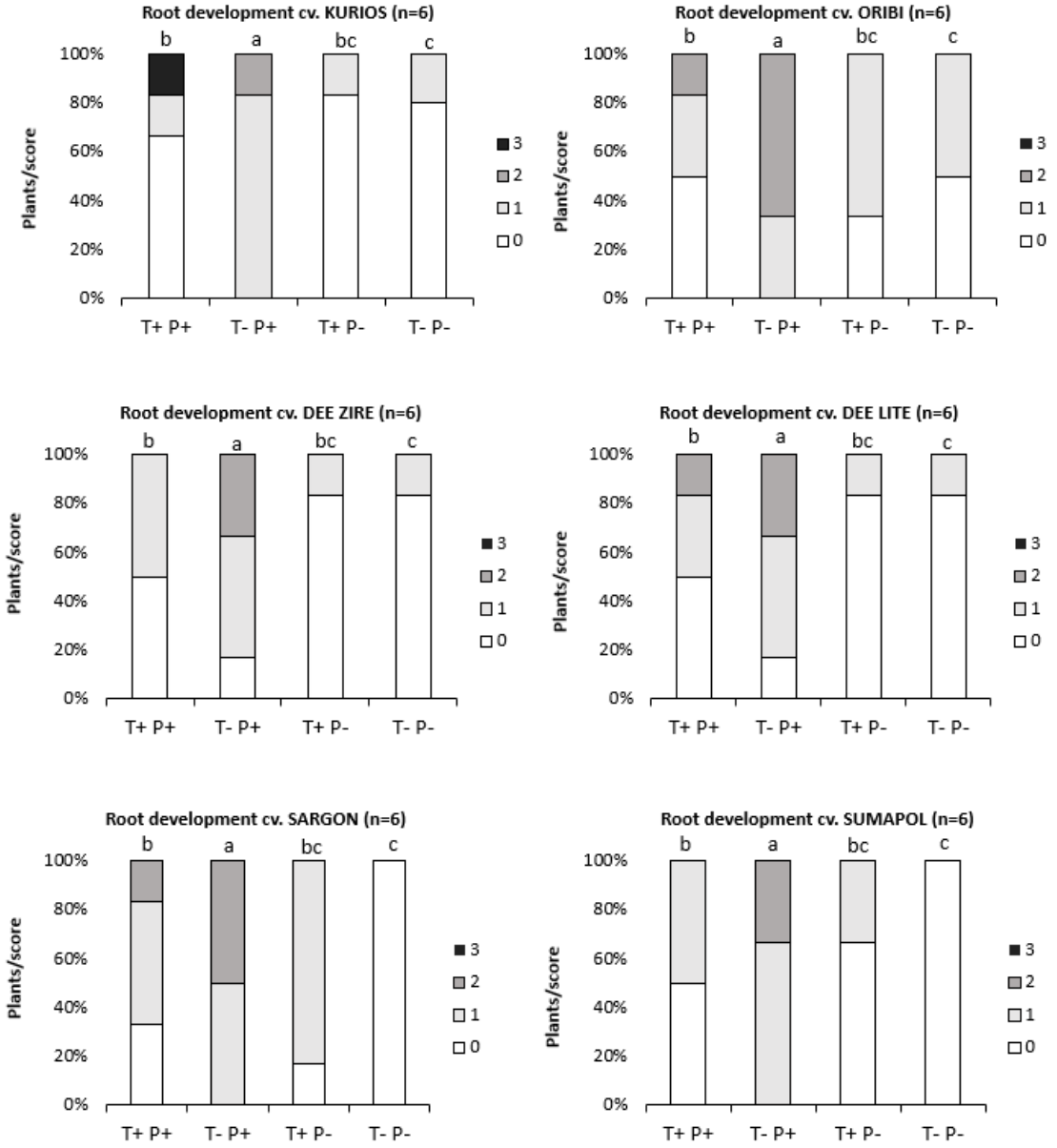

Figure 9. Root development of different cucumber cultivars under a hydroponic system inoculated with *Trichoderma asperellum* (T+) and *Pythium ultimum* (P+) or not inoculated (T- and P-) (trial D). Number of plants per score for root development (0= very well-developed root system, 1= intermediate root system, 2= small root system, 3= root system of dead plants). The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests. Different letters indicate significant difference between the treatments at a significance level of 0.05.

Disease severity assessment

Concerning the stem lesions, a significant difference was found among the treatments where *Pythium* was inoculated (X_i^2 = 5.832, df = 1, P = 0.015). All cultivars showed a level of variability towards *Pythium*, where DEE ZIRE seemed to be more susceptible whereas SARGON presented more tolerance. Additionally, the stem lesions from DEE ZIRE on T- P+ was significantly different from T+ P+ (X_i^2 = 4.125, df = 1, P = 0.042). Moreover, the presence of *Trichoderma* in combination with *Pythium* reduced the disease severity.

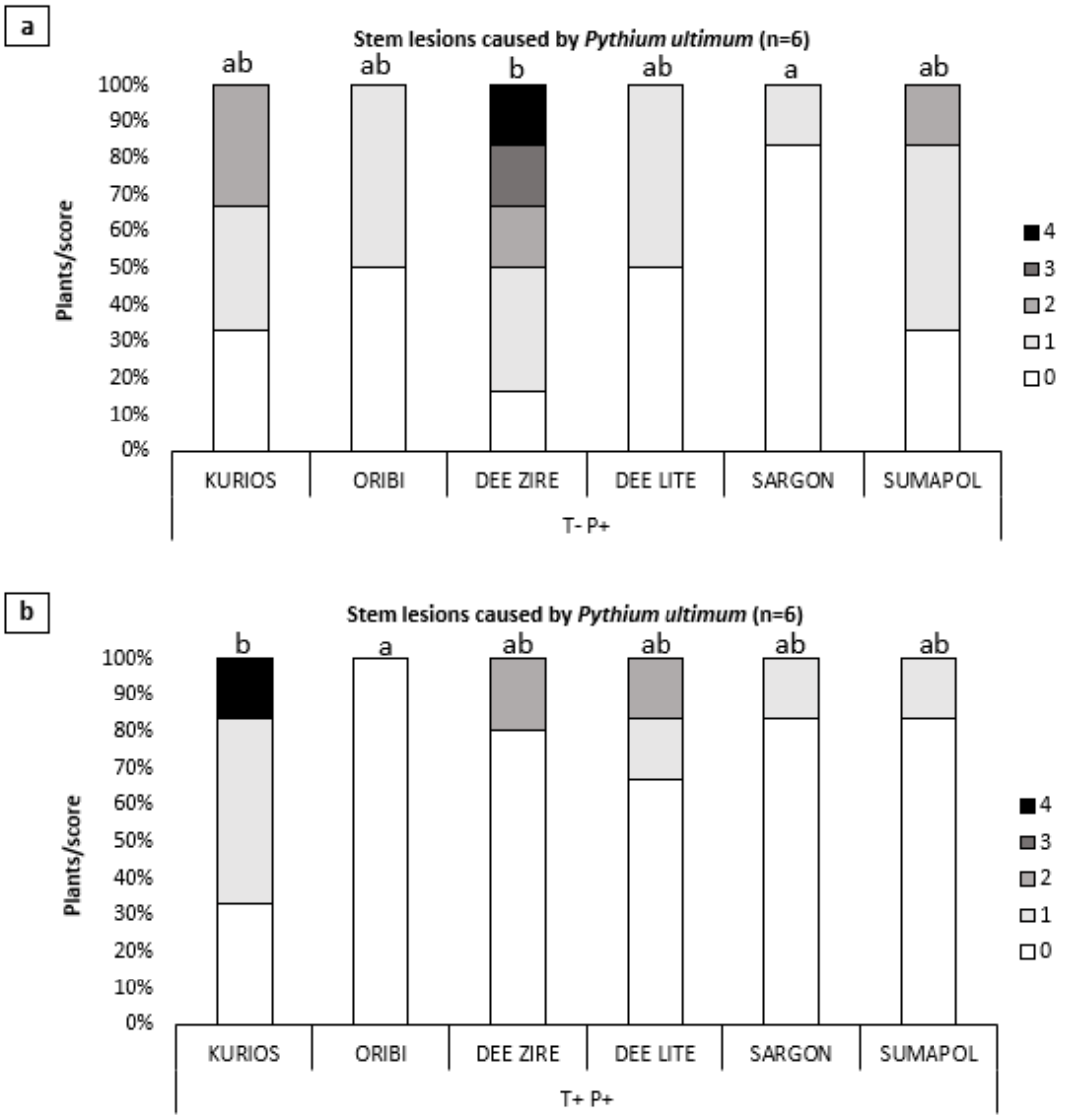

Figure 10. Disease severity on different cucumber cultivars under a hydroponic system inoculated with *Trichoderma asperellum* (T+) and *Pythium ultimum* (P+) or not inoculated (T-) (trial D). Number of plants per score for the stem lesions caused by *P. ultimum* in **(a)** T- P+ and **(b)** T+ P+ (0= healthy, 1= small lesion on the stem, 2= intermediate size of lesion on the stem, 3= big lesion on the stem, 4=severe lesion/dead plant). The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests. Different letters indicate significant difference between the cultivars at a significance level of 0.05.

Colonization assessment

The colonization of *Trichoderma* in the semi selective medium was assessed quantitatively rather than qualitatively, represented by the number of CFU/g of substrate (Table 5).

Table 5. Average of *Trichoderma asperellum* BB005 (CFU/g) in the substrate of hydroponic cucumber plants during the trial D. The cultivars were treated with *Trichoderma* alone (T+ P-) or the combination of *Trichoderma* and *Pythium ultimum* (T+ P+).

	Treatments	
Cultivars	T+ P- (CFU/g)	T+ P+ (CFU/g)
KURIOS	1.0×10^4	5.4×10^3
ORIBI	2.1×10^3	5.3×10^3
DEE ZIRE	$1.2x\ 10^4$	4.4×10^3
DEE LITE	8.1×10^3	4.2×10^3
SARGON	1.7×10^4	4.3×10^3
SUMAPOL	1.6×10^3	2.5×10^3

Overall, the plants from T+ P- had up to 4 times more CFUs/g for all cultivars, except ORIBI and SUMAPOL. These cultivars presented a decreased average of CFUs/g on T+ P- when compared to the treatment with both microorganisms (T+ P+). Additionally, the cultivar SUMAPOL was the lowest colonized in both treatments.

Regarding the colonization by the pathogen, five root pieces were plated from each plant in PDAS. For T+ P+, the cultivars KURIOS and DEE LITE had 4.3 roots (86%) positive for the pathogen, followed by DEE ZIRE with 3.6 (72%) and both ORIBI and SUMAPOL with 2.3 (46%). The cultivar SARGON presented the least amount of root pieces positive for the pathogen (1.6 – 32%). Moreover, for T- P+, SUMAPOL presented the highest average of positive roots (4.3 – 86%) followed by DEE LITE with an average of 4 roots (80%) and DEE ZIRE with 3.3 (66%). The cultivars ORIBI, SARGON and KURIOS, showed a very low number for positive roots (0.3, 1, 1.3, respectively).

TRICHODERMA - HERBIVORE INTERACTION

CUCUMBER

Greenhouse experiments

The experiments in cucumber evaluated plant responses when those were inoculated with *T. asperellum* (BB005) and the effect of this treatment on the population growth of the cotton aphid (*A. gossypii*).

Average temperature was 22.84 °C (range: 19.41 °C – 28.46 °C) and average relative humidity was 61.40 % (range: 51.67% - 74.67%).

Herbivore population growth

The number of aphids/plants was lower in plants treated with *Trichoderma* than in control plants. A significant statistical difference was found among treatments ($F = 1.342$, df = 1, 10, $P = 0.00026$) (Figure 11).

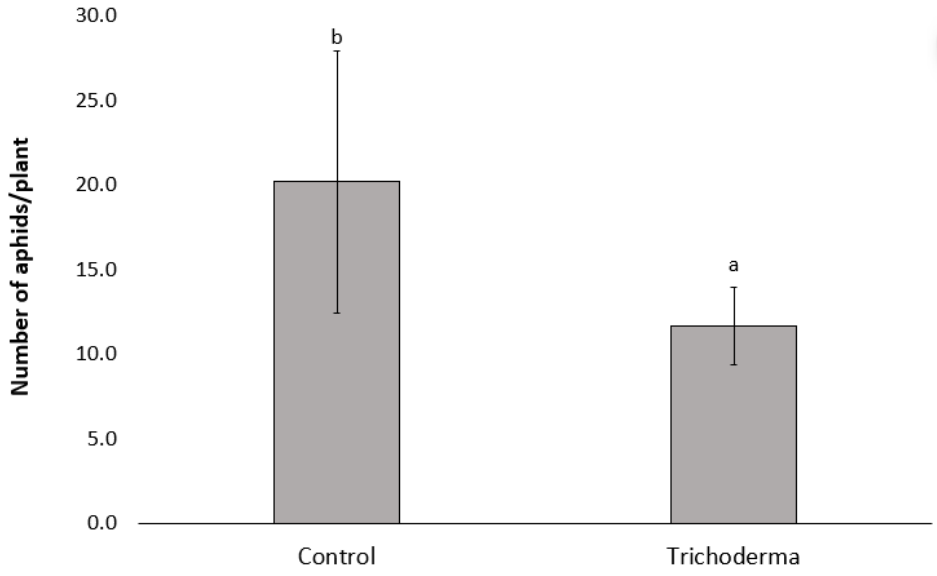

Figure 11. Number of aphids/plant (*Aphis gossypii*) in cucumber under hydroponic system counted 14 days post infestation. The data was tested by a simultaneous test for general linear hypotheses and means were compared by Tukey (Mean ± SE). Different letters indicate significant difference between the treatments at a significance level of 0.05 (n=6).

Shoot development assessment

No significant differences were for shoot fresh weight (X_i^2 = 0.103, df = 1, P = 0.747) (Annex Figure 3). However, a significant difference was found for the number of leaves/plants (X_i^2 = 9.209, df = 1, P = 0.002) (Figure 12).

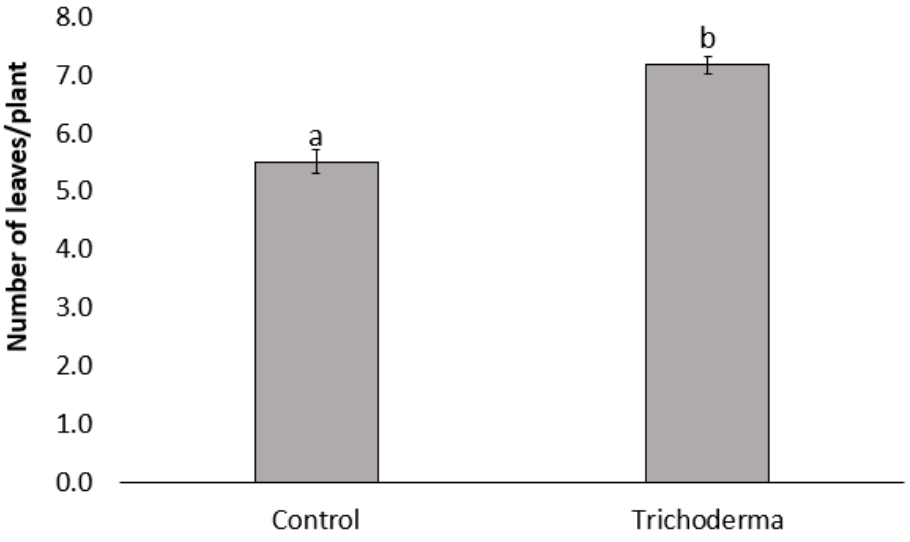

Figure 12. Shoot development of cucumber under a hydroponic system inoculated with *Trichoderma asperellum* and infested with *Aphis gossypii*. Average of number of leaves/plants. The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests (Mean ± SE). Different letters indicate significant difference among treatments at a significance level of 0.05 (n=6).

Root development assessment

The scores of root development showed a significant difference among treatments (X_i^2 = 4.6933, df = 1, *P* = 0.0302) (Figure 13).

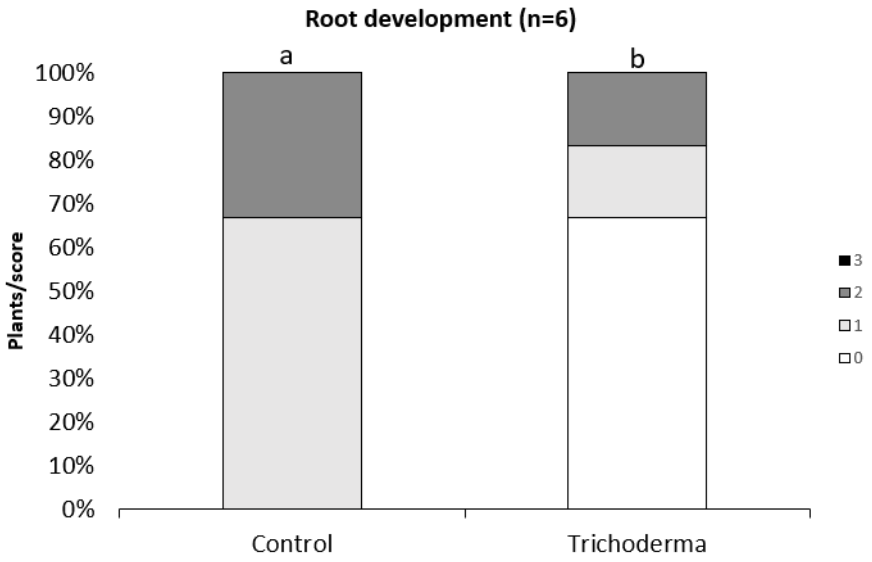

Figure 13. Root development of cucumber under a hydroponic system inoculated with *Trichoderma asperellum* and infested with the aphid *Aphis gossypii*. Number of plants per score for root development (0= very well-developed root system, 1= intermediate root system, 2= small root system, 3= root system of dead plants). The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests. Different letters indicate significant difference between the treatments at a significance level of 0.05.

Colonization assessment

The colonization of *Trichoderma* in the semi selective medium was represented by the number of CFU/g of substrate. The average *of T. asperellum* BB005 colonization of the hydroponic cucumber plants infested with *A. gossypii* was 1.64 x 10^4 CFU/g.

FAVA BEANS

Greenhouse experiments

The experiments with fava beans, evaluated the overall response of plants under different inoculation methods with *T. asperellum* BB005 and the effect of these treatments on the population growth of the pea aphid (*A. pisum*).

Two trials were performed. Plant shoot height, number of leaves and number of extra floral nectar glands per plant were recorded one day before aphid infestation and at 7 days post infestation.

The environmental conditions from both trials were quite similar. The average temperature was 23.77 °C (range: 21.53 °C – 25.66 °C) and average relative humidity was 35.01 % (range: 23.40% - 51.66%).

Spearman's rank correlation was calculated. For the first trial, the number of aphids/plants was negatively correlated to all traits, but only significant for the plant height (r = -0.350, p = 0.014),

the number of leaves/plant (r = -0.340, p = 0.017) and the root development scores (r = -0.336, p = 0.019). There was a positive correlation among the plant height and the number of leaves/plant (r = 0.426, p = 0.0025); plant height and the shoot fresh weight (r= 0.579, p < 0.0001) and the plant height and root weight (r = 0.303, p = 0.036). The number of extrafloral nectar glands/plant was positively correlated with the root weight (r = 0.289, p = 0.046); The root score development was positively correlated with the root weight (r = 0.417, p = 0.003) and with the shoot fresh weight (r = 0.384, p = 0.006); Finally, the root weight with the shoot fresh weight were positively correlated (r = 0.602, p < 0.0001).

For the second trial, the number of aphids/plants was negatively correlated with all traits except for the scores of root development. However, was only statistically significant for the plant height (r = -0.371, p = 0.025) and the number of extra floral nectar glands/plant (r = -0.349, p = 0.036). All other traits were positively correlated. The plant height was significantly correlated with all traits. The number of leaves / plants was correlated with root weight (r = 0.387, p = 0.019). The number of extra floral nectar glands/plants was correlated to the shoot fresh weight (r = 0.520, p = 0.001) and with the root weight (r = 0.346, p = 0.038), which were also correlated among each other (r = 0.629, p < 0.0001). The scores of root development was correlated to root weight (r = 0.587, p < 0.0001) and the shoot fresh weight (r = 0.750, p < 0.0001).

Herbivore population growth

The number of aphids/plants was lower in both *Trichoderma* treatments when compared to the control, for both trials (Figure 14). The first trial showed significant differences among *Trichoderma* treatments and the control in the number of aphids/plants (F = 2.944, df = 1, 46, *P* < 0.0001). In the second trial, overdispersion was detected, and the treatments were significantly different based on a quasi-Poisson distribution (F = 4.529, df = 2, 33, *P* = 0.018). These results suggest that in both trials, the treatments with *Trichoderma* had a significant negative effect on the aphid population.

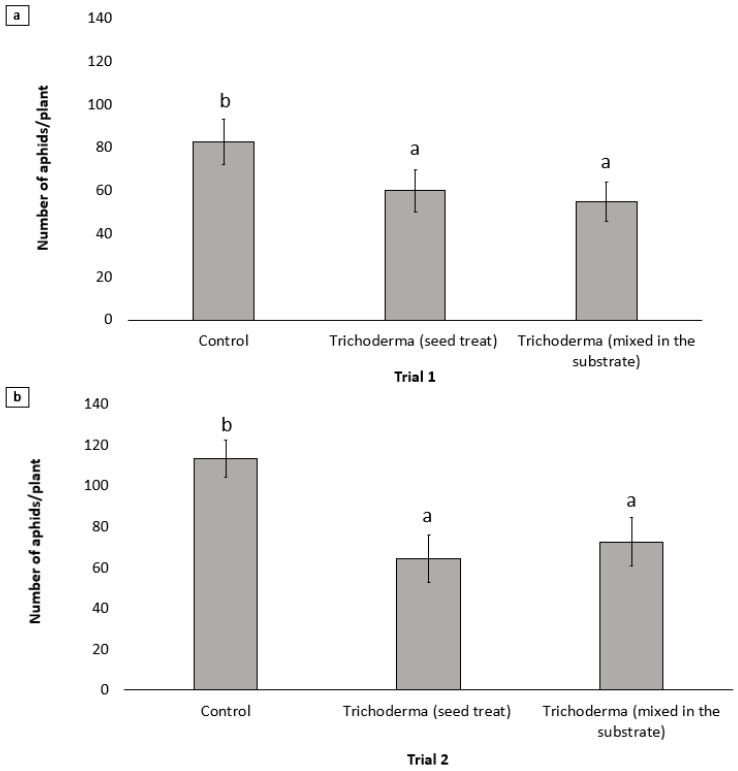

Figure 14. Number of aphids/plant (*Acyrtosiphon pisum*) in fava beans under different inoculation methods of *Trichoderma asperellum* from the **(a)** first and **(b)** second trials counted 7 days post infestation. The data was tested by a simultaneous test for general linear hypotheses and means were compared by Tukey (Mean ± SE). Different letters indicate significant difference between the treatments at a significance level of 0.05.

Shoot development assessment

In both trials, there were no differences for the number of leaves/plant (First: F = 1.830, df = 2,45, *P* = 0.172; Second: F = 1.215, df = 2, 33, *P* = 0.309), the number of extrafloral nectar glands (First: F = 0.939, df = 2, 45, *P* = 0.398; Second: F = 2.523, df = 2, 33, *P* = 0.095), plant height (First: F = 1.898, df = 2,45, *P* = 0.161; Second: F = 0.751, df = 2,33, *P* = 0.479), the shoot fresh weight (First: F = 1.956, df = 2, 45, *P* = 0.153; Second: F = 0.745, df = 2, 33, *P* = 0.483) and root weight (First: F = 0.205, df = 2, 45, *P* = 0.814; Second: F = 0.434, df = 2,33, *P* = 0.651) (Annex Figures 4 and 5).

Root development assessment

The scores of root development did not show significant differences among treatments (X_i^2 = 1.3 184, df = 2, *P*= 0.517) (Annex Figure 6).

Colonization assessment

The root pieces of approximately 1 cm from plants of each treatment were taken to be plated in the selective growth medium to check *Trichoderma* colonization. For both trials, two plants were taken and from them, fifteen root pieces were plated in semi selective medium. Comparing both treatments with *T. asperellum* the seeds treatment presented on average, 8.5 (56.6%) positive roots While, mixing *Trichoderma* into the substrate, returned on average 11.12 (74.13%) positive roots for *Trichoderma.*

DICUSSION

TRICHODERMA – PATHOGEN INTERACTION

Greenhouse experiments

In this study, the biological control potential of *T. asperellum* BB005 was analysed in hydroponically grown cucumber against *P. ultimum*. The preliminary trial (trial A) investigated plant-pathogen interactions, whereas trials B, C and D assessed an interaction involving *Trichoderma*, plant and pathogen.

In general, the results established the potential of *T. asperellum* BB005 in reducing disease severity of crown rot caused by *P. ultimu*m and promoting root growth of cucumber plants under a hydroponic system.

Trichoderma spp. is a widely used antagonistic microorganism with proven efficiency in reducing disease severity caused by plant pathogens and promoting plant growth (Harman et al., 2004). *Trichoderma* spp. may present more than one mechanism of action, depending on the species and strain. A study using *T. virens* Gv29-8 and Tv10.4 demonstrated the mycoparasitism action to control *P. ultimum* (Djonović et al., 2006) *and T. harzianum* strain T22 is a rhizosphere competent strain and capable to persist for long period. Moreover, it is efficient in controlling *Fusarium* crown and root rot in tomatoes and *R. solani* and *Pythium* in ornamentals (Paulitz & Bélanger, 2001).

Shoot and root development assessment

Trial A evaluated the disease severity among two doses of the pathogen. The plants treated with the high dose presented severe lesions on the stem (crown rot) and two plants died. In the treatment with the low dose of the pathogen only one plant exhibited a significant stem lesion while all other plants were symptomless. Interestingly, these plants presented a slightly better development than the control plants.

This response might be attributed to the phenomenon of hormesis. It is an adaptive process from plants and other living organisms. It is defined as a two-phase response to a stressor that at low doses induces a beneficial effect, whereas at high doses is toxic (Vargas-Hernandez et al., 2017). The benefits, therefore, include disease resistance, production of secondary metabolites and plant growth (Calabrese & Mattson, 2011). Even though plant development and an apparent disease resistance were observed, it is not proven, that such physiological benefits from a low dose of a pathogen can be considered hormesis. On the other hand, it might be interesting to explore the idea of activating an hormesis effect for the immunization of plants.

In addition to the beneficial effects in supressing plant diseases, some *Trichoderma* species are also able to colonize root surfaces and cause substantial changes in plant physiology. Some strains promote plant and root growth, increase nutrient availability and improve crop production (Fernández et al., 2017; Harman, 2006; Harman et al., 2004; Martínez-Medina et al., 2013; Vinale et al., 2008).

The trials B, C and D demonstrated the ability of *T. asperellum* BB005 in stimulating root development. the experiments presented significant differences among treatments regarding the root development scores. *Trichoderma* spp. can colonize roots, both externally and internally producing hormonal signals to facilitate the colonization and to promote root growth (Muckherjee et al., 2012). This phenomenon has been reported, for example, in Arabdopsis with *T. virens* in which lateral root growth was promoted (Contreas-Cornejo et al., 2009).

The enhancement of the root systems after inoculation with *Trichoderma* was clear. Additionally, there was a positive significant effect of *Trichoderma* on the root development in all trials. The enhancement of root development has positive effects for production regarding for nutrient uptake and consequently higher yields (Harman, 2006).

Regarding aerial plant growth, even though there were some positive correlations among shoot development traits there were also some negative effects in plants inoculated with *Trichoderma* compared to control plants. In many cases, when beneficial microbes are present, the shoot:root ratio might be affected in the early stages of plant development (i.e. vegetative growth). However, afterwards, the shoot resumes normal growth and might be even better when plants are inoculated with *Trichoderma* than those without. These growth depressions are also present in mycorrhizal associations (Li et al., 2008). For example, *T. asperellum* T34 and found that the fungus enhanced the availability of nutrients, leading to significant increases in dry weight, shoot length and leaf area (Segarra et al., 2007). Furthermore, Li et al (2018) reported that *T. asperellum* CHF 78 besides providing control against *Fusarium* wilt on tomato, also significantly increased plant growth.

In addition, in trial C, the combination of *Trichoderma* and glycine betaine seems to be beneficial for plant growth, however, we found no impact in reducing disease severity. The laboratory tests using different concentrations of GB on PDA plates did not show any statistical differences for the growth of the microorganisms. Still, in the plants, GB may have served as a source of nitrogen for the microorganisms allowing the combination T+ GB to help plants to recover from any negative effects caused by *Trichoderma* and GB alone. The exogenous application of GB has been studied and successfully proved to reduce the negative effects of abiotic stress and improve plant growth (Fariduddin et al., 2013).

Disease severity assessment

The results from the trials B, C and D show a reduction of disease severity promoted by *T. asperellum* BB005. The disease caused by *P. ultimum* was expressed as crown rot and was characterized by dry brownish necrotic lesions developed along the stem causing a reduction in plant growth (Menzies & Jarvis, 1994). There was a negative correlation among the plant development and the stem lesions caused by the pathogen. The higher the score of the stem lesion, the lower the development of the plants.

Depending on the species and strains of *Trichoderma*, several interactions can be observed between plants and pathogens. Numerous references in the literature have demonstrated the abilities of *Trichoderma* species to control diseases directly or indirectly, by inducing systemic resistance on plants (Djonović et al., 2006; Fernández et al., 2017; Harman, 2006; Harman et al.,

2004; Hermosa et al., 2013, 2012; Kipngeno et al., 2015; Martínez-Medina et al., 2013; Rahman et al., 2018; Segarra et al., 2013; Shoresh et al., 2005; Trillas et al., 2006; Van Wees et al., 2008; Waghunde et al., 2016; Yedidia et al., 1999).

The reduction of disease severity caused by *T. asperellum* BB005 was expressed by the lower scores the plants received. That occurred, for example in trial B, where 60% of plants in the T+ P+ treatment was scored either healthy or with a small lesion on the stem. When comparing different cultivars in trial D, four out of the six cultivars reduced stem lesions caused by *P. ultimum* were recorded when *Trichoderma* was applied.

In our experiments *T. asperellum* BB005 did not prevent plant death caused by *P. ultimum* in hydroponic cucumber, nevertheless disease severity was reduced. There are some strains of *Trichoderma* capable of supressing plant diseases by inducing systemic resistance (Van Wees et al., 2008; Pieterse et al., 2014). Although complete control of the pathogen is not achieved, a significant reduction of lesion size is possible.

In the plant immune system, the first line of defence is called Pattern Triggered immunity (PTI). It is a general immunity and possesses pattern-recognition receptors (PRRs) that evolved to recognize microbial compounds, called pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs). Upon damage caused by an invasion, plants responses are called damage-associated molecular patterns (DAMPs) (Pieterse et al., 2014). *Trichoderma* spp. are not considered as an enemy by plants. *Trichoderma* secretes proteins and secondary metabolites that act as MAMPs and enzymes that act against other fungi generating DAMPS. These are recognized by plant receptors activating a signalling cascade to stimulate plant defence as well plant growth (Hermosa et al., 2013).

Given that plant death was not completely prevented but disease was reduced, some factors such as environmental conditions, genetic variability and application method may have had an effect.

Pythium diseases can be influenced by the environment. Cooler temperatures favour *P. ultimum*. The low severity and incidence of the disease in trial D, might be explained by lower average temperature as compared to trials B and C. This is in agreement with Martin & Loper (1999) that showed variations on disease severity depending on *Pythium* spp. where *P. aphanidermatum* (Edson) Fitzp. and *P. myriotylum* (Drechsler) are more severe in higher temperatures (above 25 °C) whereas *P. ultimum* and *P. irregulare* (Buisman) present a higher severity in temperatures between 5 °C and 25 °C.

Hendrix & Campbell (1973) showed that moisture content is an important factor for *Pythium* spp. Concretely, high soil moisture favours saprophytic growth allowing fast colonization of the substrate, whereas at the same time may be detrimental to other microorganisms. These conditions could have influenced positively *Pythium* and negatively the performance of *Trichoderma* in terms of not being able to prevent plant death.

Another aspect is that, in trial D, different cultivars were used and therefore, genetic variation might have influenced the disease severity. The cultivar DEE ZIRE showed higher susceptibility to *P. ultimum* than the other cultivars while the cultivar SARGON seemed to be the most tolerant. Furthermore, all cultivars presented an improvement (in different levels) in the reduction of the

stem lesions as well as for the enhancement of root development. It has been demonstrated, in a study with *T. atroviride* P1 and *T. harzianum* T22 and four tomato genotypes, that reduction of disease and growth stimulation caused by *Trichoderma* spp. is dependent on the genotype (Tucci et al., 2011). It is important to notice that even though some variability among cultivars exists, genetic resistance is being screened, although, until the present moment the commercial availability of resistant cultivars is not known.

Regarding the application method, *Trichoderma* was applied as a drench before the pathogen inoculation. This preventive application aimed at allowing the establishment of *Trichoderma* and permit a better contact with the roots.

Colonization assessment

Colonization by *Trichoderma* spp. suggests the ability to attach on plant roots, penetrate and endure the metabolites produced by the plants in response to the invasion. The results from the colonization assessments demonstrated that, for trials B and C, the average of positive roots for *Trichoderma* was slightly higher than *Pythium*. Moreover, in trial D, the cultivars presented a good colonization (CFU/g) in T+ P+. *Trichoderma* spp. have a better ability to mobilize and uptake nutrients allowing it to overcome other microorganisms when inoculated in substrate due to its good competition ability and fast growth. In addition, it is been reported that some *Trichoderma* spp. produce highly efficient siderophores that chelate nutrients, such as iron and stop the growth of other fungi (Chet & Inbar, 1994).

The good colonization ability of *T. asperellum* BB005 was also assessed in an experimental station in Sint-Katelijne-Waver, Belgium. A semi commercial hydroponic cucumber production was set on rockwool slabs reused for 4th time. A poor colonization was expected since, in theory, the reuse of slabs would promote the presence of other microorganism and possibly inhibit the colonization of *Trichoderma*. However, *Trichoderma* was retrieved a month after application, (2.31×10^4 CFU/g) mostly in the block where it was applied, confirming the good colonization potential of this strain.

TRICHODERMA – HERBIVORE INTERACTION

Greenhouse experiments

Besides protecting plants against pathogens and promoting plant growth, some *Trichoderma* spp. have been reported to protect plants against insect pests (Menjivar, 2010; Akello and Sikora, 2012; Coppola et al., 2017). This experiment explored the potential of *T. asperellum* BB005 against aphids feeding on plants colonized by the fungus. Our results showed that the application of the root colonizing microorganism *T. asperellum* BB005 resulted in significant reduction of the population growth of *A. gossypii* on cucumber and *A. pisum* on fava beans. Moreover, in the fava beans experiment, this reduction occurred regardless of the application method.

Plants have evolved several defence strategies to respond and combat invasion by external threats such as herbivores. Plants can employ both constitutive and induced defences. Constitutive defences are normally physical barriers (e.g. leaf cuticle, cell wall, metabolites that inhibit feeding) while induced defences refer to the activation of direct and indirect mechanisms

that affect the herbivore behaviour. For example, a direct mechanism involves the synthesis of secondary metabolites that affect herbivore development and subsequent population growth whereas indirect defence mechanisms involve the release of volatiles to attract natural enemies (Dicke et al., 2003) or the food (i.e. extrafloral nectar) and shelter (e. g. acarodomatia) rewards that plants employ to attract and retain carnivorous arthropods (Pekas & Wäckers, 2017).

Beneficial root colonizing microbes such as *Trichoderma* spp. may enhance direct and indirect plant defence against insects, such as aphids. *Trichoderma* is also known to trigger ISR and priming, however very little is known about systemic resistance caused by beneficial root colonizing microorganisms towards phloem-feeding insects (Pineda et al., 2015). Plants responses triggered by *Trichoderma* could involve the synthesis of secondary metabolites which have been demonstrated as important factors in the interactions between plants and herbivores arthropods (Dicke et al., 2003; Turlings & Wackers, 2004). The tropic strategy of the fungus as well the feeding behaviour of the herbivore are important factors (Pieterse et al., 2014; Pineda et al., 2013). The interaction between plants and phloem-feeding insects can be comparable to a plant-biotrophic pathogen interaction. Meaning that phloem-feeding insects trigger a similar signalling response in plants as a biotrophic pathogen. Due to its feeding mode, it changes the plant physiology triggering a SA-mediated response, just like biotrophic pathogens do (Walling, 2008).

Thus, although the mechanism behind this is not well understood, the presence of the beneficial microbe changes the host plants physiology either by producing new metabolites of stimulating changes in concentrations resulting in resistance to aphid infestation with consequences in the population growth.

Herbivore population growth

In this study, the effects of *T. asperellum* BB005 towards the aphid population growth of the cotton aphid and the pea aphid was analysed. The results revealed that the total number of aphids significantly reduced in both experiments when they fed on plants inoculated by *T. asperellum* BB005. This result is similar to the findings from Akello & Sikora (2012) who studied the influence of different species and strains of endophytic fungi on populations of *A. pisum* and *Aphis fabae* on fava beans. They concluded that some of the studied strains can induce physiological changes in the host plant, affecting negatively the aphids, and consequently reducing population growth.

Shoot and root development assessment

Plant growth is a well-known beneficial effect from several biological control agents, including *Trichoderma* spp. However, in the trial with fava beans there were no statistical differences in plant growth, among treatments. However, in the trial with cucumber, the root development and number of leaves/plants showed a significant difference among treatments, but there were no differences for shoot fresh weight.

Given that plants presented a reduced aphid population and the differences in shoot fresh weight among treatments were not significant, we can speculate that the plants needed to trade-off between growth and defence in response to herbivore attack. Both plant growth and defence are

essential to ensure plants survival, however, defence may come at the expense of growth. It is believed that hormonal crosstalk plays a major role in regulating these growth-defence trade-offs (Huot et al., 2014).

Phytohormone signalling is influenced by these *Trichoderma*-plant interactions which alters the balance between plant growth and plant defence against biotic and abiotic stress (Hermosa et al., 2013). The diversion of resources to activate induced resistance may cause a negative impact in growth (Huot et al., 2014).

Damage caused by phloem feeding insects generally triggers the biosynthesis of SA, whereas chewing insects induce JA biosynthesis and both triggers signalling cascades to increase defence. Studies in tobacco plants demonstrated that such plant response results in a reduced photosynthesis, affecting plant growth (Halitschke et al., 2011). Similarly, in Arabidopsis was demonstrated that induction of defence may affect sugar reserves affecting the growth of roots (Machado et al., 2013).

Thus, these alterations in resources allocation due to induced resistance caused by *Trichoderma* could have reduced aphid population at the expense of plant growth and that could be a reason why plant development did not show great significant differences compared to the control.

Colonization assessment

Among treatments, the maximum positive roots were found in soil drenching (11.12) compared to the seed treatment (8.5 positive roots.)

Commercially, different methods for applying biopesticides are used (e.g. soil drenching, seed treatment, spraying, seed biopriming). Seed treatments are considered one of the most economical and attractive method to deliver BCAs into the substrate and requires small volume of inoculum (Singh et al., 2016).

Since there were no differences among treatments regarding the application method of *Trichoderma* for the reduction of aphid population, we can say that regardless the treatment, this *Trichoderma* sp. is an efficient root colonizer.

CONCLUSIONS

The study shows evidence that in hydroponically grown cucumber, *T. asperellum* BB005 did not prevent plant death caused by *P. ultimum*, however, it reduced disease severity of crown rot. Additionally, it was demonstrated that *T. asperellum* BB005 enhanced the root system development. Finally, *T. asperellum* BB005 affected negatively the population growth of two aphid species in two different crops. These results suggest that *T. asperellum* BB005 is a suitable candidate to be explored through further research as a multifunctional and powerful tool against plant pathogens and insect pests in IPM.

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ANNEX

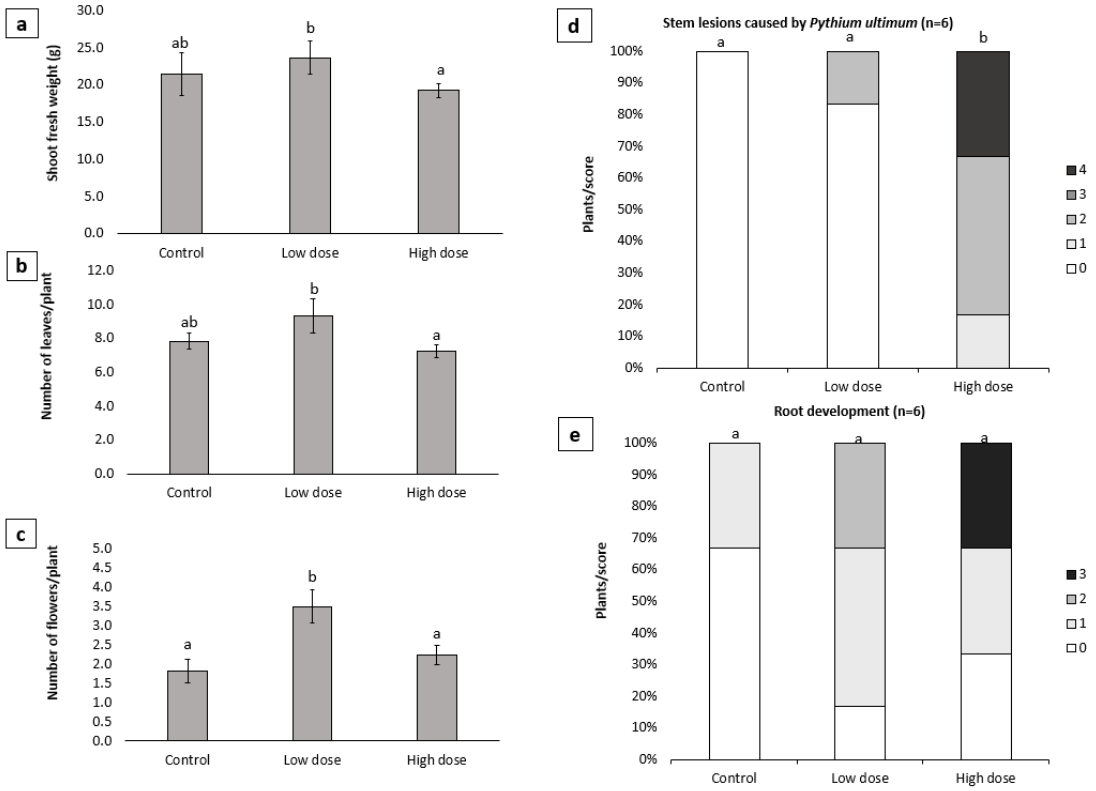

Figure 1. Plant and root development and disease severity of cucumber plants under a hydroponic system inoculated with two doses of *Pythium ultimum* (trial A). **(a)** Averages of shoot fresh weight, **(b)** number of leaves/plant, **(c)** number of flowers/plant **(d)** number of plants per score for the stem lesions caused by *P. ultimum* (0= healthy, 1= small lesion on the stem, 2= intermediate size of lesion on the stem, 3= big lesion on the stem, 4=severe lesion/dead plant), **(e)** and root development (0= very well developed root system, 1= intermediate root system, 2= small root system, 3= root system of dead plants). The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests (Mean ± SE). Different letters above columns indicate significant difference between the treatments at a significance level of 0.05. (n=6 for stem lesions caused by *P. ultimum* and root development; n vary for the other traits since dead plants were removed from the calculations).

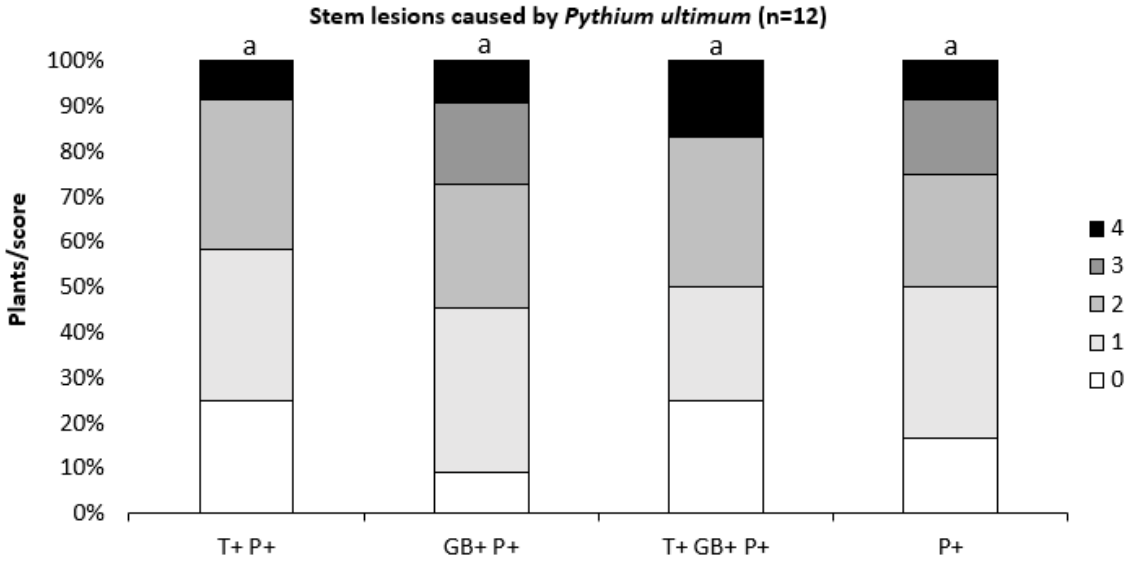

Figure 2. Disease severity of cucumber plants under a hydroponic system inoculated with *Trichoderma asperellum* (T+), *Pythium ultimum* (P+) and glycine betaine (GB+), alone or in combination (trial C). Number of plants per score for the stem lesions caused by *P. ultimum* (0= healthy, 1= small lesion on the stem, 2= intermediate size of lesion on the stem, 3= big lesion on the stem, 4=severe lesion/dead plant). The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests. Different letters indicate significant difference between the treatments at a significance level of 0.05.

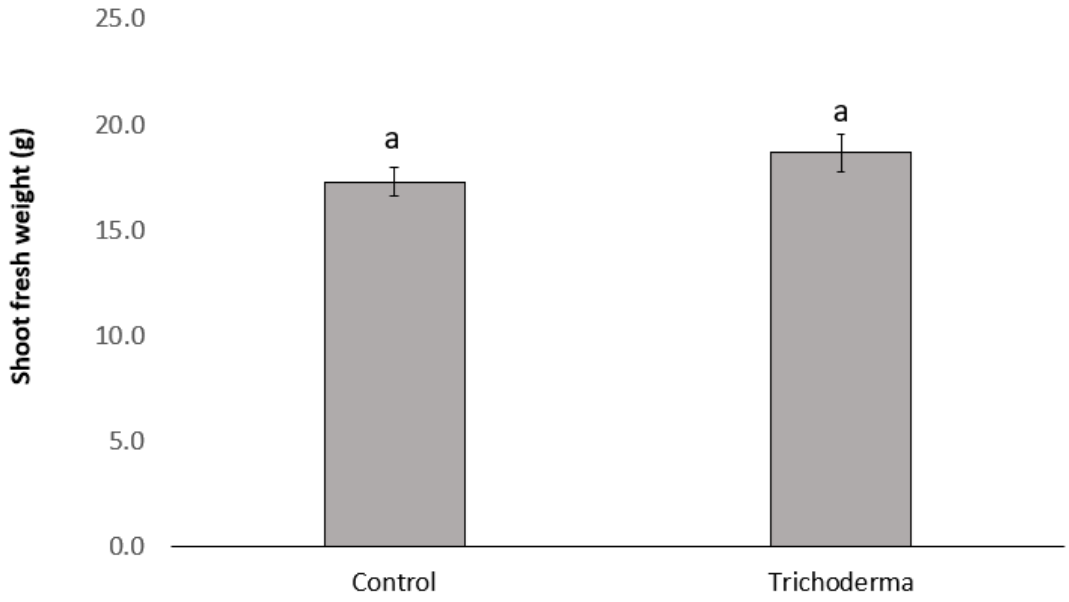

Figure 3. Plant development of cucumber plants under a hydroponic system inoculated with *Trichoderma asperellum* and *Aphis gossypii*. Average of shoot fresh weight. The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests (Mean ± SE). Different letters indicate significant difference among treatments within each cultivar at a significance level of 0.05.

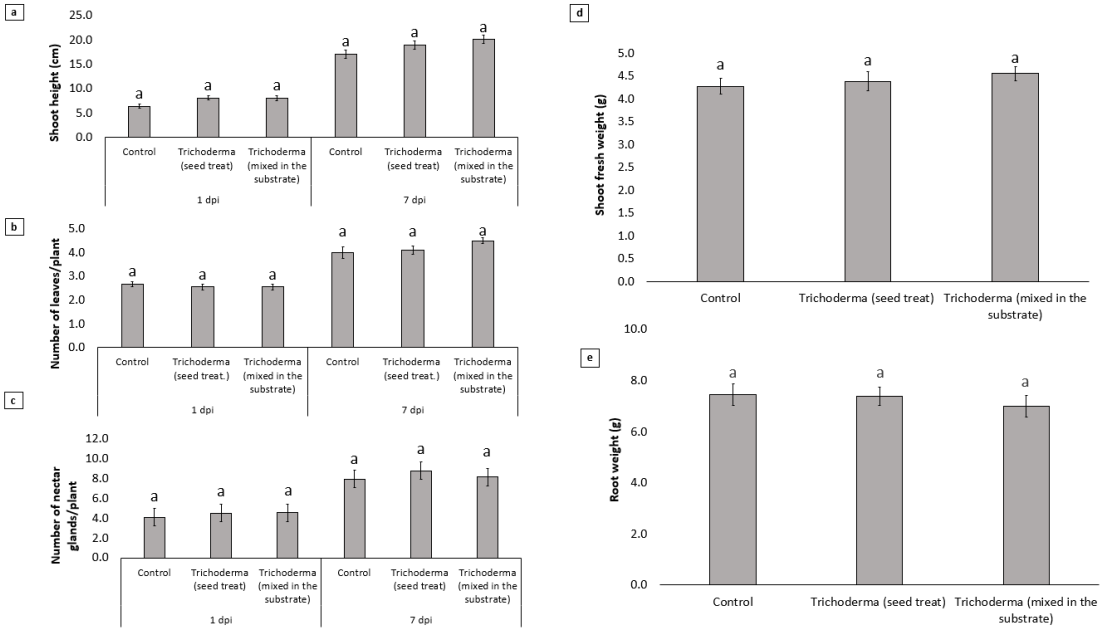

Figure 4. Plant development of fava beans under different inoculation methods of *Trichoderma asperellum* and infested with the pea aphid (*Acyrtosiphon pisum*) (trial 1). Averages of shoot height **(a)**, the number of leaves/plant **(b)**, number of nectar glands/plant **(c)**, shoot fresh weight **(d)** and root weight **(e)**. The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests (Mean ± SE). Different letters indicate significant difference between the treatments at a significance level of 0.05. (1 dpi = 1 day prior infestation; 7 dpi = 7 days post infestation).

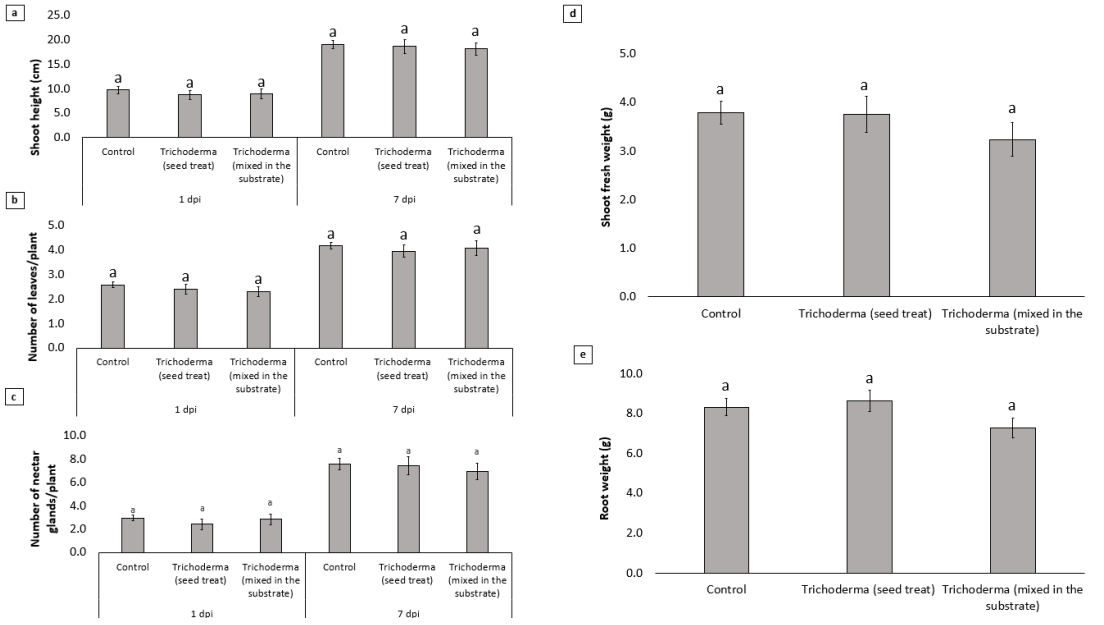

Figure 5. Plant development of fava beans under different inoculation methods of *Trichoderma asperellum* and infested with the pea aphid (*Acyrtosiphon pisum*) (trial 2). Averages of shoot height **(a)**, the number of leaves/plant **(b)**, number of nectar glands/plant **(c)**, shoot fresh weight **(d)** and root weight **(e)**. The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests (Mean ± SE). Different letters indicate significant difference between the treatments at a significance level of 0.05. (1 dpi = 1 day prior infestation; 7 dpi = 7 days post infestation).

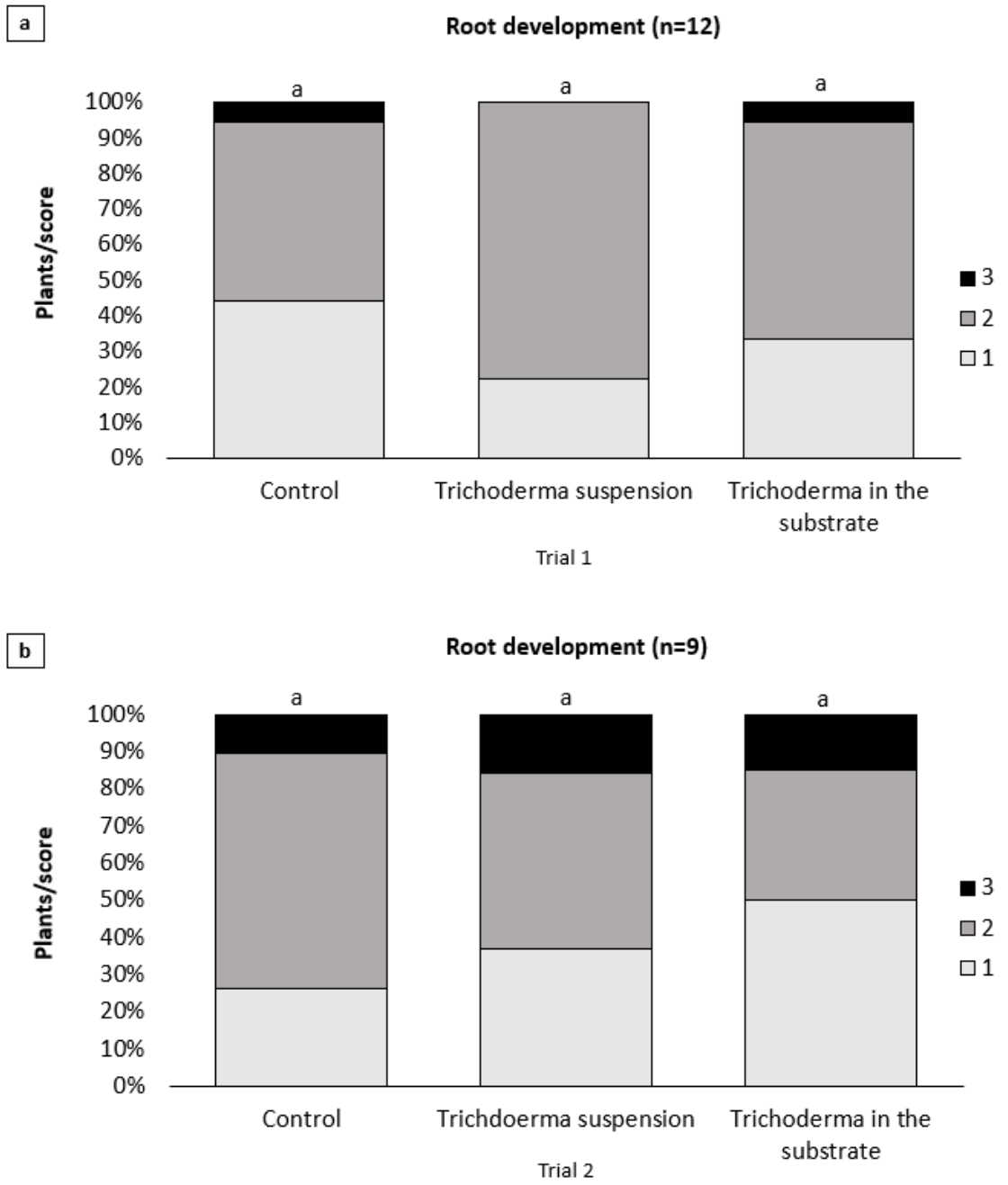

Figure 6. Root development of fava beans under different inoculation methods of *Trichoderma asperellum* and infested with the pea aphid (*Acyrtosiphon pisum*). **(a)** Trial 1 and **(b)** Trial 2. Number of plants per score for root development (1 = small root system, 2 = intermediate root system, 3 = well developed root system). The Saphiro-Wilk test was performed to check for normality. Not normally distributed data was analysed with the Kruskal-Wallis and Mann-Whitney U tests. Different letters indicate significant difference between the treatments at a significance level of 0.05.