UNIVERSITÀ DEGLI STUDI DELLA BASILICATA
SAFE - Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali

Università degli Studi della Basilicata

PhD in
Agricultural, Forest and Food Sciences

The role of *Trichoderma* in belowground-aboveground interactions between plants and insects

Scientific Area (SSD)
"AGR/11 – General and Applied Entomology"

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Cycle XXXV

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Dottorato di Ricerca in
Scienze Agrarie, Forestali e degli Alimenti

TITOLO DELLA TESI

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Settore Scientifico-Disciplinare
" AGR/11 - General and Applied Entomology "

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ACKNOWLEDGEMENTS

I would like to thank the Prof. Donatella Battaglia for being my tutor, for believing in me and supporting me even in the most difficult situations. I would like to thank my co-tutor Prof. Maria Nuzzaci for all advice and support during my PhD program. Profound thank to my research group, Dott. Vincenzo Trotta and Dott. Pierluigi Forlano for their support and help, especially in the field experiments. I would like to make a great acknowledgment to Prof. Alberto Fereres and the whole “Insect Vectors of Plant Pathogens” research group for their hospitality at the ICA-CSIC (Madrid, Spain). I am so grateful to him, Dott. Elisa Garzo and Dott. Gemma Maria Clemente Orta for giving me the great opportunity to have gained new knowledge in the field of research, but also as new life experience that I will always remember.

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SUMMARY

In recent years, the reduction of use of agrochemicals, such as synthetic fertilizers, pesticides, and herbicides, have received much attention due to environmental pollution and human health problems caused by their overuse. For this purpose, the search for green alternative practices has increased. In this context the study of microorganisms and their plant-pathogen-herbivore interactions is increasing. Aboveground and belowground communities affect plants and also influence each other in both natural and agricultural ecosystems (Bardgett & Wardle, 2010). Above-below interactions may optimize biological control of herbivore populations by natural enemies, pollination, and soil nutrient cycling (A'Bear *et al.*, 2014; Pineda *et al.*, 2017). For these reasons, these interactions can be used to improve sustainable crop production (Orrell & Bennett, 2013; Tamburini *et al.*, 2016). Above-belowground interactions improve plant nutrition, resistance to pathogens, and benefits from mutualists (Orrell & Bennett, 2013; Mariotte *et al.*, 2018). For example, arbuscular mycorrhizal fungi and growth-promoting rhizobacteria can improve plant nutrition and hence prime plant defenses to above-or belowground pests or can enhance the attraction of pollinators (Gange & Smith, 2005).

Among mycorrhizal fungi, interest for the genus *Trichoderma* has considerably increased in the last decade. Fungi of the genus *Trichoderma* are agronomically important for their beneficial effects on plant growth and development (Harman *et al.*, 2004a). Indeed, *Trichoderma* spp are a major component of many biofungicides and biofertilisers (Kaewchai *et al.*, 2009), sharing almost 50% of the market for fungal biocontrol agents (BCAs) (Whipps & Lumsden, 2001).

In recent years, the use of root symbionts has gaining popularity as an alternative practice for pathogen control, while no product has been marketed specifically for insect control to date. Several studies including root colonization by *Trichoderma* against insect herbivores were demonstrated in different plant species in greenhouses or pot experiments, little attention has been paid to the use of these fungi in open field conditions (Poveda, 2021).

In this thesis, indirect effects of *Trichoderma harzianum* T-22 has been evaluated in the laboratory and in the field. The laboratory study focused on two aphids that attack tomatoes, *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae) and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). As for *M. euphorbiae*, the negative effect of plant colonization by *T. harzianum* on aphid fitness, particularly through increased mortality rate, was already described. Instead, in this study, the response of two different clones of the aphid (referring to French and Spanish clones) to plants colonized by the fungus was investigated. *Trichoderma harzianum* results in a worsening of fitness in the French clone, but not in the Spanish clone.

The influence of *T. harzianum* on the behavior of aphids belonging to the two clones was also investigated, since this is an aspect that may have considerable importance in virus transmission. In dual choice test, aphids of both clones preferred control plants to those colonized by *Trichoderma*, probably because of the repellent effect of the volatiles produced by the latter. Feeding behavior of *M. euphorbiae*, monitored using Electrical Penetration Graph (EPG) technique, was not affected by treatment with T-22, and both clones were able to reach the phloem and feed.

As for *M. persicae*, previous studies are lacking. In laboratory conditions, *M. persicae* was attracted to tomato plants colonized by *T. harzianum* and performed better on them.

In field experiments, the indirect effects on interactions between arthropods and *Trichoderma* will be evaluate on tomato and zucchini plants, among the most important crops in Italian horticultural production. The importance of field studies lie in the fact that *Trichoderma*-plant-insect interaction occurs under conditions of greater complexity and the result may be different from that recorded in the laboratory. One aspect that cannot be reproduced in the laboratory is that soil and aboveground organisms operate in the field on different spatial scales. Aboveground organisms, such as herbivores and their natural enemies, are highly mobile and their populations can vary considerably in time and space. Moreover, plants in the field can be simultaneously attacked by different pest species, activating different resistance pathways, and are also subjected to variable abiotic conditions. In this study, plant inoculation with *T. harzianum* T-22 significantly altered arthropod community in the field.

INTRODUCTION

1. *Trichoderma* spp.

Trichoderma is a genus of filamentous ascomycete fungi that commonly colonize soils rich in organic matter and are considered to be opportunistic plant symbionts (Harman *et al*., 2004; Druzhinina *et a*l., 2011; Leylaie & Zafari, 2018). The species of *Trichoderma* are classified as either *Hypocrea*, for those species typified by a sexual (teleomorphic) stage, or *Trichoderma* when the strains show an asexual (anamorphic or mitosporic) stage (Druzhinina *et a*l., 2011). A total of 75 *Trichoderma* species are identified, most of which are considered important microbial control agents. These include *T. harzianum* Rifai, now determined *T. afroharzianum* (Chaverri *et al*., 2015), *T. hamatum* (Bonorden) Bainier, *T. koningii* Oudemans, *T. polysporum* Rifai, and *T. virens* (J.H. Miller, Giddens & A.A. Foster) (Harman *et al.*, 2004a).
Trichoderma spp. is a beneficial root-colonizing fungus (figure 1), and some isolates of these fungi are widely investigated as a biocontrol agent for plant pathogens.

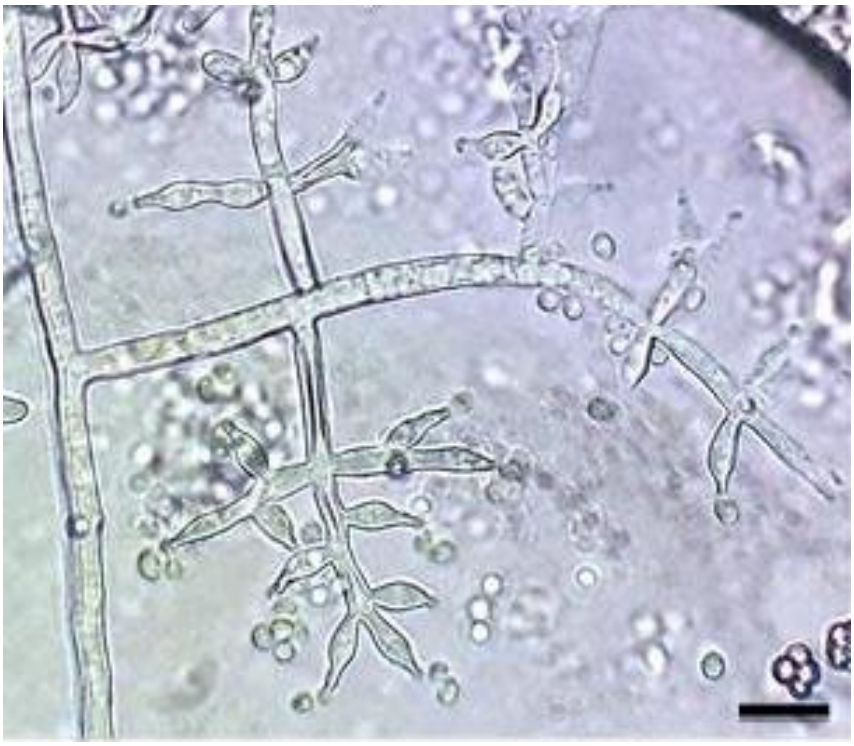

Figure 1. Microscopic features of *T. harzianum* (scale bar = 10 µm) (Benouzza *et al.*, 2020)

Trichoderma colonizes roots of mono and dicotyledonous plants, which can result in significant changes in plant metabolism, changing the content of hormones, soluble sugars, phenolic compounds, and amino acids; photosynthetic rate; transpiration; and water content (Yedidia *et*

al., 2003; Bae *et al.*, 2009; Brotman *et al.*, 2012). These fungi colonize root surfaces, sometimes with morphological features similar to those seen during mycoparasitism when hyphae invade the root epidermis (Yedidia *et al.*, 1999). The penetration into the root tissue is usually limited to the first or second layers of cells (Harman *et al.*, 2004a). During root colonization, extensive exchange of molecular messages occurs, including deposition of fungal elicitors in the root cell apoplast (Hermosa *et al.*, 2012; Contreras-Cornejo *et al*., 2014). Several studies have show that the root colonization can strongly influence the resistance to diseases, as well as plant growth and productivity. For example, root colonization of *Trichoderma harzianum* strain 22 (T-22) induces strong changes in the proteome of shoots of corn seedlings, even though T-22 is present only in roots (Shoresh & Harman, 2008). *Trichoderma* spp. positively influence crop plants. Some T*richoderma* species find to be very effective biocontrol agents. They are used to suppress plant diseases and the growth of pathogens in contact with plant tissues; they are also used as soil pathogen antagonists, both in greenhouse and field conditions (Harman *et al.*, 2004a). *Trichoderma* represents around 60% of fungal based Biological Control Agents (BCA) used nowadays globally (Harman, 2000; Verma *et al.*, 2007).

Trichoderma species operates through mixed mode of actions, involving more than one mechanism for antagonistic interaction and suppression of plant pathogens (figure 2). These mechanisms could be direct (mycoparasitism, competition, and antibiosis) or complex indirect interaction by stimulating induced systemic resistance (ISR), nutrient uptake, and enhancement of plant growth (Harman *et al.*, 2004a; Harman, 2006; Van Wees *et al.*, 2008; Pieterse *et al.*, 2014; Fernández *et al.*, 2017).

One of the important traits for agricultural crops is the capacity of *Trichoderma* strains to enhance plant growth and production (Shukla *et al.*, 2012). Soybean roots colonization by *T. harzianum* strain T-22 induces an increase to 123% in yield (Harman, 2000). The use of these fungi increases height in pea and in soybean (Lindsey & Baker, 1967; Singh *et al.*, 2015). Similarly, leaf area, shoot dry weight and root dry weight increase with the use of *T. longipile* and T. *tomentosum* in cabbage (Rabeendran *et al.*, 2000). In addition, fungus colonization increases root hairs, lateral roots, and total root biomass. Treatments with *T. asperellum* (Samuels, Lieckfeldt & Nirenberg) ceppo T42 in tobacco seeds show an increased of root hairs, of the density of lateral roots and of total root biomass, and these effects sustain up to vegetative growth (Singh *et al.*, 2018).

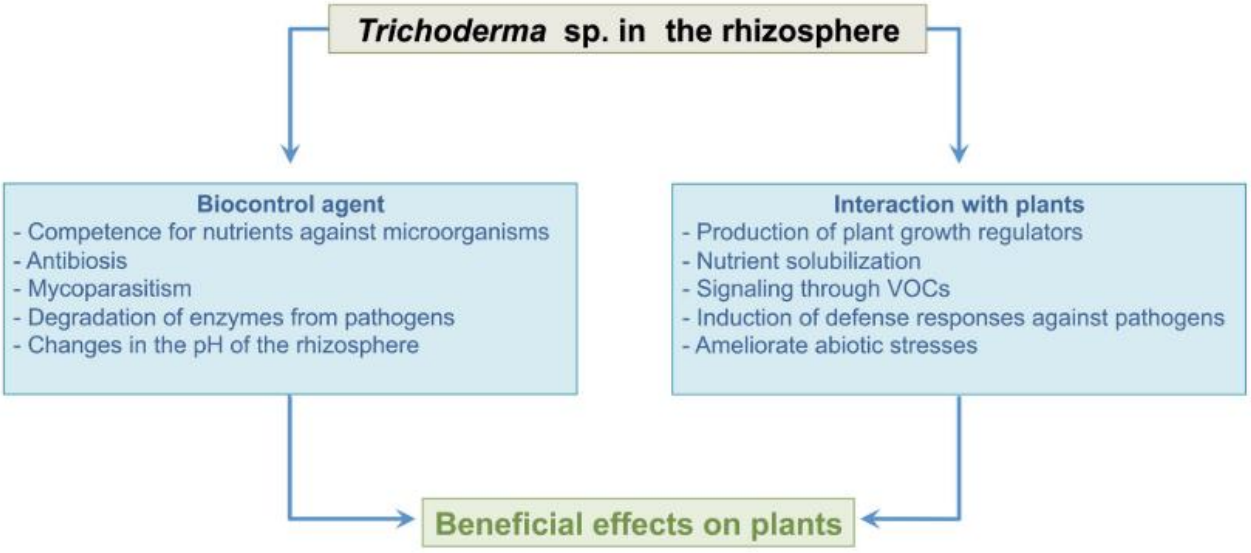

Figure 2. Schematic representation of the ecological functions of *Trichoderma* spp (Contreras-Cornejo *et al.*, 2016).

1.1 Direct mechanisms

Some *Trichoderma* species are reported to have mycoparasitic potential to attack and lyse plant pathogenic fungi such as *Alternaria alternate* (Fries) Keissler), *Botrytis cinerea (*Persoon), *Rhizoctonia solani (*Kühn), *Sclerotinia sclerotiorum (*Libert) de Bary), *Pythium* spp. (Pringsheim) and *Fusarium* spp. (Link) (Harman *et al.*, 2004; Druzhinina *et al.*, 2011). For example, disease caused by *Rhizoctonia solani* in cucumber, by *Fusarium oxysporum* f. sp. *lycopersici* in tomato, and by *Phytophthora capsici* (Leonian) in sweet-pepper are effectively controlled by *T. asperellum* strain T34 (Trillas *et al.*, 2006; Segarra *et al.*, 2010; Segarra *et al.*, 2013). The *T. harzianum* strain T-22 and the *T. hamantum* strain T17 are efficient in controlling *Fusarium* wilt and promoting growth in melon plants (Martínez-Medina *et al.*, 2014), while *T. harzianum* T-22 also efficiently protectes maize plants from *Pythium ultimum* (Harman *et al.*, 2004). *Trichoderma* mycoparasitism (figure 3) involves a combination of fungus recognition, attachment and coiling around the host fungus hyphae (Harman, 2000). This phenomenon involves the secretion of antibiotic metabolites, which results in disarmament and death of the pathogen (Harman *et al.*, 2004a; b).

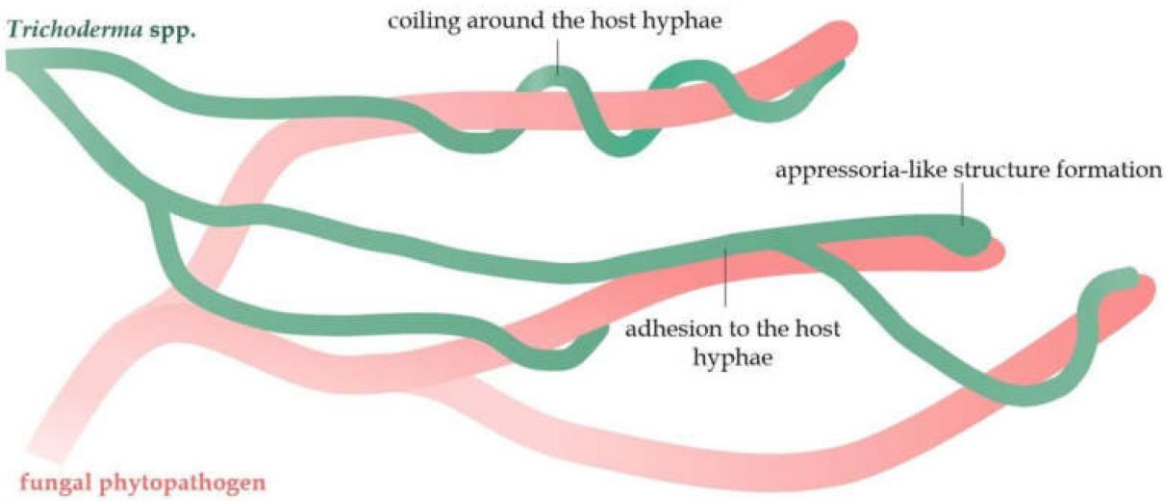

Figure 3. A schematic mycoparasitic interaction of the *Trichoderma* hyphae with the hyphae of fungal pathogens (Tyśkiewicz *et al.*, 2022).

Volatile secondary metabolites play a key role in mycoparasitism by *Trichoderma* spp. (Vinale *et al.*, 2008; Stoppacher *et al.*, 2010). However, the chemical profile differently depends on the strains. For example, *T. atroviride* produces 6-pentyl-α-pyrone, but this antibiotic compound is not produced by *T. virens* or *T. reesei*. *T. virens* produces a number of mono- and sesquiterpenes with reported antimicrobial functions. In contrast, *T. reesei* release a poor blend of Volatile Organic Compounds (VOCs) compared to the other two strains. These differences in chemical composition might influence the capacity for biocontrol (Crutcher *et al.*, 2013).

Identification of *Trichoderma* metabolites leads to the notion that, in most cases, the antagonistic process relies on the production of antibiotics and/or hydrolytic enzymes associated with possible competition for nutrients in the rhizosphere (Yedidia *et al.*, 1999; Harman *et al.*, 2004a). Competition for nutrients is considered to be a mechanism of biocontrol by *Trichoderma* (Harman, 2000). These fungi produce several siderophores that chelate iron and inhibit the growth of pathogenic microorganisms.

1.2 Indirect Mechanisms

Induction of local and systemic resistance as an indirect mechanism by *Trichoderma* species involves the recognition of the fungus by plants through ISR and systemic acquired resistance (SAR) against many phytopathogens (Harman *et al.*, 2004a). The responses is mediated by the plant-hormones salicylic acid (SA) and jasmonic acid (JA) (Woo *et al.*, 2022). The activation of the SA-mediated defenses in tomato plants (*Solanum lycopersicum* Linnaeus) colonized by

T. harzianum causes mortality rates of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) of up to 35% (Jafarbeigi *et al.*, 2020). Activation of JA- mediated systemic defensive responses in tomato plants colonized by *T. atroviride* P. Karsten causes total mortality in the cotton leafworm lepidopteran (Coppola *et al.*, 2019b; a). The activation of systemic defensive responses in the plant by *Trichoderma*-root colonization causes the accumulation of secondary defense metabolites in plant tissues. Some of these metabolites may be VOCs with repellent activity or attracting natural enemies of insect pests. Among them, terpenes (1-octen-3-ol and 6-pentyl-α-pyrone) reduce consumption of maize leaves by the fall armyworm *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) (Contreras-Cornejo *et al.*, 2018b). On the other hand, 6-pentyl-α-pyrone is confirmed to be an attractive compound for the parasitoid wasp of the fall armyworm *Campoletis sonorensis* (Hymenoptera: Ichneumonidae) (Contreras-Cornejo *et al.*, 2018a). Root colonization of tomato plants by *T. longibrachiatum* and *T. atroviride* influence the production of VOCs by the plant, which attracts the specific parasitoid wasp of *M. euphorbaie, Aphidius ervi* Haliday (Hymenoptera: Braconidae) (Battaglia *et al.*, 2013; Coppola *et al.*, 2017, 2019a).

1.3 Aboveground and belowground interactions

Above-ground and below-ground interactions in root symbioses with mycorrhizal fungi can affect the interactions between herbivores, pollinators, predators and parasitoids, whereas resistance expression in leaves can affect root herbivores as well as mutualistic or parasitic soil microorganisms (Heil, 2011). *Trichoderma*–root associations affect multi-trophic interactions, resulting in the protection of plants against agricultural arthropods pests. Several studies show the activation of defense responses is induced by arthropods of the orders Thysanoptera and Hemiptera (Battaglia *et al.*, 2013; Muvea *et al.*, 2014; Coppola *et al.*, 2019a). For example, *T. longibrachiatum* in tomato plants, can modulate the release of VOCs attacting *A. ervi*, which positively influencing the performance of the its host *M. euphorbiae* (Battaglia *et al.*, 2013). In onion plants, root colonization by *Trichoderma* spp. resultes in fewer feeding punctures caused by *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) (Muvea *et al.*, 2014). *Trichoderma atroviride* strain P1 in tomato plants affect negatively the development of larvae *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) (Coppola *et al.*, 2019a). In a recent study, Contreras-Cornejo et al (2020) found that root colonization by *T. harzianum* in maize modifies arthropod communities in above-ground part of the plant (Contreras-Cornejo *et al.*, 2020).

2. Aphids

Aphids (*Aphidoidea*) are hemimetabolous insects belonging to the order of Hemiptera. Hemiptera is traditionally divided into the suborders Heteroptera and Homoptera. Homoptera are usually split into *Sternorrhyncha* (including aphids), *Cicadomorpha*, *Fulgoromorpha*, and *Coleorrhyncha*. Aphid mouthparts are transformed to a sucking beak (Podsiadlowski & Vilcinskas, 2016) with two mandibular and two maxillary stylets, and the labium forms a sheath around them, maxillary and labial palps are reduced. The base of the sucking beak is located between the coxae of the first walking legs. The head is immovably joined to the thorax, which itself is broadly conjoined with the abdomen; consequently, the body has a compact oval shape. A morphological characteristic of the Aphididae family is the visible pair of siphons on the back. Their life cycles are highly variables, often involving alternating generations, for example, winged or wingless (apterous), sexual and parthenogenetic forms, with oviparous or viviparous females (Lars Podsiadlowski, 2016). Many aphid species are dioecious, reproducing asexually through parthenogenesis in the summer on different plant species referred to as secondary hosts. However, in response to signals such as decreasing temperatures, shortening of the duration of the day, and deterioration of the quality of the host plant, they switch to sexual reproduction in the fall. During this time the sexual morphs, the gynoparae (winged asexual females that produce the apterous, egg-laying oviparae) and males migrate to overwinter as diapausing eggs on different plant species known as primary hosts (Dixon, 1973).

Aphids represent some of the most damaging insects to agriculture in the world, and their damages are different. They remove plant sap from phloem sieve elements, which weaken the plants, resulting in lower quality and quantity of fruits. Aphids must process a large quantity of phloem to gain the products necessary for protein synthesis, which they use to produce offspring, since phloem is poor in amino acids (Perring *et al.*, 2018). Aphids are capable of shunting large amounts of phloem, which is excreted in a carbohydrate-rich exudate called 'honeydew' due to a modification of their intestinal tract into a filter chamber. They produce large amounts of honeydew, which can cover the leaves and fruits, and this sugar-rich substrate provides a suitable medium for the growth of black sooty mold fungi (belonging to the genera *Capnodium*, *Fumago*, *Scorias, Antennariella*, *Aureobasidium,* and *Limacinula*). The sooty mold fungi can become thick, reducing photosynthetic activity on the leaves that, in turn, leads to the production of less fruits and of poor quality. Finally, aphids are efficient vectors of plant viruses, causing the most dangerous plant damage (Perring *et al.*, 2018).

The main aphid species attacking tomatoes are *Macrosiphum euphorbiae* Thomas and *Myzus persicae* (Sulzer) (Blackman & Eastop, 2000). Aphids damage tomato directly, inserting their stylets into phloem sieve elements and extracting sap from the plant's phloem, and indirectly by vectoring plant viruses and by excreting honeydew on which sooty mold grows.

2.1 Macrosiphum euphorbiae

The potato aphid *M. euphorbiae* originated in North America. It is a highly polyphagous species that feeds on more than 200 plant species (Blackman & Eastop, 2007). During the winter the eggs are laid on several different primary hosts (e.g. *Rosa, Solanum, Euphorbia*, and *Lycium* spp.), while migrants spend the summer on a variety of secondary hosts. In particular, they colonize *Solanaceae*, especially potato and tomato plants (Blackman & Eastop, 2000), and plants belonging to *Cucurbitaceae* (melon), *Chenopodiaceae* (beet), *Compositae* (lettuce), *Leguminosae* (bean), and *Rutaceae* (citrus). There are two color morphs: pink and green (figure 4). The pink form prefers the younger tomato leaves, whereas the green morph prefers the younger and middle leaves (Walker *et al.*, 1984).

Figure 4. Two color morphs of *M euphorbiae* adults
(https://influentialpoints.com/Gallery/Macrosiphum_aphids.htm)

Wingless adult females (apterae) are 1.7–3.6 mm long, and pear-shaped in appearance. The body is often shiny, and the eyes are distinctly reddish. The antennae have six segments, and are generally dark apically, but sometimes are entirely dark. The legs, siphunculi, and cauda are usually of the same color of the body, but the siphunculi can be darker toward the apices. The legs are noticeably long. Wingless immature forms are rather long-bodied and lighter than

adults, with a dark spinal stripe and a light dusting of whitish-gray wax. The dark stripe on the back is sometimes seen in adults. Winged adult females are 1.7 to 3.4 mm long, although sometimes they can be larger than apterae of the same population. Different shades from green to pink characterize the color of the body. The antennae and siphunculi of alate are darker than in the apterae (Stoetzel, M.B., Miller, 1998; Blackman & Eastop, 2000).

Macrosiphum euphorbiae can be heteroecious holocyclic (alternating host with sexual reproduction) in certain temperate climates and autoecious anholocyclic (single host and parthenogenetic reproduction) in warmer tropical climates. For both types, aphid females can produce wingless and winged forms. In Europe, the life cycle is generally anholocyclic with continuous asexual reproduction on secondary hosts. Most aphids remain mobile through the winter months on weeds, on potato sprouts in storage or chitting houses, or on lettuce and other crops in greenhouses. In early May or June, winged forms migrating to potatoes or other field crops are produced. A second winged dispersal occurs in July if population densities are high, while a smaller migration occurs in the autumn. Aphid populations can survive throughout the year in warm climates and in greenhouses, infesting foliage, stems, and fruit late in the season (Perring *et al.*, 2018).

2.2 Myzus persicae

The green peach aphid or peach potato aphid *M. persicae* (figure 5) is extremely polyphagous and has a worldwide distribution (Jansson, 2003), except where there are temperature and humidity extremes. It is probably of Asian origin like its primary host, *Prunus persica* (Linnaeus). Nymphs are initially greenish, but soon turn yellowish. Adult wingless parthenogenetic females are oval-bodied, 1.2–2.1 mm in body length (Blackman & Eastop, 2000), and can vary in color from whitish green, pale yellow-green, grey green, mid-green, dark green, to pink or red (Blackman & Eastop, 2007).

Figure 5. Wingless adults of *M. persicae* (https://influentialpoints.com/Gallery/Myzus_persicae_Peach-potato_aphid.htm)

The aphid *M. persicae* is heteroecious holocyclic (host alternating with sexual reproduction) between *Prunus* and summer host plants. It is anholocyclic on secondary (summer) hosts in many parts of the world where peach is absent and where a mild climate permits active stages to survive throughout winter. While the winter host is limited to *Prunus* and close relatives, the range of summer hosts is vast with plants from over 40 families (Blackman & Eastop, 2000). These families include *Brassicaceae, Solanaceae, Poaceae, Leguminosae, Cyperaceae, Convolvulaceae, Chenopodiaceae, Compositae, Cucurbitaceae,* and *Umbelliferae*. These hosts involve many economically important plants, including tomato (Perring *et al.*, 2018).

2.3 Host plant selection

The insect that choice a plant to colonize it follows different stimuli and responses. Behavioral events have been defined primarily for aphids (Powell *et al.*, 2006), and can be extended to other hemipteran.

Before the choice, the aphid receives optical (plant color) and chemical (volatile compounds) stimuli, detected by the eyes and the olfactory sensilla of the antennae, respectively, during the search for the host (Pettersson, 1973; Perring *et al.*, 2018). A plant is accepted or rejected by various chemical attractants and repellents, plant traits such as color, morphology, and texture of the leaf surface (Powell *et al.*, 2006). After the aphids landing on a plant, they tend to walk and move their antennae from side to side in search of odours and thus evaluate the texture (topology, trichome exudates, epicuticular waxes and other signs of surface), prior to stylet penetration. Several sensilla, located on the antenna, tarsi or lip act as chemoreceptors and mechanoreceptors involved in the detection of physical and chemical stimuli respectively (Tjallingii, 1978; Backus, 1988). When the aphids land on the plant, they have the reflex to

probe the epidermal cell with the stylet automatically. The aphids probe the more superficial cells of the plant tissue by ingestion of small amounts of the cytoplasmic contents. They identify the nutrient composition of the plant by the taste buds located in the region near the precibarial valve (Wensler & Filshie, 1969; McLean & Kinsey, 1984). Before the reach of the vascular vessels, aphids examine the cells along the tissues. They begin the salivation before arriving with the stylet at the crypt element. The aphid recognises the phloem according to physical stimuli related to the turgidity of this tissue and chemical stimuli related to the composition of the sap, such as its high sugar content. Once the aphid begins phloem ingestion, and if this takes longer than 10 minutes, it is considered to be positively settled and the plant accepted as a host.

2.4 Monitoring of probing and feeding behavior

The use of the electrical penetration graph (EPG) technique makes it possible to study the probing and feeding behavior of insects, a process that involves a number of activities that cannot be observed directly. The first electronic device developed in the 1960s by McLean and Kinsey (1964) allowed the study of insect probing and feeding behavior. Kinsey (1964) made it possible to study the feeding behavior of aphids by recording electronically generated waves by applying an alternating current voltage (AC system). Subsequently, the device was modified by replacing alternating current (AC) with direct current (DC) (Tjallingii, 1978, 1985). In addition to changes in electrical resistance in the plant and the insect, this system also measures changes in the electromotive forces caused by the feeding behavior of the insect on the various plant tissues. Using the EPG technique, it has been possible to characterize the feeding behavior of more than 50 species of Hemiptera (Backus, 1994). The studies carried out with the EPG technique have been applied to advance knowledge of viral transmission processes (Powell, 1991; Prado & Tjallingii, 1994; Powell *et al.*, 1995; Collar *et al.*, 1997; Martín *et al.*, 1997; Collar & Fereres, 1998; Palacios *et al.*, 2002; Moreno *et al.*, 2005), behavior against infected plants (Moreno-Delafuente *et al.*, 2013; Carmo-Sousa *et al.*, 2014), resistance of plant varieties (Garzo *et al.*, 2002; Alvarez *et al.*, 2006), studies with transgenic plants (Liu *et al.*, 2005), action of insecticides (Nisbet *et al.*, 1993; Kaufmann *et al.*, 2004) among several other studies.

The different EPG wave patterns are mainly caused by voltage changes due to variations in the electrical resistance of the circuit depending on the position of the stylet in the different plant tissues. EPG wave patterns are also caused by voltage changes due to variations in the electromotive forces during aphid feeding in the plant, as well as their correlation with specific

aphid activities or behavioral patterns. The different EPG wave patterns are described below (Tjallingii, 1990) (figure 6 and 7):

- Waveform A: This is an irregular wave of short duration (<10 s) that occurs at the beginning of the probe and is associated with the electrical contact of the stylet with the epidermis of the plant.

- **Waveform B:** This type of wave is related to the formation of the gelling saliva required for the formation of the salivary sheath (Tjallingii, 1978) and it is an interface between waves A and C consisting of a series of regular low frequency pulses (0.2-0.3 Hz).

- **Waveform C**: This is the most complex wave pattern. It probably originates from the sum of a set of activities developed by the stylet. Like waves A and B, it is a wave with extracellular potential (between epidermis and phloem) in search of vascular tissues (Tjallingii, 1988).

- **Waveform *pd*** (*"potential drop"*): Included within the C-wave pattern, pd wave is associated with the penetration of the maxillary stylet into the cell interior.

- **Waveform E**: This is generally associated with an intracellular potential and has been correlated with the action of the salivary pump (Tjallingii, 1978), with penetration into phloem tissues (Kimmins & Tjallingii, 1985) and with ingestion (Tjallingii, 1987). In the E wave, one can differentiate two types of wave: **E1**, which is associated with salivation in the phloem and with the inoculation of persistent viruses (Prado & Tjallingii, 1994) and **E2**, which has been related to phloem ingestion and to the acquisition of persistent viruses accompanied by periodic secretion of watery saliva, ingested again by aphids (Prado & Tjallingii, 1994).

- **Waveform G**: Extracellular potential wave that correlates with stylet penetration into xylem vessels and an active ingestion process to overcome the negative pressure to which the sap circulating in the xylem of the plant is subjected (Spiller *et al.*, 1990).

- **Waveform F**: It seems to be associated with the mechanical work done by the stylet as a result of the difficulties they encounter in penetrating the plant tissue. Its potential is extracellular.

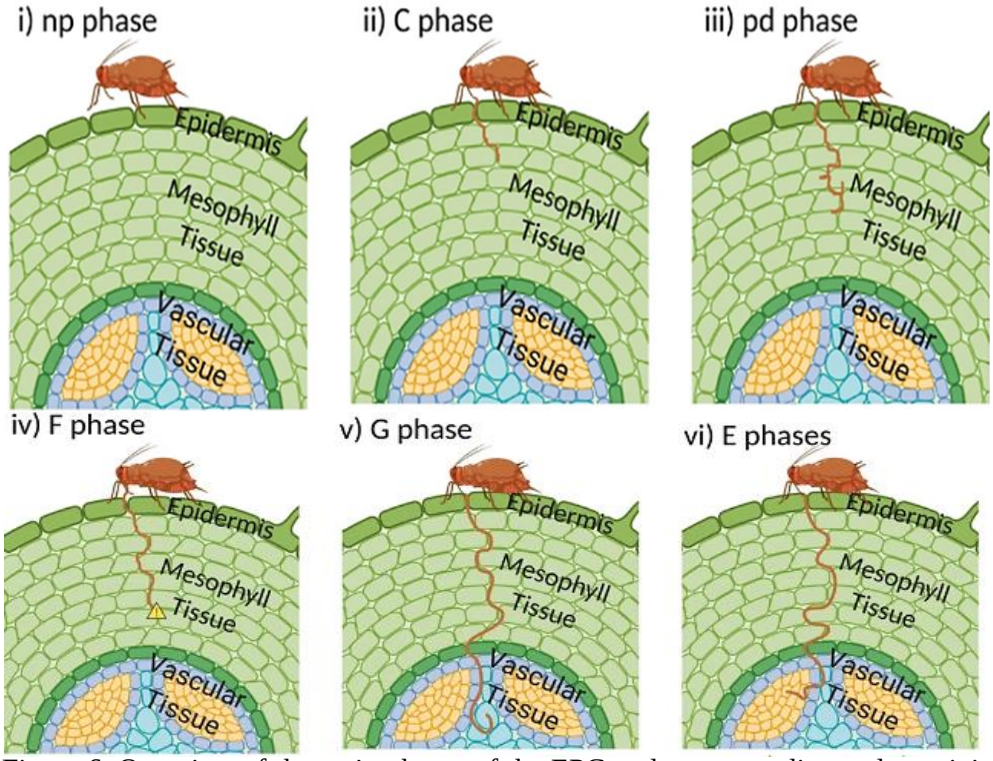

Figure 6. Overview of the main phases of the EPG and corresponding stylet activity in plant tissue. i) np phase (insects stylet not in contact with plant tissue), ii) C phase, iii) pd phase, iv) F phase, v) G phase, vi) E phase (Leybourne & Aradottir, 2021).

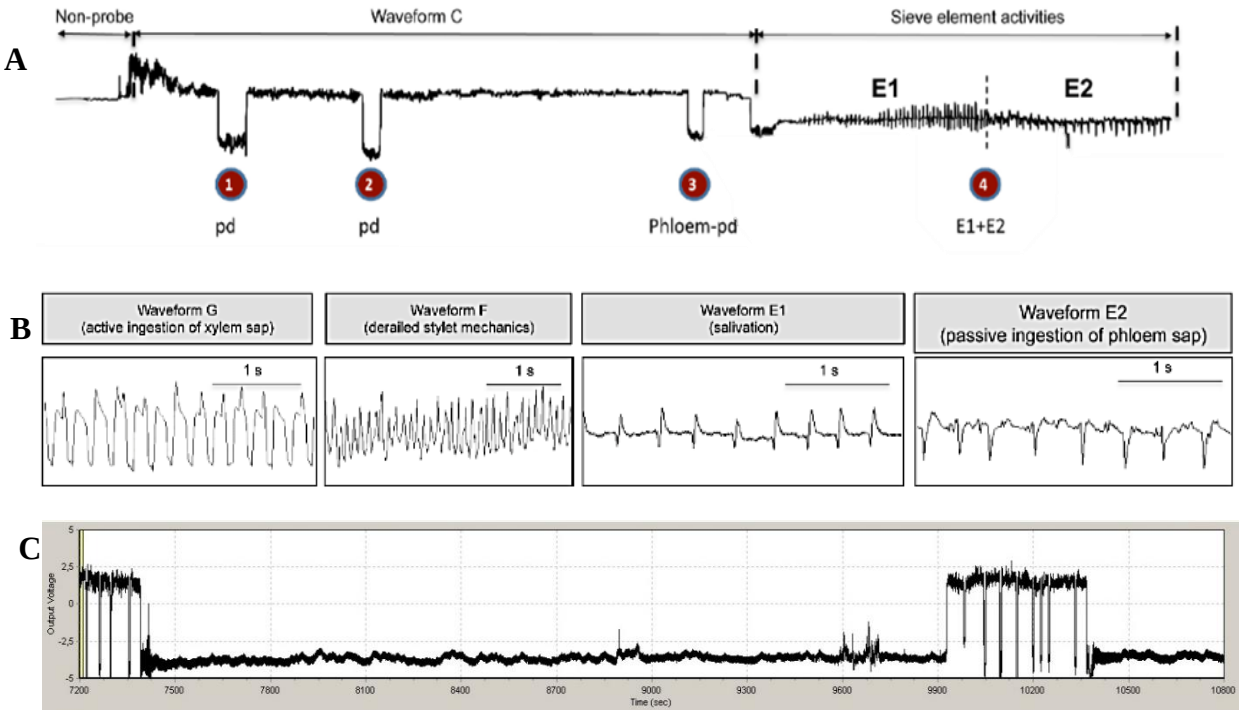

Figure 7. Electrical penetration graph (EPG). (**A**) Overview of electrical-penetration graphs in which different numbers indicate specific EPG patterns where the aphid inserts stylets into a specific cell (Garzo *et al.*, 2020). (**B**) Detail of specific waveforms (Garzo *et al.*, 2020). (**C**) Example of EPG waveforms from a feeding study (Caccavo *et al.*, unpublished data)

3. Plants

In this thesis, the indirect effects of *Trichoderma* on tomato and zucchini plants will be evaluated. Tomato is one of the most important vegetable plants in the world (Kimura & Sinha, 2008) and is the second most important vegetable crop next to potato (FAO, 2022). Zucchini squash is one of the most popular cultivated vegetable crops in the Mediterranean region, especially in Italy (Istat, 2022).

3.1 Tomato: origin, economic importance and agronomic requirements

Tomato (*genus Solanum, section Lycopersicon*, figure 8) originated in western South America (Ecuador, Peru, and Chile), and domestication is thought to have occurred in Central America (Rick, 1991). Because of its importance as food, tomato has been bred to improve productivity, fruit quality, and resistance to biotic and abiotic stresses (Kimura & Sinha, 2008).

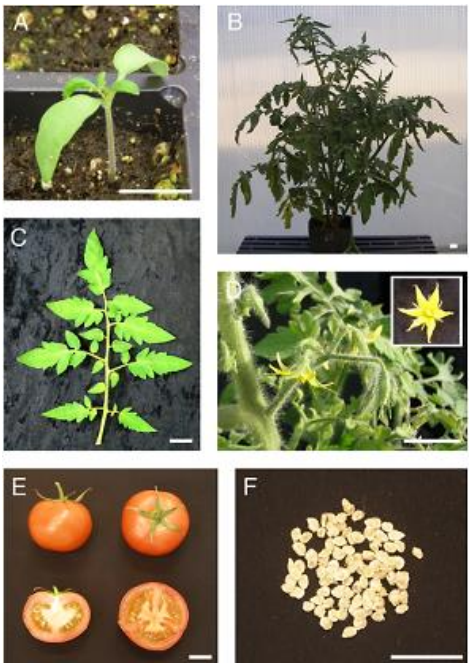

Figure 8. Tomato plants and parts: (A) seedling; (B) 40-day-old plant; (C) leaf; (D) flowers; (E) fruit; (F) seeds. (Kimura & Sinha, 2008).

The genus *Solanum* is one of the largest genera of angiosperms. It is estimated that about 1500 number of species belong to this genus. It also includes potato (*Solanum tuberosum* Linnaeus), eggplant (*Solanum melongena* Linnaeus), and wild tomato (*Solanum* L. section *Lycopersicon* [Mill.]). *Solanum* belongs to an extremely large family of plants, the *Solanaceae*, which is one of the most important to humans. Plants within this family are used as food sources (e.g., potato,

eggplant, bell peppers, chili peppers), as drugs (e.g., tobacco), and as ornamentals (e.g., petunia). The presence of many economically important plants in the *Solanaceae* family makes tomato essential as a model plant species. The tomato plant has many interesting features such as fleshy fruit, a sympodial shoot, and compound leaves, which other model plants (e.g., rice and *Arabidopsis*) do not have (Kimura & Sinha, 2008).

In Italy, during 2022, the total area designated for tomato fresh consumption production in open field was 17300 ha with a total production of about 5734900 quintals, while for tomato process production in open field the total area is about 76360 ha with a total production of 52000000 quintals (Istat, 2022).

Tomato is a rapidly growing crop with a growing period of 90 to 150 days, and it is a day length neutral plant. The optimal mean daily temperature for growth ranges from 18 to 25ºC, with night temperatures between 10 and 20ºC. The crop is very sensitive to frost and the yield is adversely affected by larger differences between day and night temperatures and also by temperatures above 25°C, if accompanied by high humidity and strong wind (FAO, 2022). Therefore, dry climates are preferred for tomato production because high humidity leads to a higher incidence of pests, diseases, and rotting of fruits. Furthermore, night temperatures above 20ºC accompanied by high humidity and low sunshine lead to excessive vegetative growth and poor fruit production.

Tomato can grow in a wide range of soils, but a well-drained, light loam soil with a pH range of 5 - 7 is preferred. Waterlogging increases the incidence of diseases such as bacterial wilt. The amount of fertilizer requirements for high-producing varieties, measured in kg/ha, range between 100 and 150 for N, between 65 and 110 for P, and between 160 and 240 for K. To reduce pests and disease infestations, tomato should alternate in a rotation plan with other crops such as maize, cabbage, and cowpea.

Tomato is also moderately sensitive to soil salinity. The most sensitive period to salinity is during germination and early plant development, and therefore necessary leaching of salts is frequently practiced during pre-irrigation or by over-watering during the initial irrigation application (FAO, 2022).

The typical Italian plum tomato is represented by the San Marzano type (figure 9), suitable for both fresh consumption and processing. San Marzano represents a type of population rather than a distinct cultivar. As many as 32 biotypes attributable to the San Marzano type are registered, divided into seven recognized varieties in the European Register (including San Marzano nano). It is thought to have originated by spontaneous hybridization between round and long-fruited varieties of the Pagani, Nocera, Angri and Scafati regions or by the accumulation of spontaneous mutations in local populations over time. As a tomato processing, San Marzano was very popular in the past, representing the 40% of the total Italian production in 1982. However, in subsequent years, its popularity declined due to the appearance of new, higher-yielding varieties and its increased susceptibility to cucumber mosaic virus (CMV). In recent years, interest in San Marzano has re-emerged and breeding work has been undertaken to improve its agronomic characteristics and yields but maintaining its excellent organoleptic and chemical-physical properties (Siviero, 2000).

Figure 9. Tomato var. San Marzano fruits (https://www.seeds-organic.com/products/san-marzano-nano)

3.2 Zucchini: origin, economic importance and agronomic requirements

Summer squash are the edible immature fruits of *Cucurbita pepo* Linnaeus, a highly diverse species of the gourd family, *Cucurbitacea* (Paris, 2008). This family consists of about 118 genera and 825 species (Jeffrey, 1990). *Cucurbita* has two cultivated subspecies, subsp. *pepo* and subsp. *texana* (Scheele) Filov, each of which includes four edible-fruited cultivar-groups (Paris, 2001). These eight cultivar-groups are distinguished on the basis of fruit shape (Paris, 1986).

The origin and early spread of all *Cucurbita* species was in the Americas. *Cucurbita ficifolia* Bouché was the most widespread cultivated species with a native range in the mountains from Mexico to northern Chile and Argentina (Whitaker & Bemis, 1975; Wilson *et al.*, 1992).

Zucchini squash (figure 10) is a vegetable crops gaining popularity for both open-field and protected cultivation in the Mediterranean region, including in Italy (Istat, 2022).

Figure 10. Adult plant with all its organs (https://www.allaboutgardening.com/zucchini-companion-plants/)

Summer squash are an easy-to-grow short-season crop best adapted to temperate and subtropical regions (Paris, 2008). This crop tolerates lower temperatures, but is very sensitive to frost, withstanding high temperatures better than other cultivated cucurbits, such as melon, watermelon, and cucumber. It grows well in environments with a temperature between 18 and 25 °C and with humidity levels between 65 and 80%. It is sensitive to waterlogging both in the seed germination phase in direct sowing and in cultivation, so much care must be taken in soils that easily percolate water. Zucchini squash adapts well to all types of soil, including sandy

soils, and grows best in loamy soils, with deep roots. The sensibility to salinity, both of soil and water, is intermediate. The availability of water is vital to obtain high yields, especially from the fruit setting and growing, although not necessarily for the quality of the fruits (López-Marin, 2017).

To respond to the high demand for fresh products on national and international markets (Youssef Rouphael, 2005), zucchini is usually grown off-season under greenhouse conditions during two growing seasons (spring–summer and summer–fall). Zucchini are harvested for culinary use when young (Lust & Paris, 2016).

In Italy, during 2022, the total area designated for zucchini production in open field was 14000 ha with a total production of about 3500000 quintals (Istat, 2022).

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OBJECTIVES

The main objective of this thesis is to investigate the indirect effect produced by the colonization of the commercial *Trichoderma harzianum* strain T-22 in tomato plants on the behavior and biological parameters of two species of aphids, *Myzus persicae* and *Macrosiphum euphorbiae,* under laboratory conditions. In addition, the effects of the fungal symbiont on the insect community associated with two different species, tomato plants (*Solanum lycopersicum)* and zucchini squash (*Cucurbita pepo),* were evaluated in an experimental field.
the complex interactions between arthropods, *Trichoderma* and plants such as tomato and zucchini, among the most important crops of Italian horticultural production, that occur in the laboratory and in the field are discussed in order to enrich our current information on the possibilities of using these fungi as an alternative green agent in agriculture.

This general objective is divided into the following specific objectives:

1. To evaluate the effect produced by the colonization of T-22 in tomato plants on probing and feeding behavior, preference, and biological performance of two different clones of *Macrosiphum euphorbiae* (original from two different geographical locations, Spain and France).

2. To evaluate the effect produced by the colonization of T-22 in tomato plants on preference and population dynamics of *Myzus persicae.*

3. To investigate the effects of T-22 colonization on the arthropod community associated with tomato plants in the field.

4. To investigate the effects of T-22 colonization on the arthropod community associated with zucchini squash, in an experimental field.

CHAPTER I

Effects of tomato plants inoculated by *Trichoderma harzianum* strain *T-22* on aphid behavior

1. Introduction

Aphids are phloem-feeding insects present in herbaceous and woody crops all over the world. They cause significant damage and great economic losses in agricultural crops as a result of their direct feeding, since they remove plant sap and inject salivary toxins, and/or indirectly, transmitting plant viruses (Emden & Harrington, 2007). One of the most important cultivated vegetable crops in the world is tomato (*Solanum lycopersicum* L.), second only to potato and one of the main foods of the Mediterranean diet (Siracusa *et al.*, 2018; FAO, 2022). Fresh and processed tomatoes are widely consumed in the Mediterranean area, and Italy is one of the main producers and suppliers of processed tomatoes in the world (Elia & Conversa, 2012; Bettini, 2019; I.Sat, 2022).

Among the insect species that attack tomato plants, the potato aphid *Macrosiphum euphorbiae* (Thomas, 1878) causes significant direct and indirect damage (Perring *et al.*, 2018). As a consequence of the direct damage, the potato aphid causes leaf curling, chlorosis, and shoot dieback (Flint, 1985; Walgenbach, 1997). Aphid infestation also promotes the growth of sooty mold on the leaves, increases the risk of weather damage to the fruit, and attracts other damaging insects (Lange & Bronson, 1981; Flint, 1985; Walgenbach, 1997; Perring *et al.*, 2018). The combined effects of direct and indirect plant damage caused by potato aphids can result in significant yield losses and reduced fruit quality (Walgenbach, 1997; Zalom, 2007). Plants damaged by herbivores show an accumulation of toxic or volatile organic compounds and a modification of their physical structures, affecting their colonization and development, nutrition, survival, and oviposition (Walling, 2000). To cope effectively with this damage, plants have developed two kinds of constitutive defense mechanisms (Zhao *et al.*, 2009). Physical barriers, including cuticle trichomes, callose, cell walls and suberin, prevent the colonization of plants, while allelochemical substances with antibiotic effects influence pest development, growth, fertility, and longevity or induce repellent effects (Ahuja *et al.*, 2011). After pest attack, the inducted plant defense responses involve the activation of Jasmonic Acid (JA), Salicylic Acid (SA), and Ethylene (ET) (Salzman *et al.*, 2005). SA activation was found to be involved against piercing-sucking insects, JA activation against chewing insects

(Lazebnik *et al.*, 2014), while ET controls various processes that are associated to the defense responses (Kunkel & Brooks, 2002; Ali & Agrawal, 2014). Interactions with phytopathogens can alter plant phenotypes, nutritional profiles, and the emission of volatile organic compounds (VOCs) (Franco *et al.*, 2017), as a consequence of the strategy to attract vectors and disseminate them (Mauck *et al.*, 2010).

Some biotic and abiotic factors, such as the root colonization of plants by microorganisms, have been reported to trigger physiological changes in plants, thus altering the insect choice of that plant as host and also altering the insect feeding behavior (Pangesti *et al.*, 2013). Plant defense responses inducted by plant-insect-symbiont fungi interactions can influence the pathogen infection directly (Karban *et al.*, 1987; Simon & Hilker, 2003; Franco *et al.*, 2017) or throughout the manipulation of the insect vector behavior (Mauck *et al.*, 2010, 2012; Bosque-Pérez & Eigenbrode, 2011; Heil, 2016). Also, VOCs produced by fungal symbionts have been investigated in recent years. The VOCs produced by *Trichoderma* species are very interesting. They are antifungal in nature (Vinodkumar *et al.*, 2017; Sunpapao *et al.*, 2018), induce systemic resistance (Kishimoto *et al.*, 2005; Yi *et al.*, 2009) and promote plant growth (Vinale *et al.*, 2008). For all these reasons, root colonization by *Trichoderma* could be considered an important factor leading to an altered aphid feeding behavior. However, to date, it is not known whether *Trichoderma* colonization can alter aphid probing and feeding behavior. The feeding behavior of aphids can be monitored using the electrical penetration graph (EPG) technique, which records signal waveforms that reflect the different penetration activities of the insect stylet (Tjallingii, 1988).

In the present study, I investigated the indirect effects produced by the colonization of the commercial *Trichoderma harzianum* strain T-22 in tomato plants (*Solanum lycopersicum*) on two different aphid clones of *Macrosiphum euphorbiae* from two geographical locations (Spain and France). Two different clones were chosen to investigate the possible differences in the host plant resistance of the two clones. It has been reported the existence of a secondary symbiont in certain clones of *M. euphorbiae*, which may contribute to interclonal variation in this aphid's responses host plant resistance (Francis *et al.*, 2010).

The outcomes of plants-*Trichoderma* interactions are species-specific and even strain-specific (Copetta *et al.*, 2006; Tucci *et al.*, 2011; Kovach-Orr & Fussmann, 2013; Soler *et al.*, 2013; Bazghaleh *et al.*, 2020), but to date we do not know whether they differ in different clones. (Francis *et al.*, 2010). This last aspect has been very often neglected in all the studies on the effect of fungal symbionts on plants.

The aim of this study was to understand if and how *T. harzianum* influences aphid preference, probing, and feeding behavior.

In the present study I investigate the differences in aphid behavior on *T. harzianum* strain T-22 colonized and non-colonized tomato plants as control measuring: i) the aphid settlement preference under a dual-choice test; ii) their probing and feeding behavior using the Electrical Penetration Graph (EPG) technique; and iii) their biological performance (fitness). The beneficial effects of *T. harzianum* on the agronomic performance of tomato plants have also been investigated.

2. Materials and methods

2.1 Plant growth conditions

Seeds of tomato *Solanum lycopersicum* cultivar 'Dwarf San Marzano' were left to germinate in a climatic chamber at 24:20 °C (D:N) and a photoperiod of 16:8 h (L:D) on filter paper inside sterile Petri dishes for 5 days in darkness until the root appeared. The plants were transplanted using a mixture of soil (Jiffy code: V4, Zwijndrecht, Netherlands) and perlite (Projar, S.A., Valencia) in a proportion of 2:1. After transplantation, all plants were kept in a growth chamber at 24:20 °C (D:N), 65 ± 5% relative humidity (RH), photoperiod of 16:8 h (L:D) and watered three times a week (two time a week with tap water and one time a week with a fertilizer solution). The nutritional solution was a 20-20-20 (N:P:K) fertilizer (Nutrichem 60, Miller Chemical & Fertilizer Corp., PE, USA) and the dosage was 3 g/l.

2.2 Aphid clonal populations rearing

Two clonal of *Macrosiphum euphorbiae* were used. The first one derived from a population adapted to laboratory of University of Naples and originally collected in Nice (France). This clone was permanently maintained on tomato plants (cv. Dwarf San Marzano) at the School of Agricultural, Forestry, Food and Environmental Sciences (SAFE-UNIBAS in Potenza, Italy). The second, originated from Torrelodones (Madrid, Spain), was maintained on tomato plants (cv. Marmande) and came from the Insect Vectors of Plant Pathogens laboratory of the Institute of Agricultural Sciences-Spanish National Research Council (ICA-CSIC in Madrid, Spain). Before the assays, the two laboratory colonies were both maintained in a growth chamber at 23:18 °C (D:N), 75% relative humidity (RH), and a photoperiod of 14:10 h (L:D) at ICA-CSIC.

For both populations, laboratory colonies were synchronised on tomato plants to ensure that the adults were of the same age (8–10 days old).

2.3 Inoculation of T-22 on tomato plants

Plant inoculation was performed using *Trichoderma harzianum* strain T-22 in a granule commercial formulation (Trianum-P, Koppert, Berkel en Rodenrijs, The Netherlands) and watering the plants with 1 gram of commercial product dissolved in 500 ml of water ($1x10^9$ cfu/g of viable *T. harzianum* T-22 spores). The treatments were performed two times. The first treatment was performed during the transplant of germinated seeds into 7x7x8 cm diameter plastic trays, watering with 20 ml of solution per seeds. The seedlings were kept in a growth chamber for 2 weeks and then were transplanted into 9x9x10 cm plastic pots containing soil and placed in a growth chamber at the same conditions above described. The second treatment was performed during the seedling transplantation, watering a single plant with 50 ml of solution. Control plants were watered only with an equal amount of water and kept in the same conditions as treated plants. The plants were used for the experiments after one week from the second treatment (5 true leaves, 30 days old).

2.4 Dual choice behavior assays

The preference of *M. euphorbiae* adults for T-22 or control plants was evaluated using a host-choice bioassay under dual-choice conditions. Dual choice behavior assays were performed using a tomato plant inoculated with T-22 (n = 10) placed in front of a control plant (n = 10) in a clear breathable cage (60x40x35 cm). The two plants were connected by a white cardboard bridge (30 × 6 cm) with the ends placed at the base of the two stems (Figure 1). Thirty wingless adult of *M. euphorbiae* (8–10 days old), previously starved for 30 minutes, were released in the center of the bridge, equidistantly from the two tomato plants. After 24h, the aphids on the two plants were counted. The experiment was repeated 10 times, using a total of 300 aphids. Bioassays were carried out on a lab bench under room conditions (23.6 ± 1 °C, 38 ± 4 % HR) under shaded conditions with no direct light starting at 14:00 p.m.

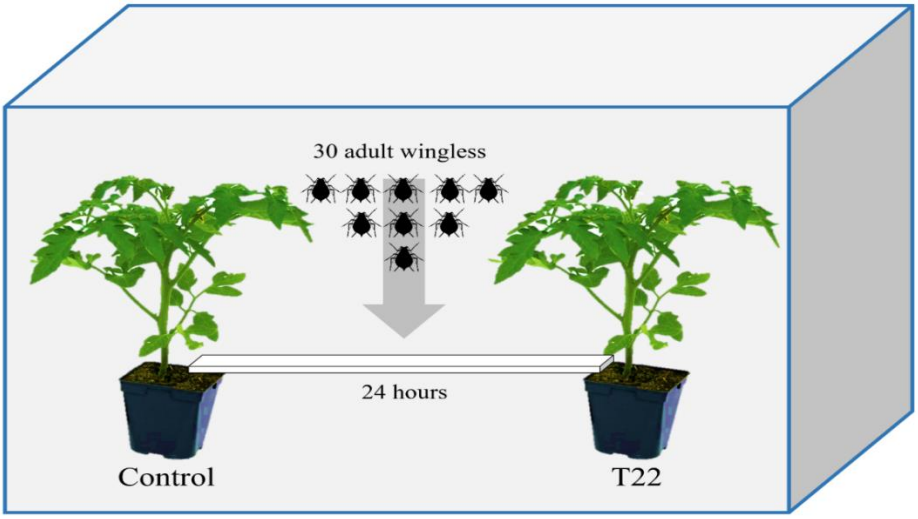

Figure 1. Schematic design of the dual choice test

2.5 Effect of the fungus on Macrosiphum euphorbiae fitness

To investigate the effect of the T-22 colonization on the reproduction and on the rate of increase in *M. euphorbiae*, two wingless adult aphids (8–10 days old) were confined inside a clip cage (3 cm of diameter) on treated and control plants. The clip cages were placed on the adaxial side of the youngest fully developed leaf of each plant (Figure 2). After 24 h, adults and surplus new-born nymphs were removed with a wet paintbrush leaving three nymphs per plant. The new nymphs were monitored daily until the adulthood stage. When the first nymph reached the adult stage, the other were removed. Then, the progeny of each adult was assessed by daily counting and removing new-born nymphs for an equal number of days to the pre-reproductive period (number of days from birth to the onset of reproduction). Aphid survivor (nymphal mortality during pre-reproductive period and adult mortality during the reproductive period), pre-reproductive period (*d*), effective fecundity (offspring produced during a period equal to the pre-reproductive one, *Md*), intrinsic rate of natural increase (r_m=0.738*($\log_e Md$)/*d*), mean generation time (*Td*=*d*/0.738) and mean relative growth rate (*RGR*=r_m/0.86) were calculated. The weight of adults was measured at the same day (day 9) for all aphids at the beginning of reproductive period. The experiment consisted of 10 replicates (plants)/ treatment/aphid clone and each replicate consisted of two aphids per plant (n = 20).

Figure 2. Clip cages placed on the adaxial side of the youngest fully developed leaf.

2.6 Electrical monitoring of aphid probing and feeding behavior

The EPG technique was used to monitor in real-time the plant penetration activities by apterous adult *M. euphorbiae* on T-22 treated tomato plants and control-untreated tomato plants. Tomato plants with 5 true leaves (30 days old) were used for the experiments. Before the onset of the bioassays, the laboratory colonies were synchronised on tomato plants (*Solanum lycopersicum* cv Dwarf San Marzano) to ensure that all individuals were of the same age. The bioassay was carried out using the two laboratory colonies. The equipment consists of a 4-channel DC amplifier (Giga-4), designed by Dr. W.F. Tjallingii (EPG-Systems, Wageningen, The Netherlands). The components of the EPG device are described in detail in Figure 3.

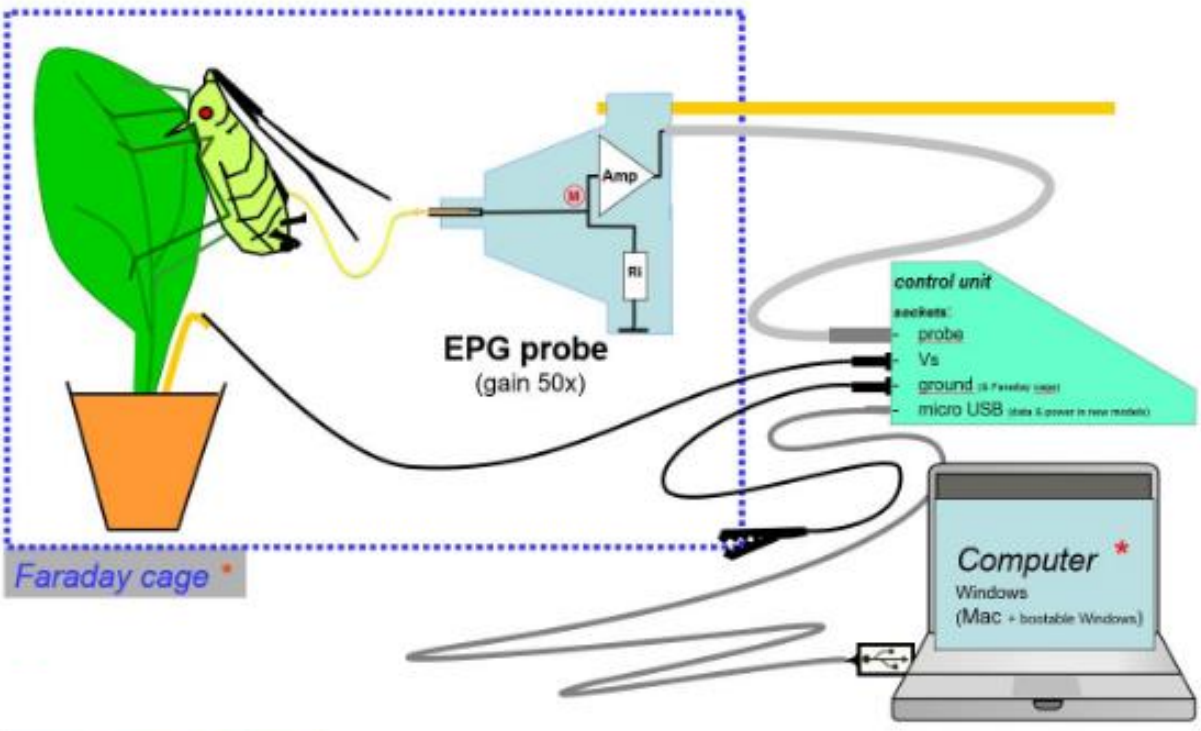

Figure 3. Graphical representation of the EPG experimental setup (https://www.epgsystems.eu/epg-measuring).

Apterous adult aphids (8–10 days old) were individually immobilized with the help of a vacuum pump and a stereo microscope adapted to cold light (Eyela Aspirator A3S, Todyo Rikakikai Co. Ltd., Japan). After this procedure, a thin gold wire (2 cm in length, 18.5 μm in diameter; Sigmund Cohn, Mount Vernon, NY, USA) was attached to the aphid dorsum with a drop of hand-mixed waterbased silver conductive paint glue (EPG Systems). The opposite extreme of the gold wire was glued to a thin copper electrode (3 cm length, 1 mm in diameter), representing the input electrode. The output electrode was a copper post (10 cm length, 2 mm diameter), which was inserted into the plant pot. Aphids with the attached gold wire were placed on the adaxial side of the youngest fully developed leaf of a tomato plant. When the aphid inserted the stylet into the plant tissue, the electrical circuit was closed and the acquisition of EPG waveforms was possible.

The acquisition and analysis of the data obtained was carried out using the computer programme Stylet+ (EPG-Systems, Wageningen, The Netherlands). The EPG waves recorded with a high resolution were visualised on the computer, allowing the identification of the types of waves described by Tjallingii (1990) (Tjallingii, 1990) that correspond to the activity of the stylet in the different tissues of the plant (based on the shape, amplitude and frequencies observed in the waves). The EPG signals were recorded for 8 h and started immediately after placing the aphids

on the tomato leaf. All aphid feeding behavior variables were processed and calculated using the MS Excel Workbook for automatic EPG data calculation, developed by Garzo et al. (2022). For the Spanish clone, a total of 19 replicate EPG recordings of 8 h on tomato control and T-22 treated plants, were recorded and analyzed. For the French clone, a total of 20 and 19 EPG recordings (8 h) were recorded and analyzed on tomato control and treated. Each EPG record was performed using a different plant and aphid.

In this study, the following aphid associated EPG waveforms were analyzed: **non-probe**, non-probing behavior (i.e., no stylet contact with the leaf tissue); **C**, intercellular apoplastic stylet pathway where insects show a cyclic activity of mechanical stylet penetration and secretion of saliva; **E1**, salivation into phloem sieve elements at the beginning of the phloem phase; **E2**, passive phloem sap uptake from the sieve elements (Tjallingii, 1988). The term "probe" refers to any type of event during the period in which the stylets of an insect were in contact with plant tissue, whereas "non-probe" refers to the event without contact between the stylets and the plant tissue. Total duration of each waveform events (non-probe, stylet penetration, as non-phloem activity, and phloem activity, as phloem salivation and phloem ingestion) was evaluated as the percentage of total time for each event.

EPG technique was carried out on a lab bench at ambient conditions (23 ± 2 °C, 25 ± 2 % HR) from 9:00 to 17:00 p.m.

2.7 Plant growth and fungus colonization

The tomato plants at the end of the dual choice behavior assays were cleaned of aphids and removed from the pots to carry out measurements of the plant growth. These measurements were performed with tomato plants inoculated with T-22 (n = 20) and control plants (n = 20). The plant height, the weights of the fresh root and shoot tissue were measured. To determine shoot tissues were frozen at -80ºC ant then were lyophilized to remove frozen water without the liquid phase, using a freeze-dryer (Telstar, Lyo Quest) at -80 °C for 72 h.

To confirm fungus colonization, tomato plants were removed from the pots at the end of the fitness experiments. These measurements were performed using tomato plants inoculated with T-22 (n = 20) and control plants (n = 20). To check the T-22 colonization on tomato plants, I followed the protocol described by Agbessenou et al. (2020) with some modifications. Roots were washed with tap water to remove soil particles. The roots were aseptically cut under a laminar flow hood into 2 cm pieces and washed in 1% sodium hypochlorite, then in 70% of ethanol 96º and finally rinsed 3 times on purified water. After that, the pieces of control and T-

22 roots were individually placed on PDA Petri plates supplemented with a 0.05% solution of antibiotic (streptomycin sulfate salt). The plates were incubated at 25 ± 2 °C for 5 days, after which the presence of endophyte was identified by morphological observations of the germination of the conidia under a light microscope (x400)(McGonigle *et al.*, 1990).

2.8 Statistical analysis

The data were checked for normality and homogeneity of variance using the Shapiro–Wilk *W* test. For the dual choice behavior assays the percentage of *M. euphorbiae* Spanish clone and *M. euphorbiae* French clone observed on control and *T. harzianum* colonized tomato plants and the measurement of plant biomass on control and colonized tomato plants was compared by a t-Student test (for Gaussian variables).

For the effect of T-22 colonization on *Macrosiphum euphorbiae* fitness the treatments were compared by a t-Student test (for Gaussian variables) or Mann-Whitney-Wilcoxon test (for non-Gaussian variables).

The EPG variables were processed for each given insect with the help of an EXCEL Data Workbook elaborated in Fereres' lab (Garzo E. et al in press). All the behavioral variables obtained by EPG recording between treatments were compared by Mann-Whitney-Wilcoxon Test. Significant differences were declared at $p < 0.05$ for all variables. These statistical analyses were analysed using SPSS (IBM SPSS Statistics 28.0 for Windows, Chicago, USA).

The percentage of total duration of each waveform events as proportions of the 8 hours of analysis of the EPG recording of *M. euphorbiae* on tomato plants treated with T-22 and on the untreated control was compared by a chi-square test and to a Fisher exact test when the expected values were lower than 5 (Statview II, abacus concepts, 1987).

3. Results

3.1 Dual choice behavior assays

Results of the dual-choice assay showed significant differences between the mean percentages of *M. euphorbiae* settled on control or T-22 plants for Spanish clone (t_{19} = 2.51, *P* = 0.02) and for the French clone (t_{19} = 2.44, *P* = 0.02), with an higher percentages of aphids settled on control plants (Figure 4).

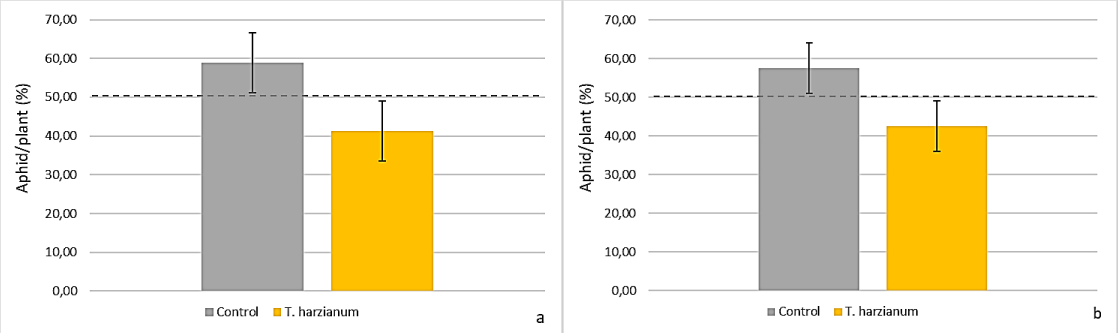

Figure 4. Dual-choice behavior assays using the two clonal population rearing under laboratory conditions. The percentage (mean ± 95% Confidence Intervals) on *T. harzianum* colonized plants was compared to the expected percentage assuming that there is no difference in the plant preference (50%) of (a.) *M. euphorbiae*-Spanish clone and (b.) Adults of *M. euphorbiae*-French clone observed on control and *T. harzianum* colonized tomato plants during dual-choice assays.

3.2 Effect of the fungus on Macrosiphum euphorbiae fitness

Table 1 shows the percentage of nymphal and adult mortality during the fitness bioassay. The percentage of nymphal mortality was not different between clones, while the percentage of adult mortality for the French clone was higher in control plants although not significant. A similar trend was also observed for the Spanish clone, but with lower values.

The results of the experiments on the fitness of *M. euphorbiae* French clone reared on T-22 and control plants are shown in Table 2, while the ones of the Spanish clone are reported in Table 3. Compared with the T-22 treated plants, a significant increase in the intrinsic rate of increase (r_m) on control plants was found for the French clone (t_{11} = 2.33, *P*= 0.042); no significant difference in the r_m was found for the Spanish clone. The same trend was observed for the mean relative growth rate (*RGR*), since it is related to r_m by the formula: r_m = 0.86 * *RGR*.

Table 1. Percentage of nymphal and adult mortality of the French and Spanish clone during the fitness assay.

Aphid clone	Treatment	*N*	Nymphal mortality (%)	Adult mortality (%) (n = 10)
French	Control	27	15%	50%
	T-22	30	13%	30%
Spanish	Control	26	4%	10%
	T-22	30	10%	0%

Table 2. Biological parameters of *M. euphorbiae* (French clone) reared on tomato plants treated with T-22 and control plants. *d*: pre-reproductive period (days); *Md*: effective fecundity; *rm*: intrinsic rate of natural increase; *Td*: mean generation time (days); *RGR*: mean relative growth rate.

Biological Parameter	**Treatment**	**Mean ± SE**	**Statistic Test**	***p*-Value**
d	Control	9.2 ± 0.2	$U_{11} = 22$	0.255
	T-22	9.57 ± 0.20		
Md	Control	32.8 ± 2.06	$t_{11} = 2.11$	0.066
	T-22	27.29 ± 1.61		
rm	Control	0.28 ± 0.01	$t_{11} = 2.33$	**0.041***
	T-22	0.26 ± 0.01		
Td	Control	12.7 ± 0.27	$U_{11} = 11$	0.255
	T-22	12.87 ± 0.27		
RGR	Control	0.47 ± 0.01	$t_{11} = 2.24$	**0.048***
	T-22	0.44 ± 0.01		
Adult weight	Control	1.05 ± 0.13	$U_{11} = 22$	0.53
	T-22	0.98 ± 0.1		

* Statistically significant *p*-values ($p<0.05$) based on the t-test for Gaussian distribution and for non-Gaussian distribution Mann-Whitney-Wilcoxon Test. Values represent mean ± SE. (Sokal and Rohlf, 1995).

Table 3. Biological parameters of *M. euphorbiae* (Spanish clone) reared on tomato plants treated with T-22 and control plants. *d*: pre-reproductive period (days); *Md*: effective fecundity; *rm*: intrinsic rate of natural increase; *Td*: mean generation time (days); *RGR*: mean relative growth rate.

Biological Parameter	**Treatment**	**Mean ± SE**	**Statistic Test**	***p*-Value**
d	Control	8,88 ± 0,13	$U_{16} = 39.5$	0.662
	T-22	8,78 ± 0,5		
Md	Control	32,13 ± 1,46	$t_{16} = 1.29$	0.217

	T-22	35,56 ± 2,22		
rm	Control	0,29 ± 0,001	U_{16} = 22	0.193
	T-22	0,30 ± 0,01		
Td	Control	12,03 ± 0,17	U_{16} = 39.5	0.662
	T-22	11,89 ± 0,20		
RGR	Control	0,49 ± 0,01	U_{16} = 21	0.162
	T-22	0,50 ± 0,01		
Adult weight	Control	0.95 ± 0.06	t_{16} = 0,21	0.831
	T-22	0.97 ± 0.06		

* Statistically significant *p*-values ($p<0.05$) based on the t-test for Gaussian distribution and for non-Gaussian distribution Mann-Whitney-Wilcoxon Test. Values represent mean ± SE. (Sokal and Rohlf, 1995).

3.3 Probing and feeding behavior of aphid clones

The analyses of the EPG behavioral variables are showed in Table 3, while the analyses of total duration of each waveform events (non-probe, stylet penetration, as non-phloem activity, and phloem activity, as phloem salivation and phloem ingestion) are showed in Figure 5.

The EPG variables evaluated indicate that both aphid-clone have not showed too much differences between treatments (untreated and *Trichoderma*-treated plants). Our results indicated that the aphid *M. euphorbiae (*clone French) showed a reduction of the number of phloem probes (number of E1 following by E2, number of E2 and number of sustained E2) on those aphids feeding on *Trichoderma*-treated plants compared with the untreated plants. However, these significant differences were not observed in the Spanish aphid-clone (Table 3). In the case of Spanish aphid clone only we observed that the total duration of the single E1 was significantly lower on those insects that feeding on *Trichoderma*-treated plant.

The percentage of the total time spent in non-probing events was significantly different only in the French clone ($\chi^2 = 59$, df = 12, $P < 0.0001$), while it was not significantly different in the Spanish clone ($\chi^2 = 0.058$, $P = 0.809$). For both clones, the phloem salivation event (E1) was not significantly different (French clone: $\chi^2 = 0.338$, $P > 0.9999$; Spanish clone: $\chi^2 = 0$, $P > 0.9999$), while the phloem ingestion event (E2) was significantly different only in the Spanish clone ($\chi^2 = 4.58$, P=0,032; French clone: $\chi^2 = 0.184$, $P = 0.668$). Similar trend observed in stylet penetration events (French clone: $\chi^2 = 0$, P > 0.9999; Spanish clone: $\chi^2 = 3.79$, $P = 0.052$).

Table 4. Mean ± standard error (min) of non-sequential and sequential electrical penetration graph (EPG) variables for the feeding behavior of *M.euphorbiae* exposed to tomato plants treated with T-22 and to control plants. Waveforms: C, intercellular stylet pathway; E shows phloem-related activities, E1: correlates with salivation into phloem elements; single E1: E1 not followed by E2; E2: is considered phloem ingestion; sE2: sustained E2 (>10 min).

Aphid clone	**EPG Variables**	**Untreated Control N=20**	***T. harzianum* T-22 N=19**	*P-value*
	Non-sequential variables			
French clone	Number of Waveform Events			
	np	13.60 ± 2.4	14.26 ± 2.7	0.966
	C	19.15 ± 2.6	18.0 ± 3.5	0.406
	Single E1	1.90 ± 0.4	1.58 ± 0.4	0.489
	E1	6.90 ± 0.9	4.53 ± 0.8	0.053
	E1 followed by E2	4.0 ± 0.5	2.63 ± 0.5	**0.035***
	E2	4.45 ± 0.7	2.68 ± 0.5	**0.027***
	E2 sustained (>10 min)	2.85 ± 0.4	1.53 ± 0.2	**0.006***
	Total Duration of Waveform (min)			
	np	37.40 ± 7.2	53.13 ± 11	0.415
	C	138.27 ± 15.2	119.13 ± 16.7	0.312
	Single E1	2.40 ± 0.7	2.07 ± 0.5	0.776
	E1	11.0 ± 3	5.40 ± 0.8	0.465
	E1 followed by E2	290.80 ± 21.3	292.0 ± 25.5	0.844
	E2	282.24 ± 22.1	288.52 ± 25.7	0.822
	E2 sustained (>10 min)	276.0 ± 23	285.5 ± 26	0.715
	Mean Duration of Waveform (min)			
	np	3.0 ± 0.3	4.10 ± 0.7	0.319
	C	8.82 ± 1.1	9.21 ± 1.7	0.694
	Single E1	1.15 ± 0.2	1.61 ± 0.5	0.49
	E1	1.28 ± 0.2	1.49 ± 0.4	0.855
	E1 followed by E2	127.80 ± 31.7	203.0 ± 34.5	0.081
	E2	127.09 ± 31.6	196.26 ± 34.7	0.092
	E2 sustained (>10 min)	162.70 ± 32.8	240.22 ± 32.6	0.06
	Sequential variables			
	Time to 1st probe from start of recording	3.10 ± 0.7	2.14 ± 0.5	0.133
	Time to 1st E from start of 1st probe	53.60 ± 9.7	88.30 ± 15.5	0.06
	Time to 1st E12 from start of 1st probe	73.08 ± 14.2	95.80 ± 17	0.238

Aphid clone	EPG Variables	Untreated Control N=19	*T. harzianum* T-22 N=19	*P-value*
Spanish clone	Number of Waveform Events			
	np	15.11 ± 2.4	11.42 ± 2.1	0.293
	C	19.42 ± 3.1	14.8 ± 2.3	0.327
	Single E1	1.15 ± 0.4	0.31 ± 0.1	0.059
	E1	4.74 ± 0.8	4.37 ± 0.6	0.953
	E1 followed by E2	3.30 ± 0.5	3.5 ± 0.5	0.745
	E2	3.47 ± 0.5	3.58 ± 0.5	0.871
	E2 sustained (>10 min)	2.50 ± 0.4	2.3 ± 0.2	0.881
	Total Duration of Waveform (min)			
	np	48.0 ± 10.3	41.40 ± 8.5	0.759
	C	152.88 ± 20.6	114.44 ± 16.2	0.157
	Single E1	1.30 ± 0.5	0.27 ± 0.1	**0.047***
	E1	4.15 ± 0.6	3.71 ± 0.6	0.693
	E1 followed by E2	248.0 ± 27.6	310.20 ± 23.4	0.112
	E2	245.14 ± 27.6	306.74 ± 23.7	0.112
	E2 sustained (>10 min)	241.80 ± 28	303.80 ± 24.3	0.112
	Mean Duration of Waveform (min)			
	np	4.03 ± 1.2	3.79 ± 0.7	0.3
	C	12.0 ± 2.6	8.88 ± 1.3	0.609
	Single E1	1.7 ± 0.8	1.10 ± 0.4	0.877
	E1	1.18 ± 0.4	0.82 ± 0.1	0.977
	E1 followed by E2	121.80 ± 27.5	158.10 ± 32.4	0.429
	E2	116.53 ± 27.6	156.38 ± 32.6	0.378
	E2 sustained (>10 min)	140.20 ± 27.3	175.0 ± 29.8	0.248
	Sequential variables			
	Time to 1st probe from start of recording	4.1 ± 1.2	2.45 ± 0.6	0.3
	Time to 1st E from start of 1st probe	90.1 ± 21.8	85 ± 15.4	0.693
	Time to 1st E12 from start of 1st probe	128.2 ± 30	93.2 ± 15.5	0.759

* Statistically significant *p*-values ($p<0.05$) based on Mann-Whitney-Wilcoxon Test. Values represent mean ± SE. (Sokal and Rohlf, 1995).

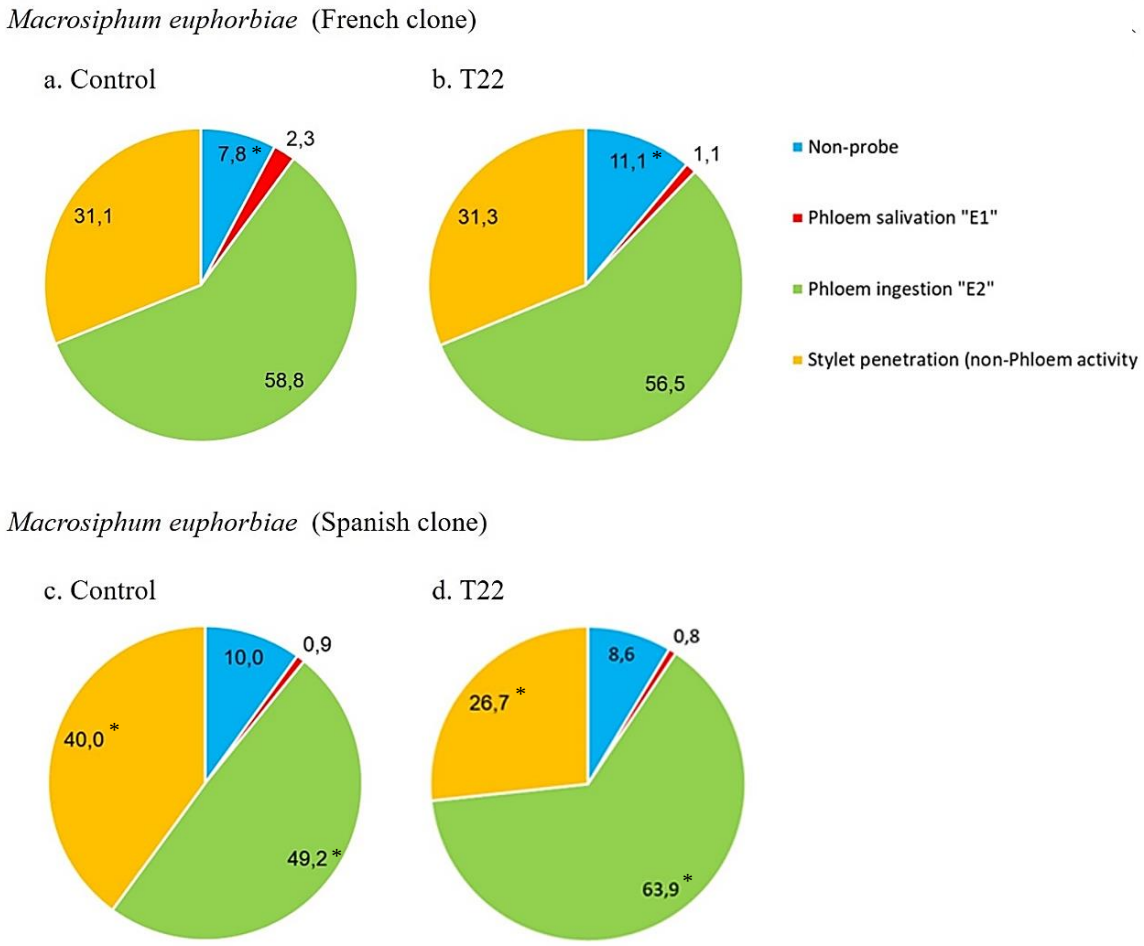

Figure 5. Total duration (%) of each waveform events as proportions of the 8 hours of analysis of the EPG recording of *M. euphorbiae* on tomato plants treated with T-22 and on the untreated control. Waveforms events: Non-probing, Phloem salivation (E1), phloem ingestion (E2), Stylet penetration (the sum of intercellular stylet pathway, probes excluding the phloem activity). Significant differences according to a chi-square test and to a Fisher exact test when the expected values were lower than 5 (Concepts A., 1987).

3.4 *Plant growth and fungus colonization*

The mean values (± standard errors) of plant biomass of control and colonized tomato plants are show in Table 4 and in Figure S1. Statistically significant differences were found between treatments for all plant growth parameters. The mean weight of fresh and biomass was greater in *Trichoderma*-treated plants than in untreated control ones, as well as the mean height in *Trichoderma*-treated plants.

Finally, the fungal colonization during all assays was confirmed since a colonization of 100% was obtained for the *Trichoderma*- treated plants, while none of the control plant showed the presence of *Trichoderma* conidia.

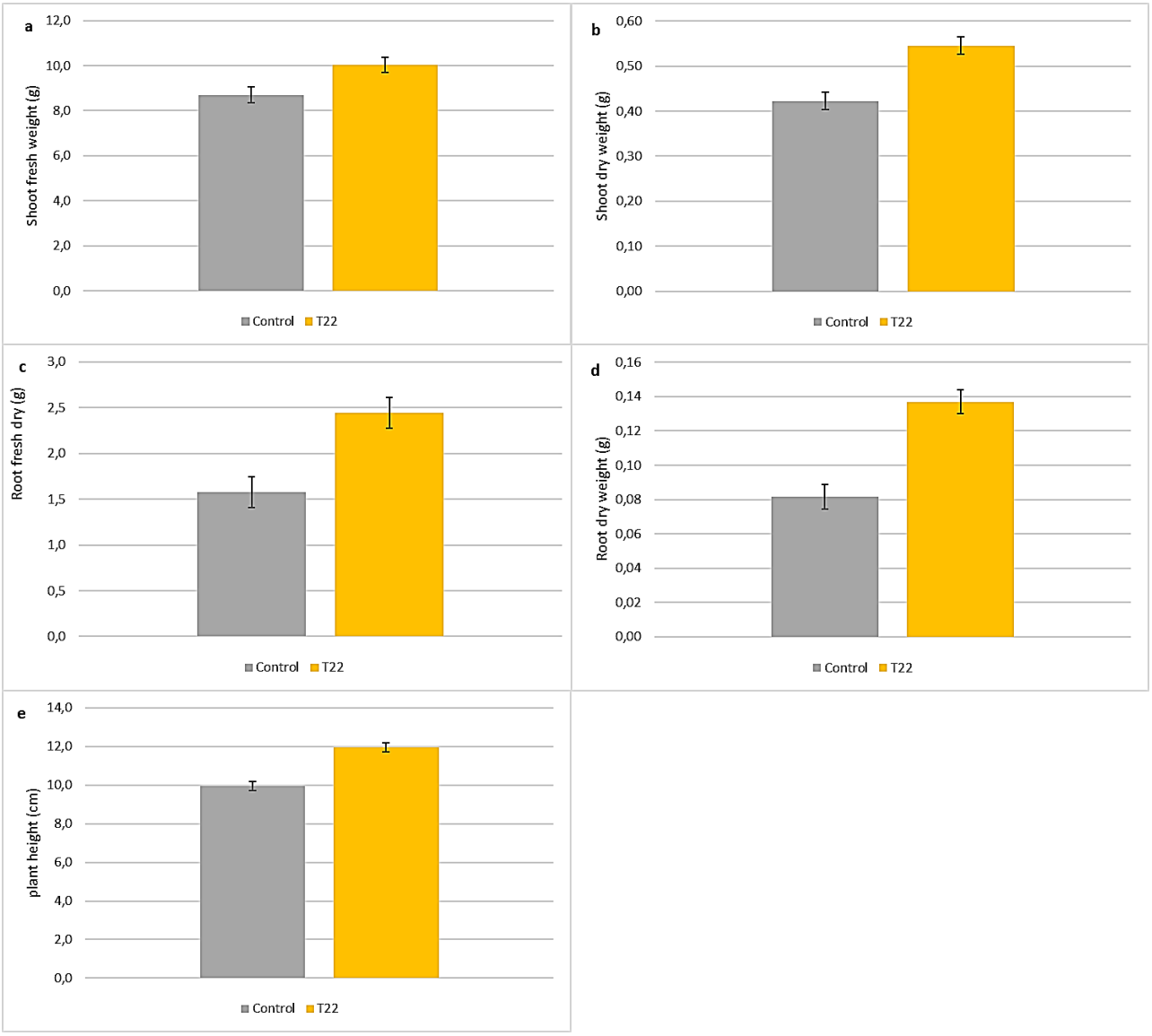

Figure 6. Measurement of plant biomass on control and colonized tomato plants. The bars show the mean values (± standard errors) for (a), (c) measure of fresh biomass (FW), (b), (d) measure of dry biomass (DW) and, (e) measure of plant height (L). * Significant differences according to t-Student test (for Gaussian variables) ($p<0.05$).

Table 5. Plant growth parameters on control and colonized tomato plants: FW: fresh weight; DW: dry weight; L: length.

	Control *N* = 20	T-22 *N* = 20	*t*	*P-value*
Shoot FW (g)	8.7 ± 0.3	10.0 ± 0.5	t_{39}=2.33	**0.025**
Shoot DW (g)	0.42 ± 0.02	0.54 ± 0.03	t_{39}=3.73	**<0,001**
Root FW (g)	1.57 ± 0.2	2.44 ± 0.1	t_{39}=4.25	**<0.001**
Root DW (g)	0.08 ± 0.01	01.14 ± 0.01	t_{39}=4.80	**<0.001**
Height L (cm)	9.94 ± 0.2	12.0 ± 0.3	t_{39}=5.29	**<0.001**

* Statistically significant *p*-values ($p<0.05$) based on the t-test for Gaussian distribution. Values represent mean ± SE.

4. Discussion

Based on current information, it is not known that *Trichoderma* alters aphid feeding behavior. In plants colonized by *Trichoderma*, it is reasonable to assume that aphids may have some difficulties in finding and reaching the phloem, as previous studies show that mycorrhizal infection induced changes in the thickening of the leaves containing higher amounts of insoluble polysaccharides and insoluble proteins (Krishna *et al.*, 1981).

The EPG technique (Tjallingii, 1988) is useful to characterize the insect stylet activities. In the present work, the EPG technique reveals that, compared to the control plants, there was no effect of T-22 on the probing behavior of *M. euphorbiae*. The results on probing and feeding behavior suggest that *T. harzianum* T-22 can slightly reduce the ability of aphids to reach the phloem (pre-phloem resistance factors might be involved), but both clones manage to reach and feed from the phloem. In this study, the number of phloem probes in the French clone were reduced in the T-22 treated plants; however, these probes have a longer mean duration, although there was no significant difference in the duration. In general, both clones fed, spending more time in the sustained phloem ingestion (sE2) phases, which lasted longer than 10 min, although the differences were not significant.

When the percentage of total time spent in each waveform was considered, the French clone did not seem to spend any more time without feeding in the control plants, but later fed without any problems from the phloem. On the other hand, the Spanish clone spent more time in phloem ingestion in T-22-treated plants, so the time spent in stylet penetration was less. These results can be explained as the Spanish clone first reaches the phloem in plants treated with T-22- and then feeds for a longer period of time.

Studies on aphid plant preference in the presence of symbiotic fungi are rare. In the dual-choice settlement experiments, both aphid clones showed a preference for the non-colonized plants. This choice behavior could be a consequence of the emission of volatile substances from T-22-treated plants that are not preferred by aphids or that have a repellent activity. A recent study showed that *T. harzianum* induces the *de novo* emission of several sesquiterpenes such as β-caryophyllene and δ-cadinene in maize plants (Contreras-Cornejo *et al.*, 2021). In another recent study (Wang *et al.*, 2020), two groups of sesquiterpenes (β-caryophyllene and α-humulene) displayed repellent activity against *M. euphorbiae* in tomato plants that interfered with their host plant choice.

The colonization of tomato plants by *T. harzianum* did not affect the performance of the Spanish *M. euphorbiae* clone. However, the intrinsic rate of natural increase (r_m) of the French clone

slightly decreased on plants colonized by *T. harzianum.* There was no difference in the number of days of pre-reproductive period (*d*), so the lower value depends on the number of nymphs was lower (effective fecundity, *Md*). This result can be explained if the control plants should be considered as better host than the treated ones for the French clone nymphs, while the adults developed on the same plants were affected more on the control plants. However, the possible differences in host quality should be attributable to the content of the foliar nutrient content.

It is known that in tomato the induction of the MeJA pathway directly affects aphid development and enhances the attraction of both parasitoids and predators (Thaler, 2002). Several studies have also shown that *Trichoderma* induces the production of hormones such as JA, SA, and ET, which are involved in the activation of plant defenses (Nawrocka & Małolepsza, 2013). The negative influence of T-22 on the fitness of aphids can be due to the production of these hormones in T-22-treated plants.

The treated plants (100% colonized by the fungus) appeared higher and with higher mean weight of the roots and the shoot than the control plants, confirming that T-22 stimulates vegetative plant growth. The effect of *Trichoderma* on the promotion of plant growth is well-documented. *Trichoderma* spp. enhance plant growth and productivity (Harman *et al.*, 2004a). In both academic research and commercial practice, strain T-22 has been shown to increase root development in maize and in numerous other plants (Harman, 2000; Harman *et al.*, 2004b). Improvements in root development are frequently associated with increases in yield and biomass. In tomato seedlings, the height, diameter, fresh and dry weight of the shoot, as well as root fresh and dry weight increased when seed were inoculated with *Trichoderma* sp. and *T. harzianum* T969 compared to the control (Azarmi *et al.*, 2011).

The effects of *Trichoderma* on plant-insect interactions depend on numerous factors, such as temperature (Di Lelio *et al.*, 2021), species and strains of *Trichoderma* (Copetta *et al.*, 2006; Tucci *et al.*, 2011; Kovach-Orr & Fussmann, 2013; Soler *et al.*, 2013; Bazghaleh *et al.*, 2020). Further studies are needed to investigate the possible effects of *Trichoderma* on different aphid species and clones.

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CHAPTER II

Tomato below ground-above ground interactions: *Trichoderma hartianum* T-22 affects the performance and behavior of *Myzus persicae*

1. Introduction

During the past decade, several studies have addressed the activation by symbiotic fungi of direct and indirect plant defenses against herbivorous insects (Battaglia *et al.*, 2013; Colella *et al.*, 2014; Coppola *et al.*, 2017, 2019b; a; Durán Prieto *et al.*, 2017; Contreras-Cornejo *et al.*, 2018; Alınç *et al.*, 2021; Caccavo *et al.*, 2022; Islam *et al.*, 2022). Many of these studies concern aphids and their natural enemies in tomato plants (Battaglia *et al.*, 2013; Coppola *et al.*, 2017, 2019b; a; Durán Prieto *et al.*, 2017). In particular, Coppola et al. (2019 b) demonstrated that tomato plants, colonized by *Trichoderma harzianum* (strain T-22) and subsequently infested by the aphid *Macrosiphum euphorbiae,* undergo a wide transcriptome reprogramming with evident expression changes of defense genes. This process results in a reduction in the survival rate of *M. euphorbiae* feeding on *Trichoderma*-treated plants. However, the reinforcement of defense barriers observed after plant colonization by *T. harzianum* (strain T-22) is not univocally associated with *Trichoderma* infection of tomato plants. Indeed, *Trichoderma longibrachiatum* strain MK1 promotes the development and reproduction of *M. euphorbiae* (Battaglia *et al.*, 2013). Therefore, it is documented that the response of the plant depends on the fungal species. Moreover, abiotic factors, such as temperature, strongly influence the outcome of tomato–*Trichoderma* interaction (Di Lelio *et al.*, 2021). The purpose of this study is to explore the possibility that the impact of plant-*Trichoderma* interaction on aphid populations also depends on aphid species. Different species of aphids, and even different clones of the same species, cause different levels of direct damage to the host plant (Perring *et al.*, 2018) and are more or less successful in overcoming the plant's defense mechanisms (Goggin *et al.*, 2001). The aphid *M. euphorbiae* has been considered a valid model species for studies investigating the tomato-*Trichoderma*-aphid interaction (Battaglia *et al.*, 2013; Coppola *et al.*, 2017, 2019b; a; Di Lelio *et al.*, 2021). Among the aphid species that attack tomato plants, *M. euphorbiae* causes the most significant direct damage that appears as foliar necrosis at the feeding site and chlorosis that extends beyond the point of infestation (Strand, 1998; Goggin, 2007). Continued feeding by large colonies results in distortion of leaves and stems, and the plant can be stunted. In

comparison, severe *Myzus persicae* infestation results in moderate wilting, which is usually not considered a problem unless plants are water-stressed (Perring *et al.*, 2018). Tomato varieties carrying the *Mi* gene appear to not affect *M. persicae*, while they have a very negative impact on some clones of *M. euphorbiae* (Kaloshian *et al*., 1995; Goggin *et al*., 2001). In view of these differences, *M. persicae* was chosen as model species to investigate the tomato-*Trichoderma*-aphid interaction. In this study, the effect of *Trichoderma harzianum* T-22 on *M. persicae* colony growth has been investigated. The preference expressed by this aphid for control or colonized plants has also been investigated in a choice test.

2. Materials and methods

Plants and Aphids

The tomato (*Solanum lycopersicum*) cultivar 'Dwarf San Marzano' was used for the experiments. Seeds of tomato were left to germinate at 20 ± 2°C, on wet cotton in the dark, inside sterile Petri dishes until the root appeared. The germinated seeds were transplanted onto polystyrene plates containing universal Naturasol® potting soil (COMPO Italia Srl, Cesano Maderno, Italy), then grown in an environmental chamber at 20 ± 2°C, photoperiod 16 h 8 h light/dark. After 20 days (with the appearance of the first four true leaves and purple hypocotyl), the tomato seedlings were transplanted into 10 cm diameter plastic pots containing universal potting soil Naturasol®. The plants were kept in a green house at 20 ± 5°C, 65 ± 5% relative humidity (RH), and 18 h of light and 6 h of darkness before use. Each plant was watered three times a week: twice with tap water and once with a 1 g/l dosage. Nutrient solution was prepared using Yaramila diamante (NPK 20.7.13) (Yara Italia spa, Milan, Italy).

The aphid *Myzus persicae* was reared on broad bean plants (*Vicia faba* Linnaeus, cultivar Aguadulce), in a climatic chamber at 20±1°C, 65%±10% RH, photoperiod 16 h: 8 h light/dark. Before all experiments, aphids were subjected to a 30 min pre-acquisition starvation period.

Treatment with T. harzianum strain T-22

Before the experiments, the viability of the commercial formulation of *T. harzianum* T-22 was evaluated in the laboratory by serial dilution. The dilutions were placed on Petri plates (9 cm in diameter) containing Potato Dextrose Agar (PDA) medium (Oxoid Ltd., Hants, UK) amended with the antibiotic streptomycin sulphate 40 mg/L (MerckKGaA, Darmstadt, Germany) until

growth could be detected. As suggested by a previous study (Pocurull *et al.*, 2020), the number of colony forming units (CFU) was counted after 24 h of incubation at 25°C in the dark.

Once the viability of the commercial product was confirmed, the *Trichoderma harzianum* strain T-22 was used as a granule commercial formulation (Trianum-P, Koppert, Berkel en Rodenrijs, The Netherlands), using the dose and methodology described in chapter I. The plants were kept in a green house at 20 ± 5°C, 65 ± 5% relative humidity (RH) and 16 h: 8 h light/dark before use. The plants were watered three times a week (2 tap water + 1 nutrient solution) and were used for the experiments after one week from the second treatment (5 true leaves, 30 days old).

Dual choice behavior assays

Aphid plant preference experiment was performed using a T-22-treated tomato plant placed in front of a control in a clear breathable cage (80x40x35 cm) using a white cardboard bridge (25 × 4 cm) that connects the base section of the plants with cross-touch leaves. Thirty wingless adults of *M. persicae* (8-10 days old), with a 30-min preacquisition starvation period, were released in the center of the bridge and allowed to choose the control or colonized plant (Figure 1). After 24 hours, the number of aphids present in each of the two types of plants was counted, as well as the number of dead aphids. This experiment was repeated 10 times, using a total of 300 aphids. Bioassays were carried out on a lab bench under ambient conditions (23 ± 2 °C, 34 ± 4 % HR) under shaded conditions with no direct light from 9:00 a.m.

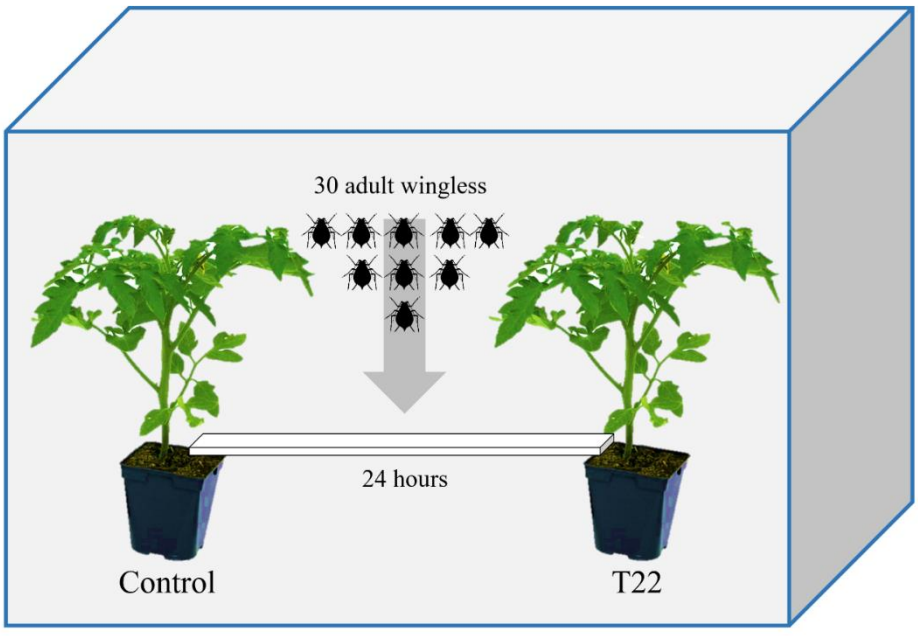

Figure 1. Schematic design of the choice design

Development of aphid colony

The experiments on the development of the aphid colony were carried out under controlled conditions (20 ± 3°C) on 20 treated and 20 control tomato plants with 5-6 fully expanded leaves (30-40 days old). Before the experiments, the aphids were subjected to a 30 min pre-acquisition starvation period. Control and treated tomato plants were infested with 10 adult wingless *M. persicae* (8-10 days old). The aphids were placed on the same upper surface of the third leaf (position 0). For each plant, adults and new-born nymphs were inspected and counted 2h (day 0) after infestation and successively every two days, until the end of the experiments (21 days after infestation). During the first five days from the beginning of the experiments, it was possible to accurately record the behavior of the 10 initial aphids, as they were easily separable from the nymphs they had produced. At the day 5, the movement of adult aphids was estimated as their different placement along the plant. If an aphid had moved upward, its position could be +1, +2 (first or second leaf above the initial one) or +3 (apex of the plant); if an aphid had moved downward, its position could be -1, -2 (first or second leaf under the initial one) or -3 (base of the plant). During this 5-day period, adult survival and production were also recorded. As the nymphs were not removed from the plants, from the seventh day it was no longer possible to distinguish the initial aphids from the new-borne ones. For this reason, the live aphids on a plant were counted every two days and their position on the plant was recorded.

Plant growth

After removing all aphids, the tomato plants were removed from the pots at the end of the experiments on the development of the aphid colony to perform measurements of the biomass of the plant. The weights of root and shoot fresh tissue, as well as plant height were recorded. Shoot tissue was placed in a muffle furnace (Hotbox oven, Gallenkamp, UK, size 2) at 72 °C for 24 h to obtain dry weight.

The measurements on plant biomass were carry out on 14 control and 14 treated plants.

Statistical analysis

Row data used in the analyses of aphids plant preference are the percentage of live aphids found on a tomato plant. The homoscedasticity and normality assumptions were checked and met on these data. The mean percentage of aphids plant preference ± 95% confidence intervals was compared with the value of 50%. The equality of the percentage of aphid plant preference of 50% is in accordance with the null hypothesis that there is no difference in the preference of aphids for a control or a *Trichoderma harzianum* strain T-22 colonized plant.

In the experiments on the development of aphid colonies, data relating adult survival and fecundity after 5 days, plant heights, fresh and dry weight of shoots and the roots were normally distributed (a ln transformation was applied for the fecundity data) and analysed using two-sample *t*-tests for differences between treatments. Data on the development of aphid colonies were also analyzed using a two-way factorial ANOVA with "Treatment", "Day" and "TREATMENT X DAY" as fixed effects. Data on aphid position on plants after 5 days were analyzed using a two-way Poisson generalized linear model (GLM) with a log-link function with "Treatment" (two levels: *T. harzianum* and control) and "Position" (7 levels: -3, -2, -1, 0, +1, +2, +3) as main fixed effects. The Poisson GLM was chosen as the best-fitting model based on AIC criteria (Burnham & Anderson, 2004).

The evolution of the aphid colony (that is, the number of live adults and nymphs present on a plant) over time was analyzed using a two-way factorial ANOVA with "Treatment" and "Day" (11 levels: from day 0 to day 22) as main fixed effects. The homoscedasticity and normality assumptions for ANOVA were checked and met on these data.

To test whether colonization with *T. harzianum* influenced aphid dispersal, the number of aphids found in a different position from the leaf where they were initially placed were recorded and standardized on the total number of individuals on the plant on a given day. The dispersion over time was then analyzed using a two-way factorial ANOVA with "Treatment" and "Day" (7 levels: from day 7 to day 21) as the main fixed effects. The homoscedasticity and normality assumptions for ANOVA were checked and met after a square root arcsin transformation of these data. Differences between treatments in plant heights, fresh and dry weight of the shoots and roots were analyzed using two-sample *t*-tests. The statistical analyses were performed in R version 4.1.2 "Bird Hippie" (R Core Team, 2021).

3. Results

Dual choice behavior assays

The mean percentages (± 95% confidence intervals) of *M. persicae* adults observed on control and *T. harzianum* colonized tomato plants are reported in Figure 2.

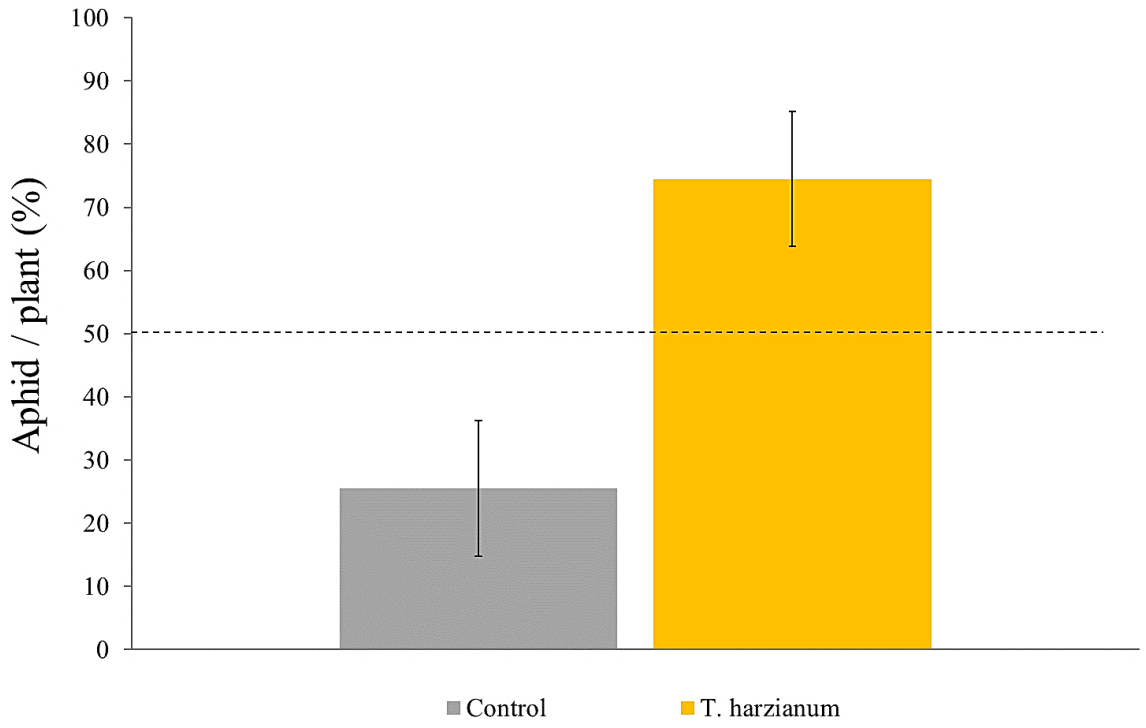

Figure 2. Dual choice behavior assays. Mean values (± 95% confidence intervals) of the percentage of *M. persicae* adults observed on control and *T. harzianum* colonized tomato plants.

The percentage (mean ± 95% CI) of aphids on *T. harzianum* colonized plants was compared to the expected percentage assuming that there is no difference in the plant preference (50%). Under our experimental conditions, the observed mean value of aphids found on *T. harzianum* colonized plants was significantly higher than its respective theoretical mean values, meaning that *M. persicae* was more attracted by plants colonized with T-22.

Behavior of adults during the first 5 days

During the first 5 days, the differences in aphid mortality between control or colonized plants were not statistically significant (37.7% ± 5.5 and 38.2% ± 6.4 respectively; $t_{32} = 0.07$, $P = 0.95$). The same trend was observed for the mean nymphs production per aphid for 5 days (7.9% ± 0.84 and 7.5% ± 0.85 respectively; $t_{32} = 0.34$, $P = 0.73$).

After 5 days from the beginning of the experiment, in the control plants, 94.3% of the aphids had not moved from their initial position; the same percentage was observed in the colonized plants (94.1%). The GLM performed on these data showed that the aphids were not uniformly distributed throughout the plant (significant differences among plant positions: $\chi^2 = 705$, df = 6, $P < 0.001$) and that there was no effect of the *T. harzianum* on aphid movement ($\chi^2 = 0.04$, df = 1, $P = 0.84$).

Development of aphid colony

In this study, the development of *M. persicae* colonies on control and *T. harzianum* colonized tomato plants were investigated for 21 days and the results are reported in Figure 3.

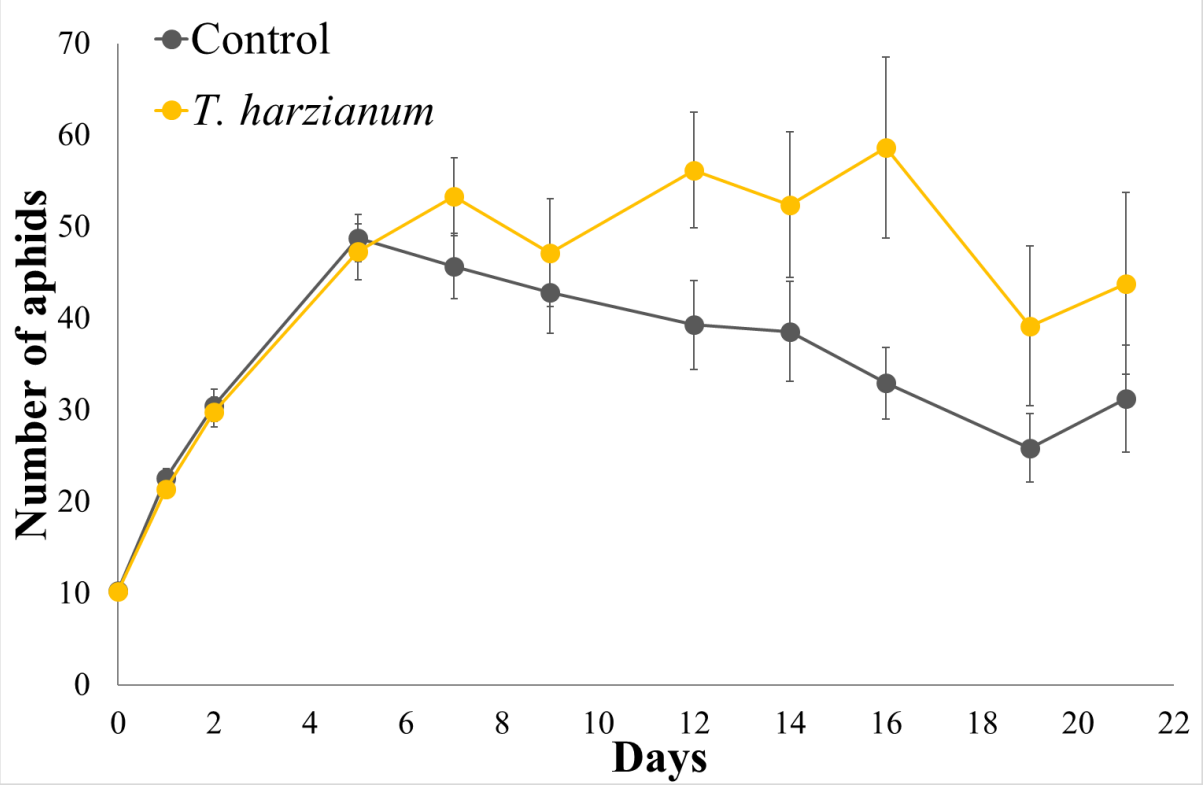

Figure 3. Development of aphid colony. Mean values (± standard errors) of the number of live *M. persicae* observed on control and *T. harzianum* colonized tomato plants over time.

Apart from the significant differences between days ($F_{10,369} = 15.2$, $P < 0.001$), aphids were more abundant, and their population reached a much higher peak on plants inoculated with *T. harzianum* compared with the control ones ($F_{1,369} = 13$, $P < 0.001$). Although the probability value was close to the threshold, the interaction "Treatment X Day" was not significant ($F_{10,369} = 1.67$, $P = 0.086$).

The percentage of aphids that abandoned the initial leaf on a given day is reported in Figure 4. The ANOVA performed on the relative *M. persicae* dispersion along the plant indicated that this trait was affected by the sampling date ($F_{6,231} = 7.38$, $P < 0.001$) but not by the treatment ($F_{1,231} = 0.0064$, $P = 0.93$) or by the interaction "treatment X date" ($F_{6,231} = 0.7$, $P = 0.65$). Regardless of the treatment, the movement of *M. persicae* along the plant was affected by the evolution of the aphid colony.

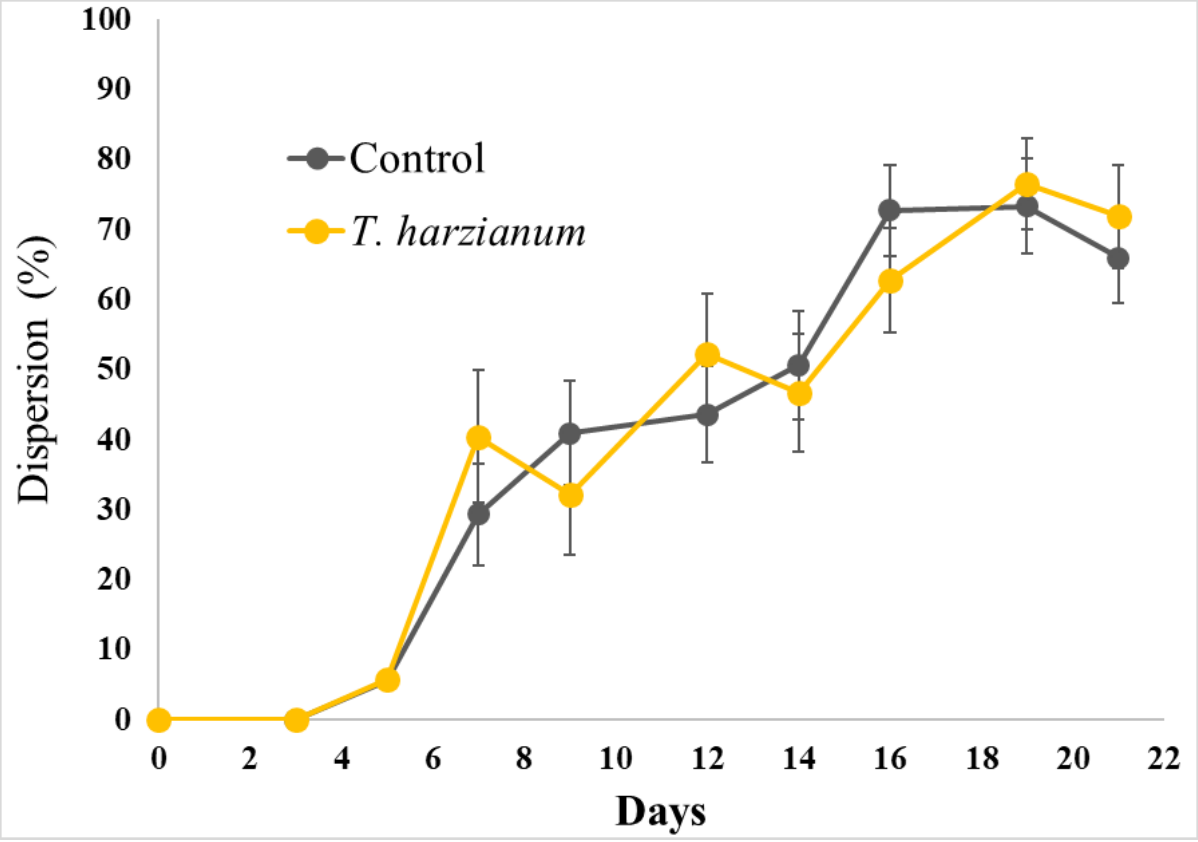

Figure 4. Mean values (± standard errors) of the percentage of *M. persicae* population that settles on parts of the plant other than initial the leaf on control and *T. harzianum* treated tomato plants over time.

Plant growth

Figure 5 shows the mean values of the fresh and dry weight of the shoots and roots and the height for control and treated tomato plants. Statistically significant differences were found between treatments for all the plant growth traits measured. The fresh and dry weight of the roots and shoots, as well as plant height was greater in T-22-treated plants than in control ones (Table 1).

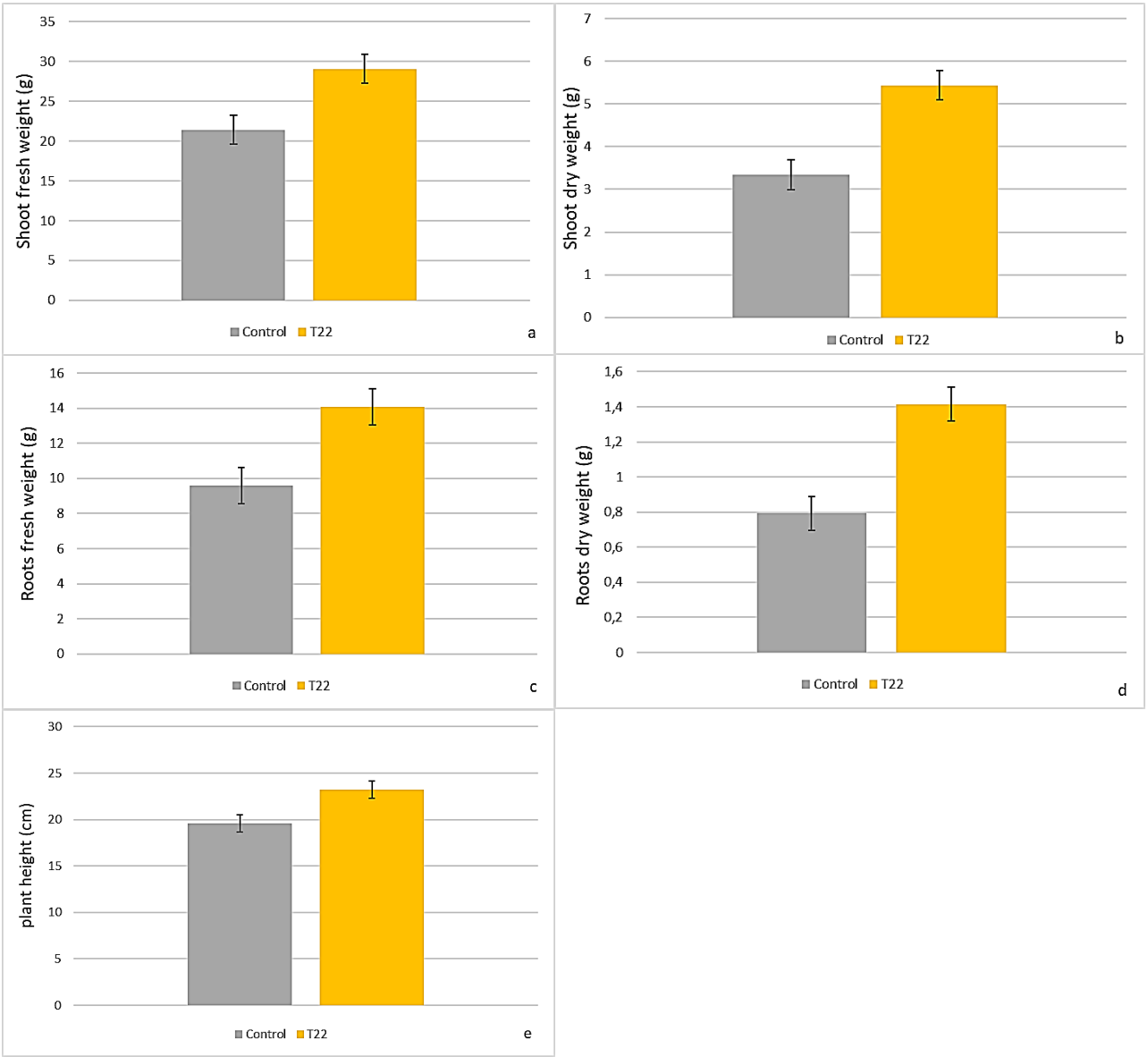

Figure 5. Measurement of plant growth on control and treated tomato plants. The bars show the mean values (± standard errors) for (a), (c) measure of fresh biomass (FW), (b), (d) measure of dry biomass (DW) and, (e) measure of plant height (L). * Significant differences according to t-Student test (for Gaussian variables) ($p<0.05$).

Table 1. Plant growth parameters of tomato plants treated: FW: fresh weight; DW: dry weight; L: length. Values represent means ± SE.

	Control *N* = 14	T-22 *N* = 14	*t*-value	*P-value*
Shoot FW (g)	21.4 ± 1.8	29.1 ± 1.6	t_{26}=2.92	**0.007**
Shoot DW (g)	3.3 ± 0.3	5.4 ± 0.2	t_{26}=2.91	**0.007**
Root FW (g)	9.6 ± 1.02	14.1 ± 1.4	t_{26}=2.62	**0.014**
Root DW (g)	0.79 ± 0.1	1.4 ± 0.2	t_{26}=2.57	**0.016**
Height L (cm)	19.6 ± 0.9	23.2 ± 1.14	t_{26}=2.48	**0.020**

Discussion

Fungi of the genus *Trichoderma* are known as plant growth promoters and as activators of plant defenses against biotic and abiotic stresses. Colonization of the rhizosphere by *Trichoderma* spp. facilitates nutrient uptake and solute transport through extension and expansion of the cell wall, development of secondary roots, production of lateral root hairs, and a higher rate of photosynthesis (Hermosa *et al.*, 2013). The positive effect of *Trichoderma* on plant growth was also very evident and statistically significant in the case of tomato plants used in the present experiments.

Trichoderma fungi, as well as other beneficial microbes in the rhizosphere, are recognized by the plant as invading organisms, resulting in the activation of resistance pathways. Symbiont organisms must actively interfere with the immune signalling network of the plant. For example, they suppress the ethylene signalling, in order to establish intimate reciprocal relationships with host roots (Kazan & Manners, 2012). As a result of the interaction with the symbiont, plants respond faster and/or with greater intensity to a pathogen or pest attack (Conrath, 2011). Thus, the *Trichoderma*-plant interaction modulates the balance between plant growth and defense against pathogens and arthropods.

The ability of fungi of the genus *Trichoderma* to suppress plant pathogens, directly through mycoparasitism and indirectly by inducing plant defense responses, has been extensively studied (Vinale *et al.*, 2008; Hermosa *et al.*, 2013; Silva *et al.*, 2019; Rajani *et al.*, 2021), and several *Trichoderma* spp. are now present in commercial formulations as biofungicides (Kumar & Ashraf, 2017).

Research on the role of *Trichoderma* spp. in modulating plant defenses against insects is a more recent topic and very few scientific studies are available. Among these, several studies have focused on the model system consisting of tomato and the aphid *Macrosiphum euphorbiae* (Battaglia *et al.*, 2013; Coppola *et al.*, 2019b). Coppola *et al.* (2019b) showed that the feeding activity of *M. euphorbiae* induces transcriptome changes in tomato plants, which, in turn, has an effect on aphid survival after the 10th day from the onset of infestation. Treatment with *T. harzianum* T-22 does not anticipate the plant reaction but amplifies the effect, causing higher aphid mortality than in the untreated control. Thus, treatment with *T. harzianum* T-22 increases the resistance level of tomato plants by negatively affecting the survival rate of *M. euphorbiae*. In the case of *Myzus persicae*, root colonization of tomato plants by *T. harzianum* T-22 did not amplify the defensive response of the host plant. *Myzus persicae* colony growth on *T. harzianum*-treated or control tomato plants proceeded identically during the first 5 days after

the onset of infestation. Subsequently, plants colonized by *Trichoderma* harboured a larger population of the aphid and appeared, therefore, more suitable for the development of *M. persicae* colonies. This seems to be confirmed by the result of the choice test, since aphids preferred to move toward plants treated with *Trichoderma,* settling on them to a greater extent. This behavior also seems to be different from the one exhibited by *M. euphorbiae,* which prefers control plants (unpublished data).

The effects of the plant-*Trichoderma* system on aphids was investigated measuring population growth, as previously reported in other scientific papers (Mcvean & Dixon, 2001; Rivelli *et al.*, 2013; Trotta *et al.*, 2021). Unlike when considering the various growth parameters individually on synchronized aphids (mortality, developmental time, and fecundity), aphid population growth over time reflects more closely, what occurs in nature. In nature, aphid colonies consist of mixed ages individuals, and not of age-synchronized ones. Also, the plant changes over time, for example because some metabolic resistance pathways have been activated by the same aphids that are feeding and breeding on the plant. In Coppola (2018) a previous infestation affects the distribution of the newly produced nymphs of *A. gossypii* on the zucchini plants (Coppola *et al*., 2018).

Consequently, each step of this change interferes differently with the survival of different aphid instars and with aphid reproduction. The effects of *Trichoderma* on aphid fitness on treated plant should take these variations into account.

The growth curve of *M. persicae* on tomato appears different from that of *M. euphorbiae*. The growth curves of *M. euphorbiae* colonies have a bell shape, with an initial growth phase, a maximum peak, and a subsequent decreasing phase (Rivelli *et al.*, 2013; Trotta *et al.*, 2021). The amplitude of the curve and the maximum peak vary depending on several factors such as plant age (Trotta *et al.*, 2021), cultivar, and water stress (Rivelli *et al.*, 2013). In the case of *M. persicae*, after an initial growth phase, we observe a tendency of the population to stabilize its abundance, as if the number of dead was compensated by the new-born. This population trend has already been described for *Aphis fabae* Scopoli in bean (Larocca *et al.*, 2011), a small aphid in a plant whose main defense mechanism is related to the presence of hooked hairs. Indeed, flattening of the growth curve of the *M. persicae* population occurs after the fifth day, when the aphids begin to leave the leaf on which they were initially placed. Furthermore, dead aphids were mainly visible along the stem of the plant. It is possible that with each wave of migration within the plant canopy there is a spike in mortality that is subsequently offset by the reproduction of surviving individuals.

The differences between *M. persicae* and *M. euphorbiae* could be partly due to the different body size and to the fact that *M. persicae*, unlike *M. euphorbiae*, does not produce significant direct damage (Perring *et al.*, 2018). The latter attitude could influence the induction of resistance in plants.

The population growth of *M. persicae* on tomato plants colonized by *T. harzianum* has the same trend of the control plants but stabilizes at a higher level. (Battaglia *et al.*, 2013)As in the case of *M. euphorbiae* on tomato plants colonized by *T. longibrachiatum* (Battaglia et al. 2013), the better performance of *M. persicae* on tomato plants colonized by *T. harzianum* could be due to the higher nutritional value of the plant. We could infer that there is a balance between the positive effects, due to the increased acquisition of nutrients by the plant, and thus available for aphid nutrition, and the negative effects due to the induction of resistance. The prevalence of positive or negative effects depends not only on the *Trichoderma* species (e.g. *longibrachiatum* or *harzianum*) but also on the aphid species, perhaps even aphid clone or abiotic conditions.

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CHAPTER III

Effects of *Trichoderma harzianum* Strain T-22 on the Arthropod Community Associated with Tomato Plants and on the Crop Performance in an Experimental Field

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops cultivated in the world, second only to potato, and one of the basic foods in the Mediterranean diet (Siracusa *et al.*, 2018; FAO, 2022). Fresh and processed tomatoes are widely consumed in the Mediterranean area, and Italy is one of the main producers and suppliers of processed tomatoes in the world (Elia & Conversa, 2012; Bettini, 2019; I.Sat, 2022). Agrochemicals such as synthetic fertilizers, pesticides, and herbicides are usually used in the tomato production to maximize yield and product quality and to achieve low production costs (Abdul-Baki *et al.*, 1996; Perring *et al.*, 2018). However, their overuse can cause environmental pollution and human health problems. There is currently a global interest in reducing off-farm input of chemical pesticides and fertilizers by using green alternative practices. Among all alternatives, numerous biological products based on beneficial plant microbes, such as bacteria (*Bacillus, Pseudomonas*) (Ferreira *et al.*, 1991; Walsh *et al.*, 2001) or fungi (*Trichoderma*, *Beauveria*, mycorrhizae) (Lopez & Sword, 2015; Rouphael *et al.*, 2015; Russo *et al.*, 2015; Sinno *et al.*, 2021) are getting a lot of attention in agricultural farming systems due to their valuable properties. These products are used for pest control and for their potential to increase crop health and fitness; they also do not have any negative impacts on the environment and guarantee food security. Fungi belonging to the genus *Trichoderma* have received much attention in recent years due to their beneficial effects on host plants (Harman *et al.*, 2004; Verma *et al.*, 2007; Woo *et al.*, 2014; Alfiky & Weisskopf, 2021; Ferreira & Musumeci, 2021; Poveda, 2021). These fungi are distributed throughout the world and are capable of colonizing plant roots, establishing chemical communication with the host plant (Tucci *et al.*, 2011). Moreover, *Trichoderma* spp. have many possible uses and have been investigated for their direct effects on the host plants, such as the increase of nutrient uptake, of the efficiency of nitrogen use, and of seed germination rate, which offer important economic benefits in agriculture. These fungi also promote plant growth and resistance against biotic and abiotic stresses (Harman *et al.*,

2004; Shoresh *et al.*, 2010; Studholme *et al.*, 2013). It has been found that the plant Systemic Acquired Resistance (SAR) and/or the Induced Systemic Resistance (ISR) against biotic and abiotic stress agents could be activated by some *Trichoderma* strains (Harman *et al.*, 2004; Shoresh *et al.*, 2010; Macías-Rodríguez *et al.*, 2020). Following the *Trichoderma* roots colonization, the plant reacts as when it is attacked by a potential root endophytic pathogen, thus activating local and systemic defense mechanisms. Therefore, the plant limits the fungus penetration inside the root, restoring its integrity and antimicrobial activity to the pre-infection levels (Shoresh *et al.*, 2010; Tucci *et al.*, 2011; Macías-Rodríguez *et al.*, 2020). Once this equilibrium is reached, the plant receives protection and more available nutrients, while the fungus receives organic compounds. In this way, *Trichoderma* activates systemic plant defenses against the attack of pests and/or pathogen.(Shoresh *et al.*, 2010; Tucci *et al.*, 2011; Ponzio *et al.*, 2013; Macías-Rodríguez *et al.*, 2020)(Pieterse *et al.*, 2009; Stam *et al.*, 2014)(Walling, 2000)(Reimer-Michalski & Conrath, 2016; Mauch-Mani *et al.*, 2017; Bürger & Chory, 2019)(Perazzolli *et al.*, 2008; Bari & Jones, 2009; Contreras-Cornejo *et al.*, 2011; Salas-Marina *et al.*, 2011). The feeding activity of phytophagous insects also elicits the release of attractive compounds (Digilio *et al.*, 2012). Volatile organic compound (VOC) blends released in response to pest attack have a direct and indirect defensive effects on insect performance (Volpe *et al.*, 2018). Plant responses to herbivores, induced by the different modes in which these organisms attack the plant, have been shown to be affected by *Trichoderma* colonization (Pieterse *et al.*, 2009; Muvea *et al.*, 2014; Stam *et al.*, 2014; Contreras-Cornejo *et al.*, 2018b). Several studies have been focused on the role of *Trichoderma* spp. (and their metabolites) on multitrophic interactions or on plant growth and defense responses (Mukherjee *et al.*, 2012; Alfiky & Weisskopf, 2021b; Poveda, 2021; TariqJaveed *et al.*, 2021).

Although direct and indirect plant defenses against insect herbivores were demonstrated in different plant species in greenhouses or in pot experiments, little attention has been paid to the use of *Trichoderma* spp. in open field conditions (Poveda, 2021). The interaction between the plant and *Trichoderma* spp. directly confers some degree of protection against nematodes (Martínez-Medina *et al.*, 2017) and against insects such as aphids (Coppola *et al.*, 2019a; b; Di Lelio *et al*., 2021), thrips (Muvea *et al.*, 2014) and caterpillars (Contreras-Cornejo *et al.*, 2018b; Coppola *et al.*, 2019a). For example, the survival of the aphid *Macrosiphum euphorbiae* in tomato plants could be significantly reduced using the P1 strain of *T. atroviride*, as a consequence of up-regulation of genes involved in the oxidative burst reaction (Coppola *et al.*, 2019a). Similar results have been obtained when *T. harzianum* strain T-22 was used in the same context (Coppola *et al.*, 2019b). The tomato defense responses against the green stink bug

Nezara viridula Linnaeus have been enhanced by the *T. harzianum* strain T-22 through an early increase in transcript levels of JA marker genes (Alınç *et al.*, 2021). In onions, the performance of *Thrips tabaci* L. has been reduced after colonization by *Trichoderma* spp. (Muvea *et al.*, 2014). Among the chewing insects, *T. atroviride* P1 strain has been associated with a reduced survival and development of *Spodoptera littoralis* larvae and with an enhanced expression of genes encoding for protective enzymes in tomato plants (Coppola *et al.*, 2019a). Maize inoculation with *T. atroviride* increased plant growth, altered the feeding pattern of *Spodoptera frugiperda* (JE Smith) larvae, and has been correlated with an increased emission of volatile terpenes and accumulation of JA (Contreras-Cornejo *et al.*, 2018b). In an in vitro assay, the secondary metabolite 6-pentyl-α-pyrone produced by the bioactivity of *T. asperellum* caused a high mortality rate in the two-spotted spider mite *Tetranychus urticae* Koch (Sholla & Kottb, 2017).

Trichoderma spp. also affect above-ground plant-insect interactions, reinforcing indirect plant defense barriers against phytophages through the production and release of VOCs that are involved in the attraction of predators and parasitoids (Macías-Rodríguez *et al.*, 2020; Poveda, 2021). For example, *T. longibrachiatum* MK1 influenced the quantity and quality of VOCs released by the tomato plant (such as methyl salicylate), improving the attractiveness and performance of the aphid parasitoid *Aphidius ervi* and of the predator *Macrolophus pygmaeus* (Battaglia *et al.*, 2013). In tomato, colonization by *T. atroviride* P1 significantly increased the attraction of *A. ervi* (Coppola *et al.*, 2019a). In a multitrophic interaction system, *Trichoderma atroviride* IMI 206040 associated with maize roots has been shown to increase the parasitism rate of *Campoletis sonorensis* (Carlson) on *S. frugiperda* (Contreras-Cornejo *et al.*, 2018a). As suggested by Battaglia *et al.* (Battaglia *et al.*, 2013), the improved attractiveness and performance of insect predators on *Trichoderma* colonized plants could be considered as a result of the "increased fitness flow" modulated through the interaction between the primary and secondary metabolism of plants (Neilson *et al.*, 2013).

However, below and above ground plant–insect–microorganism interactions are very complex and may be very different under field conditions. Contreras-Cornejo *et al.* (Contreras-Cornejo *et al.*, 2020) showed that, in a maize field, the community of native foliage arthropods could be altered after plants inoculation with *T. harzianum* strain 38. The authors found that the number of arthropods per plant did not differ between the inoculated and control plants. Nevertheless, *T. harzianum* inoculation decreased the number of piercing-sucking insects but increased the abundance of chewing herbivores and of predators. The presence of *Trichoderma* has also been

shown to influence JA-mediated VOCs production in a vineyard, attracting parasitoid wasps of the Mymaridae family (Parrilli *et al.*, 2019).

Trichoderma harzianum can also modulate soil arthropod biodiversity. For example, a higher abundance of collembolans has been found under optimal conditions but not under suboptimal or adverse ones (Sotto-Alviola *et al.*, 2017).

In many cases, the outcomes of plants-*Trichoderma* interactions are species-specific and even strain-specific (Copetta *et al.*, 2006; Tucci *et al.*, 2011; Kovach-Orr & Fussmann, 2013; Soler *et al.*, 2013; Bazghaleh *et al.*, 2020). *Trichoderma*-plant interactions with pests and their natural enemies are influenced by environmental conditions when experiments are performed in the field. Recently, it has been demonstrated that the tomato defense response against insect pests induced by diverse *Trichoderma* species is influenced by temperatures (Di Lelio *et al.*, 2021). Therefore, the use of *Trichoderma* as a biocontrol agent in agriculture depends not only on the targeted use (pests and pathogens), but also on local climatic conditions, on soil properties, on the availability of water and nutrients and on crop species.

The aim of the present study is to investigate the effects of the inoculation of a commercial *Trichoderma harzianum* strain T-22 on the arthropod community associated with tomato plants in an experimental field located in South Italy. The beneficial effects of *T. harzianum* on soil arthropod biodiversity, as well as on the agronomic performance of tomato plants (improved yield) and on the behaviuor toward downy mildew (one of the main phytopathogens of tomato in South Italy) have also been investigated. The complex tomato–arthropod–microorganism interactions that occur in the field are also discussed to enrich our current information on the possibilities of using *Trichoderma* as a green alternative agent in agriculture.

2. Materials and Methods

2.1. Crop Cultivation

The present study was performed in an experimental tomato field located in Pignola (40°34'06.2"N, 15°45'35.4"E; 780 m above sea level), Potenza, Italy during the 2021 tomato growing season. According to the world reference base for soil resources (FAO, 2022b), the soil was a dystric cambisols (Bd68-2bc), with the following characteristics: particles smaller than 2 mm in size, 935 g / Kg; particles larger than 2 mm, 65 g / Kg; apparent density, 1.294 Kg / dm^3; texture composition of sand, 481 g / Kg; clay, 149 g / Kg; silt, 370 g / kg at depth of 0-30 cm. The content of total carbonate and total organic matter was of 16 g / Kg and 32.8 g /

Kg, respectively. The composition of the soil was as follows: total N, 2 g / Kg; P, 29 mg / Kg; Ca 11.1 meq / 100 g; Mg, 4.6 meq / 100 g; Na, 1.8 meq / 100 g; soil pH (H_2O), 6.2.

The soil was left fallow the year before the experiment and then plowed to a depth of 30 cm, rotavated and levelled before planting the crop. Tomato seedlings (*Solanum lycopersicum* L.) of the commercial cultivar San Marzano Kero were used in this experiment. Tomato plants placed in alveolate containers were purchased from a nursery and were transplanted into the field on 1 June 2021. Fertigation was carried out with ammonium nitrate (YaraTera© AMNITRA™, N 34.2%) applied three times in the recovery and blossoming stage (20 kg ha^{-1} at an interval of one week from each other), calcium nitrate (YaraTera© CACINIT™, CaO 26.5%, N 15.5%) applied twice during the fruit bearing stage (30 kg ha^{-1} at an interval of one week from each other) and potassium nitrate (YaraTera© KRISTA K™ plus, N 13.7%, K_2O 46.3%) applied once (20 kg ha^{-1}) at the fruit enlargement stage. The use of fertilizers was developed during the present experiment and was appropriate to the needs of the plants, considering the soil characteristics. The tomato plants were not treated with pesticides during the entire field trial.

2.2. Meteorological Data

The Agrometeorological Service of the Agenzia Lucana per lo Sviluppo e l'Innovazione in Agricoltura (ALSIA) of the Basilicata Region provided the meteorological data for the area in which the experimental farm is located. The temperature and rainfall data recorded during the experiment are shown in Figure 1. During the period of interest, the average temperature remained less than or equal to 20°C. The temperatures reached a maximum of 30°C in August. Precipitations recorded in July, August, and September were low.

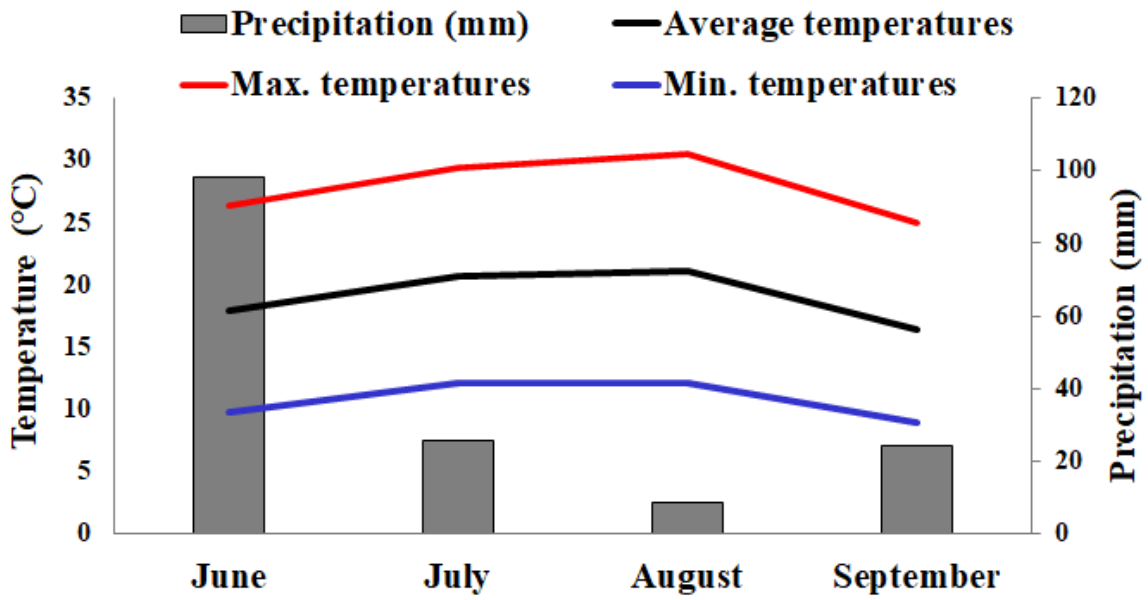

Figure 1. Meteorological data for Pignola, Potenza, Italy registered from June to September 2021. Data were provided by the Agrometeorological Service of the Basilicata Region, Italy. June was the month when *T. harzianum* T-22 inoculation and transplantation of tomato seedlings were carried out; July: vegetative growth and flowering stages; August: stages of establishment and development of fruit; September: fruit maturity stage and harvesting of tomato fruits.

2.3. Experimental Design

Two treatments were compared: non-inoculated tomato plants (control) and inoculated tomato plants with *Trichoderma harzianum* strain Rifai KRL-AG2 biocontrol agent (T-22) (KOPPERT B.V., Berkel en Rodenrijs, The Netherlands). Forty-days old tomato plants were inoculated with *T. harzianum* following the manufacturer's instructions ($1x10^9$ cfu/g of viable *T. harzianum* T-22 spores) one week before transplantation. The alveolate containers with 320 seedlings were then watered with 3 grams of commercial *T. harzianum* dissolved in 3 liter of water. Treatment was repeated after three days and then the seedlings were transplanted. The control plants were not inoculated.

The experiment was carried out on a strip of soil of about 58 meters long and 9 meters wide, divided into 6 plots of 54 m^2 (6 × 9 m) separated from each other by a strip 3.6 meters wide left without plants. Thus, 3 plots treated with *T. harzianum* T-22 and 3 control plots were obtained, alternating along the length of the field. A 10-meter strip around the field was plowed and then left uncultivated throughout the entire duration of the experiment. The plants in each plot were

manually transplanted on 1 June 2021 in 5 rows 1.8 meters apart from each other. Each row was 6 meters long with a plant spacing of 33 cm, with a total of 20 plants/row (100 plants/plot). Six root samples of control or *T. harzianum* T-22 inoculated plants (two per plot) were taken at the end of the experiment to determine the presence of the fungus. The tomato roots were dissected, mounted on a slide, and then observed with a stereomicroscope. The presence of the fungus was confirmed by the observation of hyphae in the secondary roots.

2.4. Insect Sampling

During the first month after transplantation, the tomato seedlings are very small and in a critical phase for their vegetative growth. However, seedlings are susceptible to attack by insect pests such as aphids and beetles. For this reason, the presence/absence of phytophages was recorded by direct observation of the seedlings, without damaging them, on two dates (22 June and 1 July). Ten plants were randomly sampled within each plot, with a total of 30 plants per treatment/date. The presence/absence of insect pests on the plant was recorded; some of the observed specimens were gently removed, placed in an Eppendorf tube filled with ethanol, and transported to the laboratory for identification. On 10 June, yellow sticky traps were randomly placed between two adjacent seedlings in a row (5 traps/plot) (Figure 2). After 20 days (1 July), the traps were collected and wrapped in a transparent PVC plastic film. Captured tomato pests were subsequently counted and identified using a stereomicroscope in the laboratory.

Figure 2. Yellow sticky traps in a row

After the first month, as the plants were larger in size, leaf samples were collected for observation in the laboratory under a stereoscopic microscope. This allowed a more accurate sampling of small arthropods, thus obtaining quantitative data on their abundance. Six different samplings were carried out, from 12 July to 21 September (more specifically, 12 and 22 July, 3 and 26 August, 8 and 21 September). Within each plot, ten plants were randomly sampled at 9.00 am, for a total of 30 plants/date. For each plant, a composite sample was used, consisting of three leaves taken from the apical, middle, and basal part of the tomato plant. The leaves were detached and placed in plastic cylinders (150 ml). The cylinders were then maintained in darkness at 5°C and transported to the laboratory for the identification of the arthropods. The arthropods were transferred to 50 ml sterile Falcon tubes, which were filled with ~ 30 ml of 70% ethanol in water and refrigerated at 4°C until identification. Individuals were then observed under a stereomicroscope. Arthropods from each sample were classified at order, family, and, when possible, at the genus and species level. Furthermore, the presence of damage caused by leaf miners on the leaves were noted and analysed

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2.5. Soil Sampling and Microarthropod Extraction

On the 21 September, within each experimental plot, three soil samples were randomly collected between two tomato plants with and without *T. harzianum* T-22. A sample was composed of three soil clods (10 x 10 x 15 cm depth), which were taken using a hand auger in three different rows. After collection, soil samples were placed in a plastic bag, kept in darkness at 5°C and transported to the laboratory for arthropods extraction. Microarthropod extraction was carried out by gently placing soil clods on mesh-covered funnels (mesh 2mm, 20 cm in diameter). A plastic jar containing 50 ml of hydroalcoholic solution (70%) was placed at the bottom of the funnel to store the extracted arthropods. Incandescent lamps (40 watts) were placed 20 cm above the soil clods (Figure 3). After 14 days, the extracted specimens were observed under a stereomicroscope, the biological morphs were determined, and the Ecological-Morphological Index (EMI) was assigned. Finally, the QBSar (Soil Biological Quality-arthropod) index has been computed as the sum of the EMI values (Parisi *et al.*, 2005; Menta *et al.*, 2018).

Figure 3. Microarthropod Extraction

2.6. Evaluation of Downy Mildew on Tomato Plants

We focused only on the presence of downy mildew since it is one of the diseases that can cause major damage to tomato in the considered area. All plants were screened for the presence/absence of downy mildew at the beginning of August. For this purpose, each tomato plant was visually inspected, and the presence of the above-mentioned disease was recorded if at least its initial symptoms were present, such as small pale-yellow spots with indefinite borders on the upper leaf surface. At an advanced stage of downy mildew development, other parts of the plant such as stems, flowers and fruits are also attacked and thus the damage could be very high. The evolution of the downy mildew disease during the entire period of tomato cultivation was also recorded.

2.7. Agronomic Performance Estimation

Fruit sampling was performed during the experiment. Within each experimental plot, ten plants were marked and followed throughout their development. Ripe tomato fruits were manually collected from the same plants on three different dates (30 August, 8 and 21 September). All red tomatoes on a plant were harvested, counted, and divided into marketable and non-marketable fruits. Marketable tomatoes were counted, weighed, and measured (maximum

length and width), while unmarketable fruits were divided into two groups, rotten and insect-damaged fruits, and finally counted.

2.8. Statistical Analysis

Row data used in the analyses of the presence/absence of phytophages on tomato seedlings are the number of plants/experimental plot colonized by insects. Since these data have a discrete probability distribution, a binomial Generalized Linear Model (GLM) with a logit link function has been considered as the best model for these analyses, thus avoiding transforming the data. For each of the insect species identified, the *P*-values for differences between treatments, sampling dates as well as their interactions were obtained through analyses of deviance (Type III chi-squared tests). The following model was applied:

$$Y = \mu + Treatment + Date + Treatment \times Date + \varepsilon$$

where Y is the binomial trait studied (number of plants with the presence/absence of a phytophagous), Treatment (two levels: *T. harzianum* and control) and Date (two levels: 22 June and 1 July) are the fixed effects.

Insect abundances on yellow sticky traps were analyzed by nested analysis of variance (ANOVA), since the homoscedasticity and normality assumptions for ANOVA were checked and met on these data. The following model was applied:

$$Y = \mu + Treatment + Plot\{Treatment\} + \varepsilon$$

where *Y* is the abundance of a phytophagous, Treatment (two levels: *T. harzianum* and control) is the main effect and the Plot is nested within the Treatment (three levels/treatment).

Row data used in the analyses of the the arthropod community on the tomato leaves are the number of insects per plant sampled over time. To test whether *T. harzianum* modulated the arthropod community on tomato leaves, a Poisson generalized linear mixed model (GLMM) with a log-link function fitted with ML (maximum likelihood) and Laplace approximation was used. The Poisson distribution best approximates the process that generated the observed data, since it is a discrete distribution that measures the probability that a given number of events occur in a specified period. *P*-values for differences between the treatments, sampling dates, and their interactions were obtained through analyses of deviance (Type III Wald chi-square tests). The following general model was applied:

$$Y = \mu + Treatment + Date + Treatment \times Date + Plot\{Treatment\{Date\}\} + \varepsilon$$

where *Y* is the studied group of arthropods with a Poisson distribution, Treatment and Date (six levels: 12 and 22 July, 3 and 26 August, 8 and 21 September) are the fixed factors and Plot is the random effect consisting of the three experimental plots nested in Treatment and Date. This model accounts for the non-independence of the data (pseudoreplication of measures) due to the different experimental plots (the random effect) that are part of the present design.

Data on the mean number of tomato fruits (marketable, rotten, and insect-damaged fruits) were also analyzed by Poisson GLMMs with "treatment" and "date" as fixed effects and "plot" as a random effect nested in Treatment and Date. The data on fruit weight, length, and width were analyzed by linear mixed-effects models (LMMs) fitted with REML (restricted maximum likelihood). The homoscedasticity and normality assumptions for ANOVAs were checked and met on these data. *P*-values for differences between treatments, sampling dates, and their interactions were obtained through ANOVAs (Type III Wald F tests, using the Kenward-Roger approximation for the degrees of freedom (Kenward & Roger, 1997)). The general model applied for the analysis of the arthropod community was also applied to the analysis of tomato fruits measures, but in these analyses the factor "Date" consists of three levels (30 August, 8 and 21 September).

For all the analyses described so far, the model distributions were also chosen as the best fitting, based on AIC criteria (Burnham & Anderson, 2004) and the full models were presented.

Additionally, a two-way permutation multivariate analysis of variance (PERMANOVA) was also presented as a supplemental analysis to test for differences between treatments, sampling dates or in their interaction. The PERMANOVA (based on 9999 permutations) was performed on arthropods grouped according to their feeding behavior using the software PAST version 4.0 (Hammer *et al.*, 2001).

Differences between treatments for the QBSar index were analyzed using a two-sample *t-test*.

Differences between treatments for the presence of *Peronospora* spp. on the tomato leaves were analyzed using a Pearson's chi-square test for Independence.

All statistical analyses (except the PERMANOVA) were performed in R version 4.1.2 "Bird Hippie" (R Core Team, 2021), with lme4 (Bates *et al.*, 2015), lmerTest (Kuznetsova *et al.*, 2017) packages.

3. Results

In this study, only the roots of the inoculated plants showed the presence of *Trichoderma*, while in the control plants the presence of hyphae was not observed. Furthermore, the phenotypic fruit response to inoculation is a proof for fungal establishment, as well as the differences in *Peronospora* spp. and in the associated community of arthropods that were collected at the same time from each treatment.

3.1. First Month After Transplantation: Seedling Growth Phase

During the first month after transplanting, the presence/absence of phytophages was investigated by a direct observation of the seedlings. Since the sampling method was not destructive and the plants were very small, an accurate quantitative measurement of the number of insects was not possible. At this time, we mainly found winged morphs of aphids and a several specimens of a beetle identified as *Chaetocnema tibialis* Illiger (Coleoptera: Chrysomelidae). The aphid species were identified as *Macrosiphum euphorbiae, Aphis craccivora* Koch, and *Aphis gossypii* Glover (Hom., Aphididae). No aphid colonies were detected at this stage. Since *A. gossypii* was only observed in two plants during the first sampling date, it was excluded from subsequent analyses. The percentage of tomato seedlings with *M. euphorbiae, A. craccivora* and *C. tibialis* sampled on 22 June and on 1 July (that is, during the vegetative growth stage) is reported in Figure 4.

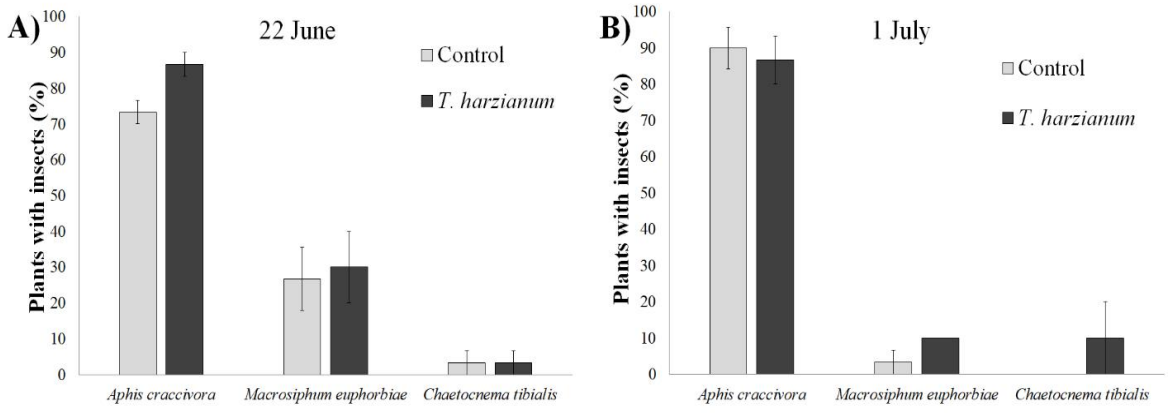

Figure 4. Presence of phytophages on tomato seedlings. Mean values (± standard errors) of the percentage of tomato seedlings showing the presence of phytophagous insects, recorded on 22 June (A) and on 1 July (B).

The binomial GLMs performed on the presence/absence of *A. craccivora*, *M. euphorbiae* and *C. tibialis* showed no significant differences between treatments (*T. harzianum vs* control), between the two sampling periods (except for *M. euphorbiae*) nor in their interaction. The number of tomato seedlings with *M. euphorbiae* was higher on the 22 June than on 1 July (χ^2 = 7.16, df = 1, $P < 0.001$).

Chaetocnema tibialis, *M. euphorbiae* and *A. craccivora* were also captured by the yellow sticky traps. From 10 June to 1 July, a total of 114 individuals of *C. tibialis*, 266 of *A. craccivora*, and 14 of *M. euphorbiae* were collected in yellow sticky traps. Because of the low number of *M. euphorbiae* specimens captured, this species was excluded from the statistical analysis. The mean numbers of *C. tibialis* and *A. craccivora* caught per trap/treatment are shown in Figure 5.

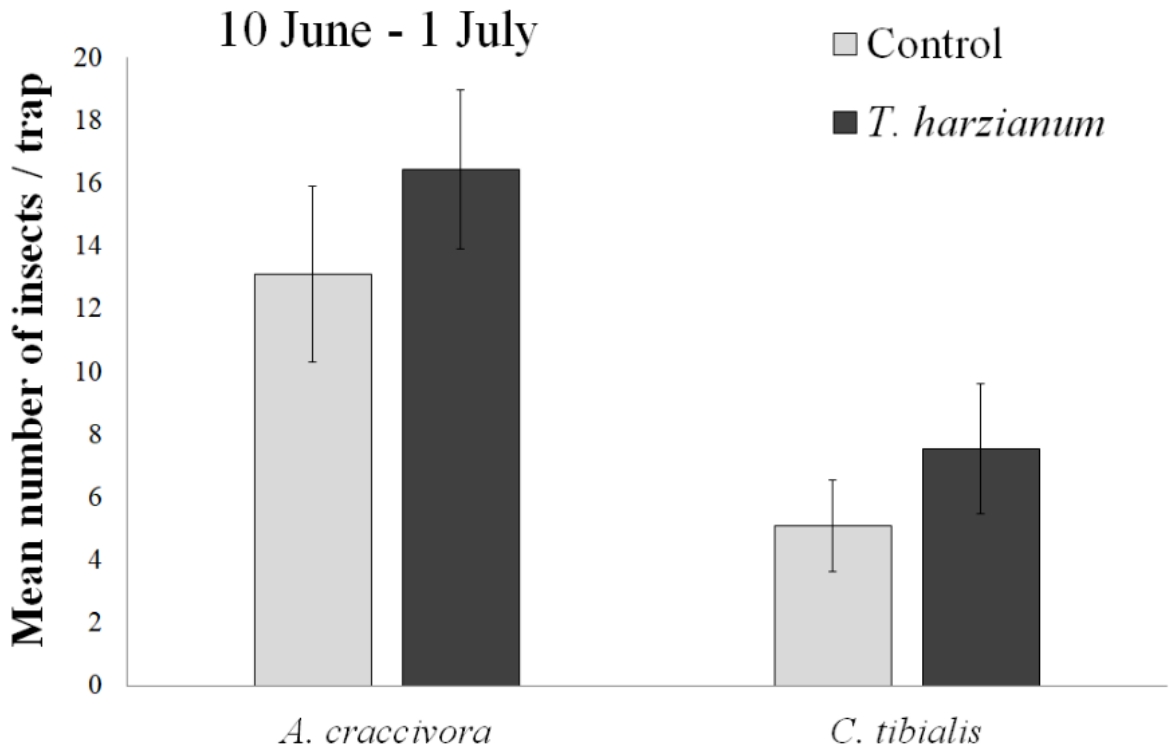

Figure 5. Presence of phytophages on yellow sticky traps. Mean values (± standard errors) of *A. craccivora* and *C. tibialis* individuals collected per trap for each treatment from 10 June to 1July.

For both *A. craccivora* and *C. tibialis*, no significant differences were found between treatments ($F_{1,12}$ = 0.82, P= 0.38 and $F_{1,12}$ = 0.9, P= 0.36, respectively) nor among experimental plots within

treatment ($F_{4,12}$ = 1.17, P= 0.36 and $F_{4,12}$ = 0.87, P= 0.50, respectively). Even if not significant, the yellow sticky traps placed in the *Trichoderma* plots captured more *A. craccivora* and *C. tibialis* than the traps placed in the control plots.

3.2. Second-Fourth Month After Transplantation: Vegetative Growth, Flowering, Fruit Set, And Fruit Ripening

At this stage, since the tomato plants were large enough to tolerate the shedding of a few leaves, leaf samples were collected for observation in the laboratory (see Materials and Methods). During this sampling period, 2473 arthropod specimens were collected, of which 1108 and 1365 were obtained from plants with and without *T. harzianum* T-22, respectively. The collected arthropods were grouped according to the following categories: natural enemies, piercing-sucking insects, chewing insects and mites. We identified four families of piercing-sucking insects (Figure S1), two families of chewing insects (Figure S2), and four families of natural enemies of herbivores (predators and parasitoids, Figure S3). Among the phytophagous mites, only the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) was recorded. The PERMANOVA indicated that the abundance of these arthropod groups was affected by the sampling date ($F_{5,348}$ = 55.2, $P < 0.001$) but not by the treatment ($F_{1,348}$ = 2.01, $P = 0.14$). More interestingly, the interaction "treatment X date" was found significant ($F_{5,348}$ = 2.7, $P < 0.03$), indicating that the arthropod community was differently affected by inoculation with *T. harzianum* T-22 in relation to the sampling period.

3.2.1. Piercing-Sucking Herbivores

The piercing-sucking insects collected on tomato leaves belonged to the families Aphididae (two species identified: winged morph of A. *craccivora* and apterous morph of *M. euphorbiae*), Cicadellidae, Thripidae, and Pentatomidae (identified as eggs). Insect abundance in the families of the piercing-sucking groups shown in Figure 6. Abundance of insects in the piercing-sucking group is shown in Figure 7. The GLMM shows that the abundance of the piercing-sucking community was affected by the sampling dates (χ^2 = 48.1, df = 5, $P < 0.001$) but not by the inoculation of *T. harzianum* T-22, although the probability value was close to be statistically significant (χ^2 = 3.48, df = 1, $P = 0.06$), nor by the interaction "treatment X date" (χ^2 = 6.69, df = 5, $P = 0.25$). It is interesting to note that during the first sampling date (12 July – flowering stage), a general increase in the abundance of piercing sucking insects was observed on control

tomato plants. During subsequent sampling dates, the abundance of piercing-sucking arthropods was extremely low.

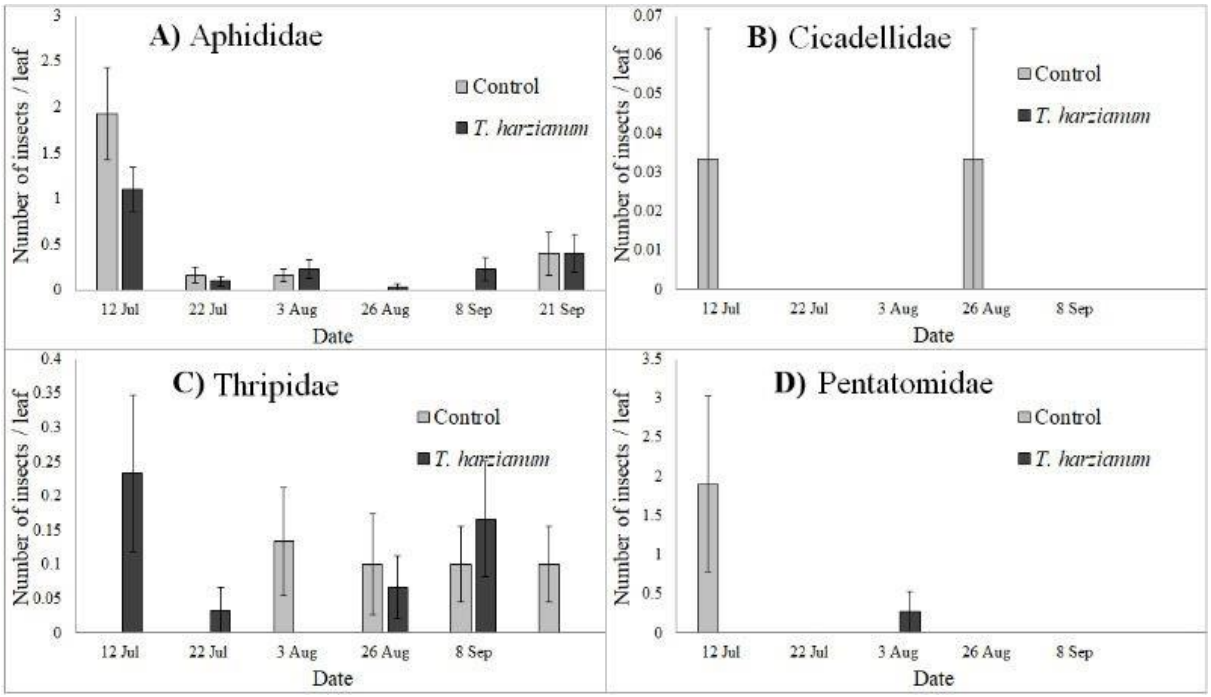

Figure 6. Families of piercing-sucking insects. Mean values (± standard errors) of insect abundance in the families of the piercing-sucking group on tomato leaves over time. At the family level, no significant differences in insect abundance were observed between treatments.

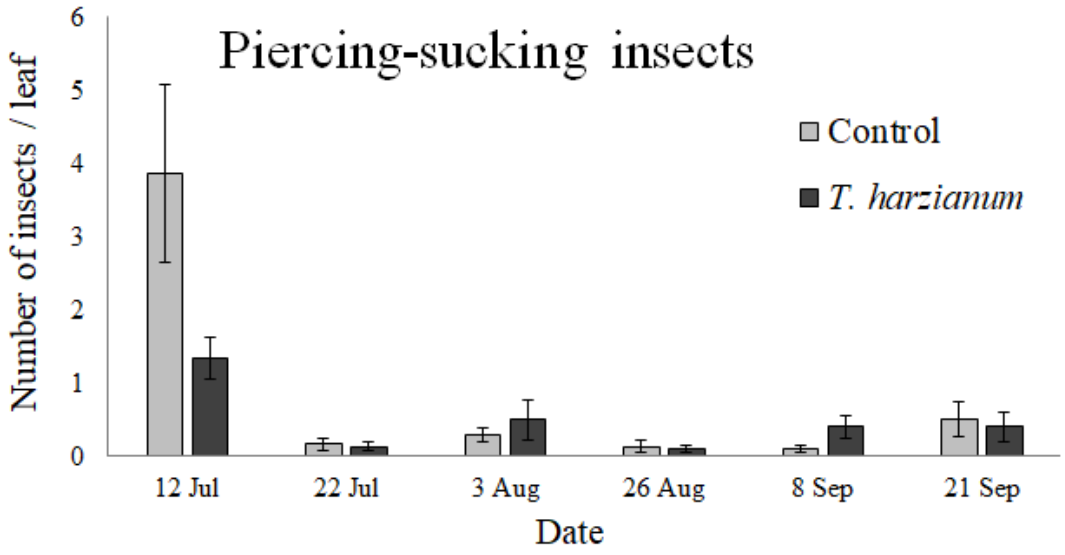

Figure 7. Mean values (± standard errors) of piercing-sucking insects collected on tomato leaves during the experiment.

3.2.2. Chewing Insects

The chewing insects collected on tomato leaves belonged to the families of Noctuidae (identified as eggs and larvae) and Chrysomelidae (one species: *Chaetocnema tibialis*). Insect abundance in the families of the chewing group is shown in Figure 8. The abundance of insects in the chewing group over time is shown in Figure 9.

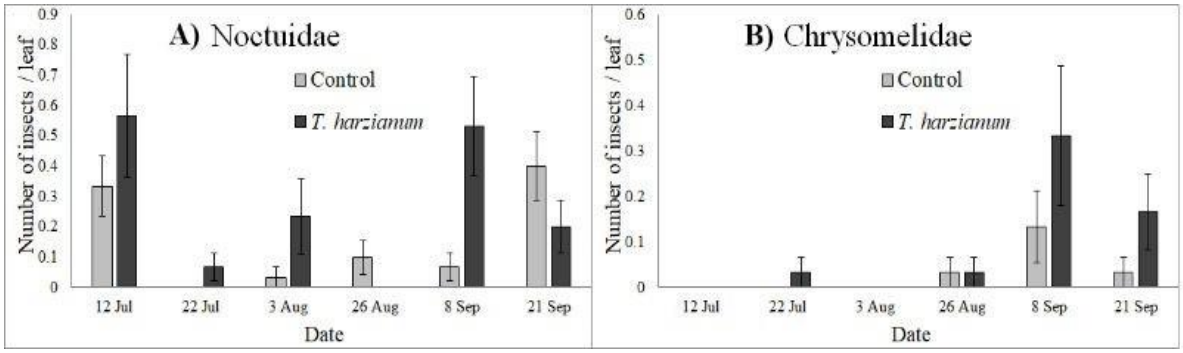

Figure 8. Families of chewing insects. Mean values (± standard errors) of insect abundance in the families of the chewing group on tomato leaves over time. At the family level, no significant differences in insect abundance were observed between treatments.

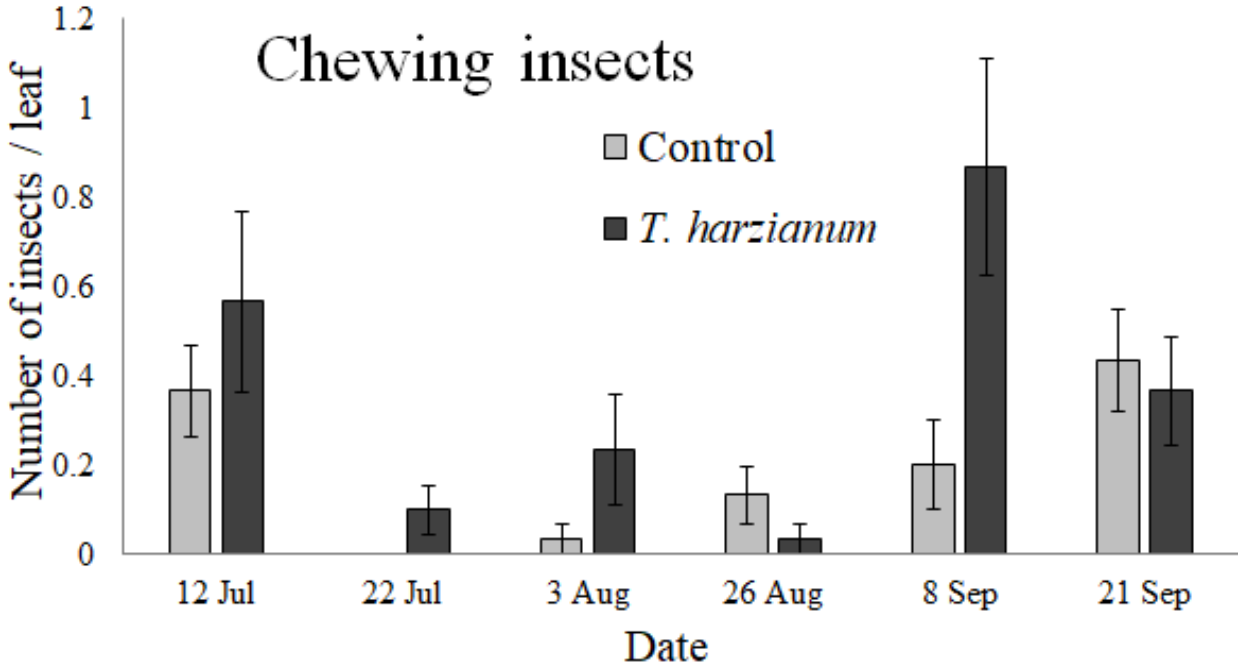

Figure 9. Mean values (± standard errors) of insect abundance in the chewing group collected on tomato leaves during the experiment.

GLMM performed on the whole chewing insect community indicated that its abundance was not affected by the inoculation of *T. harzianum* T-22 (χ^2 = 0.75, df = 1, *P* = 0.38) and by the sampling dates (χ^2 = 9.1, df = 5, *P* = 0.1). Although the probability value was close to the threshold, the interaction "treatment x date" was not significant (χ^2 = 9.8, df = 5, *P* = 0.08). In general, the abundance of chewing arthropods increased (even if not statistically significant) in plants inoculated with *T. harzianum* T-22 compared with control ones (this trend was particularly evident on 8 September).

3.2.3. Natural Enemies of Insects

The natural enemies collected on tomato leaves belonged to the families of Syrphidae (identified as eggs or larvae), Braconidae (identified as mummies), Trichogrammatidae (adults), and Miridae (adults). Insect abundance in the families of natural enemies is shown in Figure 10. We also identified 2 individuals belonging to the order of Araneae and 1 mite belonging to the family of Phytoseiidae. Abundances of natural enemies of herbivores over time are shown in Figure 11.

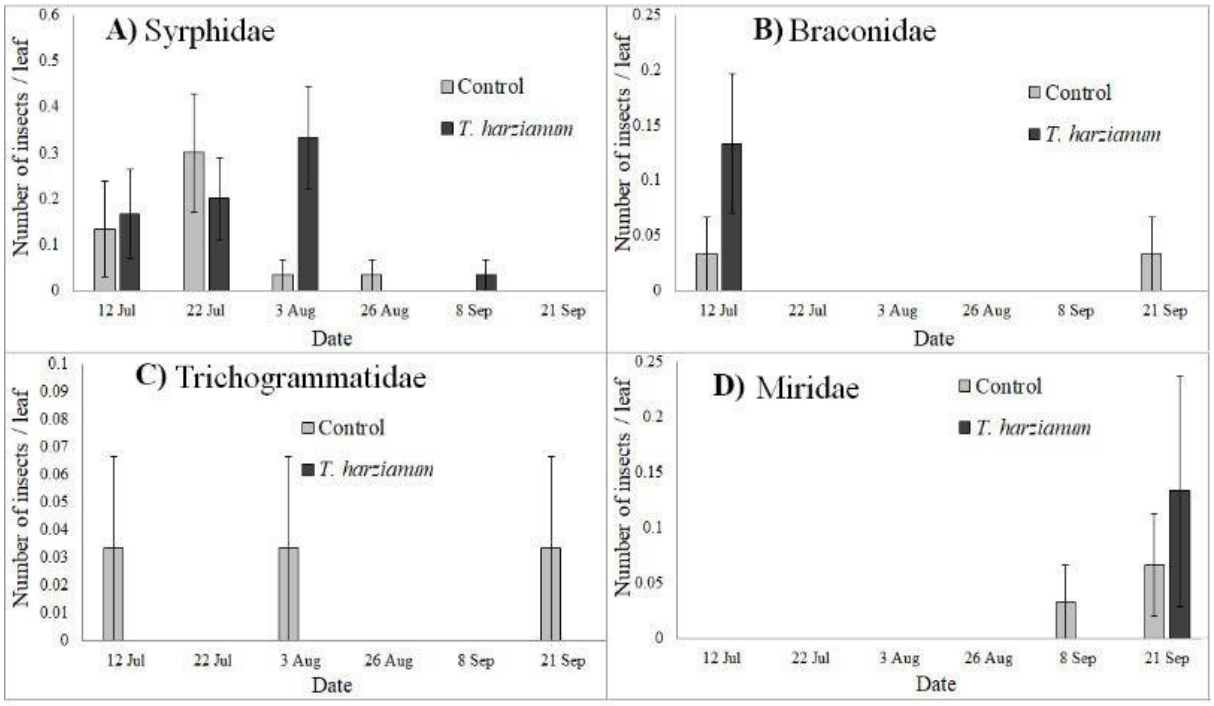

Figure 10. Families of natural enemies of insect. Mean values (± standard errors) of insect abundance in the families of natural enemies in tomato leaves over time. At the family level, no significant differences in insect abundance between treatments were observed.

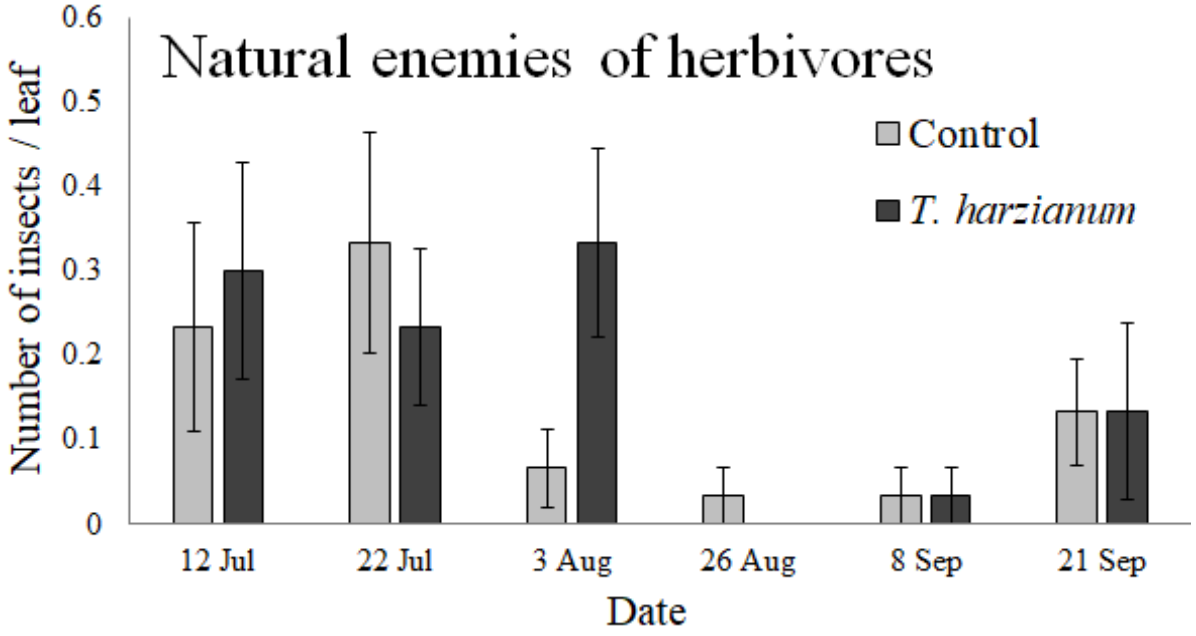

Figure 11. Mean values (± standard errors) of natural enemies of herbivores (predators and parasitoids) collected on tomato leaves during the experiment.

The GLMM performed on the entire community of natural enemies indicated that its abundance was affected by sampling dates (χ^2 = 12.9, df = 5, *P* = 0.03), but not by *T. harzianum* (χ^2 = 0.25, df = 1, *P* = 0.62), or by the interaction "treatment X date" (χ^2 = 4.7, df = 5, *P* = 0.45). In general, the abundance of predators and parasitoids was higher in July and then decreased in the following months.

3.2.4. Spider Mites

Tetranychus urticae was detected on tomato leaves only from 26 August to 21 September (Figure 12).

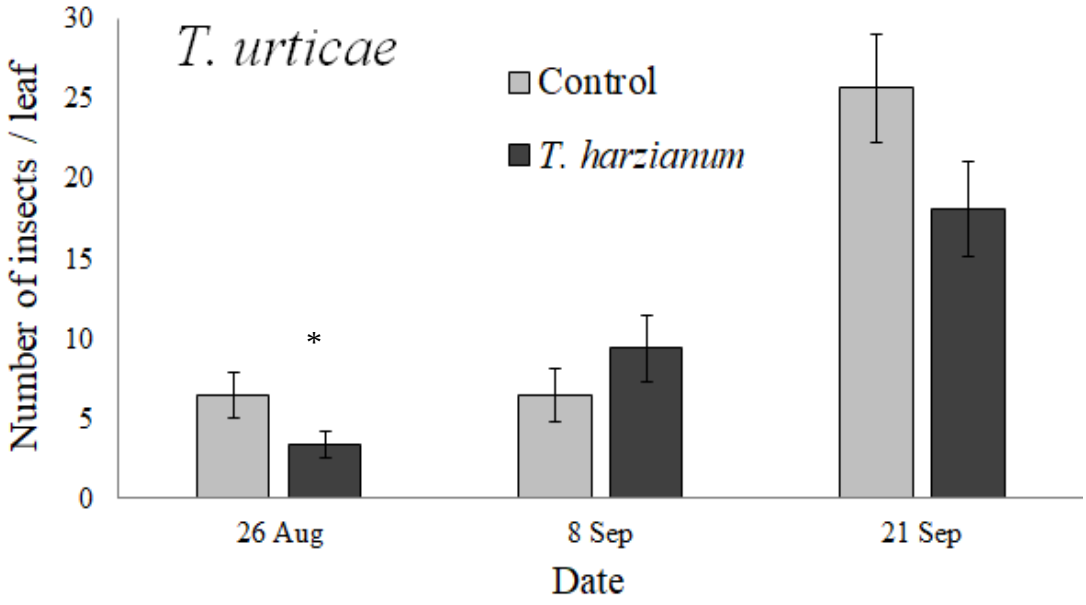

Figure 12. Mean values (± standard errors) of the abundance of *Tetranychus urticae* collected on tomato leaves during the experiment.

GLMM performed on *T. urticae* indicated that its abundance was affected by inoculation with *T. harzianum* T-22 (χ^2 = 6.9, df = 1, $P < 0.01$) and by date (χ^2 = 39.9, df = 2, $P < 0.001$); also, the interaction "treatment x date" was significant (χ^2 = 9.6, df = 2, $P < 0.01$). For this analysis, the 'date' factor consists of only three levels (fruit development and fruit ripening stages: 26 and 8 August, 21 September).

3.2.5. Leaf Miners

The mean number of leaf mines per plant observed in the tomato experimental field during the sampling period is reported in Figure 13. Both mines of *Tuta absoluta* (Meyrick) (Lepidoptera, Gelechiidae) and *Liriomyza trifolii* Burges (Diptera, Agromyzidae) were found.

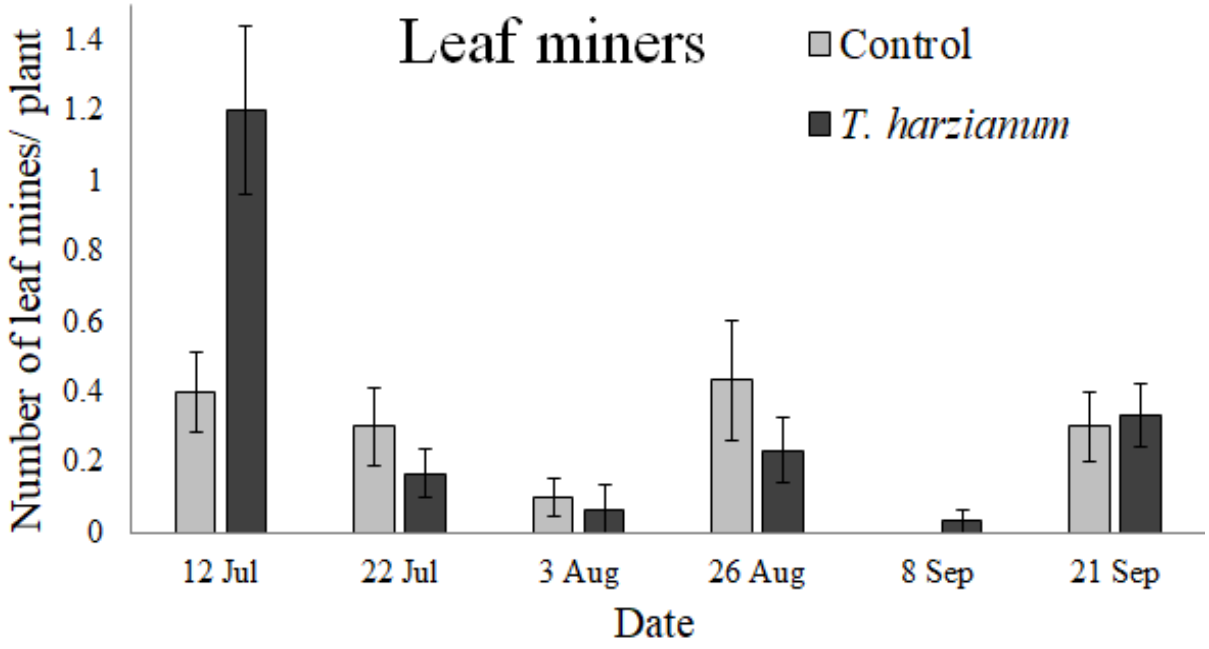

Figure 131. Mean values (± standard errors) of insects that cause leaf mine damage per plant associated with the tomato leaves over time.

The GLMM performed on leaf miners indicated that their abundance was affected by *T. harzianum* T-22 (χ^2 = 8.5, df = 1, $P < 0.01$). The sampling dates and the interaction "treatment x date" were not significant (χ^2 = 5.3, df = 5, P = 0.38 and χ^2 = 10.5, df = 5, P = 0.06, respectively). The leaf miners were more numerous on the inoculated tomato plants, especially during the first sampling date, then their number decreased.

3.3. QBSar

The soil samples contained several microarthropods belonging to the orders of Collembola, Isopoda, Protura, Diplura, and class of Arachnida. The sporadic presence of holometabolous insect larvae was also recorded. The differences in QBSar indexes calculated for the soil from control and *Trichoderma* T-22 treated plots were not statistically significant (82 ± 20 and 62 ± 5, respectively; t_4= 1.9, P = 0.13).

3.4. Crop Sampling

3.4.1. Number of Fruits per Plant

Figure 14 shows the mean number of fruits harvested per plant for the two experimental treatments during the three sampling dates. Immediately after harvesting, the tomatoes were divided into three categories: marketable, rotten, and insect-damaged fruits.

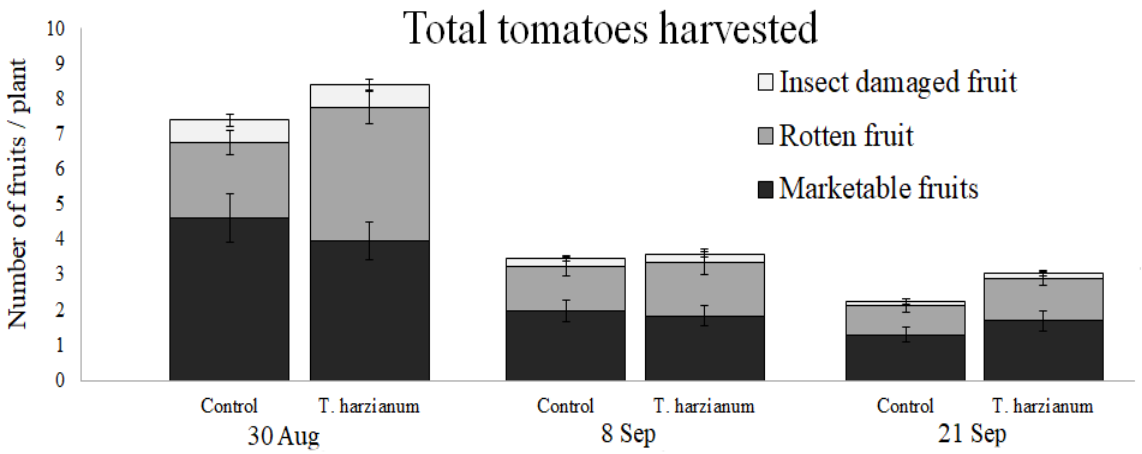

Figure 14. Mean number of tomato fruits (± standard errors) harvested per plant in the two experimental treatments during the three sampling dates.

The GLMM indicated that the numbers of marketable, rotten, and insect-damaged tomato fruits were affected by the sampling dates ($\chi^2 = 29.3$, $\chi^2 = 11.8$ and $\chi^2 = 17.6$ respectively; df = 2, $P < 0.01$ in all cases). The interaction "treatment x date" was never found significant. Differences between treatments were found only for rotten tomato fruits ($\chi^2 = 13.7$, df = 1, $P < 0.001$). In general, the number of rotten fruits was a little higher in the of *T. harzianum* T-22 inoculated plants, especially during the first sampling date.

3.4.2. Weight, Length, and Width of Marketable Tomato Fruits

Figure 15 shows the mean values of the weight, length, and width of tomato fruits harvested from plants inoculated with *T. harzianum* T-22 and control on the three sampling dates. For the fruit weight, statistically significant differences were only found between treatments ($F_{2,12} = 8.57$, $P = 0.0030$), indicating that plants inoculated with *T. harzianum* T-22 produce larger fruits over time.

The length and width of the tomato fruits were statistically different among dates ($F_{2,12}$ = 45.9 and $F_{2,12}$ = 19.76, respectively, $P < 0.001$ in both cases) and between treatments ($F_{1,12}$ = 9.86 and $F_{1,13}$ = 12.26, respectively, $P < 0.01$ in both cases). No significant interaction "date x treatment" was found for length ($F_{2,12}$ = 0.39, P = 0.69) and width ($F_{2,12}$ = 2.29, P = 0.14) of tomato fruits. As for fruit weight, plants inoculated with *T. harzianum* T-22 produced longer and wider fruits. It is interesting to note that the fruit shape of San Marzano Kero cultivar changes with time, becoming rounder and more flattened toward the end of the production season.

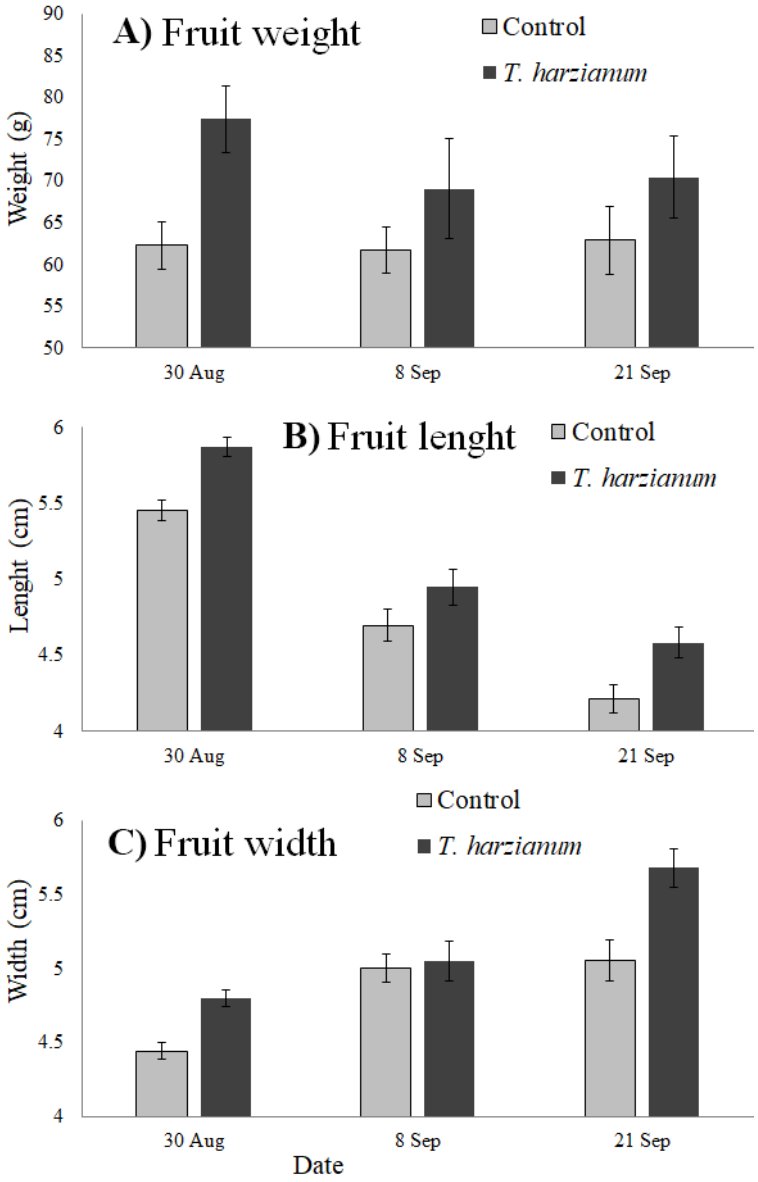

Figure 15. Mean values (± standard errors) of fruit weight, length, and width from tomato plants inoculated with *T. harzianum* T-22 and control ones at the three sampling dates.

3.4.3. Presence/Absence of Downy Mildew

At the beginning of August, all tomato plants observed in the field were in good health, despite the presence of downy mildew. Microscope observations in the laboratory performed on symptomatic leaves confirmed the presence of *Peronospora* spp. Most of the tomato leaves observed in both control and treated plants showed only initial symptoms of downy mildew and, consequently, were less damaged. Approximately 46% of the control plants (139 out of 302) showed the initial presence of downy mildew, while only approximately 25% (48 out of 193) of the inoculated plants showed similar symptoms; these differences were statistically significant ($\chi^2 = 22.4$, df = 1, $P < 0.001$).

4. Discussion

Knowledge of the adverse effects of agrochemicals on human health and the environment has led to the search for environmentally friendly methods to control plant diseases and pests. Beneficial soil microbes promoting plant defenses, such as non-pathogenic bacteria (Liu *et al.*, 2020), mycorrhizal fungi (Jung *et al.*, 2012; Pozo de la Hoz *et al.*, 2021; Sanmartín *et al.*, 2021), and plant growth-promoting fungi (Vitti *et al.*, 2015; Alfiky & Weisskopf, 2021b), are a possible alternative to pesticides. Fungi belonging to the genus *Trichoderma* are known as plant growth promoters and control agents against plant pathogens (Alfiky & Weisskopf, 2021b). *Trichoderma* spp. potentiate and stimulate plant defense responses against plant pathogens (Basińska-Barczak *et al.*, 2020). Furthermore, *Trichoderma* fungi can directly antagonize plant pathogens through competition, antibiosis, and mycoparasitism (Alfiky & Weisskopf, 2021b; Tyśkiewicz *et al.*, 2022). After a plant is attacked by pathogenic microorganisms or herbivorous arthropods, defense mechanisms involving signal transduction pathways responding to the phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are activated (Shoresh *et al.*, 2010; Tucci *et al.*, 2011; Ponzio *et al.*, 2013; Macías-Rodríguez *et al.*, 2020). Plant responses induced by herbivores depend on the mode in which these organisms attack the plant, with differences between piercing/sucking and chewing organisms (Pieterse *et al.*, 2009; Stam *et al.*, 2014). For example, JA signalling pathway is activated in response to chewing insects such as caterpillars, whereas piercing-sucking insects, like aphids, induce SA related defenses (Walling, 2000). SAR signalling is mainly mediated by SA derived compounds, while ISR is regulated by the antagonistic JA/ET signalling, but dependence on SA signalling has also been reported (Reimer-Michalski & Conrath, 2016; Mauch-Mani *et al.*, 2017; Bürger & Chory, 2019). However, although the pathways regulated by JA and ET are mutually

antagonistic, their synergistic interactions play a fundamental role in the ISR activation, but how plants coordinate these complex interactions is still unclear (Perazzolli *et al.*, 2008; Bari & Jones, 2009; Contreras-Cornejo *et al.*, 2011; Salas-Marina *et al.*, 2011). (Rojo *et al.*, 2007; Perelló *et al.*, 2009; Degani & Dor, 2021; Metz & Hausladen, 2022)(Woo *et al.*, 2014). Although the effects of *Trichoderma* spp. against plant pathogens are widely documented in the laboratory and in the field, fewer and mostly recent studies have addressed their effects on insect pests. Laboratory studies showed that *Trichoderma* spp. negatively influence both piercing-sucking (Muvea *et al.*, 2014; Coppola *et al.*, 2019a; b; Alınç *et al.*, 2021; Islam *et al.*, 2022) and chewing insects (Contreras-Cornejo *et al.*, 2018b; Coppola *et al.*, 2019a). In addition, *Trichoderma* spp. activate the plant's indirect defenses by attracting the natural enemies of pest insects (Battaglia *et al.*, 2013; Coppola *et al.*, 2017; Contreras-Cornejo *et al.*, 2018a). Field studies confirming these results are almost absent. This is an important knowledge gap because the results obtained in the laboratory (Guerrieri *et al.*, 2004; Durán Prieto *et al.*, 2017) may not be evident in more complex field conditions (Colella *et al.*, 2014). The implications from a practical perspective are significant.

Regarding *Trichoderma* spp., the only available field study investigated the effect of *T. harzianum* root inoculation on the community of pests and beneficial arthropods associated with maize foliage (Contreras-Cornejo *et al.*, 2020). This study showed that, under field conditions, the abundance of piercing-sucking herbivores decreases, while that of natural enemies increases, confirming laboratory observations. In contrast, the abundance of chewing herbivores increases, and this result seems to be inconsistent with what was previously observed in the laboratory.

Our study is the first investigation of the effects of *T. harzianum* T-22 on the above-ground arthropod community in a tomato field. In addition, we investigated whether soil inoculation with *T. harzianum* T-22 may have an effect on soil micro-arthropod biodiversity, on the presence and degree of attack of *Peronospora* spp. and on some fruit traits. This study is based on a 1-year tomato cycle in the field and, as with all experiments carried out in the field, it has intrinsic limitations due to the effects of many environmental factors that cannot be controlled. Environmental variables such as temperature can affect the performance of *Trichoderma* spp. Temperature influences the spore germination and the hyphal growth, and, consequently, the plant colonization of the biocontrol agent. Recently, Di Lelio *et al.* (Di Lelio *et al.*, 2021) investigated the impact of temperatures on the defense response induced by insects in tomato plants inoculated with T. *harzianum* T-22 and *T. atroviride* P1. Tomato plants treated with T-22 exhibited enhanced resistance, mediated by SA, toward *Spodoptera littoralis* and

Macrosiphum euphorbiae at 25°C, while *T. atroviride P1* was shown to be more effective at 20°C (Di Lelio *et al.*, 2021). Another important environmental variable is the wind. For example, depending on the turbulence and wind speed, plant VOCs rapidly become diluted within and above the plant canopy in agroecosystems (Loivamäki *et al.*, 2008), limiting plant protection against insects. Other factors such as the genotype of the plant (Rivelli *et al.*, 2013) may also significantly influence the trophic interaction. However, our results provide valid information on the complex plant–arthropod–microorganism interactions that occur during a season and can be fundamental to the correct development of sustainable organic agricultural systems.

During the first month after transplanting, tomato plants were colonized by *C. tibialis* adults and winged aphids of different species (no aphid colonies were observed at this time). The colonization of tomato plants by these species was not significantly different from the control when compared to the treatment with *Trichoderma* T-22. This seems to indicate that inoculation with *T. harzianum* does not alter plant attractiveness to aphids and flea beetles in the field. The trap catches confirmed this information. Traps placed inside the *T. harzianum* T-22 inoculated plots captured more aphids and beetles than those placed in the control plots, but the differences were not statistically significant.

In the following months, the foliage arthropod communities were quantified. We found that the total number of arthropod pests and natural enemies sampled on treated and control plants was almost the same. Unlike laboratory studies, these neutral effects are widespread in ecological above-below-ground field experiments (Heinen *et al.*, 2018), where there are many variables (and all their interactions) that can influence the final results. However, we observed that the effects of *T. harzianum* T-22 on insect abundance interact with the sampling period. This result could be related to different attractiveness of the plants with *T. harzianum* towards a particular arthropod group (Guerrieri *et al.*, 2004; Battaglia *et al.*, 2013; Contreras-Cornejo *et al.*, 2020) and to the period of its appearance. To better understand the plant-mediated influence of *T. harzianum* T-22 on these dynamics, the sampled arthropods were grouped into three groups: piercing-sucking or chewing herbivores and natural enemies of pests.

Our results indicated that the arthropod composition was affected differently by inoculation with *T. harzianum* T-22 in relation to the sampling period, although the average abundance was not significantly different from the control. Differences among different phytophagous groups, found by Contreras-Cornejo *et al.* (Contreras-Cornejo *et al.*, 2020) on maize, are confirmed in this study as trends and are highly influenced by the sampling date. This result could be related

to different attractiveness of the plants with *T. harzianum* towards a particular arthropod group (Guerrieri *et al.*, 2004; Battaglia *et al.*, 2013) and to the period of its appearance. A general increase of piercing-sucking insects was observed in control tomato plants during the flowering stage, while the abundance of chewing arthropods increased in plants inoculated with *T. harzianum* T-22, particularly in September (i.e., during the fruit maturity stage). In our experimental field, there was a slightly greater number of Noctuidae eggs in treated plants, probably due to the insect deposition preference. *Trichoderma* spp. is known to promote plant N uptake (Harman *et al.*, 2004). As a consequence, it is possible that chewing arthropods prefer hosts with high nutritional value for mating, oviposition, and food source for offspring (Contreras-Cornejo *et al.*, 2020).

Only the abundances of the spider mite *T. urticae* and the leaf miners were significantly different on plants inoculated with *Trichoderma* T-22 compared to control plants. The increased abundance of leaf mines on *Trichoderma* inoculated plants is consistent with the increase in chewing insects observed by Cornejo *et al.* (Contreras-Cornejo *et al.*, 2020). Equally, the reduction in *T. urticae* abundance on *Trichoderma*-inoculated plants is consistent with the reduction in piercing-sucking herbivores reported by Cornejo *et al.* (Contreras-Cornejo *et al.*, 2020), although spider mites, compared to other piercing-sucking herbivores such as aphids and whiteflies, produce substantial cellular damage.

To our knowledge, this is one of the first studies investigating the effects of *Trichoderma* plant inoculation on *T. urticae*. Until now, it has only been reported that the secondary metabolites of *Trichoderma* caused a high mortality rate in *T. urticae* (Sholla & Kottb, 2017) and future studies should be performed to assess the effects of *Trichoderma* on the performance of *T. urticae*.

No significant effects on natural enemies of insects and on the QBSar index are apparent from our study. In general, the number of these arthropods was not affected by *T. harzianum*, probably as a consequence of their relative low number or of uncontrolled environmental variables operating in the field. However, even if not significant, the number of natural enemies of insects increased on 3 August. In this period, the number of Syrphidae eggs and Syrphidae larvae were very abundant, probably due to the presence of aphids.

During the first sampling date, the number of rotten fruits was slightly higher in plants inoculated with *T. harzianum* T-22, but the number of marketable fruits was almost the same between treatments. In addition, the weight of marketable fruit in the inoculated plants is increased by about 20%. Taking these results together, the production of fresh marketable tomatoes in the field can increase with *T. harzianum* T-22 inoculation. Our study also confirms

the beneficial effect of *T. harzianum* T-22 against plant pathogens. Downy mildew was recorded in both control and treated tomato plants. However, even if the control tomato plants were more attacked by *Peronospora* spp. (46%) than plants treated with the *T. harzianum* T-22 strain (25%), in both cases the disease did not develop further. It has been shown that *Trichoderma* positively influences tomato fruit yields, as a consequence of an enhanced plant growth (Gravel *et al.*, 2007). The fungus increases the availability of nutrients to the host plant, lowers the ethylene level within the plant and enhanced the production of stimulatory compounds, such as plant growth regulators (Gravel *et al.*, 2007). Our results confirmed that *Trichoderma* T-22 inoculation in tomato has the potential to improve fruit yields.

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CHAPTER IV

Effects of below-ground microbial biostimulant *Trichoderma harzianum* on diseases, insect community, and plant performance in *Cucurbita pepo* L. under open field conditions

1. Introduction

Agrochemicals are at the base of intensive agricultural systems, and the increasing demand of food for humans has enhanced their use worldwide (Foley *et al.*, 2011). Agrochemicals include pesticides and other product categories that promote plant growth and preserve plant health, are used to maximize crop yield. However, the use of agrochemicals, particularly synthetic agrochemicals and inorganic fertilizers, causes toxicity to humans and ecosystems (Marschner *et al.*, 2003; Khanna & Gupta, 2018). Another problem related to the use of synthetic agrochemicals is the increasing emergence of resistant strains of pests and pathogens (Carletto *et al.*, 2010; Bass & Jones, 2018; Hawkins *et al.*, 2019).

Beneficial soil microbes, enhancing crop yield and promoting plant defenses, such as non-pathogenic bacteria (Liu *et al.*, 2020), mycorrhizal fungi (Pozo de la Hoz *et al.*, 2021) and plant-growth-promoting fungi (Alfiky & Weisskopf, 2021), are a possible alternative to the use of agrochemicals. The application of beneficial microbial inoculants in agriculture has increased over the past two decades (Batista & Singh, 2021). Fungi of the genus *Trichoderma* are among the most effective plant growth promoters in cultivated plant species (Macías-Rodríguez *et al.*, 2020). The induction of plant resistance against pests and pathogens by fungi of the genus *Trichoderma* has been much studied in tomato (Battaglia *et al.*, 2013; Martínez-Medina *et al.*, 2013; Jogaiah *et al.*, 2018; Coppola *et al.*, 2019a; Herrera-Téllez *et al.*, 2019; Alınç *et al.*, 2021; Heflish *et al.*, 2021; Caccavo *et al.*, 2022). Some *Trichoderma* strains were found to activate the plant systemic acquired resistance (SAR) and/or induce systemic resistance (ISR) against biotic and abiotic stress agents (Harman *et al.*, 2004; Shoresh *et al.*, 2010; Studholme *et al.*, 2013; Contreras-Cornejo *et al.*, 2016; Macías-Rodríguez *et al.*, 2020). For example, tomato defense responses against the green stink bug *Nezara viridula* L. were enhanced by *T. harzianum* strain T-22 through an early increase in transcript levels of jasmonic acid (JA) marker genes (Alınç *et al.*, 2021).

Zucchini (*Cucurbita pepo* L.) is the most important economically and globally widespread species among the cultivated Cucurbitaceae (Andolfo *et al.*, 2017). Zucchini includes a wide

assortment of varieties and cultivars (Paris, 1986) and is one of the most important and consumed vegetables worldwide. However, there are few research studies investigating the interaction between *Trichoderma* spp. and zucchini pathogens (Gilardi *et al.*, 2020; Formisano *et al.*, 2021).

In the field, aphids, mainly *Aphis gossypii* Glover (Homoptera: Aphididae), and some pathogens, such as phytoviruses and the powdery mildew fungal agents, namely *Golovinomyces cichoracearum* (de Candolle) Heluta, *Podosphaera fusca* (Fries) Braun & Shishkov and *Leveillula taurica* (Lév.) G. Arnaud, are the most harmful organisms that cause plant damage and production losses in zucchini crops (Hinds & Hooks, 2013; Shen *et al.*, 2018; Koné *et al.*, 2019). *Aphis gossypii* is considered the major pest of cucurbits. It is a polyphagous and destructive pest of more than twenty crop plant species. In hot regions, during the prolonged dry seasons, it produces large colonies on Cucurbitaceae, and it may survive on an ample variety of plant species, including cultivated and spontaneous Graminaceae (Blackman & Eastop, 2006). In colder temperate regions, it is restricted to glasshouses, where it is a major pest. For some plant species, its direct feeding can cause serious damage to plant tissues, such as curled leaves and stunted shoots (Ng & Perry, 2004). In zucchini plants, *A. gossypii* infestation causes a transcriptional up-regulation of genes underlying the biosynthesis of salicylic acid (SA) and of genes that modulate the SA-mediated defense response. As a consequence, aphids actively disperse on the plant, rather than starting their feeding activity where they were originally deposited, as observed in controls (Coppola *et al.*, 2018).

Although *A. gossypii* can cause direct damage to zucchini, the main damage is related to phytovirus transmission (Ebert & Cartwright, 1997). *A. gossypii* can transmit more than 50 phytoviruses, including non-persistent viruses of cucurbits, such as the *Cucumber mosaic virus* (CMV), the *Zucchini yellow mosaic virus* (ZYMV), the *Papaya ringspot virus* (PRSV), and the *Watermelon mosaic virus* (WMV) (Blackman & Eastop, 2006). These viruses can infect zucchini plants and, as in the case of ZYMV, can cause 40-50% of yield losses (Al-Shahwan *et al.*, 1995). Transmission of these viruses occurs during intracellular stylet punctures of aphids in epidermal or mesophyll cells, concomitant with saliva ejection (Ng & Perry, 2004).

In the last decades, the most used strategies to control aphid infestations and pathogens in zucchini have been primarily focused on the selection of resistant genotypes (Nováková *et al.*, 2015; Shen *et al.*, 2018; Zhang *et al.*, 2021) and on the use of pesticides (Cao *et al.*, 2008; Herron & Wilson, 2017). Although pesticides may effectively reduce aphid populations in field, their use may improve the dispersion of viruses transmitted by aphids. This is due to the dispersive effect of some pesticides on aphids that survive the pesticide treatment (Yuan &

Ullman, 1996; Desbiez & Lecoq, 1997). Moreover, in literature it is well-reported the development of resistance to insecticides in *A. gossypii* in several world regions (Herron *et al.*, 2000, 2001; Ahmad & Iqbal Arif, 2008; Cao *et al.*, 2008; Herron & Wilson, 2017).

The below–ground interactions between plants and microorganisms are very complex and it remains to be understood whether microbial biostimulants such as *Trichoderma* can be used to control harmful organisms. The aim of this study was to investigate the possibility of controlling zucchini pests and the most relevant virus diseases and powdery mildew in the field using the *Trichoderma harzianum* strain T-22. The effects of inoculation of the commercial *T. harzianum* strain T-22 on the arthropod community, on the above-mentioned plant diseases, and on the agronomic performance of zucchini squash was studied in detail for the first time in an experimental field.

2. Materials and Methods

2.1. Crop Cultivation

The present study was performed in an experimental field located in Pignola (40°34'06.2"N, 15°45'35.4"E; 780 m above sea level), Potenza, Italy, during the period June-September 2022. The soil was left fallow the year before the experiment and then plowed to a depth of 25 cm, rotavated and levelled before planting the crop. The soil characteristics are listed below: particles smaller than 2 mm in size, 935 g / kg; particles larger than 2 mm, 65 g / kg; apparent density, 1.294 kg / dm^3; texture composition of sand, 481 g / kg; clay, 149 g / kg; silt, 370 g / kg at depth of 0-30 cm. The content of total carbonate and total organic matter was of 16 g / kg and 32.8 g / kg, respectively. The composition of the soil was as follows: total N, 2 g / kg; P, 29 mg / kg; Ca 11.1 meq / 100 g; Mg, 4.6 meq / 100 g; Na, 1.8 meq / 100 g; soil pH (H_2O), 6.2. According to the world reference base for soil resources (FAO, 2022b), the soil was a dystric cambisol (Bd68-2bc).

Zucchini seedlings (*Cucurbita pepo* L.) of the San Pasquale cultivar (Pagano Domenico & Figli, Scafati, Salerno, Italy) were used in this experiment. Zucchini plants placed in alveolate containers were purchased from a nursery and transplanted to the field on 6 June 2022. No fertilizers were used during the present experiment and the zucchini plants were not treated with any type of agrochemical during the entire field trial. Water irrigation was applied through the drip irrigation system.

2.2. Meteorological Data

The temperature and rainfall data recorded during the experiment are shown in Figure S1. During the period of interest, the average temperature was about 20°C. The temperatures reached a maximum of 34°C in August. The precipitations recorded in June, July, and in the first 15 days of August were very low. The Agrometeorological Service of the "Agenzia Lucana per lo Sviluppo e l'Innovazione in Agricoltura (ALSIA)" of the Basilicata Region provided the meteorological data for the area in which the experimental farm is located. Meteorological data are shown in the Figure 1.

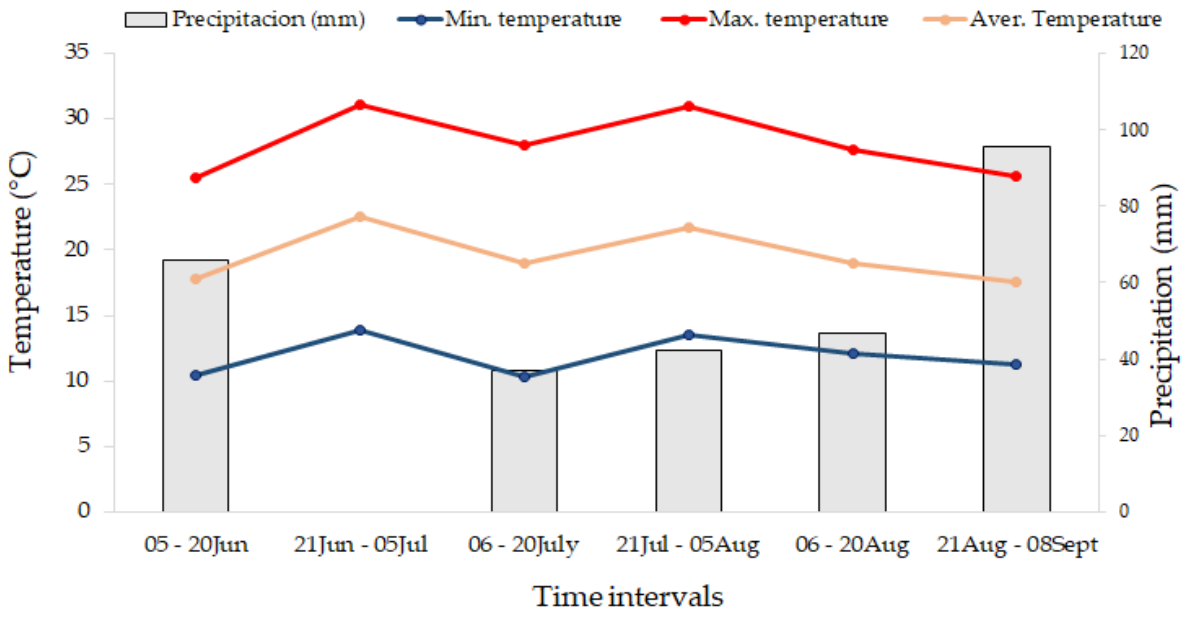

Figure 1. Meteorological data for Pignola, Potenza, Italy registered from June to September 2022. Data were provided by the Agrometeorological Service of the Basilicata Region, Italy.

2.3. Experimental Design

The effects of *T. harzianum* T-22 on diseases, insect community, and plant performance in zucchini plants were investigated. The first treatment consisted of non-inoculated zucchini plants (control), while the microbial biostimulant used in the present experiment to inoculate the plants was *Trichoderma harzianum* Rifai strain KRL-AG2 (T-22) (KOPPERT B.V., Berkel en Rodenrijs, The Netherlands), a purified strain which disperses in water.

The experiment was carried out on a strip of soil of about 40 meters long and 14 meters wide, divided into 6 plots of 22.5 m^2 (9 × 2.5 m) separated from each other by a strip 3 meters wide left without plants. Thus, 3 plots treated with *T. harzianum* T-22 and 3 control plots were

obtained, alternating along the length of the field. The plants in each plot were manually transplanted on 6 June 2022 in 2 rows 1.8 meters apart from each other. Each row was 8 meters long with a plant spacing of 66 cm, with a total of 12 plants/row (24 plants/plot). Figure 2 show zucchini plants during flowering stage.

Figure 2. Zucchini plants during flowering stage.

2.4. Fungal Inoculation

Before the experiments, the viability of the commercial formulation of *T. harzianum* T-22 was evaluated in the laboratory by serial dilution. The dilutions were placed on Petri plates (9 cm in diameter) containing Potato Dextrose Agar (PDA) medium (Oxoid Ltd., Hants, UK) amended with the antibiotic streptomycin sulfate 40 mg/L (MerckKGaA, Darmstadt, Germany) until growth could be detected. As suggested by a previous study (Pocurull *et al.*, 2020), the number of colony forming units (CFU) was counted after 24 h of incubation at 25°C in the dark.

Once the viability of the commercial product has been confirmed, zucchini seedlings were inoculated with *T. harzianum* T-22 following the manufacturer's instructions 5 days before transplantation. The alveolate containers with 24 seedlings were then watered with 3 grams of commercial *T. harzianum* T-22 (containing $1x10^9$ CFU/g of *T. harzianum* T-22) dissolved in 3 liter of water. Each plant was watered with about 41 ml of the fungal suspension. The treatment was repeated after 4 days and then the seedlings were transplanted. A total of 72 inoculated zucchini plants and 72 non inoculated control plants were transplanted into the field. No fungal inoculation was performed after the transplantation.

In addition, 9 control and 9 treated plants were transplanted into pots and placed in a greenhouse to determine the presence of *T. harzianum* T-22 in the roots. Twenty-four days after the last treatment, root samples were accurately collected and gently washed to remove soil residues. To ensure the presence of *T. harzianum* T-22 throughout the experiment, fungal colonization was also confirmed by strain isolations on PDA medium. Zucchini roots were sampled in the field 25 days (1 July), 54 days (30 July), and 85 days (30 August) after transplantation. For each sampling date, roots were collected from three control and three inoculated plants and transported to the laboratory. The roots were washed under running tap water to remove soil, then superficially sterilised using a 70% hydroalcoholic solution followed by a sodium hypochlorite solution at 1%. The roots were finally washed with sterile distilled water and dried on sterile paper. Each sample (a piece of root of about 2 x 2 cm in size) was placed on Petri plates with PDA medium amended with the streptomycin sulfate (0.05%). Plates were incubated at 25 ± 1°C for 7 days and the presence of the fungus was then determined.

2.5. Arthropod Sampling

During the first month after transplantation, the plants were very small, with few leaves and very few specimens of insects were found. From the second month after transplantation, the arthropod community on zucchini plants increased and was studied. Arthropod community survey was carried out by adopting two different sampling techniques: sampling of zucchini leaves, which mainly provided information on arthropod community colonising the plant, and capturing insects with colored pan trap sets, which mainly provided information of the winged arthropod community visiting the zucchini plant.

2.5.1. Arthropod Sampling on Zucchini Leaves

To investigate the arthropod community on zucchini plants, within each plot, six plants were randomly sampled at 9:00 a.m., for a total of 36 plants/date. Sampled leaves, fully unfolded and of about 20 centimetres, were taken from the middle part of the zucchini plant. Each leaf was gently inserted in a transparent zip lock plastic bag (40 x 30 cm) and then cut at the insertion with it stem. This procedure allows an accurate sampling of small arthropods, also collecting the ones that drop and/or jump when the plant is touched, thus obtaining quantitative data on their abundance. The plastic bags were kept in darkness at 5°C and transported to the laboratory for the identification of the arthropods. The collected arthropods were transferred to 50 ml

Falcon tubes filled with ~ 30 ml of 70% hydroalcoholic solution and refrigerated at 4°C until identification. The samples were then observed under a stereomicroscope. Arthropods from each sample were counted and classified at order, family, and, when possible, at the species level. Furthermore, the presence of damage caused by leaf miners on the leaves were noted and analysed. Five different leaf samples were carried out on 14 and 26 July, 9 and 26 August, and 8 September (that is, 38, 50, 64, 81, and 94 days after transplantation).

2.5.2. Arthropod Sampling with Colored Pan Traps

Arthropods were also sampled using pan trap sets consisting of one blue, one yellow, and one white bowl. Pan trap is a passive sampling method that provide an ample return of data for relatively short periods of time and are particularly appropriate for faunal surveys (Southwood & Henderson, 2000), without a collector effect (Leong & Thorp, 1999). The traps were made by painting plastic bowls (17 cm in diameter, 4.5 cm deep), with blue (RAL standard color codes: 5015) or yellow (RAL standard color codes: 1023) acrylic paint sprays or left white. The pan traps were placed on the ground, in the middle of each experimental plot, as close as possible to the plants of each experimental plot (Figure 3). Each trap was filled with 400 mL of water and 4 mL of dishwashing detergent with no fragrance added to break surface tension. Traps were set out early at 8:00 a.m. and collected three days later, at the same time. In case of rain, pan traps were removed and the sampled specimens were not considered; the traps were replaced 24 hours after rain stopped. Traps were collected in the order they were placed to ensure that all traps were available to insects for a similar time. Pan trap survey was carried out in four different data, with an interval of time of about two weeks among them: on 14 July, 1 and 16 August, and 8 September (that is, 38, 56, 71, and 94 days after transplantation). The arthropods were removed from the soap-water solution using a fine mesh colander and gently transferred with a soft paintbrush in 50 ml Falcon tubes, filled with 70% ethanol. Falcon tubes were stocked at 4°C until the identification of the arthropods. The samples were then observed under a stereomicroscope. Subsequently, the arthropods of each sample were counted and classified according to their order, family, and, when possible, at the species level.

Figure 3. Pan traps placed on the ground.

2.6. Evaluation of Diseases in Zucchini Plants

The present study focused on the presence of zucchini viruses and powdery mildew since these are the most important zucchini diseases in the considered area. All plants were visually inspected for the presence/absence of diseases on 6 and 24 July, 4, 8, 19 and 26 August, and 7 September. The presence of virus (chlorosis, severe mosaic, deformation, blistering and reduced leaf size) and/or powdery mildew (white powdery growth and subsequently spots or patches preferably on the leaf or on plant stems) symptoms was recorded. Figure 4 show diseases in zucchini plants.

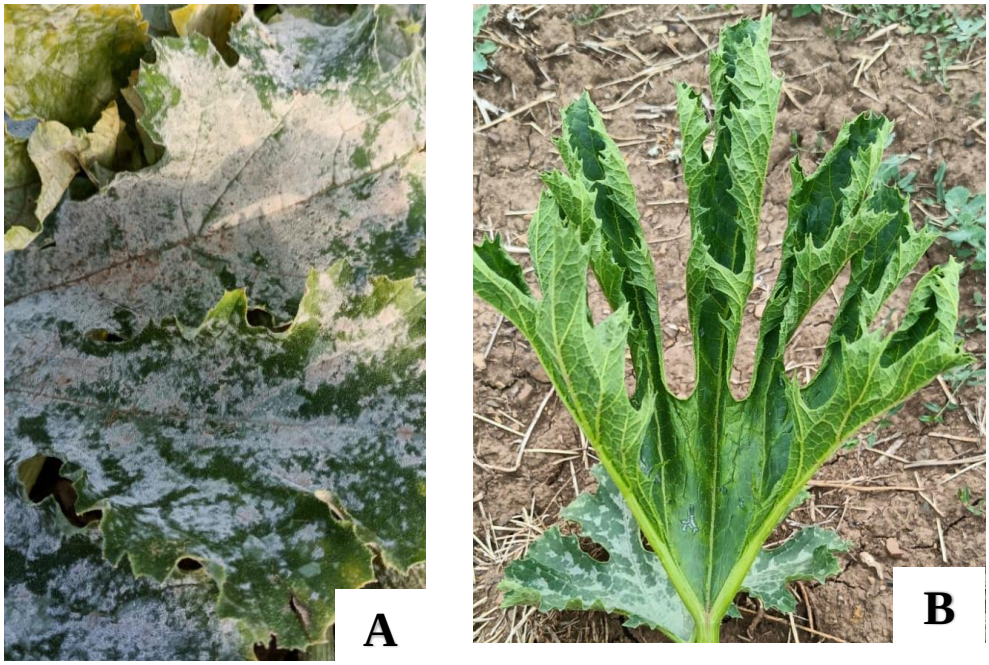

Figure 4. Diseases in Zucchini Plants: A) Powdery mildew; B) Presence of virus.

The zucchini viruses in plants were identified by enzyme-linked immunosorbent assay (ELISA), while the identification of the causal agent of the powdery mildew was done by microscopic observations.

2.6.1. Evaluation of Zucchini Viruses in the Field

The presence and development of four of the most common zucchini plant viruses, *Zucchini yellow mosaic virus* (ZYMV), *Papaya ringspot virus* (PRSV), *Cucumber mosaic virus* (CMV) and *Watermelon zucchini virus* (WMV), were evaluated throughout the entire cultivation period. The symptoms of the viruses were visually observed in the field on all parts of the plant. To assess the degree of viral attack, each plant was examined individually and the degree of attack per plot was estimated using the following formula:

$$Degree\ of\ attack\ (DA\%) = \frac{Number\ of\ symptomatic\ plants\ /\ plot}{Total\ number\ of\ plants/\ plot}\ X\ 100$$

The DA% for viruses was determined for all six plots during the experimental trial.

2.6.2. ELISA Assay

The presence of the four viruses investigated (CMV, ZYMV, PRSV and WMV) was assessed twenty days before the last harvest. For each virus, the ELISA tests were performed using specific antibodies and kits (Loewe® Biochemica GmbH, Sauerlach, Germany)following the manufacturer's instructions. Briefly, a DAS ELISA (Clark & Adams, 1977) was performed using a polyclonal antiserum rabbit for each virus. Leaves and fruits from experimental plot were placed in plastic bags, transported to the laboratory, and stored at 4°C. For the ELISA test, the sap was extracted by homogenizing 1g of sample in 10 ml of Conjugate/Sample buffer (ELISA kit) in plastic BIOREBA extraction bags (BIOREBA AG, Reinach, Switzerland) using a commercial homogenizer. Sap samples were collected in Eppendorf tubes and stored at -20°C. The DAS ELISA assays consisted in coating the Nunc™ MicroWell 96-Well Microplates (ThermoFisher Scientific Inc., Waltham, MA, U.S.A.) plates (200 µl/well) with antigen-specific antibodies (IgG) 1:200 diluted in coating buffer, incubation of the plates at 37°C for 4 h, followed by four manual washings with washing buffer at room temperature (RT), followed by samples application and overnight incubation at 4°C. Subsequently, antibody-AP-conjugate application (200 µl /well) 1:200 diluted in conjugated buffer, incubation of the plates at 37°C for 4 h, four washings and enzymatic assays using substrate buffer added with 1mg/ml of PNPP

tablets were performed. The results were evaluated by comparing the visual reaction, determined as a yellow color development, in the plate between the control (positive/negative) and samples. After 1 and 2 h of substrate incubation, plates were read photometrically at 405 nm wavelength using an ELISA Reader model A3 (DAS, Rome, Italy). All samples were run in duplicate.

2.6.3. Powdery Mildew Evaluation Assay

The percentage of powdery mildew disease attack was assessed by field observations. All leaves and fruits from control and *T. harzianum* T-22 treated zucchini plants were individually observed. To assess the percentage of powdery mildew attack in the field, the following scale was used: 0 (not infected) = 0% attack; 1 (low) = 1-25% infected tissue; 2 (medium) = 25.1-50% infected tissue; 3 (high) = 50.1-75% infected tissue; 4 (very high) = >75.1% infected tissue. Furthermore, to identify the possible pathogen causal agent responsible for the observed symptoms on zucchini plants, 25 symptomatic leaves and fruits were randomly collected from plants in the field and, on the same day, used to identify the causal agent of the powdery mildew in the laboratory. For species identification, conidia were directly obtained from the infected zucchini leaves and fruits collected in the field. The conidia were then observed under a light microscope (Axioscope, Zeiss, Germany) and also other morphological characteristics reported in literature for zucchini powdery mildew causal fungus were considered (Miazzi *et al.*, 2011; Braun & Cook, 2012).

2.7. Evaluation of Plant Growth and Productivity

Plant growth was estimated by measuring the stem length of zucchini plants, excluding the leaf. This survey was carried out on four different data: on 22 June, 6 and 22 July, and 4 August. The stem length was measured in four plants per plot.

The zucchini fruits were first harvested on 9 July (33 days from transplanting) and were successively collected every two days until 22 August. From 22 August to 30 August, zucchini fruits were collected every four days. For each harvest, marketable fruits were counted and weighed for each experimental plot. The mean values of the weight of the zucchini fruits and the cumulative number and weight of the zucchini fruits per plant harvested from the plots inoculated with *T. harzianum* T-22 and from the controls from 9 July to 30 August were then calculated.

2.8. Statistical Analysis

The number of arthropods sampled over time on the leaves, in the pan traps, and the data relating to the disease symptoms of the powdery mildew were analyzed with a Poisson generalized linear mixed models (GLMMs) with a log-link function fitted with ML (maximum likelihood) and Laplace approximation. The discrete Poisson distribution best approximates the process that generated the observed data. The *P*-values for the differences between the treatments, sampling dates, and their interactions were obtained through analyses of deviance (Type II Wald chi-square tests). The following general model was applied:

$$Y = \mu + Treatment + Date + Treatment \times Date + Plot\ \{Treatment\ \{Date\}\} + \varepsilon$$

where *Y* is the studied variable with a Poisson distribution, Treatment and Date are the fixed factors and Plot is the random effect consisting of the three experimental plots nested in Treatment and Date. This model accounts for the non-independence of the data (pseudoreplication of measures) due to the different experimental plots (the random effect) that are part of the present design.

Data on plant length, fruit weight, cumulative number and cumulative weight of zucchini fruits per plant were analyzed using linear mixed-effects models (LMMs) fitted with REML (restricted maximum likelihood). The homoscedasticity and normality assumptions for these ANOVAs were checked and met on these data. The *P*-values for the differences between the treatments, sampling dates, and their interactions were obtained through ANOVAs (type II Wald chi-square tests). To better appreciate the (possible) differences in fruit wight over time, in this analysis the sampling dates were grouped into 4 periods: 09-19 July, 20-31 July, 01-12 August and 13-25 August. The general model applied for these analyses was the same as applied for the analysis of the insect community.

The percentage of virus infected plants per plot was analyzed using a linear model (LM) after an arcsine transformation of the data. The following model was applied:

$$Y = \mu + Treatment + Date + Treatment \times Date + \varepsilon$$

where Y is the percentage (transformed) of virus-infected plants, Treatment and Date (7 levels) are fixed effects. The *P*-values for were obtained by a factorial model ANOVA (Type II sum-square tests).

To test for the influence of virosis on powdery mildew symptoms, the following model was applied:

$$Y = \mu + Virus\ class + Treatment + Virus\ classes \times Treatment + \varepsilon$$

where Y is the quantification of the disease symptoms of the powdery mildew on a plant, Virus class (four levels of degree of virus attack, 0: 0 DA%, 1: 1-25 DA%, 2: 26-50 DA%, 3: 51-75 DA%, and 4: 76-100 DA%) and Treatment are fixed effects. The P-values were obtained by a factorial model ANOVA (Type II sum-square tests).

For all the analyses described so far, the model distributions were also chosen as the best fitting, based on AIC criteria (Burnham & Anderson, 2004) and the full models were presented. All statistical analyses were performed in R version 4.1.2 "Bird Hippie" (R Core Team, 2021), with lme4 (Bates *et al.*, 2015), lmerTest (Kuznetsova *et al.*, 2017) packages.

3. Results

3.1. Trichoderma harzianum T-22 Inoculation

The zucchini plants transplanted into pots were inspected 24 days after inoculation to verify the success of the colonization of *T. harzianum* T-22 in the roots. The 9 control and 9 inoculated plants were gently removed from the pots and photographed (Figure 5).

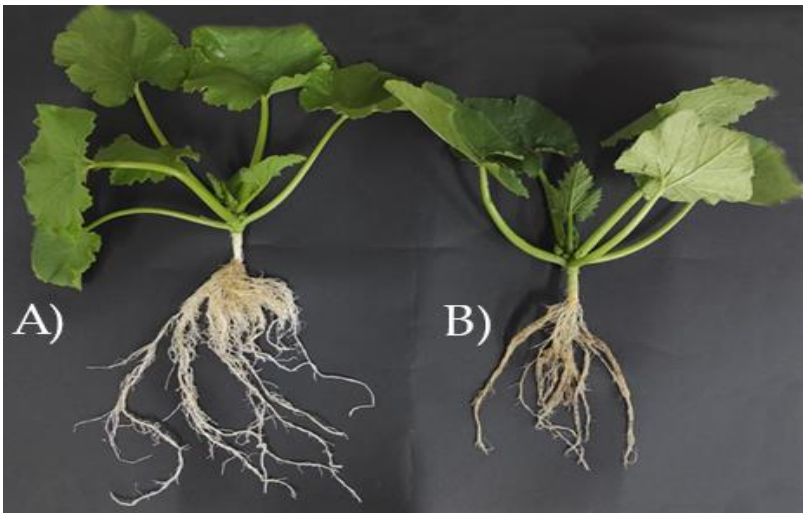

Figure 5. Zucchini plants 24 days after the inoculation. A) *Trichoderma harzianum* T-22 inoculated plant; B) not inoculated control plant.

Compared to controls, the 9 zucchini plants inoculated with *T. harzianum* T-22 showed an increased root development. In addition, light microscopy analyses showed that the colonization of the roots took place in all the 9 inoculated plants (100%). In addition, *T. harzianum* T-22 was isolated on PDA from all zucchini roots sampled in the treated plots 25

days, 54 days, and 85 days after transplantation. The presence of the fungus was not observed in the control Petri plates.

3.2. Arthropods Sampling

3.2.1. Arthropod Sampling on Zucchini Leaves

Leaf samples were collected for observation of the arthropods in the laboratory. During this sampling period, 256 arthropod specimens were collected on zucchini leaves, of which 107 and 149 were obtained from plants with *T. harzianum* T-22 and control, respectively. The arthropods on zucchini leaves belonged to the families Aphididae (one species identified: apterous morph of *Aphis gossypii*), Cicadellidae, Thripidae, Chrysomelidae, Gryllidae, Coccinellidae (adults), Syrphidae (identified as eggs or adults), Braconidae (adults), and Miridae. We also collected eggs of Lepidoptera, 9 individuals belonging to the order of Araneae, 5 individuals of *Tetranychus urticae*, and 5 leaf mines of *Liriomyza trifolii* Burges (Diptera, Agromyzidae). The abundances of Cicadellidae, Thripidae, Gryllidae, Chrysomelidae, Coccinellidae, Miridae, Syrphidae, Braconidae, Araneae, *T. urticae*, and leaf miners were very low during the whole sampling period and consequently were excluded from the analysis, and are shown in Figure 6.

The abundance of *A. gossypii* and of eggs of Lepidoptera is shown in Figure 7.

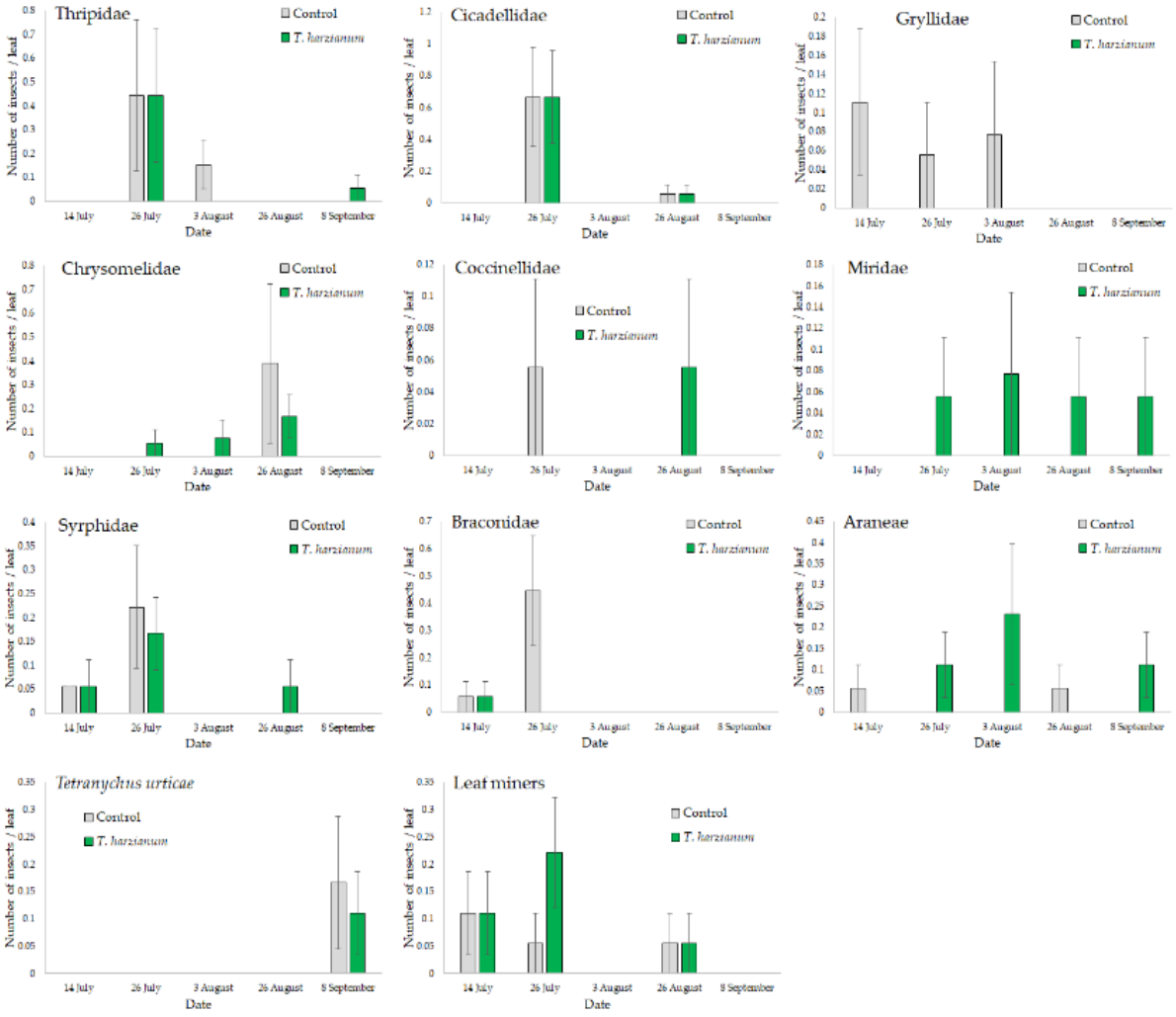

Figure 6. Leaf arthropods. Mean values (± standard errors) of insect abundance of Cicadellidae, Thripidae, Gryllidae, Coccinellidae, Miridae, Syrphidae, Araneae, leaf miners, and *Tetranychus urticae* on zucchini leaves sampled from plants inoculated with *T. harzianum* T-22 and control ones during the five sampling dates.

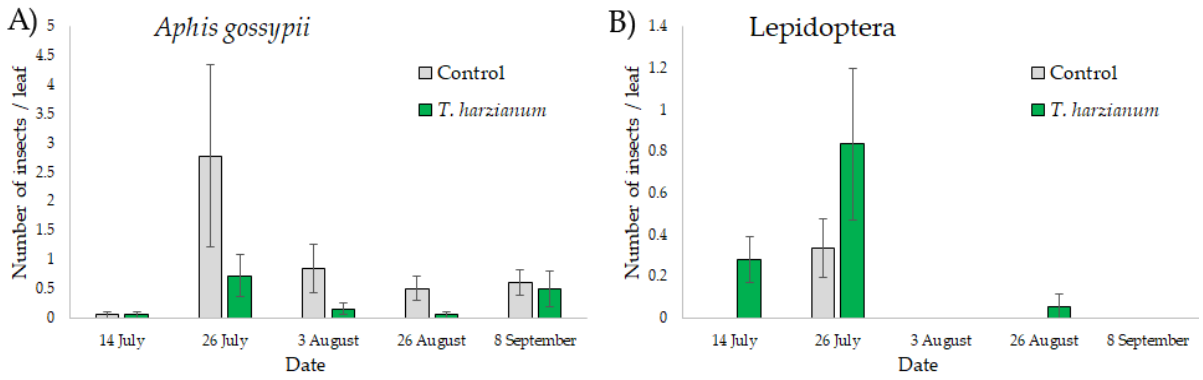

Figure 7. Mean values (± standard errors) of the number of apterous *Aphis gossypii* (A) and eggs of Lepidoptera (B) on zucchini leaves sampled from plants inoculated with *T. harzianum* T-22 and control ones during the five sampling dates.

The GLMMs showed that the sampling dates influenced the abundance of apterous *Aphis gossypii* ($\chi^2 = 20.1$, df = 4, $P < 0.001$) and of eggs of Lepidoptera ($\chi^2 = 10.2$, df = 4, $P = 0.03$). The abundance of these insects was higher in July and then decreased in the following months. The abundance of *A. gossypii* was also affected by the treatment ($\chi^2 = 6.2$, df = 1, $P = 0.01$), with more apterous individuals collected on control plants. No significant differences between control and plants inoculated with *T. harzianum* T-22 in the number of Lepidoptera eggs were observed ($\chi^2 = 3.6$, df = 1, P = 0.058). The interactions "treatment X date" were never found significant.

3.2.2. Arthropod Sampling with Colored Pan Traps

During the sampling period, 3925 arthropod specimens were collected with the pan traps, of which 2307 and 1618 were obtained from plants inoculated with *T. harzianum* T-22 and control, respectively. The arthropods collected in the traps belonged to the families Aphididae (winged morphs of *A. gossypii*), Cicadellidae, Thripidae, Chrysomelidae, Gryllidae, Coccinellidae, Miridae, and to the order Lepidoptera, Hymenoptera (Ichneumonoidea and Chalcidoidea), and Araneae. The abundances of Lepidoptera, Coccinellidae, Staphylinidae, Gryllidae, and Miridae were very low, and they have not been considered for the analysis, and are shown in Figure 8.

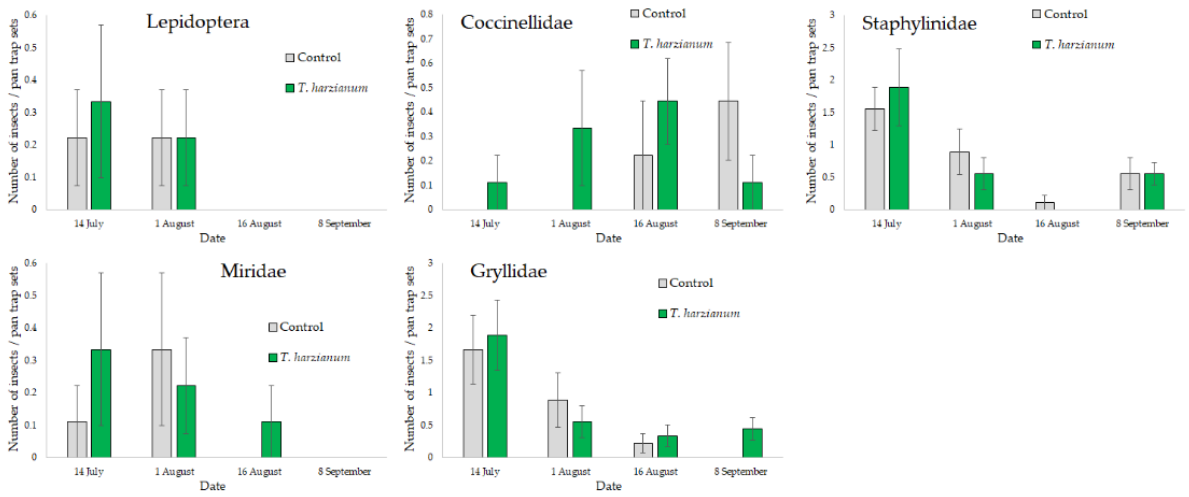

Figure 8. Pan traps samples. Mean values (± standard errors) of insect abundance of Lepidoptera, Coccinellidae, Staphylinidae, Cicadellidae, and Miridae collected with pan traps placed near zucchini plants inoculated with *T. harzianum* T-22 and control ones at the four sampling dates.

The abundances of *A. gossypii*, Chrysomelidae, Thripidae, Cicadellidae, Hymenoptera parasitoids, and Araneae are shown in Figure 9.

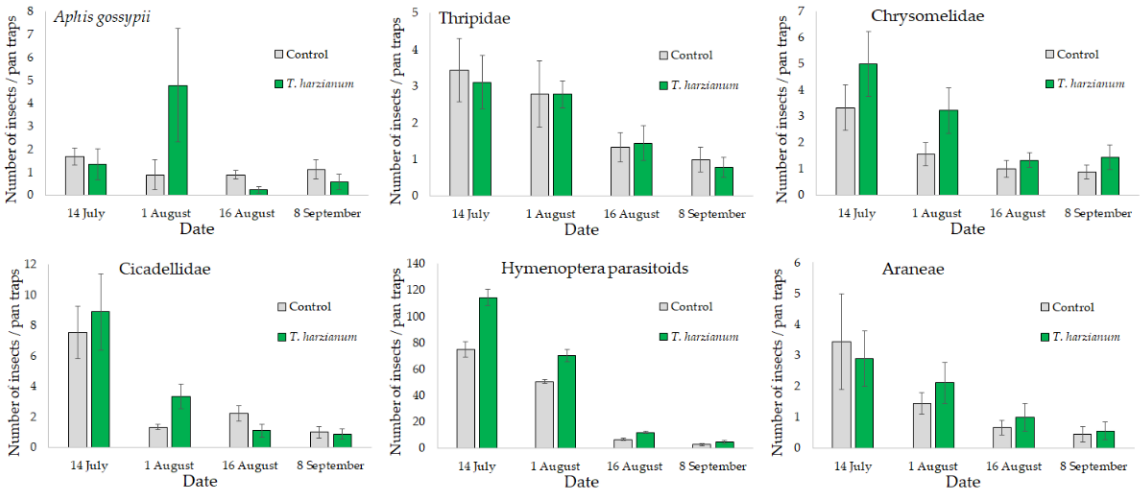

Figure 9. Mean values (± standard errors) of the number of winged *Aphis gossypii* (A), Thripidae (B), Chrysomelidae (C), Cicadellidae (D), Hymenoptera parasitoids (E) and Araneae (F) collected with pan trap sets placed near plants inoculated with *T. harzianum* T-22 and control at the four sampling dates.

The GLMMs showed that the abundances of all the arthropods collected with the pan trap sets in the experimental field were affected by the sampling dates ($P < 0.001$ in all cases). The abundance of arthropods was higher in July and then decreased in the following months. Significant differences between treatments were found for the abundance of winged *A. gossypii* ($\chi^2 = 33.8$, df = 1, $P < 0.001$), of Chrysomelidae ($\chi^2 = 5.1$, df = 1, $P = 0.02$), and of Hymenoptera parasitoids ($\chi^2 = 61.9$, df = 1, $P < 0.001$), with a higher number of insects collected in plots with zucchini inoculated with *T. harzianum* T-22. The interaction "treatment X date" was only found significant for *A. gossypii* ($\chi^2 = 439322$, df = 3, $P < 0.001$) and for the family of Cicadellidae (and $\chi^2 = 10.1$, df = 3, $P = 0.01$). Compared with the control, the abundance of aphids and Cicadellidae was higher on the 1 August on the *T. harzianum* T-22 plots.

3.3. Plant Diseases

3.3.1. Field Evaluation of Zucchini Viral Diseases

The ANOVA performed on the data relating the viral infection gave significant differences among sampling dates ($F_{6,28} = 127.6$, $P < 0.001$) but not between treatment ($F_{1,28} = 2.4$, $P = 0.13$) or for the "treatment X date" interaction ($F_{6,28} = 0.76$, $P = 0.61$) as shown in Figure 5. The viral infections, in all plots, started on 24 July on both treated and untreated plants and continuously increased over time until the end of the cultivation period, reaching the 100% of infection at the beginning of September. On 7 September there was no difference in the viral symptoms observed in the field between the untreated (control) and treated (*T. harzianum* T-22) plants per plot (Figure 10).

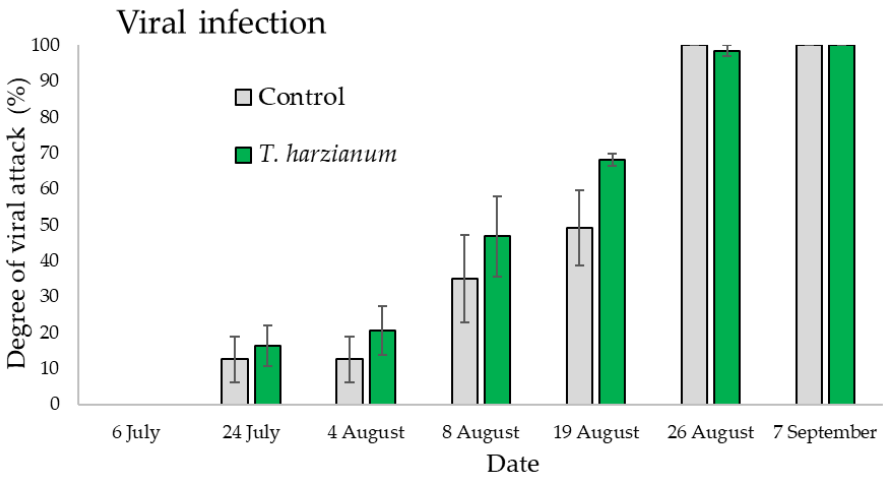

Figure 10. Mean degree of viral attack (± standard errors) in plots inoculated with *T. harzianum* T-22 and control ones over time.

Regarding the influence of the virosis on powdery mildew symptoms of the zucchini plants, it was observed that in plants with the same symptoms of virosis, the powdery mildew infection was more evident for the control plants compared with the *T. harzianum* T-22 inoculated ones (Figure 11). Furthermore, the ANOVA performed on these data gave significant differences related to the virosis classes ($F_{4,514} = 398.7$, $P < 0.001$) and between treatments ($F_{1,514} = 21.9$, $P < 0.001$) but not for the "virosis classes X treatment" interaction ($F_{4,514} = 0.6$, $P = 0.66$), as shown in Figure 11.

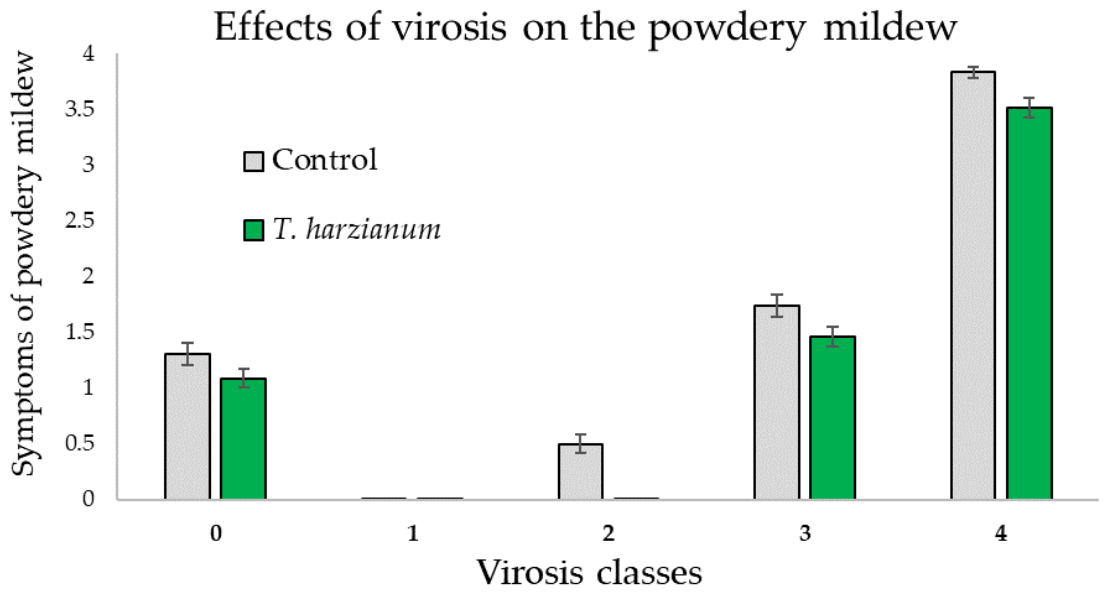

Figure 11. Mean values (± standard errors) of the symptoms the powdery mildew in relation to the virus classes in plants inoculated with *T. harzianum* T-22 and control ones.

3.3.2. ELISA Test for Viruses in Zucchini Plants

The results of the ELISA serological assay demonstrated that of the four most common zucchini viruses (CMV, ZYMV, PRSNV and WMV), only one virus (CMV) was not present in the experimental field, while all others were detected. In particular, ZYMV and PRSNV were detected at 100%, WMV had a 45% of incidence in control plants and a 44% incidence in the *T. harzianum* T-22 treated ones (Figure 12). In summary, our results showed that zucchini plants were infected by three of four most common viruses and the viral incidence was not much different between the control and *T. harzianum* T-22 inoculated plants.

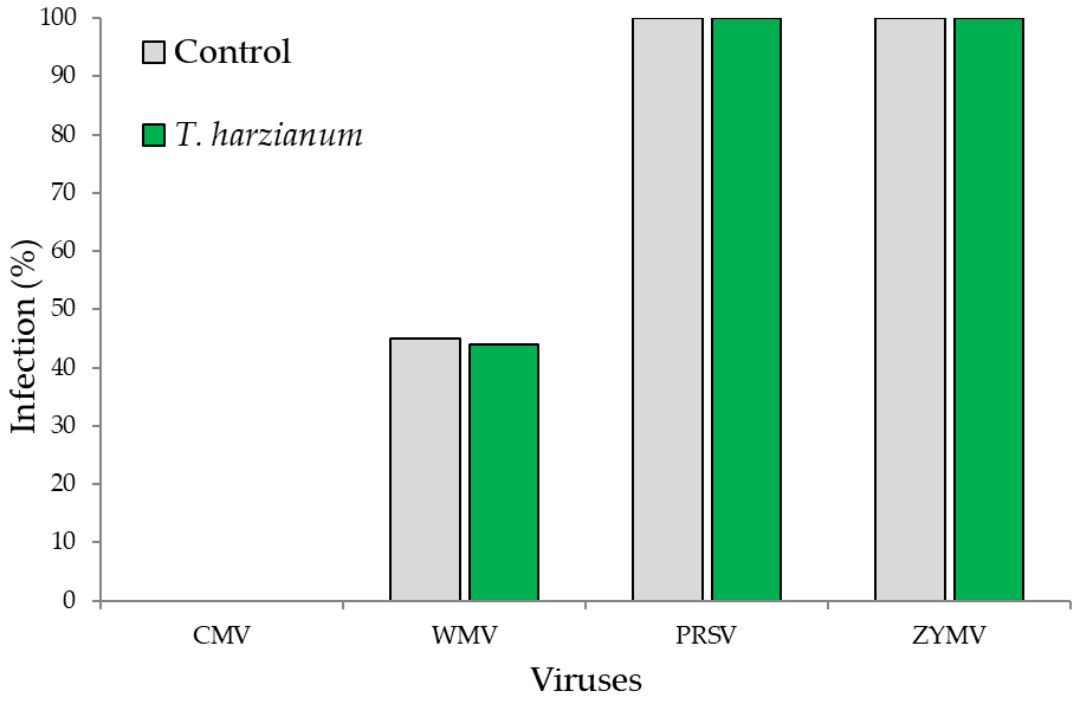

Figure 12. Percentage of infection for each virus determined by ELISA in zucchini plants.

3.3.3. Powdery Mildew

Microscopic analysis showed that the causal agent of powdery mildew attack on zucchini plants in the field was closely similar to the *P. fusca*. These results are based on the morphological features reported in literature (Miazzi *et al.*, 2011; Braun & Cook, 2012) for zucchini powdery mildew and the fibrosin bodies presence in the conidia.

The results regarding the symptoms of powdery mildew are shown in Figure 13. The symptoms were observed in the field after 19 August and the disease progressed in both controls and *T. harzianum* T-22 plots, reaching 100% of infection on 7 September. Even if the disease symptom development was similar, a small delay was observed for the *T. harzianum* T-22 treated plants compared to the control, at least in the initial and also during the disease development stages. However, the GLMMs showed that the symptoms of the powdery mildew were influenced by the sampling dates ($\chi 2 = 200$, df = 3, $P < 0.001$), but not by the treatment ($\chi 2 = 0.79$, df = 1, $P = 0.37$), nor by the interactions "treatment X date" ($\chi 2 = 1.1$, df = 3, $P = 0.78$) as shown in Figure 13.

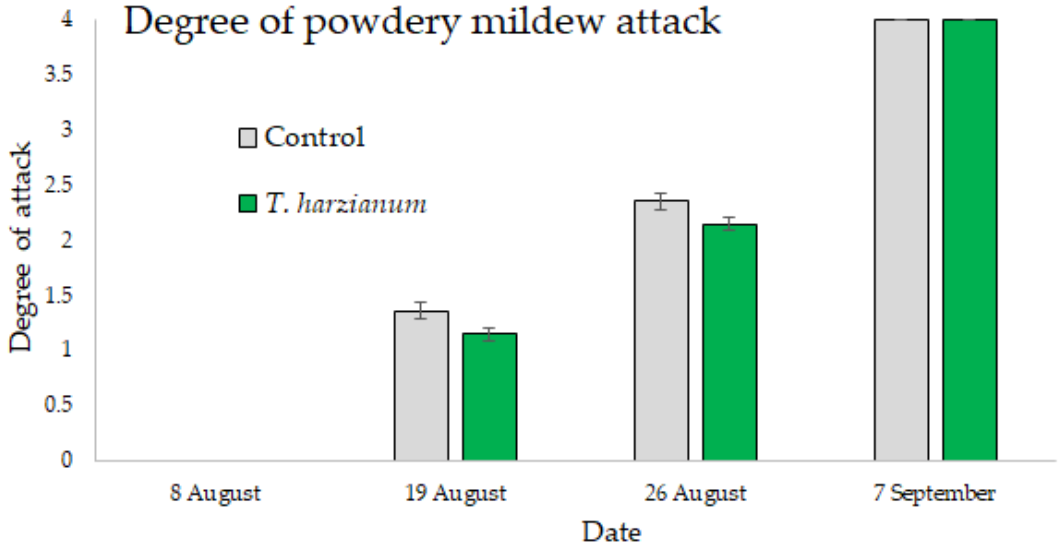

Figure 13. Mean values (± standard errors) of the powdery mildew degree of attack in plants inoculated with *T. harzianum* T-22 and control over time.

3.4. Crop Sampling

3.4.1. Plant length

Figure 14 shows the mean values of the length of zucchini plants inoculated with *T. harzianum* T-22 and control on the four sampling dates.

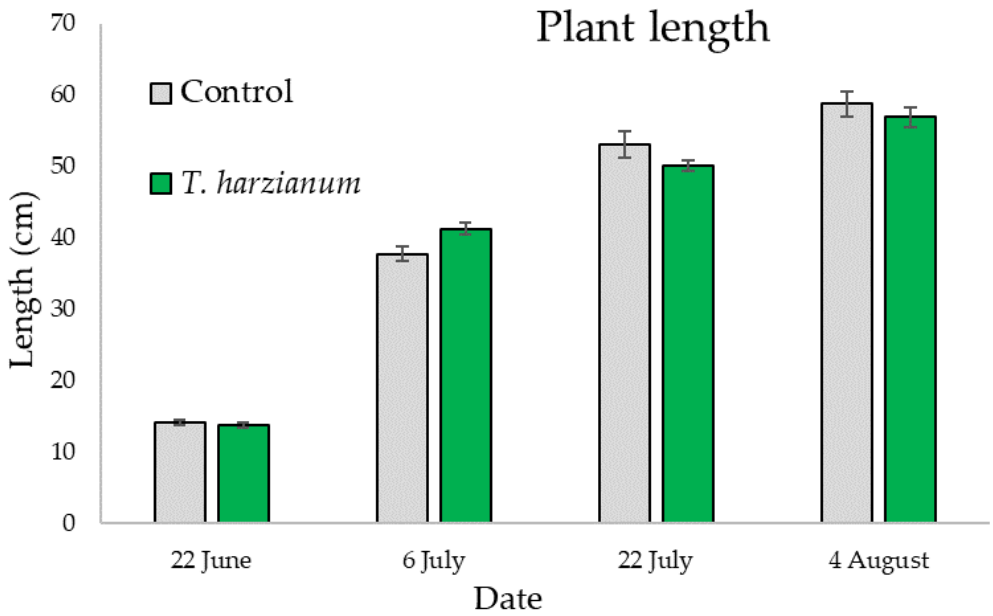

Figure 14. Mean values (± standard errors) of the length of zucchini plants inoculated with *T. harzianum* T-22 and control on the four sampling dates.

For plant length, statistically significant differences were only found between sampling dates (χ^2 = 204, df = 3, P < 0.001), indicating that plants inoculated with *T. harzianum* T-22 or not inoculated have the same growth rate over time.

3.4.2. Plant Productivity

Figure 15 shows the mean values of the weight of the zucchini fruit harvested from plants inoculated with *T. harzianum* T-22 and from the control during the four sampling periods.

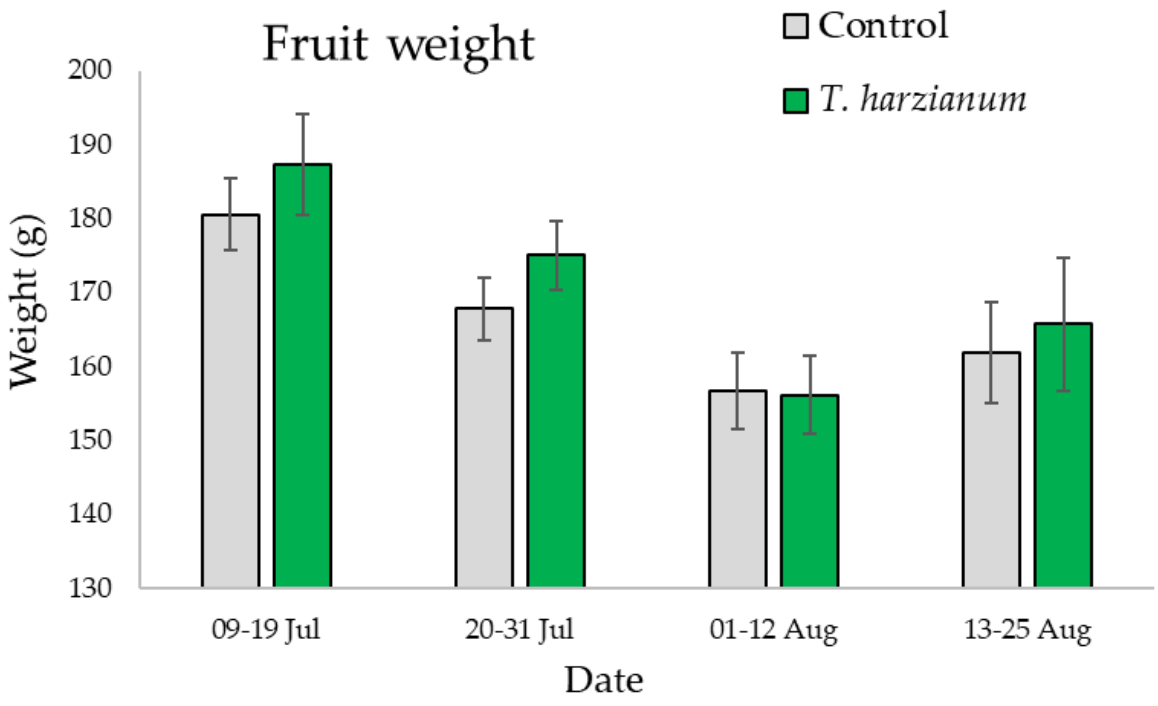

Figure 15. Mean values (± standard errors) of fruit weight from zucchini plants inoculated with *T. harzianum* T-22 and control during the four sampling periods.

For fruit weight, statistically significant differences were only found among sampling periods (χ^2 = 18.1, df = 3, P < 0.001), with heavier fruit produced during the first month. Even if the differences were not significant, during the first month plants inoculated with *T. harzianum* T-22 showed a production of heavier fruit than controls.

Figure 16 shows the number and weight of fruits recorded after each harvest accumulatively from 9 July to 30 August from plants inoculated with *T. harzianum* T-22 and from control ones.

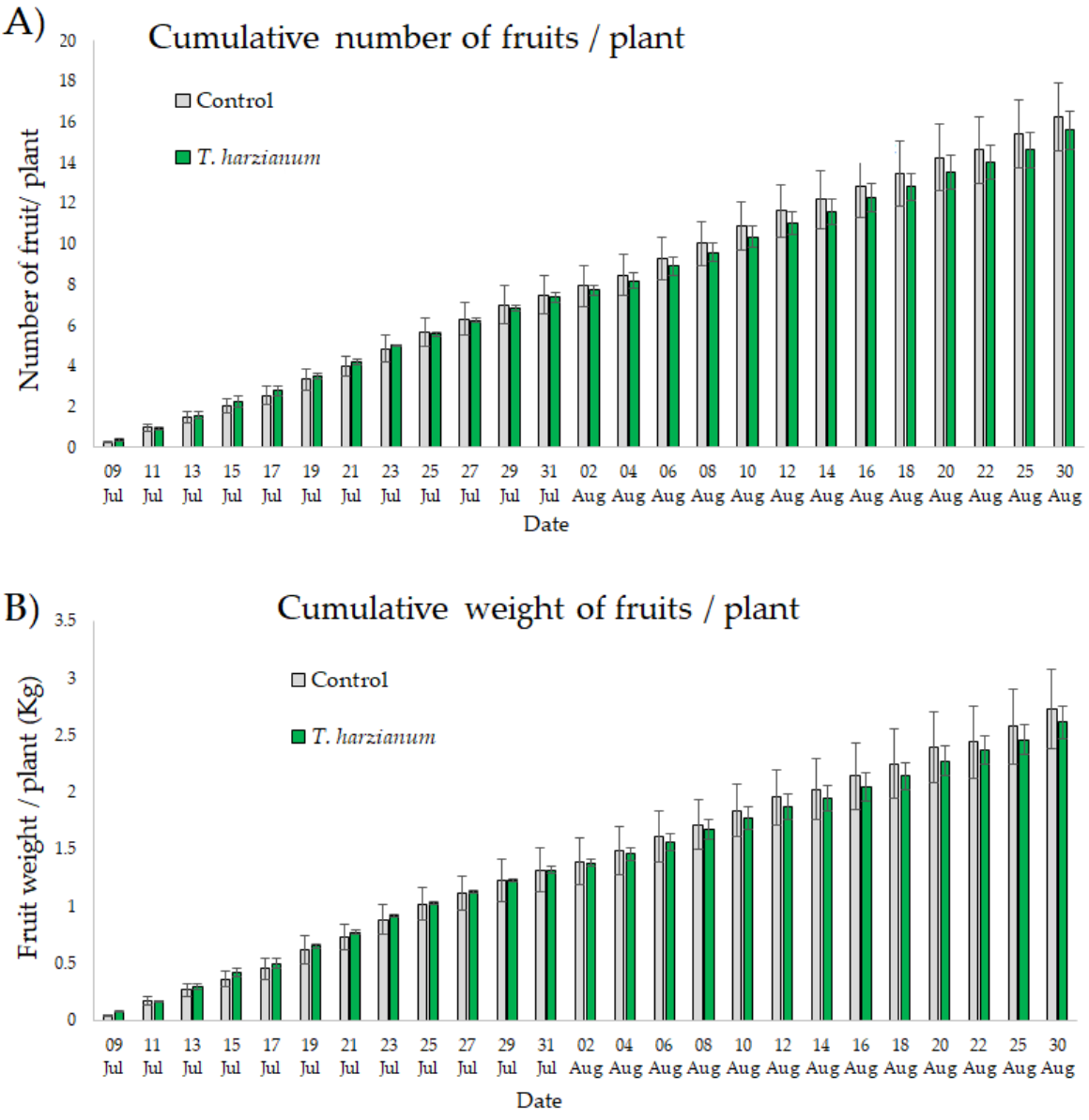

Figure 16. Mean values (± standard errors) of the cumulative number (A) and weight of zucchini fruits (B) recorded after each harvest from 9 July to 30 August from plants inoculated with *T. harzianum* T-22 and from control.

The ANOVAs performed on these data show that the cumulative number of zucchini fruits/plant and the cumulative yield/plant were not affected by inoculation with *T. harzianum* T-22 ($\chi^2 = 2.55$, df = 1, $P = 0.11$ and $\chi^2 = 0.74$, df = 1, $P = 0.39$, respectively). No significant interaction "date X treatment" was also found ($\chi^2 = 4.6$, df = 24, $P = 0.99$ and $\chi^2 = 3.2$, df = 24, $P = 0.99$, respectively).

4. Discussion

The use of beneficial microbial species in agriculture as biocontrol agents and plant growth promoters has increased in recent decades (Batista & Singh, 2021). Among them, fungi of the genus *Trichoderma* are the most widespread and effective (Woo *et al.*, 2014; Macías-Rodríguez *et al.*, 2020; Tyśkiewicz *et al.*, 2022). *Trichoderma* fungi can antagonize plant pathogens through competition, antibiosis, and mycoparasitism mechanisms. *Trichoderma* is known to induce metabolic and physiologic changes in the colonized plants (Shoresh & Harman, 2008) by activating the plant SAR and/or by inducing systemic resistance against biotic and abiotic stress agents (Harman *et al.*, 2004; Shoresh *et al.*, 2010; Studholme *et al.*, 2013; Contreras-Cornejo *et al.*, 2016; Macías-Rodríguez *et al.*, 2020). It is well accepted that plants have sophisticated defense strategies and when attacked by pathogens or pests activate signalling defense mechanisms modulated by jasmonic JA, SA or ethylene phytohormones (ET) (Walling, 2000; Shoresh *et al.*, 2010; Tucci *et al.*, 2011; Ponzio *et al.*, 2013; Macías-Rodríguez *et al.*, 2020). However, these pathways can crosstalk and their synergistic interactions can play a fundamental role in the ISR activation (Perazzolli *et al.*, 2008; Bari & Jones, 2009; Contreras-Cornejo *et al.*, 2011; Salas-Marina *et al.*, 2011).

Recently, the possibility of using fungi of the genus *Trichoderma* as pest biocontrol agents has been emphasized (Poveda, 2021). Most studies on below ground-above ground interactions involving *Trichoderma* and plant pests have used tomato as a model plant in the laboratory (Battaglia *et al.*, 2013; Martínez-Medina *et al.*, 2013; Jogaiah *et al.*, 2018; Coppola *et al.*, 2019b; Herrera-Téllez *et al.*, 2019; Alınç *et al.*, 2021; Heflish *et al.*, 2021). Few studies have verified, under far more complex field conditions, the role of *Trichoderma* fungi as a pest biocontrol agents (Woo *et al.*, 2014; Contreras-Cornejo *et al.*, 2020; Caccavo *et al.*, 2022). In the context of horticultural crops, Cucurbitaceae is the second family in terms of economic relevance after Solanaceae (Afechtal *et al.*, 2019), and zucchini is the most important economically and globally widespread species among the cultivated Cucurbitaceae (Andolfo *et al.*, 2017). The main phytosanitary problems of zucchini are aphids, phytoviruses and powdery mildews (Hinds & Hooks, 2013; Koné *et al.*, 2019). The management of these pest and pathogen infections on zucchini crops has so far been based mainly on use of resistant varieties (Nováková *et al.*, 2015; Shen *et al.*, 2018; Zhang *et al.*, 2021) and pesticides (Cao *et al.*, 2008; Herron & Wilson, 2017). The effectiveness of *Trichoderma* spp. as biocontrol agents against zucchini fungal pathogens is confirmed by several studies, especially on *Fusarium* spp. (Li *et*

al., 2019; El-Sharkawy & Abdelrazik, 2022). Inoculations with *Trichoderma* spp. inhibited *F. oxysporum* infection stimulating plant metabolism and increasing the activities of stress-resistance enzymes (Li *et al.*, 2019).

On the contrary, laboratory and field studies that report the effectiveness of *T. harzianum* as a biocontrol agent against aphids and phytoviruses in zucchini are not available. In this study, we measured the natural evolution of pests and diseases in a zucchini field by comparing plots inoculated or not inoculated with *T. harzianum* T-22.

Throughout the cultivation period, *A. gossypii* was the only pest species worth mentioning. The highest occurrence on plants was observed on 26 July. Subsequently, the infestation decreased, probably due to the increase in temperature in mid-summer. Various studies on the ecology of aphid populations report a rapid population decline during the mid-summer, with host plants without aphids or with a lower abundance compared to the population abundance in early-summer and spring (Müller *et al.*, 1999; Weisser, 2000; Karley *et al.*, 2004). A major part of the aphids sampled on zucchini leaves were apterae, and in many cases colonies were formed by just an adult aphid and a few nymphs. Interestingly, the abundance of aphids on leaves was significantly higher on control plants. *Trichoderma* is known to be involved in priming, the activation of plant defense prior to invasion, and up-regulated several Serine/threonine- and Leucine-rich repeat protein kinases that activate defense against pests (Coppola *et al.*, 2019b). *Trichoderma* colonization can generate a pre-alerted state of "priming" to face incoming pest attacks more efficiently (Reimer-Michalski & Conrath, 2016; Coppola *et al.*, 2019b), inhibiting the development and reproduction of aphids on the leaves of inoculated plants. In contrast, the winged aphids caught in pan traps were significantly more numerous in the plots inoculated with *T. harzianum* T-22. These data seem to indicate that *T. harzianum* T-22 makes zucchini plants more attractive to aphids, but this is followed by limited colony production. Winged aphids, while not producing colonies, could contribute to the spread of viruses. Another significant result is the high number of Hymenoptera parasitoid captured in pan traps placed in plots inoculated with *T. harzianum* T-22. The increased attractiveness to parasitoids and the reduced infestation of aphids, as a result of colonization by *Trichoderma*, confirm the results obtained in the laboratory, although with a different plant/aphid/parasitoid system (Coppola *et al.*, 2017). *Trichoderma* influenced the quantity and quality of the volatile organic compound (VOC) blends released by plants (Battaglia *et al.*, 2013). The attractiveness to parasitoids is associated with an enhanced release of VOCs such as methyl-salicylate and β-caryophyllene, known to be among the most active compounds in promoting parasitoids flight orientation (Battaglia *et al.*, 2013; Coppola *et al.*, 2017). *Trichoderma* spp. promotes plant nitrogen uptake

(Harman *et al.*, 2004) giving the plant a higher nutritional value which can orient insects at the time of oviposition.

Trichoderma harzianum T-22 failed to control zucchini pathogens investigated in the experimental field. Both viral diseases and powdery mildew equally attacked the control and *T. harzianum* T-22 inoculated plants, starting from the end of July for viral diseases to middle of August in the case of powdery mildew. Furthermore, severity of both the diseases worsened over time and the symptoms observed on zucchini plants changed from very mild to very strong, reaching the maximum peak at the beginning of September (expressed as 100% of infection). It may be useful to point out that laboratory experiments, testing the induction of resistance pathways by *Trichoderma* fungi, usually use young plants. In our experimental trial, in the field, it was observed that both viral infections and powdery mildew attack spread when plants had already begun to produce fruits. The ontogeny of resistance in plants has been approached with reference to insects (Boege & Marquis, 2005; Boege *et al.*, 2007; Trotta *et al.*, 2021), but still less is known about phytopathogens. For example, Vitti et al. (Vitti *et al.*, 2016), reported favorable effects of *T. harzianum* T-22 in tomato seedling artificially inoculated with cucumber mosaic virus (CMV) in laboratory experiments. The authors showed that *T. harzianum* T-22 was able to promote the induction of tomato defense responses against CMV and also demonstrated that this involves reactive oxygen species (ROS). Another study by Shen et al. (Shen *et al.*, 2018), investigating the dynamic distribution of *A. gossypii* on the incidence of viral disease in six zucchini cultivars, concluded that the ability of zucchini plants to resist aphids attack was not consistent with their capacity to resist viral diseases. Slow transformation rate varieties with a mild disease phenotype in the late growth stage showed strong resistance to the disease.

It cannot be exclude that the resistance induced by *Trichoderma* fungi observed in previous studies could be influenced by the phenological stage of the plant. The present study showed that there is no resistance effect in the field. Probably, the lack of resistance observed can be due to the higher costs of resistance for a plant that is already at the stage of fruit production. This aspect deserves future investigations. In fact, Shen et al. (Shen *et al.*, 2018) showed that the disease resistance ability of zucchini plants always differed among the different growth stages. Overall, no differences in terms of fruit yields were found between zucchini inoculated with *T. harzianum* T-22 and control plants. This is in contrast to Hazef *et al*. (Hafez *et al.*, 2018), Elsisi (Elsisi, 2019), and El-Sharkawy *et al*. (El-Sharkawy *et al.*, 2021) who found an increase in the yield of zucchini plants under greenhouse and filed conditions due to the inoculation of *T. harzianum* T-22.

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GENERAL CONCLUSIONS

The general conclusions of the studies set out in this doctoral thesis are as follows:

1. The behavior of *Macrosiphum euphorbiae*, monitored with EPG, was not affected by treatment with T-22, and both clones are able to reach the phloem and feed. Both clones are not attracted to T-22-treated plants, probably due to the type of volatiles emitted, and the French clone produced more nymphs on control plants. The small differences that were found between the clones during the EPG monitoring and in the fitness bioassays are probably due to genetic differences in the past selective history of these aphids. Therefore, future studies should focus on studying the volatiles emitted by *Trichoderm*a and the pathways activated in the case of different clones.

2. The behavior of *Myzus persicae* differed from that of *M. euphorbiae*. *M. persicae* was attracted to tomato plants colonized by *T. harzianum* and performed better on them. It is possible to assume that there is a balance between available nutrients and the negative effects of resistance induction, which depend on the *Trichoderma* species and the aphid species, or perhaps even the aphid clone.

The differences between *M. persicae* and *M. euphorbiae* could be due to the different damage they cause to the plant, influencing differently the induction of resistance. This last trait represents a gap in current knowledge, so further studies are needed to expand our understanding of it.

3. The studies exposed in the Chapter III and IV are among the few that explore the potential beneficial effects related to the use of the *Trichoderma harzianum* strain T-22 under field conditions. In the Chapter III, the results confirm that colonization with *T. harzianum* T-22 can alter the arthropod community and reduce the abundance of specific pests under field conditions. Indeed, a general increase of piercing-sucking insects was observed in control tomato plants during the flowering stage, while the abundance of chewing arthropods increased in plants inoculated with *T. harzianum* T-22, during the fruit maturity stage.

4. The results in the Chapter IV confirmed the ability of *T. harzianum* T-22 to alter the arthropod community by increasing the attractiveness of zucchini to winged aphids and hymenopteran parasitoids. Unlike the outcomes of other studies conducted in the laboratory, a reduction in pathogen infestation was not observed in zucchini colonized with *T. harzianum* T-22.

FUTURE PERSPECTIVES

In recent years, laboratory studies on the interactions of fungus colonization with insects have increased and several interesting results have been achieved. However, the interaction of the complex *T. harzianum*-plant with insects appears to be complex, due to the variation in the abiotic conditions and to the species and strain specificity of the host plant and insect.

Future researches focusing on insects-fungal symbiont relationships are necessary to better understand the role of these fungi in the interaction with other plant host species or other insect species. In addition, the possible interactions with other fungi with insecticidal or repellent properties should be investigated. Laboratory researches combined with field studies are also required to suggest the possibility of using *Trichoderma* as an alternative green agent in agriculture.

SCIENTIFIC PRODUCTION

Scientific papers on the PhD activity

- Forlano P, Mang SM, Caccavo V, Fanti P, Camele I, Battaglia D, Trotta V. Effects of Below-Ground Microbial Biostimulant Trichoderma harzianum on Diseases, Insect Community, and Plant Performance in Cucurbita pepo L. under Open Field Conditions. Microorganisms. 2022; 10(11):2242. https://doi.org/10.3390/microorganisms10112242

- Caccavo V, Forlano P, Mang SM, Fanti P, Nuzzaci M, Battaglia D, Trotta V. Effects of *Trichoderma harzianum* Strain T-22 on the Arthropod Community Associated with Tomato Plants and on the Crop Performance in an Experimental Field. *Insects*. 2022; 13(5):418. https://doi.org/10.3390/insects13050418

- Caccavo V, Vitti A, Forlano P, Trotta V, Nuzzaci M, Battaglia D, 2021. Can *Trichoderma harzianum* be used to control the aphid transmission of *Cumumber mosaic virus*?. In: Abstracts of presentations at the XXVI Congress of the Italian Phytopathological Society (SIPaV), 16-17 September 2021, University of Verona, Verona (Italy).). *J Plant Pathol* **103**, 1087–1134 (2021). https://doi.org/10.1007/s42161-021-00942-x

Other paper

- Trotta V, Forlano P, Caccavo V, Fanti P, Battaglia D, 2021. A survey of potential vectors of the plant pathogenic bacterium *Xylella fastidiosa* in the Basilicata Region, Italy. Bulletin of Insectology 74 (2): xxx-xxx, 2021 ISSN 1721-8861 eISSN 2283-0332

Conference partecipations

- Caccavo V, Garzo E, Clemente-Orta G, Battaglia D, Fereres A, 2022. Effects of tomato plants inoculated by *Trichoderma harzianum* strain T-22 on aphid behavior. The 14th Australasian Plant Virology Workshop (APVW) in conjunction with the Hemipteran-Plant Interaction Symposium (HPIS-2022) in Melbourne (Australia), 5-9 December 2022 (**oral presentation**)

- Trotta V, Caccavo V, Forlano P, Fanti P, Battaglia D, 2021. A survey of the potential insect vectors of the plant pathogenic bacterium *Xylella fastidiosa* in the Basilicata Region, Italy. XXVI Congresso Nazionale Italiano di Entomologia, Torino (Italia), 7-11 June 2021 (**oral presentation**)

- Trotta V, Caccavo V, Forlano P, Fanti P, Battaglia D, 2021. Impacts of single and repeated heat shocks applied at different developmental stages in an aphid-parasitoid system. XXVI Congresso Nazionale Italiano di Entomologia, Torino (Italia), 7-11 June 2021