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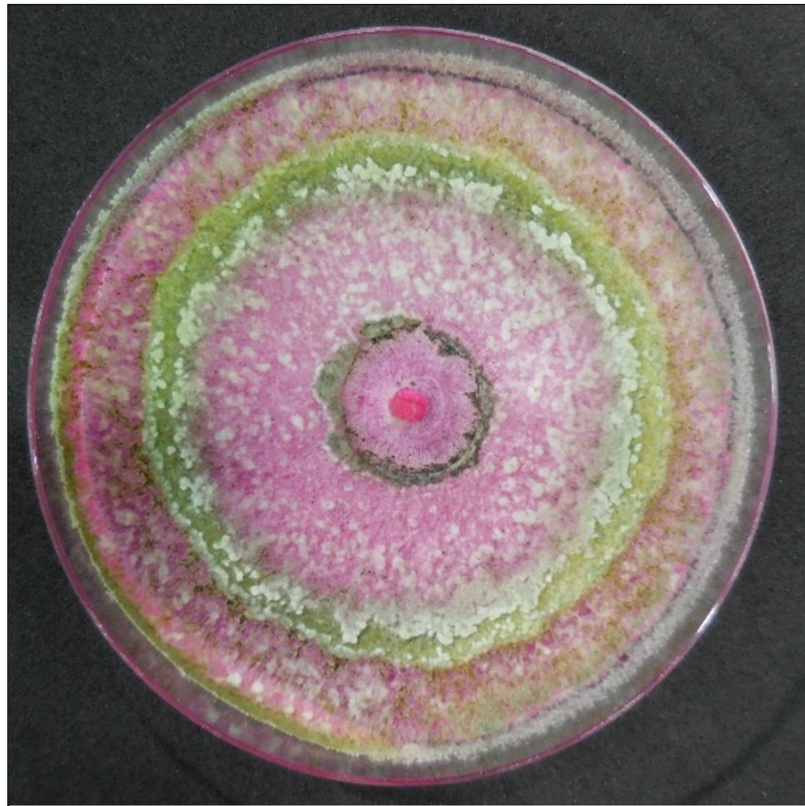
Lignocellulosic materials coated with *Trichoderma atroviride* SC1 increase its persistency in the soil and impact soil microbiota



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Lignocellulosic materials coated with *Trichoderma atroviride* SC1 increase its persistency in the soil and impact soil microbiota

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ABSTRACT:

Trichoderma atroviride SC1 (SC1) was isolated from hazelnut wood and it is effective in the biocontrol of soil-borne pathogens. However, its effectiveness decreases as its population declines in the soil over time. To improve its persistency in the soil, lignocellulosic materials (wood pellet) were tested to be used as carriers to sustain the population of SC1 and facilitate its incorporation into the soil. A method was developed to coat wood pellets of fir, beech and, chestnut with a conidial suspension to reach a preset concentration (i.e. 10^4, 10^5, and 10^6 cfu/ g of wood). The growth of SC1 on each type of wood was compared. Chestnut pellets were excluded from further experiments because they had low counts of colony-forming units (cfu) of SC1. Beech pellets were preferred over fir pellets for showing more suitable physicochemical characteristics for soil application. In addition, for the same wood type, increased initial coating concentrations did not impact the final colony counts of SC1 and no significant difference was observed between the counts of 10^4, 10^5, and 10^6 cfu/g of wood at the end of the experiment. The addition of small quantities of nitrogen increased the final cfu on all types of wood pellets. The growth of SC1 on beech pellets was then tested by adding cheap nitrogen sources namely, soy flour, soy protein isolates, and proteins that originated from animal wastes. The best results were obtained with soy protein isolates (1 g/L) and the population of SC1 reached 10^9 cfu/ g of beech wood. Finally, this carrier of coated beech pellets with soy protein isolates was tested in the soil under controlled conditions, in an experimental greenhouse at 25°C and 60% of soil humidity. The pellets were coated to reach a final concentration of 5×10^5 cfu/ g of beech and 10 g of beech coated pellets were mixed with 1 kg of soil in plastic pots to reach the final contentarion of 5×10^3 cfu/ g of soil. The carrier increased the bacterial richness and diversity of the soil and decreased the fungal ones. The total *Trichoderma* population persisted in the first month and then declined after three months with competition from other bacteria such as *Massilia* spp. and fungi such as *Stachybotrys* spp. and *Mortierella* spp.

Keywords: *Trichoderma,* soil, formulation, wood, coating, metabarcoding

Hamza CHAMMEM obtained his engineering diploma in crop protection from the National Agronomic Institute of Tunis (Tunisia). He holds the distinction *Maxima Cum Laude* for a Master of Science in Sustainable IPM Technologies for Mediterranean Fruit and Vegetable Crops from the Mediterranean Agronomic Institute of Bari (Italy).
In November 2017, he became a Ph.D. candidate in the Departement of Civil, Environmental and Mechanical Engineering at the Univeristy of Trento (Italy) carrying out his research activities at the Edmund Mach Foundation in San Michele all'Adige (TN, Italy). His Ph.D. thesis was focused on developing a new soil formulation for the biocontrol agent *Trichoderma atroviride* SC1, and testing its effect on the microorganisms of the soil.
Together with his supervisor, Prof. Dr. Ilaria Pertot and with Dr. Andrea Nesler, they participated at the first edition of Trentino Startup Valley, where the project "Trichostar" was conceptualised.
Along his research he also engaged in the international scientific communication competition for young researchers and university students FameLab Italy, where he won the third (2019) and fourth (2021) prize at the local selection organized by FameLab Trieste.
Due to his critical thinking, great communication skills and passion for research he became an active part of the executive direction of the first debating association "Tridentum Debating Team" in Italy.
His goal is to disseminate research so it can be understood by everyone.

Chapter 1: Introduction

Trichoderma species (Ascomycetes, Hypocreales) are ubiquitous, and dominante the soil microflora (Domsch et al., 1980; Gams and Bissett, 1998; Klein and Eveleigh, 1998; Killham, 1994). Considering the morphological characteristics of the genus, Bissett (1991 a, b, and c) identified four sections: *Trichoderma*, *Pachybasium*, *Longibrachiatum*, and *Hypocreanum*. To date, 256 species have been described, and some of these species are considered the anamorphs of *Hypocrea* species (Samuels 1996; Samuels, 2006; Bisset et al., 2015). *Trichoderma* spp. reproduce asexually by growing from hyphae fragments or by producing spores (Gams and Bissett, 1998).
Trichoderma spp. are important biological control agents, with specie such as *T. hamatum*, *T. harzianum*, and *T. virens* showing high potential for biocontrol (Harman et al., 2004). This is due to their high competitiveness in the soil as they can survive under various stressful conditions using a variety of enzymes and volatile compounds (Qi and Zhao, 2013; Jalali et al., 2017; Racic et al., 2017). *Trichoderma* spp. tolerate different climates from cool temperate to tropical (Danielson and Davey, 1973a; Papavizas, 1985; Kubicek et al., 2008; Kredics et al., 2014). They can be found in various agricultural soil types as well as in forests, and sandy desert soils (Domsch et al., 1980; Hagn et al., 2003; Roiger et al., 1991). This is due to their versatility in growing and degrading very diverse carbon sources such as sucrose, D-mannose, D-xylose, D-galactose, and D-fructose (Danielson and Davey, 1973b; Domsch et al., 1980; Klein and Eveleigh, 1998; Papavizas, 1985).
Species of the genus *Trichoderma* are able to increase plant growth by changing the structure of roots or releasing substances such as siderophores (to chelate iron) and organic acids. These compounds stimulate the production of auxins and increase the concentration of essential elements such as Ca, P, K, and Mg in the soil. These elements can directly influence shoot height, root proliferation and plant productivity (Lopez-Bucio et al., 2015; Pascale et al., 2017; Porras et al., 2007; Contreras-Cornejo et al., 2009). *Trichoderma* spp. can also prduce different antibiotics such as pyrones (Claydon et al., 1987) and other secondary metabolites (Sivasithamparam and Ghisalberti, 1998). These matbolites helped confer to *Trichoderma* spp. their biocontrol activity by antibiosis, mycoparasitism, or simply by competition for

space and nutrients. These activities against many fungal pathogens have rendered them a suitable substitute for chemical compounds for disease control (Benitez et al., 2004). Mycoparasitism is the direct attack of one fungus on another and is referred to, usually, as direct antagonism (Dix and Webster, 1995) with β-glucanase, chitinase and proteinases being the main lytic enzymes involved in the degradation of the host cell wall (Chet and Baker, 1981; Harman et al., 2004). *Trichoderma* spp. can also inhibit the growth of other microorganisms/pathogens by antibiosis, which is the release of toxic substances such as pyrones, terpenoids, steroids, polyketides, and non-ribosomal peptides (peptaibols) (Sivasithamparam and Ghisalberti, 1998; Howell, 1998).
The fast-growing nature of *Trichoderma* spp. along with all the above-mentioned characteristics grant these fungi the ability to outcompete less aggressive colonisers for space and nutrients (Dix and Webster, 1995; Papavizas, 1985), and makes them very prolific in the biocontrol of soilborne-pathogens. However, the short lifespan of non-native fungi of these species is a major drawback in the soil as well as in storage. Hence, formulations have been developed to solve these issues, and many substrates and carriers have been tested with different *Trichoderma* spp. to facilitate their soil incorporation, support their survival in the soil and prolong the shelf-life of their mycelia biomass, and conidia (John et al., 2011; Jin and Custis, 2011; Kumar et al., 2014; Cumagun, 2014).
Trichoderma atroviride SC1 is an isolate that was isolated from hazelnut wood in northern Italy (Pertot et al., 2008). It tolerates a wide range of temperature (5°C – 30°C) and pH (4-7) and was demonstrated to possess good biocontrol potential (Pertot et al., 2016).

Aims of the Ph.D. thesis

The aims of this thesis were to develop an easy way to introduce conidia of *Trichoderma* spp. into the soil by testing lignocellulosic materials in general, and wood pellets in particular, to be used as carriers of *Trichoderma* spp. for soil application, and to test the effect of such a carrier on the microorganisms of the soil.

Thesis format (outline)

The thesis is composed of six chapters. A general introduction, one submitted review article, two submitted original papers, a conclusion / future perspectives, and finally the references (organised by chapter, in their order of appearance in the text).
The review paper is composed of four different parts discussing the current situation of the survival of *Trichoderma* spp. in the soil with and without formulations, the biocontrol effectiveness of these fungi, and their impact on non-target microorganisms. The third and fourth chapters both comprise an abstract, an introduction, a materials and methods section, a results section, a discussion section, and a conclusion.

Chapter 2: Fate of formulated and non-formulated *Trichoderma* spp. strains after application in the soil and side effects on non-target microorganisms

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Abstract

Trichoderma spp. are saprophytic fungi that have gained increasing attention as biocontrol agents against soil borne plant pathogens. However, the decline of the population of *Trichoderma* spp. in the soil often renders field treatments inefficient. This decline depends on the applied strain of *Trichoderma* spp., the soil abiotic factors, namely temperature, moisture, and pH, and the complex interactions with the microbial biomass of a soil and the plants. Since increasing the initial inoculum concentration of *Trichoderma* spp. does not prevent the decline of the population, formulations were often proposed to overcome this obstacle. Formulations can enhance the proliferation of the fungus and increase the consistency of soil treatments but can be ineffective when the plant pathogen is present at high levels in the soil. In addition, they must avoid any excess of nutrients that can advantage soil borne plant pathogens. Understanding the behaviour of *Trichoderma* spp. and soil microbiota after the inoculation is crucial to enhancing the efficiency of the treatments. Applications of *Trichoderma* spp. can shift the microbial community of the soil, however with a transient effect. Although culture-independent analysis partially clarified the impact of introducing *Trichoderma* spp. on soil microbiota, several aspects of the complex interactions among the plants, and soil microorganisms including plant pathogens during time are still unknown. Moreover, the effect of agricultural practices on the

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survival of introduced isolates of *Trichoderma* spp. needs to be explored to improve biocontrol in practice.

Keywords: *Trichoderma* spp., soil treatment, population dynamics, survival, interaction, microorganisms.

Introduction

The application of microbial biocontrol agents (BCAs) is considered a promising alternative to the use of chemical soil fumigants (Graham and Strauss, 2021). However, many challenges still affect the practical application of BCAs against soil borne diseases on crops. The most important one is the difficulty to replicate the conditions of naturally suppressive soils, which are based on a precise combination of the physicochemical conditions of the soil and the composition of the microbial population (Weller *et al.,* 2002). Since the discovery of the antagonistic properties of *Trichoderma lignorum* (Weindling, 1932) many studies have shown that *Trichoderma* spp. can be effective in the biocontrol of soil borne plant pathogens and several isolates of the genus *Trichoderma* have been implemented for such use (Benítez *et al.,* 2004).

The importance of *Trichoderma* spp. in biocontrol emanates from their diverse mechanisms of action in antagonising soil borne plant pathogens. They are highly competitive in colonising a wide range of substrates, which contributes to the displacement of less competitive microorganisms (Papavizas, 1985). This phenomenon is often referred to as "competition for space and nutrients" and is regarded as an indirect way of antagonism, however, it is highly dependent on the ability of the fungus to proliferate and establish in the soil. Luckily, *Trichoderma* spp. possess other weapons in their arsenal. They are able to induce plant resistance when applied to the root system, by releasing elicitors that can be peptides, proteins, and/or low-molecular-weight compounds that stimulate plant defence responses (Harman *et al.,* 2004). The release of these chemicals elicits the production of ethylene or terpenoid phytoalexins, which are linked to plant resistance to plant pathogens (Howell *et al.,* 2000). Moreover, *Trichoderma* spp. can act by antibiosis through the production of certain secondary metabolites belonging to the class of diketopiperazines, isocyano derivatives, peptaibols, polyketides, pyrones, and terpenoids (Sivasithamparam, and Ghisalberti, 1998). These metabolites have antifungal and antibacterial properties and inhibit the growth of other microorganisms (Benítez *et al.,* 2004). Finally, *Trichoderma* spp. can attack other fungi directly

using a variety of cell-wall-degrading enzymes such as chitinases, glucanases, and proteases (Benítez *et al.,* 2004). The process of this mechanism of action is quite complex and involves four different steps (Chet *et al.,* 1998). The first step consists of the recognition of the plant pathogen by the BCA through the secretion of chemicals by the former that stimulate the latter. After the recognition, *Trichoderma* attaches to the plant pathogen lectins with cell-wall carbohydrates, coils around the plant pathogen, forms appressoria, then starts to produce cell-wall-degrading enzymes and peptaibols, which assist in killing the targeted plant pathogen (Benítez *et al.,* 2004).

However, when it comes to a practical large-scale application, the main challenge is to keep their concentration in the soil equal to or higher than the effective threshold for a sufficient time in order to exert their antagonistic properties against the plant pathogen(s) (Adams, 1990). Moreover, most of the studies focused on the efficacy of *Trichoderma* spp. in antagonising a specific pathogen *in vitro* or under controlled conditions (Kay and Stewart, 1994; Schoeman *et al*., 1996; Gracia-Garza *et al*., 1997; Lewis and Lumsden, 2001), while subsequent application under field conditions often yields inconsistent results. This is due to the complex interaction of the factors that affect the effectiveness of soil treatments. Soil temperature, moisture, pH, organic matter and nutrients content, as well as microbial biomass and composition can influence the rate of soil colonisation by *Trichoderma* spp. (Carreiro and Koske, 1992; Klein and Eveleigh, 1998). Therefore, good understanding of the ecology and dynamics of *Trichoderma* spp. populations in the field is crucial to enhancing the biocontrol efficiency (Chet, 1990; Paulitz, 2000; Gerhardson, 2002).

Several studies addressed the role of the above-mentioned factors on *Trichoderma* spp. survival in the soil, but, unfortunately, due to the different experimental setups and lack of monitoring of the population over time, generalizing conclusions is difficult. This chapter aims to collect and elaborate on available information on the fate of exogenous *Trichoderma* spp. isolates when applied in the soil and the role of formulations or substrates on their survival. Moreover, it provides an- overview of recent findings regarding the factors that can affect the efficiency of soil treatments with *Trichoderma* spp., and the side effects of *Trichoderma* spp. on the microbial communities of soils.

The decline of non-formulated *Trichoderma* spp. after their application in the soil

Soil temperature, moisture, pH, organic matter, nutrient content, and plant types are key factors that influence the proliferation of *Trichoderma* spp. in the soil, whereas soil texture is less relevant to influence their soil colonization (Danielson and Davey, 1973a,b; Widden and Abitbol, 1980; Papavizas, 1985; Eastburn and Butler, 1991). Although *Trichoderma* spp. tolerate a wide range of temperatures, soil temperature has a relevant impact on their enzymatic activity and volatile compound production (Tronsmo and Dennis, 1978). Temperature also affects the water availability in the soil, which in turn controls the germination of conidia and the hyphal growth of *Trichoderma* spp. (Eastburn and Butler, 1991; Dix and Webster, 1995; Clarkson *et al.,* 2004). Generally, *Trichoderma* spp. prefer humid acidic soils, with optimal growth and conidia germination in a pH ranging between 3.5 and 5.6 (Danielson and Davey, 1973c; Domsch *et al.,* 1980).

The proliferation of *Trichoderma* spp. can also be affected by biological factors that include the competition between different *Trichoderma* spp. isolates present in the soil (Widden and Hsu, 1987) and the inhibition by bacterial species such as *Pseudomonas* spp. (Hubbard *et al.,* 1983; de Boer *et al.,* 2003). Unfortunately, early studies focused only on the effect of the soil microbial communities against the antagonistic activity of *Trichoderma* spp., without providing information on the interaction with resident bacterial or fungal species (Naar and Kecskes, 1998; Kredics *et al.,* 2003). Moreover, little is known about the effect of agronomic practices on the dynamics of *Trichoderma* spp. in soil, except for the effect of a crop rotation with soybean, maize, and, potato on the increase of indigenous populations of *Trichoderma* as compared to potato monoculture (Larkin, 2003).

Since different *Trichoderma* spp. isolates can be found in different soil habitats and their biocontrol efficacy depends on the isolate that is used in relation to the targeted soil borne plant pathogen (Bell *et al.,* 1980), the goal of several studies was to transfer effective indigenous isolates from suppressive soils to soils where they do not belong naturally (Wells *et al.,* 1972; Papavizas and Lewis 1981; Lewis and Papavizas, 1991).

The level of indigenous *Trichoderma* spp. in soils, which varies between 10^1 and 10^3 colony forming units (CFU)/g of soil, depends on the combination of physicochemical and biological factors (Papavizas, 1981; Roiger *et al*., 1991; Larkin, 2003; Sariah *et al*., 2005; Longa and Pertot, 2009). When an exogenous *Trichoderma* spp. isolate is introduced into a soil, its fate depends not only on the genetic characteristics of the isolate, but also on the environmental factors that affect the dynamics of the indigenous species. For example, conidial suspensions of *T. harzianum* isolates added to a non-sterile sandy loam soil, at the rate of 10^4 CFU/g of soil, declined over time, reaching a concentration lower than 10^3 CFU/g of soil after four months, which is the natural level for that soil (Papavizas, 1981). A non-native *Trichoderma* spp. isolate can be considered established in a new soil habitat when it maintains a stable population level for a long time after the inoculation (Lewis, and Papavizas, 1984). Several studies showed that this commonly occurs at concentrations similar to those of the indigenous *Trichoderma* spp. (Leandro *et al.,* 2007; Longa *et al.,* 2008; Savazzini *et al.,* 2008; Oskiera *et al.,* 2017). However, the rate of the decline, defined as the decrease in CFU counts of *Trichoderma* spp. population in time, varies for the same isolate and depends on the abiotic and biotic conditions of the soil where it is introduced. For instance, *T. atroviride* SC1 incorporated in three soils (10^6 CFU/g of soil) with different physicochemical characteristics (e.g. two sandy loam and one clay loamy) survived at high rates (between 10^6 and 10^7 CFU/g of soil) 45 days after the inoculation (Longa *et al.,* 2008; Savazzini *et al.,* 2008). In contrast, the decline of *T. atroviride* I-1237 (inoculated at 10^5 CFU/g of soil) occurred just after 21 days in a neutral clay loamy soil, but after three months in an acidic sandy loam soil (Cordier and Alabouvette, 2009).

Increasing the concentration of the *Trichoderma* spp. inoculum commonly does not prevent the decline and/or influence the final concentration of the established population. For example, when a high concentration of *T. atroviride* SC1 conidia was incorporated into the first layer of two types of soils (1.2×10^8 CFU/g of dry soil), it decreased within 12 months to levels close to the concentration of the indigenous *Trichoderma* spp. of those soils to levels ranging from 1×10^1 to 3.9×10^2 CFU/g of dry soil (Longa and Pertot, 2009).

Although assessing the interaction of the effect of every single factor of soils and the genetic traits of each specific isolate may explain the reasons for the decline of any introduced exogenous *Trichoderma* spp. isolate in soils, this approach would be extremely expensive and time-consuming. For this reason, research commonly focuses on the

behaviour of specific *Trichoderma* spp. isolates over time under the specific conditions of the target use (Table 1).

In summary, the soil physicochemical factors influence the fluctuations of indigenous *Trichoderma* spp. populations and determine the fate of any introduced exogenous *Trichoderma* spp. isolate that needs to adapt to a new habitat. The population of the introduced *Trichoderma* spp. isolates usually ends up declining in time even if high inoculum levels are added to the soil. Therefore, more research is needed to understand the impact of the interaction among soil abiotic and biotic factors and the role of agronomic practices to enhance the proliferation of *Trichoderma* spp. in soils. We deem the information provided in Table 1 necessary to create a robust database of studies that report the decline rate of *Trichoderma* spp. and we encourage future studies to follow the same protocol by reporting the initial inoculum, the period of the experiment, the soil conditions, and the final recuperated concentration at the end of the experiment. Moreover, using a unified index for the dynamics of *Trichoderma* spp. such as “fold/week” or “order of magnitude/week” decrease/increase of the population allows comparing the population dynamics of the same *Trichoderma* spp. isolates over time in different soil conditions, which in terms can facilitate evaluating the effects of each soil parameter on the fungus. In addition, following this protocol, comparing between decline rates of two different isolates will become easier even when reported in two separate studies that share similar soil habitats.

Effect of *Trichoderma* spp. formulations and/or nutritional supplements on the fate of the fungal population

To prevent the decline of *Trichoderma* spp., various formulations have been proposed (Gašić and Tanović, 2013). Formulations are the blending of biomass of active ingredients, such as conidia or chlamydospores of *Trichoderma* with inert carriers, to improve the physical characteristics (Kumar, 2013). The biomass of *Trichoderma* spp. is normally produced by submerged fermentation, semi-solid fermentation, or on solid substrates (Lewis and Papvizas, 1991). The most common substrates used to grow *Trichoderma* spp. are agricultural by-products such as wheat bran, sawdust, bagasse, straws,

and other liquid substances such as molasses and jaggery solutions added to inert solids (Papavizas and Lewis, 1981; Lewis and Papavizas, 1991). The final product can be formulated as wettable powders, dusts, gels, emulsions, prill, pellets and granules (Lewis and Papavizas, 1991; Fravel *et al*., 1998). The formulation aims to stabilise the BCA during the production and distribution, increase its shelf life during the storage at room or controlled temperature, protect conidia from the adverse conditions of the soil and/or provide the fungal propagules with nutrients that support the growth and enhance their activity (Burges and Jones, 1998).

Within a formulation, the carrier is the component that facilitates the distribution of the active ingredient in the substrate, and it can be inert such as, Pyrax or talc, a food base, such as powdered wheat bran or soy fibre, or a combination of both (Lewis and Papavizas, 1991). A good carrier should be non-toxic to the BCAs and plants, cheap and available in sufficient quantities, and easy to sterilize to avoid contaminants (Leggett *et al.,* 2011). For the soil application inert carriers, such as, peat, talc, vermiculite, charcoal and alginate pellets, are most commonly used (Lewis and Papavizas, 1991). *Trichoderma* spp. are sensitive to soil fungistasis (Steiner and Lockwood, 1969; Lubeck *et al.,* 2004) and formulated products display enhanced longevity in time as compared to the non-formulated conidia (Table 1). For example, while non-formulated population of *T. harzianum* declined from 10^6 to 10^4 CFU/g of soil eight weeks after inoculation, bentonite-vermiculite formulated conidia of the same isolate did not (Martínez-Medina *et al.,* 2009).

If formulations may help delay the decline of fungal populations of *Trichoderma* spp. over time and therefore, extend the biocontrol effect of the introduced BCA, their effect varies among isolates. For instance, *T. virens* G1-21 and *T. harzianum* T-22, when applied as granular formulations, were still present after 15 weeks in bulk soil at a concentration of 1 - 1.2 × 10^4 CFU/g of soil in contrast to unformulated conidia, but only *T. virens* G1-21 was still detectable after one year (Larkin, 2016).

An efficient formulation may delay the decline of the population of *Trichoderma* spp., but the final outcome always depends on the soil/rhizosphere competence of the specific isolate (Harman, 2000; Sibi *et al*., 2008). Rhizosphere competence is associated to the plant and its resident microflora, which adds an additional layer of complexity to the system, suggesting that the formulation of a *Trichoderma* spp. isolate may need to be adjusted not only to the soil and the isolate, but also to

the crop. In fact, some species of *Trichoderma* spp. isolates showed rhizosphere preferences, as *T. harzianum* and *T. hamatum* that were more often associated with the rhizoplane of cucumber or tomato, respectively, which is probably related to the differences in plant exudates among these crops (Kurakov and Kostina, 2001).

In general, without the addition of a carbon source to the soil, *Trichoderma* spp. isolates tend to decline fast until stabilising at a concentration, which varies according the specific soil conditions, regardless of the initial inoculated population level (Longa *et al.,* 2008). The competitiveness of an introduced *Trichoderma* spp. isolate may increase with the addition to the soil of a carbon source that can support its growth. Several compounds have been tested, as components of the formulation, on various isolates and fungal stages providing different outcomes (Table 1). Many carriers combined with nutrients increased the populations of *Trichoderma* spp. in the soil (Renganathan *et al*., 1995; Smolinska *et al.,* 2014; Medeiros *et al.,* 2020). However, the positive effect on the fungal growth of increasing dosages of the nutritional substrate usually reaches a plateau (Lewis and Papavizas, 1984). On the contrary, increasing the *Trichoderma* spp. inoculum levels in the nutritional substrate does not increase the final concentration in the soil over time. For instance, the application of two rates of *T. harzianum* (10 and 20 g of a talc-based formulation per kg of farmyard manure) to a sandy loam soil resulted in the same proliferation pattern almost eight weeks (60 days) after the inoculation (Prasad *et al*., 2002) and the population of *T. harzianum* increased to 10^8 CFU/g of soil from an initial concentration of 10^4 CFU/g of soil with both doses. Cho and Lee (1999) also observed a similar behaviour with *T. viride* ATCC 52440 in a sandy loam soil (pH=4.6; organic matter =19.3%) by entrapping three wet biomass weights (0.4, 0.8, and 1.4 mg) of the fungus in gluten granules. The number of CFU of *T. viride* ATCC 52440 reached the level of 4.5-6.7 × 10^7 CFU/g of soil nearly 50 days after incorporation into the soil for all tested biomass of the fungus.

The type of propagules of the introduced *Trichoderma* spp. isolate, may have an effect on its fate in soil, with mycelial propagules being more active than conidia, which need to find favourable conditions to germinate. For example, wheat bran added at 1% weight/weight (w/w) did not prevent the decline of the populations of *T. harzianum* and *T. viride*, unless fresh mycelium is used instead of conidia, with an effect on the survival that varied according to the isolate. In fact, when wheat bran was added, *T. harzianum* and *T. viride* increased from an initial concentration of 10^4 to 5 × 10^7 and 10^8 CFU/g of soil, respectively,

before stabilizing at 10^4 and 10^6 CFU/g of soil after 36 weeks, respectively (Lewis and Papavizas, 1984). However, conidia and chlamydospores are preferred over fresh mycelia in the formulation process due to the sensitivity of mycelial propagules to the dry conditions in storage at room temperature (Papavizas and Lewis, 1989; Lewis *et al*., 1990).

Concentrations of nutritive substances in the substrates are important. For example, high concentrations of carbon sources in a formulation do not necessarily guarantee better *Trichoderma* spp. proliferation, because the carbon increase can nourish plant pathogens instead of the introduced inoculum (Kelley, 1976; Cook and Baker, 1983; Cummings *et al.,* 2009). Implementing formulations that can support the growth of *Trichoderma* spp. for long time is the key to guarantee a better proliferation of the fungus, and the optimization of the formulation and/or its nutritional components for a wide range of soils is the challenge for a wide use of *Trichoderma* spp. isolate for soil treatments.

To improve the development of formulations, it is important to enhance our understanding of the ecology of *Trichoderma* spp. their interactions with the microbiota of the soil, and the effect of agricultural practices on their proliferation. In addition, a good knowledge of the plant-pathogen system can improve the choice of carriers, substrates, and additives that must be suited for the mode of action and the delivery of *Trichoderma* spp. to obtain better biocontrol.

Table.1 Fate of formulated and non-formulated *Trichoderma* spp. isolates applied in the soil at a specific concentration.

Strain	**Initial concentration (cfu g^{-1})**	**Final Concentration (cfu g^{-1})**	**Duration of the experiment (week)**	**Soil type and proprieties/greenhouse conditions**	**Food support /Formulation**	**Reference and year**
T. harzianum **T-1** **T-5** **T-14** **H-54** **WT-6** **WT-6-1 (Uv-induced biotype)**	10^4	$\leq 10^3$	18	Sandy loam (pH=6)	No	Papavizas, 1981
T. viride **T-1-R4** ***T.harzianum*WT-6-24**	$10^{4\ a}$ 10^{4a}	10^{6a} 10^{4a}	36	Loamy sand (pH=6.4; 0.4% organic matter)	Bran 1% of soil t(w/w)	Lewis and Papavizas, 1984

***T.hamatum* T382**	2×10^5	10^3	34	Potting mix (75-85% Canadian sphagnum peat moss, 15-20% perlite, 5-10% vermiculite)	Compost added regularly	Leandro et al., 2007
***T. atroviride* SC1**	1.2×10^8	1.15×10^7 5.1×10^c	18	Sandy clay soil (pH=7.78) Sandy loam soil (pH=7.64)	Sterilized boiled rice	Longa and Pertot, 2009
***T.atroviride* SC1**	10^6	10^6-10^7	6	Two sandy loam and one clay loamy soil	Sterilized boiled rice	Longa et al., 2008
***T.atroviride* I-1237**	10^5	1.7×10^4 2.2×10^4	3 13	Neutral clay loam Acidic sandy loam	No	Cordier and Alabouvette, 2009
T. harzianum	10^6	10^4	8	Peat	No	Martínez-Medina et al., 2009

	10^6	10^6			Bentonite-vermiculite	
***T. virens* G1-21** ***T. harzianum* T-22**	$6.5\text{-}7 \times 10^8$	$1\text{-} 1.2 \times 10^4$	15	Bulk soil	Granular formulation	Larkin, 2016
T. harzianum	10^{4}[b] 10^4	10^{8}[b] 10^8	8	Sandy loam (pH= 6.2) Sandy loam (pH= 6.2)	Talc/molasses -based formulation	Prasad et al., 2002
***T.harzianum* th-10**	10^4	10^{13}	8	the rhizosphere soil of banana	Dried banana leaves immersed in jaggery solutions	Thangavelu et al., 2004

[a]Proliferation of fresh three-day-old mycelia

[b]Both applied doses of 10g and 20g of formulation per kg of manure yielded the same cfu counts at 8 weeks

Factors influencing the effectiveness of soil treatments with *Trichoderma* spp.

The difference between general and specific suppression is that the first is related to the biological conditions of a soil (total biomass of microorganisms in a soil acting synergistically to keep plant pathogens under control) and the latter relies on specific agents (a single microorganism that is effective against a specific pathogen, commonly known as fungal antagonism) (Weller *et al.,* 2002). The specific suppression is transferable, i.e. transferring an effective isolate of *Trichoderma* spp. from a suppressive soil to a conducive soil can stop the occurrence of a certain disease, even if the two soils do not share the same microbiota composition. However, as the specific suppression is an induced suppression, its effect is transient because it does not combine both general and specific suppression characteristics, and as the population of the BCA starts to decline, repetitive treatments are needed to control a given plant pathogen by reaching its suppression (Simon and Sivasithamparam, 1989).

Although a *Trichoderma* spp. isolate can have sufficient efficacy (capacity to produce the desired effect under optimal conditions) and effectiveness (consistency of that efficacy under real conditions) against plant pathogens, the efficiency (ability to produce the same result with minimum use of input and waste and at minimal cost) may depend on several factors, such as the abiotic and biotic characteristic of the soil, the suitability of the formulation for large scale application, and the interaction with soil microorganisms other than the targeted plant pathogen (Fig. 1). For example, Adams (1990) considered *Trichoderma* spp. inefficient BCAs because 10^5 CFU/g of soil are required to suppress a pathogen-density inoculum of 2×10^2 CFU/g of soil.

The proportion between the applied biomass of the BCA and the plant pathogen's population density in the soil is considered the most important factor affecting the effectiveness of the soil treatment. The concept that higher concentrations of the BCA result in higher biocontrol efficacy has been commonly accepted (Elad, 1980; Lewis and Papavizas, 1987; Papavizas and Lewis, 1989). However, this concept cannot be generalised. For instance, increasing concentrations of *T. harzianum* and *T. viride* (10^4, 10^6 and 10^8 CFU/g of soil) increased the control of *Meloidogyne javanica*, until it reached a plateau (at 10^8 CFU/g of soil) and further increase of the BCAs (10^{10} CFU/g of soil)

did not result in better biocontrol (Al-Hazmi and TariqJaveed, 2016). If applied under unfavourable conditions, even high inoculum levels may not yield sufficient biocontrol activity. For instance, *T. harzianum* Th-10 applied in the soil at the rate of 4×10^{31} CFU/g of dried banana leaf treated with jaggery solution, resulted in a similar plant disease reduction as a talc-based formulation of the same isolate applied with lower concentrations, with 49.9% of disease incidence reduction as compared to 40.1% (Thangavelu *et al.,* 2004). Although not discussed in the study of Thangavelu *et al*. (2004), investigating the long-term impacts of inundating the soil with high concentrations of a BCA on the dynamic equilibrium of the soil could be very informative.

When high levels of plant pathogen propagules are present in the soil increasing the concentration of the *Trichoderma* spp. BCA can be insufficient to suppress the plant disease (Chet and Baker, 1980). For example, in soil applications of *T. harzianum* to control *Fusarium udum* (the causal agent of the pigeon pea wilt) an increase dosage of the BCA (from 10 to 20 g/kg of farmyard manure) resulted in an increase of disease control (from 42. to 61.5%) at low plant pathogen inoculum (0.48 CFU/g of soil). In contrast, when the same dosages of *T. harzianum* were used with higher concentrations of the plant pathogen (0.69 and 0.72 CFU/g of soil) the increased dosage of the BCA did not result in an increased efficacy, which dropped to 32.3 and 35.3% and to 22 and 30.9%, with the two dosages, respectively (Prasad *et al.,* 2002).

The above-mentioned results suggest that the control of plant disease may depend on many other factors that need to coexist for an efficient biocontrol. These factors may be: i) the choice of a suitable *Trichoderma* spp. isolate against an adequate susceptible plant pathogen that is present in the soil at concentrations that can be managed by the BCA (Cummings *et al.,* 2009; Leggett *et al*., 2011); ii) the use of an efficient formulation that sustains the proliferation of the BCAs in the specific soil conditions without causing increase and/or emergence of other diseases (Papavizas, 1985; Lewis and Papavizas, 1991; Leggett *et al.,* 2011); iii) the outcome of the interaction of introduced *Trichoderma* spp. with the indigenous microbial population of the soil, which can inhibit its biocontrol activity (Hadar, 1984). These requirements explain the low consistency of biocontrol of soil borne plant pathogens by *Trichoderma* spp. (Lewis and Papavizas, 1991; Weller *et al.,* 2002; Cummings *et al.,* 2009) and call for a better understanding of the mechanisms of action involved in the biocontrol of each targeted plant pathogen, and of the interaction between plants, plant pathogens and *Trichoderma* spp.

When it comes to specific plant pathogens' biocontrol, the efficiency of treatments seems to be more related to the type and combination of the mechanisms of action of the *Trichoderma* spp. isolate than to the concentration of its population in the soil (Bae and Knudsen, 2005). In addition, the antibiotics released by *Trichoderma* spp., which vary according to the isolate of the fungus, may display different efficacy levels in relation to the isolate of the plant pathogen used for the elicitation (Dennis and Webster, 1971; Vinale *et al.,* 2009). This might explain the reduced effectiveness in controlling a plant pathogen through the simple increase of populations of *Trichoderma* spp. (Chet and Baker, 1980; Sivan and Chet, 1989). The choice of the appropriate *Trichoderma* spp. isolate that shows high efficacy in controlling a specific plant pathogen is very important to ensure a good disease control, and a deep knowledge of the mechanism(s) of action involved in the biocontrol plays a crucial role in this selection: if biocontrol activity relies strictly on the competition for space and nutrients or modification of the rhizosphere, higher inoculum levels of the BCA might not be effective in controlling high plant pathogen population levels (Sivan and Chet, 1989; Prasad *et al.,* 2002). This is probably due to a lack of nutrients that were already colonized by the plant pathogen, which inhibits the proliferation of *Trichoderma* spp., and/or the ability of high inoculums of the plant pathogen to cause a plant disease even with reduced propagules (Sivan and Chet, 1989). On the other hand, when the biocontrol activity relies on the production of antibiotics, lytic enzymes and/or volatiles (direct mycoparasitism) further research is needed to quantify the effect of increased population levels of *Trichoderma* spp. on the disease suppressiveness. In fact, in the case of direct mycoparasitism using lytic enzymes, higher population levels may provide better control (Ojha and Chatterjee, 2011; Wijesinghe *et al.,* 2011; Elamathi *et al.,* 2018).

In some cases, the higher the concentration of the plant pathogen in the soil, the longer it takes *Trichoderma* spp. to suppress the pathogen's inoculum. For instance, *T. asperellum,* acting by direct mycoparasitism, decreased three plant pathogen inoculum levels of *Thielaviopsis paradoxa*, (10^3 10^4 and 10^5 CFU/g of soil) to concentrations lower than 10^2 CFU/g of soil (the lowest disease causing concentration level), within six, seven and nine weeks from inoculation, respectively (Wijesinghe *et al.,* 2011). Another important aspect to be considered and that requires more studies is the possibility of conidia to migrate and thus colonize wider volumes of soils compared to the treated area. For example, *T. atroviride* SC1 migrates up to 4 m horizontally and 0.3-

0.4 m vertically, passively transported by the water movement in the soil (Longa and Pertot, 2009).

In conclusion, the factors to consider while testing the efficiency of biocontrol with a new *Trichoderma* formulation are the right choice of the BCA regarding mechanism of action, the identification of the minimum concentration of the propagule of *Trichoderma* spp. to inoculate, the selection of the concentration/quantity of additives and carriers and the application rate of the formulation to the soil and the time needed to exert the biocontrol activity for a given plant pathogen inoculum. However, further research is needed to understand the complex interactions between soil microorganisms and the applied *Trichoderma* spp. isolate in order to understand the functioning of a combined effect of general and specific disease suppressiveness on the effectiveness of disease control.

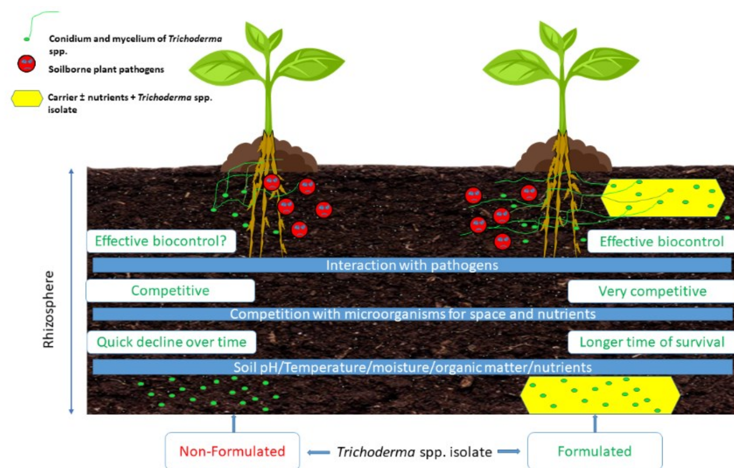

Figure 1. Factors affecting the effectiveness of soil treatments with *Trichoderma* spp. and the role of formulations in prolonging the survival of the biocontrol agent and enhancing its antagonistic activity.

Effect of *Trichoderma* spp. inoculum concentration and formulations on non-target soil microorganisms

The concentration of *Trichoderma* spp. and the formulation may influence the plant and the microflora in the soil and therefore the outcome of the soil treatment. For example, high concentrations of *Trichoderma* spp. can reduce the plant growth due to the production of volatiles that can have phytotoxic effects (Lumsden *et al*., 1990), or can result in shifting the microbial community and in the increase of diseases that were previously under the control of the indigenous *Trichoderma* spp. or other microorganisms (Papavizas, 1985). For instance, applying *T. harzianum* T22 at high rates or repeatedly, displaced *T. virens* and other fungi, which could be a disadvantage for simultaneous applications of different BCAs into the soil (Harman 2000).

Soil borne plant disease occurrence is often linked with an unbalance in the soil microbial community structure. In fact, analyses of infected soils from different studies reported reduced biodiversity in soil plots in which the plant diseaseoccurs as compared to the control (Mao and Jiang, 2020). Moreover, soil microbial communities often act synergistically to control soil borne plant pathogens and altering this equilibrium may lead to the emergence of other diseases that were previously under control. For instance, a combination of fluorescent pseudomonads and non-pathogenic *F. oxysporum* collaborate in soil suppressiveness by competing with plant pathogenic *F. oxysporum* isolates for iron and carbon, respectively (Weller *et al.,* 2002). Displacement of one or the other may lead to the development of diseases caused by plant pathogenic *F. oxysporum* that were previously under control. Therefore, studying the effects of repetitive treatments with *Trichoderma* spp. and their formulations on the microbiota of the soil is crucial. Unfortunately, only interactions between *Pseudomonas* spp. and *Trichoderma* spp. have been studied extensively, whereas little is known on the interactions between *Trichoderma* spp. and non-pathogenic *Fusarium* spp.. Except for some specific conditions, the competition between *Pseudomonas* spp. and *Trichoderma* spp. increased in the case of lack of iron in the soil, resulting in a slower growth of the latter (Hubbard, 1983; Hadar, 1984). In contrast, *Pseudomonas* spp. do not affect the biocontrol activity of *Trichoderma* BCAs when sufficient iron is available in the soil (Bin, 1991; Dandurand and Knudsen, 1993).

Repeated treatments can modify the soil microbial population. For example, the relative abundances of species of *Fusarium* and *Gibberella* decrease and *Trichoderma* and *Chaetomium* increase, after three years of repetitive application of *T. hamatum* MHT11134. This also led to an increase in Actinobacteria and an improvement in the microbial community structure of the soil that positively affected the soil quality (Mao and Jiang 2020).

The knowledge on the effect of the introduction of *Trichoderma* spp. isolates in soil increased dramatically in the last two decades in parallel with the advancements in molecular techniques that have facilitated the study of soil non-culturable microbial population. Initially, the combination of the culture-based technique with pioneering genetic fingerprinting techniques (e.g. ARDRA, DGGE, RAPD, RISA, TGGE and T-RFLP) provided information on the comparative analyses of microbial population dynamics. Using these techniques, the effect of the introduction of *T. atroviride* I1237, *T. atroviride* SC1 and *T. harzianum* T37, could be studied indicating an induced temporary increase in the bacterial community and a transient effect on the fungal community, which lasted only three months (Cordier and Alabouvette, 2009; Savazzini *et al.,* 2009; Huang *et al.,* 2016), before the re-establishment of the previous equilibrium. These studies were conducted in bulk soils and did not account for the impact of plant rhizosphere that can in turn alter the microbial communities. For instance, the addition of several isolates of *Trichoderma* spp. in Pythium infected soils resulted in the abundance of bacterial and fungal populations not because of the introduction of *Trichoderma* spp. themselves, but due to the nutrients leaking from the damaged roots (Naseby *et al.,* 2000). In fact, the selective competition between microorganisms, as well as, the concentration of antibiotics increases whenever the concentration of organic substances increases in the soil (Dennis and Webster, 1971). This should be carefully considered when developing *Trichoderma* spp. formulations.

Plants can affect the growth of *Trichoderma* spp. and can be affected themselves by *Trichoderma* spp. application in the soil as they are plant growth promoters. For instance, *T. asperellum* co-inoculated with *Bacillus* sp. provide better banana seedlings by promoting plant growth by increasing phosphate solubilisation and by favouring auxins and hydrolytic enzymes synthesis (Moreira *et al.,* 2021). *Trichoderma asperellum* M45a and biochar used as a carrier increased the tomato yields, enriched the fungal and bacterial populations by increasing the nutrients availability and soil fertility and increased the resistance of watermelon to Fusarium wilt (Sani *et al.,* 2020). The *Trichoderma* sp.

isolate RW309 inoculated with organic matter altered the fungal community in the rhizosphere, and, in parallel, stimulated nitrogen mineralization and increased soil phosphatase activity (Ye *et al.,* 2020; Asghar and Kataoka 2021).

Formulations can directly and indirectly affect microbial populations of soils, either by promoting the BCA, or stimulating the growth of plant pathogens and other competitors (Amir and Alabouvette, 1992; Smolinska *et al.,* 2016; Barua *et al*., 2018). However, since most carriers used are inert materials, with low carbon and nitrogen content, studies reported an inhibition of biocontrol activities of formulated *Trichoderma* spp. due to competition, only when nutrients were added to the carrier (Kelley, 1976). Generally, when the abiotic and biotic factors of the soil are favourable for the proliferation of *Trichoderma* spp., there is an increase in the total bacterial density, and particularly *Pseudomonas* spp. and actinomycetes (Bae and Knudsen, 2005). *Trichoderma* spp. applied with organic fertilizers also corroborate this result, showing higher abundance of fungi, bacteria and actinomycetes in the treated soil (Ye *et al.,* 2020).

Although initial culture independent techniques gave a better insight in the microbial populations, metagenomics had the most disruptive impact on the analysis of the effects of the introduction of *Trichoderma* spp. on the population dynamics of microorganisms of the soil (Xu, 2006; Friedl and Druzhinina, 2012). The application of *Trichoderma* spp. results in a selective abundance of different functional groups of agronomic importance that promote the plant growth or the development of bacterial and fungal genera with biocontrol activities (Umadevi *et al.,* 2018; Illescas *et al.,* 2020). For instance, Chammem *et al*., (2021) found that wood pellets used as carriers of *T. atroviride* SC1 and incorporated into an agricultural soil increased the richness of the bacterial population and only temporarily decreased its diversity. On the other hand, the carrier decreased both the richness and diversity of the fungal population by increasing fungal genera that were the most adapted to growing on woody substrates such as *Mortierella*, *Cladorrhinum*, and *Stachybotrys*. In another example, *T. harzianum* T-22, applied with chitosan and tea tree oil promoted the growth of the antagonistic fungi *Albifimbria* spp., *Clonostachys* spp., *Penicillium* spp., *Talaromyces* spp. and *Trichoderma* spp. in the carrot rhizosphere, which in turn increased the antagonistic activity towards *Alternaria dauci*, *A. radicina*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Patkowska *et al.,* 2020).

Treatment with *T. asperellum* significantly increased the relative abundance of *Ceratobasidium* sp. besides *T. asperellum* itself and inhibited plant pathogenic fungi such as, *Neonectria* and *Fusarium* (Fu *et al.,* 2021). Inoculation with *T. asperellum* M45a also reduced the fungal diversity of the soil whereas, it increased the bacterial diversity and the relative abundance of plant growth-promoting rhizobacteria belonging to genera such as *Actinomadura*, *Pseudomonas*, *Rhodanobacter* and *Sphingomonas* (Zhang *et al.,* 2020). In another context, *Trichoderma* spp. treatment also increased the selective abundance of beneficial bacterial genera such as, *Nitrospira*, *Sphingomonas*, and *Stenotrophomonas* (Fu *et al.,* 2019).

These studies shed the light on the selective effects of *Trichoderma* spp. and their formulations on the dynamic equilibrium at genus level. Nonetheless, further research is required to assess the functional effects of bacterial communities and their interactions with different isolates of *Trichoderma* spp.. This can lead to combined applications of various BCAs that could act synergistically to mimic conditions of naturally suppressive soils.

Conclusion:

The fate of any *Trichoderma* spp. isolate in any new habitat (transfer of a species from one soil type to another for instance) is difficult to predict as it depends on genetic factors of the *Trichoderma* spp. isolate, abiotic and biotic conditions of a soil, and the composition of the formulation used to sustain the growth of the fungus. The nutritional components of formulations must also be selected carefully considering the side effect on the plant and microbial population in the soil and their combination. A deep knowledge of the *Trichoderma* spp. isolate, the targeted plant pathogen, the crop and the soil factors are therefore crucial for an efficient biocontrol with minimal impact on non-target microorganisms. Nonetheless, further research is needed to decipher the complex interactions between the introduced *Trichoderma* spp., plants, soil borne plant pathogens and all the other soil microorganisms during time especially by investigating the different modes of actions. Moreover, the effect of agricultural practices on the proliferation of *Trichoderma* spp. must be investigated and the co-application of isolates belonging to this genus with other microbial biocontrol needs to be explored for a better biocontrol in practice.

Chapter 3: Wood pellets as carriers of conidia of *Trichoderma atroviride* SC1 for soil application

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Abstract:
The use of biocontrol agents to control soilborne diseases is a promising alternative to chemical pesticides, however, obtaining a homogeneous distribution and incorporation of conidia of fungal biocontrol agents into the soil is often difficult. Several carriers/formulations have been proposed over time, unfortunately without offering an ultimate solution. We propose the use of wood pellets as a carrier of conidia of a saprophytic fungus that has good biodegradation and biocontrol properties (*Trichoderma atroviride* SC1). The coating process is based on the direct spraying of wood pellets with a conidial suspension at different rates. Beech, fir, and chestnut wood pellets were compared in terms of relevant physicochemical traits and efficacy in supporting the growth of the fungus. Beech wood pellets displayed the best characteristics in terms of water holding capacity, swelling properties, and disintegration time. *Trichoderma atroviride* SC1 grows best on beech and fir wood pellets and reaches a plateau after nine days of incubation, regardless of the initial coating concentrations. The addition of small quantities of a nitrogen source as tryptone or soy flour, soy proteins, and a mixture of animal proteins used as pet food to the conidial suspension can increase the growth by ten-folds on all types of

wood pellets. Our results demonstrate that beech and fir wood pellets could be suitable carriers to deliver and sustain the growth of *T. atroviride* SC1.

Keywords: *Trichoderma*; coating; carrier; lignocellulosic materials; nitrogen.

1. Introduction

In recent years several microorganisms have been isolated and developed as biocontrol agents of soilborne pathogens (Idong and Sharma, 2021; Parulekar-Berde et al., 2021). Unfortunately, when an exogenous microorganism is introduced into the soil, its concentration declines rapidly, thus its biocontrol efficacy is reduced over time (Papavizas, 1981; Savazzini et al., 2008; Khare and Arora, 2015). In addition, homogeneous incorporation of small quantities of microbial propagules in a large volume of soil is very difficult to obtain, calling for the development of novel and effective formulations and/or application methodologies (Spadaro and Gullino, 2005; Glare et al., 2012). Microorganisms have a key role in the bioconversion of lignocellulosic waste and the recycling of plant biomass in nature. Wood is composed mainly of cellulose (40-50 %), hemicellulose (25-35 %), and lignin (18-35 %), with minor amounts of organic and inorganic extractives (Anderson, 1958; Pettersen and Rowell, 1984; Tarasov et al., 2018). The degradation of these molecules naturally occurs in the soil and involves a complex of enzymes produced by several microorganisms that act synergistically, with a rate of bioconversion that depends on the plant species and the structure of the microbial population of the soil (Dashtban et al., 2009). Lignin is a heterogeneous polymer with a complex structure, which makes it the hardest part of wood to decompose (Kirk and Farrell, 1987; Lopez et al., 2006). Fungi are generally more efficient than bacteria in degrading the lignin, with Ascomycota having, in general, a lower lignin degradation efficiency as compared to Basidiomycota (Dashtban et al., 2010; Janusz et al., 2017). So far, most studies have focused on white-rot fungi (Basidiomycota) such as *Phanerochaete chrysosporium* and *Coriolus versicolor*, which are the most efficient species in the complete degradation of lignin (Blanchette, 1991; Lopez et al., 2006; Dashtban et al., 2009; Janusz et al., 2017). Ascomycota belonging to the genus *Trichoderma* (Lopez et al., 2007), for example, *T. reesei* and

T. atroviride, are also known for their lignocellulolytic activity, characterized by the production of enzymes such as β-glucosidases and β-xylosidases that degrade, into their simple monomers, cellulose and hemicellulose, respectively (Perez et al., 2002). Although *Trichoderma* spp. are not the most efficient species to degrade lignin, several strains of this genus have been extensively studied because they are efficient antagonists of soilborne pathogens (Harman, 2006; Kovacs et al., 2009).

The combination of mycoparasitic and saprophytic activity of *Trichoderma* spp. has stimulated the idea of using wood bark as a substrate to apply them to the soil as biocontrol agents (Nelson et al., 1983; Kwok et al., 1987) or as mulching to prevent soilborne pathogens (Pellegrini et al., 2014). The use of wood to deliver *Trichoderma* spp. into the soil has several advantages as compared to other carriers (i.e. clay minerals, siliceous materials, etc.; Yusoff et al, 2016) , which lays mostly in the selective preference of these species for this substrate. In fact, *Trichoderma* spp. are more efficient than their microbial rivals, that do not produce cellulases, in growing on wood, which elicits their fast colonization of the soil with subsequently increased competition for space and nutrients and antibiosis activity against other microorganisms, including phytopathogens (Papavzias, 1985). In addition, woody materials are a cheap and environmentally friendly substrate that can sustain high population levels of *Trichoderma* spp. in the soil over time (Chung et al., 1990; Krause et al., 2001). Unfortunately, there are also several limiting factors in the use of barks or wood chips as carriers for *Trichoderma* spp., such as the difficulty to obtain a specific and homogeneous concentration of conidia on wood chips or barks and the slow degradation of these materials in the soil, which prevented so far their practical application in the field.

Wood pellets, made from compacted sawdust and related industrial wastes from the milling of lumber and commonly used as biofuel (Proskurina et al., 2019), could represent an alternative substrate to barks or other woody materials because they are cheap, easy to handle, highly homogeneous in size and weight, and can rapidly absorb humidity, which facilitates their fast disintegration (Acda and Devera, 2014; Artemio et al., 2018; Deng et al., 2019; Lee et al., 2020). With a view to using wood pellets as carriers of *Trichoderma* spp., the strain *T. atroviride* SC1 (Ascomycota, Hypocreales), which was originally

isolated from decaying hazelnut wood, could be a valuable candidate because it is well adapted to woody substrates (Pertot et al., 2008). In addition, wood barks have been already demonstrated to be a good growth substrate for this strain (Pellegrini et al., 2014). In addition, this strain tolerates a wide range of pH and grows in a wide range of temperatures (between 10°C and 30°C) with optimal growth at 25°C, which is a common temperature of many soils in temperate climates during the growing season (Longa et al., 2008). It can use mannose and galactose as carbon sources, which are the main components of the hemicellulose of softwood. Furthermore, it has good lignocellulolytic capacities and it was well studied as a biocontrol agent (Kovacs et al., 2009; Pellegrini et al., 2014; Pertot et al., 2016). The growth of *T. atroviride* SC1 is improved when nitrogen sources, as peptone, tryptone, and nitrate, are added at the rate of 2 g/L to Czapek Dox Liquid media (Oxoid) amended with glucose (10 g/L) or glycine (1 g/L) (Longa et al. 2008).

This research aims to validate a method for coating wood pellets with a conidial suspension of a fungal biocontrol agent. We used *T. atroviride* SC1 as a case study to optimize the method, in terms of selection of the right type of wood pellet and addition of nutrients that could be adapted to other *Trichoderma* spp. strains.

2. Materials and methods

2.1. Physicochemical characteristics of wood pellets

Three types of wood pellets representing the most frequent ones on the European market were used and specifically wood pellets of fir (Baltic Granulas, Latvia), beech (Italwood S.r.l., Italy), and white chestnut (Ledoga S.r.l. GruppoSilvateam S.p.A., Italy). These wood pellets consist of cylinders of 6 mm in diameter that vary in length from 6 to 12 mm. To assess the physicochemical characteristics of the tested wood pellets four experiments were performed to determine the water holding capacity, the swelling of the wood pellets, the disintegration time of the wood pellets, and the carbon, nitrogen, and ash content of wood pellets. All experiments were carried out twice, at room temperature.

2.1.1. Water holding capacity of pellets

The water holding capacity of pellets (WHCp) is defined as the quantity of water that pellets can hold per unit of weight. Two tests were performed to assess the maximal and minimal WHCp. The maximal WHCp is defined as the water that can be retained by pellets under normal atmospheric pressure. It was calculated by placing 1 g of dry pellets (1-3 pellet pieces as a replicate) on a filter paper that was put on the top of a glass beaker (250 mL). Water (50 mL) was poured gradually on pellets and let to freely percolate through the filter paper without applying any pressure to drain it. When the water stopped dripping below the filter (approximately 30 min) the maximal WHCp was calculated by weighing the wet wood with an analytical balance (Ohaus® Scout® Pro balance SP6000, Switzerland) and expressed in mL of water/g of wood pellets. The minimal WHCp is defined as the water that can be retained by pellets under an additional pressure (other than gravity) that in the field can be the result, for example of the impact of machinery on the soil. It was tested following the method of Lips et al. (2009) with modifications. Briefly, 1 g of pellets (1-3 pellet pieces as a replicate) was placed onto a vacuum filter (Stericup®-Merckmillipore, Italy). The device is composed of a sample funnel on top (250 mL) connected to a vacuum chamber at the bottom via a filter chamber. The vacuum chamber was attached to a vacuum pump (Knf LABOPORT® UN 816.1.2-Elettrofor, Italy) to drain the water from the sample funnel through the filter chamber. The wood pellets were kept fully immersed in 50 mL of water for 20 min before applying pressure (-0.1 bar) for 5 min to drain the water. This allows the hydrated wood sample to remain on top of the filter membrane, which was then weighed as mentioned above. The results are presented as mL of water/g of wood pellets. Five replicates per type of wood pellet were used in each of the two tests.

2.1.2. The swelling of wood pellets

The swelling of pellets in aqueous solutions (SWp) is defined as the maximum increase of the volume of a pellet when it absorbs water (maximum WHCp). The SWp is a proxy for the capacity of the pellet to break down in small pieces (sawdust) in the soil and, consequently also for the available substrate surface for the colonization by the biocontrol agent.

To assess the maximum SWp, the pellets were cut into cylinders having a height of 10 mm, while the radius is 3 mm, placed in a rectangular plastic container and a quantity of water equal to the maximal WHCp of each type of pellet was added gradually. The measure of the height (h) and the radius (r) was assessed before adding water (initial; r_i and h_i) and when the pellet stops swelling after adding it (final; r_f and h_f). The SWp was evaluated with the following formula:

$$SWp = \pi r_f^2 h_f - \pi r_i^2 h_i$$

Five replicates per type of wood pellet were used.

2.1.3. The disintegration time of wood pellets

The disintegration time (DTp) is the time required for a full disintegration of hydrated wood pellets, defined as the loss of the typical cylindrical shape of pellets and their break down into sawdust. The shorter is the DTp the faster and the better can be their incorporation into the soil, for example by harrowing. Five pellets prepared as described above were placed onto a Petri dish (90 mm) and a volume of water corresponding to their maximum WHCp was added (replicate). The Petri dishes were gently placed on a shaking rotator (Ika Werke KS 250, Germany) and the disintegration time was assessed at three different speeds of rotations (300, 400, and 500 rpm). The time of the full disintegration of each pellet type was noted when the pellets lost their shape into sawdust. The treatment was prolonged for 12 h for chestnut pellets, which did not get disintegrated also after that time. Five replicates per type of wood pellet were used for each rotation speed.

2.1.4. Carbon and nitrogen content of wood pellets

Carbon and nitrogen content of the wood pellets was measured at the Chemical Unit of the Fondazione Edmund Mach, San Michele all'Adige, Italy, according to standard protocols (ISO 16948) with the combustion method using a CN analyser for elemental analysis (Elementar, Germany) on a sample of 100 mg. The ash content was performed by the gravimetric method after ignition, using a muffle (FM 76, FORNO MAB, Italy) and an analytical balance (AE 100-Mettler Toledo, Italy), following the protocol EN 14775 (Solid biofuels - Determination of ash content).

2.2. Coating the wood pellets with *Trichoderma atroviride* SC1 conidia

2.2.1. Preparation of the pellets and the conidial suspensions of *Trichoderma atroviride* SC1

For each type of wood pellets (beech, fir, and chestnut), the average moisture content was calculated by weighing 100 g of pellets before and after drying them in an oven at 100 °C for 24 h (Dietsch et al., 2014). The moisture content serves to determine the maximum quantity of water to spray during the coating process to avoid their disintegration. Conidial suspensions of *T. atroviride* SC1 conidia produced according to Longa et al. (2008) and stored until use at 4°C were prepared in sterile distilled water (SDW) and three concentrations were adjusted to 10^5 (C1), 10^6 (C2), and 10^7 cfu/mL (C3) using a hemocytometer. The coating was repeated twice for each concentration.

2.2.2. The coating process

Each type of wood pellet was coated by continuously mixing in a mixer (MUM44R1-Bosch, Italy) at a speed of 25 rpm and contemporaneously spraying 0.1 mL/1 g of the conidial suspension at the three concentrations, with a spray bottle (volume of 50 mL). The volume of water suspension applied derived from the previously calculated moisture content of pellets (10%) and the spray lasted 1 min. In this way, while the water was absorbed by the pellets, the conidia stuck to their external surface, reaching the theoretical concentrations of 10^4, 10^5, and 10^6 cfu/g of wood pellets. The coated pellets were let to stabilise at room temperature for 4-5 min and then gently sieved (mesh < 2 mm) to clean the coated pellets from some little wood debris (< 0.2 % in weight) that originated during the process. The cfu counts recuperated after coating is the result of counting colony-forming units (cfu) of *T. atroviride* SC1 immediately after coating wood pellets. The coating accuracy of wood pellets (CAP) was calculated as follows:

$$\mathrm{CAP} = \frac{\text{cfu counts recuperated after coating}}{\text{theoretical concentration applied}} \times 100$$

2.2.3. Assessment of the growth of *Trichoderma atroviride* SC1 on wood pellets

To compare the growth of *T. atroviride* SC1 on the three types of wood pellets, subsamples of 5 g of coated pellets (replicate) per type of wood pellet and conidia concentration were transferred onto a Petri dish (90 mm) and 15 mL of SDW was added to disintegrate pellets. The wood pellets were let to swallow and then gently disintegrated using a sterile spatula. The control consisted of the corresponding conidial SDW suspensions (15 mL) of *T. atroviride* SC1 mixed as described above for the wood pellets in sterile perlite (5 g) to reach the theoretical concentrations of 10^4, 10^5, and 10^6 cfu/g of perlite. Each replicate was sampled immediately D0 (0 days) and D3 (3 days), D6 (6 days), and D9 (9 days) after coating. The assessment of *T. atroviride* SC1 on wood pellets was carried out by counting the cfu and reported as cfu/g of wood pellets. Counting was performed by collecting 4 g from the mix of 20 g of coated wood pellets and SDW or 4 g of perlite mixed with conidia of *T. atroviride* SC1 (described above) in a 50 mL falcon tube, adding 20 mL of SDW, vortexing the mixture for 1 min, performing serial dilutions, and plating 100 µL from an appropriate dilution on a *Trichoderma* semi-selective medium that contained potato dextrose agar (PDA; Oxoid-UK, 39 g/L), rose bengal (Sigma Aldrich- India, 0.1 g/L), chloramphenicol (Sigma Aldrich-China, 0.1 g/L) and streptomycin sulfate (Fluka Biochemika-Italy, 0.05 g/L). After the sampling at D0, all Petri dishes were placed in an incubator (AquaLytic, Germany) at 25 °C in the dark. Three replicates were prepared for each concentration of conidia and type of wood pellet/perlite, for each sampling time.

2.2.4. Effect of the addition of nitrogen sources to wood pellets on the growth of *Trichoderma atroviride* SC1

2.2.4.1 Effect of the addition of tryptone to beech, fir, and chestnut pellets

To assess the effect of nitrogen on the growth of *T. atroviride* SC1 on wood pellets, the above-described experiment was repeated, adding two rates of tryptone (Oxoid, UK) to the suspensions of conidia: 1 and 2 g/L (3 and 6 mg of tryptone/g of wood pellets), which are half and the full quantity recommended by Longa et al., (2008), respectively. Each treatment consisted of 5 g of pellets (replicate) sampled after coating with conidial suspensions of *T. atroviride* SC1 at the two conidia

concentrations and rate of application of tryptone. Sterile distilled water (15 mL) was added to all treatments and to the control which consisted of 5 g of perlite mixed with conidia of *T. atroviride* SC1. The sampling of coated wood pellets (with and without adding tryptone) and of perlite to assess the growth of *T. atroviride* SC1 was performed as described above at D0, D3, D6, and D9. After the sampling at D0, all Petri dishes were placed in the incubator (AquaLytic, Germany) at 25 °C in the dark. Three replicates of each wood type for each conidial concentration and tryptone application rate were considered.

2.2.4.2 Comparing the effect of the addition of cheap nitrogen sources to beech pellets

To identify cheap, but effective nitrogen sources, to be added to the pellets, beech pellets were chosen for further testing based on the fact that beech was the best type of pellet in terms of physicochemical characteristics and growth of *T. atroviride* SC1. Beech pellets were coated with a conidial suspension of *T. atroviride* SC1 (5×10^4 cfu/mL) to reach a final concentration of 5×10^3 cfu/g of wood pellets. Each treatment consisted of 5 g of coated beech pellets that were disintegrated in Petri-dishes (90 mm) by adding 15 mL of an SDW suspension of 1 g/L of soy flour (SF) (EcorNaturaSì Spa, Italy), soy protein isolates (SPI) (EcorNaturaSì Spa, Italy), and a mixture of animal proteins used as pet food (MAP) (Purina, Italy). As MAP is commercialized as solid granules, it was powdered using a coffee grinder before suspending it in water. The control consisted of coated beech pellets disintegrated with 15 mL of SDW. The first sampling was performed immediately after coating (D0), then all Petri-dishes were placed in an incubator (AquaLytic, Germany) at 25°C. The following samplings were performed after 6 (D6), 9 (D9), and 16 days of incubation (D16). The growth of *T. atroviride* SC1 population was assessed by counting cfu as described above. Three replicates for each protein source were carried out.

2.3. Statistical analysis

Statistical analysis was performed using Costat 6.451 (CoHort Software®) and R studio version 3.3.0. In all cases, significance was established at $p \leq 0.05$. The Bartlett and Shapiro-Wilk tests were applied to check the homogeneity of variances and the normality of data, respectively. All data of cfu counts of *T. atroviride* SC1 were log10 transformed before the analysis. The results of each repeated

experiment were pooled, as there was no significant difference between the experiments based on Student's T-test. ANOVA was used with Tukey's honestly significant difference HSD post hoc test ($\alpha = 0.05$) for the data that met the requirements of a normal distribution (comparing the growth of *T. atroviride* SC1 on wood pellets without tryptone/nitrogen source and the disintegration time of wood pellets), and the non-parametric Kruskal Wallis and Dunn's post-hoc (Benjamini-Hochberg *p*-adjustment method) tests ($\alpha = 0.05$) otherwise. Chestnut pellets were excluded from the analysis (ANOVA) that was performed to compare the disintegration time of beech pellets and fir pellets because they did not disintegrate.

3. Results

3.1. Physicochemical characteristics of wood pellets

3.1.1. Water holding capacity of pellets

Beech wood pellets are the best at holding water under pressure (minimal WHCp) as they can retain 1.68 ± 0.14 mL/g of wood pellets, followed by fir pellets (1.14 ± 0.20 mL/g) and chestnut pellets (0.75 ± 0.09 mL/g) (H= 25.31, df = 2, $p < 0.001$). For the maximal WHCp, significant differences between wood types were observed (H= 19.86, df = 2, $p < 0.001$). Chestnut pellets exhibited the lowest value, while there was no significant difference between beech (4.70 ± 0.29 mL/g) and fir (4.53 ± 0.27 mL/g) with p = 0.23 (Table 1).

Table 1

The mean values (± the standard deviation) of the minimal and maximal WHCp of fir, beech, and chestnut pellets.

Wood type	Minimal WHCp (mL/g)	Maximal WHCp (mL/g)
Fir	$1.14^{b} \pm 0.20$	$4.53^{a} \pm 0.27$
Beech	$1.68^{a} \pm 0.14$	$4.70^{a} \pm 0.29$
Chestnut	$0.75^{c} \pm 0.09$	$1.25^{b} \pm 0.30$

In each column, mean values followed by different letters are significantly different ($p \leq 0.05$) according to Dunn's post hoc (Benjamini-Hochberg *p*-adjustment method) test

3.1.2. The maximum swelling of wood pellets

The SWp of the three types of wood pellets differs significantly according to Kruskal-Wallis's test (H= 26.80, df = 2, $p < 0.001$). Chestnut pellets did not swell at all (Supplementary figure A). No significant difference in the height was observed between fir and beech ($p = 0.16$); however, there is an increase in the radius of beech pellets compared to fir ($p = 0.004$) and chestnut pellets ($p < 0.001$). The volume of beech and fir pellets increased 60.39 ± 12.52 and 31.09 ± 12.52 times compared to their initial volumes, respectively (Table 2).

Table 2

The mean values (± the standard deviation) of the final volume (V_f), the swelling (SWp), and the increase of the radius (r_f) and the height (h_f) of fir, beech, and chestnut pellets.

Wood type	V_f (cm^3)	SWp (cm^3)	r_f (cm)	h_f (cm)
Fir	8.78 [b] ± 2.22	8.50 [b] ± 2.20	0.63 [b] ± 0.08	1.75 [a] ± 0.23
Beech	17.06 [a] ± 3.54	16.78 [a] ± 3.54	0.92 [a] ± 0.09	1.59 [a] ± 0.08
Chestnut	0.28 [c] ± 0.00	0.28 [c] ± 0.00	0.30 [c] ± 0.00	1.00 [b] ± 0.00

In each column, mean values followed by different letters are significantly different ($p \leq 0.05$) according to Dunn's post hoc (Benjamini-Hochberg p-adjustment method) test.

3.1.3. The disintegration time of wood pellets

Chestnut pellets did not disintegrate at any of the tested rotation speeds (Fig. 1), even if the treatment was prolonged for 12 h. The DTp of fir and beech pellets significantly differed at each of the three tested rotation speeds and beech was always faster than fir to fully disintegrate ($p < 0.001$).

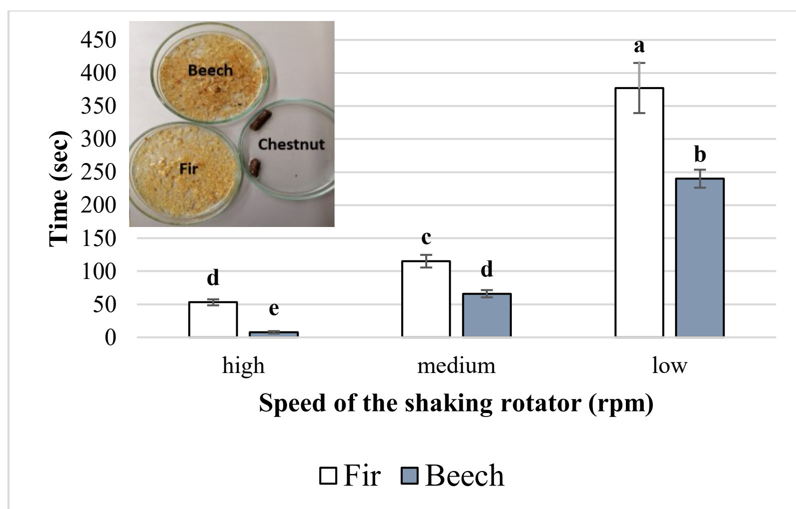


Fig. 1. The disintegration time of wood pellets tested by placing 1 g of wood pellets supplemented with water at their maximal water holding capacity on a shaking rotator. Three speeds were tested: 500 (high), 400 (medium), and 300 rpm (low). Chestnut pellets did not disintegrate even after 12 h at the highest speed of rotation (picture top left). Data from the repeated experiments were pooled. Mean values followed by different letters are significantly different ($p \leq 0.05$) according to Tukey's HSD test.

3.1.4. Carbon and nitrogen content of wood pellets

Nitrogen is two times higher in beech wood (0.23 %) than in chestnut wood pellet (0.11 %) and more than four times higher than the nitrogen present in fir wood pellets (< 0.05 %). The carbon content presented comparable results between fir and beech pellets, with 46.00 % and 46.70 %, respectively, while the chestnut results were slightly lower (43.80 %). Fir presented the lowest ash content of the gravimetric analysis by 0.36 %, followed by chestnut (0.46 %) than beech (0.65 %).

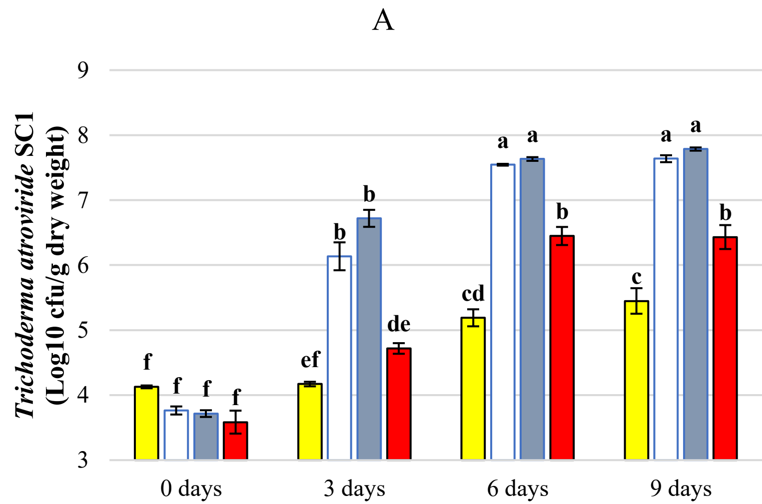
3.2. Coating the wood pellets with *Trichoderma atroviride* SC1 conidia

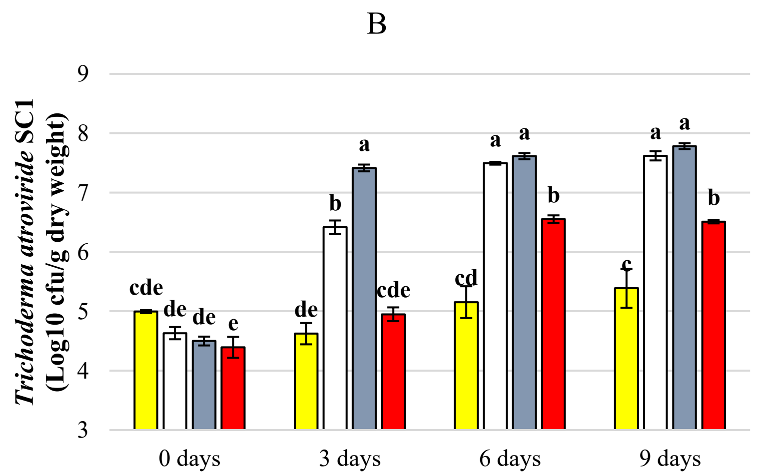
3.2.1. The growth of *Trichoderma atroviride* SC1 on wood pellets

The wood pellets were coated with conidia of *Trichoderma atroviride* SC1 at three coating concentrations C1 (10^5 cfu/mL of conidial suspension), C2 (10^6 cfu/mL of conidial suspension) and, C3 (10^7

cfu/mL of conidial suspension) in order to reach the theoretical concentrations of 10^4, 10^5, and 10^6 cfu/g of wood pellets.

The sampling of the three wood pellets at D0 confirmed cfu concentrations close to the theoretical concentrations of 10^4, 10^5, and 10^6 cfu/g of wood pellets (Supplementary table A1). For the concentrations C1 and C2, no significant difference was observed between the coated pellets and the control according to Tukey's HSD test ($\alpha = 0.05$) (Fig. 2 A, B, and C). For C3 the control reached levels higher than the theoretical concentration on perlite, namely 1.51×10^6 cfu/g and was significantly different from all the treatments (Fig. 2 C). Generally, the CAP ranged between 22 and 68 % with fir presenting the highest values in average for all concentrations (59.36 % ± 9.54 %) and chestnut the lowest (38.10% ± 16.33%) while the average CAP of beech wood pellets was (48.56 % ± 12.20 %). At D3, the growth of the population of *T. atroviride* SC1 showed a fast increase in the cfu counts in the first three days then slowed down in the last three days (D6-D9) to reach a plateau. This behaviour was observed for all wood pellets and at all tested concentrations. The final sampling revealed no significant difference between the cfu counts of *T. atroviride* SC1 of beech and fir (Fig. 2 A, B, and C). The highest colony counts reached 6.26×10^7 cfu/g of beech wood pellets and 4.50×10^7 cfu/g of fir wood pellets and were registered at C2 (Supplementary table A2). In contrast, *T. atroviride* SC1 did not reach the same levels, when growing on chestnut pellets, and the cfu counts were significantly lower than those recorded with beech and fir pellets with a maximum of 3.95×10^6 cfu/g of chestnut wood pellets (Supplementary table A2). The results also showed that, for the same type of wood pellets, the increased initial concentrations of coating did not affect the final cfu counts of *T. atroviride* SC1 at D9, except for chestnut wood pellets ($p = 0.003$). Pairwise comparisons between the cfu counts of *T. atroviride* SC1 growing on beech and fir at D9 for the three coating concentrations showed no significant difference between treatments with *p*-values equal to 0.15, 0.21, and 0.18, respectively.


A
Trichoderma atroviride SC1
(Log10 cfu/g dry weight)
9
8
7
6
5
4
3
f
f
f
f
ef
b
b
de
cd
a
a
b
c
a
a
b
0 days
3 days
6 days
9 days


B
Trichoderma atroviride SC1
(Log10 cfu/g dry weight)
9
8
7
6
5
4
3
cde
de
de
e
de
b
a
cde
cd
a
a
b
c
a
a
b
0 days
3 days
6 days
9 days

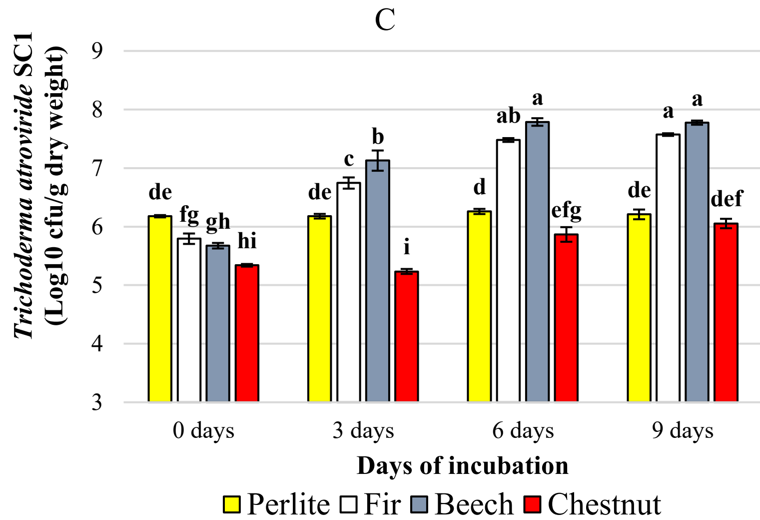


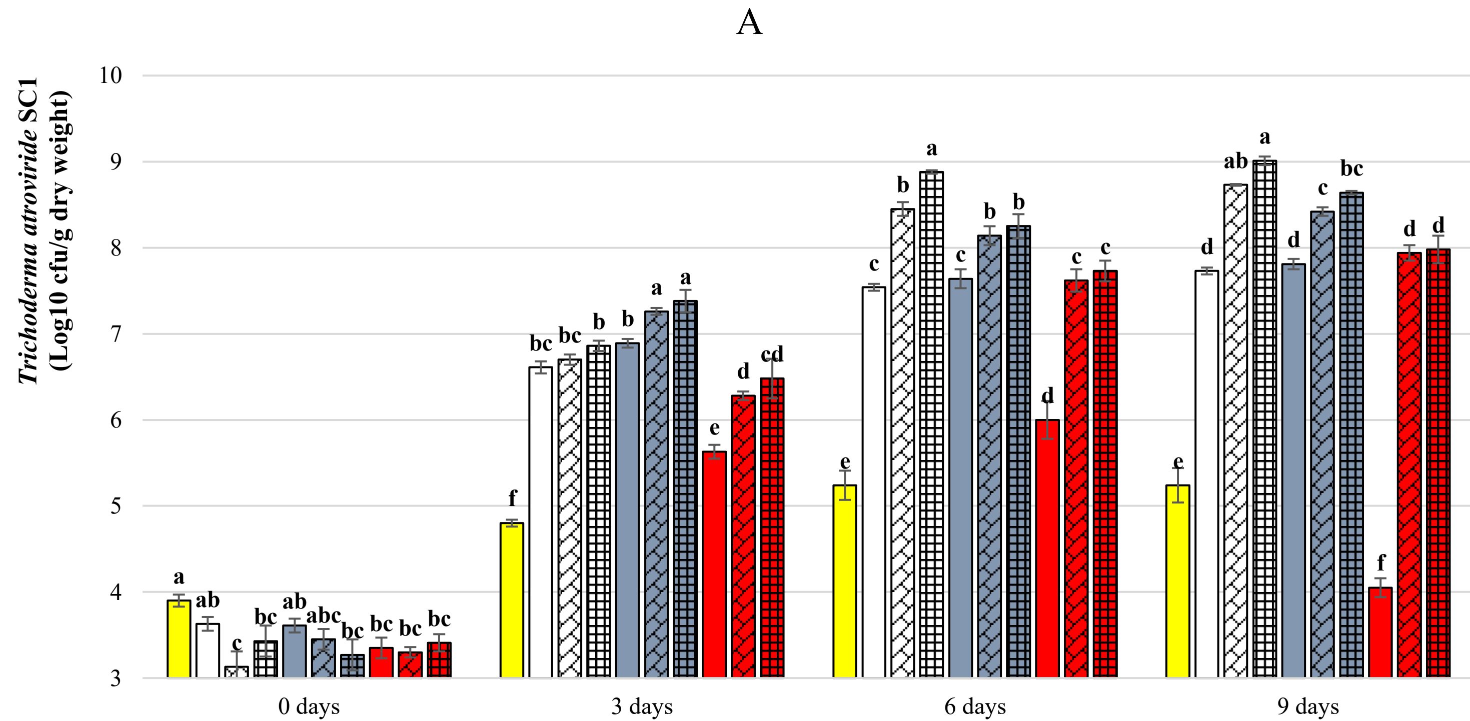
Fig. 2. The growth of *Trichoderma atroviride* SC1 over time on different types of wood pellets coated with a conidial suspension at various initial coating concentrations. Beech, fir, and chestnut pellets were sprayed with conidial suspensions of *T. atroviride* SC1. The three initial concentrations were 10^4 **(A),** 10^5 **(B),** and 10^6 cfu/g **(C)**. The population of *T. atroviride* SC1 grows better on beech and fir wood pellets than on chestnut pellets. Data from the repeated experiments were pooled. Mean values followed by different letters are significantly different ($p \leq 0.05$) according to Tukey's HSD test.

3.3. Effect of the addition of nitrogen sources to wood pellets on the growth of *Trichoderma atroviride* SC1

3.3.1. Effect of the addition of tryptone to beech, fir, and chestnut pellets

The two applied rates of tryptone (1 and 2 g/L) enhanced the growth of *T. atroviride* SC1 on all wood pellets (beech, fir, and chestnut), at both coating concentrations (10^5 and 10^7 cfu/mL), as compared to the untreated controls (perlite and wood pellets). At the end of the experiment (D9), the cfu counts of *T. atroviride* SC1 did not differ between the two concentrations of tryptone for the same type of wood pellets and for both coating concentrations (Fig. 3 A and B). The cfu counts on coated fir wood pellets were significantly higher than those recorded on beech and chestnut wood pellets when tryptone was added

at the highest tryptone concentration (2 g/L). The highest recorded cfu counts in the experiment were registered with fir at the lowest coating concentration (10^5 cfu/mL) and consisted of 1.16×10^9 cfu/g of fir wood pellets and 6.24×10^8 cfu/g of fir wood pellets for the applied concentrations of 1 g/L and 2 g/L of tryptone, respectively. The highest colony counts of *T. atroviride* SC1 on beech wood pellets reached 2.36×10^8 cfu/g of beech and 4.39×10^8 cfu/g of beech wood pellets for the same applied tryptone concentrations. Tryptone enhanced the growth of *T. atroviride* SC1 also on chestnut pellets, however, in all cases, the cfu counts remained significantly lower than the cfu counts that were registered on beech and fir wood pellets at D9.



A
Trichoderma atroviride SC1
(Log10 cfu/g dry weight)
10
9
8
7
6
5
4
3
0 days
3 days
6 days
9 days

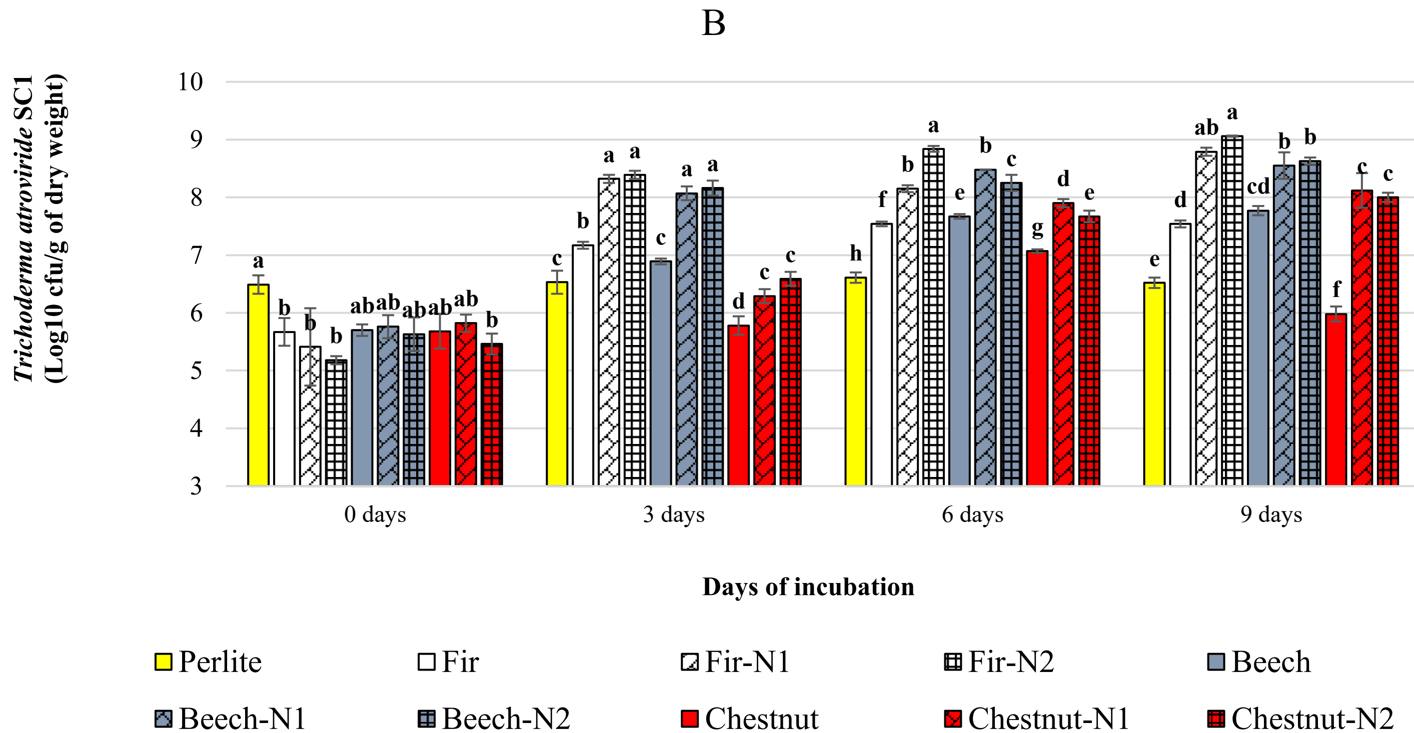

B
Trichoderma atroviride SC1 (Log10 cfu/g of dry weight)
Days of incubation
0 days
3 days
6 days
9 days
Perlite
Fir
Fir-N1
Fir-N2
Beech
Beech-N1
Beech-N2
Chestnut
Chestnut-N1
Chestnut-N2

Fig. 3. The growth of *Trichoderma atroviride* SC1 over time on different types of wood pellets coated with a conidial suspension at two concentrations, 10^4 **(A)** and 10^6 cfu/g **(B),** with tryptone added at the rates of 1 g/L (N1) and 2 g/L (N2) of conidial suspension. Data from the repeated experiments were pooled. At each time point, mean values followed by different letters are significantly different ($p \leq 0.05$) according to Dunn's post hoc test (Benjamini-Hochberg p-adjustment method).

3.3.2. Comparing the effect of the addition of cheap nitrogen sources to beech pellets

The results of cfu counts on beech wood pellets show no significant differences between treatments ($H = 0.589$, df = 3, p = 0.898) at D0. The differences between treatments started to be significant from D6 (Fig. 4) and the best results in terms of *T. atroviride* SC1 growth were obtained with SPI which remained statistically different than the control until the end of the experiment (p = 0.001). At D3, the population of *T. atroviride* SC1 reached population counts of 1.18×10^9 cfu/g of pellets when grown with SPI, followed by MAP and, then SF with 3.05×10^8 cfu/g of pellets and 2.16×10^8 cfu/g of wood pellets, respectively. The cfu counts on the control did not grow more than 1.08×10^8 cfu/g of pellets.

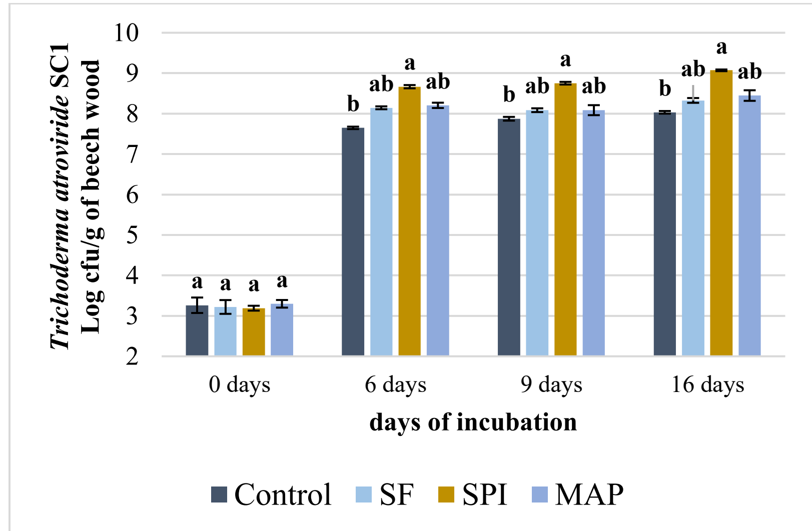


Fig. 4. The growth of *Trichoderma atroviride* SC1 over time on coated beech wood pellets (5×10^3 cfu/g of wood pellets) supplemented with (1 g/L) soy flour (SF), soy protein isolates (SPI), and a mixture of animal proteins used as pet food (MAP). The control received the same amount of water suspension. Data from the repeated experiments were pooled. At each time point, mean values followed by different letters are significantly different ($p \leq 0.05$) according to Dunn's post hoc test (Benjamini-Hochberg *p*-adjustment method).

4. Discussion

Homogeneous distribution of *Trichoderma* spp. in the soil is often hard to achieve as mixing very small quantities of conidia in large volumes of soil is difficult. This is often the reason for the limited commercial use of these biocontrol agents against soil-borne diseases (Vannacci and Gullino, 2000). Substrates such as rice are effective, but highly expensive (Longa et al., 2008; Longa et al., 2009; Longa and Pertot, 2009), and straw or wood barks, persist for a long time as non-decomposed large fragments in the soil, negatively interfering with agronomic practices as sowing or transplanting. In addition, *Trichoderma* spp. strains are usually grown on these carriers before application, and therefore they consist mainly of actively growing mycelium (Pellegrini et al., 2014; Smolinska et al., 2016). For this reason, the exact dosage of active ingredient per unit of soil is difficult to standardize and stabilize and the resulting product has commonly a limited shelf-life. The use of wood pellets as carriers of *Trichoderma spp.* strains can overcome most of these difficulties. In fact, they can help in obtaining a more homogenous distribution of conidia in the field in a simple way: a fertilizer spreader could be used to distribute the pellet on the soil, the wood pellet quickly absorbs the soil/air moisture and disintegrate rapidly, then it can be truly incorporated by harrowing the soil.

Wood pellets presented different physicochemical behaviours and characteristics, which can make them more or less suited for this application. In our experiments, the beech pellets had the highest values of minimal and maximal WHCp, the highest SWp, and the shortest DTp, which makes them the most suited for the use as *Trichoderma* spp. carriers as they can easily absorb water and break down into pieces very rapidly. The same type of experiments can be carried out not only to select the best wood type but also as a quality control test to assess different batches within the same type of wood pellet. High values of minimal WHCp are important in the field to avoid the loss of water by pressure applied by animals, heavy machinery, or simply gravity and therefore to keep optimal water availability for the growth of the fungus (Lips et al., 2009), and high values of maximal WHCp are an advantage when wood is used in fields with scarce water resources, or in sandy soils with low water-holding capacities. The results obtained with beech pellets are in concordance with literature as beech wood is reported to

be very efficient in the uptake of water in the soil (Brischke and Wegener, 2019). Differences between different wood types in the swelling of wood have been well documented (Rowell et al., 2005). Mantanis et al. (1994) reported higher swelling properties for hardwood as compared to softwood, and a negative effect of lignin and extractives on the swelling behaviour, which can explain the minimal swelling of chestnut pellets that we observed.

Wood pellets can absorb relevant volumes of water if the environmental humidity is high and they may break down easily once exposed to high humidity. Different types of pellets have different sensitivities to humidity, which is in general considered a negative trait for wood pellets used as biofuels (Deng et al., 2019). The coating process we propose involves the use of small quantities of conidial water suspension that is sprayed on completely dehydrated pellets, thus restoring a minimal level of humidity that does not interfere with its stability. Our process of coating does not hydrate the pellets enough to promote germination of conidia so they can stay vital as dry conidia (data not shown). In fact, the final moisture level of our coated pellets is about 10 %, which can be compared to formulations previously reported in the literature, such as the talc-based formulation of *T. harzianum* that had a final moisture level of 11 % after drying (Prasad et al., 2002). In general, *Trichoderma* spp. were described as organisms with low osmotolerance, which means that their conidia do not grow under conditions of low humidity (Kredics et al, 2004; Begoude et al., 2007; Longa et al., 2008).

Our coating method has several technical advantages: for example, allows to choose the most suited wood type in terms of WHCp. The high values of CAP and low variability among replicates, from a maximum of 59.00% ± 9.54 % on fir wood pellet to the minimum of 38.44 % ± 16.33 % on chestnut wood pellet, demonstrate high reproducibility and high efficiency in the coating with conidia. These CAP values could be further increased in the scale-up, for example by adapting machines for seed coating or by adding antiflocculants, wetting agents, or binders to the spray suspension. To reach an optimal coating of wood, both the type of liquid and the wood species are important (Vick, 1999). In particular, the wettability of wood depends on the presence of hydrophilic components, such as hemicellulose on the wood surface, or extractives that reduce the wettability and block

the penetration of water below the surface (Mantanis and Young, 1997). When it comes to wood pellets, the pores of woody surfaces increase the penetration of water, which is crucial for the water adsorption by the amorphous regions of cellulose (Karimi and Taherzadeh, 2016). In agreement with previous findings (Pellegrini et al., 2014), fir and beech wood pellets supported a similar growth, always higher than perlite, with beech being faster in reaching the plateau, while the growth on chestnut did not reach similar values. In addition, the growth of *T. atroviride* SC1 on beech and fir pellets is four orders of magnitude greater than those described in the literature as necessary for an effective biocontrol (Adams, 1990). The lower growth of *T. atroviride* SC1 on chestnut pellets might be due to the presence of chestnut components that have antimicrobial activities (Zivcovic et al., 2010; Hao et al., 2012). Generally, the growth of *Trichoderma* spp. on wood relies not only on their ability to produce cellulases, but also on the accessibility of these enzymes to the holocellulose of wood (Lopez et al., 2006; Dashtban et al., 2009). The heterogeneity of the chemical composition of wood and the differences between species in terms of lignin, cellulose, and hemicellulose contents may affect the growth of *T. atroviride* SC1 (Anderson, 1958; Pettersen and Rowell, 1984; Tarasov et al., 2018). *Trichoderma atroviride* SC1 grew slightly better on hardwood (beech wood pellets) than on softwood (fir pellets). This is in concordance with Janusz et al. (2017) who reported lower recalcitrance of hardwood to biodegradation as compared to softwood because the latter is more abundant in lignin and possesses a smaller size of pores. The higher nitrogen content in hardwood as compared to softwood can also stimulate *Trichoderma* spp. to produce lignases, which can explain the faster growth of *T. atroviride* SC1 on beech pellets (Lopez et al., 2006). However, this hypothesis needs to be confirmed by further in-depth analyses, for example of the secretome of the strain *T. atroviride* SC1.

The fast growth of *T. atroviride* SC1 on wood pellets and the plateau observed after nine days of incubation is in line with other studies in the literature that focused on the use of *Trichoderma* using composted hardwood bark (Kwok et al., 1987; Chung and Hoitink 1990; Krause et al., 2001,). For example, *T. harzianum* (#738) required only 14 days after the inoculation to grow on fresh hardwood bark (Nelson et al., 1983). Low initial coating concentrations of *T. atroviride* SC1 have

resulted in the same population levels as those of high coating concentrations, which are in concordance with the results obtained by Prasad et al. (2002) who observed the same behaviour in the soil. Although further studies are needed to implement the application method in the field and define the optimal quantities, these results suggest that the use of pellets as carriers may reduce the use of conidia per unit of soil, thus reducing the cost of soil treatments with *Trichoderma* spp.

The slower growth of the population of *T. atroviride* SC1 when it reaches high concentration levels might be due to the lack of nitrogen. This was confirmed by the results obtained with the addition of tryptone: this easily accessible source of nitrogen has boosted the growth of the fungal population. Our results are in concordance with the literature that reported a better growth of fungi in the presence of organic nitrogen sources (Martin et al., 1987; Hawkins et al., 2000; Rajput et al., 2014; Rajput and Shahzad, 2015) and that the scarcity of nitrogen is a limiting factor to the growth of microorganisms in the soil (Geissler et al., 2010; Kennedy, 2010). Switching from expensive tryptone to a cheap source of nitrogen, as soy protein isolates (3 mg of SPI/g of beech pellets) to beech wood pellets is promising as the population of *T. atroviride* SC1 reached levels higher than 10^9 cfu/g of beech wood pellets.

In conclusion, using wood pellets as carriers for *Trichoderma* spp. conidia can offer many advantages: as they are cheap and available worldwide, they can be easily handled and stored, and once applied in the field may sustain the growth of the fungus. The process is easy to scale-up with existing technologies and the incorporation of wood material can improve the soil quality, by increasing the organic matter content. The characteristics of good water retention and water absorption of wood pellets that lead to easier swelling and disintegration of pellets can facilitate their application in the field. However, further research is required to assess the feasibility of the use of wood pellets as a formulation for the delivery of *Trichoderma* spp. into the soil and the methods to distribute the coated pellets in the soil. Good distribution could be obtained, for example, with a common spreader of pellet fertilizer. That way, the water of irrigation can be used to humidify the pellets, let them swallow and disintegrate, and then disintegrated pellets can be mixed with the bulk soil with a disc harrow

or any other suitable tool. Another way to apply pellets can be by using a potato seeding machinery that allows laying pellets next to the seed, as beech wood was reported to be efficient in absorbing humidity not only on the surface but also when incorporated into the soil (Brischke and Wegener, 2019). Another point that can be improved is the accuracy of coating which can be enhanced by adjusting a seed coating machine, which can offer more control over the functioning of nozzles, the time required to spray a specific quantity of pellets, and the use of additives such as biodegradable binders that might improve the adherence of conidia to the surface of wood pellets.

5. Conclusions

The method of coating wood pellets by spraying a conidial suspension of *T. atroviride* SC1 is promising because it may overcome one of the most relevant difficulties of applying conidia of biocontrol agents in bulk soils in general, and obtain an even distribution of conidia in particular. An industrial advantage of this coating is that the technology of wood pellet production is already set up, we can add any ingredient in the wood to enrich the composition with nutritional factors and adapt it therefore to the needs of various strains, and, possibly also to other fungal species.

The choice of the right type of wood is relevant to obtain an optimal application, as different wood species possess different physical characteristics (water holding capacity, swelling, disintegration, etc.), but also in sustaining the growth of the biocontrol microorganism because of their different chemical compositions (lignin, hemicellulose, and cellulose contents). The wood pellet can serve as a nutritional substrate that can be further improved with the addition of nutritional components as nitrogen sources, for example during the extrusion process. Other wood types or mixtures of other cellulose/lignin sources can be further explored. Wood pellets can also contribute to maintaining organic matter high in the soil, but this needs further investigation along with its effect on plants.

Last, but not least, the use of wood, which is an optimal food base support, to support the fast growth of *T. atroviride* SC1, may increase its competitiveness in soil and reduce the growth of other microorganisms including soil-borne pathogens, but it may also increase the pathogen. Therefore, further studies assessing the impact

on the biocontrol efficacy and exploring the side effect of coated wood pellet on microbial population in the treated bulk soil are needed.

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Conflict of interest

Andrea Nesler is a development engineer at Bi-PA nv. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Chapter 4: Effect of a wood-based carrier of *Trichoderma atroviride* SC1 on the microorganisms of the soil

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Abstract: Wood pellets can sustain the growth of *Trichoderma* spp. in soil; however, little is known about their side effects on the microbiota. The aims of this study were to evaluate the effect of wood pellets on the growth of *Trichoderma* spp. in bulk soil and on the soil microbial population's composition and diversity. *Trichoderma atroviride* SC1 coated wood pellets and non-coated pellets were applied at the level of 10 g·kg^{-1} of soil and at the final concentration of 5 × 10^{3} conidia·g^{-1} of soil and compared to a conidial suspension applied at the same concentration without the wood carrier. Untreated bulk soil served as a control. The non-coated wood pellets increased the total *Trichoderma* spp. population throughout the experiment (estimated as colony-forming unit g^{-1} of soil), while wood pellets coated with *T. atroviride*

SC1 did not. The wood carrier increased the richness, and temporarily decreased the diversity, of the bacterial population, with *Massilia* being the most abundant bacterial genus, while it decreased both the richness and diversity of the fungal community. Wood pellets selectively increased fungal species having biocontrol potential, such as *Mortierella*, *Cladorrhinum,* and *Stachybotrys*, which confirms the suitability of such carriers of *Trichoderma* spp. for soil application.

Keywords: *Trichoderma*; substrate; soil; metabarcoding; diversity; community composition; wood pellets

1. Introduction

Biological control of soil-borne diseases is a valuable alternative to synthetic chemical fungicides (Rahman et al, 2018) and, within the genus *Trichoderma* (Kubicek et al, 2001), several strains have demonstrated good efficacy against soil-borne pathogens such as *Rhizoctonia solani*, *Fusarium* spp., *Pythium* spp., and nematodes of the genus *Meloidogyne* (Ferreira and Musumeci, 2021). However, the success of treatments with *Trichoderma* spp. depends highly on the physicochemical and biological traits of the soil, as well as the rhizosphere competence of the strains used. After the soil treatment, the population of *Trichoderma* spp. normally tends to decrease over time (Papavizas, 1982). This problem is usually addressed by applying high quantities of the biocontrol agent and/or by formulating the biocontrol agent (i.e., as a wettable powder, emulsion, pellets, granules, etc.) or adding nutrients to the formulation that can extend its longevity in the soil (Elad et al, 1980; Lewis and Papavizas, 1987; Papavizas and Lewis, 1989; Kumar et al, 2017). Particular formulations of biocontrol agents comprise aids that can preserve them, favor their delivery to targets, and improve their activity (Burges and Jones, 1998). Another limiting factor that prevents the widespread use of *Trichoderma* spp. in soil treatments is the difficulty in homogenously applying small quantities of conidia in large volumes of soil (Chammem et al, 2021). Although several authors have addressed the effectiveness of formulations and the addition of nutrients (Papavizas et al, 1984; Jin et al, 1992; Whipps, 1997; Shaban and El-Komy, 2001; Thangavelu et al, 2004; Kolombet et al, 2008; Al-Taweil et al, 2010; Sriram et al, 2010; Sriram et al, 2011; John et al, 2011), limited information is available on the effect of such components on the soil microbiome (Cumagun, 2014). More

particularly, the effect of the use of lignocellulosic substrates inoculated with *Trichoderma* spp. on soil fungal and bacterial populations is unknown.

The use of wood pellets coated with conidia of *Trichoderma* spp. might represent an interesting approach for soil treatments (Chammem et al, 2021). For example, *Trichoderma atroviride* SC1 can easily grow on beech wood pellets and reach high population levels (e.g., 10^9 cfu·g^{-1} of wood pellet), especially if complemented with nitrogen sources, such as soy protein isolates. The advantage of using wood pellets is double: they can be easily spread and incorporated in soil by using standard equipment (e.g., using a fertilizer spreader, followed by harrowing) and support the growth of the fungus, which colonize wood before other microbes and then outcompete them. For example, early or simultaneous inoculation of *T. viride* or *T. harzianum* with basidiomycetes that can attack coconut fibers, such as *Trametes versicolor* and *Stereum rugosum*, can protect coconuts from white rot decay, mainly by nutrient competition, but also by toxins that can inhibit the growth of the pathogens (Antheunisse and Burema, 1983). On the other hand, carriers can also modify the soil microbial communities' composition. Since soil microbial communities often act synergistically to control soil-borne pathogens, a change in the soil microbial community structure, and/or a reduction in biodiversity, may affect the occurrence of soil-borne diseases (Mao and Jiang, 2021).

Many factors can contribute to shifting the microbial populations in the soil, such as the soil type and pH, structure, salinity, and moisture, but most importantly, soil organic matter and plant exudates (Fontaine et al, 2003; Compant et al, 2019). Generally, adding organic matter to the soil enhances the microbial activity (Chirinda et al., 2008; Hou et al., 2017; Vermeire et al., 2018), while the use of mineral fertilizers can reduce fungal diversity (Bärlocher et al., 2016, Cai et al., 2019). The inoculation of *Trichoderma* spp. without organic matter has a transient effect on the microbial population of the soil (Cordier and Alabouvette, 2009; Liu et al., 2008), and combining the application of *Trichoderma* spp. with organic composts and bio-organic fertilizers has been proposed as an alternative to mineral fertilizers; in fact, adding these species to the substrate can increase soil fertility and microbial biodiversity (Ye et al., 2020; Asghar and Kataoka, 2021).

The aim of this study was to test the effectiveness of a carrier of *T. atroviride* SC1 made from wood pellets in prolonging the survival of the fungus in the soil and to evaluate its possible impact on the soil microbiota by metabarcoding analysis of the microbial communities. Although this case study is based on the use of a specific carrier (beech wood pellet) and a specific strain (*T. atroviride* SC1) in a single soil, the protocol could be replicated for other similar combinations, for future comparison.

2. Materials and Methods

2.1. Coating the Wood Pellets with *Trichoderma atroviride* SC1, Soil Treatments and Experimental Design

Beech wood pellets (Italwood S.r.l., Piovene Rocchette , Italy) were used as carriers to deliver *T. atroviride* SC1 to the soil. Wood pellets were coated with conidia, according to Chammem et al. (2021). Briefly, beech pellets (100 g) were dried in an oven at 120 °C for 12 h, sprayed with soy protein isolates (30 $mg \cdot mL^{-1}$ corresponding to a final rate of 3 $mg \cdot g^{-1}$ of pellets) and coated with 0.1 mL of *T. atroviride* SC1 conidia sterile water suspension (SDW) with a spray bottle, while continuously mixing in a mixer (MUM44R1- BSH Elettrodomestici S.p.A., Milan, Italy) at a speed of 25 rpm. Conidia were prepared according to Longa et al. (2009) and adjusted with a hemocytometer at 5×10^6 $conidia \cdot mL^{-1}$ to reach a final concentration of 5×10^5 $conidia \cdot g^{-1}$ of pellets. The wood pellets were used immediately after coating.

The experiment was carried out under controlled greenhouse conditions, at a temperature of 25 ± 1°C and relative humidity 70 ± 10%, in 2020. The coated wood pellets were applied to a bulk of sandy loam soil (69.7% sand: 26.3% lime: 4% clay, pH 8) collected in San Michele all'Adige, Italy (N 46.182315, E 11.118804), representing a typical agricultural soil of this region (apple orchards). The soil was mixed thoroughly, sieved, and then distributed into 20 plastic pots (Mongardi, Ferriera di Buttigliera Alta , Italy; 2L) at 1 kg of $soil \cdot pot^{-1}$. A randomized block design was used, with four treatments and five replicates (pots) each: bulk soil (untreated control; Ctr), soil mixed with non-coated wood pellet (Trt1), soil mixed with a conidial SDW suspension *T. atroviride* SC1 (Trt2), and soil mixed with of *T. atroviride* SC1 coated pellets prepared as described above (Trt3). The

final estimated concentration of *T. atroviride* SC1 conidia in Trt2 and Trt3 was 5 × 10^3 conidia·g^{-1} of soil. The wood pellets in Trt1 and Trt3 were applied by laying them on the soil surface (10 g·pot^{-1}), spraying 50 mL of SDW pot^{-1}, letting them swell and disintegrate (20 min), and gently mixing the broken-down pieces in the soil. The Ctr and Tr2 were sprayed with 50 mL of SDW per pot^{-1}. After calculation of the water holding capacity of the soil using the percolation method, the soil was kept at 60 ± 10% humidity, by weighing the pots every two days and adding the quantity of water that was lost by evaporation. The experiment (E1) was repeated after one week (E2).

To ensure the absence of *T. atroviride* SC1 in the soil, real-time PCR primers and probes, designed for the detection and quantification of *T. atroviride* SC1 (Savazzini et al., 2009), were used to check the bulk soil before the experiments.

2.2. Soil Sampling

The growth of *T. atroviride* SC1 was monitored by sampling the soil immediately after completing the treatments (12 h, D0), every 15 days in the first two months (D15, D30, D45, D60), and the final sampling was carried out after ninety days (D90). Samples of soil (5 g) were collected from each pot (replicate) by taking 1 g from the center of the pot and 1 g from each of its four corners. The samples were put in 50 mL sterile Falcon tubes (Merk Life Science S.r.l., Milan, Italy) and thoroughly mixed. Colony forming unit (cfu) counting was performed by suspending 1 g from each falcon tube in 10 mL of SDW, vortexing for 1 min, and plating 100–200 µL from the SDW suspension on a *Trichoderma* semi-selective medium that contained potato dextrose agar (Oxoid, Basingstoke, UK, 39 g·L^{-1}), rose bengal (Sigma Aldrich, Anekal Taluk, India, 0.1 g·L^{-1}), chloramphenicol (Sigma Aldrich, Beijing, China, 0.1 g·L^{-1}), and streptomycin sulfate (Fluka Biochemika, Milan, Italy, 0.05 g·L^{-1}). The results are reported as cfu·g^{-1} of soil ± the standard deviation.

For metabarcoding analysis, only four replicates from each treatment were considered and were chosen randomly. Samples (1 g) were collected at D0, D15, and D90, lyophilized in a freeze-dryer (HetoLyoLab 3000-Analitica De Mori, Milan, Italy) for 12 h, and stored at −80 °C until use.

2.3. DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted from 500 mg of lyophilized soil samples (96 soil samples) using a FastDNA™ Spin kit (MP Biomedicals, Irvine, CA, USA), following the manufacturer's instructions and was quantified using a NanoDrop™ 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

The library construction and sequencing were performed on the sequencing platform of the Edmund Mach Foundation. Total genomic DNA was amplified using primers specific to either the bacterial and archaeal 16S rRNA gene or the fungal ITS1 region. The specific bacterial primer set 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and the 806R (5′-GGACTACNVGGGTWTCTAAT-3′) was used (Caporaso et al., 2011), with degenerate bases suggested by Apprill et al. (Apprill et al., 2015) and by Parada et al. (2016). Although no approach based on PCR amplification is free from bias, this primer pair has been shown to guarantee good coverage of known bacterial and archaeal taxa (Walters et al., 2016). For the identification of fungi, the internal transcribed spacer 1 (ITS1) was amplified using the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns, 1993) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (White et al., 1990). All the primers included specific overhang Illumina adapters for the amplicon library construction.

For the 16S V4 region, each sample was amplified by PCR using a 25-µL reaction with one µM of each primer. In more detail, 12.5 µL of 2× KAPA HiFi HotStart ReadyMix and 10 µL forward and reverse primers were used in combination with 2.5 µL of template DNA (5–20 ng·μL^{-1}). PCR reactions were executed using a GeneAmp PCR System 9700 (Thermo Fisher Scientific) and the following cycling conditions: initial denaturation step at 95 °C for 5 min (1 cycle); 28 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; a final extension step at 72 °C for 5 min (1 cycle).

For the ITS1 region, each sample was amplified by PCR using 25-µL reaction with 10 µM of each primer. In more detail, 22 µL of premix FastStart High Fidelity PCR System (Roche) and 2 µL forward and reverse primers were used in combination with 1 µL of template DNA (5–20 ng·ul^{-1}). PCR reactions were executed using a GeneAmp PCR

System 9700 (Thermo Fisher Scientific) and the following cycling conditions: initial denaturation step at 95 °C for 3 min (1 cycle); 30 cycles at 95 °C for 20 s, 50 °C for 45 s, and 72 °C for 90 s; final extension step at 72 °C for 10 min (1 cycle).

The amplification products were checked on 1.5% agarose gel and purified using a CleanNGS kit (CleanNA, Waddinxveen, The Netherlands), following the manufacturer's instructions. Afterward, a second PCR was used to apply dual indices and Illumina sequencing adapters Nextera XT Index Primer (Illumina, Berlin, Germany), using seven cycles of PCR (16S Metagenomic Sequencing Library Preparation, Illumina, Berlin, Germany). The amplicon libraries were purified using a CleanNGS kit (CleanNA, Waddinxveen, The Netherlands), and quality control was performed on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA). Finally, all barcoded libraries were pooled in an equimolar manner and sequenced on an Illumina® MiSeq (PE300) platform (MiSeq Control Software 2.5.0.5 and Real-Time Analysis software 1.18.54.0).

2.4. Bioinformatics and Statistical Analysis

Illumina reads were filtered with Bowtie2 v2.3.4.3 (Langmead and Salzberg, 2012) to avoid the presence of Illumina phiX contamination, and quality was preliminarily checked with FastQC v0.11.8 (Andrews, 2010). Primers were stripped using Cutadapt v1.18 (Martin, 2011). Sequences were quality filtered, trimmed, denoised, and amplicon sequence variants (ASVs) were generated with DADA2 v1.14 (Callahan et al., 2016). Denoised forward and reverse ASV sequences were merged and chimeras were removed. Filtered ASVs were checked using Metaxa2 v2.2.1 (Bengtsson-Palme et al., 2016) and ITSx v1.1.2 (Bengtsson-Palme et al., 2013) for targeting the presence of the V4 16S rRNA and ITS1 regions, in archaeal and bacterial sequences and fungal sequences, respectively. Taxonomic assignment of 16S rRNA gene ASVs and ITS based ASVs was performed using a RDP classifier, reimplemented in DADA2 against the SILVA v138 database (Quast et al., 2013) and UNITE 8.2 database (Nilsson et al., 2019), respectively. BIOM objects with bacterial and fungal counts, respectively, were built and imported into the R-4.0.3 statistical environment for further analyses (R Core Team, 2021).

The data of the growth assessment of *T. atroviride* SC1 cfu counts (Figure 1) were log10 transformed to simplify the data analysis, as is commonly the case for colony counts to avoid data skewness. Bartlett's test of homogeneity of variances and Shapiro–Wilk's normality test were used to check the normal distribution of the data. ANOVA and Tukey's HSD tests were performed on log10 transformed data with a normal distribution (test of the evolution of treatments in time), and the non-parametric Kruskal–Wallis and Dunn post-hoc (Benjamini–Hochberg *p*-adjustment method $\alpha = 0.05$) tests were used otherwise (comparing the cfu counts between treatments at each sampling point).

Bacterial and fungal count tables were filtered using the RAM R package, and rare ASVs (relative abundance < 0.1%) were discarded. Relative abundance of taxa at different taxonomic ranks was calculated with the RAM R package (Chen et al., 2020).

Alpha-diversity values were calculated adopting a multiple rarefaction method, implemented in the rtk R package (Saary and Hildebrand, 2020). In detail, richness (observed ASVs) and diversity values (Simpson's index) were generated by averaging the results inferred after 999 rarefactions, starting from a minimum number of 38,256 and 13,418 reads, for 16S rRNA gene and ITS data, respectively. A regression analysis based on linear models was carried out on the richness and diversity values, for each dataset, after inspection with the fitdistrplus R package (Delignette-Muller et al., 2021). In more detail, a machine learning approach based on 9999 bootstrap resampling was adopted to evaluate models in which factors (i.e., experiment, time, and treatment) were considered only for their main effects or also with an interaction. The performance of the models was assessed by means of RMSE (root mean squared error) and R-squared, which measure the prediction error and the proportion of variation explained by each model, respectively (Kuhn, 2008). An analysis of variance (ANOVA) followed, to evaluate the linear model fit. A post-hoc analysis was carried out with pairwise comparisons, based on the estimated marginal means (EMMs) as implemented in the emmeans R package (Lenth et al., 2020). Richness and diversity values were graphically represented as boxplots, using the ggplot2 R package (Figures 2 and 3) (Wickham et al., 2021). A confirmatory analysis based on recursive partitioning (Hothorn et al., 2020) was carried out by considering richness and

diversity variables together in the same model; with experiment, time, and treatment as factors (Figure S1).

Beta diversity calculations were conducted after normalization with the median of ratios method implemented in the DESeq2 R Bioconductor package (Love et al., 2021). Exploratory non-metric multidimensional scaling (NMDS) ordinations were applied to Bray–Curtis dissimilarities. NMDS ordinations were plotted using the ggvegan and ggplot2 R packages (Figure 4) (Simpson, 2021). A multivariate analysis based on PERMANOVA was performed on Bray–Curtis dissimilarities applied to normalized bacterial and fungal count tables, respectively (adonis function, vegan R package) (Oksanen et al., 2019). To confirm the PERMANOVA results, a multivariate generalized model (mGLM) was calculated, including all available factors and based on a negative binomial distribution (confirmed by graphical inspection). The model was assessed by analysis of deviance with a likelihood-ratio-test (manyglm function, mvabund R package) (Wang et al., 2020). ASVs that had abundances significantly different ($p < 0.05$) in at least one factor were extracted from the mGLM results and were used to calculate univariate non-parametric tests for each factor (multtest.gp function, RVAideMemoire R package) (Hervé, 2020). The results of each rank test were corrected by false discovery rate (FDR), and post-hoc pairwise comparisons were performed between the levels in each factor, with a Dunn test followed by Benjamini–Hochberg adjustment (dunntest function, FSA R package) (Ogle et al., 2021). Bacterial and fungal indicator ASVs, respectively, were collapsed at genus level and relative abundances were plotted with the RAM R package (Figure 5).

3. Results

3.1. Impact of the *Trichoderma atroviride* SC1 Coated Beech Wood Carrier on the Growth of *Trichoderma* spp. in Soil

Trichoderma atroviride SC1 DNA was not detected in the original bulk soil. Since the cfu counting does not allow for distinguishing the species/strains of *Trichoderma*, *T. atroviride* SC1 and the indigenous population of *Trichoderma*, are mentioned as *Trichoderma* spp. population throughout the paper.

The *Trichoderma* spp. cfu counts increased rapidly in the first 30 days, until D45 in Ctr and Trt1. For Ctr, the cfu counts were not significantly different at D15 and D30 compared to D0, according to Dunn's post-

hoc test ($p = 0.380$; $p = 0.080$). Then at D45, the counts reached levels significantly different from the ones registered at D0 and D15 ($p = 0.001$; $p = 0.002$). No significant difference was observed between the cfu counts of the *Trichoderma* spp. population at D60 compared to D30 ($p = 0.074$) and D45 ($p = 0.370$), respectively, while it remained significant for D0 ($p = 0.002$) and D15 ($p = 0.004$). At the end of the experiment (D90), a slight decrease in the population was observed ($2.69 \times 10^2 \pm 102.87$ cfu·g^{-1} of soil) and a significant difference was recorded only with D0 ($p = 0.021$). For Trt1, the colonies started to grow faster than Ctr, and a significant difference was detected starting from D30 as compared to D0 ($p = 0.010$). The population of *Trichoderma* spp. continued to grow and became significantly different from the levels observed at D30 a month later at D60 ($p = 0.009$). The levels registered at D90 ($1.00 \times 10^3 \pm 712.57$ cfu·g^{-1} of soil) remained significantly different from the cfu counts at D30, but not different from those of D45 ($p = 0.275$) and D60 ($p = 0.338$). The treatments Trt2 and Trt3, where *T. atroviride SC1* was inoculated at the rate of 5×10^3 conidia·g^{-1} of soil, maintained the same level of cfu count in the first 30 days compared to D0, with $p = 0.065$ and $p = 0.206$ for Trt2 and Trt3, respectively. Then at D45, the population of *Trichoderma* spp. started to decline in Trt2 ($2.54 \times 10^3 \pm 689.00$ cfu·g^{-1} of soil) and Trt3 ($1.40 \times 10^3 \pm 681.09$ cfu·g^{-1} of soil), reaching levels significantly different from the ones registered at D15 ($p = 0.003$; 0.001), and the same was observed between D60 and D30 ($p = 0.012$; $p = 0.001$). The population at D90 was significantly lower than all the other sampling points for Trt2, except for D60 ($p = 0.103$), while no significant difference was observed between D45, D60, and D90 for Trt3 ($p = 0.057$; $p = 0.218$).

Between treatments, at D0, there was a significant difference in the cfu counts between Ctr/Trt1 and Trt2/Trt3 ($H = 31.538$, $df = 3$, $p < 0.001$), which persisted until D45. At D45, no significant difference was observed between the counts of Ctr and Trt1 ($p = 0.052$), Trt1 and Trt3 ($p = 0.054$), or Trt2 and Trt3 ($p = 0.050$). Trt2 and Trt3 were both significantly different from Ctr ($H = 29.067$, $df = 3$, $p < 0.001$). Trt2 registered the highest cfu count among the treatments ($2.54 \times 10^3 \pm 689.82$ cfu·g^{-1} of soil).

At D60 the cfu counts of Trt1 continued to rise and became significantly different from the control ($p = 0.003$). The cfu counts in Trt3 continued

to decrease and became significantly different from the cfu counts in Trt2 ($p = 0.011$).

At D90, the population of *Trichoderma* spp. showed no statistical difference ($p = 0.250$; $p = 0.140$; $p = 0.040$) between Trt1 ($1.03 \times 10^3 \pm 712.71$ cfu·g^{-1} of soil), Trt2 ($1.07 \times 10^3 \pm 417.05$ cfu·g^{-1} of soil), and Trt3 ($6.76 \times 10^2 \pm 232.81$ cfu·g^{-1} of soil), with cfu counts that were significantly higher than the control (H = 23.766, df = 3, $p < 0.001$) (Figure 1).

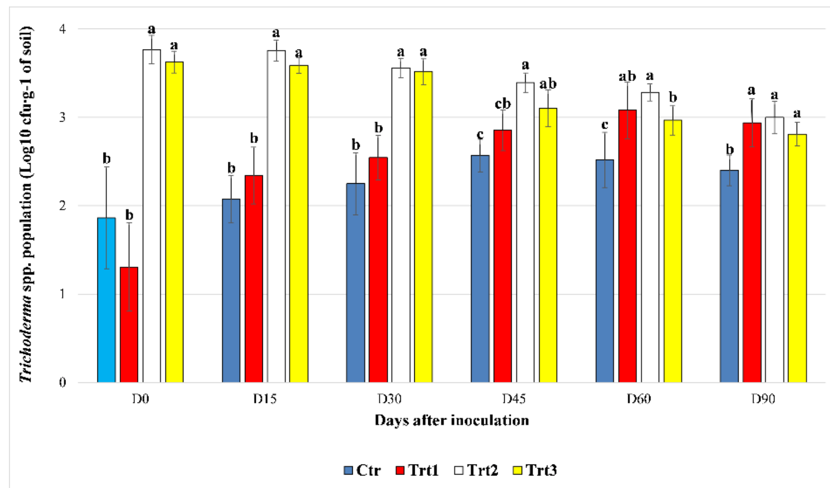

Figure 1. The effect of beech wood pellets coated and uncoated with *Trichoderma atroviride* SC1 on the *Trichoderma* spp. population in the soil at different sampling times post inoculation (D0: after 12h, D15: after 15 days, D30: after 30 days, D45: after 45 days, D60: after 60 days, and D90: after 90 days). Ctr: bulk soil; Trt1: soil with 10 g of beech wood pellets; Trt2: soil with *T. atroviride* SC1 conidial suspension (5×10^3 conidia·g^{-1} of soil); Trt3: soil with 10 g of *T. atroviride* SC1 coated beech pellets (5×10^5 conidia·g^{-1} of beech wood pellets). At each sampling point, different letters indicate significant statistical differences between treatments according to

Dunn's test (α=0.05). Colony counts of the two experiment were pooled.

3.2. Impact of the *Trichoderma atroviride* SC1 coated beech wood carrier on the microorganisms of the soil

A total of 103,970.6 bacterial/archaeal reads and 86,077.48 fungal reads were obtained. The most dominant bacterial phyla, in terms of relative abundance, were Proteobacteria (34%), Crenarchaeota (10%), Actinobacteriota (10%), Bacteroidota (10%), and Acidobacteriota (10%). At genus level, *Massilia* was the most abundant, with 12%, followed by *Pontibacter*, *Sphingomonas*, and *Gaiella,* with (2%) and finally *Microvirga* (1%). The fungal taxa were dominated by Ascomycota (82%), then Basidiomycota (6%), and Mortierellomycota (6%), followed by Chytridiomycota (3%) and Aphelidiomycota (3%). *Mortierella* (6%) was the most dominant fungal genus, followed by *Fusarium* (4%) and *Cladorrhinum* (4%), and finally *Gibberella* (3%), then *Stachybotrys* (2%).

3.2.1. Bacterial and fungal richness and diversity

The bacterial alpha diversity showed statistical differences in richness (observed ASVs) between the different sampling time points for each treatment ($F = 514.48$, $p < 0.001$) and between treatments ($F = 45.94$, $p < 0.001$). The increase in the bacterial richness occurred faster for all treatments (15 days after the inoculation) compared to the control, which showed a significant increase after 90 days (Figure 2). This shows that the introduction of the carrier components, the incorporation of *T. atroviride* SC1 into the soil, and their combined application enhanced the bacterial richness. At D90, the highest effect was observed with Trt3, which presented the highest ASVs (680) among all treatments, while no significant difference was observed between Trt1 and Trt2.

In contrast, the bacterial diversity (Simpson's index) showed differences between treatments at D0 only ($F = 245.07$, $p < 0.001$). The treatments where a carrier was applied with or without *T. atroviride* SC1 (Trt1 and Trt3) affected the bacterial population community and decreased its diversity, as is shown by the low Simpson's values (0.88 and 0.87) for Trt1 and Trt3, respectively. The addition of the carrier,

which contains carbon and nitrogen sources, clearly favored certain genera that are more adapted to those components and decreased the less competent ones (Figure 2). Generally, the introduction of *T. atroviride* SC1 did not affect the bacterial population and the most important factor that governed the bacterial dynamics was the addition of the carrier ($F = 61.40$, $p < 0.001$).

The fungal alpha diversity showed significant differences in richness between the different sampling time points for each treatment ($F = 303.58$, $p < 0.001$), but not between treatments ($F = 0.31$, $p = 0.81$). The richness decreased in time for both control and treatments, with no exceptions (Figure 3).

Simpson's diversity index was significantly different for Trt1 compared to the other treatments, including the control ($F = 5.17$, $p < 0.001$) (Figure 3). This shows the effect of combining the addition of wood to the soil with the application of *T. atroviride* SC1. The fungal diversity in the treatments where *T. atroviride* SC1 was applied with and without wood pellets were not significantly different from the control (Figure 3). In contrast, the fungal diversity in Trt1, which was supplemented only with wood, was significantly different. This shows that adding T. atroviride SC1 can counterbalance the effects of the wood on the fungal diversity. The addition of wood was the main cause for the differences observed between treatments. The recursive partitioning analysis of the bacterial and fungal richness and diversity confirmed the above-mentioned results (Figure S1).

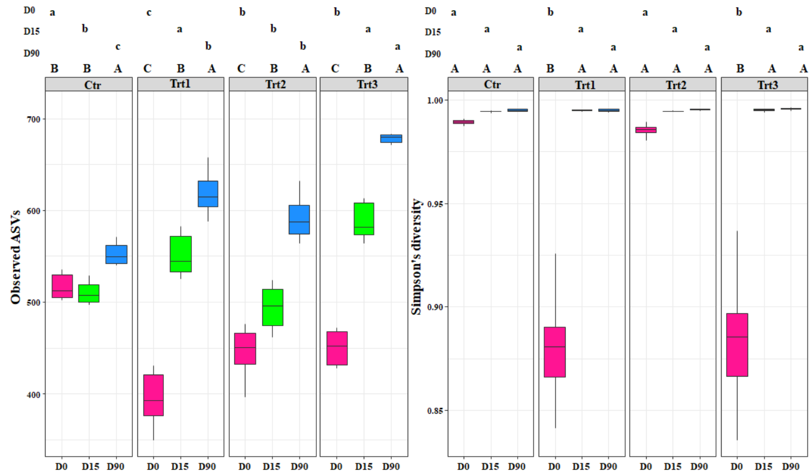

Figure 2. Boxplots of the bacterial richness (observed ASVs) and bacterial diversity (Simpson’s diversity) at different sampling times (D0: after 12 h; D15: after 15 days; D90: after 90 days). Ctr: Bulk soil; Trt1: soil with 10 g of beech wood pellets; Trt2: soil with *Trichoderma atroviride* SC1 conidial suspension at the rate of 5 × 10^3 conidia·g^{-1} of soil; Trt3: soil with 10 g of *T. atroviride* SC1 coated beech pellets (5 × 10^5 conidia·g^{-1} of beech wood pellets). Letters indicate significant differences according to emmeans package (α=0.05) between treatments at each sampling point (lower case letters) or for the same treatment over time (upper case letters). Data of the two experiments E1 and E2 were pooled.

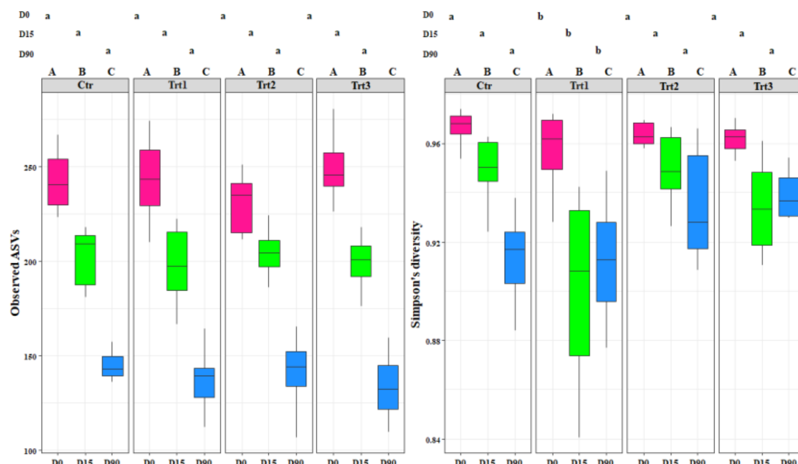

Figure 3. Boxplots of the fungal richness (observed ASVs) and fungal diversity (Simpson's diversity) at different sampling times (D0: after 12 h; D15: after 15 days; D90: after 90 days). Ctr: Bulk soil; Trt1: soil with 10 g of beech wood pellets; Trt2: soil with *Trichoderma atroviride* SC1 conidial suspension at the rate of 5 × 10^3 conidia·g^{-1} of soil; Trt3: soil with 10 g of *T. atroviride* SC1 coated beech pellets (5 × 10^5 conidia·g^{-1} of beech wood pellets). Letters indicate significant differences according to emmeans package (α=0.05) between treatments at each sampling point (lower case letters) or for the same treatment over time (upper case letters). Data of the two experiments E1 and E2 were pooled.

Unsupervised non-metric multidimensional scaling (NMDS) ordinations applied on Bray–Curtis dissimilarities showed that the dissimilarities observed among the bacterial samples were grouped (Figure 4) according to the factors, time and treatment, while it pooled the fungal communities only according to the factor, time (Figure 4). In fact, the permutational multivariate analyses of variance (PERMANOVA) on Bray-Curtis dissimilarities revealed that time was responsible for the biggest portion of the variation in microbiome beta-diversity. The bacterial community differed very significantly according to the factor time (F = 144.95, R^2 = 0.56, p < 0.001) and treatment (F = 20.38, R^2 = 0.11, p < 0.001), as well as their interaction (F = 11.37, R^2 = 0.13, p < 0.001), and less significantly with the factor

experiment (F = 3.47, R^2 = 0.006, p = 0.015). The same was observed for the fungal communities, which varied according to time (F = 21.99, R^2 = 0.23, p < 0.001), treatment (F = 8.59, R^2 = 0.14, p < 0.001), the interaction between the two factors (F = 3.35, R^2 = 0.10, p = 0.001), and finally the factor experiment (F = 2.64, R^2 = 0.014, p = 0.006). These results indicate a high consistency of the effects of the treatments over time (*T. atroviride* SC1 coated and uncoated wood pellets and *T. atroviride* SC1) on soil microbial communities.

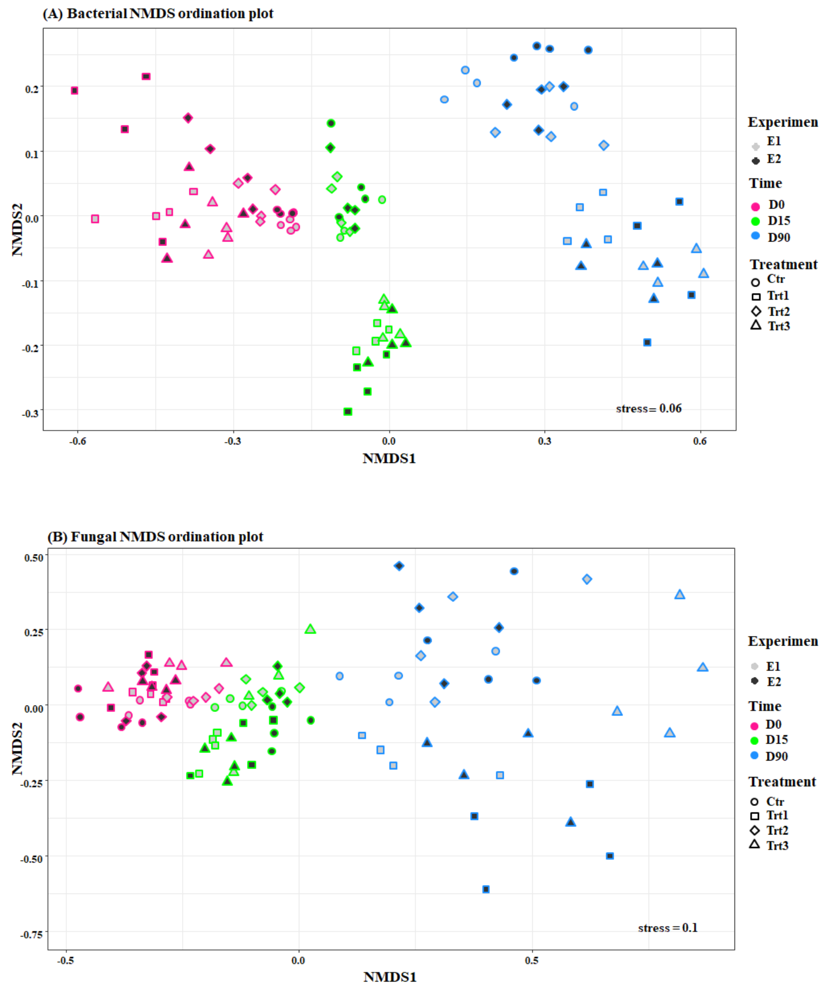

Figure 4. Ordination plots of non-metric multidimensional scale analysis (NMDS) using the Bray-Curtis dissimilarities of bacterial (A) and fungal (B) communities. Pink, green, and blue colours indicate different sampling times (D0: after 12 h; D15: after 15 days; D90: after 90 days) and shows how both fungal and bacterial communities are grouped by time. The colours of the filling grey and black represent the two experiments (E1 and E2 respectively), and different shapes represent the treatments that grouped mainly the bacterial community Ctr: bulk soil;Trt1: soil with 10 g of beech wood pellets; Trt2: *Trichoderma atroviride* SC1 applied to the soil as a conidial suspension at the rate of 5×10^3 conidia g-1 of soil; Trt3: soil with 10 g of *T. atroviride* SC1 coated beech wood pellets at 5×10^5 conidia g^{-1}.

Bacterial and fungal indicator ASVs that have significantly different abundances ($p < 0.05$) in the factor time and treatment were extracted from the mGLM results. The results of the Simpson's bacterial diversity index correspond with an increase in the population of the genus *Massilia,* which was significantly different between the two groups Ctr/Trt2 and Trt1/Trt3 (Figure 5). The results also revealed a significant increase in terms of the relative abundances of the genera *Pontibacter*, *Sphingomonas*, *Gaiella*, *Pedobacter*, and *Microvirga* (Figure 5).

For the fungal community, at D0, *Trichoderma* spp. were higher in Trt2 and Trt3 than in Ctr and Trt1, as expected. Contrarily to the cfu counts, however, this did not change until the end of the experiment, according to Dunn's post-hoc test. The carrier (Trt1/Trt3) selectively and significantly increased the total ASV's of *Cystobasidium*, *Ascobolus*, *Stachybotrys*, *Cladorrhinum*, *Preussia*, and *Stachylidium* (Figure 5).

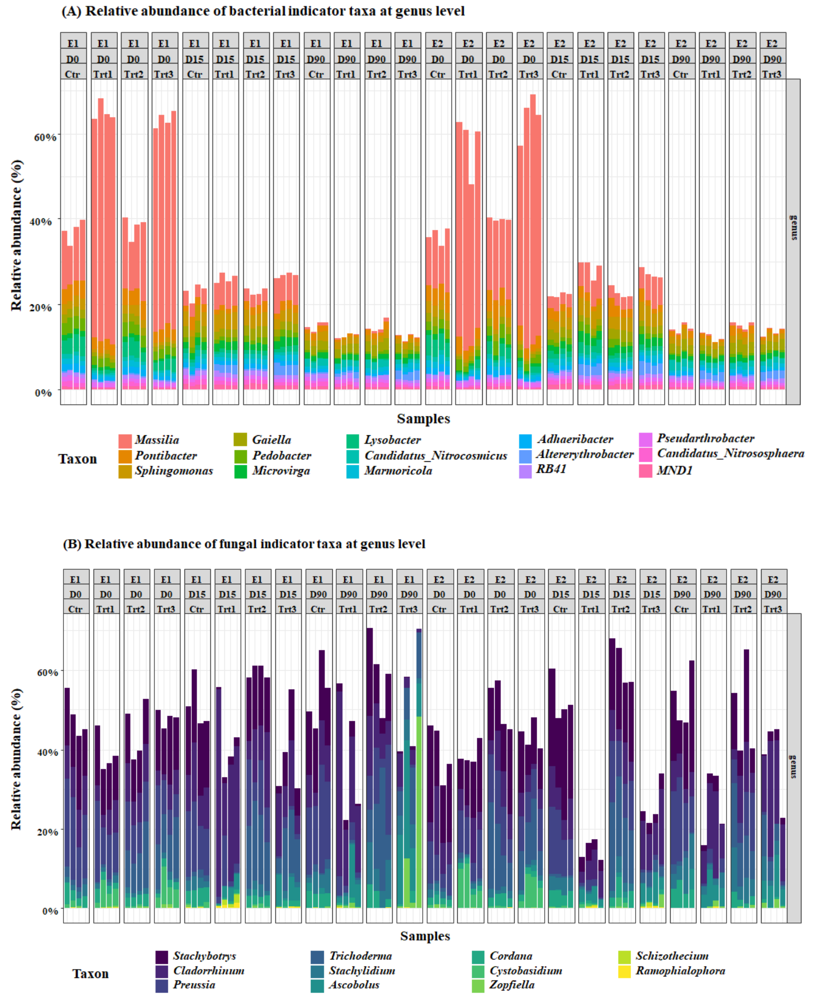

Figure 5. Relative abundance of the most important bacterial (A) and fungal (B) genera. E1and E2: two experiments with four treatments at different sampling times (D0: after 12 h; D15: after 15 days; D90: after 90 days); Ctr: Bulk soil; Trt1: soil with 10 g of beech wood pellets; Trt2: soil with *Trichoderma atroviride* SC1 conidial suspension at the rate of 5 × 10^3 conidia·g^{-1} of soil; Trt3: soil with 10 g of T. *atroviride* SC1 coated beech pellets (5 × 10^5 conidia·g^{-1} of beech wood pellets). Data of each replicate are presented in the histograms.

4. Discussion

Trichoderma spp. formulations are important, as they can delay the decline of the population of the fungus, protecting conidia from soil fungistasis [Martínez-Medina et al., 2009; Larkin, 2016], by providing nutrients to selectively stimulate their growth, or by combining both mentioned benefits (Prasad et al., 2002). In our experiment, we tested the effect of a carrier based on lignocellulosic materials (beech wood pellet supplemented with soy protein isolates) on the growth of *T. atroviride* SC1 in a sandy loam, and assessed the effect of such a carrier on the microbiota of the soil. Since it was hard to distinguish colonies of our strain from the indigenous population in the soil, we reported the results as the population of the total *Trichoderma* spp., instead of *T. atroviride* SC1.

The *Trichoderma* population showed a steady growth in the Ctr and Trt1 for the first 30 to 45 days, then it stabilized. This growth can be explained by the addition of SDW, which stimulated the indigenous population to germinate and exploit the soil organic matter. This is in concordance with studies that show that water affects the microbial growth in the soil [Griffin, 1963; Griffin, 1969; Kaisermann et al., 2015]. Trt2, where *T. atroviride* SC1 was applied as an SDW conidial suspension, showed the typical population decline that is reported in literature when *Trichoderma* spp. are applied as conidia (Papavizas, 1982; Longa and Pertot, 2009]. The population remained stable for 30 days, then started to decline gradually until D90. This result is in concordance with (Cordier and Alabouvette, 2009), who reported a decrease in the population of *Trichoderma atroviride* I-1237 after three weeks in neutral clayey soils and after 13 weeks after its inoculation into an acidic sandy loam. Their research showed that the decline of the population of *Trichoderma* spp. can be governed by the physicochemical characteristics of a soil. In fact, since the growth of *T. atroviride* SC1 on wood has been demonstrated by previous studies (Chammem et al., 2021; Pellegrini et al., 2014), we expected the fungus to grow to higher levels in Trt3 compared to Trt1 and Trt2; however, surprisingly, the total *Trichoderma* population remained stable for 30 days and then declined to levels similar to the ones registered with Trt1 and Trt2, which suggests a soil fungistatic effect that inhibited the growth of *T. atroviride* SC1. The growth of *T. atroviride* SC1 in the soil can be hindered by unfavorable soil conditions (pH 8 in our

experiment), as it grows best in acidic conditions (Longa et al., 2008). This has also been reported in other studies, which showed that the growth of *Trichoderma* spp. can be affected by soil texture and pH Brockett et al., 2012; Van Der Bom et al., 2018; Mayo-Prieto et al., 2021).

The carrier increased the *Trichoderma* spp. population in Trt1 steadily in the first 45 days and then it stabilized to levels that were not significantly different to those observed in the treatments where *T. atroviride* SC1 was added with and without coated beech pellets. Since Trt1 did not contain detectable levels of *T. atroviride* SC1 (since initially we tested the soil for the presence of this fungal strain using specific primers (Savazzini et al., 2009)) the presence of other competitive strains of *Trichoderma* spp. that are more efficient than our strain in degrading wood is highly plausible. Competition with other *Trichoderma* spp. could have played an important role in slowing down the growth of *T. atroviride* SC1 with the carrier in Trt3 as compared to Trt1, where the population of *Trichoderma* flourished in the first 45 days. Generally, *Trichoderma* spp. compete with each other in the soil for wood colonization with an effectiveness that depends on the species (Widden and Hsu, 1987; Klein and Eveleigh, 1998). Several species have been reported in the literature for their high cellulolytic activity, such as *T. reesei*, *T. viride*, *T. harzianum*, *T. virens,* and *T. longibrachiatum* (Mutschlechner et al., 2015; Bischof et al., 2016; Marecik et al., 2018). The difference that was observed between the results of the cfu counts of *Trichoderma* spp. and the results of the ITS amplicon-based analysis is in concordance with previous studies, which reported that such differences could be due to the fact that a dead propagule that still contains DNA does not develop into a cfu (Savazzini et al., 2008).

Since soil experiments can be influenced by a complex of physicochemical factors of a soil, such as temperature, texture, water availability, aeration, and light, as well as other biological factors, consisting mainly of the distribution of microorganisms in the soil and their interactions (Griffin, 1969), we repeated the experiment twice. Our results show that the experimental design consistently detected the differences occurring due to time and treatment. The incorporation of *T. atroviride* SC1 did not affect the bacterial richness and diversity. This

is in concordance with other studies that reported a transient effect of *Trichoderma* spp. on the microbial population [Cordier and Alabouvette, 2009; Savazzini et al., 2009; Huang et al., 2016; Umadevi et al., 2018). The fungal richness, however, decreased in all treatments, probably due to the growth of fungal genera that are more competitive in growing in conditions either of low organic matter or on woody substrates. Cordier and Alabouvette (2009) observed the same effect with the introduction of *T. atroviride* I-1237 on the fungal community; however, the change only lasted for three months, which was the full length of our experiment. The fungal diversity also decreased in all treatments; however, it decreased the most in Trt1, where pellets were introduced in the absence of *T. atroviride* SC1. This suggests that the main driver of the change observed between treatments was the introduction of non-coated beech wood pellets and that *T. atroviride* SC1 contributed in balancing the fungal diversity, probably by increasing the availability of nutrients to other fungi. This is in concordance with Asghar and Kataoka (2021), who found that introducing organic amendments into the soil had a negative effect on the diversity of the fungal community. Longa et al. (2009) also reported a correlation between the increase in the organic matter and the abundance of microbial functional groups with agricultural importance, such as nitrogen fixing bacteria when soil was supplied with green manure.

In our study, the tested soil was rich in wood-degrading bacteria and fungi in all treatments, as was demonstrated by the analysis of the top ten genera of bacteria and fungi, in terms of relevant abundance. These analyses revealed a dominance of the genus *Massila* for bacteria in all treatments, including the control. *Massilia,* which are saprophytic and opportunistic and were significantly more abundant in the presence of the carrier at D0. This can be explained by the early growth of these bacteria on wood pellets. In fact, *Oxalobacteraceae* in general, and *Massilia* spp. in particular, are very active in the early stages of bacterial succession in soils (Shrestha et al., 2007), and species of this genus can produce cellulases (Hrynkiewicz et al., 2010; Ofek et al., 2012) which can explain their initial growth on wood and soil amended with fresh plant residues (Cheng et al., 1996; Bernard et al., 2007). However, this effect is usually transient and only occurs when sufficient carbon and energy sources are present, and before competition with other

microorganisms becomes limiting (Ofek et al., 2012). Overall, the carrier increased the bacterial richness and had a transient effect on its diversity. This is in concordance with other studies that showed that using inorganic nitrogen fertilizers in bulk soils affects their bacterial composition (Illescas et al. 2020). Illescas et al. (2020) found that inorganic fertilizers increased bacterial genera with antagonistic activities, such as *Sphingomonas* and *Pseudomonas*, *Kaistobacter*, and *Streptomyces*. We observed the same pattern with *Sphingomonas* and *Pseudomonas*, but also *Lysobacter,* which was not reported in their study. *Lysobacter* spp., named after their lytic effects on other microorganisms and which are often good biocontrol agents, are Gram-negative bacteria that are frequently found in soils. Their increase could be a response to the increase of other microorganisms or to the availability of a nutritional substrate related to the treatments (Nakagawa et al., 2006).

The carrier also enriched the presence of bacteria involved in the soil nitrogen cycle such as *Microvirga* and *Pedobacter,* which confirms the results obtained by Longa et al. (2017) when soil was supplemented with green manure. These results suggest a selective effect of a wood-based carrier of *T. atroviride* SC1. This selective effect was more visible for fungi. In fact, non-coated beech wood pellets decreased both the richness and diversity of the fungal population. This could be the result of promoting fungi that are more adapted to the addition of wood, particularly in bulk soils (Illescas et al. 2020), such as *Mortierella,* which was the most abundant genus. These fungi are considered good degraders of toxic pollutants, such as pesticides and heavy metals (De Bruijn et al., 2015; Kataoka et al., 2010; Cui et al., 2017), and they have potential for biocontrol (Lambe and Wills, 1983; Tagawa et al., 2010; Melo et al., 2014). They are also important plant growth promoters and can enhance soil conditions under salt stress (Zhang et al., 2011; Zhang et al., 2020), but most importantly, they can degrade cellulose, hemicellulose, and organic matter in periods that range from 30 to 430 days, depending on the substrate and the soil conditions (Tamayo-Vélez et al., 2019; Ozimek and Hanaka, 2021). Tamayo-Vélez et al. (2019) reported an optimal degradation rate of organic matter by *Mortierella* 90 days after inoculation. These results can be compared to what we have observed, as 90 days were sufficient to rank *Mortierella* spp. as

the most abundant fungi in the soil. This shows that wood pellets are a suitable substrate for the growth of *Mortierella* spp. This might have caused the competition that prevented *Trichoderma* spp. in general, and *T. atroviride* SC1 in particular, from thriving in the pots supplemented with coated wood pellets.

Another genus that might have played an important role in the competition for wood degradation is *Cladorrhinum*. *Cladorrhinum* spp. have been extensively reported in agricultural soils (Mouchacca and Gams, 1993; Barrera et al., 2019). They were found in soil as saprotrophs on dung or plant material (Lewis and Larkin, 1998; Madrid et al., 2011), or in roots as endophytes (Gasoni and Stegman De Gurfinkel, 1997). It is an ammonia fungus belonging to the early successional phase of fungi involved in the saprotrophic litter decomposition in soil (Sagara, 1975). Moreover, they are more efficient than *Trichoderma* for the degradation of hardwood (Mao and Jiang 2021; Nilsson, 1973). This selective abundance of the carrier might be beneficial, as Gasoni and Stegman de Gurfinkel (2009) reported a potential antagonistic activity of *Cladorrhinum* spp. against *Rhizoctonia solani*. In fact, this is not the only fungus found in our research that can be effective against *Rhizoctonia solani*. Some species of the genus *Stachybotrys*, which is also commonly found in soils and on cellulose (Wang et al., 2015; Yasanthika et al., 2020), have a strong antagonistic activity against *R. solani* through mycoparasitism, by the means of chitinases and β-1,3-glucanases (Wang et al., 2015). One of the species of this genus, namely, *Stachybotrys chartarum* was also suspected to play a role in the development of human pulmonary diseases; however, the results are not yet conclusive (Kuhn et al., 2003; Hossain et al., 2004). Generally, drying pellets before coating, as suggested in our protocol, would eliminate any potential development of unwanted molds that could be harmful to animals or humans; however, further research is needed in different soil types and with different wood species. The results of the selective abundance of beneficial microbes is in concordance with Sani et al. (2020), Fu et al. (2021), and Zhang et al. (2020), who reported a decrease in the relative abundance of genera hosting phytopathogens such as *Neonectria* and *Fusarium,* improved soil fertility, and an increase in the relative abundance of plant growth-promoting rhizobacteria, respectively, when different *Trichoderma* isolates where inoculated into the soil. In our

research, the results of the two experiments yielded more consistent results with bacteria compared to fungi. This could be explained by the high competition between fungi in colonizing the woody substrate. The decreased prevalence of wood degrading fungi in some samples was replaced by an increase in the relative abundance of the genera *Cordana*, *Cystobasidium*, *Zopfiella*, *Schizothecium,* and *Ramophialophora*, which might be due to the heterogeneous distribution of microorganisms in the soil (Griffin, 1969).

5. Conclusions

The incorporation of *T. atroviride* SC1 coated wood pellets in the soil enriched the bacterial community and had a selective effect on the abundance of beneficial fungal species that have biocontrol potential. Although a wider screening of a combination of different wood and soil types is necessary to confirm this effect, these results are promising as they reinforce the suitability of the use of wood pellets as carriers of *Trichoderma* spp., because, in addition to being good growth substrates for the fungus, they have a mild effect on the microbial community, as they induce temporary side effects on the bacterial community, and favor the growth of fungal species with biocontrol potential. In addition, the carrier did not increase any potential human or plant pathogens. Although wood pellets can be used as carriers of conidia of *Trichoderma* spp., further studies are needed to assess their efficacy against soil-borne pathogens and to optimize application conditions and dosages in order to reach an efficient biocontrol effect. Possible side-effects on the plant, e.g., phytotoxicity, must also be tested.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Recursive partitioning analysis of bacterial richness and diversity.

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M.P. and I.P. All authors have read and agreed to the published version of the manuscript.

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Chapter 5: Conclusions and future perspectives

The effectiveness of the biocontrol of soilborne pathogens depends on one hand, on abiotic and biotic factors of a soil, and on the other hand, on the efficacy of a strain in controlling a specific pathogen, and on the abundance of the propagules of both the pathogen and the antagonist. To improve soil treatments with *Trichoderma* spp., better understanding of their ecology and interactions with other microorganisms is crucial. The review article presented in this thesis highlights the most important factors that can affect the survival of *Trichoderma* spp. in different soils. Soil temperature, pH, moisture level, and availability of nutrients as well as the presence of some microorganisms that can compete with *Trichoderma* spp. in certain conditions, such as *Pseudomonas* spp., are all limiting factors that call for the use of formulations to enhance the proliferation of *Trichoderma* spp. in the soil. Formulations can extend the longevity of *Trichoderma* and affect, therefore, the effectiveness of soil treatments when the conditions for the biocontrol activity of a *Trichoderma* spp. strain are favourable. However, the choice of carriers and the addition of nutrients to soils must be applied with care, since little is known on the effect of formulated *Trichoderma* spp. and their repetitive applications on soil microorganisms. Generally, the more specific the nutrient supplied for the antagonist, the lower the chances of the emergence of diseases that can use any excess of nutrients in the soil in their favour. In the light of this, wood pellets were chosen to be applied as carriers of *T. atroviride* SC1, which was isolated itself from hazelnut wood. Beech, fir, and chestnut wood pellets were coated with a conidial suspension of *T. atroviride* SC1, and our coating method was successful, with a maximum accuracy of 69%, however, the coating method can be improved by adding a biodegradable binder, and by adapting a seed coating machine for example to enhance the final results. Regardless, the coated pellets increased the population of *Trichoderma* from 10^4 cfu/g of wood pellets to a maximum of 10^7 cfu/g of wood pellets without the addition of a nitrogen source to moist pellets, and to 10^9 cfu/g of wood pellets, when moist coated pellets were supplied with nitrogen. Moreover, when applied in the soil, the carrier enriched the bacterial population and increased its diversity, while the fungal richness and diversity were affected in all treatments. Generally,

the carrier had a selective effect on both bacterial and fungal populations by increasing saprophytic microorganisms with cellulolytic activity. Some of these microorganisms were reported to control some soilborne diseases. These results suggest that beech wood pellets can be used as carriers for *Trichoderma atroviride* SC1, however, the development of a formulation requires testing it against several pathogens in the soil as the choice of the pathogen is key to the success of biocontrol. Moerover, the effectiveness of the formulation should be tested in different rhizospheres to assess its possible effect on plants.

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Chapter 2: Fate of formulated and non-formulated *Trichoderma* spp. strains after application in the soil and side effects on non-target of microorganisms

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Chapter 3: Wood pellets as carriers of conidia of *Trichoderma atroviride* SC1 for soil application

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Chapter 4: Effect of a wood-based carrier of *Trichoderma atroviride* SC1 on the microorganisms of the soil

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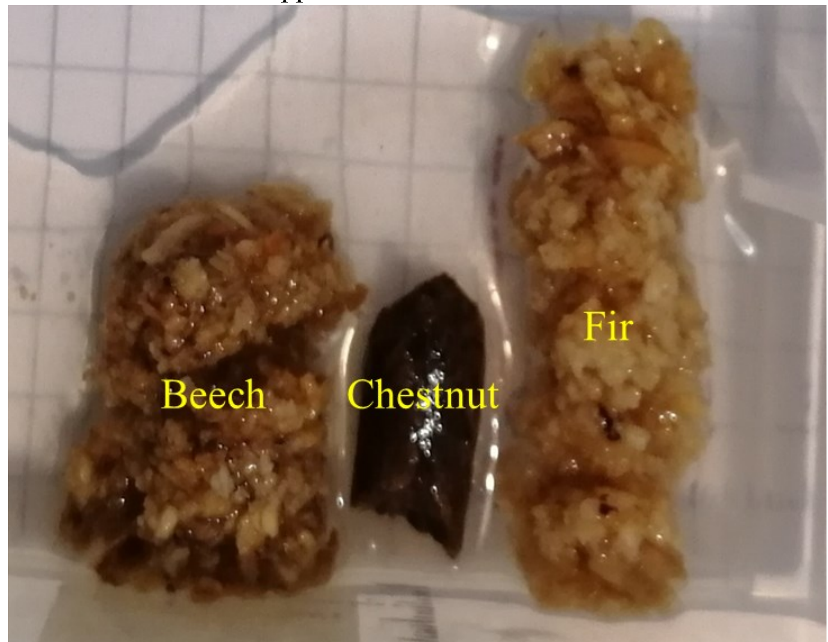
Supplementary materials

The supplementary materials of each chapter are available at the following address:

Chapter 3: Wood pellets as carriers of conidia of *Trichoderma atroviride* SC1 for soil application



Supplementary figure A. The swelling (SWp) and final increase in the volume of beech (left position), chestnut (middle position), and fir (right position) pellets after water absorption in a plastic container with graduated rulers at the sides to measure the increase in the height and the radius.

Table A1: The mean cfu counts of *Trichoderma atroviride* SC1 per gram of substrate grown on beech, fir, and chestnut pellets at D0 as compared to the control (Perlite).

Substrate \ Concentration of coating	C1[a] (cfu/g)	C2[a] (cfu/g)	C3[a] (cfu/g)
Perlite	1.34×10^4	9.95×10^4	1.51×10^6
Beech	5.46×10^3	4.35×10^4	4.85×10^5
Fir	6.10×10^3	4.91×10^4	6.80×10^5
Chestnut	5.37×10^3	3.86×10^4	2.20×10^5

[a] Coating concentrations C1 (10^5 cfu/mL of conidial suspension), C2 (10^6 cfu/mL of conidial suspension) and, C3 (10^7 cfu/mL of conidial suspension)

Table A2: The mean cfu counts of *Trichoderma atroviride* SC1 per gram of substrate grown on beech, fir, and chestnut pellets at D9 as compared to the control (Perlite).

Substrate \ Concentration of coating	C1[a] (cfu/g)	C2[a] (cfu/g)	C3[a] (cfu/g)
Perlite	4.42×10^5	5.38×10^5	8.56×10^6
Beech	6.15×10^7	6.26×10^7	6.00×10^7
Fir	4.50×10^7	4.50×10^7	3.75×10^7
Chestnut	3.95×10^6	3.27×10^6	1.22×10^6

[a] Coating concentrations C1 (10^5 cfu/mL of conidial suspension), C2 (10^6 cfu/mL of conidial suspension) and, C3 (10^7 cfu/mL of conidial suspension)

Chapter 4: Effect of a wood-based carrier of *Trichoderma atroviride* SC1 on the microorganisms of the soil

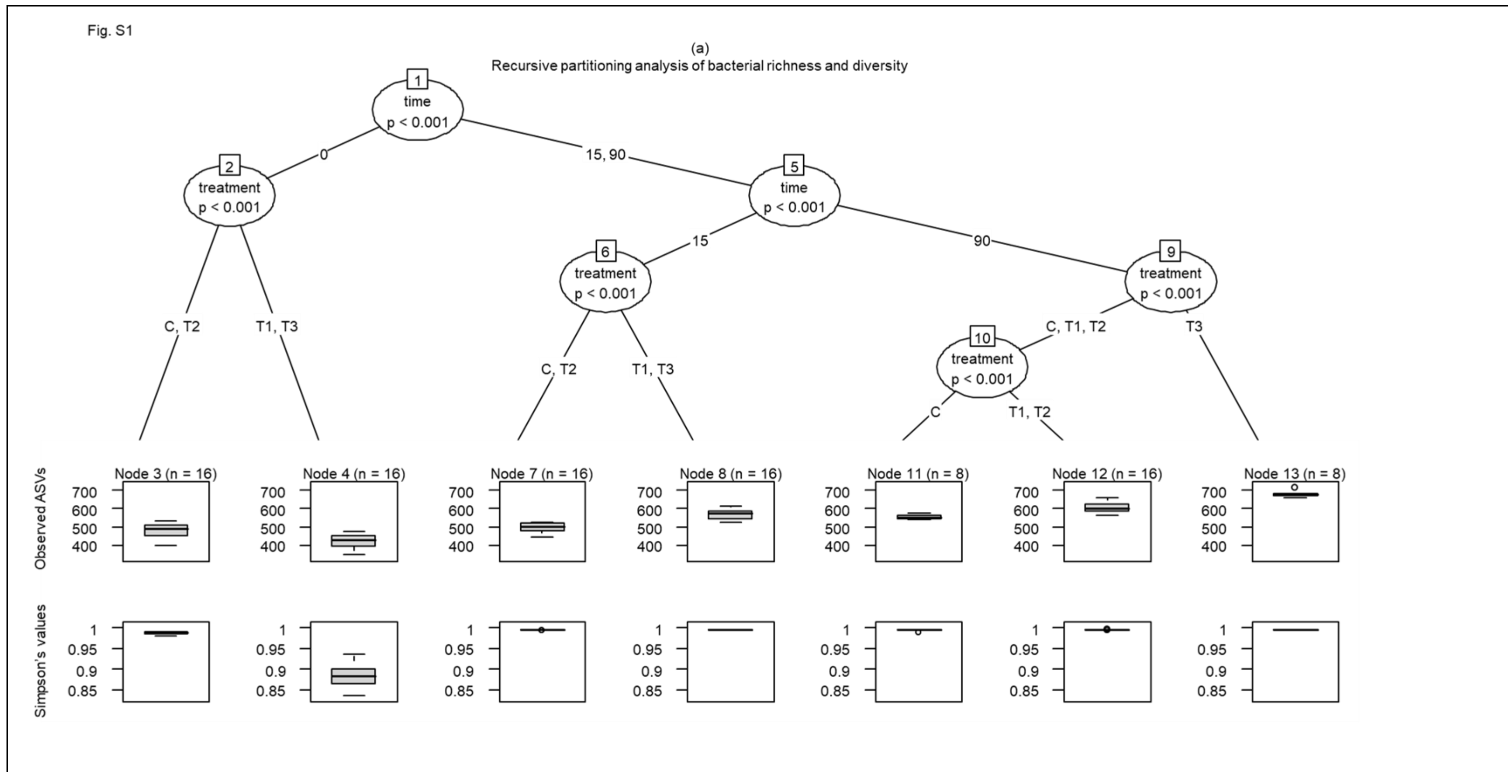


Fig. S1
(a)
Recursive partitioning analysis of bacterial richness and diversity
1
time
p < 0.001
0
15, 90
2
treatment
p < 0.001
5
time
p < 0.001
15
90
6
treatment
p < 0.001
9
treatment
p < 0.001
C, T2
T1, T3
C, T2
T1, T3
10
treatment
p < 0.001
C, T1, T2
T3
C
T1, T2
Node 3 (n = 16)
Node 4 (n = 16)
Node 7 (n = 16)
Node 8 (n = 16)
Node 11 (n = 8)
Node 12 (n = 16)
Node 13 (n = 8)
Observed ASVs
Simpson's values
700
600
500
400
1
0.95
0.9
0.85

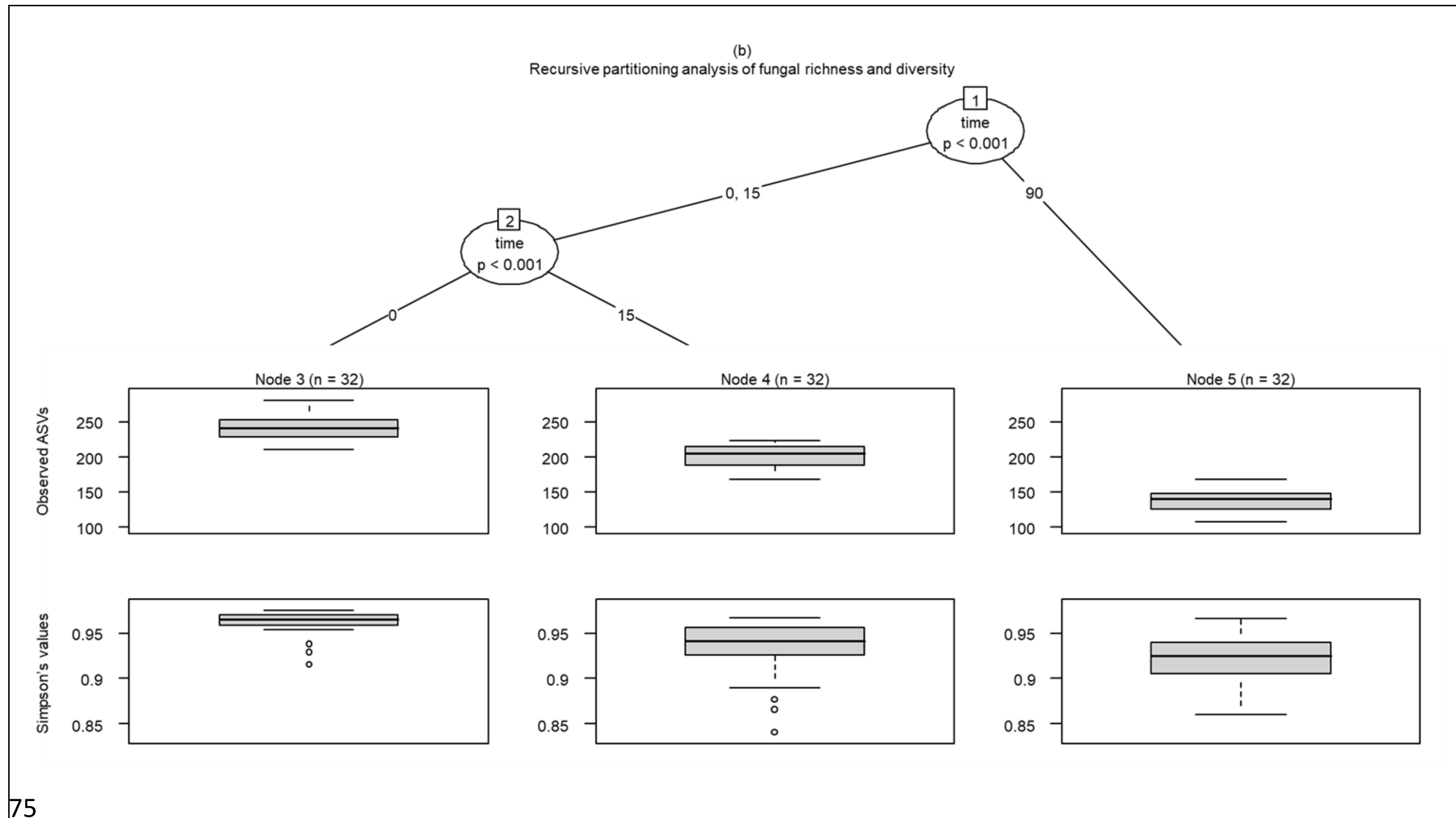

(b)
Recursive partitioning analysis of fungal richness and diversity
1
time
p < 0.001
0, 15
90
2
time
p < 0.001
0
15
Node 3 (n = 32)
Node 4 (n = 32)
Node 5 (n = 32)
Observed ASVs
250
200
150
100
Simpson's values
0.95
0.9
0.85

Figure S1: Recursive partitioning analysis of bacterial (a) and fungal (b) richness and diversity