Agricultural, Forest and Food Sciences doctoral school

Cycle XXXIV

Candidate: Alessio Bellini

Scientific Tutor: Professor Maria Lodovica Gullino

Alternative control strategies against soilbone pathogens: mechanism of action and interaction in three different pathosystems

Index

Introduction

Annual vegetable crops are constantly threatened by the presence of soilborne pathogens, whose attacks determine heavy losses (Katan, 2017). As opposed to the natural ecosystems, where soilborne pathogens are generally more unlikely to cause severe damages, in agricultural systems their outbreaks are common and can be highly disruptive. The management of the diseases caused by these pathogens is difficult to achieve due to several factors, which can be resumed as follows: (i) repeated cultural crop cycles of the same susceptible hosts in the same field which causes an enrichment of pathogens' propagules in the soil (Chellemi et al., 2016); (ii) ability of soilborne pathogens to survive, act and live in the soil even when their hosts are not present, making their control from the environment even more difficult. Other agricultural practices, i.e. the constant irrigation and the fertilization, may also result in the persistence of the pathogens, as also can implement their fitness (Katan, 2017); (iii) great efforts have been put on the selection of genetic resistance, and many are the commercial cultivars which are resistant to one or more soilborne pathogens, although these resistances can be overcome by the pathogens when the environmental conditions are particularly favorable or if the plants are subjected by severe infections (Gullino et al., 2022). As soilborne pathogen control is a major issue in the agricultural systems, the main strategy adopted has been for a long time soil disinfestation, which consists in the eradication of pests (such as nematodes, fungi, bacteria, insects, viruses) and weeds before the start of a cropping cycle, using techniques like steaming or fumigation. Several fumigants, volatile liquid-based agents, were developed and used, starting with chloropicrin in 1927-1928 against nematodes (Johnson and Godfrey 1932). Among different

molecules tested, methyl bromide (MB) became the most used one since the half of the 20th century, until its phase-out in 2005 (Katan, 1999; Gullino et al., 2003). Nowadays the list of permitted fumigants is limited, many of them were banned through the years and the selection of new ones is difficult due to the increasing attention above the toxicological and environmental issues connected with their use (Gullino et al., 2022). Nowadays, the knowledge about soil microbial composition and interactions between plants and microorganisms has greatly increased; it has been demonstrated that soil health (which also consists in the diversity and richness of soil microorganisms) can help to achieve a better control of diseases caused by soilborne pathogens (Raaijmakers and Mazzola, 2012; De Corato, 2020). To prevent fumigants' side effects on autochthon microbial community, chemical soil disinfestation cannot be used as principal control strategy for soilborne pathogens. In addition to technical and ecological impediments to a wide use of fumigants, in the last two decades, consumers' demand for organic products increased, leading the agricultural system to a rearrangement of the disease management sector (Klonsky, 2000). The study of alternative control strategies enlightened how soilborne diseases outbreaks are not simply the result of the presence of both pathogens and susceptible hosts; in fact, many factors both abiotic and biotic can alter their ability to infect the plants (Mazzola, 2002). For this reason, it is extremely important to approach their management in a holistic way that considers not only the elimination of them from the environment but also the understanding of their biology to develop methods able to obstruct their outbreaks without the impoverishment of soil health. In the attempt to find alternative control strategies to soilborne pathogens outbreaks, many were the paths studied and applied in the last decades, among which can be found: biological control agents (Cook,

1993; Vannacci and Gullino, 2000), compost able to confer suppressiveness ability to the soil (Bonanomi et al., 2007) and resistance inducers (Burketova et al., 2015; Alexandersson et al., 2016). Biocontrol agents can act in several ways to protect plants from soilborne pathogens infections i.e. antibiosis, predation, faster colonization of the rhizosphere area; many of them are already known to be effective, such as strains of *Trichoderma* (Woo et al., 2014), *Pseudomonas* (Walsh et al., 2001) and *Bacillus* (Paulitz and Bélanger, 2001). Composts are a great source of microorganisms (in terms of richness and diversity) which can act as biocontrol agents, and of organic substances able to improve plants fitness and be used by such microorganisms as source of nutrients whether they are soil autochthones or delivered by the composts themselves (Noble and Coventry, 2005). The enrichment of composts with antagonistic microorganisms is a great strategy, which can allow a better suppressiveness of the pathogens (Bonanomi, 2018). Plant resistance inducers (PRIs) are substances able to activate the plants' immune system, stimulating the salicylic acid, jasmonic acid and ethylene pathways, putting the hosts in an alert status so they are able to respond to pathogens attacks in a faster and more efficient way (Sandroni et al., 2020). In addition, also microorganisms already known as biocontrol agents and composts proved to activate the plants' defense systems (Shoresh et al., 2010). Despite the great advancement produced by the research in this sector, many gaps are still to be filled. As an example, the efficacy of alternative control agents is not always guaranteed, depending on the pathosystem and the environmental conditions in which they are applied. The consistency of their protective action can be unpredictable especially when used in field (Mercado-Blanco and Lugtenberg, 2014; Pirttilä et al., 2021; Gullino et al., 2022). As already mentioned, one single strategy to

manage soilborne pathogens cannot be considered efficient, or at least good for a long way usage in years; for such reason it is extremely important to study how alternative strategies might work in the attempt to defend crop systems from these pathogens (Katan, 2017).

In this thesis dissertation, three original studies will be presented and discussed, each one focused on a different crop: zucchini (*Cucurbita pepo*), pepper (*Capsicum annuum*) and lettuce (*Lactuca sativa*). The choice of these crops was based on their economic importance and great surface destined to the production in Italy and in other countries, having about 420630, 247620 and 735470 tons of production in Italy in 2020 (FAOSTAT, 2022). The short life cycle and reduced dimensions of both aerial and root systems guided the choice of these crops; the factors mentioned make them perfect hybrids between model plants and crops with actual importance in agriculture. For what concerns the pathogens, two main soilborne ones were chosen: *Phytophthora capsic*i (Phc) (Leonian, 1922) and *Fusarium oxysporum* f. sp. *lactucae* (Fol) (Matuo and Motohashi, 1967). Phc causes a wide range of diseases on several hosts (especially inside the families of *Solanaceae* and *Cucurbitaceae*), causing root, fruit, foliar and crown rot (Crossan et al., 1954; Erwin and Ribeiro, 1996). The only propagules of Phc able to survive in soil when the host is not present for long periods, are the thick-walled oospores. Infection can start from oospore, sporangia or zoospore that reach the plants' cuticle and penetrate it. In many cases, the infection starts with a biotrophic mode, switching to necrotrophic one (Stam et al., 2013); this behavior is described as hemibiotrophic. The life cycle of Phc is mostly associated with the host, after the collapse of the tissues in the necrotrophic phase, the oomycete can sporulate. Fol is able to cause disease specifically in lettuce,

but it can also colonize other plant species while the main host is not present in the field to persist in the environment between two crop cycles (Scott, 2014). In lettuce, Fol causes root rot and wilting; the infection starts with a direct contact with the root system, the fungus then enters the xylem and starts its biotrophic phase, colonizing the vascular system until it switches to a necrotrophic phase that determine the death of the cells; Fol also is considered hemibiotrophic. Fol has a great ability to differentiate new races, so far 4 have been identified (Matuo & Motohashi, 1967; Fujinaga et al, 2001; Fujinaga et al., 2003; Gilardi et al., 2016). The pathogen is able to develop resistance to chemical substances and to overcome the genetic resistances that are selected and inserted in lettuce varieties. Fol is also a seedborne as it is able to pass through infected seeds and spread in the field, implying the ineffectiveness of strategies only focused on the soil disinfestation before a cultural cycle. Besides the importance of the two pathogens chosen for the research here presented, *P. capsici* and *F. oxysporum* f. sp. *lactucae* belong to two different *phyla*, respectively: the fungus-like *Oomycota* and *Ascomycota* (Matuo and Motashi, 1967; Crossan et al., 1954), which makes them very interesting to be studied since they can potentially have different responses and mechanisms of interaction with the alternative control strategies. The three works presented here were focused on the study of different alternative strategies to better understand their mode of action and interaction with the plants and the soil system; each chapter of this thesis dissertation is organized in the form of papers as they were already published or submitted to scientific journals.

Outline of the works presented

Chapter I: A compost treatment acts as a suppressive agent in *Phytophthora capsici – Cucurbita pepo* pathosystem by modifying the rhizosphere microbiota

Bellini, A., Ferrocino, I., Cucu, M. A., Pugliese, M., Garibaldi, A. and Gullino, M. L.

Frontiers in plant science, 885. (2020).
https://doi.org/10.3389/fpls.2020.00885

In the first chapter the pathosystem *Phytophthora capsici* - *Cucurbita pepo* is considered and studied in a greenhouse pot experiment where twelve compost mixtures were tested to evaluate their suppressiveness. At the end of the experiment, the disease incidence was evaluated: only one compost mixture (green compost enriched with *Trichoderma* TW2 strain at 10%) was able to statistically reduce disease incidence. To understand how that mixture was able to do so, an amplicon-based Illumina sequencing (based on rRNA gene specific for fungi) was performed on the rhizosphere area of the plants treated with that compost mixture in comparison to the ones of the plants of the chemical control (treated with metalaxyl) and the inoculated untreated control. The spatial division of the taxa found in the samples analyzed showed that compost treated plants had a different rhizosphere composition compared to the controls. This fact suggests that this specific compost may confer suppressiveness to the system considered here by altering the rhizosphere microbial composition. This can be a possible explanation, but it may also be not the only way.

Chapter II: Calcium oxide, potassium phosphite and a *Trichoderma* enriched compost water suspension protect *Capsicum annuum* against *Phytophthora capsici* by priming the immune system

Bellini, A., Pugliese, M., Guarnaccia, V., Meloni, G. R.

and Gullino, L. M.

Pest Management Science, 77(7), 3484-3490. (2021).
https://doi.org/10.1002/ps.6401

In the second chapter the same compost enriched with *Trichoderma* TW2 (but as compost suspension), a calcium oxide mixture and potassium phosphite were used in *Phytophthora capsici* - *Cucurbita pepo* pathosystem to test their ability to induce systemic acquired resistance (SAR) in the host plant. To verify this, the products were applied to the root system as drench while the pathogen was inoculated with an agar plug directly to the third leaf. A positive control (acibenzolar-s-methyl), and two negative controls (untreated and untreated inoculated) were used. The treatments were applied 72 and 24 hours before the inoculation. The induction of resistance was evaluated through the analysis of the mRNA of three specific genes activated by the SAR pathway: CaBPR1, CaPO1 and CaDEF1 in three different endpoint times: immediately before the inoculation, 6 hours later and 24 hours later, by collecting the third leaf. At the same endpoint times, an upper leaf from each plant was collected to evaluate the accumulation rate of the salicylic acid through HPLC-MS/MS analysis. The analyses confirmed that all the treatments over-expressed at least one target gene or over-accumulated salicylic acid in at least one end point time. These results suggested that in the pathosystem considered, the treatments used were able to activate the SAR pathway.

Chapter III: Effects of *Trichoderma* enriched compost, BCAs and potassium phosphite on Fusarium wilt of lettuce and soil microbiome under intensive cultivation system

Bellini A., Gilardi G., Idbella M., Zotti M., Pugliese M., Bonanomi G., and Gullino M.L.

In preparation

In the third chapter, two years, two field experiments are presented. The pathosystem here considered is *Fusarium oxysporum* f. sp. *lactucae* - *Lactuca sativa*. Based on previous works, three integrated pest management strategies were applied on seedlings at the greenhouse or in the fields right before the transplantation: (i) the same compost enriched with *Trichoderma* TW2 used in the two previous works here presented, (ii) a combination of *T. gamsii* + *T. asperellum*, *Bacillus amyloliquefaciens* and potassium posphite and (iii) a combination of *T. polysporum* + *T. atroviride*. All the IPM strategies used lowered the disease severity of lettuce wilting caused by Fol and increased the productivity compared to the untreated control. To test if the products used in the trials caused shifting in the microbial community of the rhizosphere area, the microbiota was characterized by high-throughput sequencing of bacterial and eukaryotic rRNA gene markers. No differences were found between the treated and the untreated plants, yet large differences were found comparing the two fields suggesting an important microbial buffering effect triggered by the soil.

Literature cited

Alexandersson, E., Mulugeta, T., Lankinen, Å., Liljeroth, E. and Andreasson, E. (2016). Plant resistance inducers against pathogens in *Solanaceae* species—From molecular mechanisms to field application. *International journal of molecular sciences*, *17*(10), 1673.

Bonanomi, G., Antignani, V., Pane, C. and Scala, F. (2007). Suppression of soilborne fungal diseases with organic amendments. *Journal of Plant Pathology*, 311-324.

Bonanomi, G., Lorito, M., Vinale, F. and Woo, S. L. (2018). Organic amendments, beneficial microbes, and soil microbiota: toward a unified framework for disease suppression. *Annual Review of Phytopathology*, 56, 1-20.

Burketova, L., Trda, L., Ott, P. G. and Valentova, O. (2015). Bio-based resistance inducers for sustainable plant protection against pathogens. *Biotechnology advances*, *33*(6), 994-1004.

Chellemi, D. O., Gamliel, A., Katan, J. and Subbarao, K. V. (2016). Development and deployment of systems-based approaches for the management of soilborne plant pathogens. *Phytopathology*, *106*(3), 216-225.

Cook, R. J. (1993). Making greater use of introduced microorganisms for biological control of plant pathogens. *Annual review of phytopathology*, 31(1), 53-80.

Crossan, D. F., Haasis, F. A., and Ellis, D. E. (1954). Phytophthora blight of summer squash. *Plant Disease Report* 38:557-559.

De Corato, U. (2020). Soil microbiota manipulation and its role in suppressing soil-borne plant pathogens in organic farming systems under the light of microbiome-assisted strategies. *Chemical and Biological Technologies in Agriculture*, *7*(1), 1-26.

Erwin, D. C., and Ribeiro, O. K. (1996). Phytophthora Diseases Worldwide. *American Phytopathological Society*, St. Paul, MN.

FAOSTAT (2022) https://www.fao.org/statistics/en/

Fujinaga, M., Ogiso, H., Tsuchiya, N. and Saito, H. (2001). Physiological specialization of *Fusarium oxysporum* f. sp. *lactucae*, a causal organism of Fusarium root rot of crisp head lettuce in Japan. *Journal of General Plant Pathology*, 67(3), 205-206.

Fujinaga, M., Ogiso, H., Tuchiya, N., Saito, H., Yamanaka, S., Nozue, M. and Kojima, M. (2003). Race 3, a new race of *Fusarium oxysporum* f. sp. *lactucae* determined by a differential system with commercial cultivars. *Journal of General Plant Pathology*, 69(1), 23-28.

Gilardi, G., Franco Ortega, S., Van Rijswick, P. C. J., Ortu, G., Gullino M.L. and Garibaldi, A. (2017). A new race of *Fusarium oxysporum* f. sp. *lactucae* of lettuce. *Plant Pathology*, 66(4): 677-688.

Gullino M.L., Garibaldi A., Gamliel A. and Katan J. (2022). Soil Disinfestation: From Soil Treatment to Soil and Plant Health. *Plant Disease* Jan 3. doi: 10.1094/PDIS-09-21-2023-FE.

Gullino, M. L., Camponogara, A., Gasparrini, G., Rizzo, V., Clini, C. and Garibaldi, A. (2003). Replacing methyl bromide for soil disinfestation: the ltalian experience and implications for other countries. *Plant Disease*, *87*(9), 1012-1021.

Johnson, M. O. and Godfrey, G. H. (1932). Chloropicrin for nematode control. *Industrial & Engineering Chemistry*, *24*(3), 311-313.

Katan, J. (2017). Diseases caused by soilborne pathogens: biology, management and challenges. *Journal of Plant Pathology*, 305-315.

Katan, J. (1999). The methyl bromide issue. Problems and potential solutions. *Journal of Plant Pathol*ogy 81:153-159

Klonsky, K. (2000). Forces impacting the production of organic foods. *Agriculture and human values*, *17*(3), 233-243.

Leonian, L. H. (1922). Stem and fruit blight of peppers caused by *Phytophthora capsici* sp. nov. *Phytopathology*, *12*(9).

Matuo, T. and Motohashi, S. (1967). On Fusarium oxysporum f. sp. lactucae n. f. causing root rot of lettuce. *Transaction on the mycology Society in Japan* 8:13–15.

Mazzola, M. (2002). Mechanisms of natural soil suppressiveness to soilborne diseases. *Antonie van Leeuwenhoek*, *81*(1), 557-564.

Mercado-Blanco, J. and Lugtenberg, B. (2014). Biotechnological applications of bacterial endophytes. *Current Biotechnology*, 3(1), 60-75.

Noble, R. and Coventry, E. (2005). Suppression of soil-borne plant diseases with composts: a review. *Biocontrol Science and Technology*, *15*(1), 3-20.

Paulitz, T. C. and Bélanger, R. R. (2001). Biological control in greenhouse systems. *Annual review of phytopathology*, 39(1), 103-133.

Pirttilä, A. M., Mohammad Parast Tabas, H., Baruah, N. and Koskimäki, J. J. (2021). Biofertilizers and Biocontrol Agents for Agriculture: How to Identify and Develop New Potent Microbial Strains and Traits. *Microorganisms*, 9(4), 817.

Raaijmakers, J. M. and Mazzola, M. (2012). Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Annual review of phytopathology*, *50*, 403-424.

Ruano-Rosa, D. and Mercado-Blanco, J. (2015). Combining biocontrol agents and organics amendments to manage soil-borne phytopathogens. In *Organic amendments and soil suppressiveness in plant disease management* (pp. 457-478). Springer, Cham.

Sandroni, M., Liljeroth, E., Mulugeta, T. and Alexandersson, E. (2020). Plant resistance inducers (PRIs): Perspectives for future disease management in the field. *CAB Review*, *15*, 1-10.

Scott, J. C., McRoberts, D. N. and Gordon, T. R. (2014). Colonization of lettuce cultivars and rotation crops by *Fusarium oxysporum* f. sp. *lactucae*, the cause of Fusarium wilt of lettuce. *Plant Pathology*, 63(3), 548-553.

Shatilov, M. V., Razin, A. F. and Ivanova, M. I. (2019, November). Analysis of the world lettuce market. In *IOP Conference Series: Earth and Environmental Science* (Vol. 395, No. 1, p. 012053).

Shoresh, M., Harman, G. E. and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annual review of phytopathology*, *48*, 21-43.

Spadaro, D. and Gullino, M. L. (2005). Improving the efficacy of biocontrol agents against soilborne pathogens. *Crop protection*, *24*(7), 601-613.

Stam, R., Howden, A. J. M., Delgado Cerezo, M., Amaro, T. M. M. M., Motion, G. B., Pham, J. and Huitema, E. (2013). Characterization of cell death inducing *Phytophthora capsici* CRN effectors suggests diverse activities in the host nucleus. *Frontiers in plant science*, *4*, 387.

Vannacci, G. and Gullino, M. (2000). Use of biocontrol agents against soil-borne pathogens: results and limitations. In *International Symposium on Chemical and Non-Chemical Soil and Substrate Disinfectation 532* (pp. 79-88).

Walsh, U. F., Morrissey, J. P., and O'Gara, F. (2001). Pseudomonas for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Current Opinion in Biotechnology*, *12*(3), 289-295.

Wissuwa, M., Mazzola, M. and Picard, C. (2009). Novel approaches in plant breeding for rhizosphere-related traits. *Plant and Soil*, 321(1), 409-430.

Woo, S. L., Ruocco, M., Vinale, F., Nigro, M., Marra, R., Lombardi, N., Pascale, A., Lanzuise, S., Manganiello, G. and Lorito, M. (2014). *Trichoderma*-based products and their widespread use in agriculture. *The Open Mycology Journal*, *8*(1).

Chapter I: A *Trichoderma* enriched compost treatment acts as a suppressive agent in *Phytophthora capsici* – *Cucurbita pepo* pathosystem by modifying the rhizosphere microbiota

Bellini, A., Ferrocino, I., Cucu, M. A., Pugliese, M., Garibaldi, A.

and Gullino, M. L.

Frontiers in plant science, 885. (2020).
https://doi.org/10.3389/fpls.2020.00885

Introduction

Phythophthora capsici Leonian *(PHC)* is a filamentous pathogen oomycete that causes root, fruit, foliar and crown rot over a wide host range, including some economically and nutritionally important horticultural crops (cucurbits, tomatoes, pepper, eggplant) (Lamour et al., 2012). In Italy, and throughout the world, summer squash (*Cucurbita pepo* var. *cylindrica* L.) cultivation is threatened by the natural and human-derived presence of PHC inoculum (Erwin and Ribeiro, 1996). Chemical control strategies are difficult to adopt, due to the limited number of registered chemicals that are permitted and the scalar harvest system (Gilardi et al., 2015). However, many efforts have been made to find a source of genetic resistance in squash accessions to PHC strains (Michael et al., 2019; Siddique et al., 2019), but this approach is still at its beginning. For these reasons, alternative PHC control agents are being studied and adopted in agriculture. Moreover, one of the most promising and most studied techniques to prevent the infection of soil-borne pathogens is the application of organic amendments (Megavansi, 2015; Gilardi et al., 2016; De Corato et al., 2018c, 2018d). In the last few decades, interest in using organic amendments has grown throughout the world, due to the possibility of reintroducing recycled biowastes and organic matter into the

primary production industry, which is connected to the concept of circular economy. Among the various organic amendments, compost has been studied the most and has been used because of its suppressiveness activity (Pugliese et al., 2015; Bonanomi et al., 2018). Nevertheless, since each compost can be different from other composts, suppressive action is not always guaranteed and, sometimes, a compost can even play a conductive role; Bonanomi et al. (2007) redacted a list of researches in this field and showed that, out of 2,423 studies, only 54% of the studied composts showed suppressive ability. It is also known that a mature compost can play both a suppressive and a conductive role as a function of the pathosystem (Bonanomi et al., 2010). Soil microbiota plays a major role in the suppressive activity of mature composts and, in an attempt to prove this, many studies have compared sterilized composts with their not-sterilized counterparts, and have found that the inactivation of microbiota is connected to the loss of suppressive activity (Reuveni et al., 2002; Tilston et al., 2002; Papasotiriou et al., 2013; De Corato et al., 2019). Plant root exudates have been shown to have the ability to select a specific microbiota and influence the colonization of root areas by microorganisms; this means that rhizosphere microbiota is closely connected not only to the soil, but also to the plant genotype (Weller et al., 2002; Haas et al., 2005; Bulgarelli et al., 2012; Doornbos et al., 2012; Badri et al., 2013; Mazzola et al., 2015). Moreover, the microorganisms present in a mature compost are also selected by root exudates, according to the need and the genotype of the plant (Antoniou et al., 2017, Cucu et al., 2019). Thus, in order to prevent soil-borne diseases when pathogens are present in field, it is very important to know which microorganisms confer compost suppressiveness according to the type of pathosystem. Many studies have investigated the microbial communities present in

several composts, but most of them were based on *in vitro* isolation, which has the drawback of excluding non-cultivable microorganisms and therefore of not giving a full picture of the entire complexity. Moreover, recent studies have pointed out the importance of using molecular cultural independent methods (Cao et al., 2019; Carrasco et al., 2019; Fernández-Bayo et al., 2019; Sìsìc et al., 2018; Zaho et al., 2019; Zhou et al., 2019). The objectives of this work were: to investigate the microbiota populations of four different composts by using targeted (Real Time PCR) and non-targeted (amplicon based Illumina sequencing) molecular approaches; to test their ability to confer suppressiveness in a squash - PHC pathosystem in controlled greenhouse pot trials; to investigate, on the basis of the greenhouse trial results, the microbiota composition of the best compost treatment, in comparison with an untreated and a chemical treatment, by means of the same molecular tools used for the compost characterization.

Materials and methods

Composts used in this study

Four different commercial composts were used in this study: i) a green waste compost produced in a dynamic composting system for 6 months and sifted with a 10 mm sieve (ANT's Compost V - CV; AgriNewTech srl, Italy), ii) the same green compost enriched with experimental BCA "*Trichoderma* spp. TW2" (ANT's compost M - CM; AgriNewTech srl, Italy), iii) green compost produced in a dynamic composting system for 6 months and sifted with a 20 mm sieve (ANT's compost V2 – CV2; AgriNewTech srl, Italy) and iv) a municipal biowaste compost produced using green and urban organic fraction bio-wastes in a dynamic composting system for 4 months (ANT's Compost B -CB; AgriNewTech srl, Italy). At the end of the maturation process, CV, CM, CV2 and CB were analyzed in service to establish their chemical compositions (Table 1).

Table 1: Chemical composition of the tested composts

	ANT CV/CM	ANT CV2	ANT CB
pH	7.92	8.08	8.08
Humidity (%)	42.00	43.10	40.90
Organic C % w/w dry	21.00	25.80	22.00
Organic N % w/w dry	1.57	2.36	2.13
C/N ratio	13.00	9.44	9.16
Total N %	1.63	1.55	2.40
Organic N/total N ratio	96.00	88.45	88.75
Hg mg/kg dry matter	0.16	< 0.01	< 1.50
Ni mg/kg dry matter	93.10	11.6	84.00
Pb mg/kg dry matter	47.90	32.1	39.00
Zn mg/kg dry matter	143.80	140.10	206.00
Cu mg/kg dry matter	52.50	56.60	148.00

Greenhouse trials

Summer squash (*Cucurbita pepo* var. *cylindrica L*. cv Genovese) seeds were sown in seed cells in a peat substrate (Tecno 2, 70% white peat and 30% clay, pH 5.5–6, N 110–190 mg/L, P2O5 140–230 mg/L, K2O 170–280 mg/L, Turco Silvestro terricci, Bastia d'Albenga, SV, Italy) and kept in a nursery for two weeks at 26±1°C. In the meantime, substrates were prepared for potted plants, by adding different percentages of each compost (1-10-20% v/v) to the same peat used for sowing. After one week, each substrate mixture was infested with 2 g/l of one strain of PHC (Agroinnova collection) grown for two weeks in grain-hemp (60:40) flasks. A chemical treatment, in which a suspension of metalaxyl (Ridomil gold,480 g l^{-1}, Syngenta Crop Protection) and water was used in order to reach a final concentration of 50 µl/l of substrate, was carried out at the same time as the inoculation. One week after the infestation, the seedlings were transplanted into 2 l pots, with 3 plants per pot, and placed in a greenhouse kept at 24±1°C. Each treatment was replicated in three different pots per trial, with a randomized experiment design. The experiment was carried out twice independently.

Disease assessment

Disease incidence (DI) was evaluated by counting the number of diseased plants in each pot twice during the trials, according to the formula: number of diseased plants number of total plants x 100; an intermediate disease assessment was performed one week after transplantation; the final evaluation was performed one week later. The fresh biomass of the plants was also weighed. The area under the disease progress curve (AUDPC) was calculated according to Padley et al. (1989).

Sampling and DNA extraction

Each compost was collected individually from different and random parts of big bags (total volume 50 ml). Two separate DNA extractions were carried out for each compost using 100 mg of fresh compost. The rhizosphere was collected, at the end of the pot trials, three biological replicates were collected from three different pots for each trial, the plant roots were shaken to remove any excess peat and the particles that were still adhered to the root system were collected in 50 ml vials. Total microbial DNA extraction was carried out with EZNA soil DNA kit (Omega Bio-Tek, Norcross, GA), following manufacturer's instructions. DNA quantity was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham MA), while DNA integrity was verified by running 5 µl of each sample in a 1% agarose electrophoretic gel.

Real Time PCR assays

Real Time PCR assays were performed using a StepOne-Plus™ Real-Time System (Applied Biosystems, Foster City, CA, USA). The abundance of the total fungal (18S rRNA gene) and bacterial (16S rRNA gene) communities in the compost samples was determined. The abundance in the rhizosphere samples was instead assessed, for the total fungi, total bacteria and *Phytophthora capsici*, with the primers (Lan et al., 2013) and under the conditions described by Cucu et al. (2020). Real Time PCR was performed for each extraction in triplicate, and the average values were then transformed into Log of gene copies per gram of dry compost; these data were mediated between two extractions of each sample. Table 2 summarizes the primers and Real Time PCR conditions.

Table 2: Description of the primer sets and amplification conditions of the quantitative Real Time PCR assays.

Gene	Primers	Real Time PCR conditions
18s rRNA gene, total fungal abundance	FR1 (Vainio and Hantula 2019) 390FF (Vainio and Hantula 2019)	45 cycles 95° 30", 50° 30", 70° 60"
16s rRNA gene, total bacterial abundance	Eub338 (Muyzer, Waal, and Uitierlinden 1993) Eub51 (Muyzer, Waal, and Uitierlinden 1993)	40 cycles 95° 30", 55° 35", 72° 45"
PHC, pathogen abundance	Pc1F (Lan et. al., 2013) Pc1R (Lan et. al., 2013)	40 cycles 95°C 30s, 60°C 35s, 72°C 45s

Amplicon-based sequencing

The mycobiota were evaluated by amplifying the D1 domain of the 26S gene using the primers and condition described by Mota-Gutierrez et al. (2018). A library preparation was performed according to the Illumina metagenomic procedure. Sequencing was performed using a MiSeq instrument (Illumina) with V3 chemistry and 250-bp generated paired-end reads, following the manufacturer's instructions. After sequencing reads were assembled, quality filtered and processed using QIIME 1.9.0 software (Caporaso), and the pipeline described by Mota-Gutierrez et al. (2018). Centroids sequences of each cluster were manually checked by Blast tool to confirm the taxonomic assignment. QIIME was used to rarefy the OTU table at the lowest number of sequences per sample and to build the OTU table. The OTU table displays the highest taxonomy resolution that was reached; when the taxonomy assignment was not able to reach the genus level, family name was displayed. The OTU table was used to build a Principal Component Analysis as a function of the treatment. An anosim

statistical test was used, through the *vegan* function of R, to identify any significant differences as a function of the treatments. The Wilcoxon matched pairs test was used to establish the difference in OTUs abundance as a function of the treatment. The P value was adjusted by the False Discover Rate.

Statistical analyses

Statistical analyses were performed, with SPSS software (IBM SPSS Statistics, Westland, MI, USA), for the disease incidence, fresh biomass, AUDPC and Real Time PCR data. ANOVA and Tukey post hoc tests were performed to establish the statistical values of the differences ($p < 0.05$). The DI, fresh biomass and AUDPC data were unified for the two separate trials.

Availability of the sequence data

The sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information under BioProject number PRJNA580394

Results

Abundance of the total microbial community in four composts

Analyses were carried out, with Real Time PCR, to describe the microbiological assessment in four mature composts, in terms of gene abundance expressed as Log of copy^{-1} numbers per gram of dry matter (Figure 1 a-b). The fungal 18S rRNA gene copies per 1g of dry compost were between 10.29 and 10.56 for the four composts, while the bacterial 16S rRNA gene was detected at between 9.48 and 9.84. No statistical differences were observed for the fungal or bacterial communities, since the four composts showed a similar abundance for these two markers.

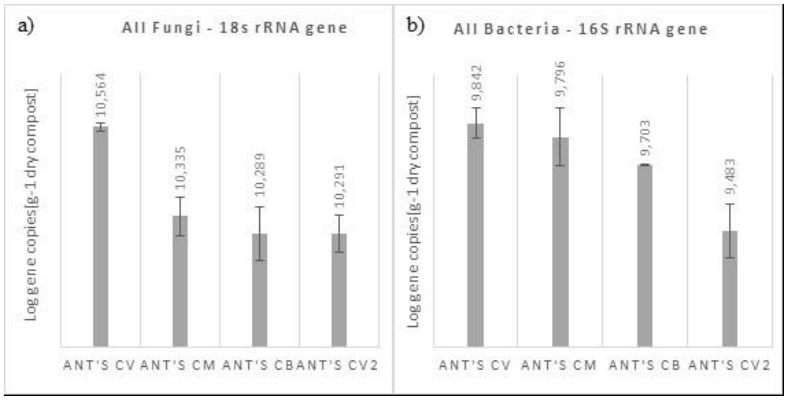

Figure 1: Abundance of the fungal 18S rRNA gene (A) and bacterial 16S rRNA gene (B) for four composts: CV, CM, CB, and CV2. Different letters indicate statistical differences between the four composts, as obtained with the ANOVA test and Tukey's post hoc test.

Mycobiota composition of the 4 mature composts used in this study

A total of 244298 raw reads (2 x 250 bp) were obtained after sequencing. After quality filtering, a total of 242702 clean reads were used, with an average value of 60675 reads/sample and an average sequence length of 386 bp. 26S rRNA gene sequencing showed differences between the four composts used in this study at a genus level. Thirty-one genera were observed (Figure 2), and it was possible to observe a clear predominance of a few taxa in all the composts. CV was mostly populated by *Phialophora* (5.5%), *Coniochaeta* (4.2%) and *Aureobasidium* (8.5%); CV2 showed *Penicillium (21.1%)*, *Miceliophthora (9.8%)*, *Coniochaeta (2.5%)*, *Cladosporium (10.7%)*, *Aspergillus* (8.5%), *Arthoderma (13.3%)* and *Pseudoeurotium* (5%); CB showed *Scopulariopsis (2.6%)*, *Pseudeurotium (4%)* and *Chaetomium* (2.9%). CM was mostly populated by three main genera: *Trichoderma* (6.4%), *Phialophora* (3.4%) and *Fusarium* (11.5%).

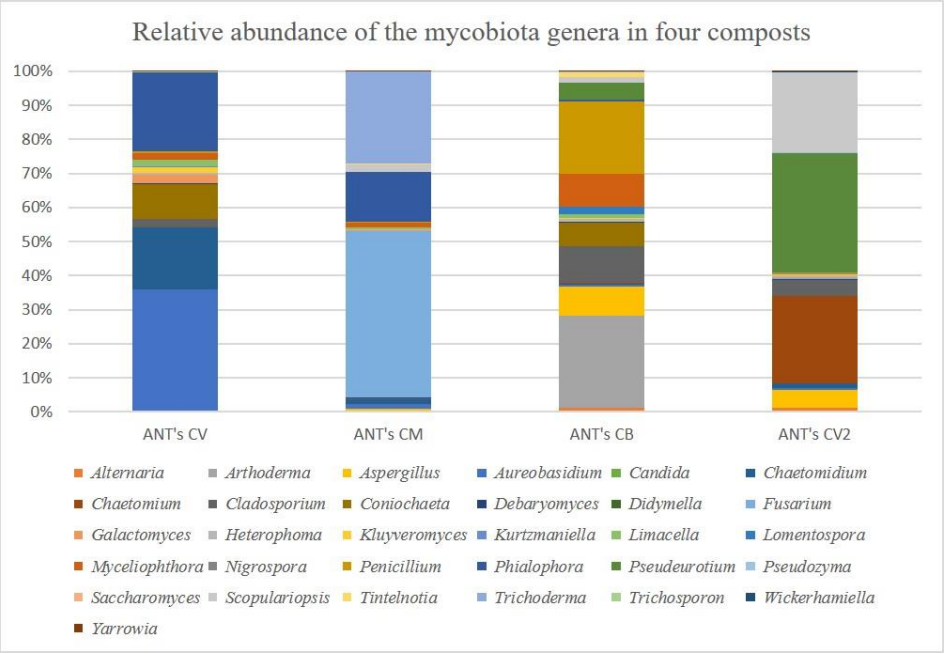

Figure 2: Relative abundance of the fungal community in the four analyzed composts: CV green compost, CV2 green compost, CM green compost with the addition of *Trichoderma* sp. TW2 and CB mixed compost. The OTUs were selected by discarding the ones that were not present in the four composts under a threshold of 0.5%.

Disease suppression by the compost mixtures

The Negative Control –non-inoculated- (NC) and Chemical control (CC) showed no disease symptoms at the end of both trials. The Inoculated Untreated control (UC) showed up to 90% of disease incidence at the end of the trials. All the treatments showed a numerical reduction in DI, compared to the UC, and all the CM mixtures had the lowest disease index, but only samples from CM – 10% showed a statistical reduction of DI, compared to the UC ($P<0.05$), that ranged from 90% to 45%. As far as the fresh biomass is concerned, the untreated control showed the lowest value of all (5.8 ± 1.9 g), while the highest was for the non-inoculated treatment (34.0 ± 2.4 g). Of all the compost mixtures, CM – 10% was the only one

that was significantly different from the UC, with an average fresh biomass of 26.3 g ($P<0.05$).

The area under the disease progress curve (AUDPC) values highlighted a similar situation as the DI and fresh biomass: the inoculated untreated control (UC) had the highest AUDPC value (351) of all the treatments, but only CM – 10% was significantly different from UC, with a value of 137. All the data are shown in Table 3, where the results of Trial 1 and Trial 2 are united.

Table 3: Efficacy of the compost mixtures to suppress *Phytophthora capsici* disease on summer squash plants expressed as disease incidence (%), fresh biomass (g) and AUDPC values. The letters refer to the Tukey post hoc test, which was performed after one-way Anova (P<0.05).

Treatments	**Disease incidence**	**standard error**	**Tukey**	**AUDPC**	**standard error**	**Tukey**	**fresh biomass**	**standard error**	**Tukey**
Non-inoculated control	0.00	0.00	c	0.00	0.00	a	34.00	2.40	d
Untreated control	90.00	4.70	a	351.30	28.80	c	5.80	1.90	a
Chemical control	0.00	0.00	c	0.00	0.00	a	32.80	3.10	cd
CV - 1%	70.00	9.40	ab	268.80	47.30	bc	9.80	3.00	ab
CV - 10%	75.00	12.50	ab	301.30	49.10	bc	17.60	5.00	abc
CV - 20%	57.50	11.60	ab	212.90	48.60	abc	21.20	2.90	abc
CB - 1%	67.50	10.60	ab	251.70	41.20	bc	16.60	2.30	abc
CB - 10%	67.50	6.60	ab	226.70	30.70	bc	21.50	2.70	abc
CB - 20%	72.50	10.30	ab	286.30	42.80	bc	18.10	5.90	abc
CV2 - 1%	72.50	13.30	ab	287.50	58.20	bc	10.60	4.20	ab
CV2 - 10%	75.00	10.30	ab	286.30	44.90	bc	17.50	3.50	abc
CV2 - 20%	70.00	6.30	ab	257.50	30.60	bc	18.70	4.30	abc
CM - 1%	55.00	9.40	ab	245.40	44.00	bc	12.40	3.10	ab
CM - 10%	45.00	9.10	b	137.10	36.70	ab	26.30	3.30	bcd
CM - 20%	55.00	12.90	ab	181.30	49.10	abc	21.70	2.90	abc

Abundance of fungi, bacteria and PHC in the rhizosphere of UC, CC and CM – 10%

Since the only effective treatment was CM – 10%, molecular analyses were carried out using rhizosphere soils collected at the end of the trials for the Untreated Control, Chemical Control and CM – 10% treatments. Data from two trials are shown in Figure 3(a-c). No differences are evident for the abundance of fungi and bacteria between the three treatments. The specific PHC gene was found in both the untreated control and in the CM – 10% treatment at levels of 3 and 2.82, respectively [Log of copy^{-1} numbers per gram of dry matter], while it was not found in the chemical treatment.

Mycobiota composition of the rhizosphere soils

The total number of paired sequences obtained from samples reached 884148 raw reads. A total of 784797 reads were obtained after quality filtering, with an average value of 35672 ± 17772 reads/sample, and a mean sequence length of 393 bp. The Alpha diversity index showed a satisfactory coverage for all the samples (> 96%) but did not show a different level of complexity on the basis of the treatment. No significant difference in mycobiota composition or in alpha diversity index was observed, and the data were therefore averaged.

Taking into account the microbiota composition at the highest taxonomic level (Figure 4), it is possible to observe a core mycobiota, composed of *Fusarium*, which reaches about 3% of the relative abundance in the control samples (UC), 1% in the compost (CM – 10%) and 2% in the chemical treatment (CC); *Glomus*, which reaches 9, 5 and 15% in UC, CM – 10% and CC, respectively; *Penicillium*, which reaches 6, 1 and 4%, of the relative abundance in UC, CM – 10% and CC, respectively;

Saccharomycetales, which reaches 4, 1, and 3 %, *Torrubiella*, which reaches 2, 2 and 1%; *Trichoderma*, which reaches 10, 12 and 7% and *Zygoascus*, which reaches 1, 1 and 3%, respectively (Figure 4). A further separation of the samples, based on the treatment, was also observed, through the Principal Component Analysis (PCA, Figure 5), and the result was confirmed by means of the ANOSIM statistical test (P= 0,003). Moreover, it was possible to observe a clear separation of the samples treated with compost, while the chemical treatment and control ones clustered together (Figure 5). By taking into account the significant difference in the OTUs among treatment (FDR < 0.05), it was possible to observe that the compost treatment (CM – 10%) was characterized by the presence of minor fraction OTUs. In other words, a higher presence of *Chaetomiaceae*, *Microascaceae*, *Arthrographis*, *Myceliophthora* and *Phialophora* was observed (Figure 6), while *Penicillium* and *Pseudeurotium* were reduced in the compost treated samples, compared with UC and with CC. It should be observed that *Didymella* was reduced by both treatments, compared with the controls (Figure 6).

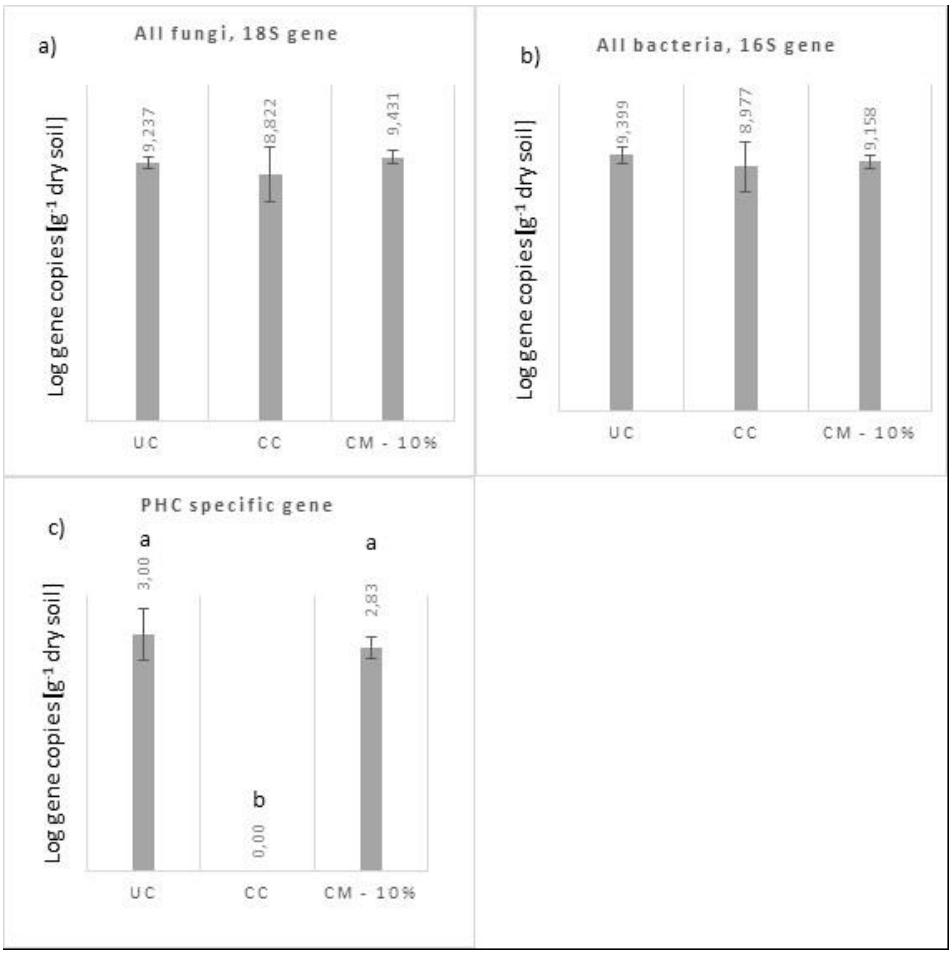

Figure 3: Abundance of the fungal 18S rRNA gene (A), bacterial 16s rRNA gene (B), and PHC specific gene (C) in the rhizosphere samples at the end of the trials: untreated control (UC), chemical control (CC), and CM – 10% treatment. Three biological replicates were collected from three different pots per treatment and for each trial. PHC means *Phytophthora capsici*. Data from the two trials were analyzed separately and then averaged. Different letters indicate statistical differences between the treatments obtained with the ANOVA test and the Tukey's *post hoc* test.

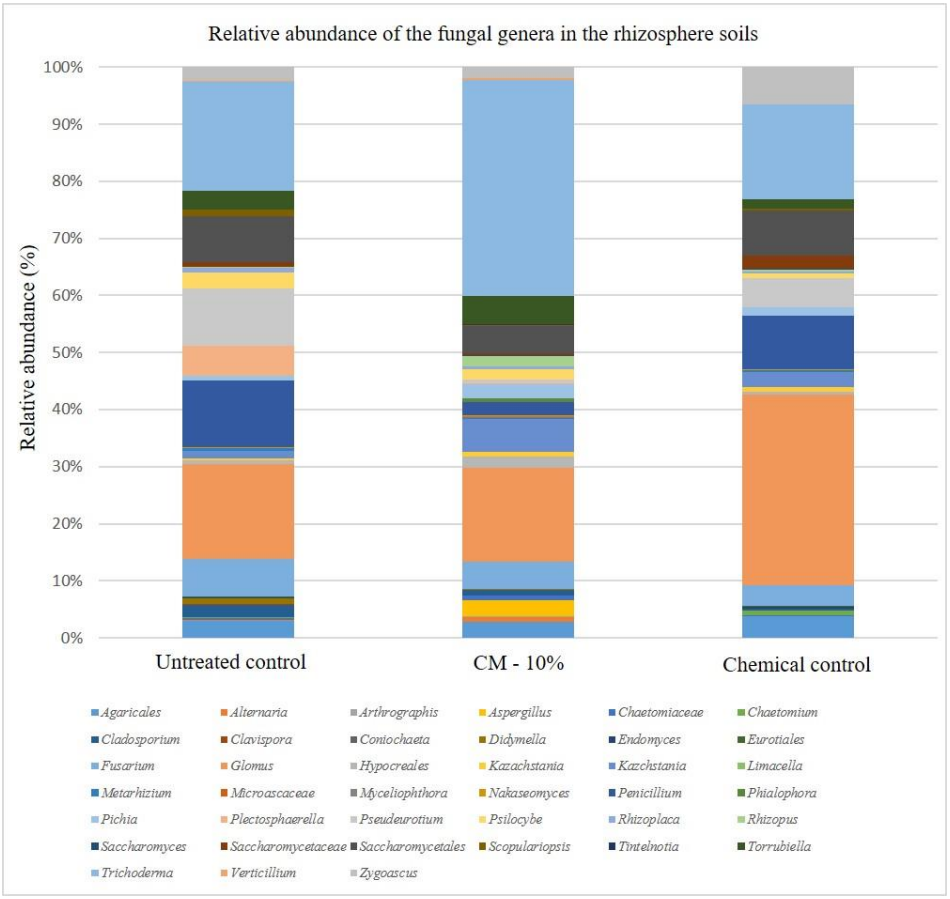

Figure 4: Relative abundance of the mycobiota in the rhizosphere samples at the end of the trials: untreated control (UC), chemical control (CC), and CM – 10% treatment. Three biological replicates were collected from three different pots per treatment and for each trial. Only OTUs which showed an incidence above 0.2% in at least two samples are shown. The data from replicates were averaged.

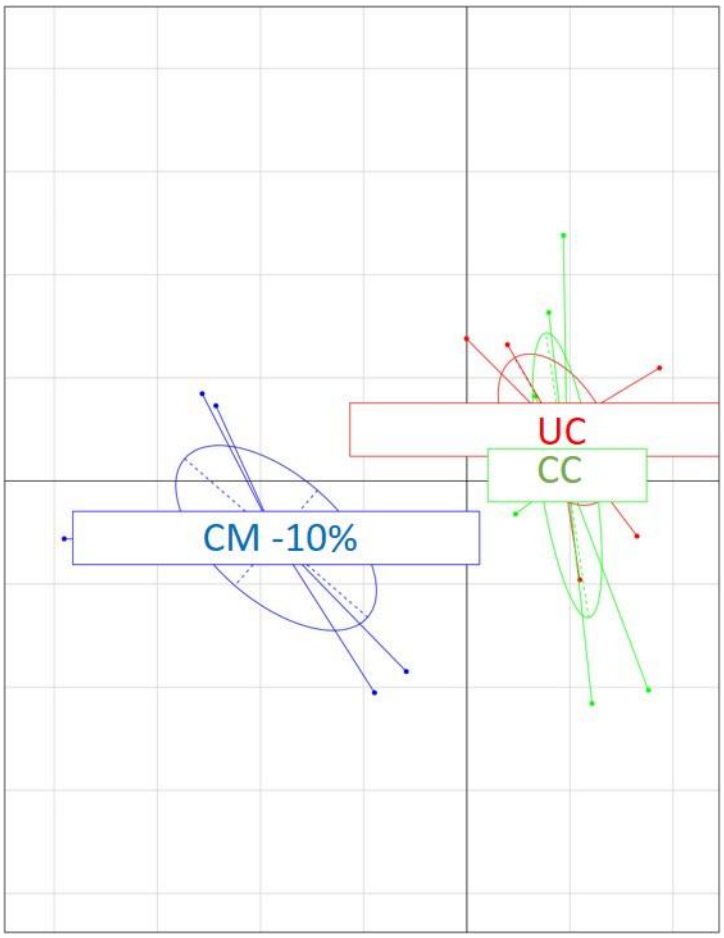

Figure 5: Principal component analysis based on the mycobiota composition referred to the rhizosphere samples at the end of the trials: untreated control (UC), chemical control (CC), and CM – 10% treatment. Three biological replicates were collected from three different pots per treatment and for each trial. The samples are color-coded according to the treatment.

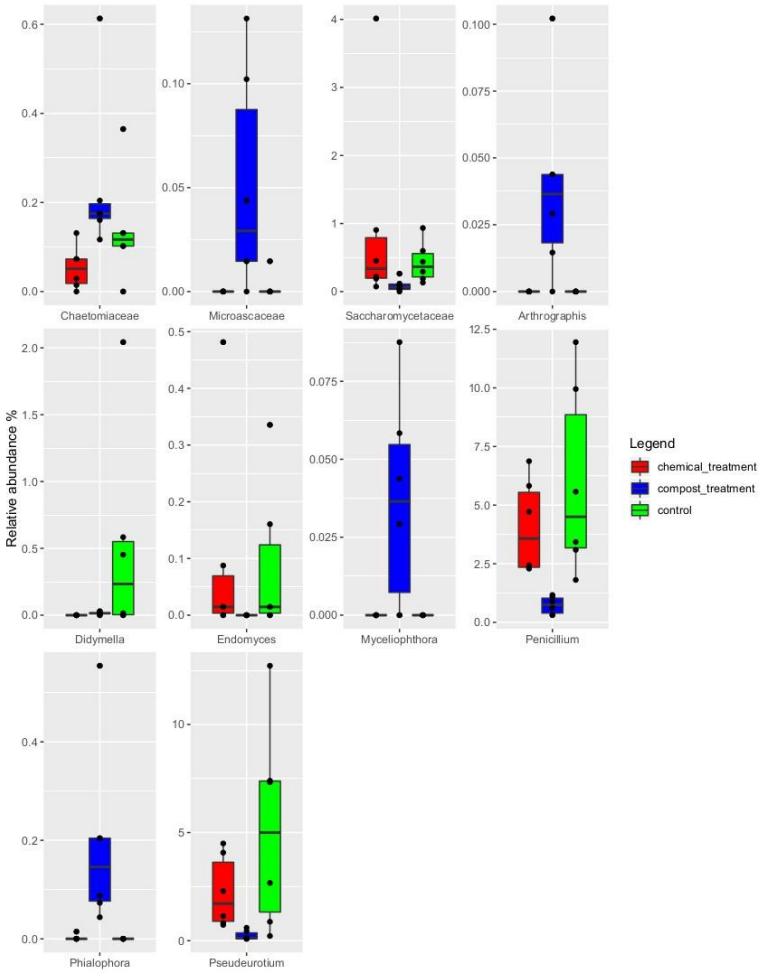

Figure 6: Boxplots showing the relative abundance of the differentially abundant OTUs based on the Wilcoxon matched pairs test (FDR ≤ 0.05) of the rhizosphere soil samples at the end of the trials: untreated control (UC), chemical control (CC), and CM – 10% treatment. Three biological replicates were collected from three different pots per treatment and for each trial. The boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2ND quartile). The whiskers denote the lowest and the highest values within 1.56 IQR from the first and third quartiles, respectively. The circles represent outliers beyond the whiskers.

Co-occurence co-exclusion analysis of the mycobiota of the rhizosphere soils

The OTU co-occurrence/exclusion pattern of the rhizosphere soils is shown in Figure 7, where only significant correlations are reported (at a false discovery rate [FDR] of <0.01). As far as the main OTUs shared in the datasets are concerned, we observed that *Trichoderma* co-occurs with *Artrhographis, Myceliophtora, Phialophora* and *Glomus*; *Verticillium* co-occurs with *Alternaria* and *Cladosporium,* while there is co-exclusion with *Rizophus*; *Penicillium* shows co-exclusion with *Artrographis*, *Limacella* and *Endomyces*, while it co-occurs with *Chaetomium*; *Aspergillus* co-occurs with *Myceliophtora* and shows co-exclusion with *Endomyces*; *Alternaria* co-occurs with *Cladosporium*, *Limacella* and *Verticillium,* while it co-excludes with *Zigoascus*; *Fusarium* co-occurs with *Hypocreales* and *Psillocybe.*

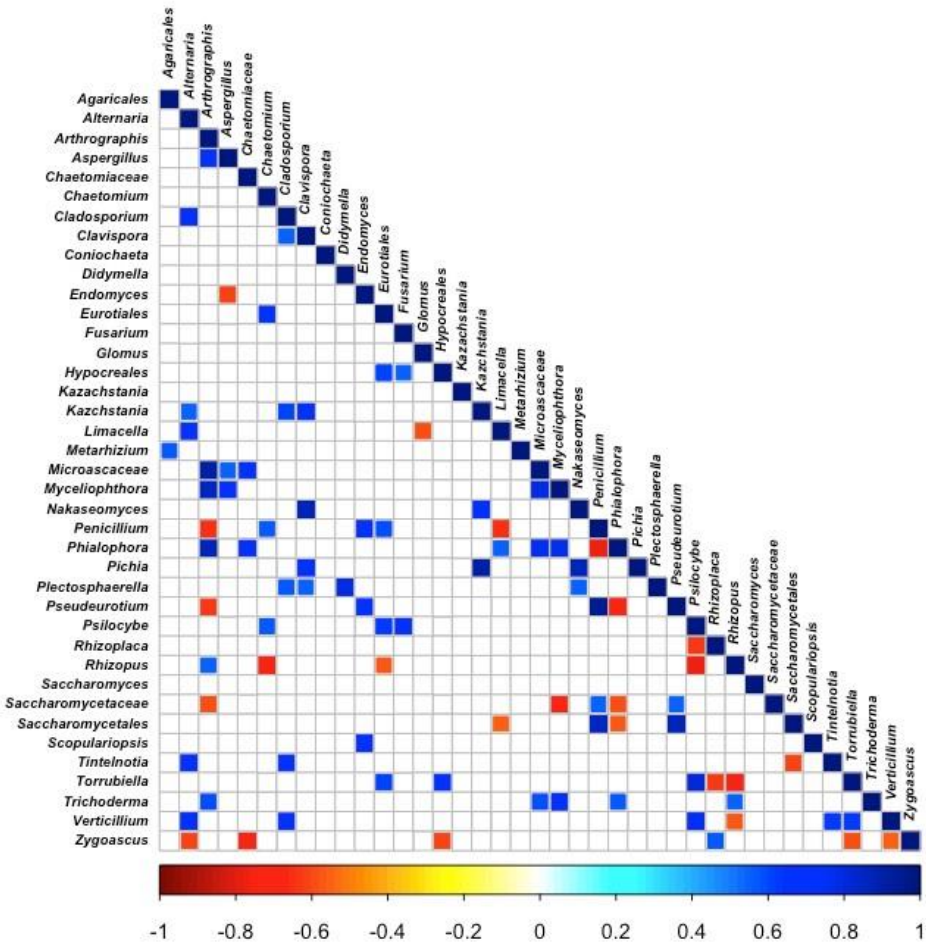

Figure 7: Significant co-occurrence and co-exclusion relationships between the OTUs of the rhizosphere soil samples at the end of the trials. Three biological replicates were collected from three different pots per treatment and for each trial. The figurepresents a Spearman's rank correlation matrix (FDR < 0.01). The color of the scale bar denotes the nature of the correlation, with 1 indicating a perfect positive correlation (dark blue) and –1 indicating a perfect negative correlation (dark red).

Discussion

Differences in the microbial load of the 4 composts used in this study

Real Time PCR assays of the absolute abundance of genes have shown that all the composts had a high number of bacteria and fungi, confirming that all of them were potential sources of inoculum for agricultural applications. The mycobiota composition analyzed with amplicon-based sequencing showed differences between the 4 composts, as already reported for composting procedures, and the choice of the wastes is involved in the selection of the mycobiota (Anastasi et al., 2005; Neher et al., 2013). CV and CM were the same green composts, but CM was added with *Trichoderma* sp. TW2 strain, it is interesting to observe the difference, in terms of the relative abundance of the fungal genera, between these two strains, which suggests that the composition of mature systems, such as a green commercial compost with a strong microbiome, can also be altered.

Disease suppression

The disease assessments showed that, of all the compost mixtures, CM was the most effective one, but only CM – 10% was able to suppress the disease in the summer squash-PHC pathosystem, and to reduce the disease incidence by 50%, compared to the untreated control. The fresh biomass and AUDPC were in accordance with the disease incidence, these results were in accordance in both experiments. CV, which is the same mature compost as CM, but without the addition of the *Trichoderma* sp. TW2 strain, was not able to control the disease in any mixture concentration. This datum suggests that the enrichment of mature composts with biological control agents may be a good strategy for this pathosystem. In addition, *Trichoderma* was effective against PHC, as already reported by

many authors (Ahmed et al., 2000; Ezziyyani et al., 2007; Lorito et al., 2010; Bae et al., 2011), but a previous study conducted in field (Gilardi et al., 2018) showed that, against *Fusarium* wilt in lettuce, the application of *Trichoderma* TW2 alone was less effective compared to use of CM compost. This datum suggested that the combination of both BCA and compost would have been a better option also for the pathosystem considered in this research.

Bonanomi et al. (2018) reviewed several works on BCA compost enrichment and pointed out that this could be the most promising way to achieve a long-term suppressiveness against soil-borne pathogens. In this context, the study of different composts, with the addition of different BCAs for several pathosystems, helps to clarify and identify the best one to use. On the other hand, biocontrol agents can be selected by composts that showed a high suppressive action and can be used as inoculum to enhance the ability of other systems to suppress soil-borne pathogens (Pugliese et al., 2008). Surprisingly CM – 20% treatment did not statistically suppress PHC disease incidence, the reason is still not clear, but it can be hypothesized that the complex interactions among compost's microbiota and rhizosphere's one could be modified when too much compost is added to the peat, regardless further analyses will be performed to investigate this phenomenon.

Differences in the microbial load of the rhizosphere

Real Time PCR of the total gene abundance showed no differences in the rhizosphere soils at the end of the trial for the CM – 10 % treatment, chemical treatment and untreated control for the bacterial and fungal communities. Interestingly, the PHC gene was found in the CM – 10% treatment and Untreated control at similar levels, while it was not found in the Chemical treatment, thus suggesting that the reduction in the disease incidence in the CM – 10% treatment was not due to less development of the oomycete, but could instead be due to either the complex interaction between *Trichoderma* and others microorganisms, or the induction of resistance, which has already been reported to be stimulated by composts and *Trichoderma* spp. on many pathosystems (Vallad et al., 2003; Sang et al., 2010; Martínez-Medina, 2017; Savitha and Sriram, 2017), or even to a combination of both. As for the mycobiota composition in the CM – 10% treatment, it was possible to observe that *Chaetomiaceae* and *Microascaceae* were higher than in the Untreated and Chemical controls. *Chaetomiaceae* is a family composed of cosmopolitan genera, commonly found in the air and soil (Rodríguez et al., 2002), that has already been related to beneficial actions, such as the suppression of *Lisianthus Fusarium* wilt (Zhou et al., 2019), antibacterial activity (Chovanová and Zámocký, 2016) and putative Entomopathogenic activity against Khapra Beetle (Mohammed et al., 2019). The high presence of *Microascaceae* in a compost is not surprising, since this family has already been reported to increase during compost maturation (Galitskaya et al., 2017). Interestingly, the levels of *Penicillium* were lower in the CM – 10% compost treatment than in the Chemical and Untreated control, thus suggesting that this compost treatment could be able to lower the percentage of this genus, which is a well-known plant pathogen and

mycotoxin producer (Olsen et al., 2019; Schmidt-Heydt et al., 2019, 2019; Vidal et al., 2019; Zinedine and El Akhdari). PCA analyses clustered the CM – 10% treatment in a separate spatial dimension from the Chemical and Untreated controls. The chemical control and untreated control clustered together in the PCA analyses, but the disease incidence was zero in the chemical control, thus suggesting that the fungicide used in this study was effective against *Phytophthora capsici* but did not alter the rhizosphere mycobiome, compared to the untreated control, while the CM – 10% treatment altered the equilibrium of the rhizosphere. This datum is further confirmation of the CM suppressive action conferred by the microbial community as a result of the interaction of the *Trichoderma* sp. TW2 inoculum.

Co-exclusion and co-occurrence

The co-occurrence and co-exclusion analyses highlighted that *Verticillium*, *Alternaria* and *Cladosporium* occurred together, which is interesting because of the well-known pathogenic activity of these three genera (Klosterman et al., 2009; Rotem, 1994; Crous et al., 2017; Benschet al., 2012). The fact that these three genera co-occurred in the rhizosphere samples suggests their potential cooperation in biotic stressed plants.

In addition, we observed that *Trichoderma* co-occurred with *Glomus*, the genus considered the one that contains the highest number of arbuscular mycorrhizal species (Schwarzott et al., 2001). This data suggests that a beneficial effect of *Trichoderma* is guaranteed in the rhizosphere environment, not only as a suppressive agent against *Phytophthora capsici*, but also by improving the rhizosphere microbiome in terms of quality.

Conclusions

This study, which involved 4 different composts in a greenhouse pot trial, points out the potential of 12 compost-peat mixtures to prevent *Phytophthora capsici* infection against summer squash. Of all the compost treatments, only CM – 10% was able to suppress PHC. An investigation was therefore conducted on the microbiome composition of: i) the rhizosphere soil at the end this treatment, ii) the chemical control and iii) the untreated control, to understand whether there was a reliable connection with suppressiveness. The mycobiota core composition of the CM – 10% treated pots clustered separately, compared to CC and UC. Further investigations are necessary to obtain a deeper understanding of how this protection is conferred to this pathosystem and how, if possible, to predict the suppressive capacity of mature composts against this disruptive oomycete.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation program under the EXCALIBUR grant agreement. The authors would like to thank Marguerite Jones for the language revision.

Conflict of Interest

The authors declare that they have no conflict of interest. However, Massimo Pugliese declares he has a financial interest as he is a shareholder in the AgriNewTech company that provided the products tested in this paper.

Literature cited

Ahmed, A. S., Sánchez, C. P., & Candela, M. E. (2000). Evaluation of induction of systemic resistance in pepper plants (Capsicum annuum) to *Phytophthora capsici* using *Trichoderma harzianum* and its relation with capsidiol accumulation. *European Journal of Plant Pathology*, *106*(9), 817-824.

Anastasi, A., Varese, G. C., & Marchisio, V. F. (2005). Isolation and identification of fungal communities in compost and vermicompost. *Mycologia,* 97(1), 33-44. doi.org/10.1080/15572536.2006.11832836

Antoniou, A., Tsolakidou, M. D., Stringlis, I. A., & Pantelides, I. S. (2017). Rhizosphere microbiome recruited from a suppressive compost improves plant fitness and increases protection against vascular wilt pathogens of tomato. *Frontiers in Plant Science*, 8, 2022. doi.org/10.3389/fpls.2017.02022

Badri, D. V., Chaparro, J. M., Zhang, R., Shen, Q., & Vivanco, J. M. (2013). Application of natural blends of phytochemicals derived from the root exudates of Arabidopsis to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *Journal of Biological Chemistry*, 288(7), 4502-4512. doi: 10.1074/jbc.M112.433300

Bae, H., Roberts, D. P., Lim, H. S., Strem, M. D., Park, S. C., Ryu, C. M., and Bailey, B. A. (2011). Endophytic *Trichoderma* isolates from tropical environments delay disease onset and induce resistance against *Phytophthora capsici* in hot pepper using multiple mechanisms. *Molecular Plant-Microbe Interactions*, *24*(3), 336-351. doi.org/10.1094/MPMI-09-10-0221

Bensch, K., Braun, U., Groenewald, J. Z., & Crous, P. W. (2012). The genus cladosporium. *Studies in mycology*, *72*, 1-401. doi.org/10.3114/sim0003

Bonanomi, G., Antignani, V., Capodilupo, M., & Scala, F. (2010). Identifying the characteristics of organic soil amendments that suppress soilborne plant diseases. *Soil Biology and Biochemistry*, 42(2), 136-144. doi.org/10.1016/j.soilbio.2009.10.012

Bonanomi, G., Antignani, V., Pane, C., & Scala, F. (2007). Suppression of soilborne fungal diseases with organic amendments. *Journal of Plant Pathology*, 311-324.

Bonanomi, G., Lorito, M., Vinale, F., & Woo, S. L. (2018). Organic amendments, beneficial microbes, and soil microbiota: toward a unified framework for disease suppression. *Annual review of phytopathology*, 56, 1-20. doi.org/10.1146/annurev-phyto-080615-100046

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., Schulze-Lefert, P. (2012). Revealing structure and assembly cues for arabidopsis root-inhabiting bacterial microbiota. *Nature*, 488(7409), 91-95. doi:10.1038/nature11336

Cao, G., Song, T., Shen, Y., Jin, Q., Feng, W., Fan, L., & Cai, W. (2019). Diversity of bacterial and fungal communities in wheat straw compost for *Agaricus bisporus* cultivation. *HortScience*, 54(1), 100-109. doi:10.21273/HORTSCI13598-18

Carrasco, J., Tello, M. L., De Toro, M., Tkacz, A., Poole, P., Péerez-Clavijo, M., & Preston, G. (2019). Casing microbiome dynamics during

button mushroom cultivation: Implications for dry and wet bubble diseases. *Microbiology* (United Kingdom), 165(6), 611-624. doi:10.1099/mic.0.000792

Chovanová, K., & Zámocký, M. (2016). Detection of the antibacterial effect of *Chaetomium cochliodes* Palliser CCM F-232 based on agar plugs and unprocessed fungal substances from cultivation media. *Biologia*, 71(11), 1204-1211. doi.org/10.1515/biolog-2016-0153

Crous, P. W., Braun, U., Schubert, K., & Groenewald, J. Z. (2007). *The genus Cladosporium and similar dematiaceous hyphomycetes*. *CBS, Centralbureau voor Schimmelcultures*.

Cucu, M. A., Gilardi, G., Pugliese, M., Matić, S., Gisi, U., Gullino, M. L., & Garibaldi, A. (2019). Influence of different biological control agents and compost on total and nitrification-driven microbial communities at rhizosphere and soil level in a lettuce - *Fusarium oxysporum* f. sp. *lactucae* pathosystem. *Journal of Applied Microbiology*, 126(3), 905-918. doi:10.1111/jam.14153

De Corato, U., Patruno, L., Avella, N., Lacolla, G., & Cucci, G. (2019). Composts from green sources show an increased suppressiveness to soilborne plant pathogenic fungi: Relationships between physicochemical properties, disease suppression, and the microbiome. *Crop Protection*, 124, 104870. doi.org/10.1016/j.cropro.2019.104870

De Corato, U., Salimbeni, R., & De Pretis, A. (2018c). Suppression of soil-borne pathogens in container media amended with on-farm composted agro-bioenergy wastes and residues under glasshouse condition. Journal of

Plant Diseases and Protection, 125(2), 213-226. doi.org/10.1007/s41348-017-0133-5

De Corato, U., Salimbeni, R., De Pretis, A., Patruno, L., Avella, N., Lacolla, G., & Cucci, G. (2018d). Microbiota from 'next-generation green compost'improves suppressiveness of composted Municipal-Solid-Waste to soil-borne plant pathogens. *Biological control*, 124, 1-17. doi.org/10.1016/j.biocontrol.2018.05.020

Doornbos, R. F., van Loon, L. C., & Bakker, P. A. (2012). Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. *Agronomy for Sustainable Development*, 32(1), 227-243. doi.org/10.1007/s13593-011-0028-y

Erwin, D. C., & Ribeiro, O. K. (1996). Phytophthora diseases worldwide. *American Phytopathological Society (APS Press).*

Ezziyyani, M., Requena, M. E., Egea-Gilabert, C., & Candela, M. E. (2007). Biological control of Phytophthora root rot of pepper using Trichoderma harzianum and Streptomyces rochei in combination. *Journal of Phytopathology*, *155*(6), 342-349. doi.org/10.1111/j.1439-0434.2007.01237.x

Fernández-Bayo, J. D., Hestmark, K. V., Claypool, J. T., Harrold, D. R., Randall, T. E., Achmon, Y., . . . VanderGheynst, J. S. (2019). The initial soil microbiota impacts the potential for lignocellulose degradation during soil solarization. *Journal of Applied Microbiology*, 126(6), 1729-1741. doi:10.1111/jam.14258

Galitskaya, P., Biktasheva, L., Saveliev, A., Grigoryeva, T., Boulygina, E., & Selivanovskaya, S. (2017). Fungal and bacterial successions in the process of co-composting of organic wastes as revealed by 454 pyrosequencing. *PLoS ONE*, 12(10) doi:10.1371/journal.pone.0186051

Gilardi, G., Demarchi, S., Gullino, M. L., & Garibaldi, A. (2015). Nursery treatments with non-conventional products against crown and root rot, caused by phytophthora capsici, on zucchini. *Phytoparasitica*, 43(4), 501-508. doi:10.1007/s12600-015-0461-6

Gilardi, G., Pugliese, M., Gullino, M. L., & Garibaldi, A. (2016). Effect of different organic amendments on lettuce fusarium wilt and on selected soilborne microorganisms. *Plant Pathology*, 65(5), 704-712. doi:10.1111/ppa.12460

Haas, D., & Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature reviews microbiology*, 3(4), 307. doi.org/10.1038/nrmicro1129

Holman, D. B., Hao, X., Topp, E., Yang, H. E., & Alexander, T. W. (2016). Effect of co-composting cattle manure with construction and demolition waste on the archaeal, bacterial, and fungal microbiota, and on antimicrobial resistance determinants. *PLoS ONE*, 11(6), 1-21. doi:10.1371/journal.pone.0157539

Klosterman, S. J., Atallah, Z. K., Vallad, G. E., & Subbarao, K. V. (2009). Diversity, pathogenicity, and management of Verticillium species. *Annual review of phytopathology*, *47*, 39-62. doi.org/10.1146/annurev-phyto-080508-081748

Lamour, K. H., Stam, R., Jupe, J., & Huitema, E. (2012). The oomycete broad-host-range pathogen phytophthora capsici. *Molecular Plant Pathology*, 13(4), 329-337. doi:10.1111/j.1364-3703.2011.00754.x

Lan, C. Z., Liu, P. Q., Li, B. J., Chen, Q. H., & Weng, Q. Y. (2013). Development of a specific PCR assay for the rapid and sensitive detection of *Phytophthora capsici*. *Australasian Plant Pathology*, 42(3), 379-384. doi.org/10.1007/s13313-012-0185-8

Lorito, M., Woo, S. L., Harman, G. E., & Monte, E. (2010). Translational research on Trichoderma: from'omics to the field. *Annual review of phytopathology*, 48, 395-417. doi.org/10.1146/annurev-phyto-073009-114314

Martínez-Medina, A., Fernandez, I., Lok, G. B., Pozo, M. J., Pieterse, C. M., & Van Wees, S. C. (2017). Shifting from priming of salicylic acid-to jasmonic acid-regulated defences by Trichoderma protects tomato against the root knot nematode Meloidogyne incognita. *New Phytologist*, 213(3), 1363-1377. doi.org/10.1111/nph.14251

Martínez-Medina, A., Fernandez, I., Lok, G. B., Pozo, M. J., Pieterse, C. M., & Van Wees, S. C. (2017). Shifting from priming of salicylic acid-to jasmonic acid-regulated defences by Trichoderma protects tomato against the root knot nematode Meloidogyne incognita. *New Phytologist*, *213*(3), 1363-1377. doi.org/10.1111/nph.14251

Mazzola, M., Hewavitharana, S. S., & Strauss, S. L. (2015). Brassica seed meal soil amendments transform the rhizosphere microbiome and improve

apple production through resistance to pathogen reinfestation. *Phytopathology*, 105(4), 460-469. doi:10.1094/PHYTO-09-14-0247-R

Meghvansi, M. K., & Varma, A. (Eds.). (2015). Organic amendments and soil suppressiveness in plant disease management (Vol. 46). *Dordrecht: Springer.*

Michael, V. N., Fu, Y., & Meru, G. (2019). Inheritance of resistance to phytophthora crown rot in *Cucurbita pepo. HortScience*, 54(7), 1156-1158. doi:10.21273/HORTSCI14021-19

Mohammed, A. A., Kadhim, J. H., & Hasan, A. M. (2019). Laboratory Evaluation of Entomopathogenic Fungi for the Control of Khapra Beetle (Coleoptera: Dermestidae) and Their Effects on the Beetles' Fecundity and Longevity. *Journal of Agricultural and Urban Entomology*, 35(1), 1-12. doi.org/10.3954/1523-5475-35.1.1

Mota-Gutierrez, J., Botta, C., Ferrocino, I., Giordano, M., Bertolino, M., Dolci, P., & Cocolin, L. (2018). Dynamics and biodiversity of bacterial and yeast communities during fermentation of cocoa beans. *Applied Environmental Microbiology*, 84(19), e01164-18. doi: 10.1128/aem.01164-18

Muyzer, G., & De Waal, E. (1993). C. and Uitierlinden, A. G,(1993) Profiling of complex microbial populations by denauturing gradient gel electrophoresis analysis of polymerase chain reaction− amplified genes coding for 16SrRKA. *Applied Environmental Microbiology*, 59,695− 700.

Neher, D. A., Weicht, T. R., Bates, S. T., Leff, J. W., & Fierer, N. (2013). Changes in bacterial and fungal communities across compost recipes,

preparation methods, and composting times. *PLoS ONE*, 8(11) doi:10.1371/journal.pone.0079512

Olsen, M., Lindqvist, R., Bakeeva, A., Su-lin, L. L., & Sulyok, M. (2019). Distribution of mycotoxins produced by *Penicillium* spp. inoculated in apple jam and crème fraiche during chilled storage. *International journal of food microbiology*, *292*, 13-20. doi.org/10.1016/j.ijfoodmicro.2018.12.003

Pandey, H. N., Menon, T. C. M., & Rao, M. V. (1989). A simple formula for calculating area under disease progress curve. *Barley and Wheat Newsletter*.

Papasotiriou, F. G., Varypatakis, K. G., Christofi, N., Tjamos, S. E., & Paplomatas, E. J. (2013). Olive mill wastes: a source of resistance for plants against *Verticillium dahliae* and a reservoir of biocontrol agents. *Biological control*, 67(1), 51-60. doi.org/10.1016/j.biocontrol.2013.07.008

Pugliese M., Gilardi G., Garibaldi A., Gullino M. L. (2015). Organic Amendments and Soil Suppressiveness: Results with Vegetable and Ornamental Crops. In: Organic Amendments and Soil Suppressiveness in Plant Disease Management (Meghvansi M. K. and Varma A. coord.), *Soil Biology* 46, Springer, 495-509. doi.org/10.1007/978-3-319-23075-7_24

Pugliese, M., Gullino, M. L., Garibaldi, A., & Marenco, M. (2014). Control of soilborne pathogens on vegetables grown in greenhouse by microorganisms isolated from compost doi:10.17660/ActaHortic.2014.1044.30

Pugliese, M., Liu, B. P., Gullino, M. L., & Garibaldi, A. (2008). Selection of antagonists from compost to control soil-borne pathogens. *Journal of Plant Diseases and Protection*, 115(5), 220-228. doi:10.1007/BF03356267.

Raper, K. B., & Thom, C. (1949). A manual of the *Penicillia*. *A manual of the Penicillia.*

Reuveni, R., Raviv, M., Krasnovsky, A., Freiman, L., Medina, S., Bar, A., & Orion, D. (2002). Compost induces protection against *Fusarium oxysporum* in sweet basil. *Crop Protection*, 21(7), 583-587. doi.org/10.1016/S0261-2194(01)00149-1

Rodríguez, K., Stchigel, A., & Guarro, J. (2002). Three new species of *Chaetomium* from soil. *Mycologia*, 94(1), 116-126. doi.org/10.1080/15572536.2003.11833254

Rotem, J. (1994). The genus *Alternaria*: biology, epidemiology, and pathogenicity. *American Phytopathological Society*.

Sang, M. K., Kim, J. -., & Kim, K. D. (2010). Biocontrol activity and induction of systemic resistance in pepper by compost water extracts against *Phytophthora capsici. Phytopathology*, 100(8), 774-783. doi:10.1094/PHYTO-100-8-0774

Savitha, M. J., & Sriram, S. (2017). Induced systemic resistance (ISR) in hot pepper against *Phytophthora capsici* infection triggered by cell wall oligosaccharide elicitors from *Trichoderma* species. *Indian Journal of Horticulture*, 74(2), 233-239. doi:10.5958/0974-0112.2017.00048.2

Schmidt-Heydt, M., Stoll, D., & Geisen, R. (2019). Whole-Genome sequencing of the fungus *Penicillium citrinum* reveals the biosynthesis gene cluster for the mycotoxin citrinin. *Microbiogical Resource Announcement*, *8*(4), e01419-18. doi: 10.1128/mra.01419-18

Schwarzott, D., Walker, C., & Schüßler, A. (2001). *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (*Glomales*), is nonmonophyletic. *Molecular Phylogenetics and Evolution*, *21*(2), 190-197. doi.org/10.1006/mpev.2001.1007

Siddique, M. I., Lee, H. -., Ro, N. -., Han, K., Venkatesh, J., Solomon, A. M., . . . Kang, B. -. (2019). Identifying candidate genes for *Phytophthora capsici* resistance in pepper (*Capsicum annuum*) via genotyping-by-sequencing-based QTL mapping and genome-wide association study. *Scientific Reports*, 9(1) doi:10.1038/s41598-019-46342-1

Šišić, A., Al-Hatmi, A. M., Baćanović-Šišić, J., Ahmed, S. A., Dennenmoser, D., de Hoog, G. S., & Finckh, M. R. (2018). Two new species of the *Fusarium solani* species complex isolated from compost and hibiscus (*Hibiscus* spp.). *Antonie van Leeuwenhoek*, 111(10), 1785-1805. doi.org/10.1007/s10482-018-1068-y

Tilston, E. L., Pitt, D., & Groenhof, A. C. (2002). Composted recycled organic matter suppresses soil-borne diseases of field crops. *New Phytologist*, 154(3), 731-740. doi.org/10.1046/j.1469-8137.2002.00411.x

Vainio, E. J., & Hantula, J. (2000). Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal

DNA. *Mycological research*, 104(8), 927-936. doi.org/10.1017/S0953756200002471

Vallad, G. E., Cooperband, L., & Goodman, R. M. (2003). Plant foliar disease suppression mediated by composted forms of paper mill residuals exhibits molecular features of induced resistance. *Physiological and Molecular Plant Pathology*, 63(2), 65-77. doi.org/10.1016/j.pmpp.2003.10.003

Vidal, A., Ouhibi, S., Gali, R., Hedhili, A., De Saeger, S., & De Boevre, M. (2019). The mycotoxin patulin: An updated short review on occurrence, toxicity and analytical challenges. *Food and Chemical Toxicology*. doi.org/10.1016/j.fct.2019.04.048

Weller, D. M., Raaijmakers, J. M., Gardener, B. B. M., & Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annual review of phytopathology*, 40(1), 309-348. doi.org/10.1146/annurev.phyto.40.030402.110010

Zhao, F., Zhang, Y., Dong, W., Zhang, Y., Zhang, G., Sun, Z., & Yang, L. (2019). Vermicompost can suppress *Fusarium oxysporum* f. sp. *lycopersici* via generation of beneficial bacteria in a long-term tomato monoculture soil. *Plant and Soil*, 440(1-2), 491-505. doi:10.1007/s11104-019-04104-y

Zhou, G., Qiu, X., Chen, L., Zhang, C., Ma, D., & Zhang, J. (2019). Succession of organics metabolic function of bacterial community in response to addition of earthworm casts and zeolite in maize straw

composting. *Bioresource Technology*, 280, 229-238. doi:10.1016/j.biortech.2019.02.015

Zhou, X., Li, C., Liu, L., Zhao, J., Zhang, J., Cai, Z., & Huang, X. (2019). Control of Fusarium wilt of lisianthus by reassembling the microbial community in infested soil through reductive soil disinfestation. *Microbiological research*, 220, 1-11. doi.org/10.1146/annurev.phyto.40.030402.110010

Zinedine, A., & El Akhdari, S. (2019). Food Safety and Climate Change: Case of Mycotoxins. In *Handbook of Research on Global Environmental Changes and Human Health* (pp. 74-97). *IGI Global*. doi: 10.4018/978-1-5225-7775-1.ch005

Chapter two: Calcium oxide, potassium phosphite and a *Trichoderma* enriched compost water suspension protect *Capsicum annuum* against *Phytophthora capsici* by priming the immune system

Bellini, A., Pugliese, M., Guarnaccia, V., Meloni, G. R.

and Gullino, L. M.

Pest Management Science, 77(7), 3484-3490. (2021)
https://doi.org/10.1002/ps.6401

Introduction

Phytophthora capsici is a filamentous soilborne oomycete considered one of the most disruptive pathogens of some vegetablecrops, representing the main problem on pepper (*Capsicum annuum*) (Leonian, 1922). The diseases caused by *P. capsici* depend on the siteof infection, ranging from root, fruit, foliar to crown rot (Ristaino, 1990). Disease management is focused on several management toolsincluding agronomical strategies, chemical fungicides and soil disinfestation (Hausbeck, 2004). A good control of the disease was guaranteed with the use of fungicides belonging to phenylamide class (PAF), in particular metalaxyl and its enantiomer mefenoxam. However, the fact these molecules have a specific site of action together with some aspects of *P. capsici* biology, i.e., the sexual recombination through oospores production when two matingtypes are paired and the mutations in zoospores genome mediated by UV light since sporangia are hyaline (Bruin and Edgington, 1982), led to the selection of some resistant strains. On the other hand, pepper plant breeding is promising but not resolutive: resistant varieties have often worse horticultural characteristics (granke et al., 2012). Moreover, it has been proved that genetic resistance might be effective only against some specific strains of *P. capsica* (Foster and Hausbeck, 2010). For these reasons, integrated management of Phytophthora blight is highly recommended.

Beyond the agronomical practices, such as increasing water draining, decreasing watering if not necessary and destroying infected plants, further control strategies need to be developed and implemented in practice. Among them, one interesting technique is the use of the so-called Plant Resistance Inducers (PRIs): inorganic salts, microorganisms and biomolecules able to prime the plant immune system guaranteeing a more efficient and quicker response to pathogens' attack (Alexandersson et al., 2016). PRIs, acting on the host plants rather than targeting directly the pathogen, have several advantages: (i) they are considered safer for both environment and human health compared to chemical fungicides, since they are not focused on biocide mode of action (ii) they can be used on several pathosystems, and (ii) they are less able to induce resistance in target pathogens (Oostendorp et al, 2001). More specifically, PRIs act triggering two different molecular pathways through different stimuli: the systemic acquired resistance (SAR) and the induced systemic resistance (ISR). SAR is salicylic acid dependent, it can be activated by both biotic and abiotic factors and it is able to prime the plant immune system against biotrophic and hemibiotrophic pathogens (Gozzo and Faoro, 2013). ISR is regulated by jasmonic acid and ethylene, it is generally activated by symbiosis with bacteria and fungi and it works against necrotrophic pathogens (Pieterse et al., 2014). The activation of salicylic acid dependent pathway leads to overexpression of genes encoding defence proteins such as: the pathogenesis related proteins (Linthorst and Van Loon, 1991), the defensins (Lay and Anderson, 2005) and the peroxidases (Do et al., 2003). Based on previous experiments showing treatments able to reduce the disease severity and incidence on a wide range of pathosystems (Pugliese et al., 2018; Gilardi et al., 2020 a-b; Gullino et al., 2020), two mineral salts (potassium phosphite and calcium oxide) and a compost enriched with a *Trichoderma* strain (Cucu et al., 2020), were selected to understand their mode of action; acibenzolar-s-

methyl was used as a positive control of resistance induction (Buonaurio et al., 2002). Potassium phosphite has already been proved to be effective in stimulating the SAR in model species like *Arabidopsis* and potato (Olivieri et al., 2012; Mohammadi et al., 2020). Several compost treatments were demonstrated to enhance the immune systems of plants against different pathogens (Sang et al., 2010; Segarra et al., 2013). Moreover, *Trichoderma* spp. were demonstrated as able to activate the systemic acquired resistance pathway (Ahmed et al., 2000; De Sousa et al., 2020). *Trichoderma* sp. TW2 enriched compost has been used to prevent *P. capsici* infection against summer squash (Bellini et al., 2020); its mode of action was related with the alteration of rhizosphere mycobiota, but induction of resistance was also hypothesized. Calcium oxide was demonstrated to be effective in different pathosystems (Pugliese et al., 2018; Gilardi et al., 2020), similarly, it is known that calcium ions are involved in the SAR pathway (Schneider-Müller et al., 1994; Guerra et al., 2020). The aim of this work was to demonstrate the ability of the treatments chosen to act as PRIs in *C. annuum* against *P. capsici*. The investigation was conducted by studying: (i) the expression level of three genes representing the most studied and important families of SAR related genes (*CaPO1* –peroxidases, *CaPBR1* – pathogenesis related proteins and *CaDEF1* - defensins) (Wong et al., 2007; Pandey et al., 2017) through RT-Real-Time PCR assays, and (ii) the accumulation rate of salicylic acid through HPLC-MS/MS analysis.

Materials and methods

Setting of the experiments

The experiment was conducted using 40 plants for each treatment. Twenty plants for each treatment were used to sample the leaves for chemical and molecular analysis, while the other 20 plants were used for the assessment at T48 (48 hours after the inoculation) of mycelium growth, which was performed measuring the expansion of the lesion. The experiment was carried out twice independently.

Statistical analyses

The homogeneity of the data (Levene's test) allowed to unify the two trials. Statistical analyses were performed using the SPSS software (IBM SPSS Statistics, Westland, MI, USA). ANOVA and Duncan post hoc tests were performed to establish the statistical values of the differences ($P < 0.05$) for all data collected (damage on the leaf, molecular and chemical analyses).

Plant material and inoculum

Capsicum annuum seeds were sown in a peat substrate (Tecno 2, 70% white peat and 30% clay, pH 5.5–6, N 110–190 mg L−1, P2O5 140–230 mg L−1 , K2O 170–280 mg L−1 , Turco Silvestro Terricci, Bastia d'Albenga, SV, Italy) and left in a growth chamber at 24 ± 1 °C for 3 weeks. After that, seedlings were transplanted with one plant for every pot (7 × 7 cm) filled with the same peat used for the germination. A strain of P. capsici from the Agroinnova collection (1/63) was grown at 24 ± 1 °C in corn meal agar plates for 1 week before the inoculation.

Treatments and inoculation

Potassium phosphite, calcium oxide and acibenzolar-s-methyl were prepared dissolving commercial products in deionized water as in Table

1. Compost water suspension (Compost w.s.) was prepared in flasks with 50% of green compost fortified with *Trichoderma* sp. TW2 (ANT's Compost M, AgriNewTech srl) and 50% of deionized water kept stirring overnight. After that, the supernatant was filtered with paper to remove the compost. The compost used was the same already applied in previous trials (Sang et al., 2010; Pagliarani et al., 2020). Treatments were applied as radical drench with an amount of 100 mL per each pot, two times: 72 and 24 h before inoculation with the pathogen; the not inoculated and the untreated control plants were drenched with water. The inoculation of *P. capsici* was carried out after 24 h from the last PRIs treatment, briefly: a 5 mm (diameter) plug disk of fresh mycelium was cut from Corn Meal Agar plates and placed on the third leaf previously moisturized with sprayed water. After the inoculation, plants were covered with plastic bags to create a moist chamber for 24 h.

Table 1. List and amount of the treatments used in the trials

Treatment	Commercial name	Dosage (a. i.) mL or g L^{-1}	% Active ingredient
Potassium phosphite P_2O_5 52%, K_2O 42%	Alexin	1.03 + 1.05 g L^{-1}	52 + 42
Acibenzolar-s-methyl	Bion	0.0125 g L^{-1}	50
Calcium oxide	Califol	1.10 mL L^{-1}	22.1
Compost water suspension	ANT's Compost M	50% compost 50% water	—
Untreated inoculated control	—	—	
Untreated not inoculated control	—	—	

Analyses of target gene expression

The third leaf of inoculated and not inoculated plants was collected in biological triplicate at three time points: immediately before inoculation

(T0), 6 h after inoculation (T6) and 24 h after inoculation (T24). The leaves were frozen with liquid nitrogen and stocked at −80 °C until the extraction. 50 mg of each sample was grinded in liquid nitrogen with pestles and mortars previously sterilized for 3 h at 170 °C to eliminate any nuclease. The samples were then extracted using the RNase-Free DNase I Kit (Norgen Biotek Corp., Canada) following the manufacturer's instructions. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA contamination was eliminated using the TURBO DNA-free™ Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the rigorous treatment as in the manufacturer's instructions. The samples were retrotranscribed with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The obtained cDNA was analysed in biological and technical triplicate using a StepOne-PlusTM Real-Time System with the Power SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Three key genes of the systemic acquired resistance were analysed to assess their expression levels: *CaPBR1*, *CaPO1* and *CaDEF1*, while *CaActin* (the actin gene) was used as housekeeping gene. The primer (Zhang et al., 2019) list and the RT Real-Time PCR conditions are described in Table 2. The expression levels of the target genes were calculated through $2^{-\Delta\Delta CT}$ method.

Table 2. Description of primer sets and amplification conditions ofRT Real-Time PCR assays

Gene	Primers	Real time PCR conditions
Pathogenesis related	CAPR1-F	40 Cycles
protein (PR)1 - *CaBPR1*	CAPR1-R	95° 10^{00}, 56° 30^{00}, 72° 30^{00}
Defensin - CaDEF1	CADEF-F	40 Cycles
	CADEF-R	95° 10^{00}, 56° 30^{00}, 72° 30^{00}
Peroxidase - CaPO1	CAPO1-F	40 Cycles
	CAPO1-R	95° 10^{00}, 56° 30^{00}, 72° 30^{00}
Actin - CActin2	CACTIN2-F	40 Cycles
	CATIN2-R	95° 10^{00}, 56° 30^{00}, 72° 30^{00}

All primers and amplification conditions were taken from Zhang et al (2019)

Chemicals and reagents

HPLC-grade acetonitrile, methanol, acetic acid and formic acid were purchased from VWR International (Radnor PA, USA) and Sigma-Aldrich (St Louis, MO, USA). Water was obtained using a Milli-Q water purification system (G. Maina, Pecetto Torinese, Italy). The standard compound salicylic acid (purity ≥99%) was supplied by Sigma-Aldrich (St Louis, MO, USA). Two stock standard hormone solutions were made in methanol at the concentration of 1000 and 10 μg mL^{-1}, respectively, and both stored in the dark at −20 °C, while a standard working solution was prepared daily by diluting the standard stock solution to obtain calibration curve.

Extraction and HPLC-MS/MS analysis of salicylic acid

Phytohormone analysis from pepper leaves was performed following the procedure previously reported by Pagliarani et al. (2020), with minor modifications. Pepper leaves samples were frozen in liquid nitrogen and homogenized with mortars and pestles. About 0.2 g of sample was accurately weighed, transferred to 2 mL centrifuge tube and dissolved in 2

mL of extract solution (methanol: water, 80:20, v/v and acidified with 0.1% acetic acid). The solution was shaken at 4 °C overnight in the dark and filtered with a 0.2 μm cellulose filter. Finally, the supernatant was analysed by HPLC-MS/MS. Quantification of salicylic acid was performed using a 1260 Agilent Technologies system consisting of a binary pump and a vacuum degasser, connected to a Varian autosampler, Model 410 Prostar (Hansen Way, CA, USA), equipped with a 20 μL loop coupled to a Varian 310-MS TQ Mass Spectrometer. A Luna 3 μm phenyl-hexyl (150 × 2 mm, Phenomenex, Torrance, CA, USA) under a flow of 200 μL min^{-1}, was used for the chromatographic separation. Solvent A was H2O, while solvent B was CH3CN, both acidified with 0.1% formic acid. The gradient elution was programmed as follows: 0–7 min isocratic 40% solvent B, followed by a linear gradient from 40% to 100% B in 5 min and holding at 100% B for 4 min. The injection volume was 10 μL and the mass spectrometer was operated in the ESI (electrospray) positive ionization mode using multiple reaction monitoring (MRM) mode. The quantification ion transitions selected was 137 > 93 (16 eV). The collision gas (Ar) pressure was set at 2 mbar for all experiments.

Results

Containment of *P. capsici* growth

The symptoms on the leaves were assessed 48 h after the inoculation, measuring the diameter of the oomycete growth (mm). All treated plants showed a statistical reduction of the oomycete growth (Fig. 1) compared to the one recorded on the leaves of untreated plants. Average values of 3, 4, 4.6 and 4.8 mm were observed in compost water suspension, acibenzolar-s-methyl, potassium phosphite and calcium oxide treated plants, respectively, compared to an average value of 18 mm measured on the leaves of untreated plants.

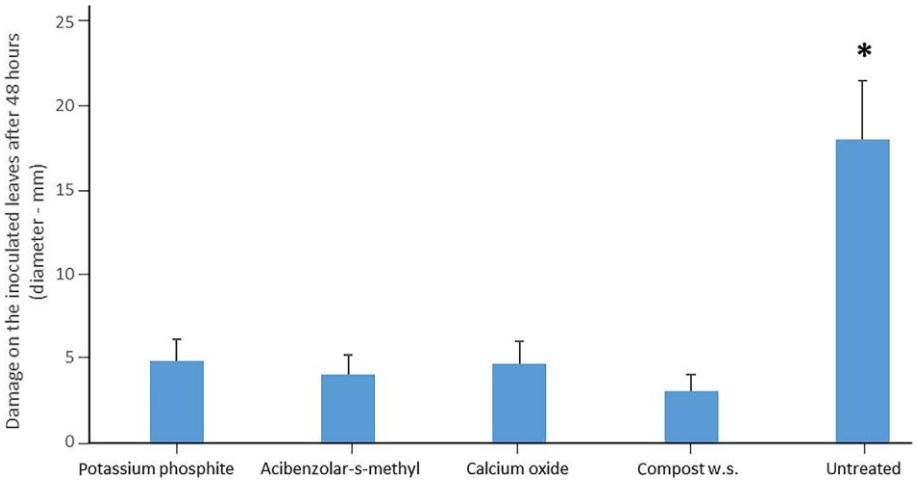

Figure 1. Containment of *P. capsici* growth in the inoculated leaves compared assessed as diameter (cm) of the damage after 48 h post inoculation. Theasterisk specifies the significant difference obtained with On-way Anova followed by the Duncan post-hoc test.

Chemical analyses

At T0, potassium phosphite and acibenzolar-s-methyl treated plants showed statistical overaccumulation of salicylic acid compared to untreated ones (respectively 760.2, 684.4 and 452.6 ng g^{-1}), while calcium oxide (548.6 ng g^{-1}) and compost suspension (431.7 ng g^{-1}) treated plants did not statistically differentiate from the control. At T6, no plant showed

statistical overaccumulation compared to the control, ranging from a minimum of 482 ng *C. annuum* (compost treated) and a maximum of 563.3 ng *C. annuum* (not inoculated untreated control). At T24, potassium phosphite, calcium oxide and compost water suspension treated plants (603.1, 528.5 and 521.6 ng g^{-1}) overaccumulated salicylic acid compared to acibenzolar-s-methyl (497.2 ng g^{-1}) and the untreated (413.9 ng g^{-1}) and not inoculated (399.9 ng g^{-1}) controls. Data shown in Fig. 2.

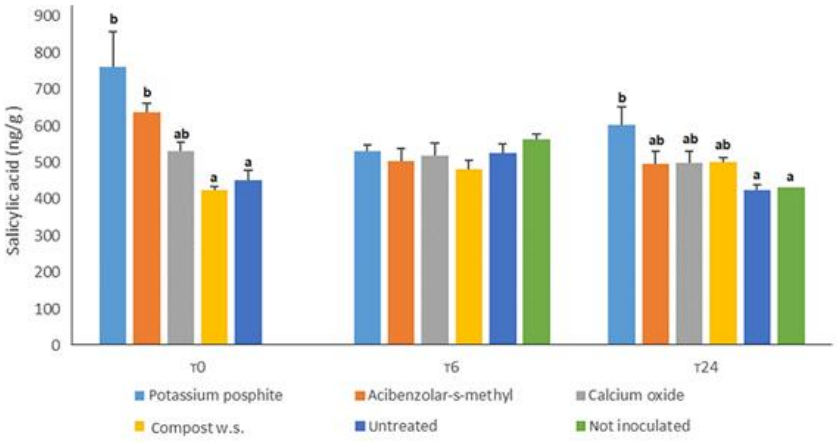

Figure 2. Accumulation of salicylic acid in the upper leaves at T0, T6 and T24 (post accumulation). Data were evaluated by HPLC-MS/MS analysis. Lettersrefers to Duncan post-hoc test performed after one way Anova. Data from first and second replication are here condensed.

Analyses of target gene expression

CaBPR1 gene was generally more expressed at T0: potassium phosphite, acibenzolar-s-methyl and calcium oxide treated plants showed overexpression compared to the untreated control (respectively 8, 5, and 4

times). At T6, calcium oxide treated plants showed no statistical difference compared to the untreated not inoculated control. All the others showed a significant reduction of *CaBPR1* gene expression. At T24, *CaBPR1* gene was overexpressed in compost water suspension treated plants, while all the other plants did not statically differentiate. Peroxidase gene was not expressed at T0 in any sample; while at T6 a great overexpression of *CaPO1* gene was observed in samples from acibenzolar-s-methyl and calcium oxide treated plants (49 and 26×) compared to the untreated control. At T24, no differences were observed. *CaDEF1* gene was overexpressed in calcium oxide treated plants at T0; whereas at T6 there was a downregulation compared to the untreated not inoculated control. At T24, acibenzolar-s-methyl treated plants showed overexpression of *CaDEF1* compared to the controls. Data were collected in Fig. 3–5, respectively for *CaPBR1*, *CaPO1* and *CaDEF1*.

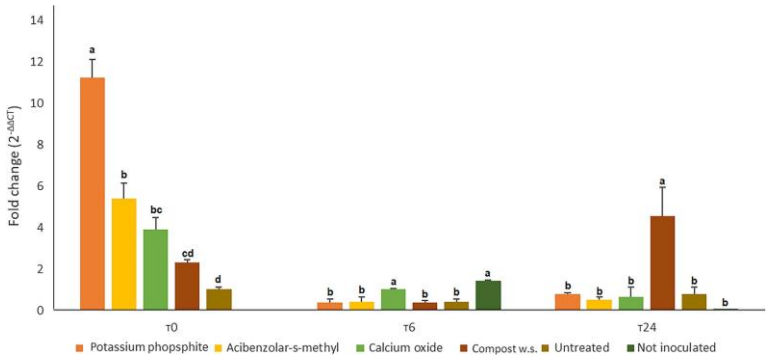

Figure 3. Expression levels of *CapPBR1* gene in inoculated leaves (third) at T0, T6 and T24 (hours post inoculation). The data were calculated with RT-Real- Time PCR assay (*CaActin* gene was used as housekeeping) and extrapolated through $2^{-\Delta\Delta CT}$ method. Letters refers to Duncan post-hoc test performedafter one way Anova. Data from first and second replication are here condensed.

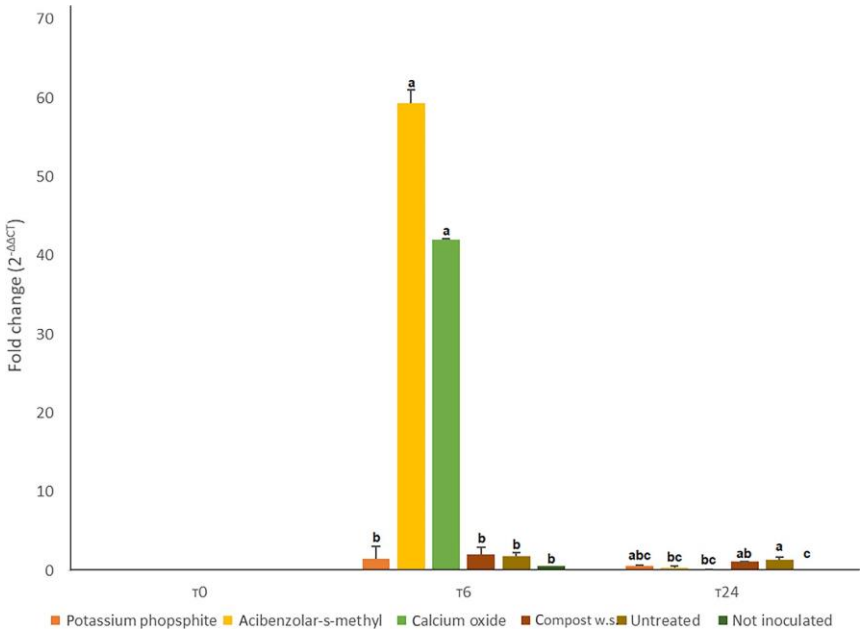

Figure 4. Expression levels of *CaPO1* gene in inoculated leaves (third) at T0, T6 and T24 (hours post inoculation). The data were calculated with RT-Real- Time PCR assay (*CaActin* gene was used as housekeeping) and extrapolated through $2^{-\Delta\Delta CT}$ method. Letters refers to Duncan post-hoc test performed after one way Anova. Data from first and second replication are here condensed.

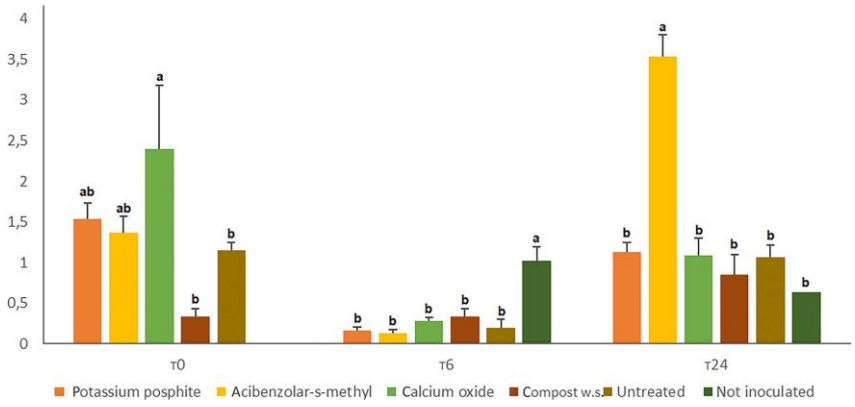

Figure 5. Expression levels of *CaDEF1* gene in inoculated leaves (third) at T0, T6 and T24 (hours post inoculation). The data were calculated with RT-Real- Time PCR assay (*CaActin* gene was used as housekeeping) and extrapolated through $2^{-\Delta\Delta CT}$ method. Letters refers to Duncan post-hoc test performedafter one way Anova. Data from first and second replication are here condensed.

Discussion

The adoption of proper management strategies against *P. capsici* has a key role for *C. annuum* cultivation both in open field and in greenhouse since this pathogen represents the major cause of significant economic losses. The aim of this study was to elucidate the mechanisms of action of the treatments here considered (potassium phosphite, calcium oxide and a water suspension from a *Trichoderma* enriched compost) in the specific pathosystem *P. capsici – C. annuum*. To avoid a possible direct biocide effect caused by the treatments, the site of inoculation and the site of treatments were spatially divided, the first one was performed on the third leaf, while the treatments were given as radical drench as in Zhang et al. (2019) The disease severity on the inoculated leaves was calculated as diameter of the lesion caused by *P. capsici*'s growth 48 h after the inoculation. The treated plants showed a significant statistical reduction of the lesions compared to the untreated ones, which could be determined by a range of several factors. To better investigate if, among these factors the activation of systemic acquired resistance was involved, both chemical and molecular analyses were conducted on tissues sampled at three endpoint times (T0, T6 and T24). RT Real-Time PCR assay was used to evaluate the expression levels of *CaBPR1*, *CaPO1* and *CaDEF1* on the third leaves. HPLC-MS/MS analysis was performed on upper leaves samples to assess the accumulation of salicylic acid in the tissues above the ones colonized by the pathogen, since it is known that the hormone generally has an acropetal movement inside the plants vascular system (Guedes et al., 1980). At T0 (72 and 24 h after the treatments) both overaccumulation of salicylic acid (in potassium phosphite and acibenzolar-s-methyl treated plants) and overexpression of *CaBPR1* and *CaDEF1* (in potassium phosphite and calcium oxide treated plants) were demonstrated. Genes expressing

pathogenesis related proteins are highly inducible by the accumulation of salicylic acid (Mandal et al., 2009) and have been proved to be overexpressed in potato plants 72 h after the treatments with potassium phosphite compared to control (Feldman et al., 2020). Defensins are proteins that exert a great antifungal activity (Thomma et al., 2002) helping the plants to cope with infections. *CaPO1* gene was not expressed at T0 in accordance with the literature, since it is highly stress inducible (Kawano, 2003). The fact that potassium phosphite and calcium oxide treatments determined an overaccumulation of salicylic acid and/or overexpression of *CaBPR1* and *CaDEF1* before the inoculation, highlights a clear priming effect, suggesting that the induction of resistance in *C. annuum* by these treatments could be helpful not only against *P. capsici* infection, but also against other biotrophic and hemibiotrophic pathogens. At T6 no overaccumulation of salicylic acid was observed in the upper leaves, in accordance with Rasmussen et al. (1991) where it was demonstrated that at least 4–6 h are needed to synthetize the salicylic acid signal in the infected tissues and then translocate it. Acibenzolar-s-methyl and calcium oxide treated plants showed a great over expression of *CaPO1* gene at T6, as already proved with other treatments by Wang et al. (2013) confirming the involvement of these treatments in the activation of SAR pathway leading to a more efficient oxidative burst compared to the untreated plants. At T24, overaccumulation of salicylic acid was detected in potassium phosphite, calcium oxide and water compost treated plants. While acibenzolar-s-methyl treatment was able to overexpress *CaDEF1* gene in accordance with Zhang et al. (2019) where *P. capsici* induced an overexpression of the defensin gene in pepper 24 h after the inoculation. Moreover, compost water suspension treated plants overexpressed *CaPBR1* gene at T24 in accordance with Asghari et al. (2020) and Esmail

et al. (2020) who demonstrated overexpression of the target gene 24 h after inoculation respectively in grapevine plants against *Agrobacterium tumefaciens* and in *Triticum aestivum* plants primed with diverse *Trichoderma* strains against *Puccinia graminis* f. sp. *tritici*. The fact that at T24 overexpression of target genes and overaccumulation of salicylic acid were here demonstrated, suggests that the immune system of the treated plants was still in an alert status. No overexpression of *CaPO1* gene was detected at T24, in accordance with Do et al. (2013). These results confirmed the ability of the treatment here considered to act as PRIs, enhancing *C. annuum* defences against *P. capsici*. The overexpression of the three target genes in inoculated leaves of treated plants proved that the molecular pathway was activated in a more efficient way, furthermore the overaccumulation of salicylic acid in upper leaves corroborated the hypothesis. The method used to prove this stimulation of the plant immune system was effective since it allowed to study the systemic response from the roots, passing by the inoculated leaf to the upper ones.

Conclusion

In conclusion, these findings highlight the priming effect of the treatments considered in this study, thus suggesting that the mode of action is not, at least only, related with the direct interaction with the pathogen. To the best of the knowledge there are not similar studies on this pathosystem which consider the treatments used in this research analyzing both the expression of resistance genes and the accumulation of salicylic acid. More research will be undertaken to test if their ability to induce the SAR is maintained in field conditions and also in other pathosystems.

Acknowledgements

This research was supported by the EU Horizon2020 NUTRIMAN project (GA 818470). The authors would like to thank AgriNewTech srl for providing two products (ANT's Compost M and Califol) used in this research.

Conflict of interest

Massimo Pugliese declares he has a financial interest as he is a shareholder in the AgriNewTech company that provided some of the products tested in this article. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Literature cited

Ahmed A. S., Sánchez C. P. and Candela M. E., (2000). Evaluation of induction of systemic resistance in pepper plants (*Capsicum annuum*) to *Phytophthora capsici* using *Trichoderma harzianum* and its relation with capsidiol accumulation. *European Journal of Plant Pathology* 106:817–824

Alexandersson E., Mulugeta T., Lankinen Å., Liljeroth E. and Andreasson E., (2016). Plant resistance inducers against pathogens in *Solanaceae* species—from molecular mechanisms to field application. *International Journal of Molecular Science* 17: 1673

Asghari S., Harighi B., Ashengroph M., Clement C., Aziz A., Esmaeel Q., (2020). Induction of systemic resistance to *Agrobacterium tumefaciens* by endophytic bacteria in grapevine. *Plant Pathology* 69:827–837

Bellini A., Ferrocino I., Cucu M. A., Pugliese M., Garibaldi A. and Gullino M. L., (2020). A compost treatment acts as a suppressive agent in *Phytophthora capsici–Cucurbita pepo* pathosystem by modifying the rhizosphere microbiota. *Frontiers in Plant Science* 11:885

Bruin G. C. A. and Edgington L. V., (1982). Induction of fungal resistance to meta-laxyl by ultraviolet irradiation. *Phytopathology* 72:476–480

Buonaurio R., Scarponi L., Ferrara M., Sidoti P. and Bertona A., (2002). Induction of systemic acquired resistance in pepper plants by acibenzolar-S-methyl against bacterial spot disease. *European Journal of Plant Pathology* 108:41–49

Cucu M. A., Gilardi G., Pugliese M., Gullino M. L. and Garibaldi A., (2020). An assess- ment of the modulation of the population dynamics of pathogenic *Fusarium oxysporum* f. sp. *lycopersici* in the tomato rhizosphere by means of

the application of *Bacillus subtilis* QST 713, *Trichoderma* sp. TW2 and two composts. *Biological Control* 142:104158

De Sousa T. P., Chaibub A. A., Da Silva G. B. and De Filippi MCC, (2020). *Trichoderma asperellum* modulates defense genes and potentiates gas exchanges in upland rice plants. *Physiological Molecular Plant Pathology* 112: 101561

Do H. M., Hong J. K., Jung H. W., Kim S. H., Ham J. H. and Hwang B. K., (2003). Expression of peroxidase-like genes, H2O2 production, and peroxidase activity during the hypersensitive response to *Xanthomonas campestris* pv. *vesicatoria* in *Capsicum annuum*. *Molecular Plant Microbe Interaction* 16: 196–205

Esmail S. M., Aboulila A. A. and Abd El-Moneim D., (2020). Variation in several pathogenesis-related (PR) protein genes in wheat (*Triticum aesti-vum*) involved in defense against *Puccinia striiformis* f. sp. *tritici*. *Physiological Molecular Plant Pathology* 112:101545.ì

Feldman M. L., Guzzo M. C., Machinandiarena M. F., Rey-Burusco M. F., Beligni M. V., Di Rienzo J., (2020). New insights into the molecular basisof induced resistance triggered by potassium phosphite in potato. *Physiological Molecular Plant Pathology* 109:101452

Foster J. M. and Hausbeck M. K., (2010). Managing *Phytophthora* crown and root rot in bell pepper using fungicides and host resistance. *Plant Disease* 94:697–702

Gilardi G., Garibaldi A. and Gullino M. L., (2020).Integrated management of downy mildew of basil. *Crop Protection* 265:109207

Gilardi G., Pugliese M., Gullino M. L. and Garibaldi A., (2020). Effect of biocontrol agents and potassium phosphite against *Phytophthora* crown rot, caused by *Phytophthora capsici*, on zucchini in a closed soilless sys- tem. *Sci Hortic* 265:109207

Gozzo F. and Faoro F., (2013).Systemic acquired resistance (50 years after discovery): moving from the lab to the field. *Journal of Agriculture and Food Chemistry* 61:12473–12491

Granke L. L., Quesada-Ocampo L., Lamour K. and Hausbeck M. K., (2012). Advances in research on *Phytophthora capsici* on vegetable crops in the United States. *Plant Disease* 96:1588–1600

Guedes M., Richmond S. and Kuć J., (1980). Induced systemic resistance to anthracnose in cucumber as influenced by the location of the inducer inoculation with *Colletotrichum lagenarium* and the onset of flowering and fruiting. *Physiological Plant Pathology* 17:229–233

Guerra T., Schilling S., Hake K., Gorzolka K., Sylvester F. P., Conrads B. (2020). Calcium-dependent protein kinase 5 links calcium signaling with N-hydroxy-l-pipecolic acid and SARD 1 dependent immune memory in systemic acquired resistance. *New Phytology* 225:310–325

Gullino M. L., Tabone G., Gilardi G. and Garibaldi A., (2020). Effects of elevated atmospheric CO2 and temperature on the management of powdery mildew of zucchini. *Journal of Phytopathology* 168:405–415

Hausbeck M. K. and Lamour K. H., (2004). *Phytophthora capsici* on vegetable crops: research progress and management challenges. *Plant Disease* 88:1292–1303

Kawano T., (2003). Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. *Plant Cell Report* 21: 829–837 https://doi.org/10.1007/s00299-003-0591-z.

Lay F. T. and Anderson M. A., (2005). Defensins components of the innate immune system in plants. *Current Protein Peptide Science* 6:85–101

Leonian L. H., (1922). Stem and fruit blight of peppers caused by *Phytophthora capsici* sp. nov. *Phytopathology* 12:401–408

Linthorst H. J. and Van Loon L. C., Pathogenesis-related proteins of plants.

Mandal S., Mallick N. and Mitra A., (2009). Salicylic acid-induced resistance to *Fusarium oxysporum* f. sp. *lycopersici* in tomato. *Plant Physiolocy Biochemistry* 47:642–649

Mohammadi M. A., Han X., Zhang Z., Xi Y., Boorboori M. and Wang-Pruski G., (2020). Phosphite application alleviates *Pythophthora infestans* by modulation of photosynthetic and physiobiochemical metabolites in potato leaves. *Pathogens* 9:170

Olivieri F. P., Feldman M. L., Machinandiarena M. F., Lobato M. C., Caldiz D. O., Daleo G. R., (2012). Phosphite applications induce molecular modifications in potato tuber periderm and cortex that enhance resistance to pathogens. *Crop Protection* 32:1–6

Oostendorp M., Kunz W., Dietrich B. and Staub T., (2001). Induced disease resistance in plants by chemicals. *European Journal of Plant Pathology* 107:19–28

Pagliarani C., Moine A., Chitarra W., Meloni G. R, Abbà S, Nerva L. Pugliese M., Gullino M. L. and Gambino G. (2020). The molecular priming of defense responses is differently regulated in grapevine genotypes following elicitor

application against powdery mildew. *International Journal of Molecular Science* 21:6776

Pandey V. P., Awasthi M., Singh S., Tiwari S. and Dwivedi U. N., (2017). A comprehensive review on function and application of plant peroxidases. *Biochemical Analysis* 6:2161–1009

Pieterse C. M., Zamioudis C., Berendsen R. L., Weller D. M., Van Wees S. and Bakker P. A., (2014). Induced systemic resistance by beneficial microbes. *Annual Review of Phytopathology* 52:347–375

Pugliese M., Monchiero M., Gullino M. L. and Garibaldi A., (2018). Application oflaminarin and calcium oxide for the control of grape powdery mil- dew on *Vitis vinifera cv. Moscato*. *Journal Plant Disease Protection* 125:477–482 https://doi.org/10.1007/s41348-018-0162-8.

Rasmussen J. B., Hammerschmidt R. and Zook M. N., (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseu-domonas syringae pv syringae*. *Plant Physiol* 97:1342–1347

Ristaino J. B., (1990). Intraspecific variation among isolates of *Phytophthora cap-sici* from pepper and cucurbit fields in North Carolina. *Phytopathology* 80:1253–1259

Sang M. K., Kim J. G. and Kim K. D., (2010). Biocontrol activity and induction of sys- temic resistance in pepper by compost water extracts against *Phytophthora capsici*. *Phytopathology* 100:774–783

Schneider-Müller S., Kurosaki F. and Nishi A., (1994). Role of salicylic acid andintracellular Ca2þ in the induction of chitinase activity in carrot suspension culture. *Physiological Molecular Plant Pathology* 45:101–109

Segarra G., Santpere G., Elena G. and Trillas I., (2013). Enhanced *Botrytis cinerea* resistance of *Arabidopsis* plants grown in compost may be explained by increased expression of defense-related genes, as revealed by microarray analysis. *PLoS One* 8:1–10 https://doi.org/10. 1371/journal.pone.0056075

Thomma B. P., Cammue B. P. and Thevissen K., (2002). Plant defensins. *Planta* 216:193–202 https://doi.org/10.1007/s00425-002-0902-6.

Wang J. E., Liu K. K., Li D. W., Zhang Y. L., Zhao Q., He Y. M. (2013). A novel perox- idase CanPOD gene of pepper is involved in defense responses to *Phytophtora capsici* infection as well as abiotic stress tolerance. *International Journal Molecular Science* 14:3158–3177

Wong J. H., Xia L. and Ng T. B., (2007). A review of defensins of diverse origins. *Current Protein Peptide Scence* 8:446–459

Zhang H. X., Ali M., Feng X. H., Jin J. H., Huang L. J., Khan A., (2019). A novel tran- scription factor CaSBP12 gene negatively regulates the defense response against *Phytophthora capsici* in pepper (*Capsicum annuum* L.). *International Journal Molecular Science* 20:48

Chapter III: Effects of *Trichoderma* enriched compost, BCAs and potassium phosphite on Fusarium wilt of lettuce and soil microbiome under intensive cultivation system

Bellini A., Gilardi G., Idbella M., Zotti M., Pugliese M., Bonanomi G., and Gullino M.L.

In preparation

Introduction

Lettuce cultivation is practised on 20,000 ha of agricultural land in Italy (ISTAT 2020). As an intensive crop, lettuce production and yield are threatened by the presence of *Fusarium oxysporum* f. sp. *lactucae* (Fol), the causal agent of Fusarium lettuce wilt, one of the most disturbing diseases affecting this crop worldwide (Matheron and Gullino 2012; Gullino et al. 2019). Characteristic symptoms of Fol infection are yellowing of leaves, slow growth accompanied by brown or red streaks of the vascular system and wilting to death. The economic losses, if the infection is not properly managed, can be significant (Matheron and Gullino 2012). In any case, Fol management is difficult for two reasons: (i) it spreads rapidly and easily as it is seed-borne (Garibaldi et al. 2004a) and (ii) has the ability to distinguish new races, in fact four are already known and reported (Fujinaga et al. 2001; 2003; Matheron and Gullino 2012; Gilardi et al. 2017). Chemical control is not considered a sufficient strategy due to the reduced availability of soil fumigants, the need to reduce chemical treatments to avoid environment and lettuce contamination, and the increasing consumer demand for organic vegetable production. For these reasons, it is important to develop and test new alternative control strategies against this pathogen. The use of biological

control agents (BCAs), organic amendments and resistance inducers are among the most studied (Bonanomi et al. 2007; 2010; Gilardi et al. 2019; Gilardi et al. 2020). BCAs can act directly against pathogens via antibiosis, parasitism or predation, or indirectly by colonising the rhizosphere environment and using resources more efficiently than pathogens (Pal and Gardener 2006). Organic amendments, and composts, have been described for decades as suppressive against various vascular pathogens, including Fol (Pugliese et al. 2015; Gilardi et al. 2016; De Corato et al. 2018a, 2018b; Bonanomi et al. 2018). Many studies have demonstrated that composts' microbiota plays an important role in suppressive action (Reuveni et al. 2002; Tilston et al. 2002; Papasotiriou et al. 2013; De Corato et al. 2019). Their mechanisms of action are similar to those of BCAs, but the complexity and richness of the compost microbiota suggest that different mechanisms could be used and act synergistically. Composts can also be enriched with BCAs to enhance their suppressive activity, as it has been indicated that this technique could be the most promising to achieve long-term suppressiveness against vascular pathogens (Bonanomi et al. 2018; Gilardi et al. 2019; Pugliese et al. 2011). Resistance inducers are compounds of various origins that have been shown to activate the plant immune system (Walters et al. 2009; Akram and Anjum 2011; Alexandersson et al. 2016); as they are non-biocidal, they are considered safer for the environment and mammals. BCAs, and by extension composts, have been shown to activate the induction of resistance in several pathosystems (Compant et al. 2005; Ongena et al. 2007; Pieterse et al. 2014; Akram et al. 2015). Integrated pest management (IPM) strategies are combinations of different measures based on the principle of synergy between them to control pathogen attacks (Barzman et al. 2015). The complex of microorganisms inhabiting the

rhizosphere can strengthen plants and protect them from both biotic and abiotic stresses (Nihorimbere et al. 2010), in fact, the rhizosphere microbiota population is considered to be one of the greatest influences on plant health (Berendsen 2012). This is even more important for the protective effect against vascular pathogens that invade plant tissues through the root system, as is the case with Fol (Hubband and Gerik 1993; Gordon 2017). The application of organic amendments and BCAs may cause a shift in the composition and diversity of the rhizosphere microbiota, leading to a change in soil suppressiveness. Because the role of the rhizosphere microbiota in plant health is well established, it is not clear how microorganisms applied as treatments interact with those already present in the soil and their ability to establish permanent colonisation. Studying the rhizosphere microbiota with amplicon-based sequencing is a powerful tool (Simmons et al. 2018; Elsayed et al. 2020) to understand the effects of IPM strategies on microbial populations at the end of the crop cycle and to determine whether the protective effect guaranteed by the strategies is related to the shift in these populations. In the present work, and based on previous studies (Gilardi et al. 2016; 2019), three IPM strategies were developed by using compost enriched with *Trichoderma* sp. strain TW2 and the combinations of different BCAs (*Trichoderma* spp. and *Bacillus amyloliquefaciens* (former subtilis) alone or with potassium phosphite. Lettuce rhizosphere composition and diversity were investigated for both bacteria (16s) and fungi (ITS) using Illumina amplicon-based sequencing. The objectives of the work were: i) to evaluate the efficacy of the three selected IPM strategies against lettuce Fusarium wilt under commercial and semi-commercial fields with different physicochemical characteristics and ii) to study the rhizosphere microbiota of treated and untreated plants.

Material and Methods

Plants material and experimental design

Field trials were conducted on two farms: a commercial farm in Moretta (CN, Italy) and an experimental farm in Carmagnola (TO, Italy). The farm in Moretta (sand:silt:clay 56:19:25, pH 7.12 and 1.37% organic matter) has a natural infestation of Fol race 1, which causes large annual losses in susceptible lettuce cultivars (Gilardi et al. 2019). The experiments here were conducted under a 360 m^2 plastic tunnel, while on the Carmagnola experimental farm a 64 m^2 plastic tunnel was used for the experiments. The soil in Carmagnola (sand:silt:clay 68.16:10.7:21.1, pH 8.2 and 0.94% organic matter) has no history of intensive lettuce cultivation nor evidence of natural occurrence of the pathogen. For these reasons, the soil there was artificially infested with a virulent strain of Fol coded as Mya 3040 (race 1) from the Agroinnova collection (Garibaldi et al. 2002). Soil infestation was carried out as follows: i) the pathogen was cultured in sterilised wheat kernels left at 23°C for two weeks, then ii) 100 g/m^2 of the colonised wheat kernels were incorporated into the soil at a depth of 10-15 cm using a rake. The experiments were repeated in two consecutive years (2019 and 2020, see Table S1) with the lettuce cultivar 'Voluski', classified as moderately susceptible to Fol (Gilardi et al. 2017). In both fields, two-week-old lettuce plants were transplanted in a mulched soil at a density of 16 plants/m^2, with a randomised design of treatments and untreated controls, with four replicates to avoid the side effect. Plants were irrigated with a drip irrigation system and grown in accordance with standard cultivation practises in the region.

Table S1: Schedule of the trials conducted in 2019 and in 2020 in both farms.

year	Farm	Sowing	Transplantation	Trial end
2019	Carmagnola	06/5/2019	30/05/2019	10/07/2019
2020		26/06/2020	21/07/2020	24/08/2020
2019	Moretta	06/5/2019	29/05/2019	01/07/2019
2020		07/05/2020	01/06/2020	06/07/2020

Integrated pest managements treatments

The three IPM strategies tested in this experiment were selected from those described in previous works (Gilardi et al. 2016; 2019), choosing the best combination for the control of Fol in greenhouse and field conditions. The strategies were designed and applied starting at the nursery level as follows: (i) a compost enriched with *Trichoderma* sp. strain TW2 (ANT's compost M; AgriNewTech s.r.l., Italy), (ii) a combination of commercial *Bacillus amyloliquefaciens* (former *subtilis*) QST 713 (Serenade Max, 15.6%, Bayer Crop Science, Italy), *Trichoderma gamsii* icc 080 + *T. asperellum asperellum* icc 012 (Remedier, 2+2%, Isagro Ricerca, Milan, Italy), and potassium phosphite (Alexin, 95PS, P_2O_5 52%, K_2O 42%, Massò, Spain), and (iii) a commercial mixture of *Trichoderma polysporum* IMI 206039 and *Trichoderma atroviride* IMI 206040 (Binab solution 1+1%, BINAB Bio-innovation AB, Florettgatan 5, 254 67 Helsingborg, Sweden). For ease of reading, the tested IPM strategies are coded here as: i) Comp_Tricho, ii) Bac_Tricho and iii) Tricho. The products Bac_Tricho and Tricho were applied as soil drench in the nursery. Comp_Tricho was given as such for T0 treatment at sowing and applied in the field immediately before transplanting at a dosage of 1 kg/m^2 for Moretta. In Carmagnola, Comp_Tricho was applied at a dosage of 0.5 kg/m^2, considering the soil mixture, the possibility of applying the treatment with

the rototiller or manually, and because of the smaller size of the tunnel, which allows less deep diffusion. The type, dosage and timing of application of the three strategies are shown in Table 1.

Table 1: Coding names, technical formulation, dosage and timing of administration of the three IPM strategies used in this work for in fields experiments. T0 refers to the day of sowing, the other timing here presented are referred in days. Dosage column refers to the amount of commercial product used to treat seedling or soil. Comp_Tricho was given as compost as such, while Bac_Tricho and Tricho were given in a volume of 1 l per treatment.

Treatment	**Formulation**	**Commercial name**	**Dosage**	**Number of applications**	**Timing**	**Application**
Comp_Tricho	Ant Compost + *Trichoderma* TW2	ANT's CM	400 g/100 seedlings	2	T0	sowing
			1 kg/m^2 for Moretta; 0.5 kg/m^2 for Carmagnola		Immediately before transplant	field
Bac_Tricho	*Bacillus amyloliquefaciens* (former subtilis) QST 713	Serenade MAX	8 ml/l	2	T0	sowing
					T10	nursery
	Trichoderma gamsii icc 080 + *T. asperellum asperellum* icc 012	Remedier	2.5 g/l	2	T5	sowing
					T15	nursery
	Potassium phosphite	Alexin	2.5 g/l	2	T0	sowing
					T15	nursery
Tricho	*Trichoderma polysporum* IMI 206039 + *Trichoderma atroviride* IMI 206040	Binab solution	1.7 g/l	3	T0	nursery
					T7	nursery
					T15	nursery

Lettuce yield and disease assessment

Yield and disease were evaluated at the end of each experiment. Plants, 64 for each treatment considering 16 plants per replicate as experimental unit, were visually evaluated, and dissected to assign rating scale 0 to 4 (Garibaldi et al. 2004). Disease severity data were calculated as follows:

DS0-100= ∑Nplants*Rating scale0-4*100Total N recorded plants4.

The same plants used for disease severity rating were weighed to measure the fresh weight of each plant at the end of the trials. The data were then analysed using SPSS 26 software by performing one-way analysis of variance (ANOVA) and *Tukey's post hoc* tests to determine the statistical values of differences at the value of $\alpha = 0.05$.

Soil collection and DNA extraction

Rhizosphere samples were collected at the end of the experiment as follows: roots were shaken to avoid any excess of soil, and the remaining particles adhering to the root surface were collected in sterile vials for an amount of 100 mg from each plant. Three biological samples, from each treatment in both fields at the two experimental years, were collected unifying the rhizosphere soil of five plants randomly chosen inside the plots. Microbial DNA extraction was performed using the "EZNA soil DNA kit" (Omega Bio-Tek, Norcross, GA), following manufacturer's instructions. DNA concentration was assessed by using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham MA). Amplicon based sequencing was performed using V3 – V4 region (16s) for bacterial community (primers: 341F - CCTAYGGGRBGCASCAG, 806R – GGACTACNNGGGTATCTAAT) and ITS2 region for fungal community (primers: ITS3 - GCATCGATGAAGAACGCAGC, ITS4-

TCCTCCGCTTATTGATATGC). The analysis was done by Novogene using Illumina NovaSeq 6000 platform (Cambridge Science Park, Cambridge, CB4 0FW, United Kingdom).

Sequence data analysis

Demultiplexed fastq files were processed using the DADA2 pipeline (version 1.16.0) (Callahan et al. 2016) in R software (4.0.4) (Team 2016). The resulting taxonomic units are referred to as amplicon sequence variants (ASVs). For bacterial sequences, forward and reverse reads were trimmed to 250 bp and primer sequences were removed using the following filter parameters: maxN = 0, maxEE for both reads = 2, truncQ = 2 (MaxEE corresponded to the maximum expected errors, TruncQ represented the parameter that truncates reads on the first occurrence of a quality score less than or equal to two, and MaxN was the maximum number of 'N' bases accepted). Nearly 4 million reads were used to estimate the error rates by learnErrors function. Sequences were dereplicated using derepFastq with default parameters and exact sequence variants were resolved using the dada algorithm. The RemoveBimeraDenovo function was then used to remove chimeric sequences. Fungal sequences were preliminary trimmed using Cutadapt software (Martin 2011) to remove adapter sequences and low-quality ends (<Q20). For both the bacterial and fungal datasets, reads with more than three errors in the forward reads and five errors in the reverse reads were removed. Taxonomy was then assigned using assignTaxonomy based on the SILVA (v132) and UNITE (v7) databases for bacterial and fungal communities, respectively (Quast et al. 2013; Nilsson et al. 2019). All sequencing data generated by this study can be found in the ENA using accession number PRJEB46355.

Statistical analysis and data visualization

Plotting was performed using PRIMER 7 software (Primer-E Ltd, Plymouth; UK). Alpha diversity metrics were calculated. Heatmaps were created to assess variation in community composition at lowest taxonomic levels including the most frequent 50 ASVs of both bacterial and fungal communities for all the samples. In heatmaps, to assess the behaviour of group of variables according to IPM strategies, clustering was made according to the index of association. Differently, samples were ordered according to non-metric multidimensional scaling (nMDS) based on Bray-Curtis dissimilarity matrices. The significance of changes in composition of the two communities analysed were tested by PERMANOVA (999 permutations, Table S2), using the treatments, field, and year as fixed factors. The significance of variation in the alpha diversity metrics of the two communities was assessed using the ANOVA test, and specific changes in IPM strategies assessed by *post-hoc* Tukey test. The level of significant differences was assessed at $\alpha < 0.05$. All statistical analyses were performed using R software. Furthermore, functional group variation for the fungal community was analysed, identifying putative fungal functional groups as well as their trophic modes using FUNGuild (Nguyen et al. 2016). Co-occurrence networks incorporating communities containing bacteria and fungi were based on single ASV and generated using only the 50 most frequent ASVs for each bacteria and fungi. The pairwise correlations between the ASVs were calculated using the Spearman correlation in R (version 3.3.2 and Hmisc package 4.0–1). Based on the statistical analysis, only strong and significant correlations were considered (Spearman's $r>0.6$ or $r<-0.6$ and $p<0.05$). The network visualisation was made using Cytoscape (version 3.8.2). Each edge

represents correlation and each node represents an ASV. A set of integrative metrics were calculated and compared to describe the network topology. For example, the average number of neighbours explains the complex pairwise connections and the average path length describes node distribution. Pearson's correlation was used to identify the correlations between disease severity and yield production and the first 50 most abundant ASVs for bacteria and fungi.

Table S2: Result of Permanova significance test across treatments during the two years of the experiment, in two different fields. Treatments were used as the fixed factor (N° of permutation 999). Here reported main significant variation between the studied treatments for fungal and bacterial communities. Test of significance is based on Bray-Curtis similarity values. Significance level is fixed for p-value below 0.05.

	Fungal community	Bacterial community
Permutation N	999	999
Total sum of squares	8.067	3.435
Within-group sum of squares	4.092	1.104
F	2.073	4.504
p	0.004*	0.001*

(*Significance level is at 0.05)

Results

Disease assessment and yield

In both years, the disease severity (DS) recorded in the control plots always showed a statistically higher infection rate compared to the treated ones in Carmagnola (DS 34.4-43.3) and Moretta (DS 43.3-40.0), with the only exception for the Comp_Tricho and Tricho strategies in the trial conducted in 2020 at the Carmagnola experimental farm (Fig. 1a). At the Carmagnola site, Comp_Tricho, Bac_Tricho, Tricho and the control provided DS of 17, 12.5, 14.3 and 34.4 in 2019 and 21.7, 10, 20.8 and 43.3 in 2020, respectively; while at the Moretta site DS of 11, 13, 13.1 and 43.3 in 2019 and 24.4, 14.4, 18.1 and 40 in 2020 were recorded. Except for the case in Carmagnola in 2020, which had a problem with water runoff due to the intense summer rains, all treatments showed a fresh weight between 3.7 and 5.3 kg/m^2, while the controls ranged between 1.4 and 4.4 kg/m^2 (Fig. 1b). In Moretta, all treated plots had statistically higher yield compared to the untreated control in 2020. The same trend was observed in Carmagnola in 2020 with the treatments Comp_Tricho and Bac_Tricho IPM compared to the control. The efficacy of both treatments and yield production are shown in Figure 1.

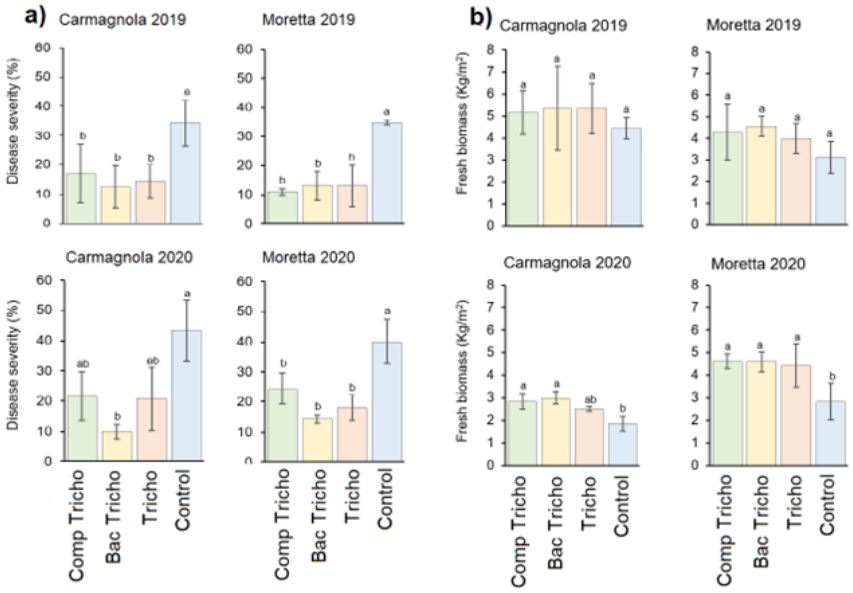

Figure 1: (a) Efficacy of the three IPM strategies to reduce *Fusarium oxysporum* f. sp. *lactucae* disease on lettuce plants expressed as disease severity (%) and (b) productivity expressed as fresh biomass (Kg/m^2). Evaluation made at the final survey. Different letters indicate statistical differences between the four composts, as obtained with the ANOVA test and Tukey's post hoc test ($p<0.05$).

Microbial diversity

Illumina amplicon-based sequencing (on 16s and ITS fragments) revealed the composition of the microbiota in the rhizosphere; diversity indices (number of species, number of reads, and Shannon index) showed no statistical differences between samples for both bacterial and fungal communities (Fig. 2-3).

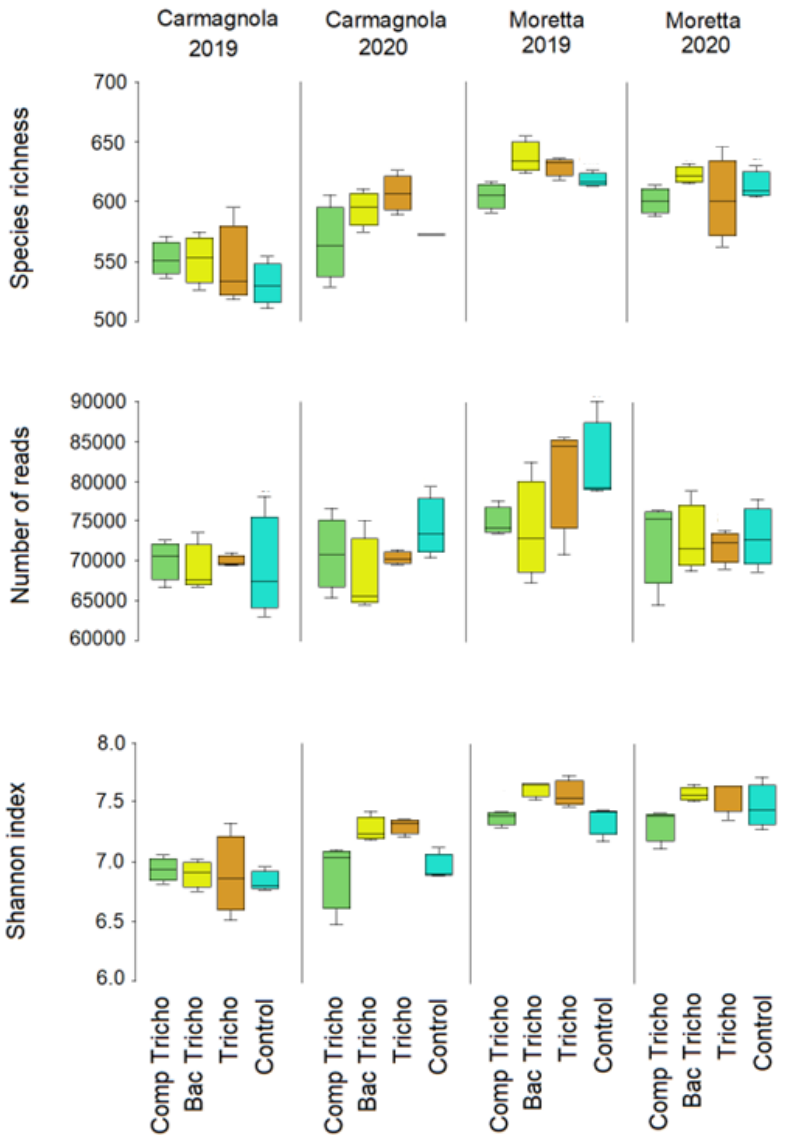

Figure 2: Box and whisker plots showing distribution of diversity indices, number of species, number of reads and Shannon Index, for bacteria community, for each treatment divided per site and year of rhizosphere sampling.

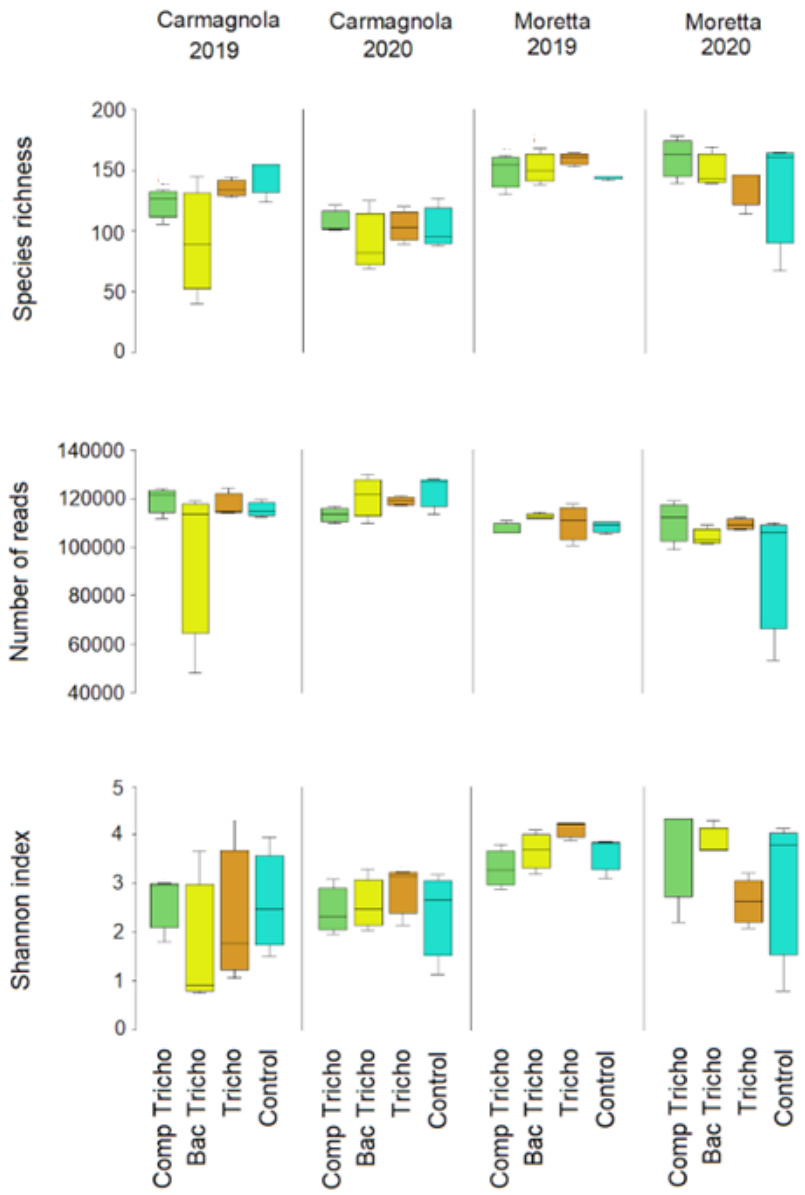

Figure 3: Box and whisker plots showing distribution of diversity indices, number of species, number of reads and Shannon Index, for fungal community, for each treatment divided per site and year of rhizosphere sampling.

Rhizosphere associated bacteria

The bacterial community showed a dominance of five phyla: *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Acidobacteria* and *Actinobacteria* (Fig. 4a) in all samples. There were observable differences only between different trials, indicating some kind of site- and year-specific pattern. No similarities were observed between the same treatments in different years or fields, nor were there any significant differences between treated plants and controls. In Moretta 2019 and in Carmagnola 2019, Comp_Tricho and control plants had a similar rhizosphere bacterial community. Overall, no major differences were observed between controls and treatments. Plotting the 50 most abundant bacterial community ASVs (Fig. 5) showed that the greatest differences in community composition existed between the two fields. Specifically, most bacterial ASVs were found in similar abundance in both Carmagnola and Moretta, while some ASVs showed some specificity for one or the other field, such as *Rodopseudomonas*, *Enterobacteriaceae*, *Paenisporosarcina*, which were found more frequently in the Carmagnola field, and *Blautia*, *Faecalibacterium*, *Escherichia* and *Ralstonia*, which were more frequent in Moretta. Bacteria of the genus *Bacillus*, which were slightly more abundant in Carmagnola, were the most abundant ASV, which underlines the analysis; surprisingly, this genus was not more abundant in the plants treated with Bac_Tricho.

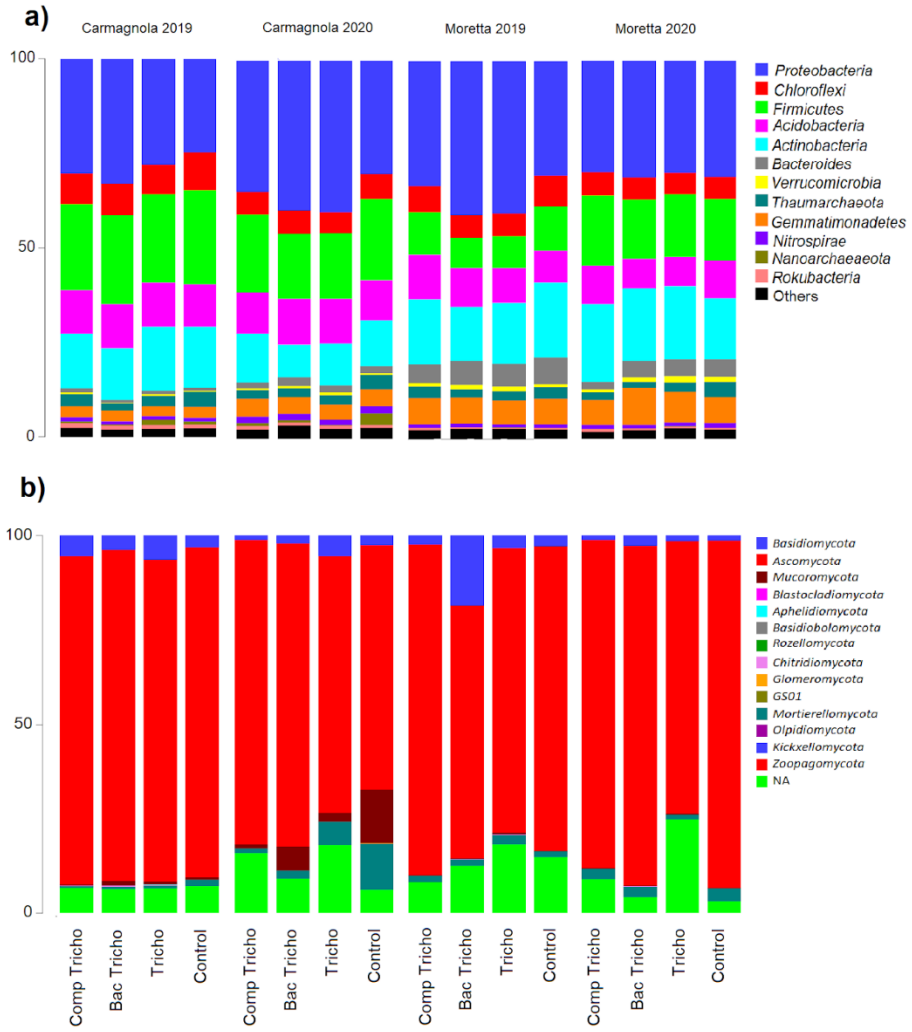

Figure 4: Stacked bar chart of relative abundance for (a) bacterial and (b) fungal communities at phylum level. Data are averaged among three biological replicates for each treatment and are here presented grouped for site and year.

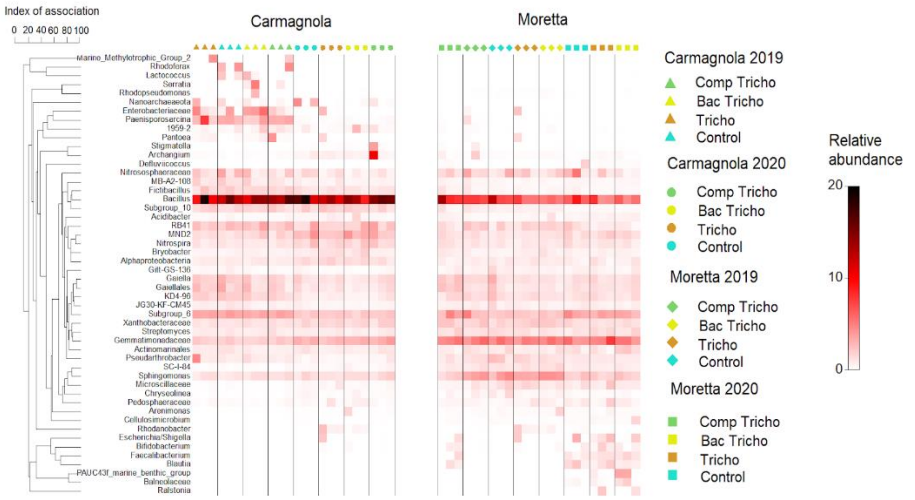

Figure 5: Heatmap showing relative abundance of the 50 most frequent amplicon sequence variant (ASV) in the bacterial community for each sample collected from rhizosphere at the end of the trials. Hierarchical clustering of variables is based on an association index. Samples follow the color coding of the legend.

Rhizosphere associated fungi

A similar situation was observed for the fungal community (Fig. 4b), where *Ascomycota* occupied 75 to 90% of the observed phyla, followed by *Basidiomycota*, *Mucoromycota*, and *Aphelidiomycota*, with variable proportions. There was also no clear separation between the rhizosphere of the treated plants and that of the control among the fungi. More in detail, Carmagnola 2019 had a clear pattern of similarity between samples, in Carmagnola 2020 there was a higher proportion of *Mucoromycota* and *Aphelidiomycota* in Bac_Tricho, in Tricho and in the control samples. In Moretta 2019, there was the highest proportion of *Basidiomycota* (about 20%) in the Bac_Tricho treatment, while Moretta 2020 had the highest proportion of unassigned sequences in the Tricho treated plants. The heatmap of the 50 most abundant ASVs in the fungal community (Fig. 6) showed that few of them dominated over the others. Ascomycota, for

example, was the most abundant, with a greater presence in the Carmagnola field compared to the Moretta field. In terms of fungi, there was also a common core of ASVs between the two sites, but also some differences: *Fusarium* and *Chaetomium* were more abundant in Moretta, while *Rhizopus* was slightly more abundant in Carmagnola. Surprisingly, *Trichoderma* did not show a greater presence in the rhizosphere of the treated plants compared to the control plants.

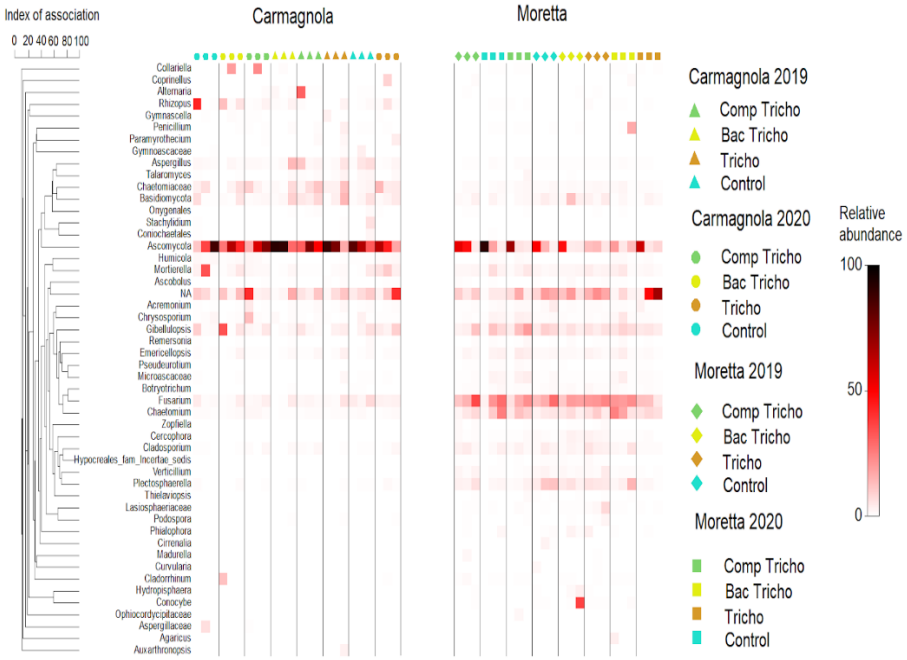

Figure 6: Heatmap showing relative abundance of the 50 most frequent amplicon sequence variant (ASV) in the fungal community for each sample collected from rhizosphere at the end of the trials. Hierarchical clustering of variables is based on an association index. Samples follow the color coding of the legend.

Bacterial and fungal distribution and co-occurrence

Considered at the whole community level with nMDS analysis, both bacteria and fungi showed no spatial segregation as a function of treatments. The ordination of the bacterial community (Fig. 7a) clearly separated by field and year, while that of the fungi (Fig. 7b) followed the site. Pearson correlations (Fig. S2) between bacterial or fungal communities and disease severity or yield production for the Carmagnola and Moretta fields generally showed no strong correlations for any individual ASV, except for Carmagnola and yield in the bacterial community. The correlation basis network (Fig. 8) showed that the Carmagnola and Moretta fields had different interactive structures when the community of bacteria and fungi were analysed together. In the Moretta field, the correlations can be explained as more open, fungi and bacteria seemed to co-occur and co-exclude each other, while in Carmagnola the trend was more closed, as fungi seemed to interact only with each other, and bacteria did the same. Figures (8a and 8b) highlighted some genera that were considered more important for the experiments, surprisingly *Fusarium* and *Trichoderma* did not correlate in a strong way either in Carmagnola or in Moretta. *Bacillus*, which dominated the population of both fields, showed few correlations in the Moretta field and had none in the Carmagnola field.

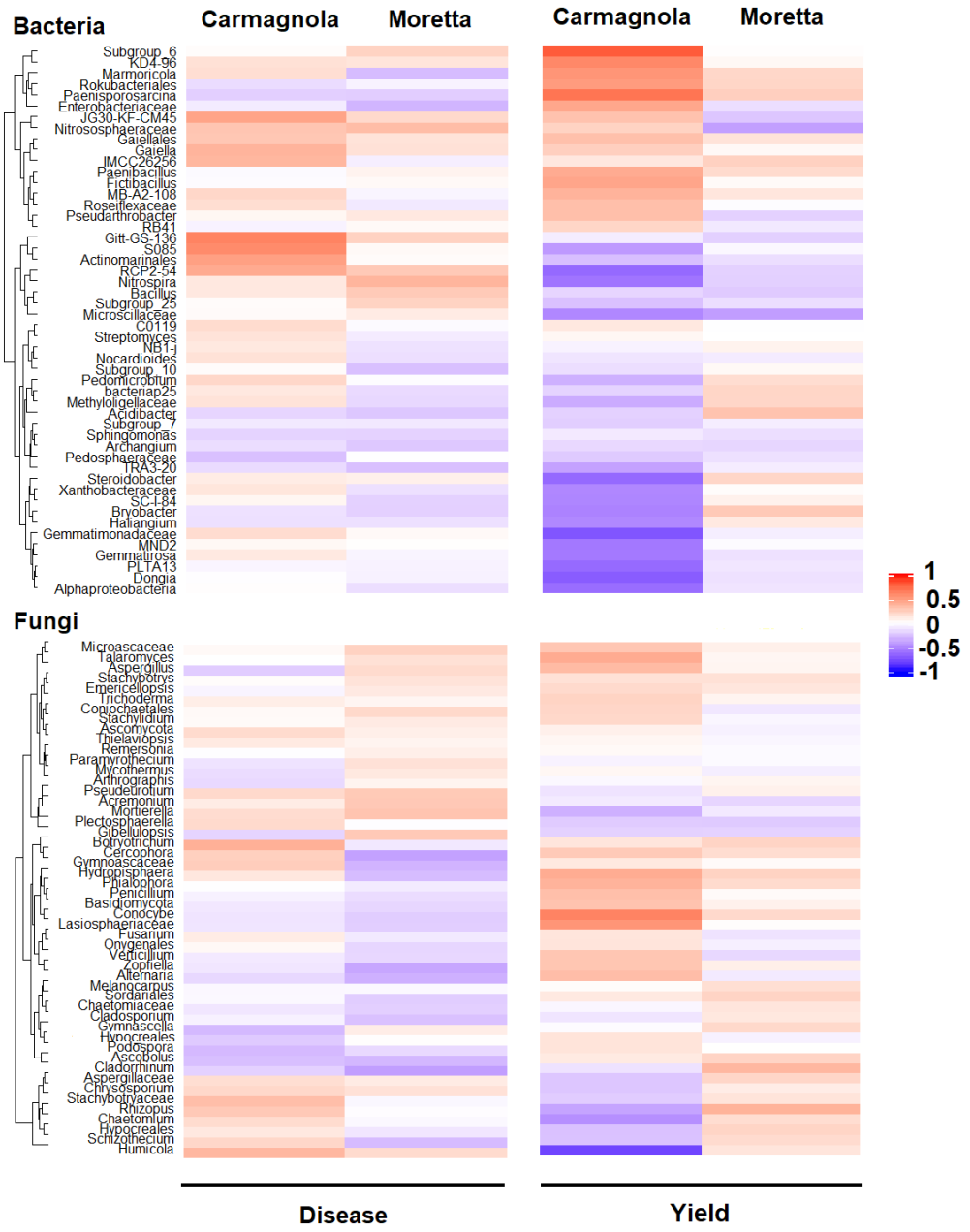

Figure S2: Heatmap of Pearson's correlation between the 50 most frequent amplicon sequence variant (ASV) in the bacterial and fungal community and the disease index and growth (fresh biomass). Data are expressed as average of every sample per each site.

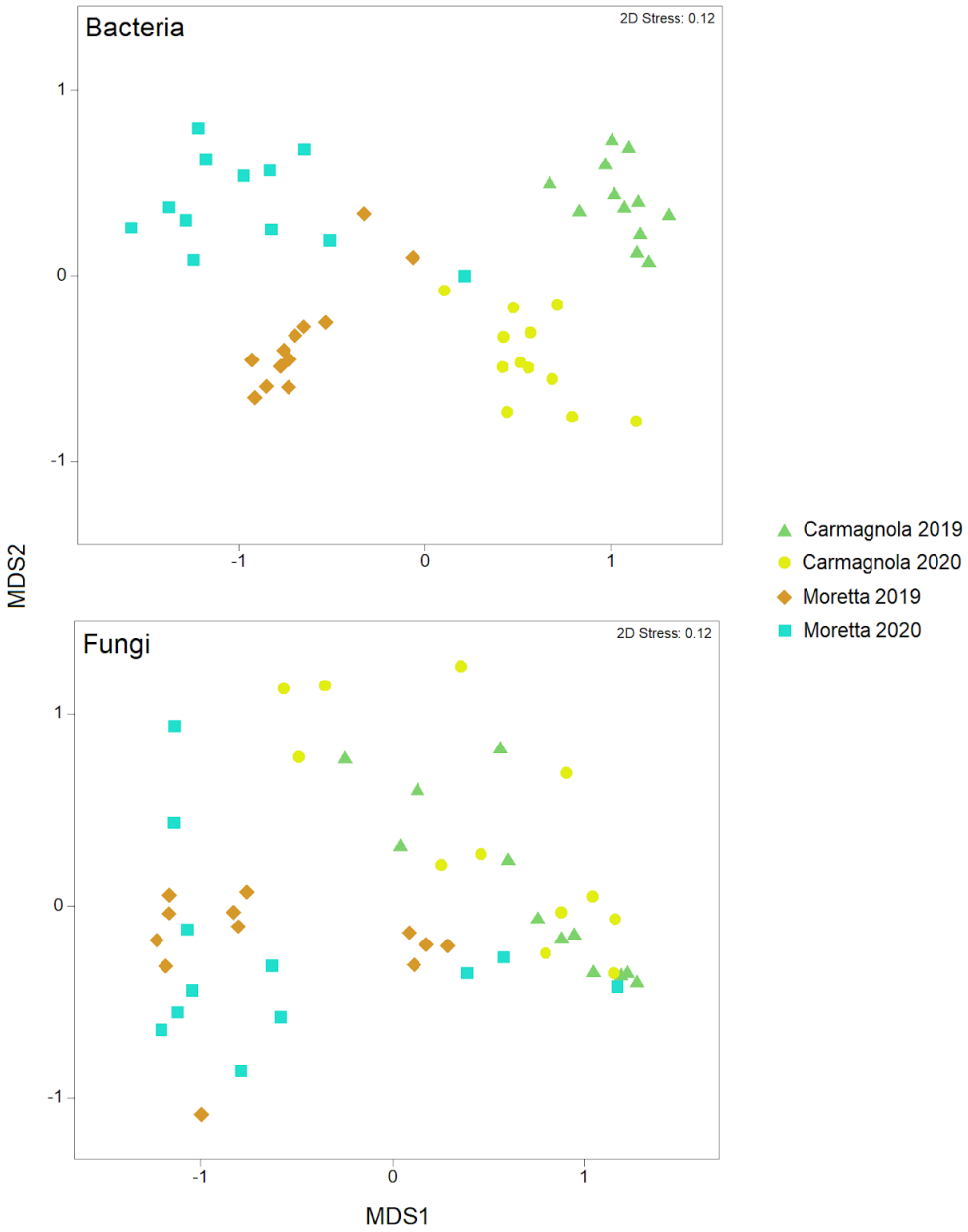

Figure 7: Bi-dimensional Non-metric MDS of the bacterial and fungal communities in the rhizosphere collected at the end of the trials. Data are averaged for year and site of sampling, following the colour coding of the legend.

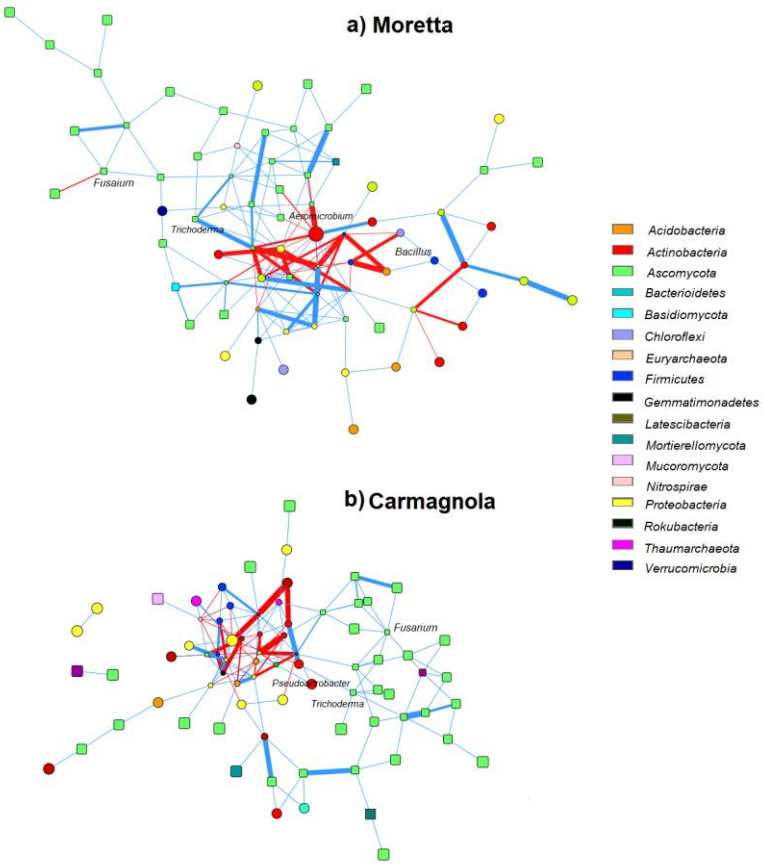

Figure 8: Correlation base network analysis showing potential interactions between bacterial and fungal families in (a) Moretta and (b) Carmagnola fields. The lines connecting nodes (edges) represent positive (blue) or negative (red) co-occurrence relationship. The intensity of the color and the length of the edges represent the strength of correlation. Square nodes correspond to fungal ASVs and circle nodes correspond to bacterial ASVs. The connection stands for a strong (Spearman's $\rho>0.6$ and $\rho<-0.6$) and significant (P-value<0.05) correlation. The size of each node is proportional to the ASV relative abundance, only the top 50 ASVs were kept. The nodes were colored by phylum level. Data of the years and the treatments were averaged to have a view of the impact made by the site.

Fungal functional guilds

The fungal community was analysed to identify the different guilds (Fig. 9). Twenty-one main guilds were found, with a predominance of plant pathogens, animal pathogens, fungal parasites, endophytes and wood saprophytes. In terms of phyla composition, there was no clear pattern identifying the treatments in the different trials, nor was there a consistent difference between treated and control plants. The Tricho treatment of Carmagnola 2019 and the Bac_Tricho treatment of Moretta 2019 showed a greater number of guilds compared to all other treatments. Seven trophic modes were found (Fig. S1) with dominance for pathotroph-saprotroph-symbiotroph in Moretta (both 2019 and 2020) and increased abundance of pathotroph-saprotroph and saprotroph-symbiotroph for Carmagnola field in both years.

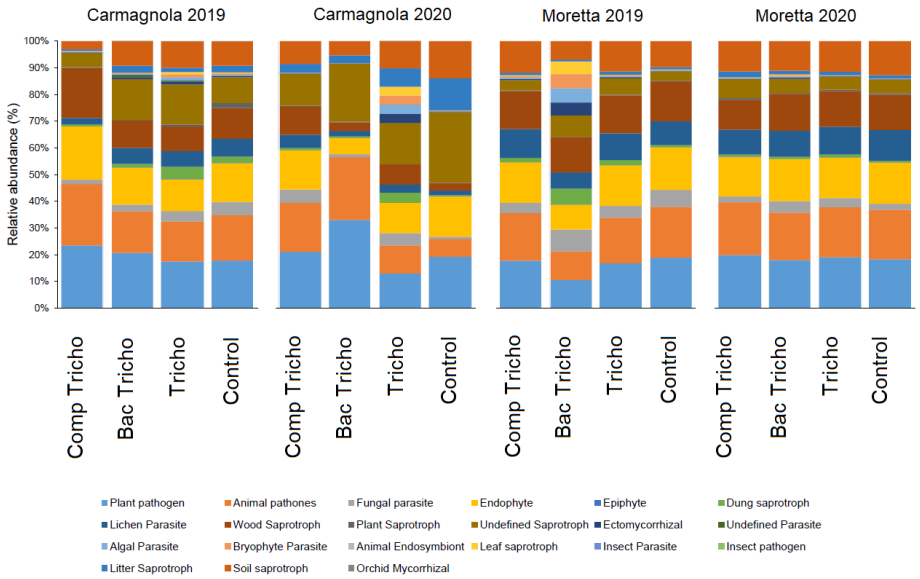

Figure 9: Stacked bar chart of relative abundance of fungal guilds based on FunGuilds. Data are averaged among three biological replicates for each treatment and are here presented grouped for site and year.

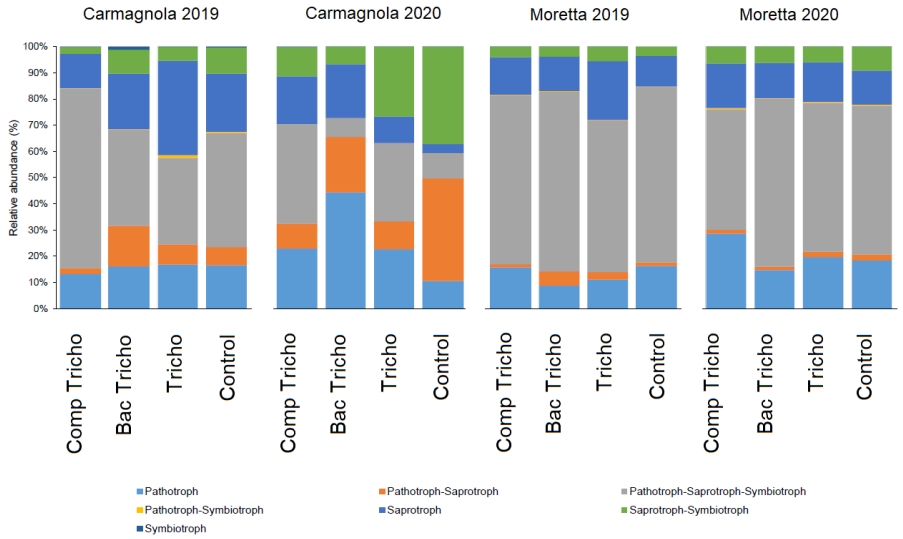

Figure S1: Stacked bar chart of relative abundance of fungal trophic mode. Data are averaged among biological replicates for each treatment and are here presented grouped for site and year.

Discussion

The introduction of environmentally friendly strategies to control lettuce Fusarium wilt is extremely important, as is a better understanding of how they behave under commercial conditions. In this work, the effectiveness of three IPM strategies against Fol was tested under commercial and semi-commercial conditions in tunnels. The three IPM strategies tested showed a great ability to statistically reduce disease severity in both fields and years, from 50% to 70% compared to untreated controls. This is in agreement with preliminary experiments conducted in both greenhouse and field conditions (Gilardi et al. 2019). On the other hand, at the phylum level, the microbiota data showed that there was no detectable treatment effect at the end of the experiment. In fact, there was no clear differentiation between treated plants and the corresponding controls, in contrast to a previous work where the same compost applied against *Phytophthora capsici* protected *Cucurbita pepo* by altering its rhizosphere composition (Bellini et al. 2020). In this case, the experiment was conducted under greenhouse/pot conditions, using peat as substrate, which was probably a much simpler study system. Some work reported a change in rhizosphere microbiota when different BCAs were used, but always in pot systems (Liu et al. 2021). While in agreement with our results, Cucu et al. (2020) reported that field application of BCAs did not alter the rhizosphere microbiome of *Cucurbita pepo*. Most ASVs were similar, but some differences were observed between the two fields. *Bacillus* was the most abundant ASV for bacteria, which is consistent with the literature (Amin et al. 2015). The Moretta field had a greater abundance of *Gemmatimonadaceae, Blautia* and *Bifidobacterium* than the Carmagnola field. These bacterial ASVs have been reported to be involved in chitin degradation and could be affected by fertilisation and the use of sludge

amendments in the soil (Vo et al. 2017; Hui et al. 2020), which is consistent with the fact that Moretta is a commercial farm subject to more intensive management and therefore conventional fertilisation. Moreover, the abundances of *Blautia* and *Bifidobacterium* could also be explained by the difference in pH between the two fields, as the two bacterial genera have a neutral pH optimum (Cui et al. 2021; Liu et al. 2021). Some of the ASVs found in greater presence in Carmagnola soil (such as *Enterobacteriaceae*, *Pantea* and *Lactococcus*) have been associated with the ability to degrade simple sugar molecules (Degelmann et al. 2009; Teuber et al. 2006) and this may indicate a richer presence of these molecules in this field compared to Moretta. The 50 most abundant ASVs of fungi showed that the greatest dominance was in *Ascomycota*, which was expected since this phylum is predominant in agricultural soils (Ma et al. 2013; Egidi et al. 2019). *Fusarium* was very abundant in the Moretta field, as expected based on the history of Fol infection at this site. *Chaetomium* was also more abundant in Moretta, again a genus containing species that degrade cellulose-rich substrates, such as components in soil, straw, or wood (Aru et al. 1997). Neither *Trichoderma* nor *Bacillus* was found in greater abundance in the rhizosphere of plants treated with these BCAs. These data suggest that the BCAs used here did not colonise either the bulk soil or the rhizosphere of the lettuce, at least at the end of the experiments. Accordingly, very low Pearson correlation coefficients were found between the 50 most abundant ASVs with yield production and disease severity. Two-dimensional nMDS analyses showed that there was no treatment effect on the spatial distribution of bacterial and fungal communities: Bacteria segregated by site and year, while for the fungal community segregation was only observed for site. Network analyses of the two fields confirmed that genera such as *Fusarium*, *Trichoderma*, and

Bacillus were not involved in large co-occurrences or co-exclusions; however, some differences were highlighted between the two fields, again suggesting that the microbiota compositions of the rhizosphere samples were mainly influenced by soil type than by experimental treatments. All microbiota composition analyses indicated that the rhizosphere of mature lettuce was not shaped by the nursery treatments, but that the native soil microbiota influenced and balanced it, which is in agreement with Tosi et al. (2021). Nevertheless, a clear protective effect of the treatments against Fusarium wilt in lettuce was observed. Two hypotheses are presented here to explain this phenomenon. The shaping of the rhizosphere could have been performed at the nursery stage and could have been involved in the protective effect against Fol in the first phase of field cultivation. After this early phase, the soil microbiota might have acted as a biological buffer and balanced the composition of the rhizosphere, which is consistent with previous studies (Dalmastri et al. 1999; Prischl et al. 2012; Lundberg et al. 2012). Franco (2021) noted that the timing of sampling could influence the results of metagenomic analyses of the rhizosphere microbiota, as it has a dynamic and plastic composition. The age of a plant also affects the microbial rhizosphere composition, as young plants have different needs (Tosi et al. 2021) and it is known that plant exudates recruit beneficial microorganisms from the soil that follow specific needs (Antoniou et al. 2017). Therefore, Fol-induced infection itself could be a factor driving the rhizosphere microbiota of lettuce, as Berendsen et al. (2012) found that infected plants tend to attract specific microorganisms as a defence coping system. The second hypothesis presented here is the involvement of the induction of resistance, which may have played a central role in these experiments by activating the molecular pathways that can protect the plant from pathogen attacks. Many studies have reported the ability of

Trichoderma spp. to activate the immune system of the plants they come into contact with (Fontenelle et al. 2011; Ramírez-Cariño et al. 2020; Sawant et al. 2020). Indeed, in a previous work with the *Capsicum annuum* - *Phytophthora capsici* pathosystem (Bellini et al. 2021), it was shown that the same *Trichoderma* TW2-enriched compost used in the first IPM strategy and potassium phosphite (used in the second IPM strategy) activate systemic acquired resistance. These two hypotheses require further confirmation with experiments conducted, although it cannot be ruled out that both or only one of them is true.

Conclusions

This study showed that the three IPM strategies tested here were able to reduce disease severity caused by Fol in two different soils under commercial tunnel conditions in two consecutive years. Surprisingly, the rhizospheric microbiota was not driven by the treatments, but was shaped by the autochthonous soil microbial populations. Induction of resistance may have been involved in the protective effect of the treatments. Further studies should be considered to evaluate whether lettuce resistance pathways can be activated by these IPM strategies.

Acknowledgements

The authors thank pathology department of University of Napoli Federico II for the collaboration and hospitality, in particular professors Felice Scala, Matteo Lorito and Sheridan Woo.

Literature cited

Antoniou, A., Tsolakidou, M. D., Stringlis, I. A., and Pantelides, I. S. (2017). Rhizosphere microbiome recruited from a suppressive compost improves plant fitness and increases protection against vascular wilt pathogens of tomato. Frontiers in plant science, 8, 2022.

Akram, W., and Anjum, T. (2011). Use of bioagents and synthetic chemicals for induction of systemic resistance in tomato against diseases. Int. Res. J. Agric. Sci. Soil Sci, *1*, 286-292.

Akram, W., Anjum, T., and Ali, B. (2015). Searching ISR determinant/s from *Bacillus subtilis* IAGS174 against Fusarium wilt of tomato. BioControl, 60(2), 271-280.

Alexandersson, E., Mulugeta, T., Lankinen, Å., Liljeroth, E., and Andreasson, E. (2016). Plant resistance inducers against pathogens in *Solanaceae* species—From molecular mechanisms to field application. International journal of molecular sciences, 17(10), 1673.

Amin, M., Rakhisi, Z., and Ahmady, A. Z. (2015). Isolation and identification of *Bacillus* species from soil and evaluation of their antibacterial properties. Avicenna Journal of Clinical Microbiology and Infection, 2(1).

Aru, A., Munk-Nielsen, L. and Federspiel, B.H. The soil fungus *Chaetomium* in the human paranasal sinuses. Eur Arch Otorhinolaryngol 254, 350–352 (1997). https://doi.org/10.1007/BF02630728

Barzman, M., Bàrberi, P., Birch, A. N. E., Boonekamp, P., Dachbrodt-Saaydeh, S., Graf, B., Hommel, B., Jensen, J. E., Kiss, J., Kudsk, P., Lamichhane, J. R., Messéan, A., Moonen, A.-C., Ratnadass, A., Ricci, P., Sarah, J.-L., and Sattin, M. 2015. Eight principles of Integrated Pest Management. Agron. Sustain. Dev. 35:1199-1215https://doi.org/10.1007/s13593-015-0327-9.

Bellini, A., Ferrocino, I., Cucu, M. A., Pugliese, M., Garibaldi, A., and Gullino, M. L. (2020). A compost treatment acts as a suppressive agent in *Phytophthora capsici–Cucurbita pepo* Pathosystem by modifying the rhizosphere microbiota. Frontiers in plant science, 11, 885.

Bellini, A., Pugliese, M., Guarnaccia, V., Meloni, G. R., and Gullino, L. M. (2021). Calcium oxide, potassium phosphite and a *Trichoderma* enriched compost water suspension protect *Capsicum annuum* against *Phytophthora capsici* by priming the immune system. Pest Management Science.

Berendsen RL, Pieterse CM, Bakker PA (2012) The rhizosphere microbiome and plant health. Trends Plant Sci 17(8):478–486. https://doi.org/10.1016/j.tplants.2012.04.001

Bonanomi, G., Antignani, V., Capodilupo, M., and Scala, F. (2010). Identifying the characteristics of organic soil amendments that suppress soilborne plant diseases. Soil Biol. Biochem. 42, 136–144. doi: 10.1016/j.soilbio.2009.10.012

Bonanomi, G., Antignani, V., Pane, C., and Scala, F. (2007). Suppression of soilborne fungal diseases with organic amendments. J. Plant Pathol. 89,311–324.

Bonanomi, G., Lorito, M., Vinale, F., and Woo, S. L. (2018). Organic amendments, beneficial microbes, and soil microbiota: toward a unified framework for disease suppression. Annu. Rev. Phytopathol. 56, 1–20. doi: 10.1146/annurev-phyto-080615-100046

Callahan B.J., P.J. McMurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson, S.P. Holmes. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods., 13 (2016), pp. 581-583.

Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Applied and environmental microbiology, 71(9), 4951-4959.

Cucu, M. A., Gilardi, G., Pugliese, M., Matić, S., Gisi, U., Gullino, M. L., and Garibaldi, A. (2019). Influence of different biological control agents and compost on total and nitrification-driven microbial communities at

rhizosphere and soil level in a lettuce-*Fusarium oxysporum* f. sp. *lactucae* pathosystem. *Journal of applied microbiology*, *126*(3), 905-918.

Cucu, M. A., Gilardi, G., Pugliese, M., Ferrocino, I., and Gullino, M. L. (2020). Effects of biocontrol agents and compost against the *Phytophthora capsici* of zucchini and their impact on the rhizosphere microbiota. Applied Soil Ecology, 154, 103659.

Cui, S., Zhu, D., Mao, B., Ma, F., Zhao, J., Zhang, H., and Chen, W. (2021). Rapid evaluation of optimal growth substrates and improvement of industrial production of *Bifidobacterium adolescentis* based on the automatic feedback feeding method. *LWT*, *143*, 110960.

Dalmastri C, Chiarini L, Cantale C, Bevivino A, Tabacchioni S. 1999. Soil type and maize cultivar affect the genetic diversity of maize root-associated *Burkholderia cepacia* populations. Microb. Ecol. 38:273–84

De Corato, U., Patruno, L., Avella, N., Lacolla, G., and Cucci, G. (2019). Composts from green sources show an increased suppressiveness to soilborne plant pathogenic fungi: relationships between physicochemical properties, disease suppression, and the microbiome. Crop Protect. 124:104870. doi: 10.1016/j.cropro.2019.104870

De Corato, U., Salimbeni, R., and De Pretis, A. (2018a). Suppression of soilborne pathogens in container media amended with on-farm composted

agrobioenergy wastes and residues under glasshouse condition. J. Plant Dis. Protect.125, 213–226. doi: 10.1007/s41348-017-0133-5

De Corato, U., Salimbeni, R., De Pretis, A., Patruno, L., Avella, N., Lacolla, G., et al. (2018b). Microbiota from 'next-generation green compost' improves suppressiveness of composted Municipal-Solid-Waste to soil-borne plant pathogens. Biol. Control 124, 1–17. doi: 10.1016/j.biocontrol.2018.05.020

Degelmann, D. M., Kolb, S., Dumont, M., Murrell, J. C., and Drake, H. L. (2009). *Enterobacteriaceae* facilitate the anaerobic degradation of glucose by a forest soil. *FEMS Microbiology Ecology*, *68*(3), 312-319.

Egidi E, Delgado-Baquerizo M, Plett JM, Wang J, Eldridge DJ, Bardgett RD, Maestre FT, Singh BK. 2019 A few *Ascomycota* taxa dominate soil fungal communities worldwide. Nat. Commun. 10, 2369. (doi:10.1038/s41467-019-10373-z)

Elsayed, T. R., Jacquiod, S., Nour, E. H., Sørensen, S. J., and Smalla, K. (2020). Biocontrol of bacterial wilt disease through complex interaction between tomato plant, antagonists, the indigenous rhizosphere microbiota, and *Ralstonia solanacearum*. Frontiers in microbiology, 10, 2835.

Fontenelle, A. D. B., Guzzo, S. D., Lucon, C. M. M., and Harakava, R. (2011). Growth promotion and induction of resistance in tomato plant

against *Xanthomonas euvesicatoria* and *Alternaria solani* by *Trichoderma* spp. Crop Protection, *30*(11), 1492-1500.

Franco, C. M. (2021). Inoculation Effects in the Rhizosphere: Diversity and Function. In Rhizosphere Biology: Interactions Between Microbes and Plants (pp. 339-356). Springer, Singapore.

Fujinaga M, Ogiso H, Tsuchiya N, Saito H (2001) Physiological Specialization of *Fusarium oxysporum* f.sp. *lactucae*, a causal organism of Fusarium Root Rot of Crisp Head Lettuce in Japan. J Gen Plant Pathol 67: 205-206.

Fujinaga M, Ogiso H, Tuchiya N, Saito H, Yamanaka S, Nozue M, Kojima M (2003) Race 3, a new race of *Fusarium oxysporum* f. sp. *lactucae* determined by a differential system with commercial cultivars. J Gen Plant Pathol 69: 23–28

Garibaldi, A., Gilardi, G., and Gullino, M. L. (2004a). Seed transmission of *Fusarium oxysporum* f. sp. *lactucae*. Phytoparasitica, 32, 61–65

Garibaldi, A., Gilardi, G., and Gullino, M. L. (2004b). Varietal resistance of lettuce *to Fusarium oxysporum* f. sp. *lactucae*. Crop Protection, 23, 845–851.

Garibaldi, A., Gilardi, G., and Gullino, M. L. (2002). First report of *Fusarium oxysporum* on lettuce in Europe. Plant Disease, 86, 1052–1052.

Gilardi, G., Franco Ortega, S., Van Rijswick, P. C. J., Ortu, G., Gullino, M. L., and Garibaldi, A. (2017). A new race of *Fusarium oxysporum* f. sp. *lactucae* of lettuce. *Plant Pathology*, *66*(4), 677-688.

Gilardi, G., Pugliese, M., Gullino, M. L., and Garibaldi, A. (2020). Effect of biocontrol agents and potassium phosphite against Phytophthora crown rot, caused by *Phytophthora capsici*, on zucchini in a closed soilless system. *Scientia Horticulturae*, 265, 109207.

Gilardi, G., Pugliese, M., Gullino, M. L., and Garibaldi, A. (2019). Nursery treatments with resistant inducers, soil amendments and biocontrol agents for the management of the Fusarium wilt of lettuce under glasshouse and field conditions. Journal of Phytopathology, 167(2), 98-110.

Gilardi, G., Pugliese, M., Gullino, M. L., and Garibaldi, A. (2016). Effect of different organic amendments on lettuce Fusarium wilt and on selected soilborne microorganisms. Plant Pathol. 65, 704–712. doi: 10.1111/ppa.12460

Gordon, T. R. (2017). *Fusarium oxysporum* and the Fusarium wilt syndrome. *Annual review of phytopathology*, *55*, 23-39.

Gullino ML, Gilardi G, Garibaldi A (2019) Ready-to-eat salad crops: a plant pathogen's heaven. Plant Dis 103: 2153-2170

Hubband JC, Gerik JS (1993) A new wilt disease of lettuce incited by *Fusarium oxysporum* f. sp. *lactucum* formae specialis nov. Plant Dis 77: 750-754

Hui, C., Jiang, H., Liu, B., Wei, R., Zhang, Y., Zhang, Q., Liang, Y. and Zhao, Y. (2020). Chitin degradation and the temporary response of bacterial chitinolytic communities to chitin amendment in soil under different fertilization regimes. Science of the Total Environment, 705, 136003.

Liu, X., Mao, B., Gu, J., Wu, J., Cui, S., Wang, G., Zhao, J., Zhang, H., and Chen, W. (2021). *Blautia*—a new functional genus with potential probiotic properties?. *Gut Microbes*, *13*(1), 1-21.

Lundberg D.S., Lebeis S.L., Paredes S.H., Yourstone S., Gehring J., Malfatti S., Tremblay J., Engelbrektson A., Kunin V., del Rio T.G., Edgar R.C., Eickhorst T., Ley R.E., Hugenholtz P., Tringe S.G., and Dangl J.L. (2012) Defining the core *Arabidopsis thaliana* root microbiome. Nature 488:86–90

Martin, M. Cutadapt removes adaptor sequences from high-throughput sequencing reads EMBnetjournal, 17 (2011), pp. 10-12.

Ma, A., Zhuang, X., Wu, J., Cui, M., Lv, D., Liu, C., and Zhuang, G. (2013). *Ascomycota* members dominate fungal communities during straw residue decomposition in arable soil. *PloS one*, *8*(6), e66146.

Matheron, M., and Gullino, M. L. (2012). Fusarium wilt of lettuce and other salad crops. Fusarium wilts of greenhouse vegetable and ornamental crops, 175-183.

Nguyen N.H., Song Z., Bates S.T., Branco S., Tedersoo L., Menke J., Schilling J.S., and Kennedy P.G. (2016) FUNGuild: an open annotation tool for parsing fungal community data sets by ecological guild. Fung Ecol 20:241–248.

Nihorimbere V., Ongena M., Smargiassi M., and Thonart P. (2011) Beneficial effect of the rhizosphere microbial community for plant growth and health. Biotechnologie, Agronomie, Société et Environnement, 15(2), 327-337.

Nilsson R.H., Larsson K-H., Taylor A.F.S., Bengtsson-Palme J., Jeppesen T.S., Schigel D., Kennedy P., Picard K., Glöckner F.O., Tedersoo L., Saar I., Kõljalg U., and Abarenkov K. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nucleic Acids Res. 2019;47: D259–64.

Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., Arpigny, J.L. and Thonart, P. (2007). Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environmental microbiology*, *9*(4), 1084-1090.

Pal, K.K., and Gardener, B.M. (2006). Biological control of plant pathogens. The plant health instructor, 2, 1117-1142.

Papasotiriou, F.G., Varypatakis, K.G., Christofi, N., Tjamos, S.E., and Paplomatas, E.J. (2013). Olive mill wastes: a source of resistance for plants against *Verticillium dahliae* and a reservoir of biocontrol agents. Biol. Control 67, 51–60.doi: 10.1016/j.biocontrol.2013.07.008

Pieterse, C.M., Zamioudis, C., Berendsen, R.L., Weller, D.M., Van Wees, S.C., and Bakker, P.A. (2014). Induced systemic resistance by beneficial microbes. *Annual review of phytopathology*, *52*.

Prischl, M., E. Hackl, M. Pastar, S. Pfeiffer, and A. Sessitsch. 2012. Genetically modified Bt maize lines containing cry3Bb1, cry1A105 or cry1Ab2 do not affect the structure and functioning of root-associated endophyte communities. Applied Soil Ecology 54: 39–48 https://doi.org/10.1016/J.APSOIL.2011.12.005

Pugliese, M., Liu, B., Gullino, M. L., and Garibaldi, A. (2011). Microbial enrichment of compost with biological control agents to enhance suppressiveness to four soil-borne diseases in greenhouse. Journal of Plant Diseases and Protection, *118*(2), 45-50.

Pugliese, M., Gilardi, G., Garibaldi, A., and Gullino, M. L. (2015). "Organic amendments and soil suppressiveness: results with vegetable and ornamental crops," in Organic Amendments And Soil Suppressiveness In

Plant Disease Management, eds M. Meghvansi and A. Varma (Cham: Springer), 495–509.doi: 10.1007/978-3-319-23075-7_24

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41: D590–6.

R.C. Team. R: A language and environment for statistical computing. R Found. Stat. Comput. (2016).

Ramírez-Cariño, H.F., Guadarrama-Mendoza, P.C., Sánchez-López, V. et al. Biocontrol of Alternaria alternata and *Fusarium oxysporum* by *Trichoderma asperelloides* and *Bacillus paralicheniformis* in tomato plants. Antonie van Leeuwenhoek 113, 1247–1261 (2020). https://doi.org/10.1007/s10482-020-01433-2

Reuveni, R., Raviv, M., Krasnovsky, A., Freiman, L., Medina, S., Bar, A., et al. (2002). Compost induces protection against *Fusarium oxysporum* in sweet basil. Crop Protect. 21, 583–587. doi: 10.1016/S0261-2194(01)00149-1

Sawant, I.S., Wadkar, P.N., Ghule, S.B. et al. Induction of systemic resistance in grapevines against powdery mildew by *Trichoderma asperelloides* strains. Australasian Plant Pathol. 49, 107–117 (2020). https://doi.org/10.1007/s13313-020-00679-8

Simmons, T., Caddell, D. F., Deng, S., and Coleman-Derr, D. (2018). Exploring the root microbiome: extracting bacterial community data from the soil, rhizosphere, and root endosphere. Journal of visualized experiments: JoVE, (135).

Teuber, M., and Geis, A. (2006). The genus lactococcus. *The prokaryotes*, *4*, 205-228.

Tilston, E. L., Pitt, D., and Groenhof, A. C. (2002). Composted recycled organic matter suppresses soil-borne diseases of field crops. New Phytol. 154, 731–740. doi: 10.1046/j.1469-8137.2002.00411.x

Tosi, M., Gaiero, J., Linton, N., Mafa-Attoye, T., Castillo, A., and Dunfield, K. (2021). Bacterial endophytes: diversity, functional importance, and potential for manipulation. In Rhizosphere Biology: Interactions Between Microbes and Plants (pp. 1-49). Springer, Singapore.

Walters, D. R., and Fountaine, J. M. (2009). Practical application of induced resistance to plant diseases: an appraisal of effectiveness under field conditions. The Journal of Agricultural Science, 147(5), 523.

Vo, N., Tsai, T. C., Maxwell, C., and Carbonero, F. (2017). Early exposure to agricultural soil accelerates the maturation of the early-life pig gut microbiota. Anaerobe, 45, 31-39

Discussion

The aim of this Ph.D. thesis was to elucidate the mode of action behind some alternative control strategies tested in different conditions in three pathosystems.

In the first one, twelve different compost mixtures were tested against Phc to protect summer squash in a greenhouse peat pot experiment. The mixtures were obtained with four different composts added at three different percentages to the peat (1-10-20%). Above all the mixtures, only one (green compost sieved at 10 mm enriched with *Trichoderma* TW2) was able to statistically reduce the disease incidence when compared to the untreated inoculated control. Therefore, mycobiota analysis was made using the 26S rRNA gene on the rhizosphere collected from that compost mixture, the chemical control (treated with metalaxyl) and the untreated inoculated control at the end of the trials. The amplicon-based sequencing data, when analyzed through PCA suggested that the composition of the mycobiota associated to the rhizosphere was altered by the presence of the compost, in fact a clear separation between the fungal communities of the compost treated rhizosphere plants and the controls ones was visible. The first work strengthened the knowledge about compost derived suppressiveness. Three are the major conclusions that can be deduced: (i) the enrichment of a known BCA strain can amplify the suppressive action, as the same compost not enriched with the *Trichoderma* TW2 strain did not statistically lower the disease incidence in the pathosystem studied, this was in agreement with Bonanomi et al. (2018); also it was previously tested in another pathosystem that the same compost was more effective than the *Trichoderma* strain used alone (Gilardi et al., 2019); (ii) the quantity of the compost used is not positively correlated with the beneficial effect against a pathogen, as it was already proven by Noble et al. (2017)

and Pugliese et al. (2011), in fact only 10% mixture reached the highest value of protection, while the same compost used at 20% decreased the protection effect against Phc; (iii) in a controlled and simple system as the peat pot greenhouse experiments conducted for this study, it is clearly evident how the compost altered the mycobiota rhizosphere composition and that this alteration is involved in the suppressive action, since the metalaxyl treated plants and the untreated inoculated ones showed the same spatial placing in the PCA analysis. A possible involvement of the induction of resistance by the compost enriched with *Trichoderma* TW2 was also hypothesized, since other strains of *Trichoderma* were already proven to act in that way (Vallad et al., 2003; Sang et al., 2010; Martínez-Medina et al., 2017; Savitha and Sriram, 2017).

To test this hypothesis, another work was conducted using the same strain of *Phytophthora capsici* in a growth chamber environment with *Capsicum annum* as host, chosen for the reduced sized and to see whether the protective action could have been exploited also for a member of the *Solanaceae* family. Together with the compost (given as water suspension), also potassium phosphite and calcium oxide were tested for their ability to activate the plant immune system, acibenzolar-s-methyl was used as a positive control as it is known to activate the systemic acquired resistance (Buonaurio et al., 2002).

To avoid any direct interaction between the treatments and the pathogen, the site of the inoculation and the site of treatments administration were spatially divided (the first one was performed with an agar plug directly on the third leaf, while the treatments were given as radical drench two times: 72 and 24 hours before the inoculation). In this case, all the treatments were able to statistically reduce the growth of the oomycete compared to the untreated control. Analyses of the accumulation of

salicylic acid on the upper leaves and of three target genes known to be involved in the SAR pathways (*CaPBR1*, *CaPO1* and *CaDEF1*) (Zhang et al., 2019) on the inoculated leaves were conducted respectively with HPLC-MS/MS and RT-Real Time PCR at three end-point times: immediately before the inoculation, six and twenty-four hours after. It was interesting to see that all the treatments used were able to statistically induce an over accumulation and/or over expression of the salicylic acid and target genes in at least one endpoint time compared to the untreated controls, in accordance with patterns of accumulation or expression found in other studies on the SAR pathway in other pathosystems (Rasmussen et al., 1991; Do et al., 2003; Kawano, 2003; Asghari et al., 2020; Esmail et al., 2020). These results confirmed the ability of the treatments applied to act as plant resistance inducers at least in this pathosystem, but they also suggested that potassium phosphite and calcium oxide are able to activate the SAR in *Capsicum annuum* regardless the pathogen chosen, since they induced respectively over accumulation of salicylic acid and over expression of *CaPBR1* and *CaDEF1* genes at time zero, therefore activating the alert status in the plant.

Lastly, to test the *Trichoderma* TW2 enriched compost, potassium phosphite and other BCAs (of the genera *Trichoderma* and *Bacillus*) on a more complex environment and against another pathogen, a two-year experiment was carried out considering the pathosystem *Fusarium oxysporum* f.sp. *lactucae* and *Lactuca sativa*. Two fields were considered: a commercial farm with an intense lettuce cultivation history and an experimental farm. Three IPM strategies (the compost enriched with *Trichoderma* TW2, a combination of *T. gamsii* + *T. asperellum*, *Bacillus amyloliquefaciens* and potassium phosphite and a combination of *T. polysporum* + *T. atroviride)* were selected for this study to test their

efficacy on the control of Fol's attacks and their involvement in the change of rhizosphere associated microbiota, an untreated control was present in both fields. The treatments were given at the nursery level and, only in the case of the compost, as an amendment directly on the field immediately before the transplantation of the seedlings. All three IPM strategies statistically reduced the disease incidence caused by Fol in both years and fields when compared to the corresponding untreated controls. The rhizospheric soil, collected at the end of the trials, was analyzed through amplicon-based Illumina sequencing targeting the 16s and the ITS rRNA genes to study the bacterial and fungal communities. Surprisingly, neither in the bacterial nor in the fungal communities there were treatment associated patterns, as the analyses of the *phyla*, the 50 most abundant ASVs and nMDS showed. Conversely, large differences were observed when comparing the two fields and years, as in the nMDS analysis the spatial distribution clearly showed how they were grouping for the same soil and year, regardless of the treatments. Based on the data collected two considerations can be made: firstly the treatments were able to reduce the disease severity in two different fields, suggesting that their employment could be considered as consistent IPM strategies; then the rhizospheric microbiota at the end of the trials were more affected by the native microbial communities of the soils than by the treatments, which is in agreement with Tosi et al (2021). Together the three works discussed can be seen as an in-depth study of the mechanisms of action of the treatments chosen, tested in different pathosystems and environments. The compost enriched with *Trichoderma* TW2 was able to exert a protective action in all the three pathosystems studied; the work done to select it in the Phc-zucchini pathosystem allowed to set a percentage (10%) of use for that specific pathosystem and to see how the rhizospheric mycobiota changed

after the plants were treated with it. In the second pathosystem (Phc-pepper), all the treatments were able to induce the systemic acquired resistance and therefore hinder the growth of the oomycete's mycelium on the inoculated leaves, also for this work the number and the amount of the administration were set (data not shown), until reaching the best settings. Lastly, the third work represents a sort of scale-up of the previous ones, because the field is the most complex, challenging and yet exciting for the study of the rhizosphere microbiota. The same compost used in the first two works, the combination of different BCAs (of the genera *Trichoderma* and *Bacillus*) and potassium phosphite were able to lower the disease incidence caused by Fol on lettuce cultivation, in both years and fields. As mentioned, the microbiota inhabiting the rhizosphere area at the end of the in field trials did not show a treatment related pattern, but showed a site and year division. This behavior of the rhizospheric microbiota suggests that the soil acted as a microbial buffer, but yet the protective effect of the treatments exerted above lettuce plants against Fol was still visible. It is clear that the treatments had an effect on the plants, but it is not certain how they acted since no major changes in the rhizospheric microbiota were registered at the end of the trials, as it was on the first work described. It is possible that the rhizosphere microbial communities were affected by the treatments at the nursery stage; this initial shaping could have been involved in the protective effect that was registered against Fol's attacks. Once in the fields, the soil microbiota acted as a biological buffer, balancing the composition of the rhizosphere, which is consistent with previous studies (Dalmastri et al. 1999; Prischl et al. 2012; Lundberg et al. 2012). Furthermore, it is known that root exudates can recruit beneficial microorganisms from the soil, following specific needs of the plants, such as when they establish contact with a pathogen (Antoniou et al. 2017;

Cucu et al. 2019). Indeed, the activation of the immune system by the treatments is a possible explanation too since it was already proven for the compost and potassium phosphite in the second work on the Phc-pepper pathosystem. As the research it is built on itself, many are the future works that can be done in future to implement this thesis. For example the selection of new BCAs from composts, the implementation of other composts with the already known BCA, the study of new IPM strategies using different combination and the study of the same strategies (such as alone or in combination) on other pathosystems.

The importance of understanding the mode of action of the alternative control strategies is crucial for their improvement and their correct use. The studies presented here were an effort to fill, whether it was possible, this gap between what is already known and what is still to be discovered.

Some knowledge was strengthened confirming the initial assumptions, while others were weakened and led to make new assumptions and hypotheses.

Acknowledgments

I would like to thank Professor Maria Lodovica Gullino for the opportunity to carry out the doctoral thesis at Agroinnova, for the scientific guide she had been to me and for the critical revision of every study, scientific paper and manuscript I wrote, including this thesis.

I would also like to thank Professors Davide Spadaro and Paola Battilani for the revision of this work, Professor Paolo Cortesi for accepting to be on the commission board, Professor Vladimiro Guarnaccia for the work we did together and for your guide, Professor Monica Mezzalama for your kindness, Professor Massimo Pugliese for the help, Dr. Giovanna Gilardi

for the guide and all the survey in field we did together, Renata for the kindness you have and you demonstrate with everyone in the lab, Domenico Bertetti for teaching me how to be precise during the surveys, Martina for the daily chats, Gabriele and Guido for your constant help.

I would like to thank Professor Giuliano Bonanomi for having me in his research group during some months of the last year of my PhD.

I would like to thank all the colleagues that helped me during these three years, I really appreciate it.

Projects

European Union's Horizon 2020 Research and Innovation Program under grant agreements n° 817946 (EXCALIBUR), n° 633999 (EUCLID) and n° NUTRIMAN (GA 818470).

Literature cited

Antoniou, A., Tsolakidou, M. D., Stringlis, I. A., & Pantelides, I. S. (2017). Rhizosphere microbiome recruited from a suppressive compost improves plant fitness and increases protection against vascular wilt pathogens of tomato. Frontiers in Plant Science, 8, 2022. doi.org/10.3389/fpls.2017.02022

Asghari S, Harighi B, Ashengroph M, Clement C, Aziz A, Esmaeel Q et al., Induction of systemic resistance to Agrobacterium tumefaciens by endophytic bacteria in grapevine. Plant Pathol 69:827–837 (2020).

Bonanomi, G., Lorito, M., Vinale, F. and Woo, S. L. (2018). Organic amendments, beneficial microbes, and soil microbiota: toward a unified framework for disease suppression. *Annual Review of Phytopathology*, 56, 1-20.

Buonaurio R, Scarponi L, Ferrara M, Sidoti P and Bertona A, Induction of systemic acquired resistance in pepper plants by acibenzolar-S-methyl against bacterial spot disease. Eur J Plant Pathol 108:41–49 (2002).

Dalmastri C, Chiarini L, Cantale C, Bevivino A, Tabacchioni S. 1999. Soil type and maize cultivar affect the genetic diversity of maize root-associated Burkholderia cepacia populations. Microb. Ecol. 38:273–84

Do HM, Hong JK, Jung HW, Kim SH, Ham JH and Hwang BK, Expression of peroxidase-like genes, H2O2 production, and peroxidase activity during the hypersensitive response to *Xanthomonas campestris pv. vesicatoria* in *Capsicum annuum*. *Mol Plant Microbe Interact* 16:196–205 (2003).

Esmail SM, Aboulila AA and Abd El-Moneim D, Variation in several pathogenesis-related (PR) protein genes in wheat (*Triticum aestivum*)

involved in defense against Puccinia striiformis f. sp. tritici. Physiol Mol Plant Pathol 112:101545.ì (2020).

Gilardi, G., Pugliese, M., Gullino, M. L., and Garibaldi, A. (2019). Nursery treatments with resistant inducers, soil amendments and biocontrol agents for the management of the Fusarium wilt of lettuce under glasshouse and field conditions. J. Phytopathol. 167, 98–110. doi: 10.1111/jph.12778

Kawano T, Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. *Plant Cell Rep* 21:829–837 (2003). https://doi.org/10.1007/s00299-003-0591-z.

Lundberg D.S., Lebeis S.L., Paredes S.H., Yourstone S., Gehring J., Malfatti S., Tremblay J., Engelbrektson A., Kunin V., del Rio T.G., Edgar R.C., Eickhorst T., Ley R.E., Hugenholtz P., Tringe S.G., and Dangl J.L. (2012) Defining the core *Arabidopsis thaliana* root microbiome. Nature 488:86–90

Martínez-Medina, A., Fernandez, I., Lok, G. B., Pozo, M. J., Pieterse, C. M., and Van Wees, S. C. (2017). Shifting from priming of salicylic acid- to jasmonic acid-regulated defences by Trichoderma protects tomato against the root knot nematode Meloidogyne incognita. New Phytol. 213, 1363–1377. doi: 10.1111/nph.14251

Noble, R. (2011). Risks and benefits of soil amendment with composts in relation to plant pathogens. Austr. Plant Pathol. 40, 157–167. doi: 10.1007/s13313-010-0025-7

Prischl, M., E. Hackl, M. Pastar, S. Pfeiffer, and A. Sessitsch. 2012. Genetically modified Bt maize lines containing cry3Bb1, cry1A105 or

cry1Ab2 do not affect the structure and functioning of root-associated endophyte communities. Applied Soil Ecology 54: 39–48

Pugliese, M., Liu, B. P., Gullino, M. L., and Garibaldi, A. (2011). Microbial enrichment of compost with biological control agents to enhance suppressiveness to four soil-borne diseases in greenhouse. J. Plant Dis. Protect. 118, 45–50. doi: 10.1007/BF03356380

Rasmussen JB, Hammerschmidt R and Zook MN, Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae pv syringae*. *Plant Physiol* 97:1342–1347 (1991).

Sang, M. K., Kim, J. G., and Kim, K. D. (2010). Biocontrol activity and induction of systemic resistance in pepper by compost water extracts against *Phytophthora capsici*. Phytopathology 100, 774–783. doi: 10.1094/PHYTO-100-8-0774

Savitha, M. J., and Sriram, S. (2017). Induced systemic resistance (ISR) in hot pepper against Phytophthora capsici infection triggered by cell wall oligosaccharide elicitors from Trichoderma species. Indian J. Hortic. 74, 233–239. doi: 10.5958/0974-0112.2017.00048.2

Tosi, M., Gaiero, J., Linton, N., Mafa-Attoye, T., Castillo, A., and Dunfield, K. (2021). Bacterial endophytes: diversity, functional importance, and potential for manipulation. In Rhizosphere Biology: Interactions Between Microbes and Plants (pp. 1-49). Springer, Singapore.

Vallad, G. E., Cooperband, L., and Goodman, R. M. (2003). Plant foliar disease suppression mediated by composted forms of paper mill residuals exhibits molecular features of induced resistance. Physiol. Mol. Plant Pathol. 63, 65–77. doi: 10.1016/j.pmpp.2003.10.003

Wang JE, Liu KK, Li DW, Zhang YL, Zhao Q, He YM et al., A novel peroxidase *CanPOD* gene of pepper is involved in defense responses to *Phytophthora capsici* infection as well as abiotic stress tolerance. *IntJ Mol Sci* 14:3158–3177 (2013).

Zhang HX, Ali M, Feng XH, Jin JH, Huang LJ, Khan A et al., A novel transcription factor CaSBP12 gene negatively regulates the defense response against Phytophthora capsici in pepper (Capsicum annuum L.). Int J Mol Sci 20:48 (2019).

Zhang HX, Ali M, Feng XH, Jin JH, Huang LJ, Khan A *et al*., A novel tran- scription factor CaSBP12 gene negatively regulates the defense response against *Phytophthora capsici* in pepper (*Capsicum annuum* L.). *Int J Mol Sci* 20:48 (2019).

Ringraziamenti

La fine di un percorso importante come il dottorato di ricerca si porta dietro molte soddisfazioni insieme ad alcune malinconie. Sono fermamente convinto che i traguardi personali siano raggiunti non solo per l'impegno costante, ma anche (e forse soprattutto) per la capacità dell'ambiente in cui si lavora di tirar fuori il meglio delle persone. Se fossimo delle monadi solitarie tutte le conquiste che facciamo sarebbero fini a se stesse; nulla avrebbe un significato che va oltre al personale appagamento. Partendo da questo punto di vista e ampliandolo spostando il focus dal singolo alla società, penso che sia fondamentale riconoscere il ruolo delle interazioni tra persone in tutti i traguardi raggiunti dall'umanità. La capacità dell'uomo, inteso come specie, di creare un avanzamento tecnologico, scientifico e umanistico è il diretto riflesso della sua attitudine di creare delle reti sociali. Del resto, non esiste nessuna materia di studio che non abbia la caratteristica di costruire nuova conoscenza partendo dalla precedente già acquisita. Alla stessa maniera, essendo anche gli studiosi e le studiose fatti e fatte non solo di testa, ma anche di cuore, ogni singolo momento di condivisione giova alla riuscita di un buon lavoro, che sia un caffè, qualche chiacchiera o anche solo il sostegno necessario per poter affrontare le giornate che non sempre sono luminose. Per questo motivo desidero dedicare i miei ringraziamenti alle colleghe e ai colleghi che mi hanno aiutato nei momenti meno facili di questo percorso, sia quando le difficoltà erano nell'ambito lavorativo che personale.
Martina, abbiamo cominciato insieme e stiamo finendo insieme, sono molto orgoglioso del percorso che abbiamo fatto, se tu fossi un personaggio di una saga, saresti decisamente Hermione Granger, perché sei incredibilmente intelligente e determinata, ma anche tanto dolce; hai

illuminato le mie giornate più buie e mi hai dato la forza di andare avanti quando non ne avevo più. Simona, a volte mi chiedo dove sarei in questo momento senza la tua guida saggia e umana, senza le nostre pause coi consigli scientifici, ma anche personali, ti devo molto amica mia, che fortuna averti incontrata e conosciuta per bene in questi tre anni. Ilaria, io "voglio provare ad esistere, la mia natura è resistere" e so che anche tu lo vuoi! Mia compagna d'ufficio, di pisolini post-prandiali, di sigarette, nervosismi e risate, sono davvero felice di averti avuta letteralmente a fianco nel periodo più complesso di questo mio percorso, un in bocca al lupo gigante per il tuo. Betta lo sai che venivo da te non solo per l'aria condizionata, ma anche e soprattutto perché confrontarmi con te era un momento di leggerezza e tranquillità che mi rigenerava le giornate, sono stato fortunato ad avere una chimica così con cui lavorare, ti ollu beni! Silvia V., mia prima collega di ufficio, che mi ha insegnato così tanto sotto molti punti di vista, a volte mi mancano le risate e i confronti in quella che, diciamocelo, era la location più sfigata, ma anche più tranquilla di tutto lo stabilimento, che fine avrei fatto se non mi avessi aperto mille volte la porta del ritardo? Se non mi avessi insegnato come comportarmi in questo ambiente? Edoardo, chi siamo noi per non essere passati da patologia vegetale? Ho davvero apprezzato tutti i momenti passati insieme, le tue battute da far agghiacciare il sangue e le risate che ne conseguivano, gli aiuti con la dry science e le torte 900; ses snart! Silvia O., che splendida sorpresa averti conosciuta, sono davvero grato di ogni scambio umano (e non subumano, quelli lasciamoli agli altri!!) che abbiamo avuto in questo tempo, sono sicuro che non mancheranno aperitivi futuri, viva i licheni. Giulia, la tua dolcezza e la capacità di preoccuparti per il prossimo sono indescrivibili, starti accanto mi ha sempre tranquillizzato. Alina, da te ho imparato che la tenacia ripaga, Francesca da te ho imparato la calma in

questo mondo folle della ricerca, con entrambe forse ci rivedremo tra i banchi di scuola! Martina S., hai appena iniziato un percorso che ti auguro sia pieno di soddisfazioni, sono contento di averti conosciuta. Athina non solo le tue torte, ma anche le tue risate mi mancheranno. Alexandra, sei stata una delle prime persone che mi abbia insegnato i valori scientifici e umani, che bello averti conosciuto! Slavica, hai reso leggere tantissime mie giornate ma ricordo ancora la tua torta sciolta di un venerdì del primo anno! Fabiano, ho sempre apprezzato le nostre chiacchiere sui massimi sistemi e su cose meno alte, la filosofia sulla scienza e sulla vita. Andrea, ti auguro di non dover prendere più uno *spostapoveri* in vita tua, sono grato degli aiuti che mi hai dato in questi anni. Cristina, dopo cinque anni di università insieme siamo finiti nello stesso percorso quasi parallelamente, il tuo sguardo attento e anche saggio mi ha aiutato a non fermarmi di fronte alle difficoltà. Viola che piacevole sorpresa averti conosciuta, tra un corso di cucito e un disagio accademico, sono stato felice di averti vicina a me. Non solo colleghe e colleghi hanno reso questo periodo più leggero per me, ma anche gli amici, quelli storici e quelli più recenti, che hanno sempre compreso i casini della mia vita, del mio lavoro, delle mie difficoltà. Matte, Lore, Paolo, Gian, Giorgia, Sic, Giuse, Robi, Michi, Ire, Hajar, Nic, Samu, Cele, Leti, Mauro, Dimi, Ale, Enri, Costi, Eli, Viola, Antonio, Scarà, Pippo, Paco, Mauro, Domenico. Un elenco, poco più di due righe (in ordine sparso giuro), ma che racchiude dentro tantissime esperienze, condivisioni, supporto e risate. Sono profondamente grato di avervi nella mia vita, grazie per avermi reso meno faticoso tutto. Infine, il ringraziamento più importante va ai miei genitori e a mio fratello, che hanno saputo comprendere chi sono, sostenere le mie scelte, seguirmi dovunque, essere orgogliosi di me e di quello che faccio, vi devo molto, vi devo tutto.