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Design and characterization of microbial consortia as inoculants for sustainable crop protection

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Design and characterization of microbial consortia as inoculants for sustainable crop protection

Memoria presentada por D. Zhivko Minchev Ivanov, graduado en Biología, para optar al título de Doctor por la Universidad de Granada dentro del Programa de Doctorado en Biología Fundamental y de Sistemas.

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El doctorando / The *doctoral candidate* **Zhivko Minchev Ivanov** y los directores de la tesis / *and the thesis supervisor/s*: **María José Pozo Jiménez y Roxina Soler**

Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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RESUMEN

La agricultura juega un papel fundamental en el mantenimiento de la población mundial que está en continuo aumento, proporcionando no solo alimentos sino también combustibles, fibras y otros materiales clave. Minimizar el impacto negativo de la intensificación de la agricultura en el medio ambiente y la salud humana es fundamental para satisfacer la creciente demanda mundial de alimentos de manera sostenible. La necesidad de soluciones naturales y respetuosas con el medio ambiente para reducir el uso de productos químicos nocivos en agricultura sin comprometer las cosechas ha sido de interés para científicos y agrónomos en los últimos años. En este sentido, los inoculantes microbianos han surgido como una alternativa viable y sostenible a los pesticidas y fertilizantes químicos para el manejo de cultivos (Barea, 2015; Trivedi *et al*., 2017). Si bien algunos productos microbianos para la protección de cultivos han estado disponibles en el mercado durante décadas, es en los últimos años cuando su uso está aumentando y despierta gran interés en el sector agrícola y biotecnológico. Sin embargo, el mercado actual de biopesticidas basados en microorganismos representa sólo el 5% del mercado de pesticidas químicos (Batista & Singh, 2021). De hecho, a pesar de las numerosas medidas para restringirlo, el uso de pesticidas químicos sigue en aumento (FAO, 2022).

Los microorganismos beneficiosos, incluidos bacterias y hongos, que viven en una asociación mutualista con las plantas, tienen gran potencial para mejorar el crecimiento, productividad y salud de la planta huésped. Los microorganismos del suelo, como las rizobacterias promotoras del crecimiento vegetal (Plant Growth Promoting Rhizobacteria, PGPR) de los géneros *Bacillus* y *Pseudomonas*, los hongos de control biológico del género *Trichoderma* y los hongos micorrícicos arbusculares (Arbuscular Mycorrhizal Fungi, AMF) se encuentran entre los grupos mejor estudiados, y son la base de múltiples productos microbianos en todo el mundo (Woo *et al*., 2014, 2022; Aamir *et al*., 2020; Basiru *et al*., 2021). Además de mejorar el crecimiento y la productividad de las plantas, estos microorganismos pueden proteger a su planta huésped contra plagas y enfermedades de forma directa, pero también indirecta.

Los efectos directos se basan en el antagonismo entre el agente de biocontrol y el organismo diana. Entre los efectos directos destaca la producción, por parte de algunos

microorganismos, de antibióticos, lipopéptidos, enzimas líticas y otros metabolitos, reduciendo la población de patógenos del suelo a través del antagonismo o parasitismo directo (Whipps, 2001; Köhl *et al*., 2019). Aunque actualmente muchos productos microbianos basados en antagonismo directo están comercialmente disponibles (Woo *et al*., 2014, 2022; van Lenteren *et al*., 2017), su eficacia en el campo aún parece ser inestable.

Además del antagonismo directo, los microorganismos beneficiosos son capaces de sensibilizar el sistema inmunitario y estimular las defensas de la planta huésped, lo que resulta en resistencia inducida (Induced Resistance, IR), normalmente eficaz frente a una amplia gama de atacantes tanto de raíz como de la parte aérea, incluidos patógenos e insectos herbívoros (Pieterse *et al*., 2014; De Kesel *et al*., 2021). A diferencia de los productos microbianos basados en antagonismo directo como modo de acción, todavía no existen productos comerciales microbianos para controlar plagas y enfermedades basados en IR.

A pesar del demostrado potencial de los microorganismos beneficiosos para mejorar el crecimiento y la salud de las plantas, la mayor parte de la investigación sobre el uso de inoculantes microbianos para la promoción y protección de cultivos se ha realizado en condiciones controladas de laboratorio, y la transferencia de esta tecnología y su adopción en agricultura todavía se enfrenta a importantes retos (Mitter *et al*., 2019; Saad *et al*., 2020).

Las condiciones ambientales variables existentes en los sistemas de producción de cultivos junto a las prácticas agrícolas a menudo limitan el éxito de los inoculantes microbianos en campo (Compant *et al*., 2019). Para superar estos desafíos, es esencial caracterizar la funcionalidad de los inoculantes en diferentes condiciones y entender los mecanismos que regulan las interacciones planta-microorganismo. Esto podría ayudar a identificar aislados microbianos más eficaces y/o más estables en diferentes contextos, lo que mejoraría el éxito de su aplicación en agricultura. En los últimos años ha surgido un interés creciente por el diseño y la explotación de comunidades microbianas sintéticas (Synthetic Microbial Communities, SynComs) en agricultura para la protección de cultivos y la mejora de cosechas de manera sostenible (Arif *et al*., 2020; Liu *et al*., 2020; Trivedi *et al*., 2020). La combinación de microorganismos complementarios, con diferentes modos de acción y requisitos, incluyendo bacterias y hongos, tiene el potencial de mejorar la consistencia de los resultados en campo y, por lo tanto, la estabilidad de las

prácticas de control biológico. Es de esperar que estos inoculantes mixtos mantengan su funcionalidad en una mayor variedad de condiciones en comparación con los inoculantes basados en un único microorganismo y, por lo tanto, soporten mejor el impacto de las condiciones ambientales variables existentes en los sistemas agrícolas (Sarma *et al*., 2015; Arif *et al*., 2020; Pozo *et al*., 2021). Diseñar SynComs eficientes, desarrollar herramientas para su estudio, monitorización y aplicación, y entender las interacciones entre los miembros de las SynComs, así como sus efectos sobre los organismos diana y la planta hospedadora son en la actualidad grandes retos para las empresas biotecnológicas del sector agrícola.

El objetivo principal de esta Tesis Doctoral, realizada como parte de la actividad de investigación y desarrollo de la empresa biotecnológica Koppert, especialista en el sector del control biológico, es diseñar un SynCom multifuncional para la protección de cultivos, compuesto por microorganismos beneficiosos del suelo compatibles, representativos de los géneros más explotados por la industria de control biológico y comparar su eficacia con la de la aplicación individual de los organismos que la componen. Para abordar este objetivo principal:

1. En primer lugar, se diseñaron diferentes SynComs realizando una selección basada en la literatura de microorganismos beneficiosos, incluyendo hongos y bacterias, previamente caracterizados en diversos aspectos. Los microorganismos seleccionados para el diseño de SynComs fueron las PGPR *Bacillus amyloliquefaciens* CECT 8238 y CECT 8237, *Pseudomonas chlororaphis* y *P. azotoformans*, los hongos *Trichoderma harzianum* T22 y ESALQ1306, y el AMF *Rhizophagus irregularis*.
2. A continuación, se abordó la compatibilidad de las cepas microbianas seleccionadas para garantizar su establecimiento y evitar las interacciones antagónicas entre los componentes de los SynComs.
3. Posteriormente, se caracterizó y probó la eficacia de los SynComs y las cepas microbianas individuales. Se realizaron múltiples experimentos/bioensayos en diferentes condiciones, desde plantas creciendo en macetas con suelo estéril bajo condiciones controladas de laboratorio hasta plantas creciendo en invernadero comercial con manejo de producción hortícola estándar. Desde la efectividad en control de plagas y enfermedades hasta la compatibilidad con el manejo de cultivos y el impacto de la aplicación en la cosecha final, probamos el efecto de

los inoculantes microbianos en plantas de tomate para el control biológico de importantes patógenos fúngicos e insectos plaga, como *Botrytis cinerea*, *Fusarium oxisporum* y el insecto minador *Tuta absoluta*, todos ellos entre las amenazas más importantes para la producción de tomate.

4. Finalmente, se recolectaron muestras de la planta para estudiar los posibles mecanismos de la resistencia inducida por microorganismos. Se realizó la identificación de marcadores asociados con una simbiosis exitosa de la planta con microorganismos particulares o con un estado de potenciación de las defensas de la planta, ya que estos marcadores serían una herramienta extremadamente útil para la selección de inoculantes eficientes.

En el **Capítulo 1**, aprovechando la colección de aislados microbianos de la empresa de control biológico Koppert Biological Systems, diseñamos SynComs con potencial para biocontrol, constituidos por bacterias y hongos beneficiosos cuidadosamente seleccionados y bien caracterizados que muestran diversos modos de acción para el control biológico. La compatibilidad de los componentes microbianos de las SynComs se confirmó en sistema planta-suelo evaluando su colonización y persistencia a través de la optimización y aplicación de métodos microbiológicos, histoquímicos y moleculares específicos.

Para caracterizar y evaluar la eficacia en control biológico de los SynComs diseñados, en el **Capítulo 2** comparamos la capacidad de las cepas microbianas seleccionadas cuando se aplican por separado o en combinación como SynComs para controlar patógenos foliares y de raíz, utilizando diferentes estrategias de aplicación -aplicación directa a suelo o pulverización de las hojas- que implican antagonismo microbiano directo o resistencia sistémica inducida en la planta. Diferentes microorganismos fueron los más efectivos individualmente en el control del patógeno radicular *F. oxysporum* o del patógeno foliar *B. cinerea*, cuando se aplicaron directamente en el suelo (efecto directo contra *F. oxysporum* o efecto sistémico contra *B. cinerea*) o en hojas (efecto directo contra *B. cinerea*). Las SynComs mostraron una funcionalidad más amplia, controlando de manera eficaz ambos patógenos en cualquiera de los métodos de aplicación, alcanzando siempre al menos los mismos niveles de protección que las cepas individuales con mejor rendimiento. Los resultados de este Capítulo ilustran el potencial de SynComs, compuestos por microorganismos beneficiosos cuidadosamente seleccionados y

compatibles, incluidas bacterias y hongos, para el desarrollo de productos de control biológico estables y versátiles para la protección de plantas contra un rango más amplio de enfermedades.

Como siguiente paso, en el **Capítulo 3**, ampliamos la investigación a condiciones reales de producción, inoculando plantas de tomate con diferentes microorganismos incluyendo PGPB, *Trichoderma*, AMF y hongos entomopatógenos (Entomopathogenic Fungi, EPF) individualmente, y con dos SynComs seleccionados. Probamos la funcionalidad de los inoculantes en un invernadero de producción con manejo de cultivos habitual para la producción de tomate, incluyendo métodos de manejo integrado de plagas. Para ello evaluamos el efecto de la inoculación en la resistencia de las plantas frente a plagas y enfermedades que aparecieron de forma natural, el crecimiento y productividad de las plantas y la calidad de los frutos. Las plantas inoculadas con *Trichoderma*, AMF y el EPF *M. robertsii*, y con la SynCom compuesta por EPF y AMF, resultaron ser más resistentes a la polilla del tomate *T. absoluta* mostrando menor incidencia natural de esta plaga. Además, no se observó efecto negativo de los inoculantes microbianos sobre el insecto beneficioso *Nesidiocoris tenuis* utilizado para el biocontrol de plagas durante la temporada de cultivo. La inoculación con el AMF *F. mosseae* y el hongo *T. harzianum* T22 aumentó la cosecha de frutos de calidad comercial, en comparación con las plantas no inoculadas. Es de remarcar que ninguno de los inóculos afectó de manera negativa a la resistencia de las plantas a plagas y enfermedades ni a la productividad de las plantas. Los resultados de este Capítulo resaltan el potencial de algunos de los microorganismos probados para mejorar la resistencia de las plantas a plagas importantes como *T. absoluta* y para mejorar la producción de frutos, siendo compatibles con otros organismos de biocontrol y con las estrategias comunes de manejo de cultivos utilizadas en la producción comercial de tomate.

La importante reducción de la incidencia de *T. absoluta* en campo nos llevó a estudiar más en detalle estos efectos, probando si la protección era consistente en diferentes condiciones experimentales, desde condiciones controladas de laboratorio hasta condiciones agronómicas. La polilla del tomate *T. absoluta* es una plaga invasora y una gran amenaza para la producción mundial de tomate que causa importantes pérdidas económicas y de cultivos. Se sabe poco sobre la eficacia de la resistencia inducida por microorganismos frente a *T. absoluta* y su potencial para controlar esta plaga devastadora.

En el **Capítulo 4** probamos la capacidad de varias bacterias y hongos beneficiosos del suelo inoculados individualmente y como SynCom para inducir resistencia en plantas de tomate frente a *T. absoluta* y exploramos los posibles mecanismos. Realizamos múltiples bioensayos para evaluar la resistencia inducida por microorganismos en condiciones controladas, semi-controladas y agronómicas. Para explorar los posibles mecanismos, realizamos análisis metabolómico no dirigido con el fin de identificar metabolitos secundarios defensivos con mayor acumulación en las plantas que muestran resistencia inducida que pudieran explicar el efecto observado en la plaga. *Trichoderma harzianum* y los AMF *R. irregularis* y *F. mosseae* mostraron su estabilidad en distintos contextos, reduciendo consistentemente el desempeño o la incidencia de *T. absoluta*, induciendo resistencia en tomate frente esta plaga en todas las diferentes condiciones probadas. Sorprendentemente, cuando los tres hongos se inocularon como parte de una SynCom, la protección frente al insecto lograda por las cepas individuales se perdió. Demostramos que estos hongos beneficiosos pueden modular las respuestas de defensa de la planta a través de una reprogramación metabólica, lo que lleva a una mayor acumulación en las plantas inoculadas de compuestos defensivos con efectos deletéreos sobre el desarrollo de *T. absoluta*. Entre estos, se confirmó que el ácido azelaico y la feruloilputrescina, sobreacumulados en las plantas inoculadas en respuesta al herbívoro, inhibían el desarrollo de *T. absoluta*. Cabe destacar que la inoculación con la SynCom no resultó en una mayor acumulación de ninguno de estos compuestos en respuesta a la herbivoría, lo que correlaciona con la falta de resistencia inducida frente a *T. absoluta* en las plantas inoculadas con esta SynCom. Estos resultados apoyan la adecuación de estos compuestos como posibles marcadores de la potenciación o "priming" de las defensas.

Además, nuestros resultados anteriores en condiciones de producción comercial (**Capítulo 3**) evidenciaron la compatibilidad de la resistencia inducida por microorganismos y las prácticas actuales de manejo de cultivos. Los resultados de este Capítulo confirmaron que la resistencia inducida por microorganismos se puede incorporar en los programas de manejo integrado de plagas, mejorando el control sostenible de *T. absoluta*.

En conjunto, los resultados obtenidos en esta Tesis Doctoral confirman el potencial de los microorganismos beneficiosos para la protección sostenible de cultivos contra plagas y enfermedades de relevancia económica, sin comprometer el rendimiento de las cosechas. Explorar la funcionalidad de diversos microorganismos bajo diferentes

condiciones, incluyendo agroecosistemas reales, permite identificar cepas microbianas estables en distintos contextos, compatibles con las prácticas habituales de manejo de cultivos, facilitando así la transferencia de esta tecnología al sector agrícola. El diseño de SynComs para la protección de plantas contra patógenos y plagas podría ser una estrategia prometedora para mejorar las prácticas de biocontrol. Sin embargo, se necesita más investigación para comprender la complejidad de las interacciones microorganismo-microorganismo y planta-microorganismo para poder desarrollar productos basados en SynCom estables y multifuncionales para la protección sostenible de cultivos frente a diversas plagas y enfermedades.

SUMMARY

Agriculture plays a pivotal role in sustaining the continuously growing world's global population, providing not only foods but also fuels, fibres and other key materials. Minimizing the negative impact of agriculture intensification on the environment and human health is fundamental to reach the increasing global food demand in a sustainable way. The need of natural and environmentally friendly solutions to reduce the use of harmful chemicals in agriculture without compromising yields got received the attention of scientists and agronomists in the recent years. In this regard, microbial inoculants have arisen as a viable alternative to chemical pesticides and fertilizers for crop management (Barea, 2015; Trivedi *et al.*, 2017). Although some microbial products for crop protection have been commercially available in the market for decades, it is only in the recent years that their use is starting to increase. However, the current market of microbial based biopesticides account for only 5% of the chemical pesticide market (Batista & Singh, 2021) In fact, the use of the chemical pesticides continues increasing (FAO, 2022).

Beneficial microorganisms including bacteria and fungi living in a mutualistic association with plants have a great potential to improve growth, productivity and health of their host plant. Soil borne microorganisms such as the plant growth promoting rhizobacteria (PGPR) from the genera *Bacillus* and *Pseudomonas*, biocontrol fungi from the genus *Trichoderma* and arbuscular mycorrhizal fungi (AMF) are among the best studied groups. And, the base of multiple microbial product globally (Woo *et al.*, 2014, 2022; Aamir *et al.*, 2020; Basiru *et al.*, 2021). In addition to improving plant growth and productivity, these microbes are able to protect their host plant against pest and diseases directly, but also indirectly.

Direct effects are based on microbial antagonism. Some microbes produce antibiotics, lipopeptides, lytic enzymes and other metabolites, reducing the population of soil pathogens through direct antagonism or parasitism (Whipps, 2001; Köhl *et al.*, 2019). Although currently many microbial products based on direct antagonism are available commercially (Woo *et al.*, 2014, 2022; van Lenteren *et al.*, 2017), their efficacy in the field still appear to be unstable.

In addition to direct antagonism, beneficial microbes can sensibilize the host plant immune system and prime plant defences leading to induced resistance (IR) to a wide

range of below and aboveground attackers, including pathogens and herbivorous insects (Pieterse *et al.*, 2014; De Kesel *et al.*, 2021). In contrast to microbial products based on direct antagonism as mode of action, there are still no microbial products available that claim IR as mechanism to control pests and diseases.

Despite the great potential of beneficial microbes to improve plant growth and health, most of the research on microbial inoculants for plant growth and crop protection has been performed under controlled conditions and the successful transfer and adoption of this technology in agriculture is still facing difficulties (Mitter *et al.*, 2019; Saad *et al.*, 2020).

The highly variable environmental conditions and agricultural practices occurring in real crop production are often limiting the success of microbial inoculants in the field (Compant *et al.*, 2019). To overcome these challenges, it is essential to further characterize he functionality of the inoculants under different conditions, and to unravel the mechanisms regulating the plant-microbe interactions. This could help to identify microbial species and isolates with improved context-stability and facilitate their application in agriculture. In the last years there is an increasing interest for the design and exploitation of synthetic microbial communities (SynComs) for sustainable crop protection and yield improvement in agriculture (Arif *et al.*, 2020; Liu *et al.*, 2020; Trivedi *et al.*, 2020). Combining complementary microorganisms, with different modes of action and requirements, across bacteria and fungi, has the potential to improve the consistency of the results in the field and so the stability of biological control practices. These mixed inoculants are expected to maintain their functionality in a greater range of conditions compared to single strain inoculants and thus to support better the impact of the variable environmental conditions in agricultural systems (Sarma *et al.*, 2015; Arif *et al.*, 2020; Pozo *et al.*, 2021). Designing efficient SynComs, developing tools for their study, monitoring and application, and understanding the interactions between the members of the SynComs, as well as their effects on the target organisms and the host plant are currently major challenges for biotech companies in the agricultural sector.

The main aim of this Doctoral Thesis, performed as part of the research and development activity of the biotechnological company Koppert, a specialist in the biological control sector, is to design a multifunctional SynCom for crop protection composed by compatible beneficial soil borne microorganisms, representative of the most exploited

genera by the biocontrol industry, and compare its effectiveness with that of the individual application of the microorganisms that compose it. To address this main aim, we have:

1. First, designed different SynComs performing a literature-based selection of plant beneficial microorganisms, including fungi and bacteria, previously characterized in diverse aspects. The selected microorganisms for the SynCom design were the PGPR *Bacillus amyloliquefaciens* strains CECT 8238 and CECT 8237, *Pseudomonas chlororaphis* and *P. azotoformans*, the fungi *Trichoderma harzianum* strains T22 and ESALQ1306, and the AMF *Rhizophagus irregularis.*
2. Afterward, addressed the compatibility of the selected strains to maximise microbial establishment and minimise antagonistic interactions between the components of the SynComs.
3. Subsequently, characterized and tested the efficacy of the SynComs and the individual microbial strains. Multiple experiments/bioassays across scales were performed, ranging from plants growing in pots with sterile soil under controlled lab conditions to plants growing in commercial greenhouses following standard production horticultural practices. From pest and disease control effectiveness to compatibility with crop management practices, we tested the effect of the inoculants on tomato plants for the biocontrol of important fungal pathogens and insect pests, as *Botrytis cinerea*, *Fusarium oxisporum* and the leaf miner *Tuta absoluta*, all among the most important threats for the commercial tomato production.
4. Finally, collected samples to dive into potential explanatory mechanisms of Microbe-IR. We achieved the identification of markers associated to successful symbiosis with particular microbes or with the primed defense status of the plant which will be an extremely useful tool for the screening of efficient inoculants.

In **Chapter 1**, exploiting the microbial library of the biocontrol company Koppert Biological Systems, we designed SynComs with potential for biocontrol, composed of carefully selected, well-characterized beneficial bacteria and fungi displaying diverse biocontrol modes of action. The compatibility of the microbial components of the SynComs was confirmed in plant-soil system by assessing their colonisation and persistence through the optimization and application of microbiological, histochemical and specific molecular methods.

To characterize and assess the biocontrol efficacy of the designed SynComs, in **Chapter 2** we compared the ability of the selected microbial strains to control shoot and root pathogens when applied separately or in combination as SynComs, using different application strategies that imply direct microbial antagonism or induced systemic plant resistance. Different individual microorganisms were the most effective in controlling the root pathogen *F. oxysporum* or the foliar pathogen *B. cinerea*, when applied directly in the soil (direct effect against *F. oxysporum* or systemic effect against *B. cinerea*) or on leaves (direct effect against *B.cinerea*). The SynComs showed an extended functionality, effectively controlling both pathogens under any of the application schemes, always reaching at least the same protection levels as the best performing single strains. The results from this Chapter illustrate the potential of SynComs, composed of carefully selected and compatible beneficial microorganisms, including bacteria and fungi, for the development of stable and versatile biological control products for plant protection against a wider range of diseases.

As next step, in **Chapter 3**, we scaled up the research to real production conditions, inoculating tomato plants with different inoculants including PGPB, *Trichoderma*, AMF and entomopathogenic fungi (EPF) individually, and with two selected SynComs. We tested the functionality of the microbial inoculants in a production greenhouse where common crop management for tomato production was applied including integrated pest management (IPM) methods. For that we evaluated the effect of the inoculation on plant resistance to naturally occurring pests and diseases, plant growth and productivity and fruit quality. Plants inoculated with *Trichoderma*, AMF and the EPF *M. robertsii*, and a SynCom composed by EPF and AMF, resulted to be more resistant to the tomato leaf miner *T. absoluta* presenting lower natural incidence of the insect, while no negative effect was observed on the beneficial insect *Nesidiocoris tenuis* used for biocontrol during the cropping season. Further, the inoculation with the AMF *F. mosseae* and the fungi *T. harzianum* T22 increased the yield of commercial quality tomatoes, as compared to the non-inoculated plants. Remarkably, none of the inocula affected negatively neither plant resistance to naturally occurring pests and diseases nor the productivity of the inoculated plants. The results from this Chapter highlight the potential of some of the tested microorganisms and SynComs to improve plant resistance to important pests such as *T. absoluta* and to enhance fruit productivity being compatible with other biocontrol

organisms and with common crop management strategies used in commercial tomato production.

The important reduction in *T. absoluta* incidence in the field lead us to study more in detail these effects, testing if the protection was consistent across experimental conditions ranging from controlled lab conditions to agronomic conditions. The tomato leaf miner *T. absoluta* is an invasive insect pest and a major threat to global tomato production causing important crop and economic losses. Little is known about the efficacy of microbe induced resistance against *T. absoluta* and its potential to control this devastating pest. In the **Chapter 4** we tested the ability of several soil-borne beneficial bacteria and fungi inoculated individually and as SynCom to trigger induced resistance against *T. absoluta* and explored possible underlying mechanisms. We performed multiple bioassays to evaluate microbe induced resistance under controlled, semi-controlled and agronomic conditions. To explore the possible underlying mechanisms, we performed an untargeted metabolomic analysis to identify defense-related secondary metabolites with primed accumulation in the plants displaying induced resistance. *Trichoderma harzianum* and the arbuscular mycorrhizal fungi *R. irregularis* and *F. mosseae* showed context stability, consistently reducing *T. absoluta* performance or incidence, activating induced resistance against this pest under all the different conditions tested. Surprisingly, when inoculated as SynCom the protection against the insect achieved by the individual strains was lost. We showed that these beneficial fungi are able to modulate plant defense responses through metabolic reprograming, leading to a primed accumulation of defensive compounds with deleterious effects on *T. absoluta* development. Among these, azelaic acid and feruloyl putrescine, over accumulated in the induced plants upon challenge, were confirmed to inhibit *T. absoluta* development. Noteworthy, the SynCom inoculation failed to trigger primed accumulation of none of these compounds upon herbivory, correlating with the lack of induced resistance against *T. absoluta* in the SynCom inoculated plants. These results point to these compounds as potential markers of primed defenses.

Further, our previous results under production conditions (**Chapter 3**) evidenced the compatibility of Microbe-Induced Resistance and current crop management practices. The results from this Chapter confirmed that Microbe-Induced Resistance can be incorporated in integrated pest management programs, improving the sustainable control of *T. absoluta*.

Overall, the results obtained in this Doctoral Thesis confirm the potential of root associated beneficial microorganisms for sustainable crop protection against economically important pests and diseases in tomato without compromising yields. Exploring the functionality of diverse microbes under different conditions, including real agroecosystems, allows the identification of strains with good context stability, compatible with the common crop production management practices, facilitating in this way their transfer and adoption in agriculture. The design of SynComs for plant protection against pathogens and pest could be a promising strategy for improving biocontrol practices. However, more research is needed to understand the complex microbe-microbe and plant-microbe interactions to obtain a stable and multifunctional SynCom based product for sustainable crop protection against pest and disease.

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. The future food security trough a more sustainable agriculture

The world's global population is in continuous increase and by the middle of this century it is expected to reach more than 9.7 billion people (FAO, 2022; accessed 28/02/2022, https://www.fao.org/faostat/en/#data/OA). This continuous increase is directly translated in a growing food demand, and consequently, in a growing demand for agricultural products. In the past 20 years the cropland surface increased considerably, however this land expansion resulted in an important environmental impact since 49% of the new croplands have replaced natural woody and herbaceous vegetation (Potapov *et al.*, 2022). The cropland extension is also occurring in protected areas dedicated to biodiversity conservation, hence evidencing a clear trade-off between conservation and the need to increase food production (Vijay & Armsworth, 2021). Thus, optimizing the use of the currently cultivable land surface is crucial to reach a balance between increasing crop production and the conservation of the environment and biodiversity.

An important part of this optimization is minimizing crop losses. For example, crop losses caused by pests and pathogens range between 20 and 30% of the total agricultural production worldwide (Savary *et al.*, 2019). Moreover, the current scenario of global warming is directly related to an increase of the relative abundance of plant pathogens worldwide. Thus, crop losses caused by pathogens are expected to continue increasing (Delgado-Baquerizo *et al.*, 2020). Similarly, it is estimated that global yield loses of three important grain crops caused by insect pests will increase by 15-25% per 1°C of global surface warming (Deutsch *et al.*, 2018).

To combat this, efficient control methods are needed. Although the use of chemical pesticides had a strong positive effect on yield in the last decades, their abuse is negatively affecting the environment and human health. Among the negative consequences are the development of resistance in target organisms leading to an increased crop susceptibility, soil degradation, biodiversity loss and toxic effects in farmers and consumers (Tilman *et al.*, 2002). Therefore, alternative approaches based on natural and environmentally friendly solutions for crop protection are needed to sustainably minimize crop losses in agriculture. In this regard, exploring and exploiting natural resources that can contribute to the reduction of pests and diseases, such as plant-associated beneficial microorganisms,

is a promising strategy to reduce the input of harmful pesticides and improve agricultural sustainability, ideally without compromising yields.

2. Beneficial microorganisms as sustainable alternative to agrochemicals

In nature plants are not alone, but live in continuous interaction with numerous organisms above- and belowground. Diverse microorganisms like bacteria, fungi, viruses, protists, and nematodes live on different parts of the plant forming the plant microbiota (Trivedi *et al.*, 2020). In the rhizosphere, the root-soil interface, plant roots interact and establish associations with great number of soil-borne microorganisms (Barea *et al.*, 2005). While some of these microorganisms are detrimental for the plant, others can establish mutualistic associations with the plant roots providing important benefits to the host plant, including improved mineral nutrition and growth and protection against diverse abiotic and biotic stresses (Mendes *et al.*, 2013; Trivedi *et al.*, 2020). Beneficial soil microbes have a great potential for improving plant health and crop yields, playing a key role in the modern crop management, and improving sustainability in agriculture (Barea, 2015; Trivedi *et al.*, 2017; Singh *et al.*, 2020). In fact, biological control of plant pests and diseases using beneficial microorganisms has emerged as an efficient and sustainable alternative to the routinary use of chemical pesticides (van Lenteren *et al.*, 2017; Ab Rahman *et al.*, 2018).

3. Biological control using beneficial microorganisms

3.1. General aspects of biological control

Definitions, mechanisms and classifications of biological control have been recently discussed by Stenberg *et al*. (2021). The authors define biological control as the use of living agents, including viruses, to combat pests and pathogens directly or indirectly, for human good, always involving a biocontrol agent, a pest, and a human stakeholder benefitting from the pest control service provided by the biocontrol agent (Stenberg *et al.*, 2021). Biological control is classified in four different types: natural, conservation, classical and augmentative biological control (**Figure 1**) (van Lenteren *et al.*, 2017; Stenberg *et al.*, 2021). In natural and conservation biological control the pest or pathogen is controlled by naturally occurring biological control agents (BCAs), without or with

human action respectively (Stenberg *et al.*, 2021). In the conservation biological control human actions are focused on the preservation of the BCA and the improvement of its performance (van Lenteren *et al.*, 2017). In contrast, in classical and augmentative biological control the BCAs are introduced artificially for permanent or temporary establishment respectively (Stenberg *et al.*, 2021).

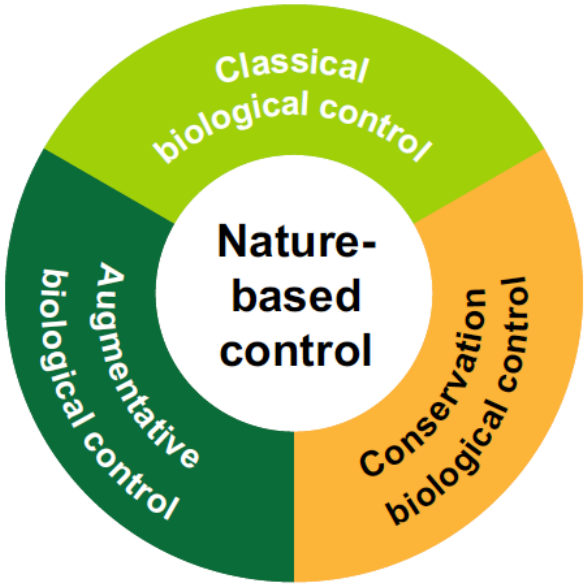

Figure 1. Classification of the different types of biological control. Natural, Conservation, Augmentative and Classical biological control (Stenberg *et al*., 2021).

3.2. Modes of action of microbial biological control agents

Biological control using microorganisms is augmentative in most cases. Microbial isolates are first screened, then, the most effective strains are selected and mass produced, and finally, they are applied once or several times during the cropping season (Köhl *et al.*, 2019). Microorganisms can act as BCA against pests and pathogens through diverse modes of action, which can be classified in two main groups: those with direct effect on the pest or pathogen (**Figure 2A and 2B**), and those with indirect or plant mediated effect (**Figure 2C**) (Whipps, 2001; Barea *et al.*, 2005).

3.2.1. Direct effects on the pathogen or pest

Direct effects mostly consist in microbial antagonism, and includes antibiosis, competition and parasitism. The antibiosis is the inhibition of the pathogen as a result of the release of antimicrobial compounds (**Figure 2A**). The competition can be for colonization sites or nutrients (**Figure 2B**), being competition for iron through the production of siderophores very common in the rhizosphere. Finally, parasitism is based on the production of lytic enzymes such as chitinase and glucanase that can lyse pathogen cell walls, allowing the parasite to obtain nutrients from the target (Whipps, 2001; Lugtenberg & Kamilova, 2009; Köhl *et al.*, 2019; Stenberg *et al.*, 2021).

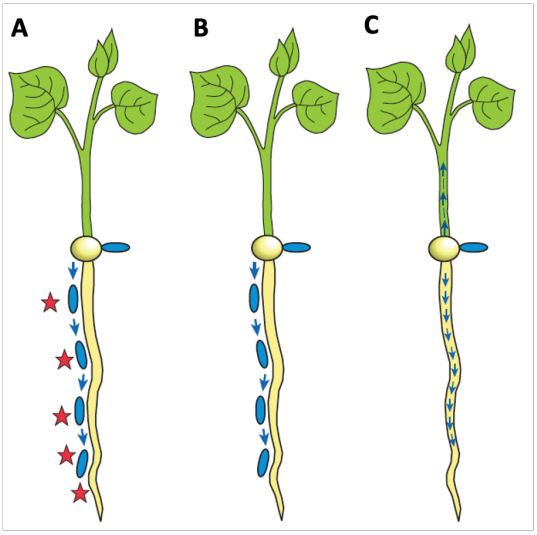

Figure 2. Most important mechanisms of biological control of plant diseases by microbes (A) Antibiosis. Microbial production of antibiotics (indicated by stars) negatively affecting pathogens around the root. (B) Competition for nutrients and niches. (C) Induced resistance (IR) triggered by the microbial root colonization. Modified from Lugtenberg and Kamilova, 2009.

Numerous microbial strains have been shown to be effective as BCA, resulting in an increasing number of registered microbial biocontrol products (Woo *et al.*, 2014, 2022; van Lenteren *et al.*, 2017). There are many examples for microorganisms as BCA with direct effect on the target pathogen or pest. For instance, plant growth promoting rhizobacteria (PGPR) and fungi from the genus *Trichoderma* are among the most studied and characterized microbial BCA with direct effect on soil or leaf pathogens.

PGPR have been widely reported as antagonists of plant pathogens and its direct biocontrol activity can be consequence of antibiosis, reduction of pathogen virulence, competition for iron (Lugtenberg & Kamilova, 2009; Barea *et al.*, 2013; Selosse *et al.*, 2014; Barea, 2015). The PGPR species more widely described as antagonists of important root pathogens are from the genera *Bacillus* and *Pseudomonas* (Haas & Défago, 2005;

Santoyo *et al.*, 2012). Some *Bacillus* spp. can produce cell wall degrading enzymes, peptide antibiotics and volatile organic compounds which have been shown to be effective in pathogen suppression. Lipopeptides from the families of iturins, fengycins and surfactines are among the most frequently produced antibiotic compounds by *Bacillus,* playing a key role in the biological control of plant diseases by bacteria from this genus (Pérez-García *et al.*, 2011). In the case of *Pseudomonas* spp.*,* they are particularly good in colonizing the rhizosphere, and this ability allows them to effectively compete for space with other microorganisms as plant pathogens (Santoyo *et al.*, 2012). Another direct biocontrol mechanism in *Pseudomonas* spp. is the production of siderophores. For example, fluorescent pseudomonads strains produce pyoverdine, contributing to disease suppression in conditions with low iron availability in the soil, by depriving pathogens of iron (Haas & Défago, 2005). In addition, most of the *Pseudomonas* strains characterized as biocontrol agents are able to produce one or several antibiotic compounds such as phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipipeptides and hydrogen cyanide for which the experimental evidence are supporting their function in biocontrol of root diseases (Haas & Défago, 2005).

Trichoderma spp. are rhizospheric fungi with well characterized biocontrol properties (Woo *et al.*, 2022). These fungi are extremely efficient to control fungal pathogens through direct antagonism based mainly on mycoparasitism but also on antibiosis and competition (Harman *et al.*, 2004; Barea *et al.*, 2013; Woo *et al.*, 2014, 2022). As a result, the great antagonistic potential of these fungi remains as the base for the effective application of *Trichoderma* strains as biofungisides against phytopatogenic fungi (Woo *et al.*, 2014, 2022). This genus is the most widely commercially exploited organism as biological control agent for plant protection. Currently there are many commercial *Trichoderma* based products available in the international market and their number has been growing continuously in the las years (Woo *et al.*, 2014, 2022).

Further, there are some groups of microbes acting as BCA with direct effect on pests, being able to infect herbivorous arthropods and to reduce their negative effect on plants. For example, entomopathogenic fungi (EPF), such as species from the genera *Beauveria* and *Metarhizium*, are important in agroecosystems because of their well-known ability for the biological control of insect and mite pests (Quesada Moraga, 2020). EPF have been used in biological control of insects for more than 150 years, and currently more than 170 species formulated as mycopesticides are commercially available (Bamisile *et al.*, 2021).

3.2.2. Indirect effects on the pest or pathogen

Besides direct effects on the pest or pathogen, beneficial microorganisms can reduce the damage caused by deleterious organisms by reducing their population or their aggressiveness indirectly through plant mediated effects. These plant mediated effects can be the result of two different aspects. On the one hand, some beneficial microorganism can improve the host plant growth and nutritional status, leading to damage compensation and better tolerance to some attackers (Barea *et al.*, 2005). On the other hand, some microorganisms can stimulate the plant immune system and potentiate plant defense responses leading to induced resistance (IR) to diverse pest and diseases (Pieterse *et al.*, 2014; De Kesel *et al.*, 2021). Beside the good antagonistic activity of PGPR, *Trichoderma* and EPF, microbes from these groups are widely reported to improve plant defenses triggering IR against broad spectrum of pests and pathogens not only locally at the colonization sites but also in distal parts of the host plant (Lugtenberg & Kamilova, 2009; Pieterse *et al.*, 2014; Pineda *et al.*, 2015; Quesada Moraga, 2020; Woo *et al.*, 2022).

Another important and well studied group of soil microbes reducing the negative effects of pests and pathogens are the arbuscular mycorrhizal fungi (AMF). They do not have direct antagonistic effect on the aggressor, but they act through plant mediated effects. AMF establish mutualistic symbiosis with the roots of most vascular plants and are able to protect the host plant against diverse deleterious organisms such as pathogens, herbivorous insects and parasitic plants, not only in roots but also in shoots (Jung *et al.*, 2012). The main mechanism implicated in plant protection by AMF seems to be the modulation of plant defenses and improvement of plant resistance, the so-called Mycorrhiza-Induced Resistance (Pozo & Azcón-Aguilar, 2007).

4. Induced Resistance and Priming of plant defenses

One of the main mechanisms operating in IR is the potentiation or priming of the plant defenses, which results in a greater capacity for the induction of defenses in response to pest or pathogen attack (**Figure 3**) (Martinez-Medina *et al.*, 2016; Mauch-Mani *et al.*, 2017). This induction of resistance is related to a higher accumulation of metabolites with antimicrobial and anti-herbivorous properties after the attack (Sanmartín *et al.*, 2020b; Rivero *et al.*, 2021). Defense priming depends on differential regulation of the

phytohormonal signaling pathways that coordinate the defense responses upon attack. In general, the IR observed after plant interaction with beneficial microorganisms depends on the jasmonic acid pathway (Pieterse *et al.*, 2014; Gruden *et al.*, 2020). Various stimuli, including microorganisms, arthropods, chemical compounds or abiotic signals, can elicit priming in the plant, which allows it to generate a more efficient defense response to a subsequent attack (Conrath *et al.*, 2006). This first stimulus triggers a temporary activation of plant defenses but leaving a 'memory' of the stress in the plant. Upon a subsequent attack, the plant generates a faster, stronger, and/or longer lasting defense response than an unprimed plant, resulting in higher resistance (Figure 3). Priming is a low energy cost adaptive immunity mechanism, since defense responses are not activated in absence of stress (Martinez-Medina *et al.*, 2016; Mauch-Mani *et al.*, 2017; Wilkinson *et al.*, 2019). It is also a long-lasting phenomenon, representing a type of plant immunological memory that can influence and be influenced by plant-microbe-pest/pathogen interactions throughout the plant's life cycle (Pozo *et al.*, 2020).

IR and priming of plant defenses by interaction with beneficial microbes have been widely described in multiple systems, including model plants and agronomically important crops. For example, it has been observed that the inoculation of tomato plants -*Solanum lycopersicum*- with AMF induces resistance against necrotrophic fungi (Sanchez-Bel *et al.*, 2016; Sanmartín *et al.*, 2020a) and lepidopteran caterpillars such as *Helicoverpa armigera* and *Spodoptera exigua* (Song *et al.*, 2013; Rivero *et al.*, 2021).

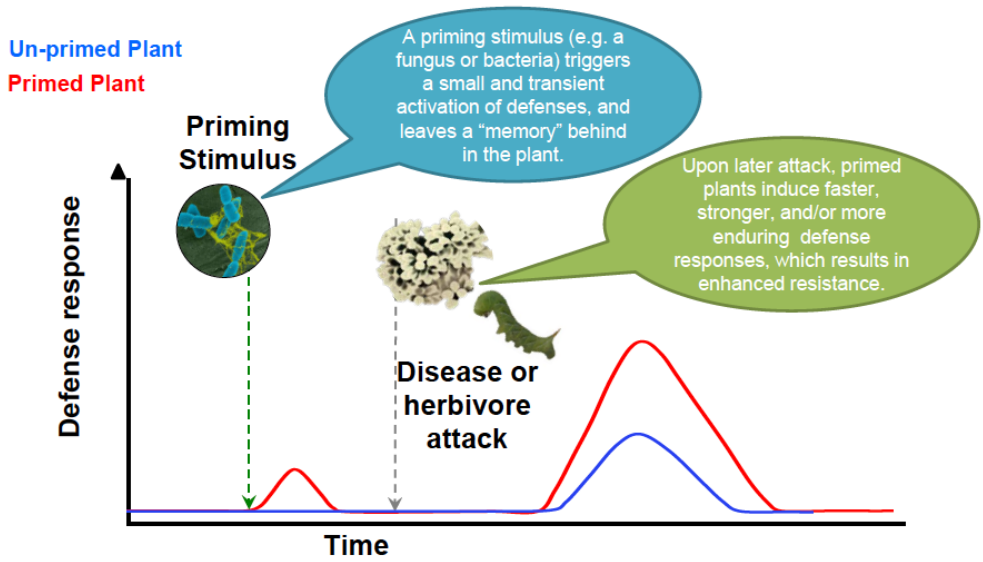

Figure 3. Preconditioning or priming of plant defenses by beneficial microorganisms. Adapted from Pozo et *al.*, (2020) "Threeway interactions between plants, microbes, and arthropods (PMA): Impacts, mechanisms, and prospects for sustainable plant protection". Teaching Tools in Plant Biology: The Plant Cell (online) https://doi/10.1105/tpc.120.tt0720

5. Microbial inoculants as part of Integrated Pest Management programs

Plants interact with a wide diversity of both beneficial and harmful organisms, including microbes and pests or pathogens. Most of these interactions have been studied only bidirectionally, between plants and microorganisms on the one hand, or between plants and pests or pathogens on the other hand. However, plants continually interact with both types of organisms, either simultaneously or sequentially, leading to three-way interactions that are much more complex (**Figure 4**) (Biere & Bennett, 2013; Gruden *et al.*, 2020; Pozo *et al.*, 2020).

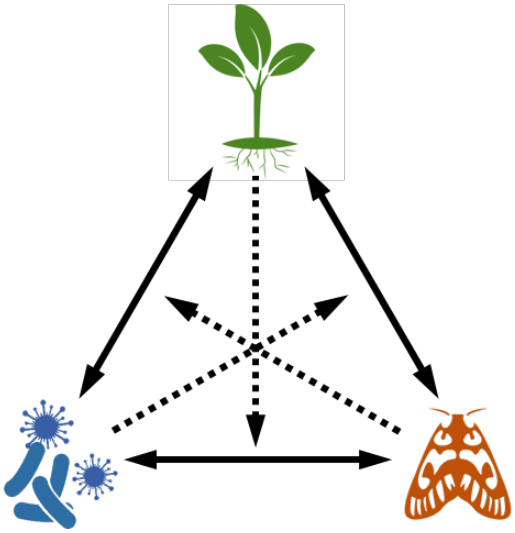

Figure 4. The three-way interactions between plants, microorganisms and arthropods are more complex that the sum of the pairwise interactions of the different components. Adapted from Pozo *et al*., (2020) "Three-way Plant-Microbe-Arthropod Interactions (PMA): Impacts, mechanisms and perspectives of a sustainable plant protection". Didactic tools in plant biology: the plant cell (online) https://doi.org/10.1105/tpc.120.tt0720

These three-way interactions have important consequences for all the organisms involved, which go beyond the simple sum of the bidirectional interactions between each pair (Gruden *et al.*, 2020). In fact, the multidirectional interactions in real agroecosystems are very complex, thus understanding their functioning is crucial for optimizing the use of natural resources in crop management. The impact of three-way interactions can be detrimental for the plant -for example, in the case of arthropods acting as vectors of phytopathogenic microorganisms- as well as beneficial -for example, beneficial microorganisms that improve the growth and resistance of plants against pests (Pineda *et al.*, 2010; Pozo *et al.*, 2020). This last type of three-way interaction has a great potential for its application in agriculture and horticulture and its implementation can lead to a

more sustainable production, reducing the input of chemical fertilizers and pesticides. Indeed, interactions of plants with beneficial microbes are very common and can help fight possible pests directly, stimulating plant defenses or improving their ability to attract natural enemies of pests (Pozo *et al.*, 2020). Managing these interactions can reduce the impact of agricultural pests in a sustainable way, reducing the need for agrochemicals.

However, the implementation of these three-way interactions in integrated pest management (IPM) programs is still in its infancy. To take advantage of these interactions as environmentally friendly biotechnology in IPM programs, it is necessary to understand its complexity from the agroecological scale to the molecular scale. This requires close collaboration between all sectors involved, including scientists, farmers, plant breeders and agricultural suppliers (Pozo *et al.*, 2020).

Biological pest control based on arthropod-arthropod interactions through the use of their natural enemies is widespread in agricultural production and in IPM systems (van Lenteren *et al.*, 2017). However, despite the potential of microbial inoculants for the biological control of pests and diseases, they are currently barely taken into account in crop management and IPM strategies. IPM is a strategy that aims to minimize or even avoid chemical pesticides application by the combined use of all available sustainable preventive and curative methods (Stenberg, 2017; Karlsson Green *et al.*, 2020), being biological control one of its main pillars (Naranjo *et al.*, 2015). IPM is often presented as a pyramid (**Figure 5**) where the largest area consists in preventive (e.g mechanical, physical, cultural) and sustainable control measures (e.g. biocontrol, plant resistance and tolerance, "plant vaccination") and chemical pesticides are applied only if the economic injury level (EIL) is reached (**Figure 5**) (Stenberg, 2017; Karlsson Green *et al.*, 2020). Remarkably, Microbe-IR is considered as important part of biological control (Köhl *et al.*, 2019) and also as an individual element of the IPM pyramid (Stenberg, 2017; Karlsson Green *et al.*, 2020) referred as "plant vaccination" in this case (**Figure 5**).

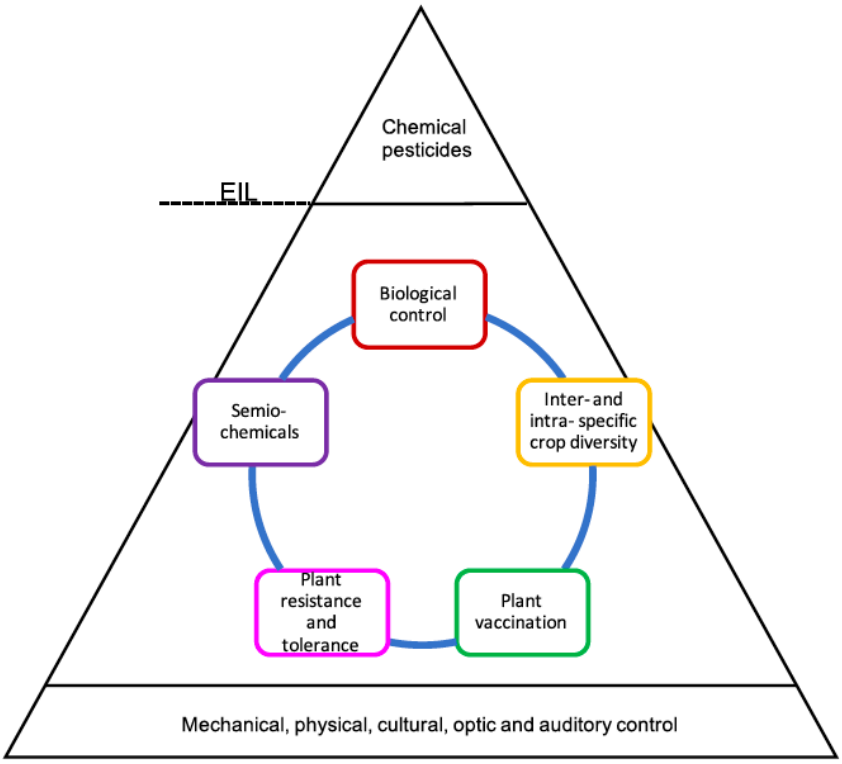

Figure 5. IPM pyramid with its largest area of sustainable preventive and curative control methods and a smaller top of chemical pesticide control that could be applied if the Economic Injury Level (EIL) has been reached. The base of the pyramid includes mechanical and physical actions, while the large mid-section exemplifies ecologically based methods, among them biological control and "plant vaccination" (Karlsson Green *et al.*, 2020).

Despite its potential for plant protection, Microbe-IR seems to be highly context-dependent, and its efficacy is often hampered by diverse biotic and abiotic factors frequently occurring in real crop production; thus its application in the field frequently results in unpredictable outcomes (Lee Díaz *et al.*, 2021). In addition, it is important to consider that Microbe-IR based methods do not reach the protection levels of chemical pesticides, specially in conditions not favoring plant-microorganism interactions. Hence, the adoption and practical implementation of Microbe-IR in real agroecosystems is still facing difficulties, and more knowledge is needed to optimize its efficacy and consistency.

6. Synthetic microbial communities (SynComs)

The use of beneficial microorganisms for the biological control of pests and diseases is a relatively young field of research that arouses great interest in the market. One of the main challenges for the wider adoption and application of this microbial inoculants is the

variability of the results obtained in real agricultural settings. The functionality and persistence of the inoculants can be affected by environmental conditions and the indigenous microbial community of the soil (Trivedi *et al.*, 2020; Pozo *et al.*, 2021). It is necessary to better understand the biology of the microorganisms to be applied, their survival in variable environmental conditions and their interaction with the plant to guarantee satisfactory and safe phytosanitary results. In this regard, there is growing interest in design and exploitation of microbial consortia or synthetic communities (SynComs) for sustainable crop protection and yield improvement in agriculture (Arif *et al.*, 2020; Liu *et al.*, 2020; Trivedi *et al.*, 2020). The use of SynComs, combining microorganisms with different modes of action and requirements, could improve the reproducibility of the results and the stability of biological control, since they are expected to be more resilient than single microorganism inoculants: maintaining their functionality in a greater range of conditions, and therefore better supporting the variability of environmental conditions in agricultural systems (Arif *et al.*, 2020; Pozo *et al.*, 2021). However, the design of effective SynComs is not an easy task and successful examples are scarce. The selection of the microbial components of SynComs is crucial and needs to be done carefully. In this regard, several microbe selection strategies have been proposed including selection based on phylogeny, classification, interaction networks or desired function deducted from experimental observations (**Figure 6**) (Vorholt *et al.*, 2017).

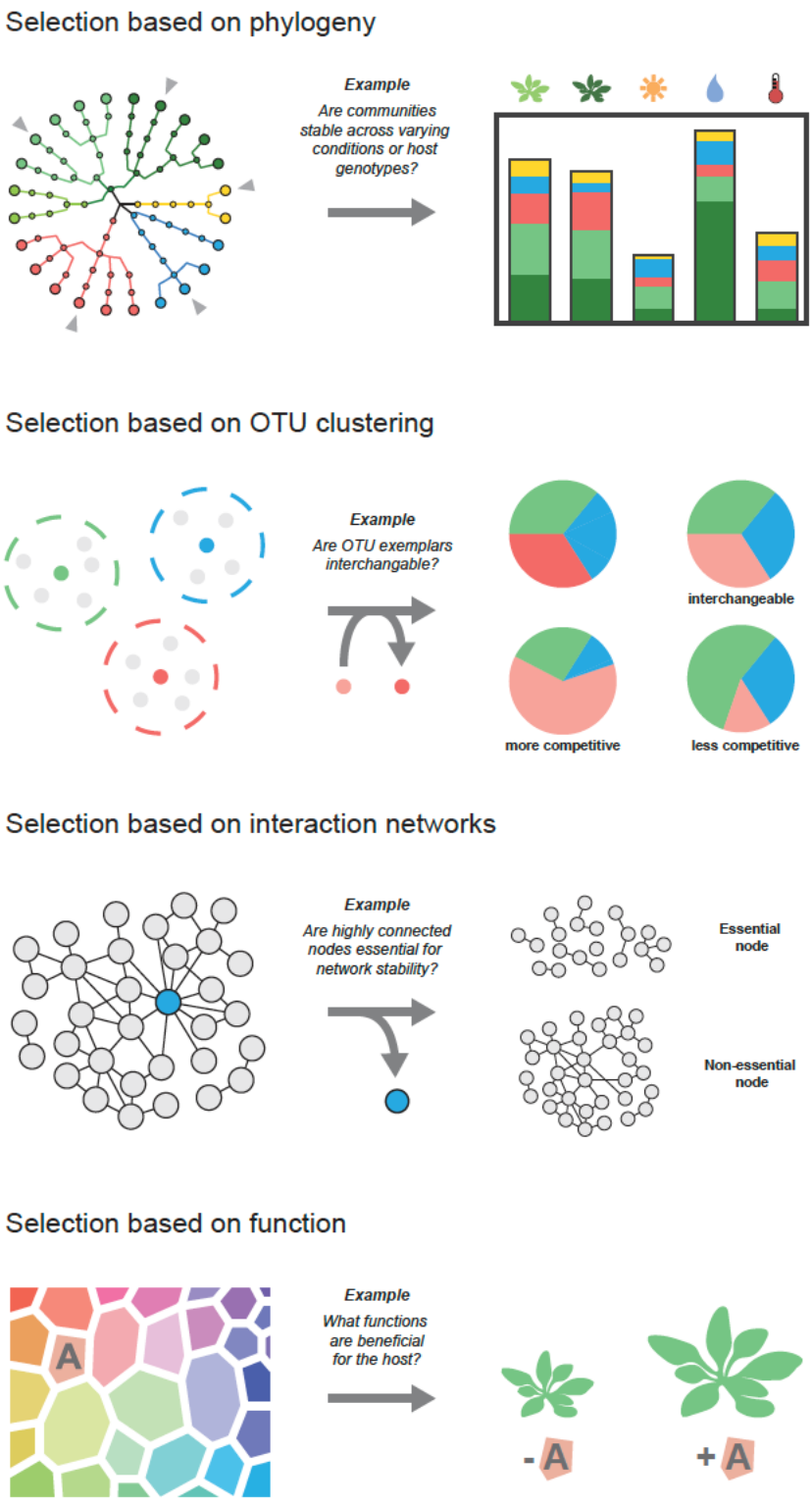

Figure 6. Proposed methods for microbial strain selection for SynCom design. Selection based on phylogeny, classification, interaction networks, or specific functions (Vorholt *et al.*, 2017).

Besides the selection of good candidates for the SynCom design, microbial compatibility of the selected strains is another important aspect to be taken into account. Microbe-

microbe interactions are complex and antagonisms between microbes can occur (Pozo *et al.*, 2021). Thus, microbial compatibility should be addressed to avoid antagonisms between the selected strains.

All in all, SynComs appear to be a promising tool to improve biocontrol and IPM programs, but more research effort is needed to fine tune the design and characterization of microbial communities to obtain stable and versatile multistrain microbial products.

7. Study system

Tomato (*Solanum lycopersicum*) is the second most produced vegetable crop worldwide, only surpassed by potato. In 2020 the global tomato production reached more than 186 million tons, dedicating for this more than 5 million ha of tomato cultivated surface worldwide, and being China, India, European Union, Turkey and the United States the major producers (FAO 2022, accessed 01/03/2022; https://www.fao.org/faostat/es/#data/QCL).

Currently several diseases and pests represent a serious threat to tomato production worldwide. *Botrytis cinerea* and *Fusarium oxysporum* are considered among the ten most important fungal phytopathogens (Dean *et al.*, 2012). *Botrytis cinerea*, a necrotrophic airborne pathogen, is the causal agent of the grey mold disease and causes important economic losses in many crops including tomato (Dean *et al.*, 2012; Bardin & Gullino, 2020). The infection by *B. cinerea* is promoted by high humidity which favors germination of the conidia and the penetration of the fungus in the plant tissues (Castañé *et al.*, 2020). The fungus can infect leaves, stems, flowers and fruits forming a characteristic grey mold on the diseased tissues (Bardin & Gullino, 2020). *Fusarium oxysporum*, a ubiquitous soil borne pathogen, causes vascular wilt in numerous plant species and can cause important crop losses in tomato among others (Dean *et al.*, 2012). In particular, *F. oxysporum* formae specialis (f. sp.) *radices-lycopersici* can disseminate trough the irrigation causing root and crown rot diseases on the host plant. The symptoms develop on base of the stem causing the wilt and death of the affected plant (Bardin & Gullino, 2020; Castañé *et al.*, 2020).

Whiteflies and the tomato leafminer *Tuta absoluta* are considered the current key pests of tomato (Castañé *et al.*, 2020). *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), recently reinstated as *Phthorimaea absoluta* (Chang *et al.*, 2021), is a devastating invasive

pest native to South America (Desneux *et al.*, 2011), being tomato -*S. lycopersicum*- its main host plant (Desneux *et al.*, 2010). After being detected in Spain in 2006, *T. absoluta* has rapidly spread across the Mediterranean basin and Europe (Desneux *et al.*, 2010, 2011). Currently has been detected in more than 100 countries across South America, Europe, Africa and Asia (EPPO, 2021), and is considered a major threat to global greenhouse and open-field tomato production (Desneux *et al.*, 2010; Biondi *et al.*, 2018). The insect larvae feed and cause damage on leaves, stems and fruits, and without control measures the pest can origin up to 80-100% of crop losses in tomato (Desneux *et al.*, 2010), thus causing important economic losses.

This doctoral thesis focuses on the potential of diverse beneficial microorganisms such as *Bacillus*, *Pseudomonas*, *Trichoderma* and AMF applied individually or as part of SynComs, as inoculants for the sustainable crop protection against three of the current major threats to tomato production such as *B. cinerea*, *F. oxysporum* f. sp. *radices lycopersici* and *T. absoluta* (**Figure 7**) under controlled and agronomic conditions.

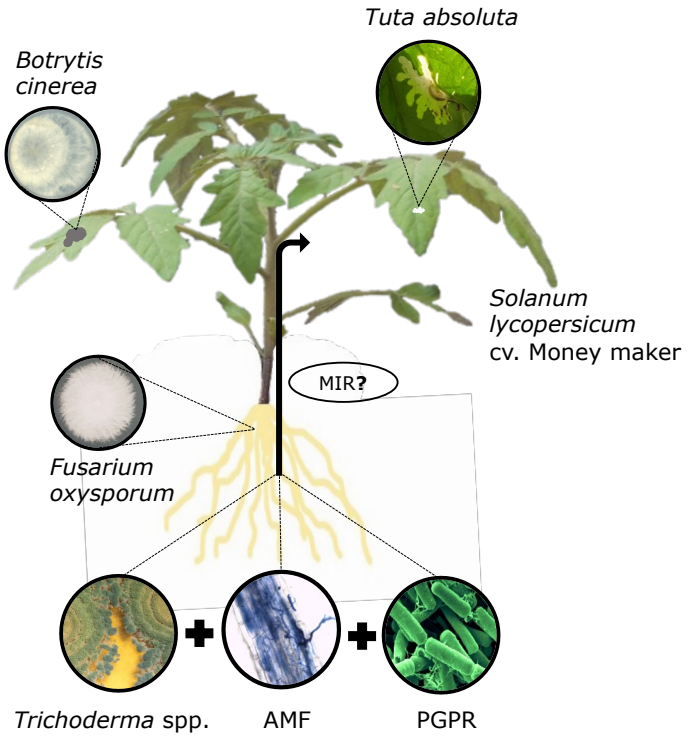

Figure 7. Schematic representation of the study system of the present Doctoral Thesis. Abbreviations: Arbuscular mycorrhizal fungi (AMF), Plant-growth promoting rhizobacteria (PGPR), Microbe-Induced Resistance (MIR).

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INTEREST OF THE STUDY AND OBJECTIVES

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In the las years there is an increasing social awareness on the negative impact of human activity on the environment and biodiversity. Agricultural production plays an important role in reaching the global food demand and sustain the world´s human population. Thus, minimizing the negative impact of the current intensive agricultural practices on environment and human health is of high priority.

Exploring natural and environmentally friendly alternatives to the abuse of agrochemicals to improve sustainability in agriculture without compromise yields is currently a major research topic. In this regard, bioinoculants for crop protection and yield enhancement based on beneficial microorganisms have the potential to improve agricultural sustainability and reduce the use of the harmful agrochemicals. Yet, the outcome of microbial inoculant´s application is often highly dependent on environmental conditions, such as soil type, nutrient availability, climate, and crop management practices. This context dependency often results in inconsistent results in the field and hamper the faster and wider adoption of microbial inoculants in agriculture. Thus, large-scale application of microbial inoculants for plant protection requires optimization and perhaps the development of particular solutions for specific agroecosystems. The identification of microbial strains with context stability and the design of synthetic microbial communities (SynComs) appears as promising strategies to improve consistency and reproducibility of the results, and thus, boost the success of microbial inoculant application in the field. In particular, the design of SynComs by combining phylogenetically diverse but compatible microorganisms with different functions have the potential to settle the base for the development of multifunctional, stable and versatile biocontrol and biostimulat products. The scientific and market interest in this area of research and the growing scientific and technical advances from ecological to molecular scale augur a boom in the use of microbial inoculants for plant protection in the context of sustainable agriculture.

Therefore, the general objective of this PhD Thesis is **to design a multifunctional consortium composed by compatible beneficial soil borne microorganisms with complementary modes of action for sustainable tomato crop protection against soil borne and leaf fungal pathogens, and leaf herbivorous insects.**

To accomplish this general objective the following specific objectives were defined:

1. To design a multifunctional microbial consortium through the combination of different biocontrol agents with complementary modes of action (Chapter 1).

2. To test root and rhizosphere colonization and persistence of the microbial inoculants when applied individually or as consortium (Chapter 1).

3. To test the biocontrol activity of the consortium and the individual inoculations against soil borne and leaf fungal pathogens in soil-plant systems (Chapter 2).

4. To test the effect of the microbial inoculants under tomato production conditions (Chapter 3).

5. To test the impact of selected microorganisms and consortia on the performance of the insect pest *Tuta absoluta* across different experimental systems (Chapter 4).

6. To explore the mechanisms underlying the Microbe-Induced Resistance against *Tuta absoluta* triggered by the best performing microorganisms and/or consortia (Chapter 4).

GENERAL MATERIAL AND METHODS

GENERAL MATERIAL AND METHODS

Plant material and seed surface sterilization

For the bioassays performed *in planta*, *Solanum lycopersicum* cv Money maker (Vreeken's Zaden, The Netherlands) was used as a model plant. Seeds were surface sterilized by immersion in 5% Sodium hypochlorite solution for 10 min followed by at least 3 washing steps in sterile water for 10 min each.

Beneficial microorganisms

The beneficial microbes used in the present PhD Thesis were:
From bacteria, two *Bacillus amyloliquefaciens* strains CECT 8238 and CECT 8237, formerly known as *Bacillus subtilis* UMAF6614 and UMAF6639 respectively, and *Pseudomonas chlororaphis* MA 342 and. From fungi, *Trichoderma harzianum* strains T22, ESALQ1306 and T78, the entomopathogenic fungi (EPF) *Beauveria bassiana* 1339 and *Metarhizium robertsii* 1235, and the arbuscular mycorrhizal fungi (AMF) *Rhizophagus irregularis* MUCL 57021, *Funneliformis mosseae* BEG12 and *Claroideoglomus etunicatum* EEZ163.

Microbe growing conditions and inoculum preparation

B. amyloliquefaciens strains were grown on tryptone soya agar (TSA, Oxoid) for 24h at 28°C. After that, a single colony from TSA culture was inoculated in 25ml of DSM (Difco sporulation medium) (Nicholson & Setlow, 1990) and incubated for 48h at 28°C in a rotatory shaker (200rpm). Spores were quantified using a Bürker-Türk counting chamber, then centrifuged at 5000 rpm for 15min and after discarding the supernatant, the pellet containing the spores was re-suspended in sterile tap water to a final concentration of 1 x 10^7 spores/ml.
P. azotoformans and *P. chlororaphis* were grown on TSA for 24h at 28°C. Liquid pre-culture was prepared using tryptone soya broth (TSB, Oxoid) inoculated with a single bacterial colony from TSA culture and incubated overnight at 28°C with rotary shaking at 200rpm. After that, 1ml of pre-culture was inoculated in 25ml of TSB media and placed in a rotatory shaker (200rpm) at 28°C. After 150mins of incubation, with bacterial growth

in exponential phase, the cell concentration was calculated measuring the O.D. (620nm) of the bacterial culture on Shimadzu UVmini-1240 Spectrophotometer. The bacterial culture was centrifuged at 5000rpm for 15min, and after discarding the supernatant, the pellet containing the bacterial cells was re-suspended in sterile tap water to a final concentration of 1 x 10^7 cfu/ml.

T. harzianum T22 and ESALQ1306 strains were cultured on potato dextrose agar (PDA, Difco) for 7 days at room temperature. Spores were collected from sporulating plates in sterile tap water, the concentration of the spore suspension was quantified using a Bürker-Türk counting chamber and adjusted to 1 x 10^7 spores/ml.

T. harzianum strain T-78 was cultured on PDA and re-cultured every two months. The fungal inoculum was prepared by adding aseptically a square piece of the fungal culture on a sterile mix of vermiculite and oat (Martínez-Medina *et al.*, 2009). The inoculum was incubated at 28°C and in the dark for 5 days. The inoculum was mixed with the substrate in a proportion of 1g per Kg of substrate.

The entomopathogenic fungi *B. bassiana* and *M. robertsii* were cultured in Sabouraud dextrose agar (SDA) and grown at 24° C in darkness for 3 weeks. The sporulated plates were scraped using a sterile spatula and the spores were recovered in a sterile solution of Triton X (0.05 %). The spore concentration was quantified using a Neubauer hemocytometer and adjusted to 1 x 10^8 spores/ml. For inoculation 1 ml of spore suspension per plant was applied to the root system during transplanting (Zitlalpopoca-Hernandez *et al.*, 2022).

The AMF *R. irregularis* was grown in a monoxenic culture on minimal (M) medium and using *Agrobacterium rhizogenes* - transformed carrot (*Daucus carota*) roots as a host root (St-Arnaud *et al.*, 1996). To extract the AMF spores, citrate buffer 0.01M (pH=6) was added to a sporulating AMF culture in a proportion 3:1 (v/v) and placed in a rotary shaker for one hour to dissolve the agar. AMF spores were recovered from the solution using sieves with different sizes (250 and 53 μm) and re-suspended in sterile tap water at final concentrations 1000 spores/ml.

The AMF *F. mosseae* and *C. etunicatum* were maintained as living inocula on mixed cultures of *Trifolium repens* and *Sorghum vulgare* in vermiculite-sepiolite substrate. The inoculants consisted of substrate containing colonized root fragments, mycelia and spores. For inoculation, 10% (v/v) of mycorhizal inocula were mixed with the substrate at transplanting (Rivero *et al.*, 2018).

Statistical analysis

Data were analysed using R statistical language, versions 4.0.5 and 4.1.1 (R Development Core Team 2021) and figures were produced using the package ggplot2 (Wickham, 2009). Details on the models used to assess the effects of the different variables evaluated are described in each Chapter of the present PhD Thesis. Model validation was performed graphically by inspecting the residuals and fitted values (Zuur & Ieno, 2016).

Selection of plant beneficial microorganisms for SynCom design (See Chapter 1)

Pathogenic fungi, growing conditions and inoculum preparation (See Chapter 1 and 2)

***In vitro* antagonism of the selected microbial strains (See Chapter 1)**

Substrate and plant growing conditions used in in planta bioassays (See each Chapter for detailed information)

Strains-Compatibility assessment (See Chapter 1)

Quantification of microbial and root mycorrhizal colonization (See Chapter 1 and 4)

***In planta* bioassays with pathogens (See Chapter 2)**

Biological control, pheromone and pollinator application

Evaluation of plant growth, nutritional status, yield, fruit quality and nutraceutical value, and natural pest and disease incidence (See Chapter 3)

***Tuta absoluta* rearing (See Chapter 4)**

In planta bioassays with *T. absoluta* (See Chapter 4)

Functional analysis of primed compounds (See Chapter 4)

Untargeted metabolomics: LC-ESI full scan mass spectrometry and data analysis (See Chapter 4)

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CHAPTER 1

CHAPTER 1:
Design of synthetic microbial communities for biocontrol of pathogens and pests

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ABSTRACT

The use of beneficial microorganisms for the biological control of plant diseases and pests has emerged as a viable alternative to chemical pesticides in agriculture. Traditionally, microbe-based biocontrol strategies for crop protection relied on the application of single microorganisms. However, the design of synthetic microbial communities (SynComs) for improving the reliability of current biological control practices is now a major trend in biotechnology, and it is already being exploited commercially in the context of sustainable agriculture.

In the present study, exploiting the microbial library of the biocontrol company Koppert Biological Systems, we designed SynComs composed by carefully selected, well characterized beneficial bacteria and fungi displaying diverse biocontrol modes of action. Finally, we addressed the colonization and persistence of the microbes using microbiological and molecular methods, confirming their compatibility when applied as SynCom.

INTRODUCTION

Plant microbiome engineering and the design of synthetic microbial communities (SynComs) for sustainable crop protection and productivity is a major research topic in this decade (Arif *et al.*, 2020; Liu *et al.*, 2020; Trivedi *et al.*, 2020). SynComs may improve the stability of biocontrol practices as microbial consortia are expected to deal better than single strain microbial inoculants with the large diversity of environmental challenges encountered in practice (Sarma *et al.*, 2015; Arif *et al.*, 2020; Pozo *et al.*, 2021). Besides this plasticity, the consortium can combine diverse biocontrol modes of actions, likely providing a better pest or disease control than single microorganisms with their specific abilities (Sarma *et al.*, 2015). Yet, most SynComs studies focus exclusively on bacteria, while fungi are major biocontrol agents and are considered to be more resilient to environmental chenges (Pozo *et al.*, 2021). Including fungi in the consortia would likely expand the range of functions and potential colonization niches of these mixed inoculants (Srivastava *et al.*, 2010; Pozo *et al.*, 2021). Thus, combining both, bacteria and fungi in SynComs design is expected to result in a multifunctional and more resilient product for biocontrol, and this is the aim of this study.

Among rhizospheric beneficial microorganisms, plant-growth promoting rhizobacteria (PGPR), fungi from the genus *Trichoderma*, and arbuscular mycorrhizal fungi (AMF) have been shown to effectively protect plants against diverse pests and diseases through different mechanisms including microbial antagonism and induced systemic resistance (ISR) (Pozo & Azcón-Aguilar, 2007; Barea *et al.*, 2013; Pieterse *et al.*, 2014; Barea, 2015; Pineda *et al.*, 2015; Woo *et al.*, 2022).

PGPR have been shown to control plant pathogens through antibiosis, reduction of pathogen virulence, competition for iron, plant growth promotion and ISR (Lugtenberg & Kamilova, 2009; Barea, 2015). Most reported PGPR antagonists are from the genera *Bacillus* and *Pseudomonas* (Haas & Défago, 2005; Pérez-García *et al.*, 2011; Santoyo *et al.*, 2012; Fira *et al.*, 2018; Dimkić *et al.*, 2022).

Regarding fungi, *Trichoderma* spp. are the most widely used BCA in agriculture and many *Trichoderma* based products are available in the market (Woo *et al.*, 2014, 2022). These fungi are extremely efficient for the of control of fungal pathogens mainly through direct antagonism, but also stimulating plant defenses (Harman *et al.*, 2004; Martínez-Medina *et al.*, 2014; Woo *et al.*, 2022). Finally, AMF are commercialized as biostimulants

in agriculture. These obligate biotrophs improve plant nutrient uptake and tolerance/resistance to multiple stresses, being able to protect the host plant against diverse pathogens and pests (Jung *et al.*, 2012; Sanmartín *et al.*, 2020; Rivero *et al.*, 2021). AMF do not produce antibiotics, but compete with the pathogens for nutrients and colonization sites and boost the plants defensive capacity, leading to ISR (Pozo & Azcón-Aguilar, 2007; Jung *et al.*, 2012).

The selection of microbial strains for SynCom design is a crucial step and needs to be approached carefully. Several strain selection strategies have been proposed based on either phylogenic criteria or phenotypic traits of potential interest deducted from experimental observations of the individual strains (Vorholt *et al.*, 2017). However, microbe-microbe interactions are complex and synergisms or antagonisms between microbes can occur (Pozo *et al.*, 2021). Thus, another crucial aspect in SynCom design is to address the compatibility between the selected microbial strains to be combined in the SynCom (Kong *et al.*, 2018; Arif *et al.*, 2020; Pozo *et al.*, 2021).

In this Chapter the design of different SynComs with potential for the biocontrol of pests and diseases is approached. First, exploring the microbial collection of the biocontrol company Koppert Biological Systems, a careful selection of diverse and well characterized microbial biocontrol agents was performed, based on phenotypical experimental observations of the individual strains available in the literature and aiming to integrate different biocontrol mechanisms. The selected microbial strains for the SynCom design include bacteria from the genera *Bacillus* and *Pseudomonas*, biocontrol fungi from the genus *Trichoderma* spp., and the AMF *Rhizophagus irregularis.* Next an *in vitro* antagonism assay is performed to additionally test the antagonistic capacity of the selected strains. Finally, the compatibility of the selected strains is tested comparing their rhizosphere or root colonization when applied individually or as consortia.

MATERIAL AND METHODS

1. Selection of plant beneficial microorganisms for SynCom design

A careful selection of beneficial microorganisms to create synthetic microbial consortia was performed focusing on the main groups of rhizospheric beneficial microorganisms such as PGPR, mycoparasitic fungi from the genus *Trichoderma* and AMF. An extensive

literature review on biocontrol studies of known BCAs was performed, taking also into account as potential candidates the microbial strains available at Koppert Biological Systems. The most relevant studies considered are summarized in **Table 1**.

As a result, we chose two *Bacillus amyloliquefaciens* strains CECT 8238 and CECT 8237, formerly known as *Bacillus subtilis* UMAF6614 and UMAF6639 respectively (Magno-Perez-Bryan *et al.*, 2015), and *Pseudomonas chlororaphis* MA 342 and (Abuamsha *et al.*, 2011a; Levenfors *et al.*, 2014). From fungi, we selected *Trichoderma harzianum* strains T22 and ESALQ1306 (Geraldine *et al.*, 2013; Coppola *et al.*, 2019) and the AMF *Rhizophagus irregularis* MUCL 57021.

Table 1. Summary of the most relevant literature on BCAs used in the present study.

Microorganism	Assay	Pathogen/Pest	Effect	Reference
Bacillus amyloliquefaciens CECT8238	*In vitro* antagonism	*Botrytis cinerea*	+	García-Gutiérrez et al., 2012
		Fusarium oxysporum	+	
		Acidovorax avenaesubsp. Avenae	0	Zeriouh et al., 2011
		Pectobacterium carotovorum subsp. Carotovorum	+	
		Xanthomonas campestrispv. Cucurbitae	+	
		X. campestris pv. Melonis	+	
	In planta antagonism	*Pectobacterium carotovorum*	+	Zeriouh et al., 2014,
		Xanthomonas campestris	+	
		Podosphaera fusca	+	Romero et al., 2004; 2007; Zeriouh et al., 2014
		Pectobacterium carotovorum subsp. Carotovorum	+	Magno-Perez-Bryan et al., 2015
		Podospaera xanthii	+	
	ISR	*Podosphaera fusca*	+	García-Gutiérrez et al., 2012
Bacillus amyloliquefaciens CECT8237	*In vitro* antagonism	*Botrytis cinerea*	+	García-Gutiérrez et al., 2012
		Fusarium oxysporum	+	
		Acidovorax avenaesubsp. Avenae	+	Zeriouh et al., 2011
		Pectobacterium carotovorum subsp. Carotovorum	+	
		Xanthomonas campestris pv. Cucurbitae	+	
		X. campestrispv. Melonis	+	
	In planta antagonism	*Podosphaera fusca*	+	Romero et al., 2004; 2007
		Pectobacterium carotovorum subsp. Carotovorum	+	Magno-Perez-Bryan et al., 2015
		Podospaera xanthii	+	
	ISR	*Podosphaera fusca*	+	García-Gutiérrez et al., 2012; 2013
Pseudomonas azotoformans	ISR	*Colletotrichum orbiculare*	+	Sang et al.,2014
		Botrytis cinerea	+	Bouaoud et al.,2017
Pseudomonas chororaphis MA342	*In planta* antagonism	*Verticillium longisporum*	+	Abuamsha et al., 2011b
		Drechslera teres	+	Tombolini et al., 1999
		Pseudomonas syringae pv. Syringae	0	Gilardi et al., 2010
	ISR	*Leptosphaeria maculans*	+	Abuamsha et al., 2011a
Trichoderma harzianum T22	*In planta* antagonism	*Rhizoctonia solani*	+	Wilson et al., 2008; Roberti et al., 2015; Fatouros et al.,2018
		Pythium ultimum	+	Fatouros et al.,2018
		Sclerotinia sclerotiorum	+	
		Armillaria mellea	+	Percival et al, 2011
	ISR	*Fusarium oxysporum*	+	Martinez-Medina et al., 2014
		Botrytis cinerea	+	Tucci et al., 2011; Aprile et al., 2022
		Cucumber mosaic virus	+	Vitti et al., 2016
		Meloidogyne incognita	+	Pocurull et al., 2020
		Nezara viridula	+	Alınç et al., 2021
		Macrosiphum euphorbiae	+	Coppola et al., 2017, 2019
		Spodoptera littoralis	+	di Leilo et al., 2021
		Tuta absoluta	+	Aprile et al., 2022
Trichoderma harzianum ESALQ1306	*In vitro* antagonism	*Leucoagaricusgongylophorus*	+	Nascimento et al., 2017
	In planta antagonism	*Sclerotinia sclerotiorum*	+	Geraldine et al., 2013
	ISR	*Tetranichus urticae*	+	Canassa et al., 2019
			0	
Rhizophagus irregularis	ISR	*Botrytis cinerea*	+	Sanchez-Bel et al., 2016; Sanmartín et al., 2020;de la Hoz et al., 2021

2. *In vitro* antagonism of the selected microbial strains

The antagonistic activity of the individual strains *B. amyloliquefaciens* CECT8238 and CECT8237, *P. azotoformans, P. chlororaphis*, *T. harzianum* T22 and ESALQ1306 was further evaluated *in vitro*, in confrontation assays against pathogens. Two major fungal pathogens causing important crop losses worldwide were selected: *Fusarium oxysporum* f.sp. radicis-lycopersici a soil pathogen, and the necrotrophic shoot pathogen *Botritis cinerea* strain B05.10. *Fusarium oxysporum* was grown on PDA at 25°C for 4 days, and *B. cinerea* was cultured on PDA at 20°C and grown for 4 days.

A schematic representation of the experimental set up is shown in **Figure 1**. For *Trichoderma*, one PDA plug (4mm) of *Trichoderma* culture and one of the pathogen cultures were placed on PDA plates with 4cm of distance from each other. For *Bacillus* and *Pseudomonas* 10µl drop of TSB liquid culture grown overnight was used instead of PDA plugs. As a control, a plug of the pathogen culture was placed in the petri dish without any antagonist. All treatments were replicated three times and all the plates were incubated at 25°C for 7 days. The radius of the pathogen colony in the confrontation plates was measured and compared to the radius of the pathogen colony in the control plates.

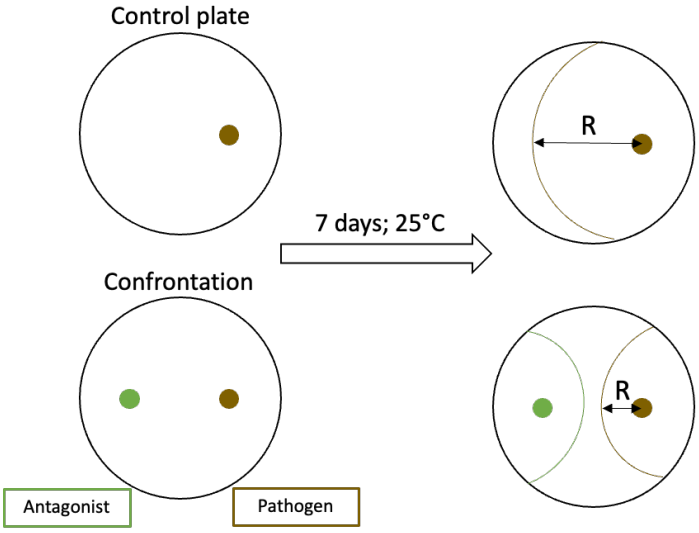


Figure 1. Schematic representation of the *in vitro* antagonism experimental set up.

3. Strains-Compatibility assay *in planta*

3.1 Microbe growing conditions and inoculum preparation

B. amyloliquefaciens strains were grown on tryptone soya agar (TSA, Oxoid) for 24h at 28°C. After that, a single colony from TSA culture was inoculated in 25ml of DSM (Difco

sporulation medium) (Nicholson & Setlow, 1990) and incubated for 48h at 28°C in a rotatory shaker (200rpm). Spores were quantified using a Bürker-Türk counting chamber, then centrifuged at 5000 rpm for 15min and after discarding the supernatant, the pellet containing the spores was re-suspended in sterile tap water to a final concentration of 1 x 10^7 spores/ml.

P. azotoformans and *P. chlororaphis* were grown on TSA for 24h at 28°C. Liquid pre-culture was prepared using tryptone soya broth (TSB, Oxoid) inoculated with a single bacterial colony from TSA culture and incubated overnight at 28°C with rotary shaking at 200rpm. After that, 1ml of pre-culture was inoculated in 25ml of TSB media and placed in a rotatory shaker (200rpm) at 28°C. After 150mins of incubation, with bacterial growth in exponential phase, the cell concentration was calculated measuring the O.D. (620nm) of the bacterial culture on Shimadzu UVmini-1240 Spectrophotometer. The bacterial culture was centrifuged at 5000rpm for 15min, and after discarding the supernatant, the pellet containing the bacterial cells was re-suspended in sterile tap water to a final concentration of 1 x 10^7 cfu/ml.

T. harzianum strains were cultured on potato dextrose agar (PDA, Difco) for 7 days at room temperature. Spores were collected from sporulating plates in sterile tap water, the concentration of the spore suspension was quantified using a Bürker-Türk counting chamber and adjusted to 1 x 10^7 spores/ml.

R. irregularis was grown in a monoxenic culture on minimal (M) medium and using *Agrobacterium rhizogenes* - transformed carrot (*Daucus carota*) roots as a host root (St-Arnaud *et al.*, 1996). To extract the AMF spores, citrate buffer 0.01M (pH=6) was added to a sporulating AMF culture in a proportion 3:1 (v/v) and placed in a rotary shaker for one hour to dissolve the agar. AMF spores were recovered from the solution using sieves with different sizes (250 and 53 μm) and re-suspended in sterile tap water at final concentrations 1000 spores/ml.

3.2 Microbial treatments

Rhizosphere or root colonization capacity of the different individual microorganisms and microbial consortia was tested (**Table 2**). Microorganisms tested individually were inoculated at a concentration of 1 x 10^7 cfu or spores/seed. The first microbial consortium, SynCom1A included one strain from each genus (*B. amyloliquefaciens* CECT8238*, P azotoformans* F30A *and T. harzianum* T22). The second one, SynCom2A, was composed by all selected microorganisms (*B. amyloliquefaciens* CECT8238 and CECT8237, *P.*

azotoformans F30A, *P. chlororaphis* MA 342, and *T. harzianum* T22 and ESALQ1306). The same concentration of each microorganism was used in both consortia (1 x 10^7 cfu each, that is a total of 3x 10^7 cfu per seed for SynCom1, 6 x 10^7 cfu per seed for SynCom2). For treatments including AMF, 1000 spores of *R. irregularis* were applied per seed.

Table 2. Composition of synthetic microbial consortia and concentration of beneficial microorganisms used in the strains-compatibility assays.

	Strains - Compatibility				
	Single	SynCom1A	SynCom1A +AMF	SynCom2A	SynCom2A +AMF
B. amyloliquefaciens CECT 8238	1 x 10^7 cfu/ plant (4 x 10^5 cfu/g of soil)	1 x 10^7 cfu/ plant (4 x 10^5 cfu/g of soil)	1 x 10^7 cfu/ plant (4 x 10^5 cfu/g of soil)	1 x 10^7 cfu/ plant (4 x 10^5 cfu/g of soil)	1 x 10^7 cfu/ plant (4 x 10^5 cfu/g of soil)
P. azotoformans F30A					
T. harzianum T22					
B.amyloliquefaciens CECT 8237		NA	NA		
P. chlororaphis MA 342					
T. harzianum ESALQ1306					
R. irregularis MUCL 57021	1000 spores/ plant		1000 spores/ plant	NA	1000 spores/ plant

3.3 Substrate, seed surface sterilization and plant growing conditions

Solanum lycopersicum cv Money maker seeds (Vreeken's Zaden, The Netherlands) were surface sterilized by immersion in 5% Sodium hypochlorite solution for 10 min followed by at least 3 washing steps in sterile water for 10 min each. The surface sterilized seeds were dried in a laminar flow cabinet and used for the experiments. The growing substrate was gamma irradiated nutrient poor peat soil (BVB, The Netherlands). All experiments were performed in a growing chamber at Koppert B.V. (Berkel en Rodenrijs, The Netherlands) under controlled conditions (25°C:23°C day:night with photoperiod 16h:8h light:dark and 60% of relative humidity).

3.4 Experimental set up

Rectangular plastic containers (18cm x 13cm x 6cm length x width x height) were filled with 300 g of soil previously moistened with tap water (300ml/1000g of soil). Then, 12 surface sterilized tomato seeds were sown in each container in a regular grid (**Figure 2**). The seeds were inoculated with the different microbial treatments (**Table 2**) by pipetting the microbial suspension to each seed. Each microbial strain (except *R. irregularis*) was

initially inoculated at 1 x 10^7 cfu/plant, resulting in a total concentration of 4 x 10^5 cfu/g of soil for each strain (12 plants/300g of soil). Finally, the seeds were covered with sterile vermiculite to avoid desiccation and undesired contaminations (**Figure 2**). A control treatment without any microbial inoculation was included. Each treatment was replicated five times. A randomized complete block design was used. Microbial colonization was evaluated 15 days after sowing (**Figure 2**) using the methods described in the next section.

Figure 2. Strains-compatibility experimental set up.

3.5 Quantification of microbes and root mycorrhizal colonization:

3.5.1 Microbiological methods

For the different bacteria and *Trichoderma*, we estimated for each genus the number of colony forming units (cfu) per gram of rhizospheric soil. For this, one gram of rhizospheric soil was sampled, diluted in 9 ml of sterile tap water and homogenized in a horizontal shaker at 350 rpm for one hour. Serial dilutions were plated on PDA + igepal (11ml/L) + tetracycline (50µg/ml) when targeting *Trichoderma,* and on TSA + natamycin (0.1g/L) when targeting bacteria. The plates were then incubated at 25°C and cfus were counted after 24h for bacteria and after 48h for *Trichoderma*. In consortia treatments, *Bacillus* spp., *Pseudomonas* spp. and *Trichoderma* spp. were distinguished morphologically, as they are well characterized strains in the Koppert collection (**Figure 3**). Microbial identity in representative colonies from each type was confirmed by PCR using specific primers for *Trichoderma, Bacillus* or *Pseudomonas* spp. (See section 3.5.3).

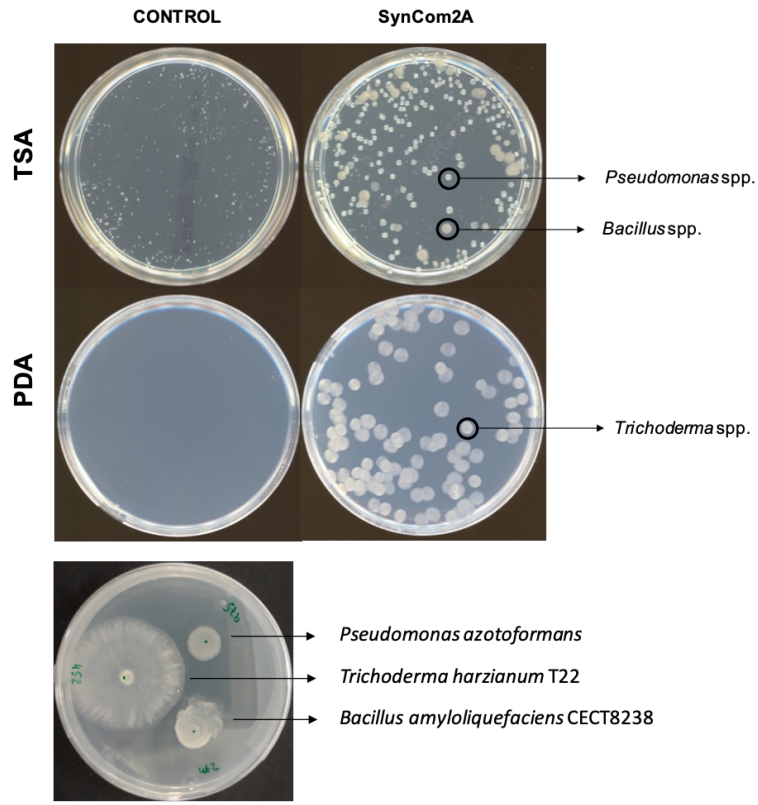


Figure 3. (A) Soil samples from Control and SynCom2 treatments plated on TSA medium amended with natamycin for bacteria determination, and PDA medium amended with igepal and tetracycline for fungi determination. Pictures illustrate the appearance of *Pseudomonas* spp, *Bacillus* spp. and *Trichoderma* spp. colonies in SynCom2 treatment, and the absence of indigenous species from these genera in the control treatment. **(B)** Colonies of *P. azotoformans*, *B. amyloliquefaciens* CECT8238 and *T. harzianum* T22 after 72h of growth on PDA. Picture illustrates morphological differences between colonies.

3.5.2 Histochemical methods

For treatments including AMF, mycorrhizal colonization was estimated by ink staining of fungal structures within the roots. For that, roots were washed and sampled upon harvesting, cleared in 10% KOH and the AMF structures were stained with 5% ink in 2% acetic acid (García *et al.*, 2020). The percentage of root length colonized by the AMF was quantified using the gridline intersection method (Giovannetti & Mosse, 1980) under a stereo microscope.

3.5.3 Molecular methods

Detection and quantification of the microbes in rhizospheric soil from the single microbe and the SynCom treatments was performed by real time quantitative PCR (qPCR). Rhizospheric soil was sampled and immediately frozen in liquid nitrogen and stored at -20ºC. DNA extraction from soil was performed using DNeasy PowerSoil Pro Kit (Qiagen, Germany) following the manufacturer's instructions. For standard curves generation, DNA extraction from pure cultures of each microorganism was performed

using a DNA extraction kit (Xtrem Biotech, Spain) following the manufacturer's instructions. DNA concentration was measured with Nanodrop 1000 (Thermo Fisher Scientific, United Statets). qPCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, United States). The following qPCR conditions were used both for standard curves and for soil DNA quantification: initial denaturation at 95ºC for 40 seconds followed by 40 cycles of denaturation at 95ºC for 5 seconds, and annealing and extension at 58ºC for 30 seconds. The strain- or species-specific primers used are shown in **Table 3**. *Bacillus amyloliquefaciens* CECT 8238 strain-specific primers and *P. azotoformans* species-specific primers for qPCR were designed with Primer3 (https://primer3.ut.ee/) and analyzed *in silico* with NetPrimer (https://www.premierbiosoft.com/netprimer/). For *B. amyloliquefaciens,* the pair of primers were designed on the *BAMY6614_00315* gene in *B. amyloliquefaciens* CECT8238 based on the previous study of Magno-Perez-Bryan *et al*. (2015). For *P. azotoformas,* the pair of primers were designed on the RNA polymerase sigma factor *RpoD* gene in *P. azotoformans* LMG21611(gene ID: 57376261). For *R. irregularis* and *T. harzianum* we used species-specific primers available in the literarure (Thonar *et al.*, 2012; Martínez-Medina *et al.*, 2017). For quantification of microbial DNA from soil samples we generated standard curves (**Figure 4**) using dilutions of DNA from each microorganism with known concentration (ng/µl) for the conversion of the qPCR cycle threshold values (Ct) into a ng/µl of microbial DNA.

Table 3. Primers for qPCR for microbial DNA identification in rhizosphere samples.

Microorganism	Gene	Primers (5'- 3')	Reference
Bacillus amyloliquefaciens CECT 8238	*Bamy6614_00315*	ACAAGGGTGGTTTATGGGCT GCTCTCGGCCTGCAGATTAT	This study
Pseudomonas azotoformans	*RpoD*	AAGGACATCAACCGTCGCAT CCGATGTTGCCTTCCTGGAT	This study
Trichoderma harzianum	*Tef-1α*	GGTACTGGTGAGTTCGAGGCTG GGGCTCGATGGAGTCGATAG	(Martínez-Medina et al., 2017)
Rhizophagus irregularis	*nLRS 28S*	TTCGGGTAATCAGCCTTTCG TCAGAGATCAGACAGGTAGCC	(Thonar et al., 2012)

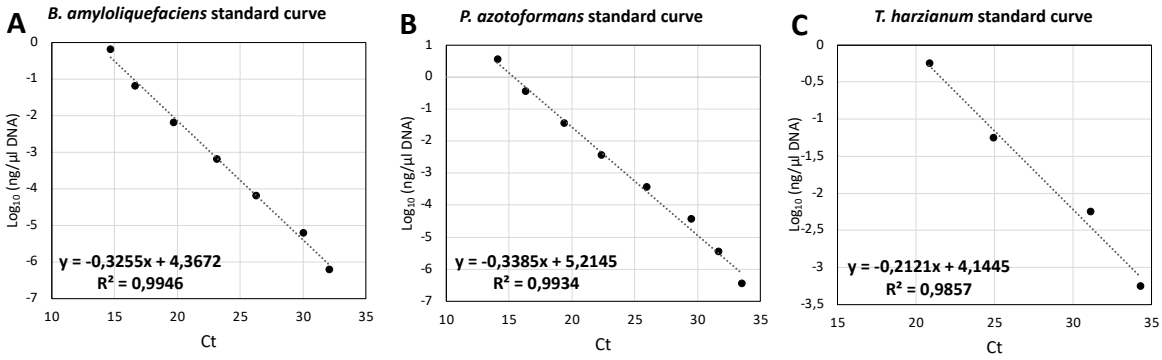


Figure 4. Standard curves generated with known concentrations of genomic DNA from **(A)** Bacillus amyloliquefaciens CECT8238, **(B)** *Pseudomonas azotoformans* and **(C)** *Trichoderma harzianum* T22. Linear regressions for the conversion of the qPCR cycle threshold values into a microbial DNA concentration expressed in $\log_{10}$ (ng/µl). Primers used for qPCR amplification are shown in Table 3.

3.6 Statistical analysis

Data were analysed using R statistical language, version 4.0.5 (R Development Core Team 2021) and figures were produced using the package ggplot2 (Wickham, 2009). The effect of single strains on *B. cinerea* and *F. oxysporum* radial growth and microbial colonisation evaluated by cfu counting after single and combined inoculations was assessed using a general linear model with blocks as an error term and microbial treatments as fixed effect, followed by Tukey HSD post-hoc test. Model validation was performed graphically by inspecting the residuals and fitted values (Zuur & Ieno, 2016) and if linear model assumptions were not met, a Kruskal-Wallis test was used. Microbial DNA quantification by qPCR after single and combined inoculations was assessed using Kruskal-Wallis test with microbial treatments as fixed effect, followed by Dunn's test for multiple comparisons.

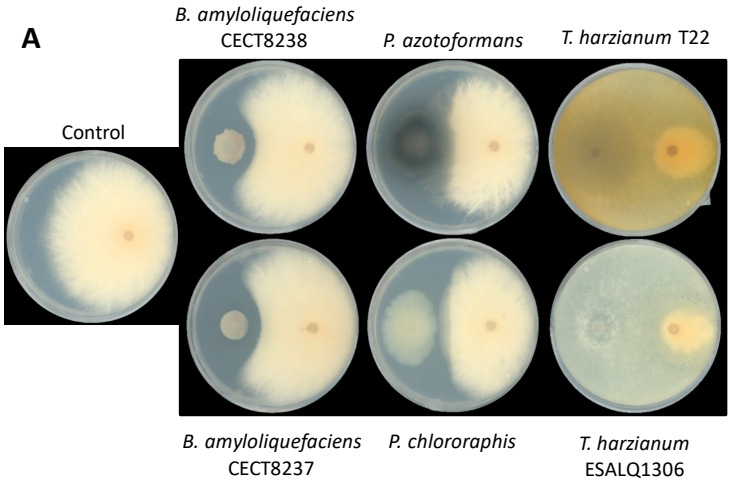
RESULTS

1. The first step: Consortia design

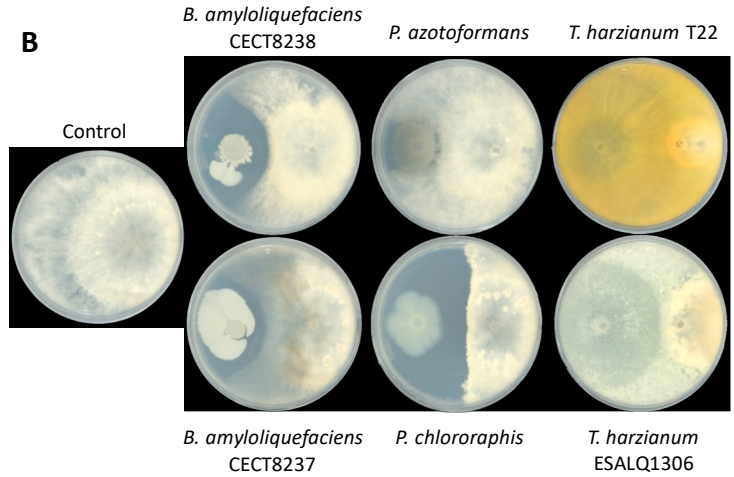
Upon a thorough literature review, we selected bacterial and fungal groups/genera with well documented potential to control plant pathogens, trying to compile diverse mechanisms including antibiosis, competition for iron and other nutrients and colonization sites, mycoparasitism and induction of plant resistance. Strains from the selected groups and available at the Koppert microbial collection were: *Bacillus amyloliquefaciens* strains CECT 8238 and CECT 8237, *Pseudomonas chlororaphis* MA 342, and *Pseudomonas azotoformans* F30A; *Trichoderma harzianum* strains T22 and ESALQ1306, and the AMF *Rhizophagus irregularis* MUCL 57021 (**Table 1**). Two synthetic communities were designed, one combining one strain for each genera (SynCom1A) and another in which all selected microbes were included (SynCom2A).

Exploring *in vitro* antagonistic activity against soil and leaf pathogens

As a first screening to move into the biocontrol potential of the selected individual strains, their antagonistic activity was tested in an *in vitro* dual confrontation assay. All selected BCA strains decreased *F. oxysporum* radial growth compared to the control plates ($p<0.05$; **Figure 5A**). Both *T. harzianum* strains showed the strongest antifungal activity, with about 80% reduction of the pathogen radial growth ($p<0.05$; **Figure 5A**). Similarly, all individual strains reduced *B. cinerea* radial growth compared to the control, and *T. harzianum* T22 was the most effective strain with a 90% reduction of pathogen growth ($p<0.05$; **Figure 5B**).



Beneficial microorganism	*Fusarium oxysporum* radial growth (mm)
Control	46.00 ± 1.04 d
B. amyloliquefaciens CECT8238	26.33 ± 0,44 b
B. amyloliquefaciens CECT8237	26.67 ± 0.33 bc
P. azotoformans	31.83 ± 3.35 c
P. chlororaphis	27.33 ± 0.33 bc
T. harzianum T22	8.17 ± 0.17 a
T. harzianum ESALQ1306	8.33 ± 0.17 a



Beneficial microorganism	*Botrytis cinerea* radial growth (mm)
Control	63.17 ± 0.44 g
B. amyloliquefaciens CECT8238	22.33 ± 0,44 d
B. amyloliquefaciens CECT8237	24.67 ± 0.44 e
P. azotoformans	33.00 ± 0.76 f
P. chlororaphis	15.33 ± 0.33 c
T. harzianum T22	7.33 ± 0.67 a
T. harzianum ESALQ1306	11.5 ± 0,29 b

Figure 5. In vitro confrontation assay of the selected microorganisms against **(A)** the soil pathogenic fungus *Fusarium oxysporum* and **(B)** the leaf pathogenic fungus *Botrytis cinerea*. For all plates, BCA on the left, pathogen on the right. Values are means of radial growth (mm) ± SE. Treatments not sharing a letter in common are significantly different based on general linear model and Tukey HSD test ($p<0.05$, $n=3$).

2. Microbial compatibility

To investigate the compatibility of the microbial components within the consortia we performed an experiment aiming to compare the colonization of each microorganism in the single or SynCom treatments after interacting in the tomato rhizosphere for 15 days. The absence of indigenous species from any of the inoculated genera (*Bacillus*, *Pseudomonas* and *Trichoderma*) in the soil was confirmed in the control treatment plates (**Figure 3**). Each microbial strain (except *R. irregularis*) was initially inoculated at a total concentration of 4 x 10^5 cfu/g of soil for each strain (both in the individual microbial treatments and in the consortia).

2.1 Microbiological and histochemical microbial quantification

First, microbial abundance in rhizospheric soil was determined by classical cfu counting for *Bacillus* spp., *Pseudomonas* spp. and *Trichoderma* spp., or root staining for *R. irregularis*.

Bacillus spp. abundance at the end of the experiment was similar to that initially inoculated both in single strain and SynCom1 treatments (**Figure 6A**). In SynCom2 treatments, were both *Bacillus* strains were co-inoculated, the abundance of *Bacillus* spp. in the soil was even higher, around 6 x 10^5 cfu/g of soil (**Figure 6A**). These results confirm the successful establishment of both *B. amyloliquefaciens* strains both when inoculated individually or in consortia.

In the single strain treatments, *Pseudomonas* spp. abundance increased compared to the initial inoculation (up to 1.5 x 10^6 and 1.2 x 10^6 cfu/g of soil in *P. azotoformans* and *P. chlororaphis*, respectively) (**Figure 6B**), evidencing the good colonisation ability of *Pseudomonas* spp. Remarkably, *Pseudomonas* spp. abundance in soil increased more than four times in SynCom1 (containing *P. azotoformans*) compared to the individual *P. azotoformans* treatment (around 6.5 x 10^6 cfu/g of soil) (**Figure 6B**). Regarding SynCom2 treatments, in the absence of AMF *Pseudomonas* spp. abundance was 2.7 x 10^6 cfu/g of soil, corresponding to the sum of both inoculated *Pseudomonas* species in SynCom2, while in SynCom2+AMF their abundance was more than double (6.9 x 10^6 cfu/g of soil), suggesting a promoting effect of AMF presence in this consortium (**Figure 6B**).

Trichoderma spp. abundance in the individual treatments was 9.3 x 10^5 and 6.3 x 10^5 cfu/g of soil in *T. harzianum* T22 and ESALQ1306 respectively, which in the case of T22 is more than double of the concentration inoculated (**Figure 6C**). Regarding the consortia, *Trichoderma* spp. abundance was similar than in the individual inoculations: 1 x 10^6 cfu/g of soil in SynCom1 (where only T22 was present) and around 1.5 x 10^6 cfu/g of soil, in SynCom2 treatments equivalent to the sum of both *Trichoderma* strains co-inoculated in this consortium (**Figure 6C**). The presence of AMF did not impact *Trichoderma* abundance.

The percentage of root length colonized by *R. irregularis* was 1.2% when applied individually (**Figure 6D**). Root colonization was similar in both consortia treatments, (SynCom1+AMF and SynCom2+AMF) (**Figure 6D**), confirming that mycorrhizal colonization was not significantly affected when inoculated in consortia. The low percentages are common in early very stages of colonization (only 2 weeks upon AMF inoculation).

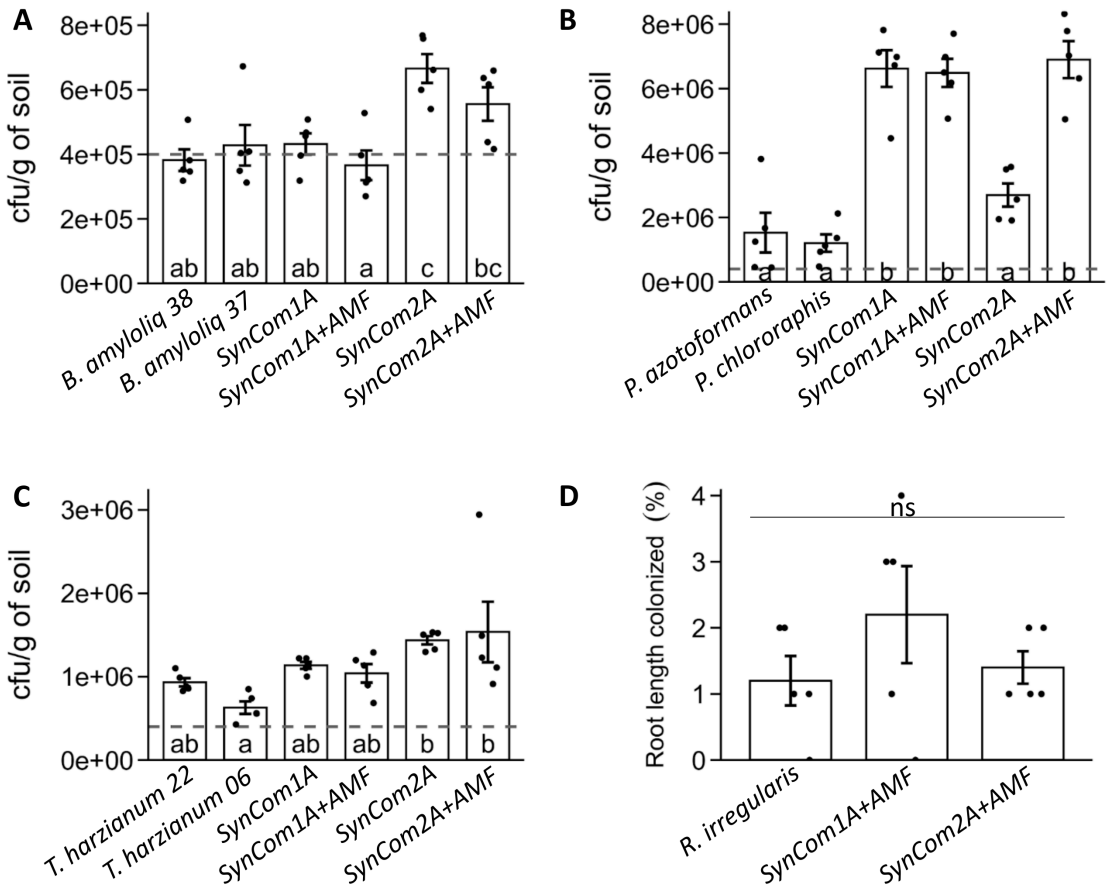


Figure 6. Rhizospheric soil colonization by **(A)** *Bacillus* spp., **(B)** *Pseudomonas* spp., and **(C)** *Trichoderma* spp., expressed as cfu/g of soil, and **(D)** mycorrhizal colonization by *Rhizophagus irregularis* represented as percentage of root length colonized by the fungus. Plants were inoculated at sowing with the individual or consortia treatments (see Table 2) and grown for 15 days. +AMF indicates consortia co-inoculated with 1000 spores/plant of *Rhizophagus irregularis.* Bars represent means ± SE. Dashed lines represent the initial concentration inoculated for each microorganism (4 x 10^5 cfu/g of soil). Treatments not sharing a letter in common are significantly different based on general linear model and Tukey HSD test ($p<0.05$, $n=5$).

To compare the treatments in more advanced stages of the mycorrhizal symbiosis mycorrhizal colonization was quantified in the roots of plants grown with the AMF for 5 weeks. Mycorrhizal colonization reached 40% in the individual treatment, and these levels remained unaltered in both SynCom1 and SynCom2 treatments at any of the tested doses (**Figure 7**).

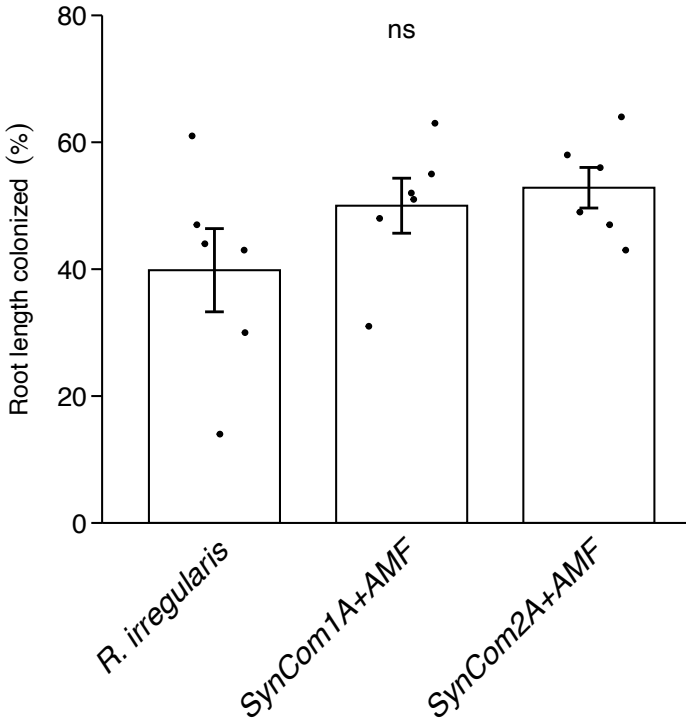


Figure 7. Mycorrhizal colonization in plant roots from the *Botrytis* ISR bioassay represented as percentage of root length colonized by *R. irregularis.* Bars represent means ± SE. Treatments not sharing a letter in common are significantly different based on general linear model and Tukey HSD test ($p<0.05$; n=6).

2.2 Molecular microbial quantification

Further, using specific primers, we quantified microbial abundance in the soil through microbial DNA quantification by qPCR comparing single inoculations with the SynCom1A with or without AMF. None of the microorganisms was detected in the non-inoculated control treatments. Remarkably, DNA quantification confirmed the results previously obtained by cfu counting or root staining (**Figure 8**).

Bacillus amyloliquefaciens CECT 8238 abundance was not significantly affected when inoculated as consortium as compared with its single inoculation (**Figure 8A**). In contrast, *P. azotoformans* DNA concentration in the rhizospheric soil from SynCom1A and SynCom1A+AMF treatments was higher than in the soil from the single inoculated treatment (**Figure 8B**), indicating the better performance of this strain when inoculated as consortium. The abundance of the fungal strains, *T. harzianum* T22 and *R. irregularis* was not significantly affected in the consortium compared with their single inoculations (**Figure 8C and D**).

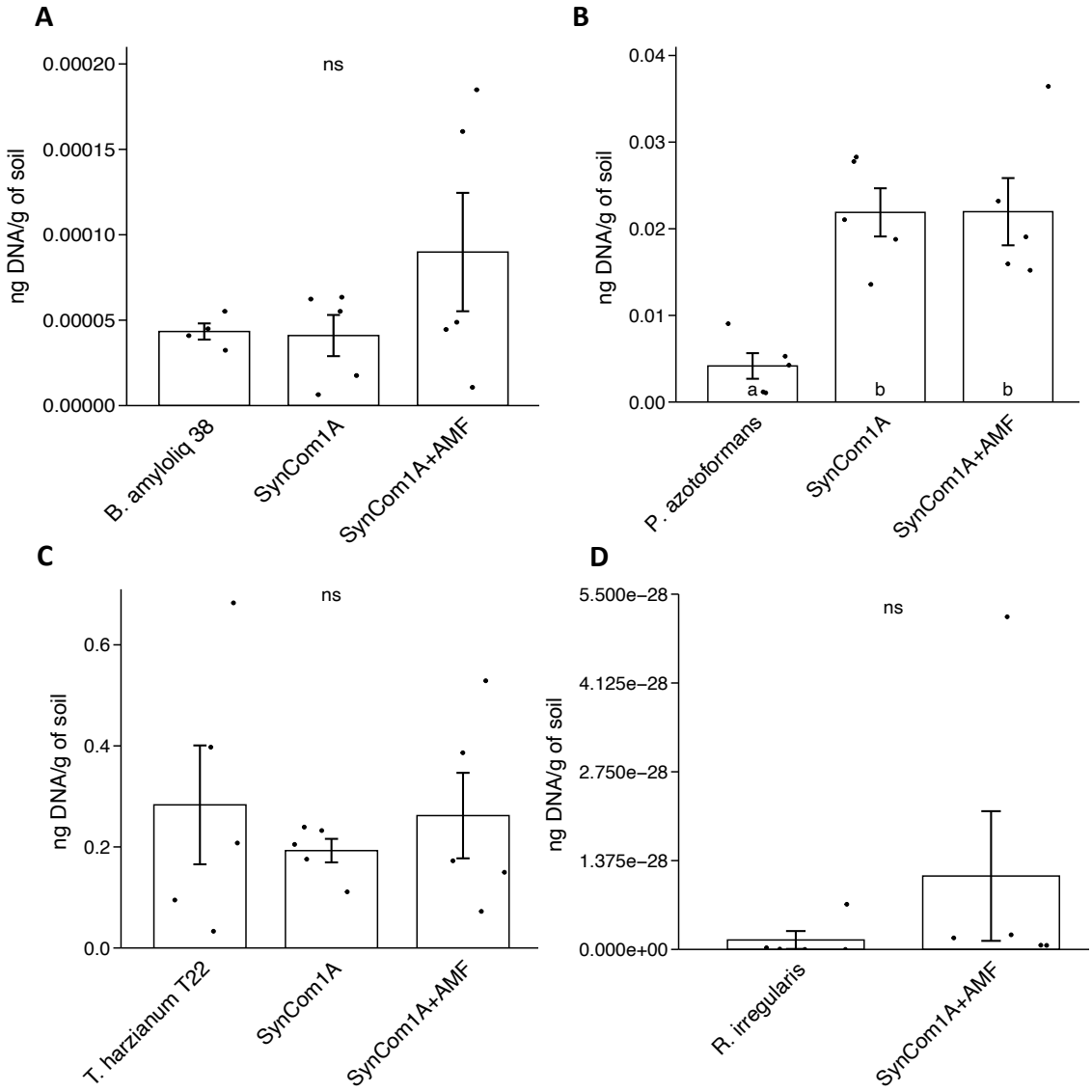


Figure 8. Quantification of DNA from **(A)** *Bacillus* amyloliquefaciens CECT8238, **(B)** *Pseudomonas* azotoformans, and **(C)** *Trichoderma harzianum* T22 and **(D)** *Rhizophagus irregularis*, in rhizospheric soil quantified by qPCR and expressed as ng DNA/g of soil. Plants were inoculated at sowing with the individual or consortia treatments (see Table 2) and grown for 15 days. +AMF indicates consortia co-inoculated with 1000 spores/plant of *Rhizophagus irregularis.* Bars represent means ± SE. Treatments not sharing a letter in common are significantly different based on Kruskal-Wallis test followed by Dunn-test for multiple comparisons ($p<0.05$, $n=5$), and "ns" indicates not significant.

To confirm the suitability of the primers for quantification of the organisms under agronomic conditions, we analyzed the abundance of the different microorganisms in complex biotic environments using natural non-sterile soil. We performed an experiment (Lee, Minchev et al., in review) inoculating tomato plants in a natural soil with the single microbe inoculants *B. amyloliquefaciens* CECT8238, *P. azotoformans*, *T. harzianum* T22 and *R irregularis*, and SynCom1A with and without AMF, and growing them for eight weeks.

The analyses of soil DNA confirmed the detection of the different organisms by the specific primers, and the compatibility of the selected microbes when applied as SynCom. None of them showed a reduction when applied in consortia (**Figure 9**). Remarkably, *B. amyloliquefaciens* CECT8238 DNA and *R. irregularis* DNA were not detected in the non-inoculated control treatment confirming the specificity of the primers used and the absence of these strains in the control treatment rhizosphere (**Figure 9A and D**). In contrast, *P. azotoformans* DNA and *T. harzianum* DNA were detected in the non-inoculated treatment, indicating the presence of these two species in the natural soil used for the experiment (**Figure 9B and C**).

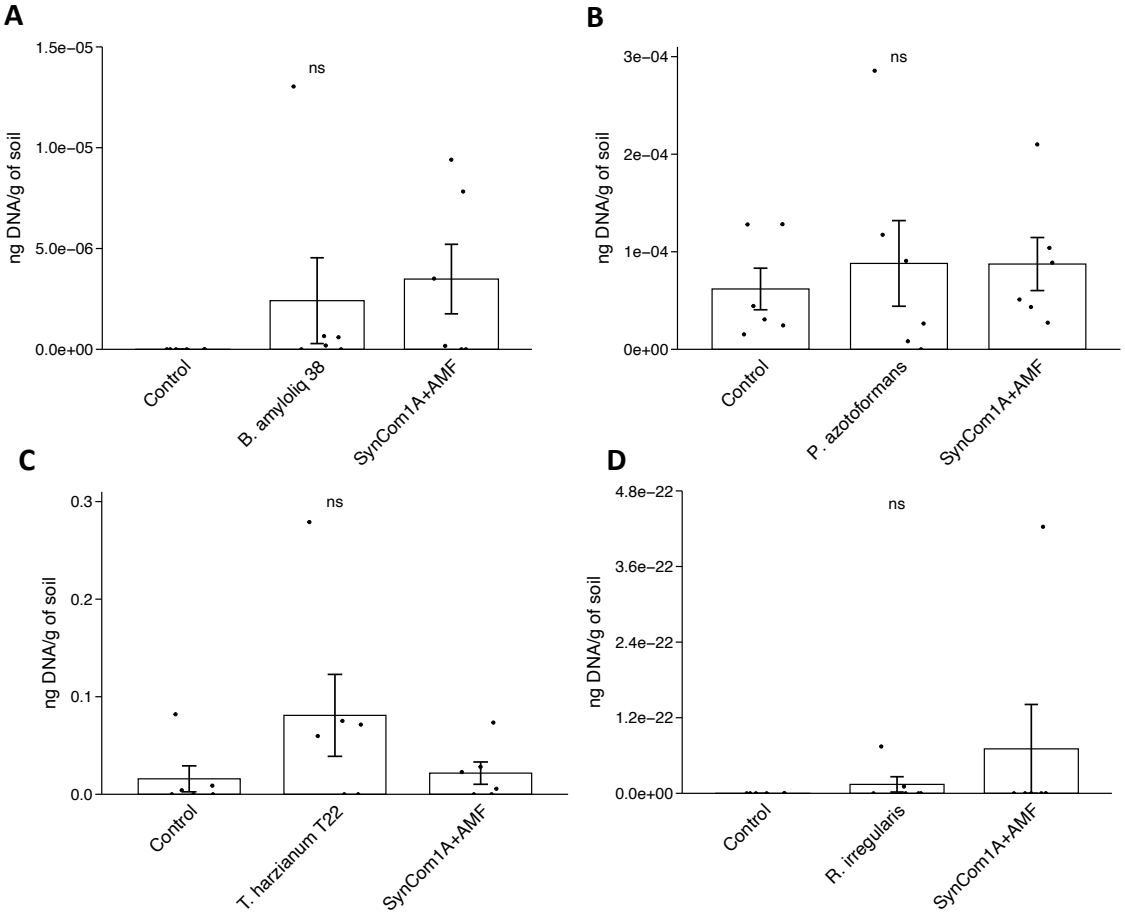


Figure 9. Quantification of DNA from **(A)** *Bacillus* amyloliquefaciens CECT8238, **(B)** *Pseudomonas* azotoformans, and **(C)** *Trichoderma harzianum* T22 and **(D)** *Rhizophagus irregularis*, in rhizospheric soil quantified by qPCR and expressed as ng DNA/g of soil. Plants were inoculated at sowing with the individual or consortia treatments (see Table 2) and grown for 8 weeks days. +AMF indicates consortia co-inoculated with 1000 spores/plant of *Rhizophagus irregularis.* Bars represent means ± SE. Treatments not sharing a letter in common are significantly different based on Kruskal-Wallis test followed by Dunn's test for multiple comparisons ($p<0.05$, $n=5$), and "ns" indicates not significant.

DISCUSSION

Selecting a potentially powerful pool as step one.

For the design of the synthetic microbial consortia we selected different strains aiming to combine different mechanisms for biocontrol, from competition and the production of diverse antimicrobial metabolites, through mycoparasitism to ISR.

Bacillus amyloliquefaciens strains CECT 8238 and CECT 8237 have been shown to promote plant growth and effectively control diverse microbial pathogens through direct antagonism or indirectly through ISR (Romero *et al.*, 2007; García-Gutiérrez *et al.*, 2012; Magno-Perez-Bryan *et al.*, 2015). *Pseudomonas chlororaphis* MA342 has been described to effectively control seed and soil pathogens via direct antagonism (Tombolini *et al.*, 1999; Abuamsha *et al.*, 2011b) and protecting against leaf pathogens through seed priming (Abuamsha *et al.*, 2011a). *Pseudomonas azotoformans* F30A effectively enhance plant emergency and growth (Levenfors *et al.*, 2014), and can also induce ISR to leaf pathogens (Sang *et al.*, 2014; Bouaoud *et al.*, 2018). *Trichoderma harzianum* strain T22 is one of the best characterized and commercialized *Trichoderma* strains. It effectively antagonizes soil pathogens (Wilson *et al.*, 2008; Percival *et al.*, 2011; Roberti *et al.*, 2015; Fatouros *et al.*, 2018), and can trigger ISR against diverse above- and belowground attackers (Tucci *et al.*, 2011; Vitti *et al.*, 2016; Coppola *et al.*, 2017, 2019; Di Lelio *et al.*, 2021; Aprile *et al.*, 2022). Besides promoting plant growth, *T. harzianum* ESALQ1306 has been shown to highly reduce *Sclerotinia sclerotiorum* disease severity through parasitism, and to induce ISR against spider mites (Geraldine *et al.*, 2013; de Oliveira *et al.*, 2018; Barroso *et al.*, 2019; Canassa *et al.*, 2020). In addition, the results from *in vitro* confrontation assay further confirmed the antagonistic potential of these bacterial and fungal strains against two important fungal pathogens such as *B. cinerea* and *F. oxysporum*.

In contrast, *R. irregularis* is not a direct antagonist of plant pathogens but is able to induce ISR against root and foliar pathogens (Pozo *et al.*, 2002; Martínez-Medina *et al.*, 2011; Sanchez-Bel *et al.*, 2016; Campo *et al.*, 2020; Sanmartín *et al.*, 2020; de La Hoz *et al.*, 2021).

All in all, we selected a potentially powerful pool of microbes, already well characterized in multiple aspects. A number of them are either under development into microbial products, or, like *T. harzianum*, already commercialized as BCA by Koppert Biological Systems all over the world from vegetable and ornamental to field and row crops.

Exploring the compatibility of the components of the SynComs.

Microbial compatibility is a key factor when designing a microbial consortium, essential for the successful establishment and functionality of the included microorganisms and the success of SynCom products (Kong *et al.*, 2018; Arif *et al.*, 2020). We tested microbial compatibility in our consortia by assessing the microbe survival in a plant-soil based experiment, and we did not find any negative interaction between them. Instead, while *Bacillus* and *Trichoderma* performed in the consortia as good as when individually inoculated, *Pseudomonas* benefited from the combination with the other organisms, as they performed better in the SynComs than when inoculated alone. It is important to note that *R. irregularis* was not negatively affected in early nor late symbiosis stages by the presence of *Trichoderma* spp., as demonstrated by the similar mycorrhizal colonization in roots and presence of the AMF in soil when inoculated alone or as part of the consortia. This is remarkable, as the compatibility of *Trichoderma* species with mycorrhizal fungi is frequently questioned because of the high mycoparasitic potential of the biocontrol fungi. In fact, *Trichoderma* is able to parasite AMF *in vitro* (Rousseau *et al.*, 1996) but other studies proved their compatibility under more realistic scenarios (i.e. rhizospheric soil) as observed here (Martínez-Medina *et al.*, 2011). Even more, *Trichoderma*-AMF synergistic effects have been reported (Poveda *et al.*, 2019). Although microbe compatibility remains poorly studied, understanding the compatibility between groups or key BCA genera is required for informed decisions in the selection of suitable candidates for SynComs development in biocontrol programs in agriculture.

Yet, quantification of microbial abundance in rhizosphere samples through conventional culture-dependent microbiological methods is time consuming and it is generally useful to track a particular microbe in sterile conditions. The natural microbial populations present in natural or agricultural soils hinder the morphological identification and the quantification of a particular microbe (Romano *et al.*, 2020). Thus, alternative methods are needed for the fast and reliable tracking of particular microbial strains applied as inoculant in natural and agricultural soils. In this regard microbial DNA detection or quantification by qPCR using specific primers is a viable strategy (Romano *et al.*, 2020). Indeed, the molecular quantification results through DNA quantification by qPCR in this Chapter are highly consistent with those obtained by classical microbiology methods by cfu counting. This is remarkable, and confirms microbial DNA quantification by qPCR

as reliable method to track microbial colonization and persistence in rhizosphere and soil. Moving to natural soil, the results confirmed the suitability of the primers to detect and track microbial inoculants in this complex biotic environment. Indeed, the primers used for *B. amyloliquefaciens* CECT8238 and *R. irregularis* quantification showed to be highly specific amplifying DNA only in treatments where the microbe was inoculated alone or in consortium. However, the primers used for *P. azotoformans* and *T. harzianum* detected microbial DNA from these species also in the non-inoculated control, indicating the presence of these microbes in the natural soil used for the experiment in agreement with their reported wide distribution across different soils and ecosystems. The results also point to the need for more specific primer to a strain level when tracking a particular microbe is required, particularly in natural or agricultural soils where other strains from the same species could be already present.

Overall, in this Chapter we designed different SynComs by combining previously well characterized and taxonomically diverse microbes, including bacteria and fungi, with potential for the biocontrol of pests and diseases. Antagonistic activity of the individual strains against foliar and root fungal pathogens was confirmed in an *in vitro* confrontation assay. Finally, the compatibility of the selected strains within the consortia was addressed through microbiological and molecular methods optimized in this study. The results confirm the microbial compatibility within our designed SynComs disregarding any antagonistic interactions between the microbial components within the SynComs.

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CHAPTER 2

CHAPTER 2:

Microbial consortia for effective biocontrol of root and foliar diseases in tomato

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ABSTRACT

Biological control of plant pathogens and pests using microorganisms has emerged as sustainable alternative agrochemicals. However, the variability of the results under field conditions are hampering its wider adoption in agriculture. Currently, the design of synthetic microbial communities (SynComs) to improve the efficacy and consistency of biocontrol practices is receiving increasing attention. Previously we designed potentially multifunctional SynComs by combining compatible and well-characterized microbial biocontrol agents including bacteria and fungi displaying diverse mode of actions. Here we compare their ability to control shoot and root pathogens when applied separately or in combination as SynCom, and across different application strategies for direct microbial antagonism or induction of systemic plant resistance. We hypothesized that consortia will be more versatile than the single strains, displaying an extended functionality, as they will be able to control a wider range of plant diseases through diverse mechanisms and application methods. Our results validated our hypothesis, revealing that while different individual microorganisms were the most effective in controlling the root pathogen *Fusarium oxysporum* or the foliar pathogen *Botrytis cinerea* in tomato, the consortia showed an extended functionality, effectively controlling both pathogens under any of the application schemes, always reaching at least similar protection levels as the best performing single strains. Our findings illustrate the potential of SynComs, composed by carefully selected and compatible beneficial microorganisms including bacteria and fungi, for the development of stable and versatile biological control products for plant protection against a wide range of diseases.

INTRODUCTION

A plethora of soil-borne microorganisms live associated with plant roots, and although some are detrimental, others provide important benefits to the host plant, from improved nutrition through growth and protection against multiple abiotic and biotic stresses (Bakker *et al.*, 2018). In fact, nowadays soil microbes are considered key players in modern crop management programs aiming to increase sustainability in agriculture (Barea, 2015; Trivedi *et al.*, 2017; Compant *et al.*, 2019). The use of plant beneficial microorganisms as biological control agents (BCAs) of pests and diseases emerges as a viable alternative to the abusive use of agrochemicals (Ab Rahman *et al.*, 2018; Rändler-Kleine et al., 2020). A strong increase in registered microbial biocontrol agents worldwide in recent years serves as good evidence (van Lenteren *et al.*, 2017). Yet, while the use of insects and mites to control pests is well established and used in practice for decades, microbes to control pests and diseases are in an earlier developmental phase (Mitter *et al.*, 2019).

The ability of microorganisms to control pests and diseases has been well documented, but the variability of results often recorded under field conditions is one of the major challenges for a wider adoption in agriculture (Trivedi *et al.*, 2017; Mitter *et al.*, 2019). Originally, biocontrol research focused on the application of single microorganisms (Sarma *et al.*, 2015; Trivedi *et al.*, 2020). The inoculant´s functionality and persistence is strongly influenced by their complex interactions within the soil microbiota and the environment (Barea *et al.*, 2005; Trivedi *et al.*, 2020; Pozo *et al.*, 2021). In fact, inconsistent or ineffective performance of single strain inoculants can be related to limited competitiveness against indigenous microbes and the varying environmental conditions (Trivedi *et al.*, 2020). It has been proposed that a way to overcome these issues is by combining different strains to cover a wider range of target organisms and conditions (Faust, 2019; Mitter *et al.*, 2019). Yet, successful examples of better performance for microbial consortia are comparatively limited and usually relates to growth or yield promotion (Bradáčová *et al.*, 2019).

Diving deeper mechanistically, two main groups of biocontrol mechanisms are described: i) those with direct effects on the attacker and ii) those with indirect -usually plant mediated- effects. Direct effects are mostly based on microbial antagonism through antibiosis, competition for nutrients or colonization niches, and /or parasitism (Whipps, 2001). Indirect mechanisms reducing pathogen proliferation, aggressiveness or damage

commonly involve plant mediated effects. Beneficial microorganisms can improve the plant nutritional status leading to damage compensation and tolerance, as well as stimulate the plant immune system, priming plant defenses and leading to induced systemic resistance (ISR) to diverse aggressors (Pieterse *et al.*, 2014; Barea, 2015; Pineda *et al.*, 2015; Gruden *et al.*, 2020; De Kesel *et al.*, 2021).

Microbiome engineering and the design of synthetic microbial communities (SynComs) for the improvement of biocontrol practices is currently a major research topic. SynComs are expected to outperform single strain inoculants as they would adapt better to the variable environmental conditions occurring in agroecosystems, thus, are likely to be more resilient. In addition, SynComs including bacteria and fungi with diverse biocontrol mode of actions could be more versatile, potentially controlling a wider range of plant aggressors.

In this Chapter we test the hypothesis that microbial consortia are more versatile than individual microbial inoculants, displaying an extended functionality in the biocontrol of a wider range of plant diseases and application methods. To test this hypothesis, we compared the ability to control root and shoot pathogens when applied individually or in combinations as SynComs. Using different inoculation methods and two agronomically relevant pathosystems (tomato plants challenged with *Fusarium oxysporum* or *Botrytis cinerea* as root and shoot pathogens, respectively), we demonstrate the advantages of targeting microbial consortia as versatile products for efficient biocontrol of diverse plant diseases.

MATERIAL AND METHODS

1. Microbe growing conditions and inoculum preparation

B. amyloliquefaciens strains were grown on tryptone soya agar (TSA, Oxoid) for 24h at 28°C. After that, a single colony from TSA culture was inoculated in 25ml of DSM (Difco sporulation medium) (Nicholson & Setlow, 1990) and incubated for 48h at 28°C in a rotatory shaker (200rpm). Spores were quantified using a Bürker-Türk counting chamber, then centrifuged at 5000 rpm for 15min and after discarding the supernatant, the pellet containing the spores was re-suspended in sterile tap water to a final concentration of 1 x 10^7 spores/ml.

P. azotoformans and *P. chlororaphis* were grown on TSA for 24h at 28°C. Liquid pre-culture was prepared using tryptone soya broth (TSB, Oxoid) inoculated with a single bacterial colony from TSA culture and incubated overnight at 28°C with rotary shaking at 200rpm. After that, 1ml of pre-culture was inoculated in 25ml of TSB media and placed in a rotatory shaker (200rpm) at 28°C. After 150mins of incubation, with bacterial growth in exponential phase, the cell concentration was calculated measuring the O.D. (620nm) of the bacterial culture on Shimadzu UVmini-1240 Spectrophotometer. The bacterial culture was centrifuged at 5000rpm for 15min, and after discarding the supernatant, the pellet containing the bacterial cells was re-suspended in sterile tap water to a final concentration of 1 x 10^7 cfu/ml.

T. harzianum strains were cultured on potato dextrose agar (PDA, Difco) for 7 days at room temperature. Spores were collected from sporulating plates in sterile tap water, the concentration of the spore suspension was quantified using a Bürker-Türk counting chamber and adjusted to 1 x 10^7 spores/ml.

R. irregularis was grown in a monoxenic culture on minimal (M) medium and using *Agrobacterium rhizogenes* - transformed carrot (*Daucus carota*) roots as a host root (St-Arnaud *et al.*, 1996). To extract the AMF spores, citrate buffer 0.01M (pH=6) was added to a sporulating AMF culture in a proportion 3:1 (v/v) and placed in a rotary shaker for one hour to dissolve the agar. AMF spores were recovered from the solution using sieves with different sizes (250 and 53 µm) and re-suspended in sterile tap water at final concentrations 1000 spores/ml.

2. Pathogenic fungi, growing conditions and inoculum preparation

Two major fungal pathogens causing important crop losses worldwide were tested: *F. oxysporum* f.sp. radicis-lycopersici as soil pathogen, and the necrotrophic shoot pathogen *B. cinerea* strain B05.10.

F. oxysporum was grown on PDA at 25°C for 4 days. For spore production, 25 plugs of 4mm diameter with new growing mycelia were removed from the PDA plates and transferred to 500ml Erlenmeyer containing 200ml of czapek dox broth (OXOID) and placed in a rotary shaker (110rpm) at room temperature. After 4 days of incubation the liquid culture was filtered using a sterile miracloth filter and the spore concentration was quantified using a Bürker-Türk counting chamber. The resulting spore suspension was centrifuged at 9500rpm for 15 min and after discarding the supernatant, the pellet containing the spores was re-suspended in sterile tap water to a final concentration of 1 x 10^8 spores/ml.

B. cinerea was cultured on PDA at 20°C. Spores were collected from sporulating 14 days old plates in potato dextrose broth (PDB, Difco), the concentration of the spore suspension was quantified using a Bürker-Türk counting chamber and adjusted to 1 x 10^6 spores/ml.

3. *In planta* bioassays

Biocontrol potential was tested in planta through several bioassays including diverse inoculation methods and targeting different pathogens. This strategy allows testing *in vivo* different modes of action ranging from direct antagonism to indirect -plant mediated- effects. Thus, we tested through seed inoculation suppression of the root pathogen *F. oxysporum* and ISR against the foliar pathogen *B. cinerea*, and suppression of *B. cinerea* by foliar spray application.

3.1 Microbial treatments

In all bioassays individual microorganisms and different synthetic consortia were tested (**Table 1**). All microorganisms tested individually were applied at 1 x 10^7 cfu or spores/plant in the seed application, and at 1 x 10^7 cfu or spores/ml in the foliar application. For the AMF treatments, a suspension of 1000 spores of *R. irregularis* were applied per plant. Regarding the consortia, the first microbial consortium, SynCom1, was composed of one strain from each genus (*B. amyloliquefaciens* CECT8238, *P*

azotoformans F30A *and T. harzianum* T22). The second one, SynCom2, was composed by all selected microorganisms (*B. amyloliquefaciens* CECT8238 and CECT8237, *P. azotoformans* F30A, *P. chlororaphis* MA 342, and *T. harzianum* T22 and ESALQ1306). Both consortia were tested at two doses**:** A: same amount of each microorganism in both consortia (1 x 10^7 cfu each, that is a total of 3x 10^7 cfu per seed or ml for SynCom1, 6 x 10^7 cfu per seed or ml for SynCom2.) or B: same total cfu per consortia: (3.33 x 10^6 cfu per microorganism in SynCom1 or 1.67 x 10^6 cfu in SynCom2, for a total of 1 x 10^7 cfu per seed or ml in both.

Table1. Composition of synthetic microbial consortia and concentration of beneficial microorganisms used in *in planta* bioassays.

<table>
<tr><th></th><th colspan="5">Suppression F. oxysporum</th><th colspan="5">Suppresion B. cinerea</th><th colspan="5">ISR against B. cinerea</th></tr>
<tr><th></th><th>Single</th><th>SynCom1 A</th><th>SynCom 1B</th><th>SynCom2 A</th><th>SynCom2 B</th><th>Single</th><th>SynCom1 A</th><th>SynCom 1B</th><th>SynCom2 A</th><th>SynCom2 B</th><th>Single</th><th>SynCom1 A +AMF</th><th>SynCom1 B +AMF</th><th>SynCom2 A+AMF</th><th>SynCom 2B +AMF</th></tr>
<tr><td>B. amyloliquefaciens CECT 8238</td><td rowspan="6">1 x 10^7 cfu/ plant</td><td rowspan="3">1 x 10^7 cfu/ plant</td><td rowspan="3">3.3 x 10^6 cfu/ plant</td><td rowspan="6">1 x 10^7 cfu/ plant</td><td rowspan="6">1.7 x 10^6 cfu/ plant</td><td rowspan="6">1 x 10^7 cfu/ ml</td><td rowspan="3">1 x 10^7 cfu/ ml</td><td rowspan="3">3.3 x 10^6 cfu/ ml</td><td rowspan="6">1 x 10^7 cfu/ ml</td><td rowspan="6">1.7 x 10^6 cfu/ ml</td><td rowspan="6">1 x 10^7 cfu/ plant</td><td rowspan="3">1 x 10^7 cfu/ plant</td><td rowspan="3">3.3 x 10^6 cfu/ plant</td><td rowspan="6">1 x 10^7 cfu/ plant</td><td rowspan="6">1.7 x 10^6 cfu/ plant</td></tr>
<tr><td>P. azotoformans F30A</td></tr>
<tr><td>T. harzianum T22</td></tr>
<tr><td>B.amyloliquefaciens CECT 8237</td><td rowspan="3">NA</td><td rowspan="3">NA</td><td rowspan="3">NA</td><td rowspan="3">NA</td><td rowspan="3">NA</td></tr>
<tr><td>P. chlororaphis MA 342</td></tr>
<tr><td>T. harzianum ESALQ1306</td></tr>
<tr><td>R. irregularis MUCL 57021</td><td>NA</td><td colspan="2">NA</td><td>NA</td><td colspan="2">NA</td><td colspan="2">NA</td><td colspan="4">1000 spores/plant</td></tr>
</table>

3.2 Substrate, seed surface sterilization and plant growing conditions

Solanum lycopersicum cv Money maker seeds (Vreeken's Zaden, The Netherlands) were surface sterilized by immersion in 5% Sodium hypochlorite solution for 10 min followed by at least 3 washing steps in sterile water for 10 min each. The surface sterilized seeds were dried in a laminar flow cabinet and used for the experiments. The growing substrate was gamma irradiated nutrient poor peat soil (BVB, The Netherlands). All experiments were performed in a growing chamber at Koppert B.V. (Berkel en Rodenrijs, The Netherlands) under controlled conditions (25°C : 23°C day : night with photoperiod 16h:8h light:dark and 60% of relative humidity).

3.3 Bioassay: Suppression of *Fusarium oxysporum in planta*

Rectangular plastic containers of 18cm x 13cm x 6cm (length x width x height) were filled with 300 g of soil previously moistened with tap water (300ml/1000g of soil) and infected with 1 x 10^6 conidia/g of soil *F. oxysporum* f.sp. radicis-lycopersici conidia. The *F. oxysporum* conidia were carefully mixed through the soil by hand. Then, 12 seeds were

sown in each container in a regular grid and inoculated with the microbial treatments (**Table 1**) by pipetting the microbial suspension to each seed. Finally, the seeds were covered with sterile vermiculite to avoid desiccation and undesired contaminations. We included two control treatments: a "non-diseased control" using the same soil and conditions but without the addition of *F. oxysporum* and microbial treatments, and a "disease control" using the same pathogen-infected soil but without beneficial microbes. Each treatment was replicated five times. We used randomized complete block design. Each treatment was randomly assigned to each block. Plant survival was evaluated 15 days after sowing by counting the number of healthy tomato plantlets in each container (**Figure 1**).

Figure 1. Experimental set up of the *Fusarium oxysporum* supression bioassay.

3.4 Bioassay: Suppression of *Botrytis cinerea in planta*

Tomato seeds were sown in pots filled with 250ml of soil (one seed per pot). Plants were grown for 7 weeks, watered twice per week with water and once per week with Long Ashton nutrient solution (Hewitt, 1966). The individual and the consortia treatments described above (**Table 1**) were applied to one fully developed leaf by spraying their surface until runoff. The disease control treatment was treated similarly, applying the same amount of sterile water but lacking any BCA microbial propagules. Each treatment was replicated six times. Treated leaves were detached after the application, using a scalpel and used for the bioassay. Each leaflet of the detached leaves was inoculated with one 4µl drop of *B. cinerea* conidia suspension (1 x 10^6 conidia/ml). The leaves were placed in six sealed boxes with high humidity at 20°C, locating one replicate from each treatment in each box. 60h after infection the diameter of the resulting necrotic lesions was measured using a digital caliper.

3.5 Bioassay: Induced Systemic Resistance against *Botrytis cinerea*

Tomato seeds were sown in pots containing 250 ml of soil (one seed per pot) and the microbial treatments (**Table1**) applied by pipetting the microbial suspension to the seeds. In this experiment, the AMF *Rhizophagus irregularis* was also included, both, individually and in the consortia. A disease control treatment was included where the seeds only received water without any BCA microbial addition. Each treatment was replicated 12 times. We used a randomized complete block design. Plants were watered twice per week with water and once per week with Long Ashton nutrient solution (Hewitt, 1966) but with reduced phosphorous concentration (50% of the standard concentration) to ensure mycorrhizal establishment. After five weeks, one fully developed leaf from each plant was detached using a scalpel, and each leaflet was inoculated with one 4µl drop of *B. cinerea* conidia suspension (1 x 10^6 conidia/ml). The leaves were placed in twelve sealed boxes with high humidity at 20°C and locating one replicate from each treatment in each box. 48h after infection the diameter of the necrotic lesions were measured using a digital caliper.

4. Statistical analysis

Data were analysed using R statistical language, version 4.0.5 (R Development Core Team 2021) and figures were produced using the package ggplot2 (Wickham, 2009). The effect of microbial treatments (single strains and synthetic communities) on the necrotic lesions caused by *B. cinerea* was assessed using a general linear model with blocks as an error term and microbial treatments as fixed effect. To examine whether microbial treatments influenced the probability of the tomato seedlings to survive to the soil pathogenic fungus *F. oxysporum,* a generalized linear model with binomial distribution and logit link function and blocks as an error term was performed. Post-hoc comparisons among microbial treatments were based on a Tukey HSD. Model validation was performed graphically by inspecting the residuals and fitted values (Zuur & Ieno, 2016).

RESULTS

1. Assessing the potential to directly suppress soil diseases *in planta*

The research was scaled up using a tomato-*Fusarium*-soil system, comparing the biocontrol activity of the individual microbial strains and the different designed consortia

(SynCom1, SynCom2). The pathogen fully compromised plant survival, as no plants survived in the disease control, while almost 100% survival was found in the absence of the pathogen (non-diseased control) (**Figure 2**). None of the individual bacterial strains significantly increased plant survival compared to the disease control. In contrast, both *T. harzianum* strains, as well as all of the SynComs were able to efficiently suppress *F. oxysporum*, increasing plant survival above 80% ($p<0.05$, **Figure 2**). In fact, plant survival in the *T. harzianum* and consortia treatments reached the levels of the non-diseased control ($p<0.05$, **Figure 2**). These results not only show the potential of *T. harzianum* but also indirectly the compatibility/tolerance of the other isolates as this high protection level was maintained in the consortia treatments (**Figure 2, Figure 3**).

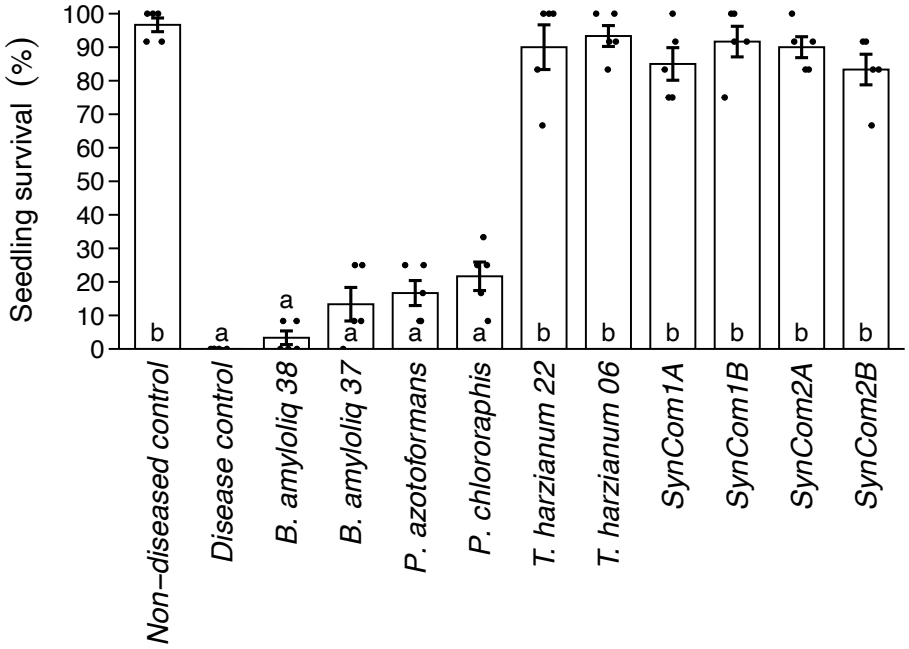

Figure 2. Effect of microbial inoculation on disease caused by the soil borne pathogen *Fusarium oxysporum.* Survival of tomato plants after 15 days of growth in *F. oxysporum* infected soil. Seeds were either water-inoculated ("disease control") or inoculated with the individual or consortia treatments (see Table 1). A "non-diseased control" was also included, where water-inoculated seeds were sown in soil without *F. oxysporum*. Single strains were inoculated at $1x10^7$ cfu/plant, and the consortia were inoculated at the same concentration for each microorganism (SynCom1A, SynCom2A) or at $1x10^7$ cfu/plant total microbial concentration (SynCom1B, SynCom2B). Bars represent predicted means ± SE of probability of seedling survival based on generalized linear model with binomial distribution and logit link function. Black dots represent raw data points. Treatments not sharing a letter in common are significantly different based on Tukey HSD test ($p<0.05$, $n=5$).

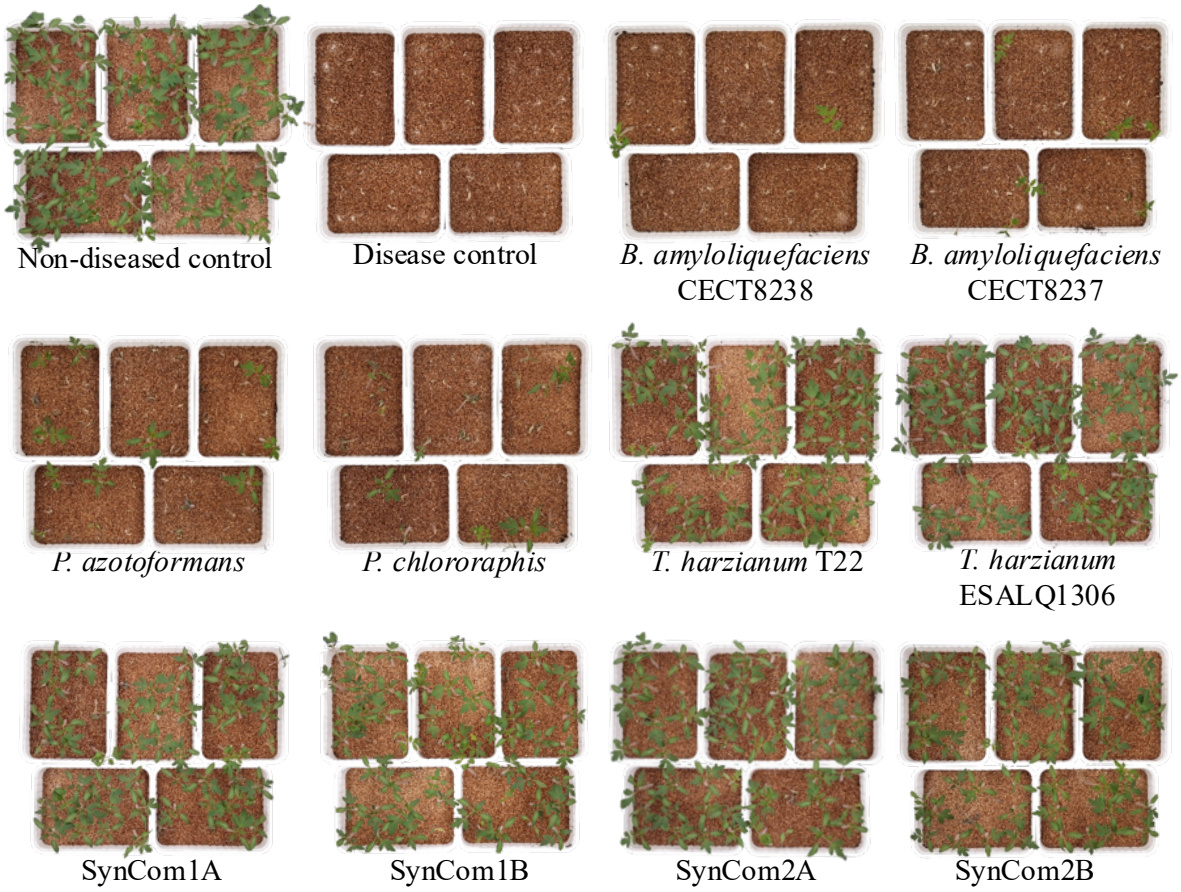

Figure 3. Survival of plant seedlings in *F. oxysporum* infected soil. Pictures illustrate plant survival in non-diseased and disease control, and BCA single strain and SynCom treatments.

2. Assessing the potential to directly suppress foliar diseases *in planta*

The antagonistic potential of single strains and consortia against the foliar pathogen *B. cinerea* was also tested *in planta*, applying the BCA treatments by spraying the leaves before *B. cinerea* infection. Among single microbial treatments, *P. chlororaphis*, *P. azotoformans* and *T. harzianum* T22 were able to reduce the area of the necrotic lesion caused by *B. cinerea* by 56%, 45% and 38% respectively compared to the control treatment ($p<0.05$, **Figure 4**). Remarkably, all the microbial consortia treatments reduced *B. cinerea* lesion area in about 50% as compared to the disease control, reaching up to a 70% reduction in SynCom2B ($p<0.05$, **Figure 4**). The higher antagonistic effect against *B. cinerea* was therefore achieved by *P. chlororaphis* (56%) and the SynCom2B (**Figure 5**).

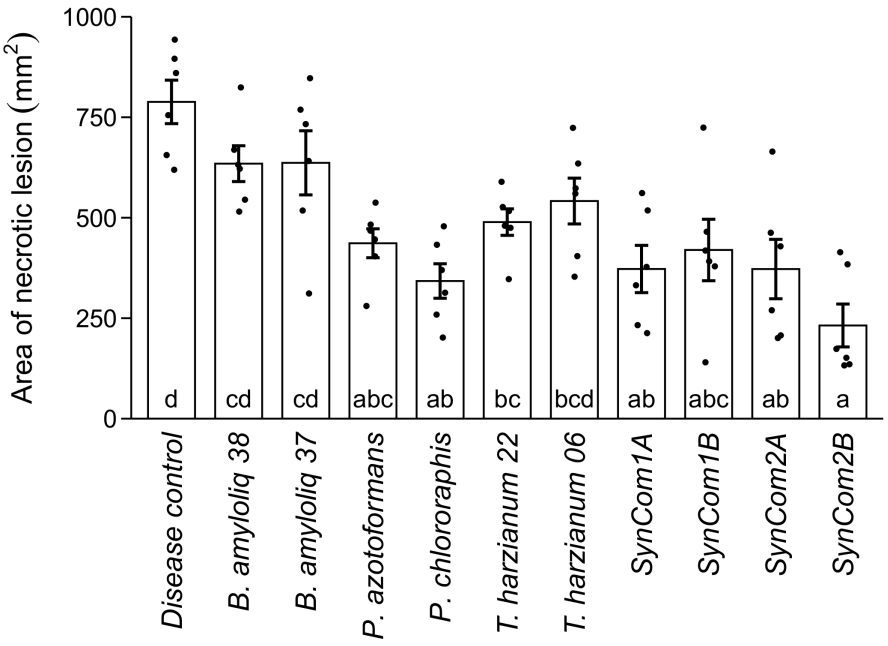

Figure 4. Area of necrotic lesions caused by *Botrytis cinerea* in plants pre-treated by foliar spray with single strains or consortia treatments (see Table 1). Water-treated plants (no BCA treatment) were included as disease control. Single strains were applied at $1x10^7$ cfu/ml, and the consortia were applied at the same concentration for each microorganism (SynCom1A, SynCom2A), or at $1x10^7$ cfu/ml total microbial concentration (SynCom1B, SynCom2B). Bars represent means ± SE and black dots represent raw data. Treatments not sharing a letter in common are significantly different based on general linear model and Tukey HSD test ($p<0.05$, n=6).

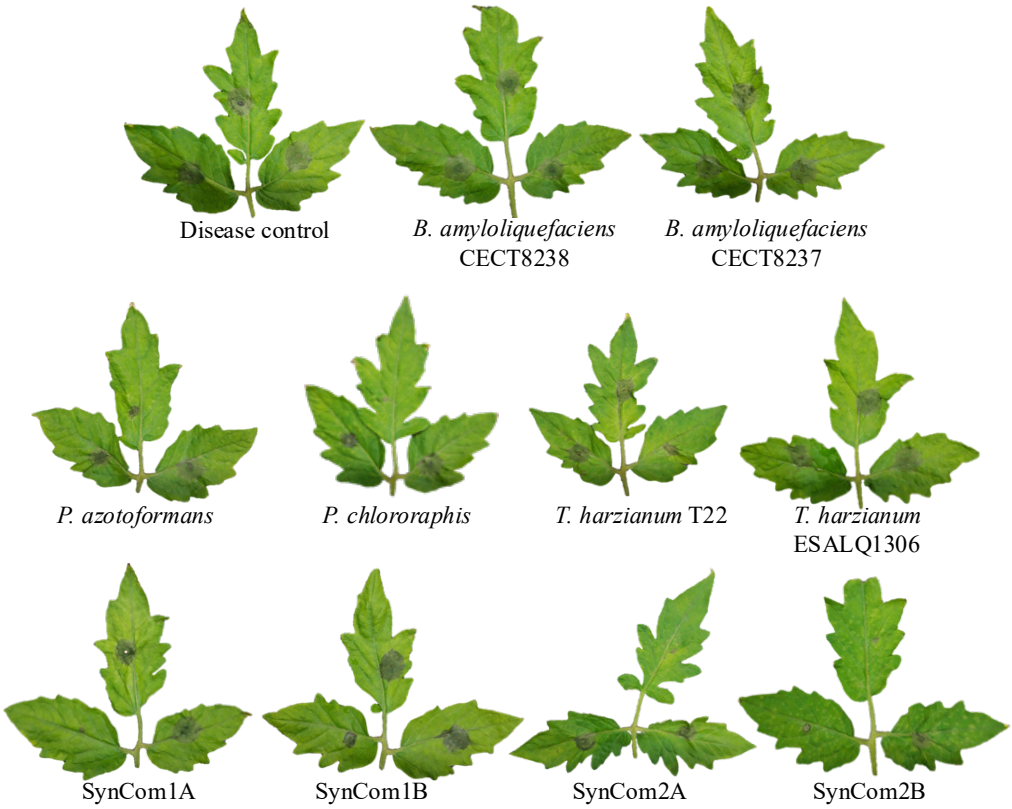

Figure 5. Representative pictures of *B. cinerea* lesions in disease control and BCA single strain and SynCom treatments, after foliar application.

3. Moving into plant mediated control: Inducing Systemic Resistance

In addition to the direct antagonistic effect of the foliar application against *B. cinerea*, we evaluated the capacity of the microbial treatments to activate plant systemic resistance. We tested the potential plant mediated effects by avoiding direct contact between the BCAs and the pathogen. In this experiment the AMF *R. irregularis* was included both, individually and in the consortia due to the reported capacity of AMF to induce ISR and their current interest as inoculants in agriculture. Among the individual treatments only *B. amyloliquefaciens* CECT8238 and *R. irregularis* were able to induce ISR against *B. cinerea*, reducing the area of the necrotic lesions by 38% and 44%, respectively, as compared to the control treatment ($p<0.05$, **Figure 6**). The consortia also achieved a significant plant mediated protection against *B. cinerea,* with SynCom1A, SynCom1B and SynCom2A reducing lesions by 33-37 % as compared to the control ($p<0.05$, **Figure 6**). Again, a similar reduction in disease symptoms was achieved by the consortia and the best performing individual treatments in this pathosystem.

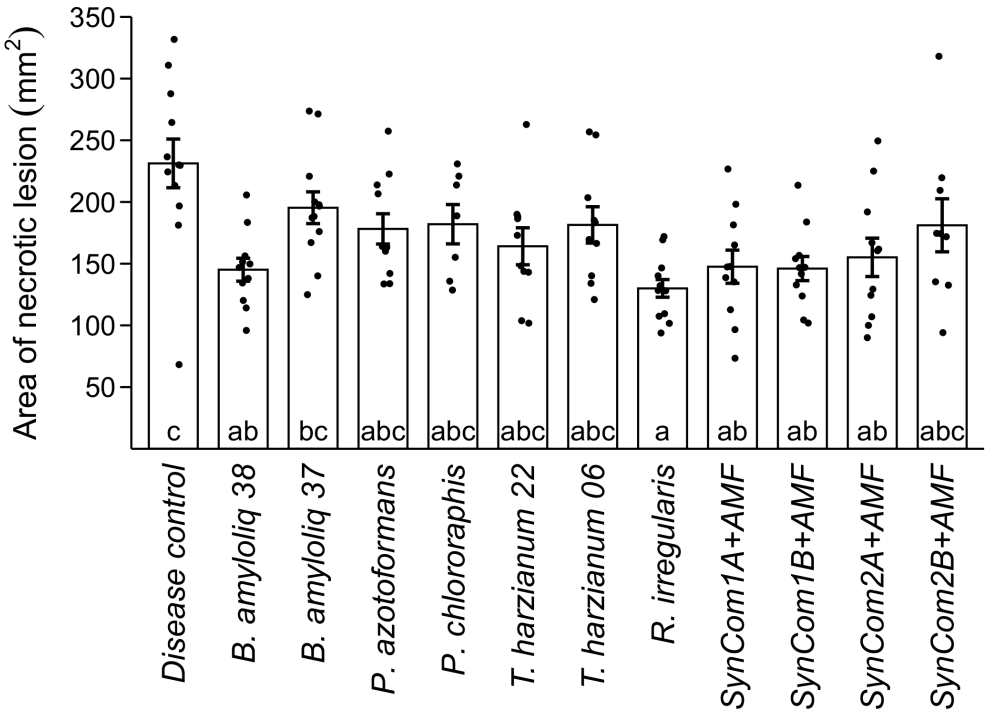

Figure 6. Area of necrotic lesions caused by *Botrytis cinerea* to determine ISR in plants inoculated at sowing either with water (disease control) or with the different microbial treatments (see Table 1). Single strains were inoculated at $1x10^7$ cfu/plant, and the consortia were inoculated at the same concentration for each microorganism (SynCom1A, SynCom2A) or at $1x10^7$ cfu/plant total microbial concentration (SynCom1B, SynCom2B). +AMF indicates consortia co-inoculated with 1000 spores/plant of *Rhizophagus irregularis.* Bars represent means ± SE and black dots represent raw data. Treatments not sharing a letter in common are significantly different based on general linear model and Tukey HSD test ($p<0.5$; $n=12$).

Taking into account all the bioassays performed, SynComs were more versatile than the individual strains, showing effective biocontrol across the different pathosystems and inoculation methods, as summarized in **Table 2**.

Microbial treatment	Suppression *F. oxysporum*	Suppression *B. cinerea*	ISR against *B. cinerea*
B.amyloliquefaciens CECT8238	**o**	**o**	**+**
B.amyloliquefaciens CECT8237	**o**	**o**	**o**
P.azotoformans	**o**	**+**	**o**
P.chlororaphis	**o**	**+**	**o**
T.harzianum T22	**+**	**+**	**o**
T.harzianum ESALQ1306	**+**	**o**	**o**
R.irregularis	nt	nt	**+**
SynCom1A	**+**	**+**	**+**
SynCom1B	**+**	**+**	**+**
SynCom2A	**+**	**+**	**+**
SynCom2B	**+**	**+**	**o**

Table 2. Effects of the microbial treatments tested in the different *in planta* bioassays. "+" and "**o**" indicates statistically different effect from the control treatment and no effect, respectively, based on Tukey HSD. "**nt**" indicates that the microbial treatment was not tested.

DISCUSSION

In the present study, by combining well characterized and compatible microorganisms, including bacteria and fungi, we demonstrated the potential of SynComs to effectively control fungal pathogens with different lifestyles through direct and plant mediated disease suppression and using different application methods. Our findings pinpoint the design of synthetic microbial consortia for biocontrol of plant pathogens as a potential strategy to extend the functionality and versatility of microbial biological control.

A dilemma to face. Across the different experiments, different individual microorganisms were the most effective in the different scenarios, depending on the type of pathogen or the strategy used for its control. Remarkably, the consortia effectively controlled all pathogens in all different bioassays, both through direct antagonism by seed or foliar

application, or inducing plant systemic resistance against foliar pathogens by seed inoculation (results summarized in **Figure 7**). The bioprotection achieved by the consortia was always similar to that of the best performing single strains. Although no significant synergism was detected, no negative interactions were observed, in contrast to some studies reporting positive and negative effects by the combination of BCAs (Freeman *et al.*, 2004; Abo-Elyousr *et al.*, 2009; Elliott *et al.*, 2009; Ruano-Rosa *et al.*, 2014). Our results illustrate the topical dilemma of selecting single beneficial microbes versus SynComs for biological control. Strictly from the potential efficacy point of view, SynComs offered the widest protection after comparing the single components and a number of consortia across soil and foliar threats and through direct and indirect actions. Yet, the efficacy was not higher than that of the best performing single strain and, in most cases, more than one individual microbe provided an effective control. Considering the current high costs and outstanding long process for registering microbial products, targeting single strain or SynCom products is a tough dilemma to face from the commercial point of view. Nevertheless, the advantage of SynComs as a more versatile tool may become more apparent under field conditions, considering the variability of growing conditions and the uncertainty of the potential challenges to be faced -what pathogens or pests would be threatening the crop. We postulate that in the field, under commercial conditions, the benefits for the SynComs would further differentiate to the individual components. Thorough validation of results in field conditions will give the answer.

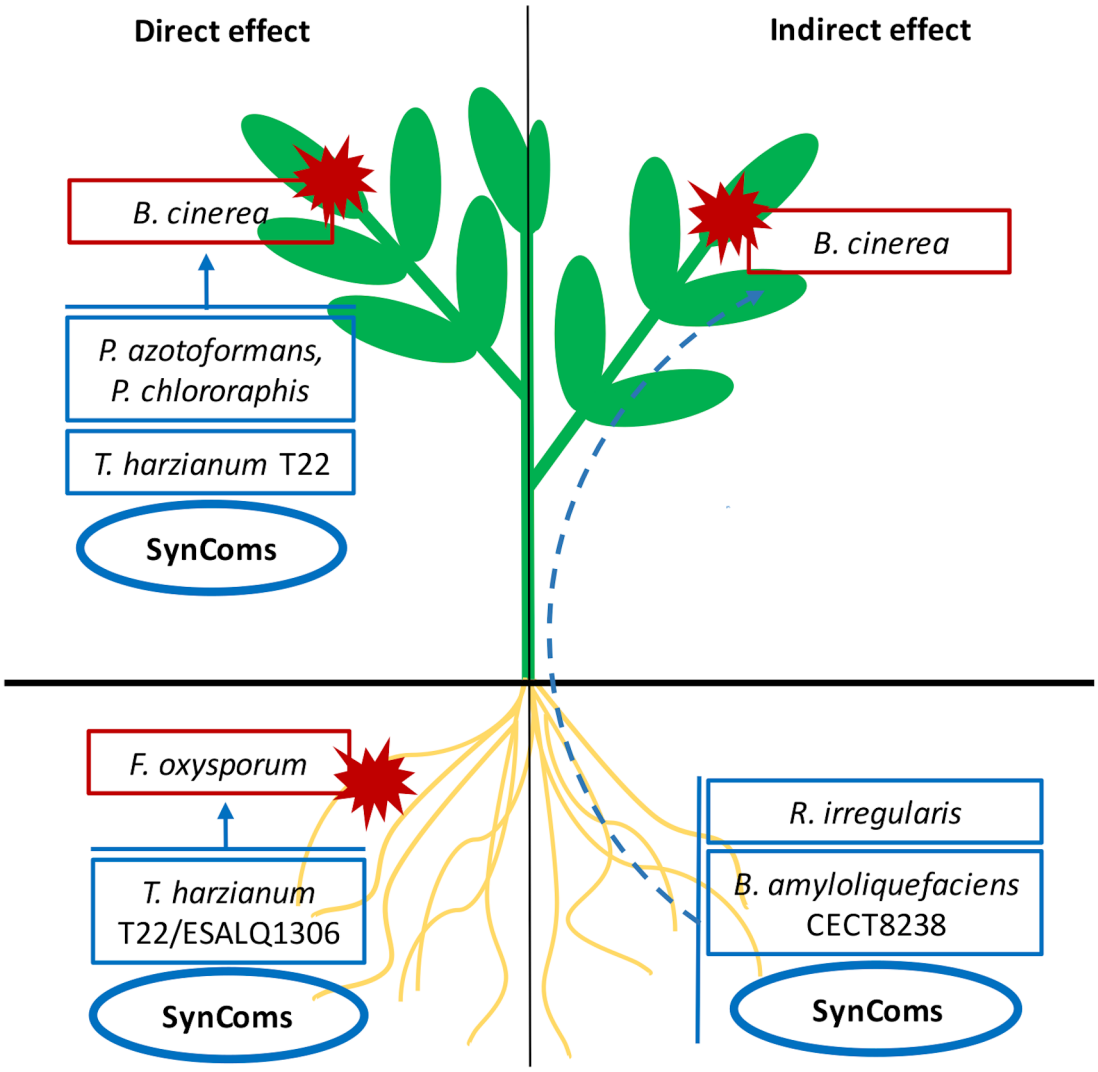

Figure 6. Summary of the microbial treatments showing suppressive effects on *Botrytis cinerea* and *Fusarium oxisporum* through direct antagonism (arrows) or through the induction of plant of systemic resistance (dashed arrow) after foliar spray and seed application.

Single strains vs *SynComs, variable outcomes so far.* Most studies focusing on the use of microbial consortia for disease control are looking for synergistic or additive effects, aiming to achieve a higher pest or disease control than their individual components. While some of these studies have indeed reported positive effects (Guetsky *et al.*, 2001, 2002; Srivastava *et al.*, 2010; Singh *et al.*, 2013; Ruano-Rosa *et al.*, 2014; Sylla *et al.*, 2015) many others showed similar or even less effectiveness in disease control when applying consortia as compared to the application of the individual microbes (Freeman *et al.*, 2004; Abo-Elyousr *et al.*, 2009; Elliott *et al.*, 2009; Ruano Rosa & López Herrera, 2009). However, most of these studies focused on one model system. In contrast, we intended to

extend the scope by including an array of target diseases –soil and foliar-, and possible mechanisms -direct and indirect control via ISR. The SynComs performed consistently well across the different pathosystems. Yet, differences between the SynComs and the individual components were relatively mild in terms of efficacy/degree of control.

In our study, the conservation of the biocontrol effectiveness in the SynComs to the same levels as the best performing individual isolates supported the compatibility between the coexisting microorganisms as previously established in **Chapter 1**.

Overall, our findings highlight the potential multifunctionality of SynComs for biological control. Combining compatible beneficial microorganisms with complementary effects on different targets, direct and indirect mechanisms of control and/or effective under different conditions will lead to the development of biocontrol products with increased versatility. To became commercial products, consistency of the outcomes needs to be tested and finally validated across multiple field trials in the geographical regions where is aimed to be used. This is a key step for the successful application of this sustainable technology in agriculture.

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CHAPTER 3

CHAPTER 3:
Evaluating the potential of microbial inoculants as part of integrated crop management practices under agricultural settings

ABSTRACT

Beneficial soil-borne microorganisms are a valid alternative to agrochemicals to sustainably protect crops without compromising yield. Their potential to enhance plant growth, productivity and health has been widely demonstrated under lab and controlled conditions. However, plant-microbe interactions and their outcome for the host plant are highly dependent on the environmental context. The highly variable abiotic and biotic conditions in the field or non-controlled greenhouse conditions, and current crop management practices occurring in conventional agronomical settings may limit the success of the microbial inoculants and their wider adoption in agriculture. Here we report the results of a field study in which several research groups collaborated under the frame of an H2020 funded EU project, MIRA, to investigate plant-microbe-pathogen/pest interactions under crop production conditions. We selected 11 previously characterized bacterial and fungal strains, and tested their impact as single inoculants or microbial consortia on plant growth, resistance, and yield in a commercial greenhouse under standard tomato crop management practices. Further, we addressed the compatibility of the microbial inoculants with common practices for integrated pest management. Our results showed that the fungal strains were more efficient than bacterial ones in reducing the incidence of the leaf mining pest *Tuta absoluta*. There was no negative impact of inoculations on yield, and *Trichoderma harzianum* T22 and *Funneliformis mosseae* even increased marketable tomato yield. Our results support the compatibility of microbial inoculants with commonly used tomato crop management practices. Identifying microbial strains with context stability that are compatible with common agricultural practices will contribute to the wider adoption of microbial inoculants for crop production, improving agricultural sustainability.

INTRODUCTION

In the last decades the urgent need to improve agricultural sustainability has prompted scientists, agroindustry, growers and customers to explore alternative solutions for the use of agrochemicals without compromising yields (Arora, 2018). In this regard, the potential of microbial inoculants based on plant associated beneficial microorganisms to improve plant growth, productivity and resistance in a sustainable manner have been widely reviewed (Berg, 2009; Barea, 2015; Trivedi *et al.*, 2017; Ab Rahman *et al.*, 2018; Compant *et al.*, 2019; Singh *et al.*, 2020). Accordingly, microbial inoculants constitute a very promising strategy aiming to meet the future global food demands while reducing the use of harmful chemicals in agriculture.

Many soil-borne microorganisms are living in association with plant roots, some of them being detrimental and others providing the host plant with important benefits (Mendes *et al.*, 2013). For example, root-associated mutualists including bacteria and fungi can help plants to deal with diverse biotic and abiotic stresses as well as to improve their nutrient uptake from the soil and thus enhance plant growth and production (Bakker *et al.*, 2018). Furthermore, several of these microbes such as *Bacillus*, *Pseudomonas*, *Trichoderma* and arbuscular mycorrhizal fungi (AMF) are able to trigger the immune system of their host plants and enhance their defense response to a broad spectrum of pests and diseases, a phenotype known as induced resistance (IR) (Pieterse *et al.*, 2014; De Kesel *et al.*, 2021).

Plant growth promoting rhizobacteria (PGPR) are described to act as efficient biological control agents, either by direct pathogen or disease suppression, or through IR, in addition to their ability to promote plant nutrition, growth and yield (Orozco-Mosqueda *et al.*, 2021). Bacteria from the genera *Bacillus* and *Pseudomonas* are among the most studied and best characterized microbial inoculants (Santoyo et al., 2012; Orozco-Mosqueda et al., 2021; Elnahal et al., 2022), as evidenced by the high number of commercial biofertilizer and biocontrol products in the market containing them (Aamir *et al.*, 2020). Other well characterized root-associated microbes with a great potential for biocontrol are fungi from the genus *Trichoderma*. The capacity of these fungi to enhance plant growth, development and resistance to pests and pathogens has been widely reviewed and recognized during the last decades (Harman *et al.*, 2004; Guzmán-Guzmán *et al.*, 2019; Poveda, 2021; Woo *et al.*, 2022; Modrzewska *et al.*, 2022). However, their current

success as bioinoculants in the market is mainly based on their mycoparasitic capacity, with 64.8% of the *Trichoderma* products available on the market claiming to be fungicidal (Woo *et al.*, 2014).

AMF are obligate biotrophs establishing symbiotic associations with the roots of most terrestrial plants, known as mycorrhizas, that constitute one of the most studied plant-fungal interactions (Pozo *et al.*, 2021). This symbiosis improves plant nutrient uptake and increases plant tolerance to biotic and abiotic stresses. Accordingly, it is considered to play a key role in sustainable agriculture (Smith & Smith, 2011; Jeffries & Barea, 2012; Barea, 2015). AMF-based inoculants are commercially available in the market and the number of companies selling AMF products has steadily increased in the last years (Bitterlich *et al*., 2020). The commercial products containing AMF are mostly used in agriculture as biofertilizers, mainly for nutrient and growth promotion benefits, but also for stress alleviation (Basiru *et al.*, 2021).

Entomopathogenic fungi (EPF) are another important group of microorganisms in agroecosystems because of their well-known ability to biologically control insect and mite pests (Quesada Moraga, 2020). Besides the direct interaction of these fungi with insects, they can interact and colonize plants endophytically, promoting plant growth and negatively affecting pathogens and phytophagous insects without a direct contact with them (Gange *et al.*, 2019; Quesada Moraga, 2020; Rasool *et al.*, 2021; Bamisile *et al.*, 2021). EPF have been used in biological control of insects for more than 150 years, and they are currently commercially available, with more than 170 species formulated as mycopesticides (Bamisile *et al.*, 2021).

Besides single microbe applications, the design of synthetic microbial communities (SynComs) for improving plant growth and health is receiving increasing interest within the scientific community and in the market (Liu *et al.*, 2020; Trivedi *et al.*, 2020; Batista & Singh, 2021; Minchev *et al.*, 2021). Recently, SynComs were shown to have an extended functionality compared to the single microorganisms for the biocontrol of foliar and soil pathogens through the combination of different mechanisms (Minchev *et al.*, 2021). Similarly, combined application of EPF and AMF showed functional complementarity for plant protection and growth (Zitlalpopoca-Hernandez *et al.*, 2022).

Despite the great potential of these microbes to improve plant growth and health, most of the research has been performed under highly controlled conditions, and the successful

transfer and adoption of this technology in agriculture is still challenging (Mitter *et al.*, 2019; Saad *et al.*, 2020). Plant-microbe interactions and their effect on plant growth and health are often conditioned by environmental factors (Saad *et al.*, 2020; Lee Díaz *et al.*, 2021). For example, abiotic factors such as temperature (di Lelio *et al.*, 2021), nitrogen or phosphorous fertilization (Ramírez-Serrano *et al.*, 2022; Dejana *et al.*, 2022), soil water content (Orine *et al.*, 2022), and light intensity (de La Hoz *et al.*, 2021) and quality (Saha *et al.*, 2022) have been reported to impact plant-microbe interactions and their outcome for the host plant. This evidences the high complexity and the context dependency of these interactions. In this regard, the highly variable environmental conditions and agricultural practices occurring in commercial production settings may limit the success or reproducibility of the results of microbial inoculation in the field (Compant *et al.*, 2019). Thus, it is crucial to test previously characterized plant beneficial microorganisms in real agrosystems, not only to evaluate their beneficial effects on the crop under production conditions, but also to address their compatibility with other consortium members (when applied as consortia) and with commonly used crop management practices.

Understanding the context dependency of plant-microbe interactions has been the focus of research in the EU ITN project MiRA ("Microbe Induced Resistance to Agricultural Pests", https://mira.ku.dk/). In this project several European academic institutions and companies collaborated to investigate Microbe-Induced Resistance against pests, its context dependency, mechanisms, and impacts on other biocontrol organisms. During the MiRA project, several rhizosphere microorganisms and microbial consortia were characterized under controlled laboratory conditions for their capacity to protect tomato plant against diverse antagonists such as herbivorous insects and phytopathogens. For example, Minchev *et al*. (2021) found that the PGPRs *B. amyloliquefaciens* and *P. azotoformans*, the biocontrol fungus *T. harzianum* strain T22 and the AMF *R. irregularis* are able to suppress *Fusarium oxysporum* or *B. cinerea* in tomato through direct antagonism or induced resistance in the plant. The authors showed that while different single microorganisms were the most effective in suppressing different pathogens, the SynCom including all microbes together effectively protected the plant against both pathogens to the same extent as the best performing single strains, pinpointing an extended functionality of the microbial consortia (Minchev *et al.*, 2021). In another study, Zitlalpopoca-Hernandez *et al*. (2022) studied the individual and combined effect of the

AMF *F. mosseae* and the EPFs *B. bassiana* and *M. robertsii* on tomato plant growth and resistance to *B. cinerea*, showing a functional complementarity of EPF and AMF in pathogen suppression and plant growth promotion (Zitlalpopoca-Hernandez *et al.*, 2022). The individual inoculation of tomato with *T. harzianum* strain T78 impacted the plant metabolome, negatively affecting the performance of the specialist insect *Manduca sexta* (Papantoniou *et al.*, 2021). Similarly, the AMF *F. mosseae* can induce resistance against the chewing generalist insect *Spodoptera exigua* in different plant species, and the resistance is dependent on P and N availability (Rivero *et al*., 2021, Dejana *et al*. 2022, Ramírez-Serrano *et al*. 2022).

While the abovementioned studies provide valuable insight in Microbe-Induced Resistance and its underlying mechanisms, they were all carried out under controlled laboratory conditions and do not necessary provide information on effects under commercial production conditions. Therefore, in this study, through the collaborative MiRA project, we explored the effect of microbial inoculation on plant pathogen/pest interactions under commercial crop production conditions. For this, we focused on tomato, *Solanum lycopersicum*, the second most produced vegetable crop worldwide. We selected bacteria, fungi and SynComs previously characterized under controlled conditions, and tested their impact on plant growth, resistance to pests and diseases, and fruit production and quality in a commercial greenhouse where common tomato crop management practices were used. We show that microbial inoculation can increase plant resistance to pests without compromising yield, thus supporting the inclusion of Microbe-Induced Resistance in integrated pest management programs.

MATERIAL AND METHODS

1. Microbial treatments

To address the effect of microbial inoculation on tomato plants, we performed a large-scale commercial greenhouse experiment with a total of 12 microbial treatments, including 2 bacteria (the plant growth promoting bacteria *Bacillus amyloliquefaciens* CECT8238 and *Pseudomonas azotoformans* F30A), 4 free living fungi (*Trichoderma harzianum* strains T22 and T78, the entomopathogenic fungi *Beauveria bassiana* 1339 and *Metarhizium robertsii* 1235) and 3 arbuscular mycorrhizal fungi, *Rhizophagus irregularis* MUCL57021, *Funneliformis mosseae* BEG12 and

Claroideoglomus etunicatum EEZ163). In addition, two microbial consortia (M1, M2), explained below, and a control treatment without soil microbe addition were included.

The bacterium *B. amyloliquefaciens* was cultured on tryptone soy agar (TSA) and grown at 28°C for 24 hours. For spore production, liquid Difco sporulation medium (Nicholson & Setlow, 1990) was inoculated with a single bacterial colony and incubated at 28°C for 48 hours with a rotary shaking at 200rpm. Spore concentration of the liquid culture was quantified using a Neubauer hemocytometer, then the culture was centrifuged for 15min at 5000 rpm to separate the spores from the growing medium. Finally, the recovered spores were resuspended in sterile water to a concentration of 1 x 10^7 spores/ml. For inoculation, 1 ml of spore solution was applied to each plant in the root system during transplanting (Minchev *et al.*, 2021).

The bacterium *P. azotoformans* was cultured on TSA and grown at 28°C for 24 hours. A pre-culture was prepared in tryptone soya broth (TSB) inoculated with a single colony and incubated overnight at 28°C with rotary shaking at 200rpm. Next, 1ml of pre-culture was added to 25ml of TSB and incubated at 28°C for 2h30min with rotary shaking at 200rpm to reach the exponential growth phase. Then, the cell concentration was quantified measuring the optical density (620nm) of the bacterial culture using a spectrophotometer. The bacterial culture was centrifuged for 15min at 5000rpm to separate the bacterial cells from the growing medium. Finally, the obtained cells were resuspended in sterile water to a concentration of 1 x 10^7 cfu/ml. For inoculation, 1ml of bacterial solution per plant was applied to the root system during transplanting (Minchev *et al.*, 2021).

The fungus *T. harzianum* strain T22 was cultured on potato dextrose agar (PDA) and grown at room temperature for 7 days. The sporulated plates were scraped using a sterile spatula and sterile water. The resulting spore suspension was filtered using a sterile miracloth filter to remove remaining mycelia and the spore concentration was quantified using a Neubauer hemocytometer and adjusted to 1 x 10^7 spores/ml. For inoculation 1ml of spore suspension was added to the root system of each plant during transplanting (Minchev *et al.*, 2021).

The fungus *T. harzianum* strain T-78 was cultured on PDA and re-cultured every two months. The fungal inoculum was prepared by adding aseptically a square piece of the fungal culture on a sterile mix of vermiculite and oat (Martínez-Medina *et al.*, 2009). The inoculum was incubated at 28°C and in the dark for 5 days. The inoculum was mixed with the substrate in a proportion of 1g per Kg of substrate.

The entomopathogenic fungi *B. bassiana* and *M. robertsii* were cultured in Sabouraud dextrose agar (SDA) and grown at 24° C in darkness for 3 weeks. The sporulated plates were scraped using a sterile spatula and the spores were recovered in a sterile solution of Triton X (0.05 %). The spore concentration was quantified using a Neubauer hemocytometer and adjusted to 1 x 10^8 spores/ml. For inoculation 1 ml of spore suspension per plant was applied to the root system during transplanting (Zitlalpopoca-Hernandez *et al.*, 2022).

The AMF *R. irregularis* was grown *in vitro* on a minimal (M) medium with *Agrobacterium rhizogenes*-transformed carrot (*Daucus carota*) roots as host (St-Arnaud *et al.*, 1996). Spore extraction was performed by adding citrate buffer (0.01 M, pH = 6) to the AMF culture in a proportion of 3:1 (v/v) and maintained for 1 hour on a rotary shaker to dissolve the agar. The spores were recollected using sieves with mesh size of 250 and 53 µm and resuspended in sterile water at 1000 spores/ml. For inoculation 1ml of spore solution was applied to the root system of each plant (Minchev *et al.*, 2021).

The AMF *F. mosseae* and *C. etunicatum* were maintained as living inocula on mixed cultures of *Trifolium repens* and *Sorghum vulgare* in vermiculite-sepiolite substrate. The inoculants consisted of substrate containing colonized root fragments, mycelia and spores. For inoculation, 10% (v/v) of mycorhizal inocula were mixed with the substrate at transplanting (Rivero *et al.*, 2018).

Further, two synthetic microbial communities (SynComs) were used. The M1 inoculum included *B. amyloliquefaciens*, *P. azotoformans* and *T. harzianum* T22 at concentration of 1 x 10^7 cfu/ml each, and *R. irregularis* at a concentration of 1000 spores/ml (Minchev *et al.*, 2021). The M2 inoculum included *M. robertsii* and *B. bassiana* both inoculated at a concentration of 1 x 10^8 conidia/ml, and *R. irregularis* at a concentration of 1000 spores/ml. For both SynComs 1ml/plant was applied to the root system during transplanting.

2. Plant material and growing conditions

Solanum lycopersicum cv Money maker seeds (Vreeken's Zaden, The Netherlands) were surface sterilized by immersion in 5% Sodium hypochlorite solution for 10 min followed by at least 3 washing steps in sterile water for 10 min each. The surface sterilized seeds were sown in sterile vermiculite and incubated for 7 days in a greenhouse at 24°C : 16°C day : night with a photoperiod 16 h: 8 h light : dark and 70% of relative humidity.

3. Experimental set up

One week old seedlings were transferred to starting trays -with cell dimensions 2,9 x 2,9 x 6,8cm- containing blond seedling peat (Kekkilä LSM 0 R8406, Projar, Valencia, Spain): sepiolite: perlite (1:1:1) mixture and inoculated with the microbial treatments described previously. Inoculated seedlings were grown in a commercial nursery (ACRENA SAT 251, El Ejido, Spain; 36°, 47', 52.9"N; 2°, 43', 36.3"W) for 4 weeks. On September 3rd, 2020 the plants were transplanted to a commercial production greenhouse (Estación experimental Cajamar, Paraje las Palmerillas, El Ejido, Almería; 36°, 47', 36.3"N; 2°, 43', 15.2"W) and maintained during the whole crop cycle from September 2020 to March 2021. The greenhouse consisted of a typical "raspa y amagado" type (Ávalos-Sánchez *et al.*, 2022), 37.8 m long and 23.2 m wide with a total area of 877 m^2 and usable area of 720 m^2, passive ventilation (25.0% window surface) with side windows (north and south sides) and zeniths, covered with anti-trip mesh. The microbial inoculation treatments were organized following a randomized complete block design, with four blocks. Each block contained all 12 treatments, and each treatment in all blocks was replicated with six plants (**Figure 1;** N = 12 treatments x 4 blocks x 6 replicates = 288 plants).

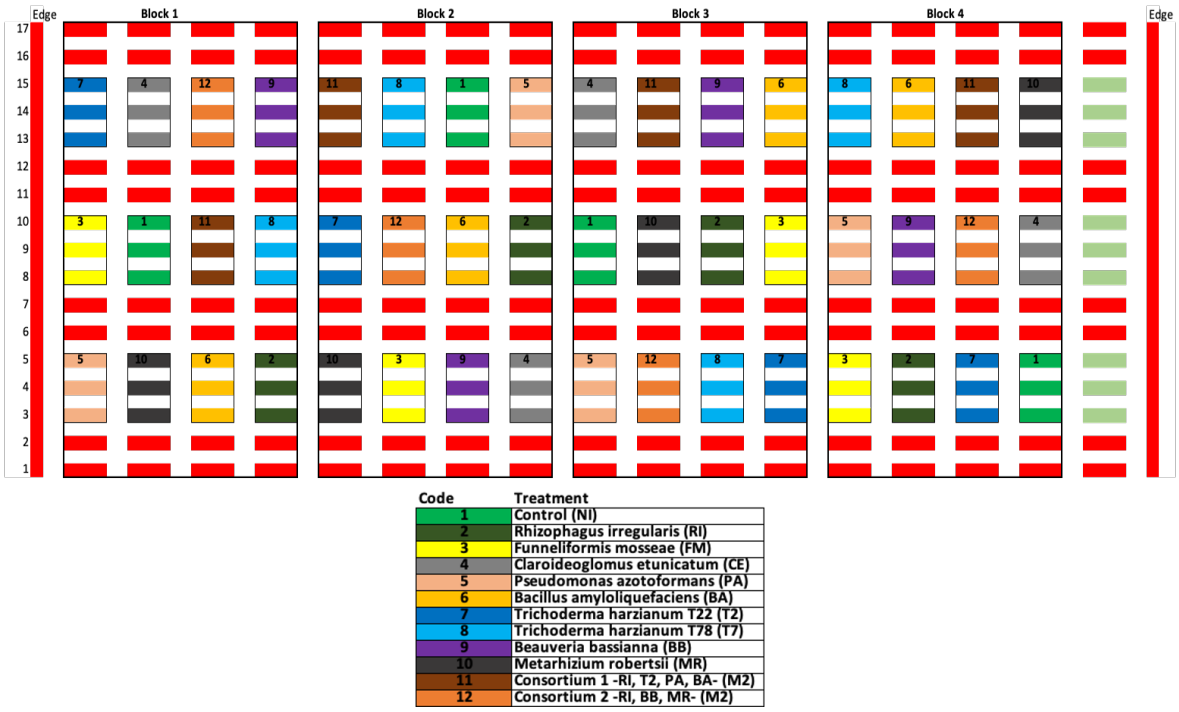

Figure 1. **Treatment distribution within the greenhouse**, consisting of 12 treatments, and four blocks.

4. Biological control, pheromone and pollinator application

Two weeks after transplanting, the predatory mirid bug *Nesidiocoris tenuis* (Hemiptera: Miridae) was released in the greenhouse with a density of 0,5 – 1,5 individuals/m2, so to inhibit the unintentional spread of whiteflies (Hemiptera: Sternorrhyncha) and *Tuta absoluta* (Lepidoptera: Gelechiidae) to tomato plants. In addition, pheromones for the mating disruption of *T. absoluta* were released during the whole cropping season. For insuring pollination of tomato flowers, *Bombus terrestris* bumblebees were released 3 weeks post transplantation (wpt) to the crop once the plants started flowering.

5. Irrigation and fertilization

The irrigation scheme during the whole cropping season and nutrient supply are shown in **Supplementary Table 1**. Nutrient content in soil and irrigation water (nutrient solution) was evaluated periodically to adjust to the crop needs for nutrient supply.

Specifically, phosphorus was measured by visible spectrophotometry using the compound phosphorous vanadate molybdate (Tandon *et al.*, 1968). Nitrates were measured spectrophotometrically at 220 and 275 nm (Norman & Stucki, 1981). Ammonia was measured by the Nessler reagent method (Yuen & Pollard, 1954). Sodium, calcium, potassium, magnesium, iron, copper, manganese and zinc, were determined by atomic absorption / emission (Isaac & Kerber, 2015). Carbonates and bicarbonates were measured by titration with 0.01 N sulfuric acid (Allison et al., 1954). Chlorides were also measured by volumetry with silver nitrate between 0.01 and 1 N using potassium chromate as an indicator (Mohr's titration). Boron was determined by spectrophotometry with the azomethine reaction (John *et al.*, 2006). Sulfates were measured by precipitation of barium sulfate.

6. Evaluations

In total we evaluated 17 response variables related to plant growth and yield, and plant resistance to pathogens and insect pests (**Supplementary Table 2**).

6.1 Plant growth, nutritional status and yield

As a proxy of plant growth, plant height from soil surface to the top of the shoot of each plant was measured on December 3rd, 2020 (12 wpt). As a proxy for plant productivity, we quantified the number of inflorescences per plant on October 26th, 2020 (8 wpt). Total

carbon and nitrogen content in leaves was measured on leaves sampled on the January 21st, 2021 (19 wpt), using a Flash1112 (Thermo Scientific).

Fruit productivity and quality were evaluated every week between November 12th and February 4th. Tomato fruits were classified by size (size GG 82-102 mm; size G 67-82 mm; size M 57-67 mm; size MM 47-57 mm) and by categories (first, second and non-commercial). Fruits were considered non-commercial when their size was too small (<45 mm of diameter), when they showed presence of pathogen damage, cracks, Blosson-end rot or blotchy ripening, or when they were misshapen.

Further, parameters such as fruit dry weight (determined after drying the fruits in a forced air stove at 70 ºC for 48 hours), acidity % (Acid-base volumetry using 1 N NaOH as base and phenolphthalein indicator), ºBrix or total soluble solids (manual refractometer), pH (pH meter), maturity index (the relationship between the content of total soluble solids and assessable acidity) were assessed during the period of fruit recollection.

6.2 Fruit quality and nutraceutical value

Polyphenol and carotenoid content in fruits were evaluated twice, on December 16th, 2020 (14 wpt) and February 25th, 2021 (23 wpt). Polyphenols were measured by the spectrophotometric method of Folin- Ciocalteau (Georgé *et al.*, 2005) using a standard curve of Gallic Acid from 0 to 1000 ppm at 760 nm (double ultraviolet-visible beam, Unicam brand; Helios Alpha model) and expressed as mg of gallic acid/100g dry fruit. Lycopene and beta-carotene content of fruits was measured with an acetone-hexane extraction and spectrophotometric determination at 487.5 nm (Sadler *et al.*, 1990) with modifications (Rousseaux *et al.*, 2005) and expressed as mg/100g fresh fruit.

6.3 Pest and disease incidence

The incidence of *N. tenuis*, thrips, *T. absoluta*, whiteflies and powdery mildew was evaluated on December 3rd, 2020 (12 wpt). For thrips, incidence was evaluated by counting the number of leaves presenting lesions caused by thrips. The incidence of *T. absoluta* was evaluated as the percentage of plants presenting damage (mines) caused by *T. absoluta* larvae. Whiteflies and *N. tenuis* incidence was evaluated using yellow sticky traps for a period of 4 weeks, placing one sticky trap per treatment per block.

7. Statistical analysis

Data were analysed using R statistical language, version 4.1.1 (R Development Core Team 2021) and figures were produced using the package ggplot2 (Wickham, 2009). The effect of the 12 microbial treatments (including the control) on the different response variables was analyzed using linear (lm) or generalized linear models (glm) following the details as shown **in Supplementary Table 2**. Treatment effects were always compared to the control (non-inoculated) treatment, as shown by the asterisks in Figures presented in the results section.

RESULTS

1. Impact of microbial inoculants on plant growth, nutritional status and flowering

To get an insight in how the different root microbial inoculations affect plant growth, nutritional status and flowering, we evaluated plant heigh, the ratio of total carbon/nitrogen (C/N) leaf content and the number of inflorescences. For plant height, we found a significant effect of microbial inoculation ($F_{11,264} = 2.29$, $p = 0.01$). Specifically, we observed that the treatment with *C. etunicatum* decreased tomato plant height on average by 12.96 cm compared to the control plants ($t = -2.65$, $p = 0.009$; **Figure 2A**). Leaf C/N ratio was not altered by microbial inoculation ($F_{11,116} = 1.3$, $p = 0.23$; **Figure 2B**). Further, our results showed no effect of microbial inoculation on the number of inflorescences produced across treatments ($F_{11,130} = 1$, $p = 0.45$; **Figure 2C**).

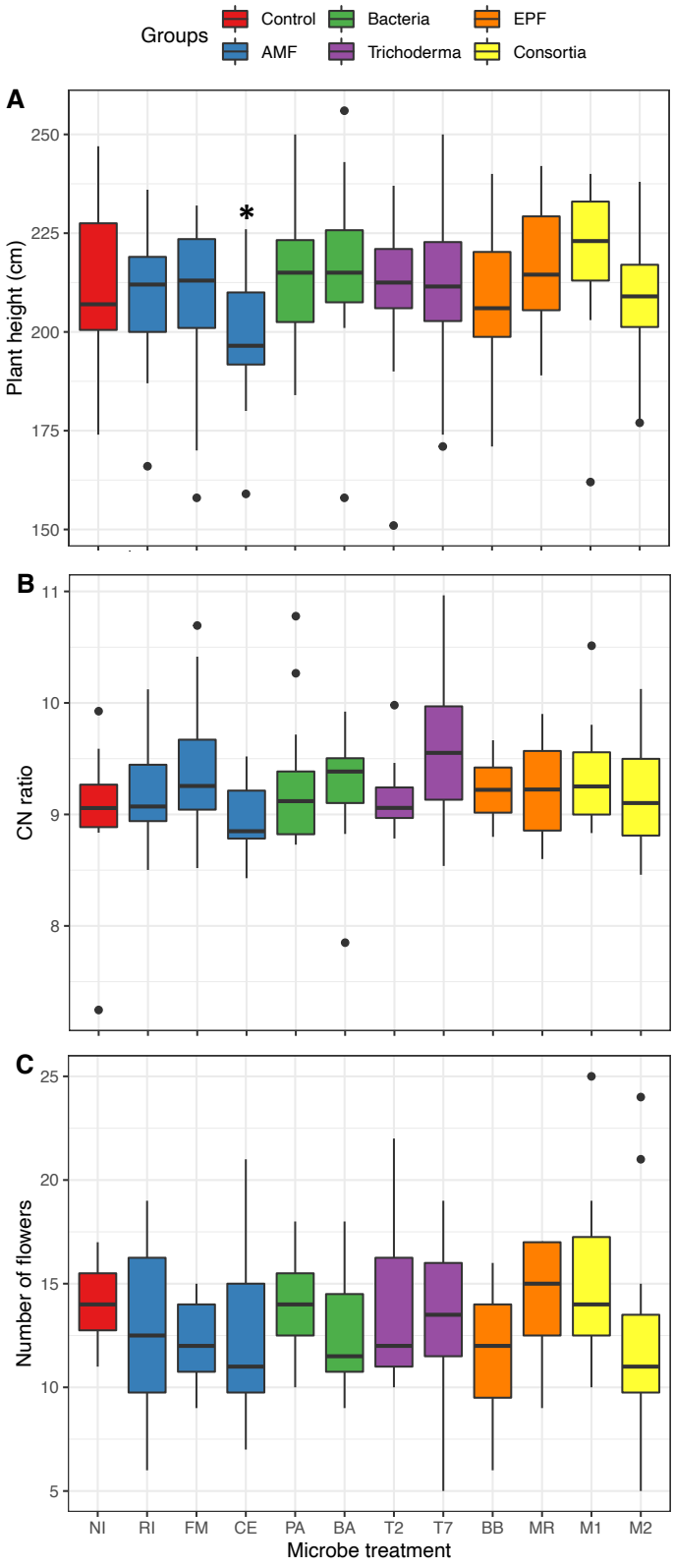

Figure 2. Impact of microbial inoculation on **(A)** plant height, **(B)** leaf carbon-nitrogen ratio, and **(C)** number of inflorescences. Plants were inoculated with: *R. irregularis* (RI), *F. mosseae* (FM), *C. etunicatum* (CE), *P. azotoformans* (PA), *B. amyloliquefaciens* (BA), *T. harzianum* T22 (T2) and T78 (T7), *B. bassiana* (BB), *M. robertsii* (MR), consortium 1 (M1) including RI+PA+BA+T2 and consortium 2 (M2) including RI+BB+MR. Non-inoculated plants were included as a control (NI). Boxes represent the interquartile range, black lines represent the median, whiskers represent maximum and minimum within 1.5 times the interquartile range, and black dots represent outliers. Asterisks indicate statistically significant differences compared to the control (*p<0.05).

2. Impact of microbial inoculation on fruit yield

To evaluate the impact of the microbial inoculants on total fruit production, we assessed the fruit yield during the whole fruit production period, quantifying the total production as well as the commercial quality production. Microbial inoculation had a prominent effect on the total tomato production ($Chisq_{11,464} = 23.1$, $p = 0.017$; **Figure 3A**) and even a stronger impact on the commercial quality tomato production ($Chisq_{11,464} = 25.1$, $p=0.009$; **Figure 3B**). Indeed, *F. mosseae* and *T. harzianum* T22 inoculated plants showed significantly higher total productivity ($t = 2.48$, $p = 0.01$ and $t = 2.64$, $p = 0.01$ respectively; **Figure 3C**) and most importantly, an increased commercial quality fruit production ($t = 2.24$, $p=0.03$ and $t = 2.69$, $p=0.007$ respectively) as compared to the non-inoculated control plants (**Figure 3D**).

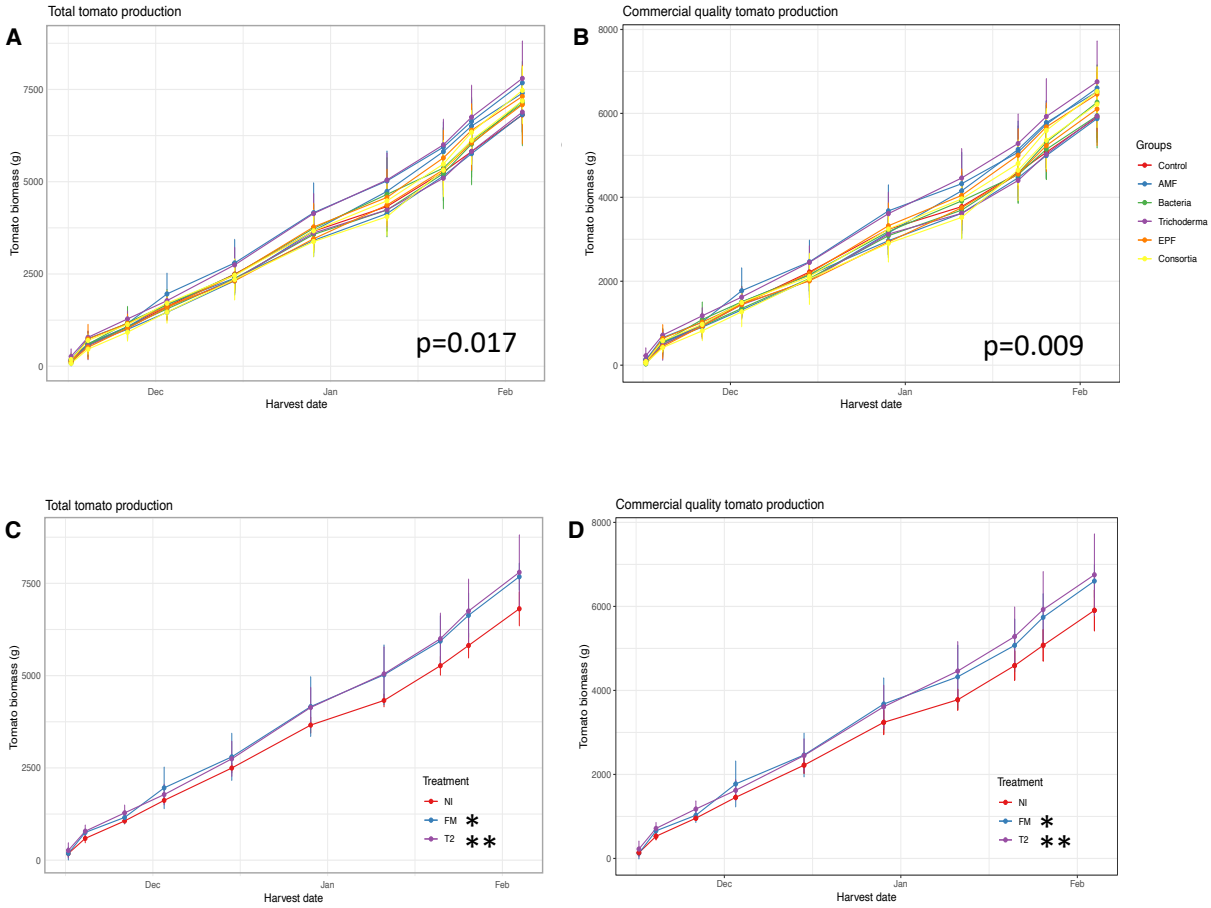

Figure 3. Impact of microbial inoculants on tomato production. A and C, total tomato production; B and D, commercial quality tomato production. Plants were inoculated with: *R. irregularis* (RI), *F. mosseae* (FM), *C. etunicatum* (CE), *P. azotoformans* (PA), *B. amyloliquefaciens* (BA), *T. harzianum* T22 (T2) and T78 (T7), *B. bassiana* (BB), *M. robertsii* (MR), consortium 1 (M1) including RI+PA+BA+T2 and consortium 2 (M2) including RI+BB+MR. Non-inoculated plants were included as a control (NI). Lines represent the average yield increase across time, dots represent the mean tomato biomass, and error bars represent ± the standard deviation. Asterisks indicate statistically significant difference compared to the control (*p<0.05, **p<0.01).

3. Effect of beneficial microbes on fruit quality and nutraceutical value

Fruit quality was evaluated by assessing different parameters: ºBrix, % Acidity, Maturity index and % Dry weight. The only fruit quality parameter significantly affected by the microbial treatments was the % Acidity ($F_{11,33}$ = 2.46, p=0.02), where fruits from *C. etunicatum* inoculated plants showed an increase of % Acidity compared to the control treatment (t = 2.25, p = 0.03; **Table 1**).

Table 1. Effect of microbial inoculation on tomato fruit quality parameters. Plants were inoculated with: *R. irregularis* (RI), *F. mosseae* (FM), *C. etunicatum* (CE), *P. azotoformans* (PA), *B. amyloliquefaciens* (BA), *T. harzianum* T22 (T2) and T78 (T7), *B. bassiana* (BB), *M. robertsii* (MR), consortium 1 (M1) including RI+PA+BA+T2 and consortium 2 (M2) including RI+BB+MR. Non-inoculated plants were included as a control (NI). Numbers are mean value ± standard error. Asterisks indicate statistically significant difference compared to the control (*p<0.05).

Treatment	ºBrix	% Acidity	Maturity index	% Dry weight
Ni	4.18 ± 0.08	0.43 ± 0.01	9.70 ± 0.43	5.29 ± 0.13
Ri	3.93 ± 0.08	0.43 ± 0.03	9.30 ± 0.44	5.16 ± 0.05
Fm	4.03 ± 0.08	0.42 ± 0.01	9.69 ± 0.05	5.37 ± 0.05
Ce	4.13 ± 0.13	0.47 ± 0.01 *	8.80 ± 0.28	5.29 ± 0.09
Pa	4.03 ± 0.06	0.45 ± 0.01	9.04 ± 0.22	5.38 ± 0.13
Ba	4.13 ± 0.09	0.45 ± 0.01	9.28 ± 0.36	5.40 ± 0.13
T2	4.03 ± 0.05	0.41 ± 0.01	9.86 ± 0.26	5.32 ± 0.07
T7	3.88 ± 0.08	0.42 ± 0.01	9.26 ± 0.23	5.22 ± 0.13
Bb	3.88 ± 0.17	0.43 ± 0.01	8.98 ± 0.44	5.10 ± 0.11
Mr	3.98 ± 0.06	0.44 ± 0.01	9.07 ± 0.21	5.41 ± 0.12
M1	4.05 ± 0.10	0.45 ± 0.004	9.02 ± 0.20	5.28 ± 0.04
M2	3.95 ± 0.18	0.41 ± 0.02	9.67 ± 0.25	5.31 ± 0.13

Further, we evaluated the nutritional and nutraceutical properties of the fruits, analyzing the content of metabolites that act as antioxidants such as phenolic compounds and carotenoids in fruits at two time points, at 14 (data not shown) and 23 weeks post transplantation, obtaining similar results. In particular, we analyzed total polyphenols, beta-carotene, lycopene and the sum of both represented as total carotenoids content. Fruit polyphenol content was not significantly affected by microbe treatment ($F_{11,36}$ = 0.61, p=0.81, **Figure 4A**). Furthermore, carotenoid contents of fruits (lycopene, beta-carotene and total carotenoids) were not significantly altered bymicrobial treatments ($F_{11,36}$ = 1.25,

p=0.29, **Figure 4B;** $F_{11,36} = 0.66$, p=0.77, **Figure 4C and** $F_{11,36} = 1.18$, p=0.34, **Figure 4D,** respectively).

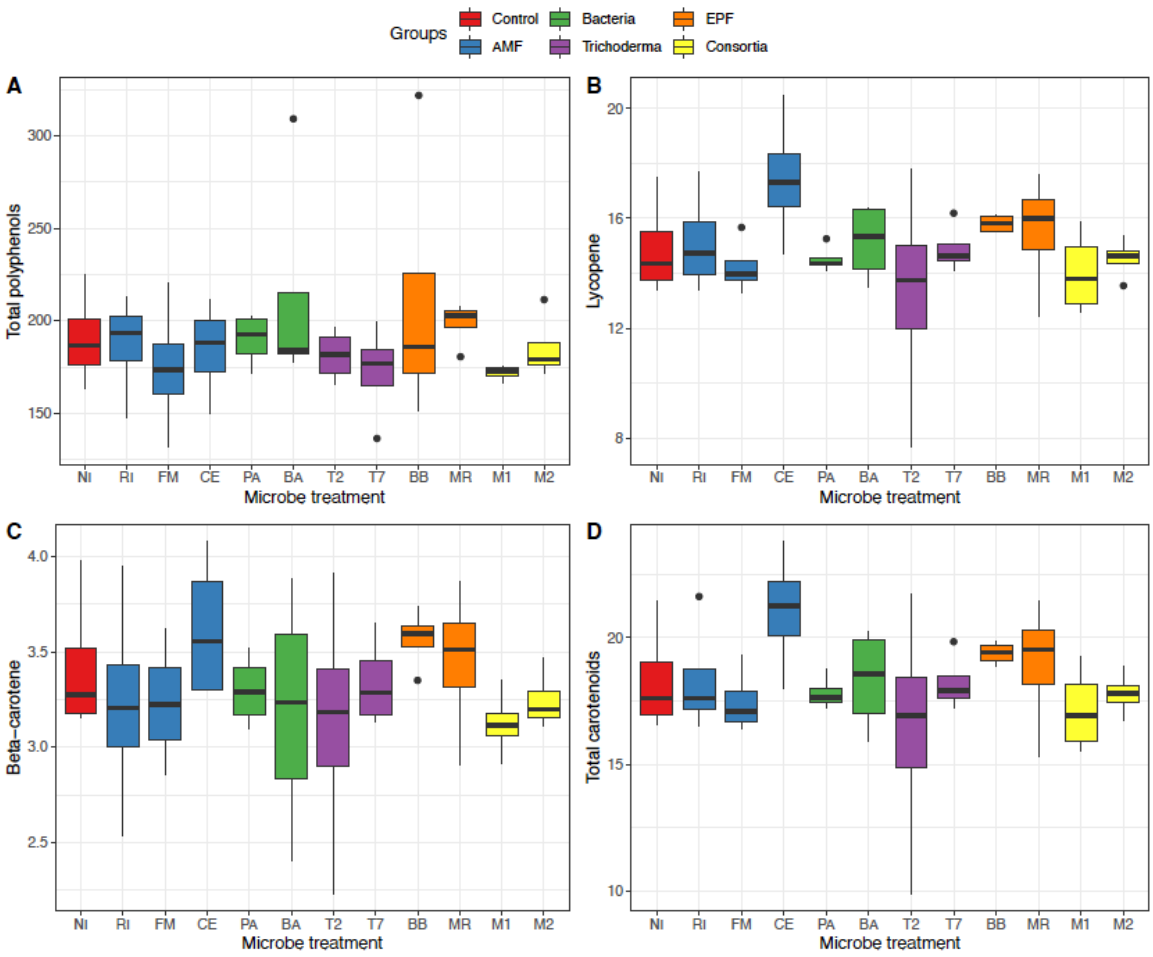

Figure 4. Effect of the microbial treatments on fruit nutritional properties. Tomato fruit content in **(A)** total polyphenols, **(B)** lycopene, **(C)** beta-carotene, and **(D)** total carotenoids. Plants were inoculated with: *R. irregularis* (RI), *F. mosseae* (FM), *C. etunicatum* (CE), *P. azotoformans* (PA), *B. amyloliquefaciens* (BA), *T. harzianum* T22 (T2) and T78 (T7), *B. bassiana* (BB), *M. robertsii* (MR), consortium 1 (M1) including RI+PA+BA+T2 and consortium 2 (M2) including RI+BB+MR. Non-inoculated plants were included as a control (NI). Boxes represent the interquartile range, black lines represent the median, whiskers represent maximum and minimum within 1.5 times the interquartile range, and black dots represent outliers.

4. Impact of microbial inoculation on natural pest and disease incidence

To evaluate the ability of the different beneficial microorganisms to trigger induced resistance we assessed the natural incidence of different pests and diseases which appeared during the cropping season. Regarding pests, the incidence of thrips, whiteflies and the tomato leaf miner *Tuta absoluta* were assessed. The incidence of *T. absoluta* was significantly impacted by microbial inoculation ($Chisq_{11,273} = 24.37$, p=0.01). In particular

R. irregularis (z = -2.698, p = 0.007), *F. mosseae* (z = -2.31, p = 0.02), *C. etunicatum* (z = -2.31, p = 0.02), *T. harzianum* T22 (z = -2.31, p = 0.02), *T. harzianum* T78 (z = -1.98, p = 0.05), *M. robertsii* (z = -2.69, p = 0.007) and the consortium M2 (z = -1.98, p = 0.05) treatments significantly decreased the percentage of plants damaged by the leaf miner as compared to the control treatment, with average reductions ranging from 60% for *T. harzianum* T78 up to 90% for *R. irregularis* and *M. robertsii* (**Figure 5A**). In contrast, thrips and whitefly incidences were not significantly affected by the microbial treatments ($Chisq_{11,131} = 15.82$, p=0.15; **Figure 5B** and $F_{11,36} = 1$, p = 0.46; **Figure 5C**). Regarding diseases, we assessed the incidence of powdery mildew which was the only pathogen that appeared naturally on the crop. No significant effect of the microbe treatments was observed on the incidence of powdery mildew ($Chisq_{11,132} = 9.01$, p = 0.62; **Figure 5D**).

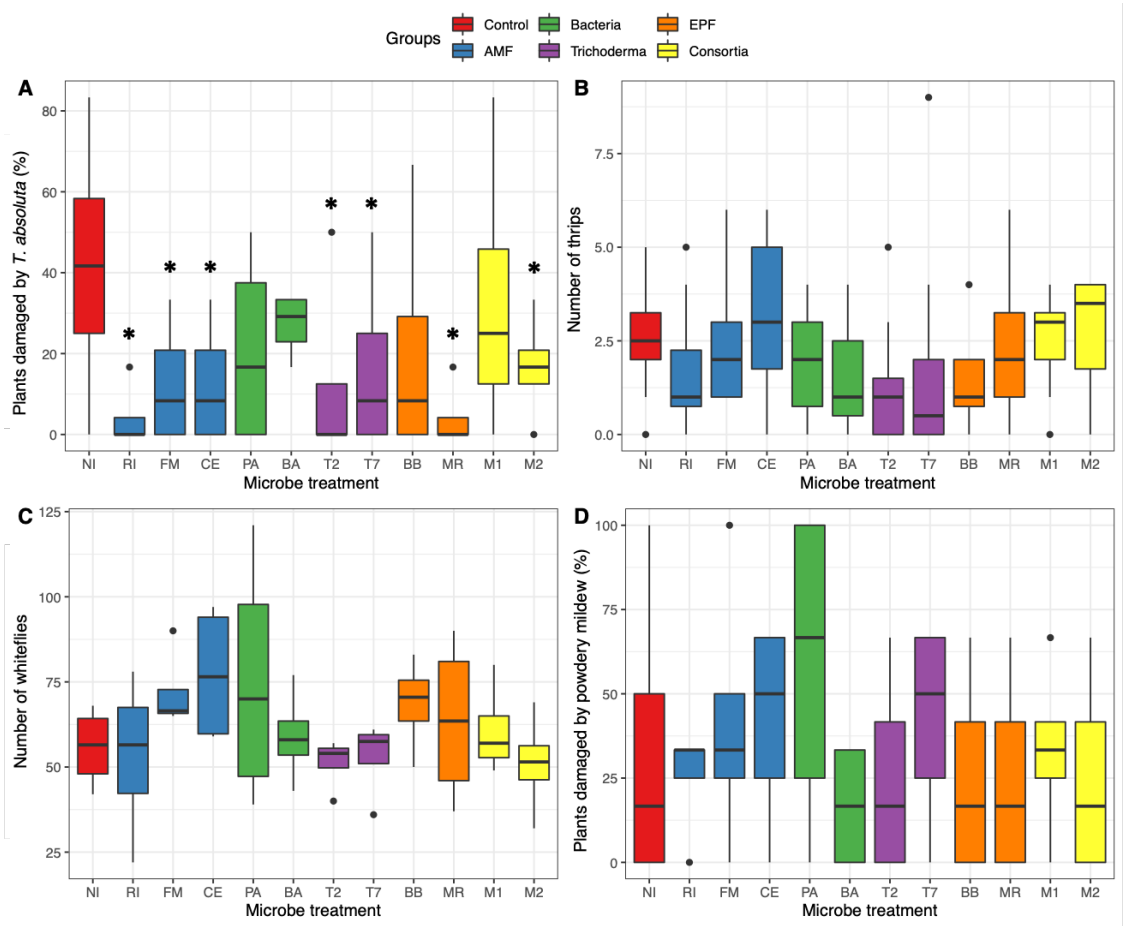

Figure 5. Impact of microbial inoculation on natural incidence of **(A)** *T. absoluta*, **(B)** thrips, **(C)** whiteflies, and **(D)** powdery mildew. Plants were inoculated with: *R. irregularis* (RI), *F. mosseae* (FM), *C. etunicatum* (CE), *P. azotoformans* (PA), *B. amyloliquefaciens* (BA), *T. harzianum* T22 (T2) and T78 (T7), *B. bassiana* (BB), *M. robertsii* (MR), consortium 1 (M1) including RI+PA+BA+T2 and consortium 2 (M2) including RI+BB+MR. Non-inoculated plants were included as a control (NI). Boxes represent the interquartile range, black lines represent the median, whiskers represent maximum and minimum within 1.5 times the interquartile range, and black dots represent outliers. Asterisks indicate statistically significant difference compared to the control (*p<0.05, **p<0.01).

5. Impact of microbial inoculants on natural enemies

To evaluate any potential impact of the microbial inoculants on other beneficial organisms, we evaluated the incidence of the predatory mirid bug *Nesidiocoris tenuis,* released in the greenhouse at the beginning of the cropping season for the control of whiteflies and *T. absoluta*. We did not find significant differences in *N. tenuis* incidence among the different microbial treatments, indicating there is no negative effect of the microbial inoculants on this predator ($F_{11,36} = 0.55$, p=0.86; **Figure 6**).

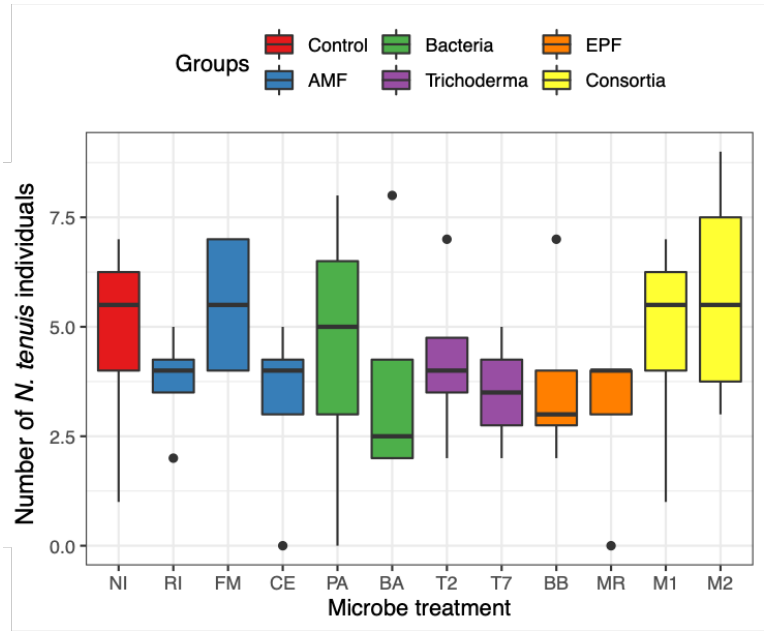

Figure 6. Impact of microbial inoculation on *N. tenuis* incidence. Plants were inoculated with: *R. irregularis* (RI), *F. mosseae* (FM), *C. etunicatum* (CE), *P. azotoformans* (PA), *B. amyloliquefaciens* (BA), *T. harzianum* T22 (T2) and T78 (T7), *B. bassiana* (BB), *M. robertsii* (MR), consortium 1 (M1) including RI+PA+BA+T2 and consortium 2 (M2) including RI+BB+MR. Non-inoculated plants were included as a control (NI). Boxes represent the interquartile range, black lines represent the median, whiskers represent maximum and minimum within 1.5 times the interquartile range, and black dots represent outliers.

DISCUSSION

In this study, by testing diverse plant beneficial microorganisms under commercial settings, we demonstrated the potential of microbial inoculants for crop protection and yield improvement in tomato crop production. We have identified microbial strains able to perform efficiently as biostimulants and bioprotectors under non-controlled, real production conditions, suggesting their compatibility with common tomato crop management practices, and confirming their potential to improve agricultural sustainability.

We monitored several parameters and plant traits to evaluate microbial performance including plant growth, productivity, fruit quality and biochemistry, as well as plant resistance to pests and diseases during the whole cropping season. Our results point to a more prominent effect of fungal inoculants promoting plant resistance, and in some cases, improving crop yield, while bacterial inoculants did not show any significant effects on the evaluated parameters.

Plant beneficial microbes such as PGPR, *Trichoderma*, AMF and EPF have been widely reported to improve plant growth and nutritional status (Quesada Moraga, 2020; Orozco-Mosqueda *et al.*, 2021; Salomon *et al.*, 2022; Woo *et al.*, 2022). Hence, we first evaluated the impact of microbial inoculants on plant growth and nutritional status in terms of plants height and carbon-nitrogen content respectively. In general, microbial inoculation did not impact plant height nor leaf C-N ratio, and accordingly, we did not find evidence for plant growth, nor for nutritional promotion under our experimental conditions. Exception to this general trend were the plants inoculated with the AMF *C. etunicatum* which presented reduced plant growth, although this reduction did not negatively affect tomato yield. It should be noted that crop management included fertilization following the crop demands, so no nutritional deficiencies appeared and a role of the microbial inoculants on nutrient deficiency alleviation could not be tested.

However, when evaluating the potential of microbial inoculants as biostimulants, crop yield and fruit quality are the most economically relevant parameters. Plant beneficial microorganisms have been reported to improve crop productivity and fruit quality. For example, a recent meta-analysis based on 97 peer-reviewed research articles analyzed the effect of different microbial inoculants -mostly PGPR- on crop productivity (Li *et al.*, 2022). The authors concluded that microbial inoculants can improve crop productivity mainly by stress alleviation or by improving plant nutrient availability (Li *et al.*, 2022). Our results show that, while none of the treatments had an impact on flower production, both total and marketable tomato yield during the cropping season was increased by the AMF *F. mosseae* and the fungus *T. harzianum* T22. However, whereas earlier studies have shown that AMF and PGPR can also improve tomato fruit quality under field conditions (Bona *et al.*, 2017), we did not find any impact of the inoculation on the fruit quality and composition parameters evaluated in this study.

Assessing Microbe induced resistance against different attackers

Soil-borne beneficial microbes are widely reported to improve plant defenses triggering induced resistance against a broad range of attackers including pathogens and herbivorous insects (Pieterse *et al.*, 2014). Here, we evaluated the impact of microbial inoculation on the incidences of powdery mildew, the phloem and cell-content feeders whiteflies and thrips respectively, and the leaf miner *Tuta absoluta*. We found no significant effect on the incidence of powdery mildew, thrips or whiteflies, but the percentage of plants damaged by the leafminer *T. absoluta* was significantly reduced by most of the fungal inocula. This is in agreement with some recent studies showing induced resistance against *T. absoluta* by AMF (Shafiei *et al.*, 2022) and *T. afroharzianum* (Aprile *et al.*, 2022). Remarkably, in the present study most fungal inoculants, including all mycorrhizal strains, both *T. harzianum* strains, the EPF *M. robertsii* and the M2 SynCom (a fungal consortia including the EPFs *B. bassiana* and *M. robertsii*, and the AMF *R. irregularis*) reduced the natural incidence of *T. absoluta* with up to 90% of reduction in the case of *R. irregularis* and *M. robertsii*. These results highlight the great potential of these fungi to enhance plant defenses and protect plants against insect pests and thus, to improve or complement current IPM practices in tomato crop management.

The differences in the extent to which successful protection was achieved against different attackers is likely related to their different lifestyles and feeding guilds. Mechanistically, induced resistance triggered by beneficial microbes such as PGPRs, *Trichoderma* or AMF has been shown to rely on the primed regulation of the jasmonic acid (JA) and ethylene (ET) related defense signaling pathways (Pozo & Azcón-Aguilar, 2007; Pieterse *et al.*, 2014). Accordingly, Microbe-Induced Resistance has been shown to have a negative effect on plant attackers susceptible to JA/ET related defenses such as necrotrophic pathogens and generalist chewing insects, but generally fail to protect against biotrophic pathogens and cell-content or phloem feeding insects (Pineda *et al.*, 2010, 2013; Pieterse *et al.*, 2014; Pozo *et al.*, 2020). In agreement with this reported pattern, the microbial treatments tested here had no effect on the phloem and cell-content feeders (whiteflies and thrips respectively), but most fungal inoculants prominently reduced the incidence of *T. absoluta*, that is, a chewing-biting insect that triggers JA related plant responses (D'Esposito *et al.*, 2021).

Compatibility of MIR with IPM practices

The successful implementation of Microbe-Induced Resistance in agriculture does not only rely on its effectiveness to control pests and pathogens but also on its compatibility with other strategies regularly used in integrated pest management (IPM) (Stenberg, 2017). As this study was performed applying the commonly used pest management practices based on integrated pest management, we also evaluated the effect of the microbial inoculation on other biocontrol agents such as the polyphagous mirid bug *Nesidiocoris tenuis*. Our results did not show any effect of the inoculations on the incidence of the predator, suggesting that Microbe-Induced Resistance is compatible with the release of this predator, which is commonly used IPM programs.

Fungal inoculants increased plant resistance to Tuta absoluta without compromising plant yield

Plant defense responses often result in fitness costs for the plants, as plants need to fine-tune resource allocation and prioritize between defense or growth and reproduction depending on the environmental conditions, which is commonly known as defense-growth trade-off (Züst & Agrawal, 2017; He *et al.*, 2022). Our results showed a significant reduction in the incidence of *T. absoluta* in fungal-inoculated plants, but remarkably, this enhanced resistance was not associated with significant costs in terms of plant growth nor yield. In fact, none of the microbial treatments negatively impacted fruit production. As no differences were found regarding the nutritional status of the plants, it is tempting to speculate that the increased yield in those treatments may be associated with the enhanced stress tolerance/resistance conferred by the microbial symbionts to the inoculated plants. Indeed, not only biotic, but also abiotic stresses such as temperature changes, including heat or cold shocks, that affect fruit production, are common during the crop cycle. Thus, an increased plant resilience may underlie the observed better performance of microbe-inoculated plants.

Overall, this study highlights the potential of rhizosphere microorganisms to improve crop productivity and resistance to important pests such as the devastating leaf miner *T. absoluta* in real agricultural settings. Testing microbial strains previously characterized under controlled lab conditions under real commercial production conditions allow us to identify beneficial microbes that are competent, stable and functional under varying

conditions. The identification of microbes effectively improving plant health and productivity under real crop production settings will contribute to a faster and wider adoption of the use of microbial inoculants for crop protection and to improve agriculture sustainability.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Irrigation scheme and nutrient supply during the cropping season.

Watering				Nutrients supplied with watering																	
Date	Days after transplantation	Water supply L/day/plant	Accumulated water supply (L/plant)	Period	CE ds/m	pH	HC03 mg/L	SO4 mg/L	NO3 mg/L	Cl mg/L	Na mg/L	K mg/L	Ca mg/L	Mg mg/L	PO4 mg/L	NH4 mg/L	Fe mg/L	Cu mg/L	Mn mg/L	Zn mg/L	B mg/L
2/9/20	0	0,755	0,755	2/9/20 - 22/9/20	3,01	6,2	85,42	284,89	359,3	525,76	181	264	160	70	40,27	24,44	1,33	0,16	1,22	0,53	0,17
3/9/20	1	0,755	1,510																		
6/9/20	4	0,755	2,265																		
9/9/20	7	0,755	3,020																		
12/9/20	10	1,510	4,530																		
16/9/20	14	1,510	6,040																		
18/9/20	16	1,512	7,552																		
20/9/20	18	1,855	9,407																		
22/9/20	20	1,855	11,262	22/9/20 - 9/10/20	2,99	6,2	73,22	288,89	423,08	508,01	181	247	156	70	25,72	23,23	1,08	0,13	0,97	0,41	0,09
24/9/20	22	1,658	12,919																		
26/9/20	24	0,341	13,260																		
28/9/20	26	1,243	14,503																		
29/9/20	27	1,243	15,746																		
1/10/20	29	1,699	17,446																		
3/10/20	31	1,492	18,938																		
5/10/20	33	1,866	20,804																		
7/10/20	35	1,867	22,671																		
9/10/20	37	1,859	24,529	9/10/20 - 23/10/20	2,93	6,2	82,37	296,84	374,86	508,01	182	253	152	76	47,3		1,29	0,1	0,97	0,41	0,16
11/10/20	39	2,126	26,656																		
13/10/20	41	2,126	28,782																		
14/10/20	42	2,253	31,035																		
17/10/20	45	2,253	33,288																		
19/10/20	47	2,439	35,727																		
21/10/20	49	2,439	38,167																		
23/10/20	51	2,388	40,555	23/10/20 - 12/11/20	3,1	5,9	64,07	282,94	419,96	561,26	183	254	171	77	16,97	21,6	1,35	0,12	1,05	0,48	0,26
25/10/20	53	3,053	43,608																		
27/10/20	55	3,053	46,662																		
29/10/20	57	3,032	49,694																		
31/10/20	59	3,032	52,726																		
2/11/20	61	2,624	55,350																		
4/11/20	63	2,624	57,974																		
6/11/20	65	2,722	60,696																		
8/11/20	67	2,253	62,949																		
10/11/20	69	2,253	65,202																		
12/11/20	71	2,407	67,609	12/11/20 - 24/11/20	3,34	6	73,22	280,69	356,19	561,26	221	268	100	79	18,64	22,23	0,82	0,1	0,76	0,35	0,16
14/11/20	73	2,407	70,015																		
16/11/20	75	2,407	72,422																		
18/11/20	77	2,393	74,815																		
20/11/20	79	2,409	77,225																		
22/11/20	81	2,411	79,636																		
24/11/20	83	2,526	82,162	24/11/20 - 30/12/20	3,49	5,8	61,02	448,8	331,31	614,51	221	465	112	68	39,11	30,51	1,04	0,06	1,14	0,45	0,31
26/11/20	85	2,036	84,198																		
29/11/20	88	2,144	86,343																		
1/12/20	90	2,144	88,487																		
3/12/20	92	2,144	90,632																		
6/12/20	95	2,144	92,776																		
8/12/20	97	2,144	94,921																		
10/12/20	99	2,144	97,065																		
13/12/20	102	2,144	99,210																		
15/12/20	104	2,156	101,365																		
17/12/20	106	2,156	103,521																		
19/12/20	108	1,627	105,148																		
21/12/20	110	1,627	106,775																		
23/12/20	112	1,919	108,694																		
25/12/20	114	1,919	110,613																		
28/12/20	117	1,919	112,532																		
30/12/20	119	1,883	114,414	30/12/20 - 26/1/21	3,34	6,4	85,42	311,24	517,96	561,26	190	503	116	70	35,16	19,16	1,73	0,27	1,36	0,92	0,36
2/1/21	122	1,883	116,297																		
4/1/21	124	1,883	118,180																		
6/1/21	126	1,574	119,754																		
10/1/21	130	1,289	121,043																		
13/1/21	133	1,289	122,332																		
15/1/21	135	1,289	123,620																		
17/1/21	137	1,289	124,909																		
19/1/21	139	1,289	126,197																		
21/1/21	141	1,139	127,336																		
24/1/21	144	1,139	128,475																		
26/1/21	146	1,139	129,613	26/1/21 - 21/2/21	3,49	6	79,32	319,59	552,18	561,26	192	485	121	70	33,01	18,31	1,71	0,21	1,25	0,85	0,36
28/1/21	148	1,181	130,794																		
30/1/21	150	1,930	132,724																		
1/2/21	152	1,930	134,654																		
3/2/21	154	1,918	136,572																		
5/2/21	156	2,101	138,673																		
8/2/21	159	2,101	140,773																		
11/2/21	162	2,101	142,874																		
13/2/21	164	2,101	144,975																		
15/2/21	166	2,101	147,075																		
17/2/21	168	2,183	149,258																		
19/2/21	170	1,680	150,938																		
21/2/21	172	1,680	152,618																		

Supplementary Table 2. Overview of the statistical analyses performed on each of the 17 variables measured during the experiment.

Theme	Variable	Unit	N	Model	Distribution	Blocking factors
Plant growth, nutritional status and flowering	Plant height	Length (cm)	284	lm	Normal	block
	CN	Ratio	144	lm	Normal	block
	Flowers	Count	144	lm	Normal	block
Fruit yield	Tomato production	Weight (g)	480	glm	Quasipoisson	day + day/block
						block
Fruit quality and nutraceutical value	ºBrix	Relative amount	48	lm	Normal	block
	%Acidity	Percentage	48	lm	Normal	block
	Maturity index	Ratio	48	lm	Normal	block
	%Dry weight	Percentage	48	lm	Normal	block
	Total polyphenols	Relative amount	48	lm	Normal	block
	Lycopene	Relative amount	48	lm	Normal	block
	b-carotene	Relative amount	48	lm	Normal	block
	Caroteoinds	Relative amount	48	lm	Normal	block
Insect incidence	Thrips	Count	144	glm	Quasipoisson	block
	T. absoluta	Presence/absence	284	glm	Binomial	block
	N. tenuis	Count	48	lm	Normal	block
	Whiteflies	Count	48	lm	Normal	block
Pathogen incidence	Powdery mildew	Presence/absence	144	glm	Binomial	block

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CHAPTER 4

CHAPTER 4:

Microbe-Induced Resistance against the tomato leaf miner *Tuta absoluta:* a novel addition to the integrated management toolbox?

ABSTRACT

The tomato leaf miner *Tuta absoluta* is an invasive insect pest and one of the major threats to the global tomato production. The combination of several strategies to manage this pest, including pesticides, natural enemies, mass trapping and mating disruption using pheromones, as well as agronomic and cultural control, is not sufficient to lower its incidence under the targeted economic threshold. Despite widely documented on diverse model systems, little is known about the potential of Microbe-Induced Resistance against this devastating leaf miner. We hypothesize that Microbe-Induced Resistance could be a valuable tool to include in the integrated pest management programs currently used in modern agriculture, to lift its control below the targeted threshold. In this study we tested the potential of an array of diverse soil beneficial bacteria and fungi alone or combined to enhance plant resistance against *T. absoluta*, and explored the possible underlying mechanisms. As a first step, we performed different bioassays ranging from controlled detached leaf assays, semi-controlled whole plant bioassays to tomato production under commercial conditions, and identified inoculants that consistently reduced *T. absoluta* performance. Subsequently, to identify the possible mechanisms mediating the increase in plant resistance, we performed an untargeted metabolomics analysis to identify defense related metabolites with primed accumulation in the plants displaying induced resistance. Lastly, a functional analysis of some of the identified primed metabolites was performed to test their potential anti-herbivory activity. Our results showed that the fungal inoculants, including *Trichoderma harzianum,* and the arbuscular mycorrhizal fungi *Rhizophagus irregularis* and *Funneliformis mosseae,* significantly reduced the performance or incidence of this foliar pest under all the conditions tested, including standard crop management in commercial greenhouses in Southern Europe. We found that these beneficial fungi can enhance plant defense responses through metabolic

reprograming and primed accumulation of defensive compounds. Among the primed compounds, azelaic acid and feruloyl putrescine, over-accumulated in plants displaying induced resistance, were shown to inhibit *T. absoluta* development when exogenously applied to tomato plants. These results add to the growing body of evidence on the potential of Microbe-Induced Resistance in biocontrol programs, specially against key pests like *T. absoluta* in tomato production.

INTRODUCTION

The tomato leaf miner *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), recently reinstated as *Phthorimaea absoluta* (Chang *et al.*, 2021), is a devastating invasive pest native to South America (Desneux *et al.*, 2011), being tomato -*Solanum lycopersicum*- its main host plant (Desneux *et al.*, 2010). After being detected in Spain in 2006, *T. absoluta* has rapidly spread across the Mediterranean basin and Europe (Desneux *et al.*, 2010, 2011). Currently it has been detected in more than 100 countries across South America, Europe, Africa and Asia (EPPO, 2021), and is considered a major threat to global greenhouse and open-field tomato production (Desneux *et al.*, 2010; Biondi *et al.*, 2018). The insect larvae feed and cause damage on leaves, stems and fruits, and without control measures the pest can origin up to 80-100% of crop losses in tomato (Desneux *et al.*, 2010), thus causing important economic losses. In The Netherlands, even with correctly executed control measures against *T. absoluta*, fruit damage levels of 1-5% are expected with an estimated economic loss of 5-25 million euros per year (Potting *et al*., 2013). The control measures used are additionally increasing tomato crop production costs by 3.7 million per year in the case of The Netherlands (Potting *et al*., 2013) and 240-480 million euros (100-150 euros per hectare) per year in Spain (Desneux *et al.*, 2011).

Among the pest management practices currently used to control this insect, chemical pesticides are still widely used (Biondi *et al.*, 2018; Desneux *et al.*, 2021). However, the development of insecticides resistance in the pest is seriously compromising the efficacy of chemical control (Biondi *et al.*, 2018; Guedes *et al.*, 2019). In addition, the intensive use of chemical pesticides has a negative impact on non-target insects such as pollinators and natural enemies. Numerous studies have demonstrated the side effects of several chemical insecticides, commonly used to control *T. absoluta*, on beneficial insects used in biocontrol like predators or parasitoids (Arnó & Gabarra, 2011; Soares *et al.*, 2019; Nozad-Bonab *et al.*, 2021). Alternative, new approaches aim to exploit plant-insect-microbe interactions for the biocontrol of this pest. Nozad-Bonab *et al*. (2021) showed that the entomopathogenic fungus *Metarhizium anisopliae* is safer for the parasitoid *Trichogramma brassicae* than chemicals when applied simultaneously with the parasitoid for the control of *T. absoluta*. There was also a synergistic effect of the combined use of both BCAs on the pest´s egg viability, thus, providing more efficient control than the combination of chemicals and the parasitoid (Nozad-Bonab *et al.*, 2021). The

implementation of integrated pest management (IPM) programs has led to a significant reduction of chemical pesticides in the management of this pest in affected areas worldwide, and especially in the early-invaded areas as the Mediterranean region and Central Europe (Desneux *et al.*, 2021). IPM programs integrate to chemical control an array of sustainable measures, from biological control using entomopathogenic microorganisms and natural enemies, to pest monitoring, mass trapping and mating disruption using pheromones together with numerous agronomic and cultural practices (Biondi *et al.*, 2018; Desneux *et al.*, 2021).

Early after *T. absoluta* invaded Europe, polyphagous predators like the commercially available mirid bugs *Nesidiocoris tenuis* or *Macrolophus pigmeus* has been demonstrated to prey on *T. absoluta* eggs and larvae (Urbaneja *et al.*, 2009). The release of these predators in seedling nurseries was shown to be an effective strategy for the biological control of the pest (Calvo *et al.*, 2012; Urbaneja *et al.*, 2012). Egg parasitoids from the family of Trichogrammatidae and larval parasitoids such as *Necremnus tutae*, *Dineulophus phthorimaeae*, *Pseudapanteles dignus* and *Dolichogenidea gelechiidivoris* have also shown their effectiveness as BCAs for *T. absoluta* management (Desneux *et al.*, 2021). Commercial formulations of the entomopathogenic bacterium *Bacillus thuringiensis* have been demonstrated to be highly effective to control *T. absoluta* and are currently widely used in IPM programs in tomato production (Biondi *et al.*, 2018; Desneux *et al.*, 2021). Microorganisms can also reduce *T. absoluta* performance through mechanisms independent of direct pathogenicity, for example by stimulating the plant defense mechanisms. Surprisingly, the possible contribution of Microbe-Induced Resistance (Microbe-IR) to IPM programs remains unexploited.

Many soil-borne microorganisms live in association with plant roots, establishing mutualistic interactions and improving plant health (Mendes *et al.*, 2013). The association of plants with some of these beneficial microorganisms can stimulate plant defenses leading to Microbe-IR to a broad spectrum of pests and pathogens (Pieterse *et al.*, 2014; De Kesel *et al.*, 2021). Plant-growth promoting rhizobacteria such as *Bacillus* spp and *Pseudomonas* spp, fungi from the genus *Trichoderma* and arbuscular mycorrhizal fungi (AMF) are among the most studied plant-associated microorganisms able to trigger Microbe-IR to diverse pests and diseases (Pozo & Azcón-Aguilar, 2007; Jung *et al.*, 2012; Pieterse *et al.*, 2014; Pineda *et al.*, 2015; Woo *et al.*, 2022). Microbe-IR commonly involve a faster and a more effective plant defense responses upon biotic or abiotic

challenges, a phenomenon known as defense priming (Mauch-Mani *et al.*, 2017). Usually, defense priming by beneficials is mediated by the jasmonate (JA) signalling pathway (Song *et al.*, 2013; Pieterse *et al.*, 2014; Gruden *et al.*, 2020). This pathway promote the synthesis of antifeedant proteins and the activation of the secondary metabolism, often contributing to the production of defensive compounds toxic to herbivores (Erb & Reymond, 2019). Primed accumulation of anti-herbivore chemical defenses has been confirmed to contribute to Microbe-IR against insect herbivores. For example, root colonization by the AMF *Funneliformis mosseae* primes the accumulation in leaves of defensive compounds including alkaloids, fatty acid derivatives and phenylpropanoid-polyamine conjugates upon attack by the herbivore *Spodoptera exigua* (Rivero *et al.*, 2021). Similarly, *Trichoderma harzianum* T22 inoculation lead to the over-accumulation of several alkaloids, phenolic acids and flavonoids in response to the aphid *Macrosiphum euphorbiae*, likely contributing to the reduced aphid survival observed (Coppola *et al.*, 2019). Recent reports also suggest the efficacy of Microbe-IR against *T. absoluta*. For example, tomato and eggplant seed inoculation with *Trichoderma asperellum*, *Beauveria bassiana* and *Hypocrea lixii* reduced *T. absoluta* performance under controlled conditions, but the underlying mechanisms were not explored (Agbessenou *et al.*, 2020). Recently, enhanced plant resistance to *T. absoluta* was observed in tomato upon root colonization by AMF (Shafiei *et al.*, 2022) or *T. harzianum* T22 (Aprile *et al.*, 2022).

Although Microbe-IR potential for plant protection is well documented under controlled conditions, studies confirming its efficacy under agronomic conditions are scarce. Microbe-IR appears to be highly context-dependent, and its functionality is often influenced by diverse biotic and abiotic factors. As a consequence, its application under the ever-changing field conditions frequently results unpredictable (Lee Díaz *et al.*, 2021). Therefore, efficacy trials under real production conditions are crucial to further validate the potential of microbial-IR and its compatibility and synergism with commonly used IPM practices for crop protection. Although Microbe-IR is presumably a suitable strategy to further improve current IPM programs (Stenberg, 2017), field research is required to properly evaluate its efficacy and full potential in agriculture. Therefore, further research is needed on the efficacy and consistency of Microbe-IR against *T. absoluta* under different conditions and on the underlying mechanisms, aiming to optimize Microbe-IR potential to control this devastating pest.

In the present study we aimed to investigate the ability of different well characterized, soil-borne beneficial bacteria and fungi, individually or in combination, to induce resistance against the major pest *T. absoluta* under different settings, and to explore the possible underlying mechanisms. For this, Microbe-IR bioassays were first performed under controlled conditions, reproduced under semi-controlled conditions and finally validated under real tomato production conditions incorporating standard IPM strategies for *T. absoluta* control. Following an untargeted metabolomics approach we explored the potential differential accumulation of defensive compounds. Primed accumulation of defense-related secondary metabolites were identified in the induced plants. A functional analysis of these metabolites confirmed their negative impact on *T. absoluta* development. Thus, our results pinpoint consistent IR inducing microbial strains with fungi performing better, efficiently controlling *T. absoluta*, and revealed the role of some bioactive compounds in this IR. The results contribute to our understanding of microbe IR and highlight their potential as useful tools to be integrated in IPM programs for crop protection.

MATERIAL AND METHODS

1. Beneficial microorganisms, growing conditions, and inoculum preparation

B. amyloliquefaciens CECT8238 was cultured on tryptone soya agar (TSA, Oxoid) for 24h at 28°C. 25ml of DSM (Difco sporulation medium) (Nicholson & Setlow, 1990) was inoculated with a single colony from the TSA culture and incubated for 48h at 28°C in a rotatory shaker (200rpm). The spore concentration was quantified using a Bürker-Türk counting chamber, centrifuged at 5000 rpm for 15min and the spores were re-suspended in sterile tap water to a final concentration of 1 x 10^7 spores/ml (Minchev *et al.*, 2021).

P. azotoformans F30A was cultured on TSA for 24h at 28°C. Liquid pre-culture was prepared using tryptone soya broth (TSB, Oxoid) inoculated with a single bacterial colony and incubated overnight at 28°C with rotary shaking at 200rpm. 25ml of TSB media was inoculated with 1ml of pre-culture and placed in a rotatory shaker (200rpm) at 28°C. After 150mins of incubation, with bacterial growth in exponential phase, the cell concentration was calculated measuring the O.D. (620nm). The bacterial culture was centrifuged at

5000 rpm for 15 min and the bacterial cells were re-suspended in sterile tap water to a final concentration of 1 x 10^7 cfu/ml (Minchev *et al.*, 2021).

T. harzianum T22 was grown on potato dextrose agar (PDA, Difco) for 7 days at room temperature. Spores were collected from sporulating plates in sterile tap water, the concentration of the spore suspension was quantified using a Bürker-Türk counting chamber and adjusted to 1 x 10^7 spores/ml (Minchev *et al.*, 2021).

R. irregularis MUCL 57021 was grown *in vitro* on minimal (M) medium using Agrobacterium rhizogenes - transformed carrot (*Daucus carota*) roots as a host (St-Arnaud *et al.*, 1996). For spore extraction, citrate buffer 0.01M (pH=6) was added to a sporulating culture in a proportion 3:1 (v/v) and placed in a rotary shaker for one hour to dissolve the agar. *R. irregularis* spores were recovered from the solution using sieves with different sizes (250 and 53 µm) and re-suspended in sterile tap water at final concentrations 1000 spores/ml (Minchev *et al.*, 2021).

Funneliformis mosseae BEG12 was maintained in an open pot culture of *Trifolium repens* mixed with *Sorghum vulgare* plants growing in a vermiculite-sepiolite (1:1) substrate in a greenhouse. The inoculum consisted of substrate containing infected root fragments, mycelia and spores (Rivero *et al.*, 2021).

2. Plant material and growing conditions

Solanum lycopersicum cv Money maker seeds (Vreeken's Zaden, The Netherlands) were surface sterilized by immersion in 5% sodium hypochlorite solution for 10 min followed by at least 3 washing steps in sterile water for 10 min each. The surface sterilized seeds were sown in a sterile vermiculite and incubated for 7 days in a greenhouse at 24°C : 18°C day : night with a photoperiod 16 h: 8 h light : dark and 60% of relative humidity. Tomato seedlings were transferred to 300 ml pots containing gamma irradiated nutrient poor sandy soil (BVB, The Netherlands): sterile vermiculite (1:1) mixture and inoculated with the microbial treatments described below. Inoculated plants were randomly distributed and grown for 6 weeks in a greenhouse with the same climatic conditions described above. Plants were watered once per week with water and twice per week with Long Ashton nutrient solution (Hewitt, 1966) but with reduced phosphorous concentration (50% of the standard concentration) to ensure mycorrhizal establishment.

3. *Tuta absoluta* rearing

Tuta absoluta colony was maintained, at 22°C with photoperiod 16h:8h day:night and 60% of relative humidity, in rearing cage of 60cm x 60cm x 60cm (length x width x

height) with tomato (*Solanum lycopersicum* cv Money maker) plants as a host. New tomato plants were exposed to *T. absoluta* adults for 24h for oviposition. After egg hatching,larvae were left to reach L2 instar and used in the bioassay.

4. Microbial treatments

B. amyloliquefaciens (Ba), *P. azotoformans* (Pa) and *T. harzianum* (Th) were inoculated by pipetting the microbial suspensions to the roots during transplantation at a concentration 1 x 10^7 cfu/plant. *Rhizophagus irregularis* (Ri)was applied by pipetting the spore suspension to the roots at a concentration 1000 spores/plant. *Funneliformis mosseae* (Fm) inoculation was done by mixing the growing substrate with 10% (v : v) of *F. mosseae* inoculum. The microbial consortium treatment (SynCom) was composed by a combination of *B. amyloliquefaciens*, *P. azotoformans*, *T. harzianum* and *R. irregularis* applied to the roots at the same concentration as described for the single microbial inoculations. A non-inoculated control treatment was included where only water without any microbial propagules was added to the roots.

5. Root mycorrhizal colonization

Roots were washed upon harvesting, cleared with 10% KOH and stained with 5% ink (Lamy, Germany) in 2% acetic acid (García *et al.*, 2020). The percentage of root length colonized by the AMF was quantified using the gridline intersection method (Giovannetti & Mosse, 1980) under a stereomicroscope Motic SMZ.

6. Plant and insect bioassays

6.1 Controlled conditions

A total of nineteen six week old plants per treatment were used. The third true leaf of each plant was detached using a scalpel and placed in a petri dish (150 mm diameter) with filter paper on the bottom previously moistened with 3 ml of sterile water to prevent desiccation. Each leaf was infested with two second instar *T. absoluta* larvae. All petri dishes with the infested leaves were maintained at 22°C until the emergence of the *T. absoluta* adults (**Figure 1**). The percentage of larvae that reached adult stage was evaluated for each treatment.

6.2 Semi-controlled conditions

A total of twelve six week old plants per treatment were placed in individual rearing cages (30 cm x 30 cm x 30 cm) and infested with three second instar *T. absoluta* larvae on the third true leaf of each plant. All cages with the infested plants were placed in a greenhouse without any control of the climatic conditions (**Figure 1**). Plants were maintained until the end of the bioassay with the same watering regime and nutrient supply as described above. 48 hours after infestation, leaflets from the infested plants presenting damage by *T. absoluta* and leaflets from non-infested plants from all treatments were collected separately, immediately frozen in liquid nitrogen and stored at -80°C until their use for metabolomic analysis. Three weeks after infestation, when all survived larvae reached the pupal stage, pupae from each plant were collected, placed separately in plastic cups and incubated at 22°C until adult emergence. The percentage of larvae reaching the pupal and adult stages were evaluated for each treatment.

6.3 Commercial production conditions

One week old seedlings were transferred to starting trays -with cell dimensions 2,9 x 2,9 x 6,8cm- containing blond seedling peat (Kekkilä LSM 0 R8406, Projar, Valencia, Spain) : sepiolite : perlite (1:1:1) mixture and inoculated with the microbial treatments described previously. Inoculated seedlings were grown in commercial nursery (ACRENA SAT 251, El Ejido, Spain; 36°, 47', 52.9"N; 2°, 43', 36.3"W) for 4 weeks. The plants were then transplanted to a commercial production greenhouse (Estación experimental Cajamar, Paraje las Palmerillas, El Ejido, Almería; 36°, 47', 36.3"N; 2°, 43', 15.2"W). Two weeks after transplanting, common IPM for *T. absoluta* were introduced consisting of release of the predatory mirid bug *Nesidiocoris tenuis* (Hemiptera: Miridae) in a density of 0,5 – 1,5 individuals/m2 weeks after, and mating disruption using pheromones. Three months after transplantation, when the plants were in mature fruiting stage, the natural infestation of *T. absoluta* was evaluated as the percentage of plants presenting damage by the leaf miner for each treatment (**Figure 1**).

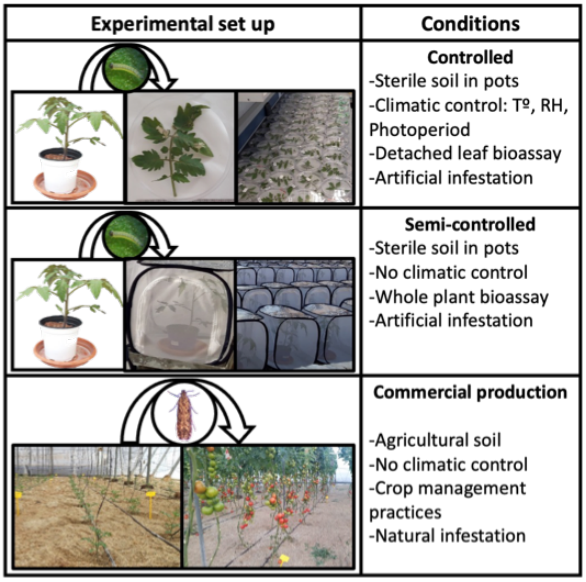

Figure 1. Schematic representation of the plant bioassays performed under controlled, semi-controlled and commercial production condition.

6.4 Functional analysis of primed compounds

Compounds with primed accumulation in microbial inoculated plants in response to *T. absoluta* were purchased to test their effect on the insect development. The treatments tested were azelaic acid (AZA, Sigma Aldrich, Germany) and feruloylputreacine (FP, AKos Consulting & Solutions Deutschland GmbH, Germany). Six weeks old plants without any microbial inoculation were used for the bioassay. One fully developed leaf from each plant was detached and the petiole dipped in 2ml of aqueous solution containing 100 ppm of the compounds. Control treatment was mock treated with water and without any of the tested compounds. Leaves were maintained until the full absorption of the aqueous solution, after that were placed in petri dishes (150 mm diameter) with filter paper on the bottom previously moistened with 3 ml of sterile water to prevent desiccation. Ten biological replicates were used for each treatment and each replicate was infested with three second instar *T. absoluta* larvae. All petri dishes with the infested leaves were maintained at room temperature (≈ 22°C) until the emergence of the *T. absoluta* adults. The percentage of larvae that reached adult stage was evaluated for each treatment.

7. Untargeted metabolomics:

7.1 LC-ESI full scan mass spectrometry

Thirty milligrams of freeze-dried leaf material (six biological replicates per treatment) were homogenized at 4°C in 1 ml of MeOH : H2O (30 : 70) containing 0.01% of HCOOH.

After that, the homogenate was centrifuged at 15 000g for 15 min at 4°C, the supernatant was recovered and filtered through 0.2 µm cellulose filters (Regenerated Cellulose Filter, 0.20 µm, 13 mm D. pk/100; Teknokroma). 20µl of the filtered supernatant were injected into an Acquity ultra performance liquid chromatography system (UPLC, Waters, Mildford, MA) interfaced with a hybrid quadrupole time-of-flight instrument (QTOF-MS Premier, Waters, Mildford, MA). Six independent biological replicates per treatment were randomly injected. To elute analytes, a gradient of methanol and water containing 0.01% HCOOH was used. The LC separation was performed using an UPLC Kinetex 2.6 µm particle size EVO C18 100 A, 50 × 2.1 mm (Phenomenex). Subsequently, a second fragmentation function was introduced into the TOF analyser to identify the signals detected. This function was programmed in a t-wave ranging from 5 to 45 eV to obtain a fragmentation spectrum of each analyte (Gamir *et al.*, 2014). Chromatographic conditions and solvent gradients were established as described by Gamir *et al*. (2014).

7.2 Full scan data analysis

Positive and negative electrospray ionization (ESI) signals were analysed independently to obtain a global view of the data conduct. For ESI positive, the instrument detected 3387 signals and, for ESI negative, 1878 signals. The raw data files acquired with the Masslynx 4.1 software (Masslynx 4.1, Waters) were transformed into .cdf files with Databridge tool. Chromatographic data files were processed with the software R using the XCMS algorithm (Smith *et al.*, 2006) to obtain the peak peaking, grouping and signal corrections. Metabolite amounts were analysed based on the normalized peak area units relative to the dry weight. Adduct and isotope correction, Kruskal–Wallis test ($p < 0.05$), filtering and clustering were carried out with the packages-MarVis filter and MarVis cluster that are integrated in the Marvis suit 2.0 (Kaever *et al.*, 2015). To obtain the overall behavior, data obtained in positive and negative ESI were combined using Marvis suit 2.0. Metabolite identification was carried out based on exact mass accuracy and fragmentation spectra matching with different online database. The database kegg (https://www.genome.jp/kegg/) was used for exact mass identity and for fragmentation spectrum analysis the Massbank and the Metlin databases were used (www.massbank.jp; www.masspec.scripps.edu).

8. Statistical analysis

Data were analysed using R statistical language, version 4.0.5 (R Development Core Team 2021) and figures were produced using the package ggplot2 (Wickham, 2009). The effect of the microbial treatments on the percentage of larvae reaching adult stage in the detached leaves bioassay and the comparisons between the microbial treatments and the control was assessed using a Pearson's Chi-squared test with Yates' continuity correction. In the whole plant bioassay, to test the effect of the microbial treatments on the percentage of larvae reaching pupal and adult stage, and the percentage of pupae reaching adult stage, and on the percentage of plants damaged by the insect in the commercial greenhouse experiment a generalized linear model with binomial distribution and logit link function and blocks as an error term was performed. The effect of the exogenous leaf application of the pure compounds on the percentage of larvae reaching adults was assessed with exact binomial test. Post-hoc comparisons among microbial treatments were based on a Fisher's LSD. Differences in the percentage of root length colonized by AMF, and the effect of microbial treatments and infestation on metabolite accumulation were assessed using a linear modeling. Model validation was performed graphically by inspecting the residuals and fitted values (Zuur & Ieno, 2016).

RESULTS

Different beneficial microbes including bacteria and fungi applied alone or as a microbial consortium (Syncom) were selected because of their confirmed ability to protect tomato plants against different pathogens (Minchev *et al.*, 2021). Here, we tested their ability to induce resistance against the tomato leaf miner *T. absoluta* under different growing conditions and experimental setups.

Microbial establishment in the rhizosphere and mycorrhizal colonization was confirmed at the end of each experiment, being all microbes detected in the soil samples of the treatments in which they were inoculated. Regarding mycorrhizal symbiosis establishment, root colonization was confirmed for all mycorrhizal treatments. Under controlled conditions, *R. irregularis* inoculated plants showed 83% and 76% of their root length colonized by the fungus in the Ri or SynCom treatments, respectively, while *F. mosseae* root colonization was 40% (**Figure 2A**). Under semi-controlled conditions, was

66% and 51% of the root systems were colonized for Ri and SynCom treatments respectively, whereas the *F. mosseae* colonization was 56% (**Figure 2B**). Intriguingly, while *T. absoluta* herbivory did not impact AMF colonization in the individual treatments, *R. irregularis root colonization* in the SynCom treatment significantly increased with the herbivory (**Figure 2B**).

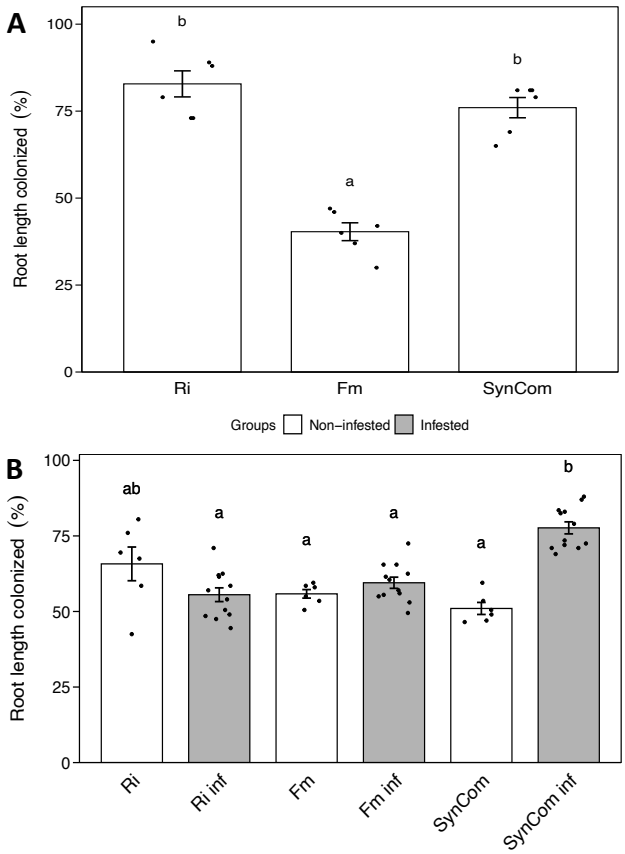

Figure 2. Mycorrhizal colonization under **(A)** controlled conditions and **(B)** semi-controlled conditions. Percentage of root length colonized by *R. irregularis* in plants inoculated with the fungus alone (Ri) or in consortium (SynCom) and by *F. mosseae* (Fm), infested (inf) or not with *T. absoluta*. Bars represent the mean percentage of root length colonized and error bars represent the standard error of the mean. Treatments not sharing a letter are statistically different based on (A) ANOVA followed by LSD ($p<0.05$, n=6) and (B) Kruskal-Wallis followed by Dunn test ($p<0.05$, non-infested n=6, infested n=12).

1. Microbial inoculation induces resistance against *Tuta absoluta* under controlled conditions

As a first step, we tested the capacity to induce systemic resistance in plants of the different beneficial soil-borne microorganisms by inoculating them in the soil at transplanting and growing the plants in a climate-controlled greenhouse, and addressing *T. absoluta* performance in a detached leaf bioassay. All microbial treatments reduced the percentage of *T. absoluta* larvae that reached the adult stage; although for *B. amyloliquefaciens, P. azotoformans*, *R. irregularis* and SynCom treatments this reduction

was not significant. Remarkably, *T. harzianum* and *F. mosseae* inoculations had a significant impact on the insect development reducing the percentage of larvae reaching adult stage by 38% compared to the non-inoculated control (**Figure 3**).

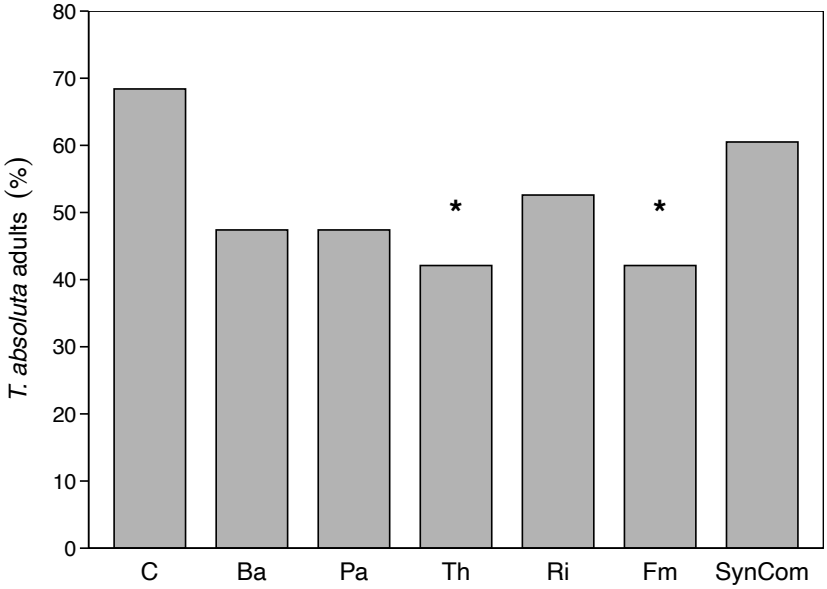

Figure 3. Microbe-Induced Resistance against *Tuta absoluta* under controlled conditions. Effect of microbial inoculation on the development of *T. absoluta* evaluated as percentage of larvae reaching adult stage. Plants were inoculated with *Bacillus amyloliquefaciens* (Ba), *Pseudomonas azotoformans* (Pa), *Trichoderma harzianum* (Th), *Rhizophagus irregularis* (Ri), *Funneliformis mosseae* (Fm) and microbial consortium (SynCom) including Ba + Pa + Th + Ri. Non inoculated plants were included as control. Plants were grown in a greenhouse with controlled climatic conditions and insect bioassay performed on detached leaves. Bars represent the percentage of *T. absoluta* larvae reaching adults for each treatment. Asterisks indicate statistically significant differences compared to the control based on Pearson's Chi-squared test with Yates' continuity correction ($p<0.05$, $n=38$).

2. Microbe-Induced Resistance under semi-controlled conditions

To test the induced resistance capacity under more realistic conditions, we tested the same microbial treatments inoculating tomato plants and growing them in a greenhouse without any climatic control, and *T. absoluta* infestation was performed on whole plants. Again, all the microbial treatments reduced the percentage of larvae that reached adult stage, but in *P. azotoformans* and the SynCom treatments the reduction was not significant (**Figure 4**). In contrast *B. amyloliquefaciens*, *T. harzianum*, *R. irregularis* and *F. mosseae* significantly reduced the percentage of larvae reaching the adult stage with 53%, 70%, 60% and 83% respectively as compared to the non-inoculated control (**Figure 4**), confirming the efficacy of the fungal inoculants to enhance plant resistance against *T. absoluta.*

This reduction in the number of adults resulted from the cumulative negative effect of the microbial inoculations on the insect life cycle, with a non-significant reduction on the proportion of individuals reaching the pupal stage by 22%, 38%, 38% and 44% reduction

in *B. amyloliquefaciens*, *T. harzianum*, *R. irregularis* and *F. mosseae* treatments, respectively, (**Figure 5A**), and a significant reduction of the percentage of pupae reaching adult stage by 32%, 47% and 65% in *B. amyloliquefaciens*, *T. harzianum* and *F. mosseae* respectively, compared to the non-inoculated control (**Figure 5B**).

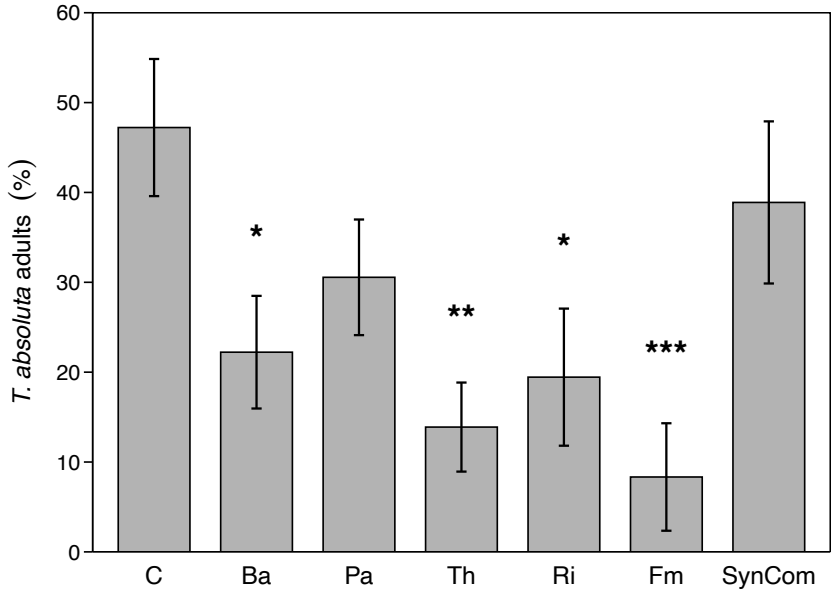

Figure 4. Microbe-Induced Resistance against *Tuta absoluta* under semi-controlled conditions. Effect of microbial inoculation on the development of *T. absoluta* evaluated as percentage of larvae reaching adult stage. Plants were inoculated with *Bacillus amyloliquefaciens* (Ba), *Pseudomonas azotoformans* (Pa), *Trichoderma harzianum* (Th), *Rhizophagus irregularis* (Ri), *Funneliformis mosseae* (Fm) and microbial consortium (SynCom) including Ba + Pa + Th + Ri. Non inoculated plants were included as control. Plants were grown in a greenhouse with controlled climatic conditions and the insect bioassay was performed on whole plants in insect cages in a greenhouse without any climatic control. Bars represent mean percentage of *T. absoluta* larvae reaching adults for each treatment and error bars represent standard errors of the means. Asterisks indicate statistically significant differences compared to the control based on generalized linear model with binomial distribution and logit link function followed by LSD (*p<0.05, **p<0.01, ***p<0.001, n=12).

3. Microbe-Induced Resistance against *Tuta absoluta* occurs under production conditions

Finally, we evaluated the efficacy of the microbial inoculations to reduce the incidence of *T. absoluta* natural infestation under tomato production conditions incorporating different IPM strategies. In average 42% of the non-inoculated control plants presented leaves with damage by *T. absoluta* feeding, indicating a moderate incidence of the pest on the crop. Remarkably, the pest incidence was reduced significantly reduced in *T. harzianum*, *R. irregularis* and *F. mosseae* inoculated plants. In particular, only 13% of the plants presented damage by *T. absoluta*, in the case *T. harzianum* and *F. mosseae* treatments, and the percentage of damaged plants was drastically reduced to only 4% in *R. irregularis* inoculated plants (**Figure 6**). Therefore, fungal inoculants showed again a

prominent negative impact on *T. absoluta,* even in a context where other control methods were already in use, confirming their efficacy to control the pest not only under controlled conditions but also under real tomato production conditions.

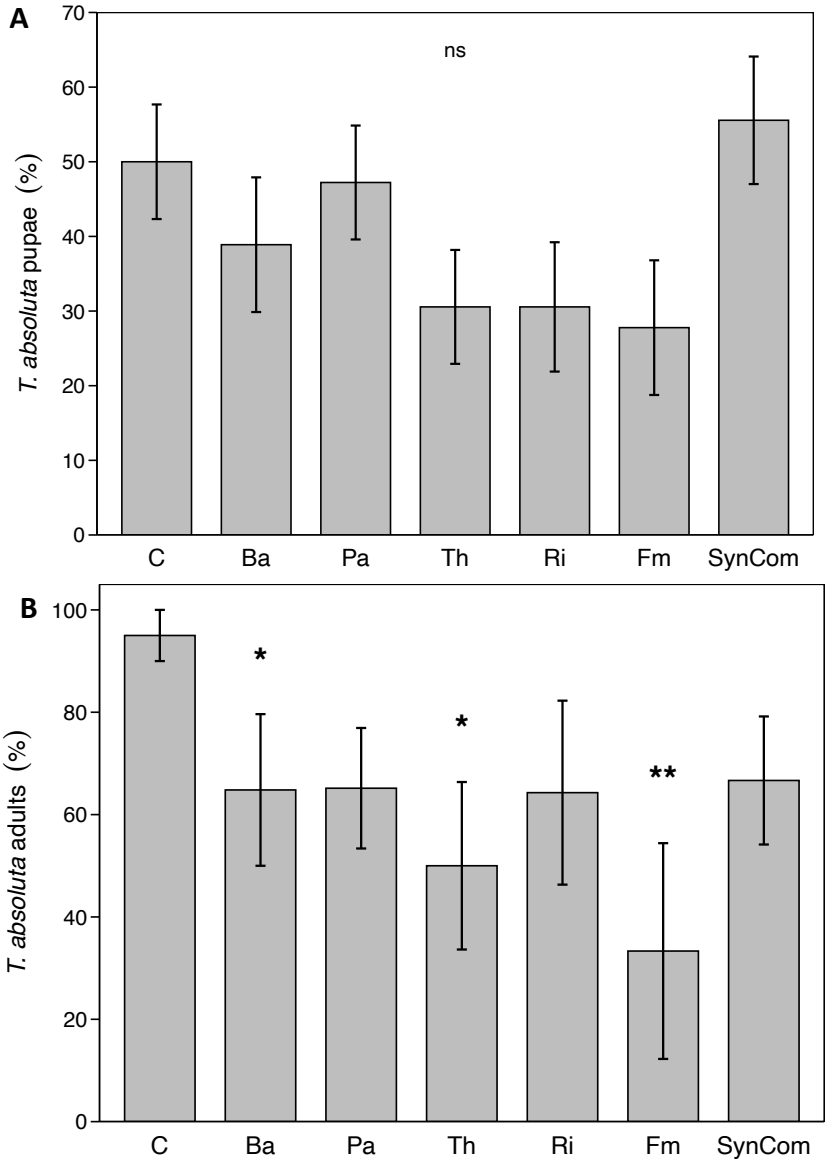

Figure 5. Microbe-Induced Resistance against *Tuta absoluta* under semi-controlled conditions. Effect of microbial inoculation on the development of *T. absoluta* evaluated as **(A)** percentage of larvae reaching pupal stage and **(B)** percentage of pupae reaching adult stage. Plants were inoculated with *Bacillus amyloliquefaciens* (Ba), *Pseudomonas azotoformans* (Pa), *Trichoderma harzianum* (Th), *Rhizophagus irregularis* (Ri), *Funneliformis mosseae* (Fm) and microbial consortium (SynCom) including Ba + Pa + Th + Ri. Non inoculated plants were included as control. Plants were grown in a greenhouse with controlled climatic conditions and the insect bioassay was performed on whole plants in insect cages in a greenhouse without any climatic control. Bars represent mean percentage of *T. absoluta* larvae reaching adults for each treatment and error bars represent standard errors of the means. "ns" means non-significant and asterisks indicate statistically significant differences compared to the control based on generalized linear model with binomial distribution and logit link function followed by LSD (*p<0.05, **p<00.1, n=12).

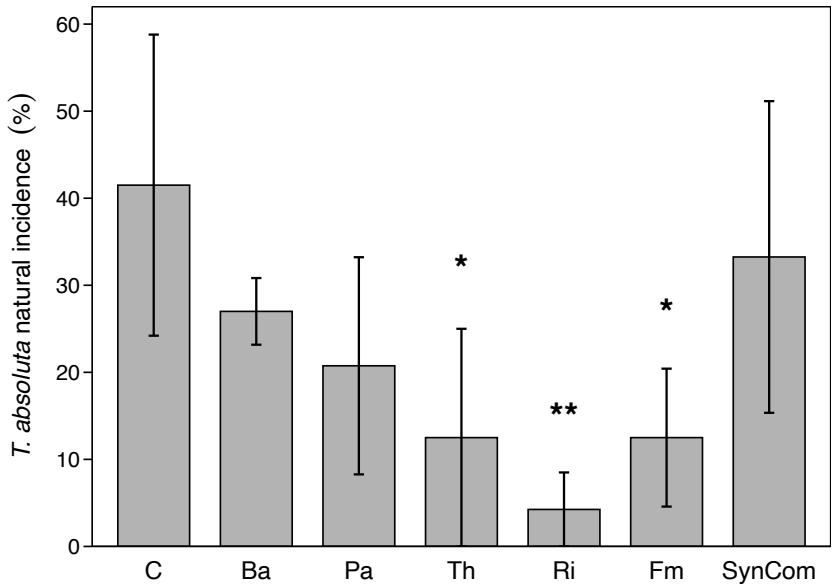

Figure 6. Microbe-Induced Resistance against *Tuta absoluta* under production conditions. Effect of microbial inoculation on the natural incidence of *T. absoluta* evaluated as percentage of plants damaged by insect feeding. Plants were inoculated with *Bacillus amyloliquefaciens* (Ba), *Pseudomonas azotoformans* (Pa), *Trichoderma harzianum* (Th), *Rhizophagus irregularis* (Ri), *Funneliformis mosseae* (Fm) and microbial consortium (SynCom) including Ba + Pa + Th + Ri. Non inoculated plants were included as control. Plants were grown in a production greenhouse without any climatic control. Bars represent mean percentage of plants presenting damage by *T. absoluta* larvae for each treatment and error bars represent standard errors of the means. Asterisks indicate statistically significant differences compared to the control based on generalized linear model with binomial distribution and logit link function followed by LSD (*p<0.05, **p<00.1, n=4).

4. Differential metabolic reprograming in response to *Tuta absoluta* in microbe-inoculated plants

In search of possible mechanisms related to the consistent plant resistance conferred by the fungal inoculants against the herbivore, we conducted an untargeted analysis of the leaf metabolic profile 48h after *T. absoluta* infestation. We analysed leaf samples from non-inoculated control plants, and *T. harzianum*, *R. irregularis*, *F. mosseae* and SynCom inoculated plants, infested or not with the herbivore. After combining data from positive and negative ESI and performing a Kruskal-Wallis test ($p<0.05$), 485 signals with statistically significant changes among treatments were detected. Heatmap analysis of these signals revealed an overall metabolic rearrangement in leaves 48h post infestation with *T. absoluta* (**Figure 7**).

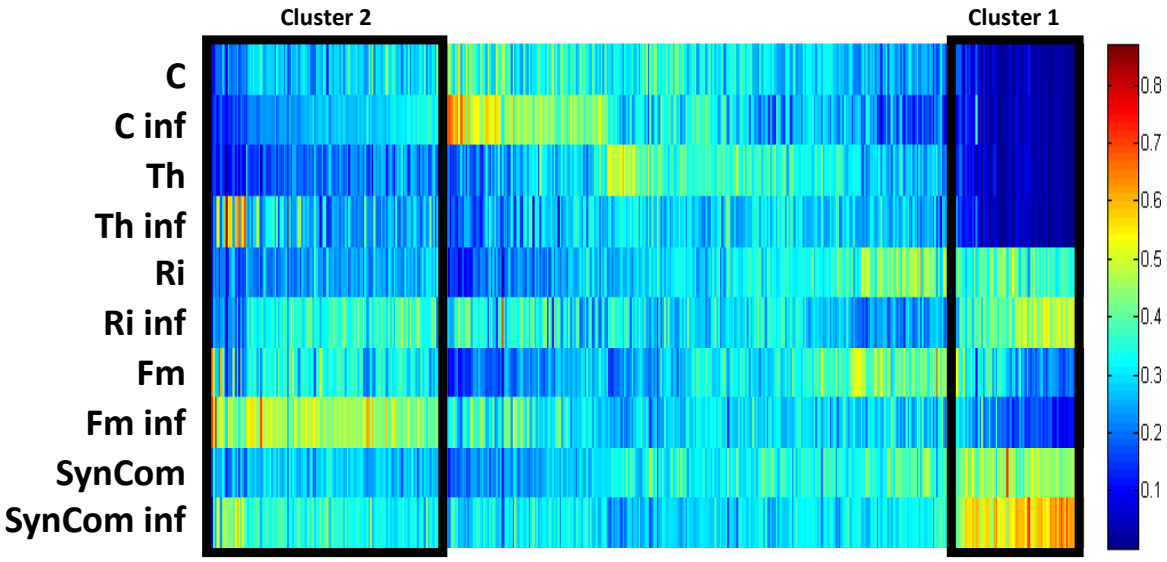

Figure 7. Overview of metabolic rearrangement in leaves in response to *T. absoluta* (48 hours post infestation) in the treatments with efficient protection from the semi-controlled assay. Heatmap analysis representing 485 signals from positive and negative ESI with statistically significant differences among treatments based on Kruskal-Wallis test ($p<0.05$, $n=6$). Non inoculated control (C), *Trichoderma harzianum* (Th), *Rhizophagus irregularis* (Ri), *Funneliformis mosseae* (Fm) and microbial consortium (SynCom) including Ba + Pa + Th + Ri. Treatments infested with *T. absoluta* larvae are indicated with "inf". Cluster of primed metabolites showing an over-accumulation pattern in response to *T. absoluta* in the microbe-inoculated plants is highlighted.

The analysis revealed a cluster of compounds more accumulated only in the mycorrhizal treatments independently of the herbivory stress (**Figure 7, cluster 1**). Some of these metabolites showed very low or any accumulation in leaves of non-inoculated and *T. harzianum* plants while their higher accumulation in *R. irregularis*, *F. mosseae* and SynCom plants followed similar pattern pointing to their possible relation with the root colonization by AMF (**Figure 8**).

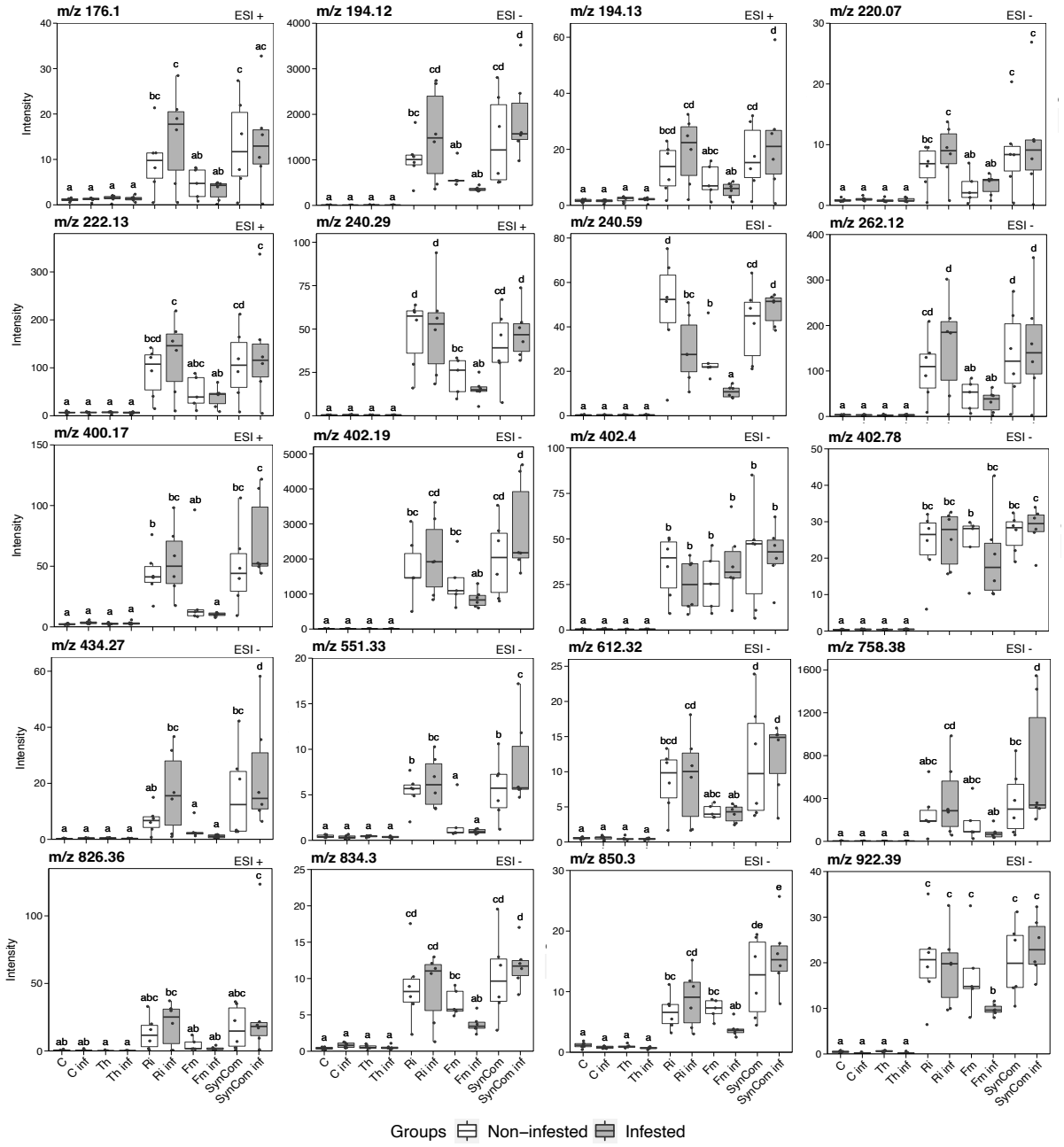

Figure 8. Signals more accumulated only in mycorrhizal plants independently of *Tuta absoluta* infestation. Non inoculated control (C), *Trichoderma harzianum* (Th), *Rhizophagus irregularis* (Ri), *Funneliformis mosseae* (Fm) and microbial consortium (SynCom) including Ba + Pa + Th + Ri. Treatments infested with *T. absoluta* larvae are indicated with "inf". Boxes represent the interquartile range, black-lines represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range, and black dots represent raw data points. Treatments not sharing a letter are statistically different based on ANOVA followed by LSD ($p<0.05$, $n=6$).

Next, we focused our analysis on a cluster of metabolites showing an over-accumulation pattern in response to *T. absoluta* in the IR displaying, microbe-inoculated plants (**Figure 7, cluster 2**). The signals in this cluster showed different patterns depending on the microbial inoculant. While some of them were primed by only one of the microbes in response to *T. absoluta*, other showed primed accumulation in more than one microbial treatment (**Figure 9**). A detailed analysis through exact mass and fragmentation spectra determinations allowed us to identify some known defense related compounds with primed accumulation by different microbial inoculations. In particular, the indole derivative indole-3-carboxaldehyde and the alkaloid lycodine were more accumulated only in *F. mosseae* inoculated plants in response to the herbivory (**Figure 9**). The fatty acid derivative azelaic acid (AZA) was significantly more accumulated in response to *T. absoluta* in *T. harzianum* and *F. mosseae* inoculated plants, while its levels remained unaltered upon infestation in non-inoculated controls (**Figure 9**). In addition, the phenolamide feruloylputrescine (FP) was only overacumulated in response to the infestation in plants inoculated with the AMF species *R. irregularis* and *F. mosseae* (**Figure 9**).

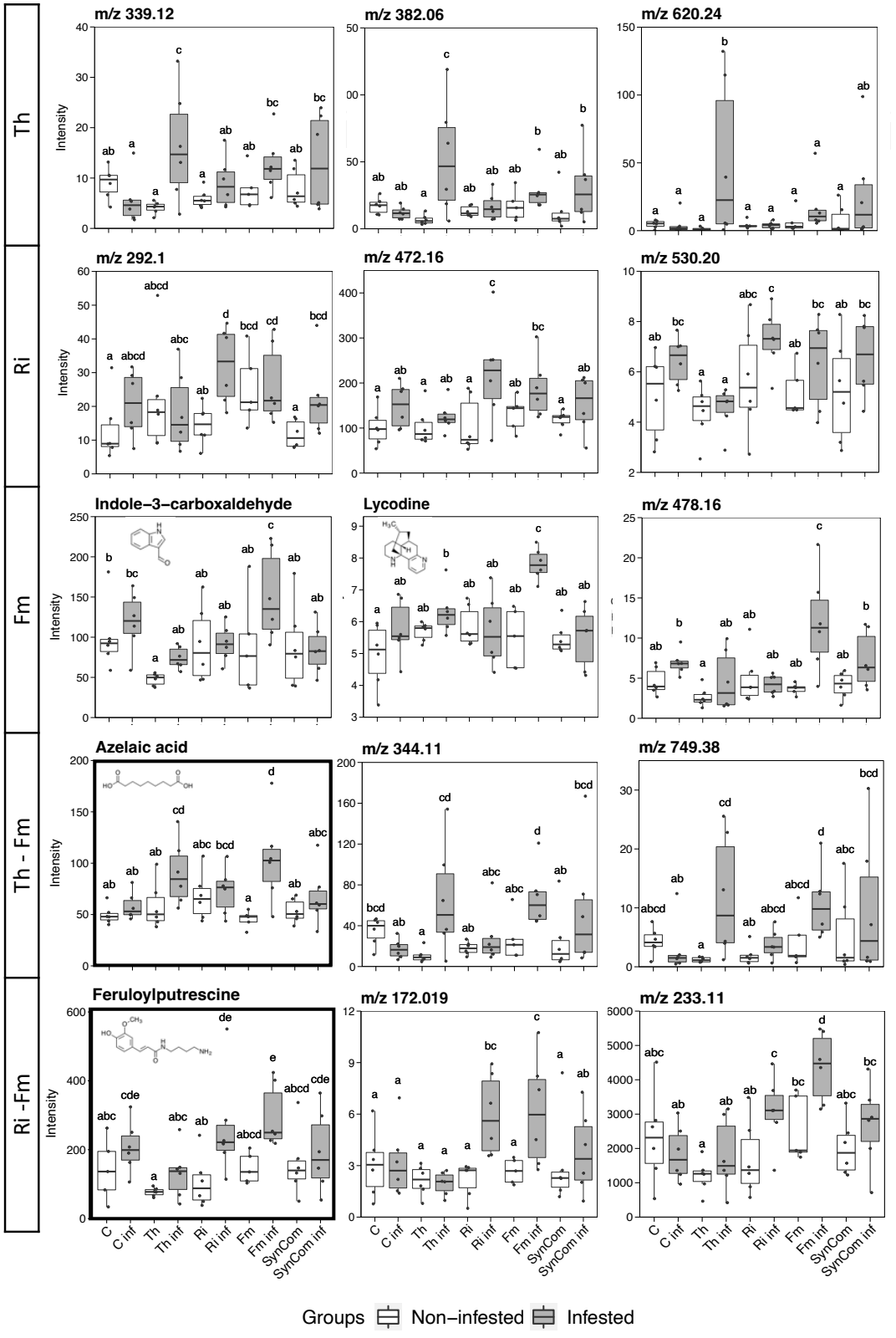

Figure 9. Primed metabolites in microbe treated plants in response to herbivory by *Tuta absoluta*. Non inoculated control (C), *Trichoderma harzianum* (Th), *Rhizophagus irregularis* (Ri), *Funneliformis mosseae* (Fm) and microbial consortium (SynCom) including Ba + Pa + Th + Ri. Treatments infested with *T. absoluta* larvae are indicated with "inf". Boxes represent the interquartile range, black-lines represent the median, whiskers represent maxima and minima within 1.5 times the interquartile range, and black dots represent raw data points. Treatments not sharing a letter are statistically different based on ANOVA followed by LSD ($p<0.05$, $n=6$).

5. Primed metabolites display anti-herbivory activity on *Tuta absoluta*

To address the potential contribution of the primed compounds to the observed negative impact on *T. absoluta* performance, we tested the effect of the previously identified primed metabolites AZA and FP on the development of the pest. For that, we applied the pure chemicals on detached leaves before *T. absoluta* infestation. In the control treatment, 70% of the larvae reached the adult stage, but the percentage was significantly reduced to 44 and 50% when the larvae fed on leaves treated with AZA and FP, respectively. Thus, a 37 and 29% reduction as compared to the control, respectively, was achieved by the chemical treatments (**Figure 10**). Thus, the pharmacological approach confirmed the negative impact of AZA and FP on *T. absoluta* development.

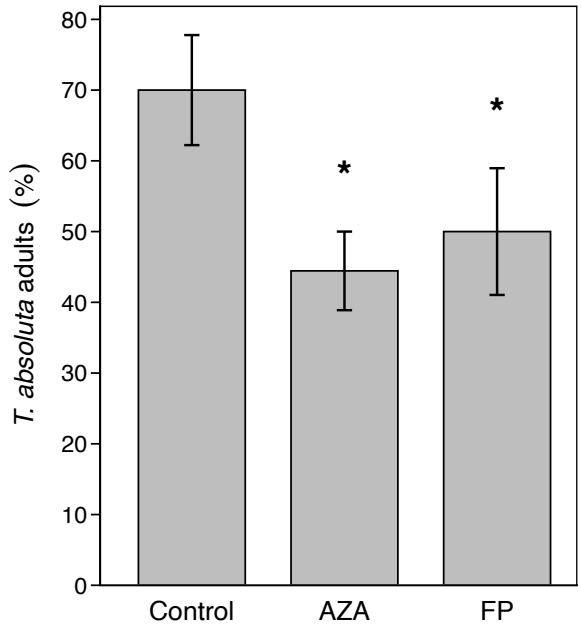

Figure 10. Functional analysis of the identified primed metabolites in microbe inoculated plants. Effect of exagenous application of the pure compounds (100ppm) on *T. absoluta* development evaluated as percentage of larvae reaching adult stage. Mock treated (Control), azelaic acid (AZA) and feruloylputrescine (FP). Asterisks indicate statistically significant differences compared to the control based on Exact binomial test ($p<0.05$, $n=30$).

DISCUSSION

In the present study we reveal the potential of the biocontrol fungus *T. harzianum* T22, and the arbuscular mycorrhizal fungi *R. irregularis* and *F. mosseae* to trigger Microbe-IR in tomato and efficiently protect the plants against the insect pest *T. absoluta* across different experimental scales, ranging from controlled conditions to tomato production

conditions. Further, we showed that root inoculation with these beneficial fungi modulate plant defense responses through a plant metabolic rearrangement resulting in the primed accumulation of defensive compounds for which we demonstrated their deterrent effect on *T. absoluta*, likely contributing to the Microbe-IR against this insect.

Microbe-IR has been well documented and its potential for plant protection against diverse pathogens and insects is widely reported (Pieterse *et al.*, 2014; Coppola *et al.*, 2019; Rivero *et al.*, 2021; de La Hoz *et al.*, 2021; Minchev *et al.*, 2021). Plant associated beneficial microorganisms are known to induce plant resistance generally through the priming of JA-dependent plant defenses, being mainly effective against necrotrophic pathogens and chewing herbivores (Pieterse *et al.*, 2014; Gruden *et al.*, 2020). *Tuta absoluta*, the major threat to the tomato production worldwide, is a leaf miner and chewing-biting insect and has been shown to activate JA signaling and related defense responses in tomato (D'Esposito *et al.*, 2021). Furthermore, overexpression of two barley proteinase inhibitors -JA inducible proteins- in transgenic tomato increased its resistant to *T. absoluta* (Hamza *et al.*, 2018). Thus, we hypothesized that Microbe-IR, usually associated to priming of JA regulated defenses, would be effective against this pest. However, studies demonstrating Microbe-IR against *T. absoluta* are scarce. To our knowledge, only few recent studies show a negative impact of microbial inoculation on *T. absoluta* performance. Tomato seed inoculation with endophytic fungi has a negative impact on the performance of this pest (Agbessenou *et al.*, 2020), but wether direct or plant mediated effects are responsible for such effect was not addressed. Further, root colonization of tomato plants with a mixture of AMF or with *Trichoderma harzianum* T22 protect the plant against *T. absoluta*, but the mechanisms remain poorly studied (Aprile *et al.*, 2022; Shafiei *et al.*, 2022).

To test whether microbial inoculants can prime plant defenses effective against *Tuta absoluta*, we explored the potential of different beneficial soil microbes, including biocontrol bacteria -*Bacillus amyloliquefaciens* and *Pseudomonas azotoformans*- and fungi -*Trichoderma harzianum*, *Rhizophagus irregularis* and *Funneliformis mosseae*- to trigger IR against *T. absoluta.* Our results demonstrated that *T. harzianum* T22 and *F. mosseae* root inoculation negatively affect *T. absoluta* development, leading to a reduction in the proportion of larvae that reach the adult stage under controlled conditions in a bioassay performed on detached leaves. This is consistent with the reduced survival

of *T. absoluta* larvae previously observed in *T. harzianum* T22 inoculated tomato plants (Aprile *et al.*, 2022). In addition, *T. harzianum* T22 and *F. mosseae* have been shown to induce resistance in tomato against other lepidopteran species such as *Spodoptera littoralis* and *S. exigua*, increasing larval mortality (Rivero *et al.*, 2021; Di Lelio *et al.*, 2021). Many reports point that environmental variables can seriously compromise the efficacy of the inoculants to induce resistance against insect pests, as demonstrated for temperature changes by di Lelio *et al*. (2021). This context dependency of Microbe-IR can hamper its practical application in agricultural systems, where environmental fluctuations are common. Indeed, progress on Microbe-IR research, evidencing its potential as a suitable strategy for plant protection, relies mostly on studies performed under controlled conditions, very different from those occurring in agroecosystems (Lee Díaz *et al.*, 2021). Thus, trials conducted under more realistic, variable conditions are required to test the consistency of the results obtained under controlled conditions.
With this aim, we scaled up our experimental system to more complex set ups, including bioassays with artificial *T. absoluta* infestation on whole plants growing in pots in a greenhouse without any climate control nor artificial light. Our results showed that only *B. amyloliquefaciens* and the fungal inoculants *T. harzianum*, *F. mosseae* and *R. irregularis* had a significant negative impact on *T. absoluta* development and/or incidence.

To validate the results on Microbe-IR against *T. absoluta* as a pest control method for crop protection, we evaluated the natural incidence of the pest in a trial performed under commercial production conditions. Crop management included well stablished IPM strategies like mating disruption by pheromones and the use of predatory mirid bugs -*N. tenuis*-. The natural incidence of the pest was not high overall (less than 40% of plants showing any damage), confirming the efficacy of the IPM management. Remarkably, a prominent reduction of the leaf miner natural incidence was further observed on the plants inoculated with *T. harzianum*, *R. irregularis* and *F. mosseae*. Thus, our results pinpoint the compatibility, and likely further synergy, of Microbe-IR with commonly used IPM practices in the control of this pest. As a whole, the different experiments carried out confirm the consistency of these beneficial fungi in enhancing plant resistance across different experimental scales and growing conditions, effectively protecting the plants against *T. absoluta,* not only under controlled conditions but also under real agronomic set ups.

Noteworthy, the protection was not achieved by the inoculation of the microbial consortia. The use of synthetic microbial communities for sustainable crop protection is considered as a viable strategy to improve the efficacy and consistency of microbial inoculants, considering potential functional complementarity and increased chances for microbial survival under varying environmental conditions. Indeed, we recently showed that microbial consortia can be more versatile than single microbes in the control of different plant pathogens (Minchev *et al.*, 2021). However, the results of the present study showed that while *R. irregularis* or *T. harzianum* single inoculation consistently protected plants against *T. absoluta* in the different settings, no negative effect on insect performance was observed when plants were inoculated with both of them as part of the microbial consortium. From the methodological point of view, the consistency of the results, whether positive (for the individual fungal inoculants) or negative (for the consortia) validates the different bioassays and support the suitability of detached leaf assays for quick preliminary screenings. From the scientific and agronomic point of view, these observations illustrate the complexity of microbe-microbe and plant-microbe interactions occurring in the rhizosphere and their impact on plant defenses, and highlight the challenges of designing and predicting the functionality of SynComs. SynComs design for agricultural applications require detailed analysis in the compatibility of the organisms involved, their rhizospheric competence in the inoculated areas, and potential interactions between the responses activated in the plant by the different microbes. Regarding compatibility, we previously showed that the isolates combined here are compatible (**see Chapter 1**) and microbial colonization was similar in the individual inoculations and in the consortium. Thus, potential antagonism among them is unlikely. Yet, further research is needed to disentangle the complex microbial interactions occurring in the SynCom and how these impact the host plant physiology and defenses against the insect pest.

Previous studies on Microbe-IR against phytophagous insects revealed that microbial inoculations trigger important metabolic changes in the plant upon herbivory, which may explain the enhanced plant resistance to the herbivore. *Funneliformis mosseae* primed the accumulation of defensive compounds like alkaloids, fatty acid derivatives and phenylpropanoid-polyamine conjugates in response to *S. exigua* (Rivero *et al.*, 2021). *Trichoderma harzianum* T22 inoculation also resulted in a higher accumulation of alkaloids, phenolic acids and flavonoids in response to *Macrosiphum euphorbiae*

(Coppola *et al.*, 2019). Accordingly, here we tested if the Microbe-IR against *T. absoluta* observed in *T. harzianum*, *R. irregularis* and *F. mosseae* inoculated plants was associated with a differential metabolic reprograming in the plant upon *T. absoluta* attack. We performed an untargeted metabolomic analysis to explore overall metabolic changes occurring in leaves when attacked by larvae of *T. absoluta*. We found 485 signals presenting significant changes in their concentrations in leaves among the different treatments. Remarkably, a group of these metabolites were highly accumulated only in AMF plants independently of the herbivory stress but not accumulated in *T. harzianum* and non-inoculated control plants, pointing to their specificity in the mycorrhizal symbiosis. Indeed, the mass -mz 402.19- of one of these compounds matches with 11-carboxyblumenol C-Glc, a compound reported as a shoot marker of arbuscular mycorrhizal symbiosis (Wang *et al.*, 2018), and previously described in mycorrhizal tomato (Rivero *et al.*, 2021).

Further, we identified a cluster of compounds showing a primed accumulation in the microbial inoculated plants in response to the herbivore, including fatty acid derivatives, phenolamides, alkaloids and indole derivatives. A more detailed analysis allowed the identification of metabolites previously described to play an important role in plant defenses as azelaic acid (AZA) and feruloylputrescine (FP). AZA, a fatty acid derivative, has been shown to operate in plant systemic immunity playing a role in defense priming (Jung *et al.*, 2009). We also found AZA to be more accumulated in *T. harzianum* and *F. mosseae* inoculated plants only upon herbivory. The phenolamide FP was over accumulated only in *R. irregularis* and *F. mosseae* inoculated plants challenged with *T. absoluta*. FP and other phenolamides have been reported to be inducible in response to different pathogens (Morimoto *et al.*, 2018), and most importantly in response to *T. absoluta* (Roumani *et al.*, 2022) as well as in response to simulated herbivory, applied as wounding plus oral secretion from *Manduca sexta* (Gaquerel *et al.*, 2014). Remarkably, primed accumulation of AZA and FP was also found in leaves of *F. mosseae* inoculated tomato plants after attack by the generalist chewing insect *S. exigua* (Rivero *et al.*, 2021). Thus, these compounds appear as good candidates to mediate Microbe-IR in tomato, as both showed primed accumulation in mycorrhizal plants upon challenge with very different attackers.

To test whether the enhanced accumulation of these compounds in the leaves of plants showing IR may have a functional relation with the reduced performance of *T. absoluta*

feeding on those plants, we analyzed the activity of the purified compounds when applied to non-inoculated plants on *T. absoluta* performance. Exogenous application of AZA and FP negatively impacted the development of *T. absoluta,* significantly reducing the number of individuals reaching the adult stage. The result confirms the potential contribution of their elevated levels in the Microbe-IR plants against *T. absoluta*. Remarkably, Rivero *et al*. (2021) also found that AZA was effective in reducing *S. exigua* performance. Thus, Microbe-IR is likely associated to the impact of the fungal inoculations on plant metabolic reprogramming upon herbivory that leads to the primed accumulation of defense related secondary metabolites. It is noteworthy that AZA and FP levels were not elevated upon herbivory in the SynCom treatment, and this lack of priming correlated with a lack in IR. Taken together, the results point to FP and AZA as possible biomarkers of microbe-mediated defense priming against chewing herbivores. This potential function as biomarkers of primed defenses can be of great interest for biotech applications as screenings for IR inducing microbes, and would be addressed in follow up studies.

Overall, this study reveals that beneficial fungi like *Trichoderma* and AMF can efficiently activate IR in tomato protecting the plants against the devastating insect pest *Tuta absoluta*, consistently from highly-controlled–lab settings to commercial production conditions. We show that these beneficial fungi can modulate plant defense responses through metabolic reprograming and primed accumulation of defensive compounds with a confirmed deleterious effect on *T. absoluta* development. These compounds may serve as potential biomarkers of primed defenses and future research may explore their potential application in screening systems for IR inducing microbes or microbes combinations. Our results also highlight the complexity of predicting the performance of microbial consortia in plant protection, since despite compatibility among the strains, their interaction with the plant immune system and the triggered responses may differ of those triggered by the individual microbes. Nonetheless, the present study demonstrate the potential of microbial inoculation for crop protection, the suitability of small scale, pilot experiments for selecting strains, and finally, that the application of Microbe-IR under production conditions is compatible with commonly used IPM practices. In summary, microbial-IR should be considered as an efficient tool complementing current IPM programs to improve the control of severer pests as *T. absoluta* in a sustainable manner.

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GENERAL DISCUSSION

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THE FRAMEWORK I OPERATED IN

Microbial inoculants based on plant associated beneficial microorganisms keeps gaining ground in the journey to increase sustainability of agriculture as an alternative to the large number of agrochemical products that are being massively removed from the markets all over the world, and especially in Europe. Yet, a wider adoption of biologicals in general, and microbials in particular, is still facing of reaching the significant place they could have given their high potential regarding efficacy, and their low impact on the environment and the health of the animals including us as humans. One of the main challenges keeps being their one on one comparison with agrochemicals; the variability of the results and limited predictability obtained in real agricultural settings its today a true drawback (Personal communication Koppert BV, Kaminsky et al., 2019; Batista & Singh, 2021; O'Callaghan et al., 2022). Indeed, unless the vast majority of agrochemicals, the efficacy of microbial inoculants can be challenged by the ever-changing environmental conditions present in the field and non-favorable crop management practices (Trivedi *et al.*, 2017, 2020; Mitter *et al.*, 2019). The successful establishment and persistence, niche colonization and relevant biological control or plant growth traits are key aspects to be considered to understand what are the conditions where the microbial products can relatively consistently thrive. Similarly, microbial colonization and establishment in the field can be limited by the competition with the native microbiota, compatibility with the plant species and cultivars, and the abiotic and biotic conditions of the different regions where they need to play. I believe that a better understanding of the ecology and biology of the microbial products on the market, so that they can be less broadly but more specifically recommended in terms of targets and markets or areas of the world, is key to gain the trust this novel tool deserves.

A way to address this challenging expectation of being as consistent as agrochemicals, can be gathering of diverse microbes; one or the other will always do the job. This has been exploited in the industry and has also been a popular area of interest for the scientific community. In the last decade microbiome engineering and the design of microbial consortia or synthetic microbial communities (SynComs) for agricultural applications is stirring great interest within the scientific and agroindustry communities (Mitter *et al.*,

2019; Compant *et al.*, 2019; Trivedi *et al.*, 2020; Batista & Singh, 2021). SynComs including diverse microbes may improve the stability of microbial inoculants and biocontrol practices as they are expected to outperform than single-microbe based inoculants under the highly variable environmental conditions occurring in crop production systems (Trivedi *et al.*, 2017; Saad *et al.*, 2020). Further, the combination of microbes with different functions could result in the multifunctionality of the inoculum (Arif *et al.*, 2020; Saad *et al.*, 2020; Batista & Singh, 2021) providing the host plant with different benefits such as protection against diverse aggressors and growth promotion (Compant *et al.*, 2019). However, also in this case, a thorough understanding of the potential and limitations of these gathered communities is key. Importantly, the methods used to do so are essential: research that builds up from laboratory conditions to build a base on the potential efficacy, accounting for the key sources of variability, scaled up to the field scale is the way to gain trust and move from a concept and innovative idea to a reality further adopted and trusted microbial product.

My PhD Thesis focuses on the design and characterization of microbial inoculants for sustainable crop protection with a special emphasis on the characterization of the SynComs. Therefore, the main purpose of the present Thesis is to design a multifunctional SynCom composed by compatible beneficial soil borne microorganisms with complementary modes of action for the biological control of economically important soil and leaf fungal pathogens, and herbivorous insect pests.

THE APPROACH

SynCom design

As first step, in **Chapter 1**, I approached the design of a multifunctional SynComs based on an extensive literature review. My aim was to explore and exploit the existing abundant scientific knowledge and combined it with the relevant existing commercial material to assemble a SynCom with a, theoretically, high potential. My aim was to combine different biocontrol agents with complementary modes of action and distinct tolerance to abiotic conditions. The aim was to select different strains combining different mechanisms for biocontrol, from the production of diverse antimicrobial metabolites, through mycoparasitism, to the stimulation of the plant defenses or the so-called Induced Resistance (IR) (Pieterse *et al.*, 2014; De Kesel *et al.*, 2021). For this, a selection of key

microorganisms for the design and assembly of SynComs was performed based on their experimentally deducted biocontrol functional traits, as previously shown in the literature (Vorholt *et al.*, 2017; Saad *et al.*, 2020; Batista & Singh, 2021). A potentially powerful group of microbes were selected, including taxonomically diverse bacteria and fungi already well characterized in their biocontrol potential. Some of them, like the fungus *T. harzianum*, are already commercialized worldwide as biocontrol agent (BCA) by Koppert Biological Systems, and others are in a product development stage. The selected BCA were further characterized for their biocontrol activity in *in vitro* confrontation assays against the two fungal phytopathogens used as a model in the present Doctoral Thesis, *Botrytis cinerea* and *Fusarium oxysporum,* confirming their antagonistic potential against both pathogens.

Microbial compatibility within the SynCom

A key factor in the design of a SynCom is microbial compatibility which is fundamental for the successful establishment and functionality of the combined microorganisms and the success of SynCom based inoculants (Trivedi *et al.*, 2017; Kong *et al.*, 2018; Arif *et al.*, 2020; Saad *et al.*, 2020). Microbe-microbe interactions can be positive or negative and synergisms or antagonisms between microbial groups can occur (Barea *et al.*, 2005; Pozo *et al.*, 2021). Thus, exploring compatibility between microbes is essential for the selection of the microbial components of the SynCom. In **Chapter 1**, microbe compatibility within the designed SynComs was evaluated, analyzing the root or rhizosphere colonization and persistence of the microbial inoculants when applied individually or as SynCom, through microbiological and molecular methods. First, we optimized detection and quantification methods to monitor the rhizosphere colonization by the different microbes. Microbial compatibility was confirmed within the SynComs, showing no reduction of rhizosphere or root colonization for any of the microbes when applied as a consortium as compared to their individual inoculation. Indeed, *Bacillus*, *Trichoderma* and *Rhizophagus* performed in the consortia as good as when applied as single-microbe inoculants, supporting the absence of microbial antagonism between these groups. As mentioned above, microbial interactions in the rhizosphere can be not only negative but also positive, and some groups of microbes can be benefited from the activity of others (Barea *et al.*, 2005). Indeed, we found that *Pseudomonas* even benefited from the combination with the other microorganisms since they performed better in the SynComs than alone. Other example for microbial co-operation in the rhizosphere is the

case of the so-called “mycorrhiza helper bacteria” that can benefit the formation or functioning of the mycorrhizal symbiosis (Barea *et al.*, 2005, 2013; Frey-Klett *et al.*, 2007). However, our results did not show any negative nor positive effect by the rest of microbial components of the SynCom on the mycorrhizal symbiosis. An example for antagonism between microbes is the interaction of the fungal mycoparasite *Trichoderma* with other fungi (Atanasova *et al.*, 2013). In fact, *Trichoderma* is able to parasite arbuscular mycorrhizal fungi (AMF) *in vitro* (Rousseau *et al.*, 1996; De Jaeger *et al.*, 2010) however, both species can coexist in the rhizosphere (Martínez-Medina *et al.*, 2011) or AMF can be even benefit by the presence of *Trichoderma* (Poveda *et al.*, 2019). Noteworthy, although compatibility between (AMF) and *Trichoderma* species has been frequently questioned, the symbiosis tomato-AMF was not negatively affected in early nor late stages by the presence of the mycoparasitic fungi *Trichoderma harzianum*. These findings contribute to understand the compatibility between key BCA groups or genera which is required for knowledge driven decisions in the selection of appropriate candidates for SynCom design. Naturally, interactions can interplay differently in the soil, but this is a solid first approximation, and a key element to always consider when designing SynComs.

Tools for tracking microbial survival and persistence in the rhizosphere

Microbial establishment is fundamental to ensure the efficacy of microbial inoculants for biocontrol or plant growth (Sessitsch *et al.*, 2019; Mitter *et al.*, 2019). Thus, developing and optimizing reliable microbe detection methods is crucial for tracking specific microbes in soil and for the proper evaluation of the survival and persistence of bioinoculants in agricultural setting (Manfredini *et al.*, 2021). In this regard, my contribution consists on optimising two different methods for microbial detection and quantification, a culture dependent method based on colony forming unit counts and a culture independent method based on microbial DNA quantification by qPCR. The results of the microbial abundance in rhizosphere obtained through both methods were highly consistent, indicating their reliability for tracking microbial colonization in rhizosphere in sterile experimental conditions. This is remarkable, as in non-sterile natural or agricultural soils, the impossibility to morphologically differentiate the inoculated microbe from native microbiota, culture-dependent methods are not always possible (Romano *et al.*, 2020). Thus, optimized culture independent molecular methods such as microbial DNA quantification by qPCR using strain specific primers becomes highly

valuable tool to track the persistence of microbial inoculants in natural and agricultural soils. We tested the primers for microbial DNA quantification in natural soil. Specificity of the primers used for *B. amyloliquefaciens* CECT8238 and *R. irregularis* showed to be suitable for DNA quantification in natural soil samples. In contrast, *T. harzianum* and *P. azotoformans* -for which the primers used were specific to species level- were also detected in the non-inoculated control samples indicating the presence of these species in the soil. This evidence the challenge to go up to strain level in the design of specific primers, specially in the case of microbes commonly present in natural soils as for example *Pseudomonas* and *Trichoderma*.

SynCom characterization

Once the SynComs were assembled, the characterization of the SynComs regarding their biocontrol potential was addressed in the following chapters of my PhD Thesis. In **Chapter 2** I focused on its biofungicide potential, both below and above ground. The biocontrol activity and efficacy of the designed consortia as compared to the individual strains was evaluated against *F. oxysporum* -a soil borne pathogen- and against *B. cinerea* -a leaf fungal pathogen- in soil-plant systems under controlled conditions. Further, in **Chapter 3**, I focused on scaling up these results. We scaled up the experimental system and tested the efficacy of SynComs and individual strains under real commercial tomato production conditions, using common crop management practices. And in **Chapter 4**, attention was paid to its potential as Bioinsecticide. The potential of the microbial inoculants to trigger IR against the devastating leaf miner pest *Tuta absoluta* was assessed in different conditions ranging in complexity, from controlled laboratory conditions, through semi-controlled conditions and to commercial production conditions. Importantly, in this last Chapter, I started exploring possible mechanisms underlying the Microbe-IR against foliar insects.

RESULTS HELICOPTER-VIEW

Biocontrol of fungal pathogens- Biofungicide potential

By testing the inoculant's efficacy against different pathogens and through different application forms, in **Chapter 2**, we found that a number of components / individual microorganisms were significantly effective in controlling the soil pathogen *F.*

oxysporum or the foliar pathogen *B. cinerea* in tomato, depending on the type of pathogen or the strategy used for its control.

Trichoderma harzianum, as expected, have effectively controlled *F. oxysporum.* This is a mycoparasite for which is well known to efficiently antagonize soil fungal pathogens (Wilson *et al.*, 2008; Roberti *et al.*, 2015; Fatouros *et al.*, 2018; Woo *et al.*, 2022). Unexpectedly, I have not seen the well documented potential effects on IR. This fungus is also able to trigger IR against diverse pathogens and pests (Tucci *et al.*, 2011; Martínez-Medina *et al.*, 2014; Coppola *et al.*, 2019; Aprile *et al.*, 2022) but in our study its soil application did not protect the plants systemically against *B. cinerea*.

Pseudomonas chlororaphis is also known to suppress fungal pathogens through direct antagonism (Abuamsha *et al.*, 2011). Indeed, our study this bacterium, applied in foliar spray, was the most efficient in suppress the leaf pathogen *B. cinerea.* But when applied in the soil was not able to suppress *B. cinerea* systemically nor the soil pathogen *F. oxysporum* through direct antagonism.

Bacillus amyloliquefaciens and *R. irregularis* applied in soil as single inoculants were the most effective in trigger IR against *B. cinerea*, in agreement with previous studies demonstrating their ability to induce resistance against foliar pathogens (García-Gutiérrez *et al.*, 2012, 2013; Sanchez-Bel *et al.*, 2016; Sanmartín *et al.*, 2020). However, none of these two microbes showed direct effect against the pathogens.

Noteworthy, the designed SynComs showed an extended functionality, effectively controlling both pathogens through direct antagonism and Microbe-IR, always achieving at least the same protection levels as the best performing single strain for each pathosystem. This is remarkable considering agricultural application, as root application of microbial inoculant based on consortia with extended functionality can provide versatile protection to important soil and leaf pathogens at the same time.

Naturally, numerous factors can lower or mask its potential, from the intensity of the disease, through the conditions that can differentially favor the disease or the beneficial, to the dose and application time of the beneficial microbe. Thus, thorough basic characterization and optimization of the SynComs would be needed to go further in the pipeline of developing commercial microbial products based on them.

Efficacy of the SynCom in the field

The relatively low consistency of the effects of microbial products documented in numerous studies (Mitter *et al.*, 2019; Batista & Singh, 2021; O'Callaghan *et al.*, 2022) and experienced by farmers is one of the major challenges on the way of their massive adoption in modern agriculture. Besides a solid basic characterization, following up with field validation is essential to address their functionality under the suboptimal and variable environmental conditions present in agricultural settings and their compatibility with the common crop management practices (O'Callaghan *et al.*, 2022). In **Chapter 3** the research was scaled up, performing an experiment under real agricultural settings in a commercial tomato production greenhouse in south of Spain, currently one of the biggest tomato production areas in Europe. Through the close collaboration of 8 early stage researchers in the frame of the Horizon 2020 funded EU-ITN project called MiRA "Microbe-Induced Resistance to Agricultural Pests" (https://mira.ku.dk/), we tested diverse bacteria, fungi and SynComs previously characterized under controlled lab conditions, for their impact on plant growth, resistance to pests and diseases, and fruit production and quality in a commercial greenhouse within the "Cajamar Experimental Station" (https://www.fundacioncajamar.es/es/comun/estacion-experimental-palmerillas/).

Our field validation showed no effect of bacterial inoculants nor SynCom on the plant, while some fungal single strain inoculants improved plant resistance and increased productivity. A total of 11 microbial inoculants were tested under these non-controlled commercial setting and we could identify microbial strains performing as efficient bioprotectors and biostimulants. Most of the fungal strains inoculated individually -the AMF *R. irregularis*, *F. mosseae* and *C. etunicatum*, the *T. harzianum* strains T22 and T78, and the entomopathogenic fungus *M. robertsii*- strongly reduced the natural incidence of *T. absoluta*, one of the major threats of tomato production worldwide. Furthermore, while none of the microbial inoculants had a negative effect on the crop protection nor productivity, *F. mosseae* and *T. harzianum* T22 increased tomato fruit production during the cropping season. Thus, the results point to a more prominent beneficial effect of fungal inoculants enhancing plant resistance and, in some cases, increasing crop productivity, whereas the bacterial strains tested did not show any effect on the crop. These results agree with previous finding suggesting that fungal networks are more stable than bacterial ones under variable conditions (de Vries *et al.*, 2018) and pinpoint the importance to consider fungi as components of SynComs. Surprisingly, the

negative impact of *R. irregularis* and *T. harzianum* T22 on the pest incidence was only achieved by their single inoculation but not when were inoculated as part of the SynCom. Despite the advantages that SynCom can provide over a single strain inoculants supported by the outcomes in **Chapter 2**, these results illustrate the complexity and the effort needed in the development of multi-strain inoculants where the microbial interactions and their impact on the plant becomes difficult to predict. Thus, detailed knowledge on how, when and where SynComs are effective is essential for the optimization of such mixed microbial products.

Microbe-IR against the tomato leaf miner T. absoluta

The results obtained in **Chapter 3** showed that the natural incidence of the tomato leafminer *T. absoluta* was highly reduced by the single strain inoculations with *R. irregularis* and *T. harzianum* T22, but unexpectedly not when these were inoculated as part of the SynCom. This observation motivated us to explore more in detail (**Chapter 4**) the Microbe-Induced Resistance against *T.* absoluta. We tested the capacity of single strain inoculants and the SynCom to trigger IR against the leaf miner in different settings under highly controlled lab conditions and semi-controlled conditions and compared them to their performance under agronomic conditions. Indeed, we confirmed that while *R. irregularis* and *T. harzianum* T22 consistently reduced the insect performance, the SynCom failed to trigger IR against the leaf miner in all experimental set ups. One of the possible explanations for this loss of efficacy against the leaf miner could be the antagonistic interactions between the microbes when inoculated together. However, as shown in **Chapter 1** the compatibility of the microbes was demonstrated confirming that all of them colonize the rhizosphere when inoculated as SynCom as well as when inoculated separately. We then hypothesized that multiorganism interactions turn more complex in the SynCom and consequently plant-microbe interactions also change probably leading to different plant responses to *T. absoluta*. Indeed, recent metanalysis showed that multiple interactions trigger more complex responses in the plant than the combination of the separate interactions, differing in time or intensity, or with new pathways being activated (Gruden *et al.*, 2020).

Mechanisms underlying Microbe-IR against T. absoluta

Previous results from our group showed that mycorrhizal symbiosis primes the accumulation of defensive compounds in response to herbivory (Rivero *et al.*, 2021). Plant protection conferred by *T. harzianum* T22 against aphids was also shown to be related to the higher accumulation of defense related secondary metabolites (Coppola *et al.*, 2019). Thus, to understand the mechanisms behind the Microbe-Induced Resistance against *T. abosluta* we explored the leaf metabolic rearrangement in microbe-inoculated plants in response to the herbivore. Indeed, we found that the IR against this pest is associated to the primed accumulation of certain defensive compounds in response to the herbivore.

Interestingly, the plant metabolic response to the herbivory differed depending on the microbial inoculant. Some of the compounds were primed only by one of the microbes while others were primed by more than one microbial inoculant. Among the primed compounds we focused on azelaic acid (AZA) and feruloylputrescine (FP), primed by more than one microbe and for which a precise identification was achieved. Both, AZA and FP have been previously reported to play a role in plant defense responses to pathogens and pests (Jung *et al.*, 2009; Morimoto *et al.*, 2018; Rivero *et al.*, 2021; Roumani *et al.*, 2022). Through a functional analysis we demonstrated that these compounds negatively impact the insect development. Noteworthy, both metabolites were only over accumulated in the microbial treatments displaying IR and the SynCom inoculation failed to trigger their primed accumulation, supporting their potential role in the Microbe-IR against *T. absoluta*. Yet, further research is needed to disentangle the loss of the capacity of the SynCom to trigger MiR against this pest.

Identifying and using markers for tracking Microbe-IR and microbial inoculants in the field

Intriguingly, previous studies have shown that Mycorrhiza-IR is associated with the primed accumulation of azelaic acid and feruloylputrescine in response to the generalist herbivorous insect *Spodoptera exigua* (Rivero *et al.*, 2021). These results together with the ones obtained in **Chapter 4** point to these two compounds as metabolic markers of Microbe-Induced Resistance against insect pests. This is remarkable, as the identification of such markers can be a highly valuable tool for tracking of Microbe-Induced Resistance in real agronomic settings and evaluate the efficacy microbial inoculants claiming to trigger IR. Yet, these findings still need a proof of concept under commercial production

conditions to confirm the reliability of these two compounds as Microbe-Induced Resistance markers.

Furthermore, the leaf metabolic profile revealed a group of metabolites only accumulated in mycorrhizal plants but not in non-inoculated nor in *Trichoderma*-inoculated plants, suggesting the specificity of these compounds for mycorrhizal symbiosis. This is in agreement with previous studies showing the accumulation of some metabolites from the grop of the blumenols in leaves of mycorrhizal plants (Wang *et al.*, 2018; Rivero *et al.*, 2021). The identification of such leaf metabolic markers of mycorrhizal symbiosis would be of high importance as they can facilitate tracking the functionality of mycorrhizal inoculants in the field. So far only some of these markers, as for example the 11-carboxyblumenol C-Glc, have been proposed as reliable for tracking mycorrhizal colonization in a few plant species independently of abiotic and biotic stresses such as drought, herbivory and pathogen infection (Wang *et al.*, 2018). However, more research is needed to show that these compounds are specific only for mycorrhizal symbiosis, and not for other plant-microbe associations, and also if they are reliable markers of arbuscular mycorrhizal symbiosis in all plant species able to form it. Developing methods for easy and accurate detection of the compounds can be an excellent tool to improve monitoring of microbial inoculants performance in the field and should be the target of biotech applications.

Microbe-IR as a promising component of Integrated Pest Management (IPM)

Microbe-IR is considered an important mechanism of biological control using beneficial microorganisms (Köhl *et al.*, 2019; Stenberg *et al.*, 2021). It is also considered its potential as part of the IPM programs (Stenberg, 2017). Yet, most of the research on Microbe-IR is performed under very controlled laboratory conditions and its exploration and implementation of in the practice is still in its infancy. Despite the numerous evidences on the efficacy of Microbe-IR for biocontrol of wide range of pathogens and pests (Pineda *et al.*, 2010; Jung *et al.*, 2012; Pieterse *et al.*, 2014; De Kesel *et al.*, 2021), remarkably, to my knowledge, there is no microbial product on the market claiming exclusively IR as mode of action. Compatibility of Microbe-IR with common crop management practices is essential for the wider adoption of microbial inoculants for crop protection triggering IR. The findings shown in **Chapter 3** and **4** fully support that Microbe-IR can be a valuable tool in biological control of pests in real crop production settings. Major concern of using IR in agriculture is the potential cost in production and

possible negative effect on non-targeted organisms. Pollinators and natural enemies of pests are commonly used now in crop production. In our study we found no reduction in yield by any of the microbial inoculants, and in some cases there was an yield increase. We did not measure the effect on pollinators, but the good fruit set indicate no negative impact. Also, our results did not show any impact of microbial inoculants on other biocontrol organisms such as the natural enemy *Nesidiocoris tenuis*, commonly used in tomato production for the integrated management of important pests like *T. absoluta* and whiteflies. These findings suggest that Microbe-Induced Resistance is compatible with common crop management and IPM practices, and its implementation in the current IPM strategies could help to reduce agrochemicals input and improve agricultural sustainability.

However, Microbe-Induced Resistance appears to be highly context-dependent and its functionality can be influenced by the environmental conditions including diverse abiotic and biotic factors (Lee Díaz *et al.*, 2021). For example, temperature alternations can negatively impact *Trichoderma*-IR against insect pests (Di Lelio *et al.*, 2021). Nutrient availability in soil is also a key abiotic factor influencing Microbe-Induced Resistance. Indeed, recent studies have found that the IR triggered by AMF against the generalist herbivorous insect *Spodoptera exigua* depends on nitrogen and phosphorous fertilization (Ramírez-Serrano *et al.*, 2022; Dejana *et al.*, 2022). These results highlight the importance of understanding regulatory factors to improve crop management to promote IR. In **Chapter 4** we identified some microbial strains, such as *T. harzianum* T22, *R. irregularis* and *F. mosseae* with high context-stability. We showed that these fungi can trigger IR in tomato and efficiently protect the plant against *T. absoluta* from very controlled laboratory conditions through semi-controlled condition and finally under tomato production conditions. This is remarkable as the identification of such microbial strains and their ability to trigger IR under different conditions will be a step forward to overcome the context dependency of Microbe-Induced Resistance and prompt its faster and wider adoption in agriculture.

Single strain vs SynCom? A dilemma to face

Overall, the results of the present Doctoral Thesis illustrate the challenges faced in the production of stable biocontrol inoculants and the current dilemma of targeting single microbial strains versus SynComs for biological control. From the efficacy perspective, our results support the idea that combining microbes with different mechanisms will

increase the versatility: SynComs provided the widest plant protection after comparing the single strains and several consortia across soil and foliar pathogens through different application methods implying direct and indirect biocontrol mechanisms. Yet, although no negative interactions were detected, there were no synergistic effects observed. The efficacy of the SynComs was not higher than that of the best performing single strain and, in general, more than one individual microbe provided an effective control. Still, SynCom offer an extended functionality in the biocontrol of certain leaf and soil pathogens under our controlled experimental set up. Accordingly, under real crop production conditions there is an uncertainty of which pests or pathogens would appear and threaten the crop. Indeed, scaling up the research to real agronomic settings, our crop faced different challenges from those tested previously under controlled conditions. In particular, the tomato crop was threatened by insect pest such as *T. absoluta*, thrips and whiteflies, as well as the powdery mildew disease. Interestingly, the Microbe-Induced Resistance achieved by *T. harzianum* T22 and *R irregularis* as single strain inoculants against the devastating pest *T. absoluta* was abolished when the same microbes were inoculated as part of the SynCom. Whether this is specific to *T. absoluta* or general to other insects remains to be determined. However, testing efficacy of microbial inoculants in the field is challenging and studies are scarce in general (O'Callaghan *et al.*, 2022) and even more including SynComs and insect pests. In addition, *T. harzianum* T22 single inoculation resulted in a yield improvement while its application as part of the SynCom did not show any effect on crop yield. Having these limitations in mind, together with the high production and registration costs and extremely long duration of the registration process of microbial products, which are even higher in the case of multi-strain products, targeting single strain or SynCom products is a tough dilemma to face from the commercial point of view. Thus, it is critical to characterize SynComs and validate results by testing them in robust field trials and comparing them with their single strain components to evaluate pros and cons of single strain or SynCom applications.

All in all, this Thesis provides evidences of the potential of microbial inoculants to control pests and diseases in agricultural settings. It generated tools to monitor and quantify microbial prevalence and compatibility and explores potential mechanisms of Microbe-Induced Resistance. It set the bases for developing markers to monitor microbial inoculants performance and Microbe-IR in agronomic settings. Finally, it shades light on the complexity of the design of multi-strain microbial inoculants and the need of thorough

characterization, optimization and field validation for the successful development of an efficient mixed microbial product.

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CONCLUSIONS

CONCLUSIONS

1. SynCom conformed by bacteria and fungi with different biocontrol capabilities was designed and compatibility between the microbial strains was confirmed. No reduction of rhizosphere or root colonization was observed for any of the microbes when applied as SynCom as compared to their individual application. *Bacillus*, *Trichoderma* and *Rhizophagus* performed in the SynCom as good as when individually inoculated, and *Pseudomonas* even benefited from the combination with the other microorganisms, as they performed better in the SynCom than when inoculated alone.

2. Different individual microorganisms were the most effective in controlling the root pathogen *Fusarium oxysporum* or the foliar pathogen *Botrytis cinerea* in tomato. The consortia showed an extended functionality, effectively controlling both pathogens through direct antagonism and Microbe-Induced Resistance, always reaching the same protection levels as the best performing single strain for each pathosystem.

3. Root inoculation with *Rhizophagus irregularis*, *Funneliformis mosseae*, *Claroideoglomus etunicatum*, *Trichoderma harzianum* strains T22 and T78, *Metarizium robertsii* and the SynCom M2 triggered Microbe-Induced Resistance in tomato plants, reducing the natural incidence of *T. absoluta* in commercial tomato production conditions. None of these microbial treatments negatively impacted tomato fruit production, improving plant resistance without compromising plant growth and productivity.

4. *Funneliformis mosseae* and *Trichoderma harzianum* T22 inoculation increased total and commercial quality tomato production under commercial conditions.

5. Microbial inoculation did not have any negative impact on other biocontrol agents such as the polyphagous mirid bug *Nesidiocoris tenuis* applied for pest control in the commercial tomato production conditions, supporting the compatibility of Microbe-Induced Resistance with the release of this predator, commonly used in integrated pest management programs.

6. Root inoculation with the biocontrol fungus *Trichoderma harzianum* T22, and the arbuscular mycorrhizal fungi *Rhizophagus irregularis* and *Funneliformis mosseae* triggers Microbe-Induced Resistance and efficiently protect tomato plants against the insect pest *Tuta absoluta*, under diverse experimental conditions ranging from controlled conditions to commercial tomato production conditions, suggesting context stability of these fungal strains and their ability to trigger induced resistance.

7. Root inoculation with *Trichoderma harzianum* T22, *Rhizophagus irregularis* and *Funneliformis mosseae* modulate plant defense responses to *Tuta absoluta* infestation through a metabolic rearrangement resulting in the primed accumulation of defensive compounds such as azelaic acid and feruloylputrescine.

8. Exogenous application of azelaic acid and feruloylputrescine on non-inoculated tomato plants has a negative effect on *Tuta absoluta* development, supporting that their primed accumulation in microbe inoculated plants contributes to the Microbe-Induced Resistance achieved against this pest.

9. The SynCom failed to trigger induced resistance against *Tuta absoluta* in all conditions tested. This lack of protective effect related to differential activation of defenses by the SynCom and the individual strains. For example, the SynCom failed to trigger the primed accumulation of the defensive compounds azalaic acid nor feruloylputrescine.