Tesi Doctoral

Endophytic communities in *S. lycopersicum* genotypes: Isolation of a novel fungal strain and its application to plant

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Memòria presentada per Luisa Liu Xu
per a optar al grau de doctora per la Universitat Jaume I

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- A new endophytic Leptobacillium sp. strain isolated from S. lycopersicum: implications and significance. Liu-Xu L, Vicedo B, García-Agustín, Llorens E (Manuscript in progress)
- Endophytic Leptobacillium sp. isolate confers resistance to tomato plants against P. syringae through multiple approaches. Liu-Xu L, Scalschi L, Vicedo B, García-Agustín, Llorens E (Manuscript in progress)

Participation in conferences:

- Traditional genotypes, a richer source of potential fungal endophytes for tomato improvement.
  Authors: Luisa Liu Xu; Loredana Scalschi; Atefeh Farvardin; Lorena Sánchez Giménez; Gemma Camañes Querol; Begonya Vicedo Jover; Pilar García Agustín; Eugenio Llorens Vilarrocha.
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- Enhancing Tomato Resistance to P. syringae through the use of a novel fungal endophyte**.**
  Authors: Luisa Liu Xu; Loredana Scalschi; Atefeh Farvardin; Lorena Sánchez-Giménez; Gemma Camañes Querol; Begonya Vicedo Jover; Pilar García Agustín; Eugenio Llorens Vilarrocha.
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- Biological control of P. syringae infection by a tomato fungal endophyte.
  Authors: Luisa Liu Xu; Loredana Scalschi; Emma Fernández Crespo; Atefeh Farvardin; Gemma Camañes Querol; Begonya Vicedo Jover; Pilar García Agustín; Eugenio Llorens Vilarrocha.
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- Abundance and distribution of microbial communities in tomato plants: traditional versus commercial genotypes
  Authors: Luisa Liu Xu; Loredana Scalschi; Emma Fernández Crespo; Ana Isabel González Hernández; Atefeh Farvardin; Gemma Camañes; Begonya Vicedo; Pilar García Agustín; Eugenio Llorens.
  Conference: IS-MPMI Congress: Esymposia Series. Plant-microbe interactions in the environment – Navigating a complex world
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  Authors: Luisa Liu Xu; Loredana Scalschi; Atefeh Farvardin; Ana Isabel González Hernández; Emma Fernández Crespo; Gemma Camañes; Begonya Vicedo; Pilar García Agustín; Eugenio Llorens.
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- Exploiting tomato genotypes to study heat stress tolerance
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- Plant growth promoting ability of a fungal endophyte from traditional tomato
  Authors: Luisa Liu Xu; Loredana Scalschi; Emma Fernández Crespo; Ana Isabel González Hernández; Atefeh Farvardin; Gemma Camañes; Begonya Vicedo; Pilar García Agustín; Eugenio Llorens.
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  Authors: Luisa Liu Xu; Loredana Scalschi; Emma Fernández Crespo; Ana González Hernández; Carlos Albert; Gemma Camañes Querol; Begonya Vicedo Jover; Pilar García Agustín; Eugenio Llorens Vilarrocha.
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CONTENTS

SUMMARY

SUMMARY

Nowadays, due to the consequences of climate change and global overpopulation research in the agricultural sector has become more crucial than ever. Although plants are sessile, they are not unprotected. It is known that plants are part of a large ecological unit that includes their microbiota - the microorganisms that live in contact with them. The presence of these microorganisms such as fungi and bacteria varies depending on biological and environmental factors. Different plant species interact with different microbial communities, and geological, climatic and biotic conditions can influence these interactions. The microbiota can affect plant growth, as well as its interaction with the environment and its resistance to abiotic and biotic stresses. Within this context, endophytes, the microorganisms that live inside plant tissues, are gaining prominence as ecological alternatives to combat various stresses that plants may face.

The use of endophytic organisms in agronomic improvement is a broad field of study which has made significant advances in recent decades. However, most literature reviews tend to focus on specific aspects of the research, such as a particular species. For this reason, we have conducted a comprehensive analysis of the general study on endophytic fungi found in the scientific literature up to 2021. The analysis has shown that the vast majority of studies to date have focused on the use of ascomycetes. More than half of the analyzed studies are related to herbaceous plants and abiotic stress. Meanwhile, despite several articles proving the use of endophytic fungi in dicotyledonous crops and their potential effect as inducers of resistance against pests, this field of research remains relatively unexplored. These results have allowed us to direct our research towards less frequently studied areas that have promising potential for exploitation.

On the other hand, previous studies have demonstrated that different genotypes of the same plant species can exhibit distinct behaviors in their development and in response to adverse conditions. This information is particularly relevant, since the use of varieties with greater tolerance to environmental stresses could be highly beneficial in contemporary agriculture. In light of this, we characterized six

different genotypes of tomato (*Solanum lycopersicum*) from several Mediterranean regions, including four traditional varieties (ADX2, TH-30, ISR-10, MO-10) and two commercial varieties (*Moneymaker* and *Ailsa Craig*) that resulted from an extensive human selection. The tomato genotypes were subjected to heat stress, and the leaf damage was evaluated phenotypically. The transcriptome was then studied to identify possible tolerance mechanisms, with emphasis on genes related to defense against high temperatures, such as HSP70 and HSP90. This enabled us to demonstrate and classify the genotypes as more susceptible (TH-30, ADX2) or more resistant (Moneymaker, MO-10) against heat stress. Our results revealed that the tolerance of MO-10 plants is mostly based on the control of the leaf cooling mechanism, as well as the rapid activation of RBOHB and ABA signaling pathways. In contrast, the MM variety exhibited a strategy based on the activation of HSP70 and HSP90, in addition to the accumulation of phenolic compounds that are correlated with early induction of PAL expression.

As previously mentioned, plant genotypes can influence the presence of different microbial communities, which in turn can affect genotype characteristics. Therefore, a study was conducted to analyze the microbiota of different tomato genotypes. This involved studying the genome of the microbiota present in plant tissue, also known as the microbiome, to identify the quantity and structure of the communities of endophytic fungi and bacteria present in each genotype. This analysis was able to confirm the hypothesis that traditional tomato varieties have broader and more diverse microbial communities in their endosphere. Additionally, traditional varieties harbor certain endophytic microorganisms that are not present in commercial varieties. Similarly, the study demonstrated the impact of conventional agronomic practices, such as the use of fungicides, on the reduction of microbiota diversity.

Then, the isolation of a collection of endophytic fungi from several tomato genotypes was performed. Among the cultivable fungi, some showed great potential for study due to the lack of scientific reports in the literature. Several tests were carried out with some of them and finally, we characterized one of the isolated fungi named Sl27 whose identity had not been clearly defined. Sl27 is a

whitish and slow-growing ascomycetous. The strain was shown to belong to *Leptobacillium* sp., a genus that has gained attention in recent years and has been found with both environmental and endophytic species. Due to the lack of bibliographic data, a study focused on its characterization as an endophytic species was conducted. First, its morphology, optimal growth conditions, and possible production of other compounds of interest were characterized, which revealed that the endophyte could synthesize siderophores and indoleacetic acid. Once its optimal behavior was known, its interaction with the tomato host plant was examined under controlled conditions. This served to rule out the pathogenic behavior of the study strain and, at the same time, to evaluate its ability to promote plant growth. In this way, its neutral role in plant growth has been confirmed, as well as a possible effect on the development of the TH-30 genotype and the improvement of chlorophyll and lycopene production in fruit.

Afterwards, the potential of the endophyte to confer resistance to biotic stress on tomato plants was evaluated by studying the infection caused by the pathogenic bacterium *Pseudomonas syringae* pv. tomato DC3000 (Pst). Given the known antagonism between fungi and bacteria, the initial hypothesis predicted that if any antagonism existed, it could be observed through the use of fungal exudates. Thus, an *in vitro* assay was conducted using different fungal culture filtrates that contained exudates of the SI27 isolate. The studied filtrates showed a clear inhibition of Pst growth in the presence of SI27 exudates, allowing subsequent experiments in plants to assess whether the endophyte could induce resistance to stresses. Two approaches were used in the experiments: inoculating the endophyte in seeds and treating the plants with exudates from the endophyte cultures. The results revealed that both methods were effective in reducing the levels of Pst infection in tomatoes. Additionally, a transcriptomic and hormonal analysis of plant samples was conducted, which indicated that different plant responses occurred based on the approach. Finally, a metabolomic analysis of the filtrates used in these experiments was performed to identify possible antimicrobial compounds.

In summary, this thesis has investigated the relationship between different tomato genotypes and their microbiomes, as well as their response to high-temperature stress. Additionally, an endophytic isolate has been characterized, and its potential role in enhancing and protecting economically valuable tomato crops has been explored.

RESUM

Actualment les conseqüències ocasionades pel canvi climàtic i la sobrepoblació mundial han fet que la investigació al sector agroalimentari siga més necessària que mai. Encara que les plantes siguen sèssils, això no vol dir que estiguen desprotegides. Actualment es coneix que una planta és part d'una gran unitat ecològica que engloba també la microbiota, és a dir, els microorganismes que hi viuen en contacte. La presència d'aquests microorganismes, que inclou fongs i bacteris, varia segons factors biològics i ambientals. Diferents espècies de plantes es relacionen amb diferents comunitats microbianes i les condicions geològiques, climàtiques i biòtiques poden afectar estes interaccions. La microbiota pot afectar el creixement de la planta, així com la seua interacció amb el medi i la seua resistència davant d'estressos abiòtics i biòtics. En aquest context, els microorganismes que viuen dins dels teixits de les plantes, coneguts com a endòfites, estan adquirint cada vegada més protagonisme i han estat estudiades com a alternatives ecològiques per combatre diferents estressos que les plantes poden patir.

L'ús d'organismes endòfits en la millora agronòmica és un camp d'estudi ampli, els avenços del qual han estat de gran rellevància en les últimes dècades. No obstant això, la majoria de les revisions de la literatura solen estar enfocades a certs aspectes de la investigació com a espècies concrets. Per això, hem realitzat una anàlisi detallada de l'estudi general sobre fongs endòfits present a la literatura científica fins al 2021. Això ha demostrat que la gran majoria dels estudis fins ara se centren en l'ús d'ascomicets. Els models d'estudi més comuns trobats han estat aquells referits a plantes herbàcies i estrès abiòtic, que superen la meitat dels estudis analitzats. Mentrestant, tot i que diversos articles van demostrar l'ús de fongs endòfits en cultius de dicotiledònies i el seu efecte prometedor com a inductors de resistència contra plagues, aquest camp de recerca encara està poc explorat. Aquests resultats ens han permès centrar la recerca en àmbits estudiats amb menys freqüència i que són potencialment explotables actualment.

D'altra banda, estudis previs han demostrat que diferents genotips d'una mateixa espècie vegetal poden presentar comportaments diferents en el

desenvolupament i davant de condicions adverses. Aquestes dades són de gran rellevància ja que l'ús de varietats amb més tolerància als estressos del medi podria ser molt útil en l'agricultura contemporània. Basant-nos en això, hem caracteritzat sis genotips diferents de tomaques (*Solanum lycopersicum*) procedents de diverses regions mediterrànies. D'aquests, quatre són varietats tradicionals (ADX2, TH-30, ISR-10, MO-10) i dues són varietats comercials (Moneymaker i Ailsa Craig) que són resultat d'una selecció forta per part de l'home. Aquests genotips de plantes de tomaques han estat sotmesos a estrès per altes temperatures i s'han avaluat els danys fenotípicament. Posteriorment, s'ha estudiat el transcriptoma per identificar els possibles mecanismes de tolerància observats, amb èmfasi en la importància de gens relacionats amb la defensa davant d'altes temperatures com són HSP70 i HSP90. Tot això ha permès demostrar i classificar els genotips com a més susceptibles (TH-30, ADX2), o més resistents (Moneymaker, MO-10) davant d'estrès per altes temperatures. Els resultats han mostrat que la tolerància de les plantes MO-10 es basa principalment en el control del mecanisme de refredament de la fulla, així com l'activació ràpida de RBOHB i vies de senyalització d'ABA. En contrast, la varietat MM va mostrar una estratègia basada en l'activació de HSP70 i HSP90, a més de l'acumulació de compostos fenòlics correlacionats amb la primerenca inducció de l'expressió de PAL.

Com hem esmentat prèviament, el genotip de la planta pot influir en la presència de diferents comunitats microbianes, i aquestes, a l'hora, poden influir en les característiques del genotip. Per això, s'ha dut a terme un estudi de la microbiota dels diferents genotips de tomaques. Per a aconseguir-ho, s'ha recorregut a estudiar el genoma de la microbiota present en el teixit vegetal, és a dir, el microbioma, per identificar la quantitat i estructura de les comunitats de fongs i bacteris endòfits presents a cada genotip. Les seves anàlisis han pogut confirmar la hipòtesi que les varietats tradicionals abasten comunitats microbianes més àmplies i diverses a la seva endosfera. A més, aquestes varietats tenen un nombre de microorganismes endòfits que no estan presents en les varietats comercials. De la mateixa manera, també s'ha volgut demostrar l'efecte que tenen

les pràctiques agronòmiques convencionals com és l'ús de fungicides en la reducció de diversitat de la microbiota.

Tot seguit, s'ha realitzat l'aïllament d'una col·lecció de fongs endòfits de diversos genotips de tomaques. Entre els fongs cultivables que es van poder aïllar, uns quants van presentar un gran potencial d'estudi a causa de l'absència de treballs disponibles a la bibliografia. Diversos assaigs s'han realitzat amb alguns d'ells, i finalment s'ha caracteritzat un dels fongs aïllats, anomenat SI27, la identitat del qual no havia estat clarament definida. L'aïllat SI27 és un fong ascomicet d'aspecte blanc i de creixement lent. Ha estat classificat com un cep pertanyent al gènere *Leptobacillium* sp., un gènere que està guanyant atenció en els darrers anys i que s'ha observat tant amb espècies ambientals com endòfites. A causa de la manca de dades bibliogràfiques, s'ha realitzat un estudi centrat en la seva caracterització com a espècie endòfita. Primer, se n'ha caracteritzat la morfologia, les condicions òptimes de creixement i la possible producció d'altres compostos d'interès, cosa que ha revelat que l'endòfita és capaç de sintetitzar sideròfors i àcid indolacètic. Un cop conegut el seu comportament òptim, s'ha examinat la interacció amb la planta hoste, la tomaca, en condicions controlades. Això ha servit per descartar el comportament patogen del cep d'estudi, i, alhora, per avaluar la seva capacitat de promoure el creixement de la planta. D'aquesta manera, s'ha confirmat el paper neutre en el creixement de les plantes, un possible efecte sobre el desenvolupament del genotip TH-30 i la millora de clorofil·les i producció de licopè en fruit.

Posteriorment, s'ha avaluat el potencial de l'endòfit per conferir resistència davant d'estrès biòtic a plantes de tomàquet, estudiant la infecció causada pel bacteri patògen *Pseudomonas syringae* pv. tomato DC3000 (Pst). A causa del conegut antagonisme entre fongs i bacteris, la hipòtesi inicial va indicar que, si hi havia algun tipus d'antagonisme, seria possible observar-lo mitjançant l'ús d'exsudats fúngics. Basat en això, s'ha realitzat un assaig *in vitro* utilitzant diferents filtrats de cultius fúngics, en els quals hi ha la presència d'exsudats de l'aïllat SI27. Els diferents filtrats estudiats han demostrat una clara inhibició del creixement de Pst en presència dels exsudats de SI27. Això ha permès fer posteriors experiments

en planta per avaluar si l'endòfita pot induir resistència davant d'estressos en planta. Els experiments s'han realitzat amb dos enfocaments: inoculant l'endòfita en llavor i tractant les plantes amb exsudats procedents dels cultius d'endòfita. Els resultats han revelat que tots dos mètodes són eficaços per reduir els nivells d'infecció per Pst a tomaques. També s'ha realitzat una anàlisi transcriptòmica i hormonal de les mostres vegetals, mostrant respostes de planta diferents depenent de l'enfocament utilitzat. Finalment, es va realitzar una anàlisi metabolòmica dels filtrats utilitzats en aquests experiments per cercar possibles compostos de caràcter antimicrobià.

En resum, en aquesta tesi s'ha estudiat la relació entre diferents genotips de tomaques i el seu microbioma, la caracterització d'aquests davant d'estrès per altes temperatura, la caracterització d'un aïllat endòfit i el paper que aquest pot tenir com a alternativa per a la millora i protecció de cultius de gran interès econòmic com la tomaca.

RESUMEN

Hoy en día las consecuencias ocasionadas por el cambio climático y la sobrepoblación mundial han hecho que la investigación en el sector agroalimentario sea más necesaria que nunca. Aunque las plantas sean sésiles, eso no significa que estén desprotegidas. Actualmente se conoce que una planta es parte de una gran unidad ecológica que engloba también su microbiota, es decir, los microorganismos que viven en contacto con ella. La presencia de estos microorganismos, que incluyen hongos y bacterias, varía en función de factores biológicos y ambientales. Diferentes especies de plantas se relacionan con diferentes comunidades microbianas, y las condiciones geológicas, climáticas y bióticas pueden afectar a estas interacciones. La microbiota puede afectar al crecimiento de la planta, así como su interacción con el medio y su resistencia frente a estreses abióticos y bióticos. En este contexto, los microorganismos que viven dentro de los tejidos de las plantas, conocidos como endófitas, están adquiriendo cada vez más protagonismo, y han sido estudiadas como alternativas ecológicas para combatir diferentes estreses que las plantas pueden sufrir.

El uso de organismos endófitos en la mejora agronómica es un amplio campo de estudio, cuyos avances han sido de gran relevancia en las últimas décadas. Sin embargo, la mayoría de las revisiones de la literatura suelen estar enfocadas a ciertos aspectos de la investigación, como especies concretos. Por ello, hemos realizado un análisis pormenorizado del estudio general sobre hongos endófitos presente en la literatura científica hasta 2021. Esto ha demostrado que la gran mayoría de los estudios hasta la fecha se centran en el uso de ascomicetos. Los modelos de estudio más comunes encontrados han sido aquellos referidos a plantas herbáceas y estrés abiótico, que superan la mitad de los estudios analizados. Mientras tanto, a pesar de que varios artículos demostraron el uso de hongos endófitos en cultivos de dicotiledóneas y su efecto prometedor como inductores de resistencia contra plagas, este campo de investigación aún está poco explorado. Estos resultados nos han permitido centrar la investigación en ámbitos estudiados con menor frecuencia y que son potencialmente explotables en la actualidad.

Por otra parte, estudios previos han demostrado que distintos genotipos de una misma especie vegetal pueden presentar comportamientos diferentes en su desarrollo y frente a condiciones adversas. Estos datos son de gran relevancia puesto que el uso de variedades con mayor tolerancia a los estreses del medio podría ser de gran utilidad en la agricultura contemporánea. Basándonos en esto, hemos caracterizado seis genotipos diferentes de tomate (*Solanum lycopersicum*) procedentes de varias regiones mediterráneas. De ellos, cuatro son variedades tradicionales (ADX2, TH-30, ISR-10, MO-10) y dos son variedades comerciales (*Moneymaker* y *Ailsa Craig*) que son resultado de una fuerte selección por parte del hombre. Estos genotipos de plantas de tomate han sido sometidos a estrés por altas temperaturas, y se han evaluado los daños fenotípicamente. Posteriormente, se ha estudiado el transcriptoma para identificar los posibles mecanismos de tolerancia observados, con énfasis en la importancia de genes relacionados con la defensa frente a altas temperaturas como son HSP70 y HSP90. Todo ello ha permitido demostrar y clasificar los genotipos como más susceptibles (TH-30, ADX2), o más resistentes (Moneymaker, MO-10) frente a estrés por altas temperaturas. Los resultados han mostrado que la tolerancia de las plantas MO-10 se basa principalmente en el control del mecanismo de enfriamiento de la hoja, así como la activación rápida de RBOHB y vías de señalización de ABA. En contraste, la variedad MM mostró una estrategia basada en la activación de HSP70 y HSP90, además de la acumulación de compuestos fenólicos correlacionados con la inducción temprana de la expresión de PAL.

Como se ha mencionado previamente, el genotipo de la planta puede influir en la presencia de diferentes comunidades microbianas, y éstas, a su vez, pueden influir en las características del genotipo. Por esta razón, se ha llevado a cabo un estudio de la microbiota de los diferentes genotipos de tomate. Para ello se ha recurrido a estudiar el genoma de la microbiota presente en el tejido vegetal, es decir, el microbioma, para identificar la cantidad y estructura de las comunidades de hongos y bacterias endófitos presentes en cada genotipo. Los análisis de este han podido confirmar la hipótesis de que las variedades tradicionales abarcan comunidades microbianas más amplias y diversas en su endosfera. Además, estas variedades poseen un número de microorganismos endófitos que no están

presentes en las variedades comerciales. Del mismo modo, se ha querido también demostrar el efecto que tienen las prácticas agronómicas convencionales como es el uso de fungicidas en la reducción de diversidad de la microbiota.

Seguidamente, se ha realizado el aislamiento de una colección de hongos endófitos de diversos genotipos de tomate. Entre los hongos cultivables que se pudieron aislar, unos pocos presentaron un gran potencial de estudio debido a la ausencia de trabajos disponibles en la bibliografía. Varios ensayos se han realizado con algunos de ellos, y finalmente se ha caracterizado uno de los hongos aislados, nombrado como Sl27, cuya identidad no había sido claramente definida. El aislado Sl27 se trata de un hongo ascomiceto de aspecto blanco y de crecimiento lento. Ha sido clasificado como una cepa perteneciente al género *Leptobacillium* sp., un género que está ganando atención en los últimos años y que se ha observado tanto con especies ambientales como endófitas. Debido a la falta de datos bibliográficos, se ha realizado un estudio centrado en su caracterización como especie endófita. Primero, se ha caracterizado su morfología, las condiciones óptimas de crecimiento y posible producción de otros compuestos de interés, lo cual ha revelado que la endófita es capaz de sintetizar sideróforos y ácido indolacético. Una vez conocido su comportamiento óptimo, se ha examinado su interacción con su planta huésped, el tomate, en condiciones controladas. Esto ha servido para descartar el comportamiento patógeno de la cepa de estudio, y, al mismo tiempo, para evaluar su capacidad de promover el crecimiento de la planta. De este modo, se ha confirmado su papel neutro en el crecimiento de las plantas, un posible efecto sobre el desarrollo del genotipo TH-30 y la mejora de clorofilas y producción de licopeno en fruto.

Posteriormente se ha evaluado el potencial de la endófita para conferir resistencia frente a estrés biótico a plantas de tomate, estudiando la infección causada por la bacteria patógena *Pseudomonas syringae* pv. tomato DC3000 (Pst). Debido al conocido antagonismo entre hongos y bacterias, la hipótesis inicial indicó que, si existía algún tipo de antagonismo, sería posible observarlo mediante el uso de exudados fúngicos. Basado en esto, se ha realizado un ensayo

in vitro utilizando diferentes filtrados de cultivos fúngicos, en los cuales se encuentra la presencia de exudados del aislado Sl27. Los diferentes filtrados estudiados han demostrado una clara inhibición del crecimiento de Pst en presencia de los exudados de Sl27. Esto ha permitido realizar posteriores experimentos en planta para evaluar si la endófita puede inducir resistencia frente a estreses en planta. Los experimentos se han realizado con dos enfoques: inoculando la endófita en semilla, y tratando las plantas con exudados procedentes de los cultivos de endófita. Los resultados han revelado que ambos métodos son eficaces para reducir los niveles de infección por Pst en tomate. También se ha realizado un análisis transcriptómico y hormonal de las muestras vegetales, mostrando respuestas de planta diferentes dependiendo del enfoque utilizado. Finalmente, se realizó un análisis metabolómico de los filtrados utilizados en estos experimentos para buscar posibles compuestos de carácter antimicrobiano.

En resumen, en la presente tesis se ha estudiado la relación entre diferentes genotipos de tomate y su microbioma, la caracterización de éstos frente a estrés por altas temperatura, la caracterización de un aislado endófito y el papel que puede tener este como una alternativa para la mejora y protección de cultivos de gran interés económico como el tomate.

摘要

随着气候变化和全球人口过剩的影响，农业部门的研究变得比以往任何时候都更加重要。尽管植物是固着的，但它们并非不受保护。目前，众所周知，植物是一个大型生态系统的组成部分，其中包括与其相互作用的微生物群。这些微生物（例如真菌和细菌）的存在因生物和环境因素而异。不同的植物物种与不同的微生物群落相互作用，地质、气候和生物条件会影响这些相互作用。微生物群可以影响植物生长，以及植物与环境的相互作用及其对非生物和生物胁迫的抵抗力。在这种情况下，内生菌，即生活在植物组织内的微生物，作为对抗植物可能面临的各种压力的生态替代品，正变得越来越重要。

应用内生生物进行农艺改良是一个广泛的研究领域，在近几十年来取得了重大进展。然而，大多数文献综述往往侧重于特定方面的研究，如特定物种。因此，我们对截至 2021 年的科学文献中关于内生真菌的研究进行了一般性综合分析。分析表明，迄今为止的绝大多数研究都集中在子囊菌的使用上。超过一半的研究与草本植物和非生物胁迫有关。同时，虽然有几篇文章证明了内生真菌在双子叶作物中的用途及其作为抗害虫诱导剂的潜在作用，但这一研究领域仍相对未被探索。这些结果使我们能够将研究的重点引向那些较少被研究但具有开发潜力的领域。

另一方面，先前的研究表明，同一植物物种的不同基因型在其发育和对不利条件的反应中可以表现出不同的行为。该信息特别重要，因为使用对环境压力具有更高耐受性的品种在当代农业中非常有益。鉴于此，我们鉴定了来自几个地中海地区的六种不同基因型的番茄（*Solanum lycopersicum*），包括四种传统品种（ADX2、TH-30、ISR-10、MO-10）和两种商业品种（Moneymaker、Ailsa Craig），这是广泛的人类选择的结果。对番茄基因型进行热胁迫，对叶片损伤进行表型评估。然后研究转录组以确定可能的耐受机制，重点是与防御温度相关的基因，例如 HSP70 和 HSP90。这使我们能够表征基因型并将它们分类为更易感（TH-30、ADX2）或更耐热应激（Moneymaker、MO-10）。我们的结果表明，MO-10 植物的耐受性主要基于叶片冷却机制的控制，以及 RBOHB 和 ABA 信号通

路的快速激活。相比之下，除了与 PAL 表达的早期诱导相关的酚类化合物的积累外，Moneymaker 品种还展示了一种基于 HSP70 和 HSP90 激活的策略。

前文提到，植物基因型对不同微生物群落的存在有影响，进而影响基因型特征。因此，我们进行了一项研究来分析不同番茄基因型的微生物群。该研究涉及研究植物组织中存在的微生物群的基因组，也称为微生物组，以确定每种基因型中内生真菌和细菌群落的数量和结构。该分析证实了传统番茄品种在其内生环境中具有更广泛和更多样化的微生物群落的假设。此外，传统品种含有某些商业品种中不存在的内生微生物。同时，该研究证明了传统农艺实践（例如使用杀菌剂）对微生物群多样性的减少有影响。

接着，从几种番茄基因型中分离出了一组内生真菌。在可培养的真菌中，由于文献中缺乏科学报道，一些真菌显示出巨大的研究潜力。我们对其中一些进行了多项测试，最终对一种分离的真菌进行了表征，并将其命名为 SI27，其身份尚未明确。SI27 是一种生长缓慢的白色子囊菌。该菌株被证明属于 Leptobacillium sp.，该属近年来受到关注，并已在环境和内生物种中被发现。由于缺乏书目数据，我们进行了一项侧重于将其描述为内生物种的研究。首先，对其形态、最佳生长条件以及可能产生的其他感兴趣的化合物进行了表征，揭示了内生菌可以合成铁载体 和吲哚乙酸。一旦了解了其最佳行为，就会在受控条件下检查它与寄主植物番茄的相互作用。这有助于排除研究菌株的致病行为，同时评估其促进植物生长的能力。通过这种方式，已经证实了它在植物生长中的中性作用，并且可能对 TH-30 基因型的发育和水果中叶绿素和番茄红素产量的提高产生影响。

随后，我们通过研究由病原菌 *Pseudomonas syringae* pv DC3000（Pst）引起的感染来评估内生菌对番茄植物生物胁迫抗性的潜力。感染采用的是番茄。鉴于已知的真菌和细菌之间的拮抗作用，我们最初假设如果存在任何拮抗作用，可以通过使用真菌渗出物来观察。因此，我们使用了含有 SI27 分离物渗出液的不同真菌培养滤液进行了体外测定。实验结果表明，研究所用的滤液在存在 SI27 渗出物的情况下能够明显抑制 Pst 的生长。这使得我们能够在植物中进行后续实验，以评估内生菌是否能够诱导抗逆性。我们使用了两种方法进行实验：将内生菌接种到种子中和使用内生菌培养物的渗出物处理植物。结果表明，这两种方法均可有效降

低番茄中 Pst 感染的水平。此外，我们还对植物样本进行了转录组学和激素分析，发现基于这种方法会发生不同的植物反应。最后，我们对这些实验中使用的滤液进行了代谢组学分析，以确定可能的抗菌化合物。

总结起来，本研究旨在探究不同番茄基因型与其微生物组之间的关系，以及它们在高温胁迫下的响应。此外，我们还通过鉴定内生分离物，探索了其在增强和保护具有经济价值的番茄作物方面的潜在作用。

INTRODUCTION

Plants are more indispensable than we give them credit for. They are fundamental to humankind as we know it, since they provide not only oxygen and food, but many other resources such as medicines, timber, textiles, and fuel. Moreover, the unstoppable growth of human society and population in the last decades has caused a rate of resource consumption and contamination that we need to address. The consequences of intensive agronomic practices, deforestation, desertification, and more will impact our global natural resources, and this will in turn affect our life. That is why society needs to prioritize the study of plants for a better future.

Feeding the increasing world population of 9.7 billion estimated by the year 2050 will be a significant challenge for agronomy due to the declining resources and the need to avoid detrimental impacts on the environment. Current studies focus on finding alternatives without increasing the current crop area (Erb et al., 2016). With limited land and water resources, climate change and increasing occurrence of pests and diseases, it is crucial to improve plant resistance to ensure food security. Therefore, developing and implementing effective strategies to improve plant resistance should be a top priority for the agricultural sector. For instance, Spain is one of the countries that produce the most tomatoes, with over 4 million tons of tomatoes per year. The area dedicated to tomato production in 2021 was 5,167,090 hectares, with an average yield per square meter of 3.66 kg of tomatoes (FAOSTAT, 2021). Improving plant resistance against stresses can help reduce crop losses, increase yields, and decrease dependence on synthetic pesticides and fertilizers. This will not only address the global food security challenge but also contribute to the sustainability of agriculture and the preservation of natural resources. However, traditional methods to obtain resistant crops are mainly through breeding programs or even genetic engineering, which takes a long time or are not always well regarded by consumers and legislators.

On the other hand, another greatly forgotten area is the one around microorganisms. For these, attention has usually been on pathogenic species we want to dispose of. However, microorganisms are of utmost importance in life on

Earth. They are present in all environments, shaping them and constantly interacting with each other and other organisms (Gunatilaka, 2006). Microorganisms accompany us in our daily lives and can act as harmful organisms, but many are neutral or even beneficial to animals and plants. It's been theorized for decades how big of an influence microbials have on humans based on cell number (Abbott, 2016). Likewise, plants are also intimately associated with countless microorganisms. The variety and identity of these microorganisms are known as the microbiota and together with their interaction and biology, they constitute the microbiome (Porras-Alfaro & Bayman, 2011). Thus, the microbial world is a world of complex relationships between macroscopic and microscopic life that is much more complex than previously imagined (Gilbert et al., 2012)

1. PLANT RESPONSES TO ADVERSE ABIOTIC CONDITIONS

Plants are constantly exposed to a wide range of abiotic and biotic stresses that they must overcome in order to survive and thrive (Fig.1). Like most living organisms, plants have an immune system to overcome stresses. However, as sessile organisms they must resort to several non-motile strategies to counter a disease.

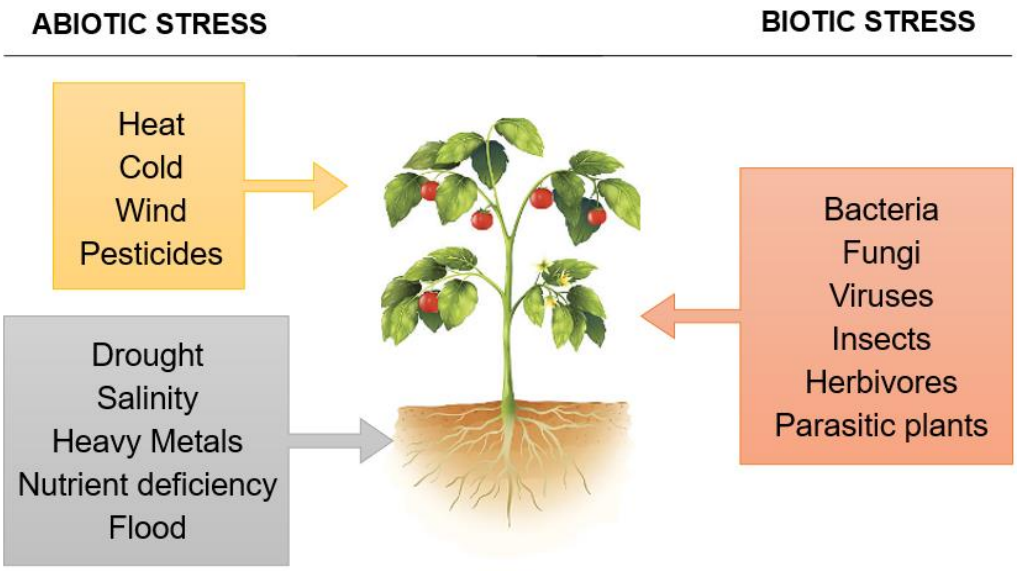

Figure 1. Main abiotic and biotic sources of plant stress and disease.

Adverse abiotic conditions can be, for instance: drought, extreme temperatures, salinity, heavy metal toxicity or nutrient deficiency or immobility. These stresses can affect plant development and productivity, so plants have evolved many defense strategies to cope with them. These include mechanisms that can be at molecular, physiological, or morphological levels. The knowledge about stress adaptation will be crucial for agronomic practices in the progressively severe climate change.

Plant defense strategies mainly revolve around water conservation, hormonal signaling and regulation, accumulation of compatible osmolytes, antioxidant production or regulation of root development. It is currently known that phytohormones, which have been described as key regulators of plant development, may also be fundamental in plant defense. Hormone signaling in abiotic stress is mainly thought to be caused by ABA, JA, Auxins, Cytokinins, Gibberellins, Ethylene, and Brassinosteroids (Bari & Jones, 2009).

In addition, researchers are studying several techniques to enhance abiotic resistance in plants. From the application of exogenous compounds, breeding strategies by exploiting tolerance traits from plant genotypes, use of beneficial microorganisms to advanced biotechnology like genome editing techniques, many techniques are being considered (Bashir et al., 2021)

Drought Stress

Drought is widely considered to be the most significant stress factor. It can affect many aspects of plant growth and metabolism (Chaves et al., 2003). The specific morphological and physiological challenges depend on both the stress intensity and the plant's sensitivity to the stress (Mukarram et al., 2021). Plant responses to drought stress can be classified into two categories: morphophysiological and biochemical processes.

The morphophysiological mechanisms are based on drought avoidance, escape, tolerance, or recovery (Fang & Xiong, 2015). For instance, tolerance is mainly achieved through the adjustment of osmotic processes, while drought avoidance

is through the regulation of stomata or root development (Ilyas et al., 2021). On the other hand, biochemical processes involve plant hormonal signaling where ABA is believed to be key. These processes usually activate other biochemical pathways and physiological processes.

Salinity stress

Increased salinization of the land is also a severe problem. High salinity is mostly caused by high concentrations of Na^+ and Cl^- in the soil. This causes plants to experience both hyperionic and hyperosmotic stress (Hasegawa 2000), causing growth inhibition by metabolic toxicity, accumulation of ROS, inhibition of photosynthesis and reduction of nutrient acquisition.

In this case, the main known defense mechanisms are through the regulation and activation of stress-related genes (Wang et al., 2003). Similar to drought stress, ABA-signaling is a central pathway that is believed to take part in plant response against salt stress (Zhu, 2016).

Heat stress

While climate change causes imbalance and extreme temperatures, either low or high, heat stress is considered a severe problem for agriculture in many areas of the world. Heat stress is usually regarded as an increase in temperatures of 10-15ºC above the ambient temperature, and is dependent on the intensity and the duration, as well as the speed of the rise (Willits & Peet, 1998). The severity of the stress can be accentuated in the presence of drought or high radiation.

Extreme temperatures might cause irreversible damage and cell death in a very short period of time, while more moderate temperature rises might cause tissue damage, and death only over a long period of time. Plant adaptation and resistance are also variable, and it depends on plant species and genotype, as well as the developmental stage (Chen et al., 1982). Particularly susceptible stages of plant growth are seed germination, flowering and anthesis, which are known to present lower heat-threshold levels.

High temperatures cause many quantitative impacts on plant development. The heat stress impairs photosynthesis and respiration, disturbs water relations and membrane stability, and changes hormone and metabolites levels. Heat stress reduces photosynthetic pigments, soluble proteins, and rubisco binding proteins (RBP) (Demirevska-Kepova et al., 2005). In terms of water status, a plant can maintain its water levels when moisture is abundant. However, high temperatures are frequently associated with a decrease in water availability (Simões-Araújo et al., 2003), which can cause more severe damage to the plant. In addition to dehydration, production, and accumulation of reactive oxygen species (ROS) (Ashaf & Harris, 2020), including singlet oxygen (O_2), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) cause oxidative stress that can severely damage the plant tissues.

In front of stress, plants resort to many defense mechanisms. The ability to tolerate high temperatures (thermotolerance) is a trait that entails a complex network of physiological, biochemical, and molecular mechanisms. Various mechanisms with metabolic and morphologic changes take part to maintain membrane and cell stability, triggered by a signaling cascade when stress is perceived (Iba, 2002). They mainly involve the synthesis of heat shock proteins (HSP), the regulation of defense gene expression through transcription factors, induction of mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) cascades, ROS scavenging to mitigate oxidative damage, and accumulation of compatible osmolytes.

HSPs can be found across all known organisms and are known to act as chaperones in molecular signaling. Their synthesis is quickly induced when the plant experiences high-temperature stress and helps improve physiological processes like photosynthesis and water regulation. The most relevant types of proteins are HSP90, HSP70 and small HSP (smHSP), which are small or low molecular weight proteins. Heat stress transcription factors (Hsf), which are the activators of the HS gene expression have also been studied for decades in the role to help plants to survive (Scharf et al., 2012).

ROS are regarded as toxic to plants and ROS scavenging is essential to prevent oxidative damage during heat stress. This process mainly relies on the action of the antioxidative system (AOS) (Dumanovic 2021), which involves antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT). Nevertheless, it has been discovered that ROS are also necessary for numerous biological processes and signaling during plant stress (Mittler, 2017).

In addition, compatible osmolytes have been shown to be crucial to mitigate stress effects by regulating osmotic activities (Bita & Gerats, 2013; Ghosh et al., 2021). These include organic compounds of low molecular mass like glycinebetaine (GB), proline or γ-4-aminobutyric acid (GABA).

The process of heat stress has been widely studied in crops like wheat and maize, but in dicot plants of economic relevance is still under research (Aleem et al., 2020). Current main approaches to mitigate heat stress are based on breeding and genomic techniques to obtain thermotolerant plants. The plant physiological responses and defense mechanisms are broadly studied and molecular aspects of thermotolerance are well understood, yet a throughout knowledge of the effect of factors and phenotype is needed to improve biotechnological techniques.

Finally, it is necessary to remember that many abiotic stresses can take place simultaneously, and this is believed to be the most common situation in the field. The plant response to two or more stress conditions is reported to differ from that of the addition of individual stresses, with a complex system that intertwines several signaling and molecular pathways and defense mechanisms (Suzuki et al., 2014).

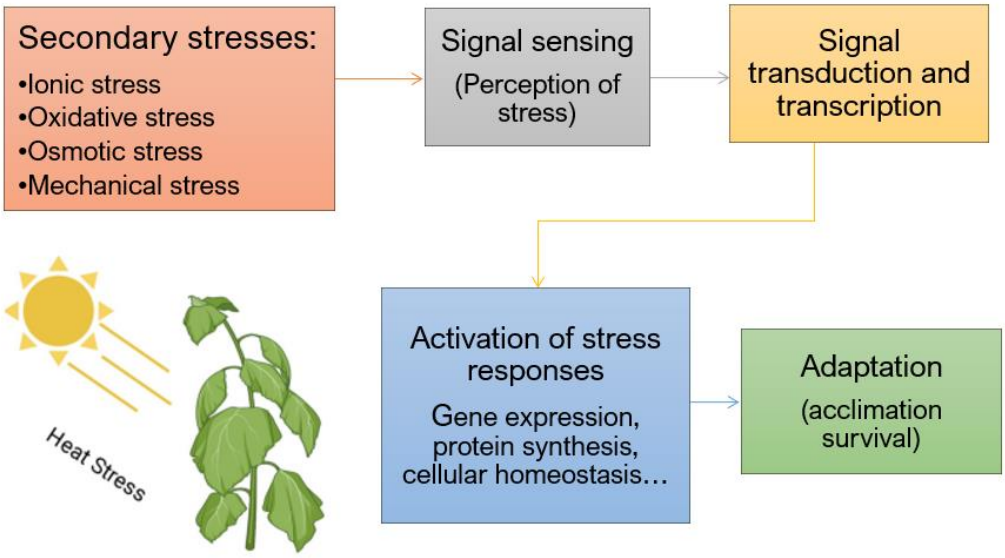

Figure 2. Generic diagram of plant stress signaling and response against heat stress.

2. PLANT DEFENSE AGAINST PATHOGENS

In addition to environmental stress, plants may also suffer biotic stress from many organisms, such as bacteria, fungi, oomycetes, viruses, nematodes, insects or animals. Plants lack adaptive immunity and specialized immune cells, so they have a complex innate immune system to confront a wide spectrum of pathogens. They possess physical and structural barriers as the first line of defense (Reina-Pinto & Yephremov, 2009) but if the pathogen manages to surpass them, a signaling cascade takes place in the plant to trigger its defense mechanisms. To activate the plant innate immunity, it is necessary to have cell surface receptors that can recognize exogenous molecules and carry out the signaling to activate defense-related pathways (Andersen et al., 2018). The plant immune system can be seen as comprised of two branches: one that functions at the transmembrane level and another that operates intracellularly (Jones & Dangl, 2006).

The intercellular responses are triggered by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs are conserved microbial features that are also known as microbe-associated

molecular patterns (MAMPs) because this stage does not the source as mutualistic or pathogenic (Staal & Dixelius, 2007). Recent studies have also introduced the term NAMPS to describe nematode-derived molecular patterns (Ali et al., 2018). Conversely, DAMPs refer to molecules that are released by stressed or damaged cells; sometimes termed HAMPs in case of herbivore injuries (Mithöfer & Boland, 2008). Many MAMPs and DAMPs contain carbohydrates. MAMPs, such as flagellin, lipo-oligosaccharides, glucans and chitin are widely recognized (Zhang & Zhou, 2010) while DAMPs include extracellular ATP, systemin and other elicitor peptides (Choi & Klessig, 2016).

To detect these molecules, plants have a diverse array of receptors, including pattern recognition receptors (PRRs), which are surface-localized receptor-like kinases that react to MAMPS, and wall-associated kinases (WAKs) that recognizes DAMPs (Kaur et al., 2022). The recognition of PAMPs and DAMPs by PRRs and WAKs is a critical step in the plant immune response. PRRs elicit a signaling cascade that leads to PAMP-triggered immunity (PTI) or MAMP-triggered immunity (MTI). DAMP-triggered immunity shares signaling compounds and functional mechanisms with PTI (Hou et al., 2019).

PTI can be effective against many different types of pathogens, so evolution has driven pathogens to develop strategies to suppress or evade PTI. Plants in turn resorts to the second immunology branch, which focuses on intracellular recognition of virulence factors that pathogens use for infection (effectors). The recognition process is mediated by nucleotide-binding domains with leucine-rich repeats (NLRs or NB-LRRs). NLRs activate what we know as effector-triggered immunity (ETI) (Andersen et al., 2018; Dodds & Rathjen, 2010). ETI has been shown to be effective against biotrophic or hemibiotrophic pathogens and is somehow considered an enhanced version of PTI (Glazebrook, 2005; Jones & Dangl, 2006).

The interaction between PTI and ETI has been traditionally recognized in a zigzag system (Jones & Dangl, 2006). First, PAMPs are recognized by PRR which triggers PTI. If a pathogen deploys effectors to evade or suppress PTI, it causes effector-triggered susceptibility (ETS). Then, these effectors may be recognized

by specialized NB-LRR proteins, activating ETI. Finally, evolution can make pathogens to diversify their effectors to surpass ETI. Nevertheless, recent studies have suggested additional interactions beyond this model, indicating that ETI may potentially enhance PTI (Ngou et al., 2021).

Many signaling mechanisms take place to regulate plant defense, which includes hormones, activation of the defense-associated mitogen-activated protein kinases (MAPKs), transcriptional factors (TF), callose deposition, ubiquitin and fluctuation of calcium ions (Choi & Klessig, 2016). For instance, plant hormones vastly affect plant growth and response functions. Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are all known to play a central role in the regulation of plant immune responses. In addition, cytokinins (CK), gibberellins (GA), abscisic acid (ABA), nitric oxide (NO), brassinosteroids (BR) and auxins have also been reported to act after pathogen detection and to be key in plant immunology (Andersen et al., 2018; Denancé et al., 2013). SA is crucial for local and systemic resistance to biotrophic pathogens, while JA and ET have central roles against insects and necrotrophic pathogens (Alazem & Lin, 2015; Carvalhais et al., 2013; Corina Vlot et al., 2009; Han & Kahmann, 2019; Pieterse et al., 2012). Plant hormones also interact with each other in their signaling, which influences plant response in several ways. These interactions are complex networks to balance plant development and defense mechanisms against many stresses (Yang et al., 2019). Thus, pathogenic organisms can target hormones to disrupt this finely tuned balance to evade plant defense mechanisms.

The downstream mechanisms to prevent further pathogenic infection include the closure of stomata, cell wall modification, localized cell death or hypersensitive response (HR) and production of compounds such as chitinases, protease inhibitors, pytoalexins (Andersen et al., 2018; Balint-Kurti, 2019).

In the end, the plant’s immune response depends on many factors, including its development stage and the microbiome (Li et al., 2020), so understanding each one of the processes is crucial to regulate plant-pathogen interactions as a whole.

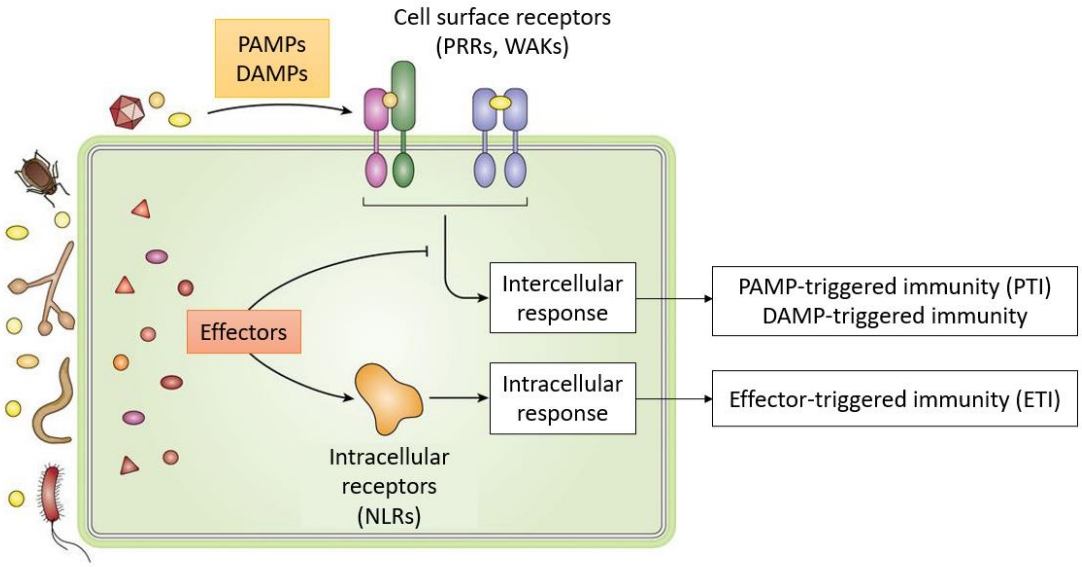

Figure 3. Simplified diagram of plant's immunity response against pathogens. Pathogenic signals are perceived by plant receptors, which trigger different signaling cascades to activate plant defense mechanisms. Adapted from Bentham et al. (2020).

2.1. Pseudomonas syringae

P. syringae is a gram-negative bacteria that causes bacterial speck disease in a wide range of economically important crop species. For instance, the disease in tomato is mainly caused by the pathovar tomato DC3000 (Mansfield et al., 2012). It is a widely studied plant pathogen since it serves as a model to study plant-pathogen interactions (Buell et al., 2003; Xin & He, 2013). Currently, more than 60 pathovars are known, mostly specialized in a narrow host range (Hirano & Upper, 2000), and new *P. syringae* isolates continue to be a threat to global crop production.

P. syringae is believed to have evolved from a non-pathogenic ancestor and have become a successful plant pathogen by acquiring crucial virulence traits. This is supported by the fact that *P. syringae* also has many nonpathogenic strains that have been reported to be environmentally relevant (Spiers et al., 2000). Here we focus on pathogenic *P. syringae*, which is an hemibiotroph that has two biological stages: an epiphytic one and an endophytic one. In the first one, it lives on plant

surfaces (biotrophy) while in the second, it invades plant tissue and multiplies causing cell death (necrotrophy).

The epiphytic stage duration depends on the bacterial strain. For instance, *P. syringae* pv. *syringae* are strong epiphytes, but *P. syringae* pv. tomato DC3000 are relatively weak. The infection can only take place when bacteria is able to enter plant tissue, usually through wounds and natural openings such as stomata. However, plants can recognize PAMPs such as flagellin, and activate PTI causing stomatal guard cells to close stomata (Melotto et al., 2006). To overcome this, *P. syringae* has a Type III secretion system (T3SS). This is a complex protein structure that is essential to inject effectors into the plant cells and is conserved in all pathogenic *P. syringae* strains (Xin et al., 2018).

Plants have in turn evolved to recognize T3SS effectors and trigger ETI to inhibit bacterial growth (Jones et al., 2016). To surpass plant immune responses, *P. syringae* uses T3Es (Type III Effector proteins) and other virulence factors like coronatine (Waite et al., 2017). Some T3Es can induce an aqueous apoplastic environment to enhance bacterial growth. As for coronatine, it is a molecular mimic of jasmonyl isoleucine (JA-Ile), the active form of jasmonic acid in the plant (Katsir et al., 2008). This molecule exploits the antagonistic interaction between JA and SA signaling, suppressing SA-induced defense mechanisms, and inhibiting stomatal closure (Ishiga, 2017; Uppalapati et al., 2005). It also has been reported to inactivate MAPKs (Freeman & Beattie, 2009).

In the end, this is a survival competition and besides the plant and pathogen responses, environmental factors also affect the outcome. For the disease to develop, high humidity and mild temperatures are required. For *P. syringae* pv. tomato, it is believed to be 20-25ºC and 90% humidity (Hirano & Upper, 2000; Lindow & Brandl, 2003). Humidity allows bacteria to survive as epiphyte and is also essential for bacterial multiplication in the apoplast. Temperatures over the optimal 28ºC are reported to affect plant immunity and potentially reduce disease by limiting the production of phytotoxins.

Finally, it is currently known that the plant microbiome can also affect the interaction between plant and pathogen. It has been proven that the addition of

certain microorganisms, known as biocontrol agents, reduces the virulence or epiphytic fitness of *P. syringae*. These microorganisms have been shown to confer resistance by direct and indirect methods previously discussed, such as competition or production of antibiotics, cytokinins or siderophores (Xin et al., 2018). Root microbiota is shown to also induce ISR in plants against pathogen infection. However, the interaction between *P. syringae* and plant endophytes is still poorly understood (Pieterse et al., 2014).

3. PLANT SYMBIONTS: THE PLANT MICROBIOME

As previously mentioned, plants are complex organisms that interact with a wide range of microorganisms in their surrounding environment known as the "microbiome". In this context, the plant microbiome is also known specifically as the "phytobiome". Plant and microorganisms might also be considered as a single large organism under the term "plant holobiont". This concept acknowledges that plants are not isolated entities but rather part of a complex ecological system that includes microorganisms. This has expanded our understanding of the plant-microbe interactions to a more holistic view that encompasses the entire community of microorganisms that interact with plants. Therefore, studying plants involves much more than just studying the plant organism. The knowledge about the interactions that occur within the plant holobiont can provide us with great natural alternatives for a diversity of functions, both in the ecosystem and for society.

On the other hand, they can be considered as symbiotic organisms in the traditional view of a host (the macroorganism) and the symbionts (the microorganisms). Furthermore, these microorganisms also interact with each other and might be mutual or antagonistic towards each other. It is also thought that symbiotic relationships often occur with more than two parties, forming complex symbiosis such as tripartite systems in bacteria-fungus-plant (Yang et al., 2012).

In recent years, recognition of the crucial role that beneficial microorganisms play in plant performance and health has risen. These microbes constitute an

important part of the plant holobiont: the collective set of organisms interact with the plant, both internally and externally, to influence its growth, development and survival. In fact, beneficial microorganisms have been found to promote plant growth, improve nutrient uptake, enhance stress tolerance and protect plants from pests and diseases (Turner et al., 2013). These microorganisms are present in the exterior or interior of plants, above or below ground. This is known as the phytosphere (Yang et al., 2012), and is comprised of the phyllosphere, the endosphere and the rhizosphere.

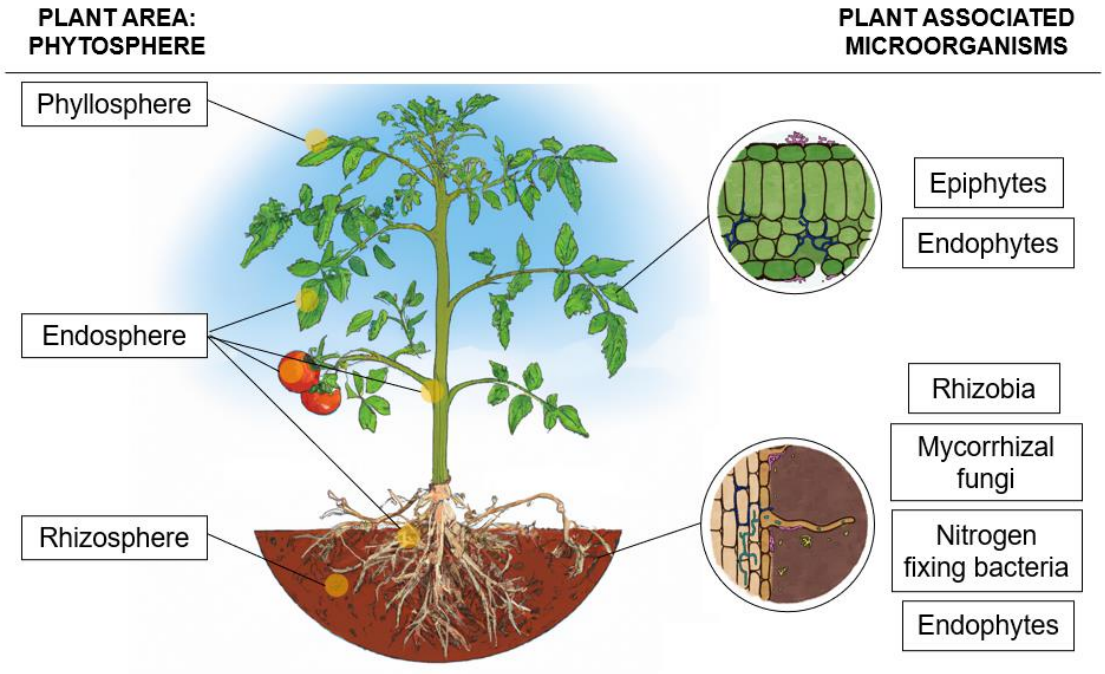

Figure 4. Diagram of plant microbiota according to plant area in aerial and underground tissue. Bacterial and fungal microorganisms can be found on or inside plant tissue and are in constant interaction with the plant.

The rhizosphere has been reported to harbor a diverse community of beneficial microorganisms that form mutualistic relationships with plants, exchanging nutrients and signals that promote plant growth. These communities are soil-born or closely related to the environment. One of the most well-known examples is mycorrhizal fungi. These fungi form a mutually beneficial relationship with plants where the fungi receive carbohydrates from the plant and, in return, provide the plant with increased access to soil nutrients like phosphorus (Van Der Heijden et

al., 2008). Another well-known symbiont is nitrogen-fixing bacteria (Hayat et al., 2010) which live in nodules on the plant roots and convert atmospheric nitrogen into a biologically available form for the plant.

On the other hand, there are plant symbionts that live inside the plant tissues. In this work, we want to focus on these microorganisms that live in the plant endosphere, known as endophytes. This term is usually referred to the organisms that live inside the plant tissues without causing any harm to the host plant (Jia et al., 2016; Porras-Alfaro & Bayman, 2011). Endophytic bacteria and fungi have been found to be abundant in all plant species, from crops to wild plants and have been shown to have important ecological roles. Endophytic fungi and bacteria have been found to colonize various plant tissues, including roots, stems, leaves and flowers.

In summary, main fungal symbionts can be classified as mycorrhizal, either arbuscular mycorrhizal fungi (AMF) or ectomycorrhizal fungi (EMF) and epiphytic or endophytic fungi. On the other hand, bacterial symbionts are usually being grouped in terms like plant growth-promoting bacteria (PGPB), plant growth-promoting rhizobacteria (PGPR), mycorrhization helper bacteria (MHB), rhizobia, and nitrogen-fixing bacteria (NFB), where overlapping is frequent according to Gilbert et al**.** (2012).

In particular, endophytic microorganisms of plants, including bacteria and fungi, have been reported as important roles in the growth, health and adaptation of their host plants. They have been shown to produce plant growth-promoting substances, protect plants from stress and stimulate the plant's immune response against pathogens. They can also fix nitrogen, break down organic matter, and influence gene expression in the host plant leading to changes in plant physiology, metabolism, and development. Their interactions with host plants are complex and they have significant potential for use in sustainable agriculture and natural resource management a field that is still under research.

4. FUNGAL ENDOPHYTES

Plant-associated fungi have been the subject of attention since the last century. These can be categorized as pathogenic, saprotrophic, epiphytic, mycorrhizal or endophytic (Porras-Alfaro & Bayman, 2011). The classification is diverse depending on the characteristics shown under the study parameters. As for symbiotic fungi, they are widely believed to exist in almost all plants, whether they are epiphytic, endophytic, or mycorrhizal in nature (Porras-Alfaro & Bayman, 2011)

As previously mentioned, fungal endophytes (from the greek words *endon*: within, and *phyton*: plant) are those microorganisms that live inside the plant tissues, whether intercellular or intracellular parts of roots, stems, and leaves, without causing any harm to their host plants. The definition of this term has evolved over time since its initial use, expanding from encompassing all living organisms inside a plant to specifically referring to those that do not cause damage. Thus, though the relationship between the host and the endophyte ranges from a continuum between mutualism, commensalism and parasitism, the term “endophyte” is commonly used in literature as a synonym for beneficial and symbiotic endophytes (Schulz & Boyle, 2005).

It is currently believed that fungal endophytes are equally or even more abundant than mycorrhizal fungi (Mandyam & Jumpponen, 2014). Thanks to modern technology, which includes advanced molecular and genomic tools, significant progress has been made in discovering and characterizing endophytes.

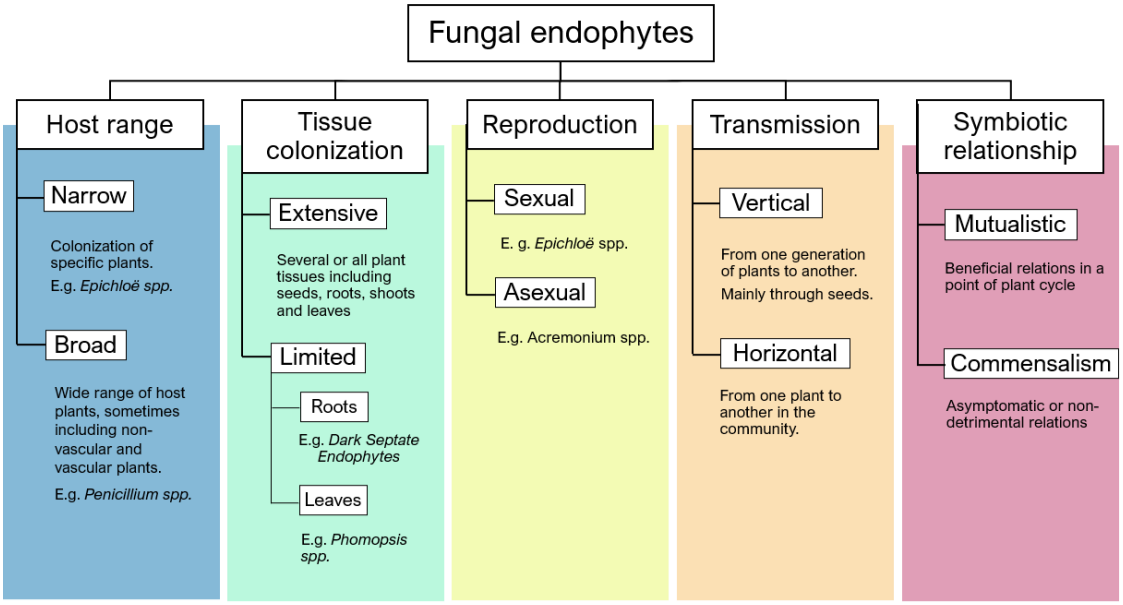

Figure 5. Main biological traits of fungal endophytes with relevance for plant research. Adapted from Rodriguez et. al (2009) and Gautam and Avasthi (2019).

4.1. Endophyte diversity

Fungi have been present on Earth for over a billion years and have played a significant role in altering the structure of Earth's ecosystems. Due to their long history of evolution and adaptation, these organisms are considered as one of the most critical mutualistic symbionts, as noted by Berbee et al. (2017). This long evolution of symbiotic fungi might be evidenced by the phylogenetic background of many symbionts which are closely related with some pathogenic species. Furthermore, fungal endophytes are a group of diverse ecological and symbiotic characteristics. Some fungi, such as mycorrhizal fungi or endophytic *Epichloë* sp., are obligate symbionts while others are considered opportunistic symbionts due to their ability to live and proliferate alone (Harman & Shoresh, 2007).

The diversity of fungal endophytes is vast, with different species being found in different plant hosts and in different parts of the plant. Fungal endophytes have been isolated from almost every plant species studied so far, including trees, herbs, shrubs, and crops. Thus, determining the diversity is a major challenge, with around 100.000 species described. However, experts consider there might be around 1.5 million species (Fröhlich et al., 2000; Gamboa et al., 2003). Every

plant species harbors their own communities of endophytes. Some studies showed that endophytes might be more diverse and abundant based on latitude (Arnold, 2007). However, that is not always the case. Communities of endophytic fungi can vary greatly in a single host species in different sites, climates, seasons and environments because plants and endophytic communities might be shaped based on the environmental factors (Arnold, 2007; Porras-Alfaro & Bayman, 2011).

Despite the vast diversity, several attempts of classification have been done. For instance, Schulz and Boyle (2005) divided fungal endophytes in mycorrhizal, balansiaceous and non-balansiaceous. Later, Rodriguez et al. (2009) provided a comprehensive basic classification of fungal endophytes. These authors considered fungal endophytes to pertain to two big categories: clavicipitaceous and non-clavicipitaceous; and four classes characterized by the symbiotic functions, host range, tissue specificity, colonization and transmission.

Something relevant to understand is that studies were previously limited to those endophytes that were cultivated and fast-growing, leaving unculturable or more specialized ones to be poorly known (Sun et al., 2012; Unterseher & Schnittler, 2009). Another issue was that many studies were based on morphological characterization, leaving the differences below species level to not be estimated (Aly et al., 2011). Thus, the diversity seems to be much higher than previously thought and thanks to the advances of DNA-based techniques we now have a better understanding of the diversity and ecology of the fungal endophytes (Arnold, 2007).

4.2. Isolation and identification of fungal endophytes

The isolation of fungal endophytes is inevitably associated with their cultivability *in vitro*, so many currently unculturable endophytes are unexplored. Common fungal culture media include Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Czapex Dox Agar (CDA), Sabouraud Dextrose Agar (SDA) or Nutrient Agar (NA) (Sharma & Pandey, 2010; Syamsia et al., 2019).

In addition, identification of fungi has always been a complex task due to the diversity of fungal species and the high specialization it requires. Traditionally, morphologic characterization was the main way to identify fungi. Mycologists were able to characterize fungal strains by its phenotypic traits such as reproductive structures and the spores. However, this was not reliable, especially for a high classification grouping at species level (Raja et al., 2017) because crucial parts of morphologic identification might differ under laboratory conditions. For instance, the characteristics shown might be highly variable or lack sporulation phase (Hyde & Soytong, 2008).

Nowadays, genomic advances have allowed a more accurate identification of fungal strains based on sequencing the Internal Transcriber Spacer (ITS) region. ITS-based sequence identification of fungi has been widely used for over two decades and is considered the official DNA barcode marker for fungi due to its rapid evolution (Mitchell & Zuccaro, 2006). However, ITS region might not work in some genera with narrow to no gaps in their ITS regions. Thus, other genes are being reported to be used in combination with ITS for further identification such as large ribosomal subunit (LSU), beta-tubuline II (TUB2) or translation elongation factor 1 (TEF1) (Mitchell & Zuccaro, 2006; Stielow et al., 2015). The sequences are usually used to perform a Basic Local Alignment Search Tool (BLAST) in Genbank or other specialized databases in order to verify the fungal identity (Hennell et al., 2012; Raja et al., 2017).

Nevertheless, the use of DNA sequencing for identification must be carried out with sequences that are representative of the study regions, in several reliable databases, because not all deposited sequences might be correctly annotated, authenticated, or have taxonomic names up to date (Nilsson et al., 2006). Thus, the use of genomic sequencing in fungi represents an essential tool for the advances in the field of fungal endophytes, yet it is crucial to resort to highly specialized databases such as UNITE or Greengenes for correct identification.

4.3. Endophyte-host interaction

As previously mentioned, endophytes do not cause any visible harm to plant tissue. However, it is important to consider that all fungi in plant tissues have an asymptomatic period that varies from imperceptibly short (pathogens) or lifelong lasting (Saikkonen et al., 2004).

Endophytic fungi are usually treated as mutualistic associated with the host plant but the term is mostly referred to the state of the detection of the fungi, regardless of future interactions (Schulz & Boyle, 2005). The symbiosis is a difficult balance between forces among the host and the microorganisms. This relationship is determined by the specific factors that intervene such as environmental or host conditions and a change in those might cause a shift in the interaction. For instance, vulnerability of the host, presence of other competitive endophytes or the longevity of the interaction (Patle et al., 2018; Rodriguez et al., 2009). Because of that, some authors prefer to use the term “true endophytes” for those long life mutualistic endophytic associations (Mostert et al., 2000). In the end, although endophytism can be a mutualistic relationship, it might not be the epitome behavior of the microorganisms but rather an adaptation to the certain conditions, which is dynamic in time (Saikkonen et al., 2004).

Root endophytes have been poorly studied in comparison to mycorrhizae and differ from these in that they don’t make the nutrient transfer interfaces (Porras-Alfaro & Bayman, 2011). Sometimes endophytes were reported as contaminants in mycorrhizal studies especially dark septate endophytes (DSEs) since they occur in mycorrhizal roots (Li & Guan, 2007) or might also form mycorrhiza-like structures (Hou & Guo, 2009).

Some endophytes can have a wide range of hosts while others are really specialized in a particular host range. Their successful colonization depends on several factors, such as the plant genotype, plant tissue, microbial strain type and environmental conditions (Hardoim et al., 2015). This initial stage can be either intercellularly or intracellularly. Fungal endophytes need to secrete metabolites to colonize their host and compete with other microorganisms and also regulate

the host metabolism to induce certain changes in plant metabolic pathways (Harman & Shoresh, 2007; Schulz et al., 2002). Though some studies report similarity of pathogenic and endophytic fungi in their initial trigger of an immune response from plant, the colonization of endophytic fungi can avoid the host defense responses and is more localized in plant tissue, and often intercellular (Gautam & Avasthi, 2019)

Finally, fungal endophytes can be transmitted either vertically or horizontally. In the case of vertical transmission, the endophytes proliferate in successive generations of the host. On the other hand, horizontal transmission involves the interaction between individuals or through the environment. It is thought that vertically transmitted endophytes, such as *Epichloë* (anamorph: *Neotyphodium*), have more standing beneficial effects than horizontally transmitted ones, since these could be local or dormant pathogens (Saikkonen et al., 2004).

4.4. Production of secondary metabolites

Medicinal plants have been used for their bioactive metabolites for a long time. However, as knowledge of endophytic organisms grow, questions have arisen about the origin of antimicrobial compounds found in these plants. It is possible that when an infection occurs in plant tissue, the antimicrobial compounds produced to withstand it may not be generated by the plant's own metabolism but rather by microorganisms that inhabit it.

As a result, the focus of drug research has shifted from plants to microorganisms and the production of secondary metabolites by endophytes have been researched. These fungal secondary metabolites are biologically active compounds that are not essential for growth or reproduction but are involved in other functions like signaling and defense (Hardoim et al., 2015). Studies have reported that compounds like taxol or cyclosporine were produced by both endophyte and host but absent when no endophyte was present (Nisa et al., 2015).

These metabolites have been targeted towards bacterial and fungal pathogens, cancer cells, glucose transport and other purposes (Porras-Alfaro & Bayman,

2011). Recently, many endophytic fungi have been found to produce compounds with medicinal properties, including alkaloids, terpenoids, flavonoids and phenolic compounds (Shevchuk et al., 2023; Strobel, 2003). Thus, these natural compounds have been found to be promising for current agricultural and medical progress (Gautam & Avasthi, 2019; Raja et al., 2017). Nevertheless, these compounds are the result of several environmental, evolutionary and ecological factors (Aly et al., 2011). Since the relationship between endophytes and their host plants is not yet well understood, further research is needed to prove the potential for producing bioactive compounds from these fungal endophytes.

5. THE ROLES OF FUNGAL ENDOPHYTES FOR PLANT IMPROVEMENT

Fungal endophytes can provide a wide spectrum of effects to their host so they can also be classified based on its functional role. Some beneficial endophytes produce compounds that can protect plants from herbivores and pathogens while others can improve nutrient uptake and enhance the plant's ability to tolerate drought or other environmental stressors. We here consider a functional classification based on beneficial endophytes, and not regard mycorrhizal fungi or dormant pathogens and saprobes. Commensal endophytes that do not confer any effect on plant performance are also not considered. In the following sections the main functional roles of plant endophytes will be introduced.

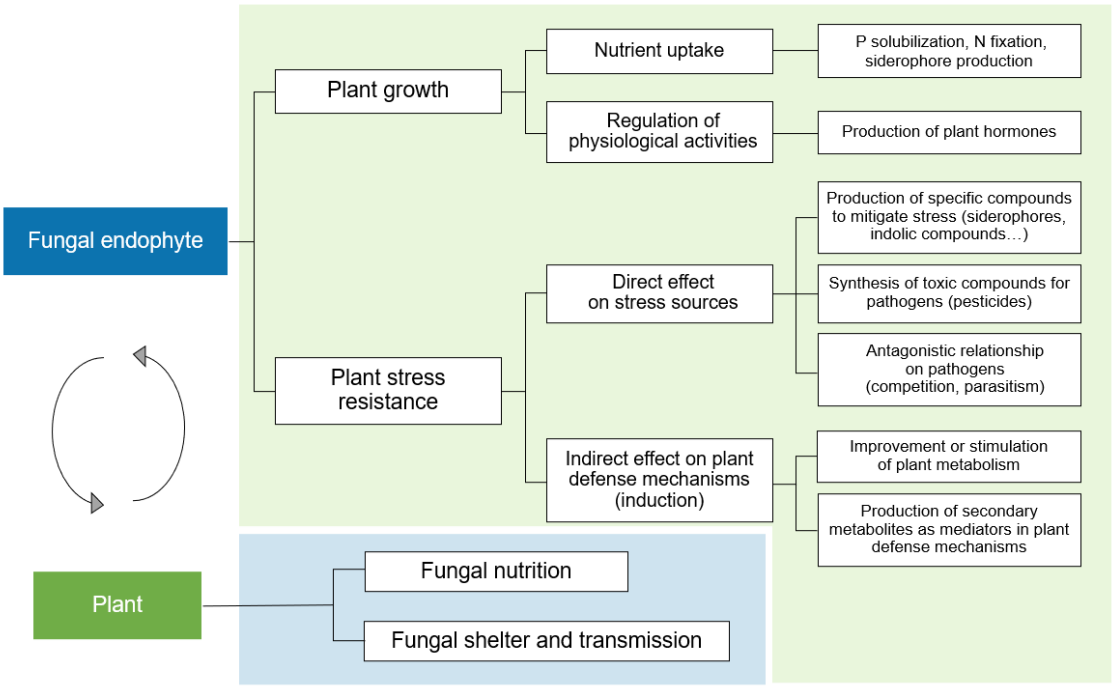

Figure 6. Beneficial effects in a symbiotic relationship between a host plant and a fungal endophyte.

5.1. Promotion of plant growth and development

There are various mechanisms through which a fungal endophyte can enhance plant performance. Beneficial endophytes are believed to influence plant growth by accelerating physiological activities, taking place in processes such as photosynthesis and carbon fixation (Hardoim et al., 2015). They can produce compounds to regulate plant growth or produce antimicrobial compounds to improve the fitness of the host plant to overcome unfavorable situations (Sudha et al., 2016).

In exchange for obtaining food and shelter, these microorganisms can help by improving nutrient uptake or producing growth-related phytohormones. Nutrient uptake is improved by the ability to solubilize phosphates, fix nitrogen, produce siderophores and more. Moreover, they can produce hormones that are relevant for plant growth, such as auxins, gibberellins and ethylene (Hardoim et al., 2015), which have been extensively studied and is a common trait of root endophytes.

5.2. Resistance to plant under abiotic stress

The presence of endophytic fungi may also help plants to withstand adverse environmental conditions. Many studies have examined these interactions under plant stress and how they change. The stress could be caused, for instance, by drought, high temperatures or salinity of the soil (Rodriguez & Redman, 2008), so endophytes could help plants adapt to climate change (Selim, 2012; Trivedi et al., 2016). Not only that, but they can also have a role in phytoremediaton, in the degradation or accumulation of heavy metals (Porras-Alfaro & Bayman, 2011).

Endophytes can confer stress resistance to the host plant by acting as a biological trigger and inducing the plant defense mechanisms or by direct synthesis of compounds that mitigate the stress effects (Singh et al., 2011). For instance, fungal species of the *Epichloë* or *Curvularia* genera have been extensively studied against drought and heat (Rodriguez et al., 2008; L. Xu et al., 2017)

5.3. Biocontrol agents in plant biotic stress

Fungal endophytes have been known for their capability to inhibit plant pathogens and combat insects and herbivores (Bamisile et al., 2018; Gao et al., 2010). For instance, some endophytes can produce compounds that can be applied as pesticides and others can stimulate the plant's immune system to help defend against fungal and bacterial pathogens.

There are many mechanisms known to improve host resistance, that can be divided into direct and indirect mechanisms. Known direct mechanisms include competition, antibiosis and parasitism. Fungal endophyte can directly antagonize with a plant pathogen for colonization or produce toxic compounds with direct effect on plant pathogen (Porras-Alfaro & Bayman, 2011). Many endophytic species have been reported to produce antibiotic substances which can be applied in agriculture against several fungal pathogens (Gautam & Avasthi, 2019). Some fungal endophytes, such as *Trichoderma* sp., are also able to act as a parasite of plant pathogens (Guzmán-Guzmán et al., 2019).

Indirect mechanisms are mainly an improvement or stimulation of plant metabolism. For instance, endophyte can produce secondary metabolites that serve as mediators for specific interactions with host plants and enhance the plant production of compounds with antimicrobial capabilities. Endophytes could also induce plant defense responses or improve plant growth and physiological functions to overcome stress (Johnson et al., 2014; Sudha et al., 2016).

These indirect mechanisms are commonly studied under the concept of plant-induced resistance, which has gained significant attention in recent years. Previously mentioned plant defenses involved PTI and ETI, which often trigger an induced resistance to enhance defense in distal plant tissues to protect against further infection. This is known as systemic acquired resistance (SAR) (Pieterse et al., 2014; Shoresh et al., 2010). SAR is characterized by SA-mediated pathways and might be triggered by pathogen attack or treatment with some chemical compounds (Corina Vlot et al., 2009). Another plant systemic resistance is provided by induced systemic resistance (ISR). ISR is triggered by the colonization of non-pathogenic microorganisms (Pieterse et al., 2014; Walters et al., 2013), which induces a physiological state called “priming”. This “priming” allows plants to respond faster against subsequent pathogenic attacks, providing an enhanced level of protection against a broad spectrum of attackers (Walters et al., 2013; Yu et al., 2022). ISR is a complex and multifaceted process that involves various biochemical and molecular interactions. Though ISR was firstly thought to be similar to pathogen-induced SAR, it was found to be independent of SA and dependent on JA and ET signaling pathways (Van Oosten et al., 2008; Walters et al., 2013). In addition, the combination of ISR and SAR has been suggested to provide further protection to plants against pathogens (Panpatte et al., 2020).

In summary, the diversity of functions of fungal endophytes is vast, and their importance in plant health and potential applications in medicine and agriculture make them a fascinating area of study: a promising sustainable approach to enhance plant defense mechanisms.

OBJECTIVES

Solanum lycopersicum (tomato) is considered a model plant for researchers, in addition to being one of the most significant crops globally. Our study was based on the hypothesis that this species harbors countless endophytic microorganisms that are still relatively unknown to the scientific community which may constitute a promising area of study in the search of more sustainable alternatives to current agronomic practices.

Therefore, the present thesis aims to:

1. Analyze the current knowledge about endophytic fungi by reviewing research literature up to 2021. Special attention to be paid into less frequent studied areas.

2. Characterize tomato genotypes of different background under high temperatures which is an increasingly common stress factor plants need to withstand.

3. Determine the microbiota of the different tomato genotypes and compare the microbial composition and structure among them and under fungicide treatment.

4. Isolate and characterize a fungal endophytic strain from a traditional tomato plant and study its influence on normal plant growth.

5. Evaluate the effects of the endophytic isolate against a common plant pathogen, *Pseudomonas syringae* pv. tomato, and its potential to confer biotic resistance to tomato plants.

CHAPTER 1

Advances in endophytic fungi research: A data analysis of 25 years of achievements and challenges

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ABSTRACT

Research on fungal endophytes has demonstrated the ability to improve crop performance and protect host plants against diverse biotic and abiotic stresses. Yet, despite the exponential growth of this topic, a whole outline to reflect the relevance and extent of each study type is missing. Hence, we performed an analysis of all available literature to expose the characteristics and limitations of this research field. Our results suggested that, overall, there is still a tendency to study the most known models in plant-fungal-stress combinations (ascomycetous fungi, grasses, abiotic stress). Fungal endophytes in dicot plants or against biotic stress, though promising, are still quite unexplored. All these data could lead future studies to assess less considered study factors that might help discern the beneficial effects of fungal endophytes with more extent and accuracy.

KEYWORDS: fungal endophytes, beneficial microorganisms, agronomic improvement, bibliographic research.

1. INTRODUCTION

Current studies about global food and agriculture revealed that world production may need to be increased by 60%–110% before 2050 to avoid food shortage. However, yields are no longer improving on 24–39% of most important cropland areas (Edgerton, 2009; Schmidhuber & Tubiello, 2007). Along with the demand for increased production, we need to preserve the environment and promote biodiversity in agronomic ecosystems, mainly by reducing the use of pesticides and making better use of agricultural inputs and resources: land, water, fertilizers, and energy. In this context, the use of beneficial microorganisms to improve crop performance and reduce the need for chemical inputs has become a real alternative that is gaining interest among researchers and the industry (Llorens et al., 2019; Lugtenberg et al., 2016; Singh & Trivedi, 2017).

Microorganisms are widely reported to be naturally associated with plants. Despite the fact that the presence of living microorganisms inside plants has been known since the beginning of the last century, its attention only increased in the last decades with the discovery of their ecological significance and their ability to produce metabolites that could modulate the physiology of the host plant or be of pharmacological interest (Mattoo & Nonzom, 2021). Given these circumstances, the term “plant microbiome” (Hardoim et al., 2015) is on the rise, and the living microorganisms that are in association with plants are investigated with rising intensity.

In this way, one of the emerging areas of study is around fungal endophytes. Plant-associated fungi are typically classified as either pathogenic, saprotrophic, epiphytic, mycorrhizal, or endophytic (Porras-Alfaro & Bayman, 2011). Yet, most, if not all, plants have symbiotic fungi, either epiphytic, endophytic, or mycorrhizal (Rodriguez et al., 2009).

With the evolution of research regarding endophytic microorganisms, fungal endophytes have held several definitions (Hyde & Soytong, 2008; Schulz & Boyle, 2005). In this case, the most accepted consideration establishes endophytic fungi as those fungi that reside entirely within plant tissues, without causing apparent

symptoms of disease (Rodriguez et al., 2009; Tan & Zou, 2001). These endophytes also differ from mycorrhizae in that there's no localized and specialized hyphae or synchronized plant-fungus development (Brundrett, 2006).

Despite the broad diversity of the group, fungal endophytes have been conventionally divided into two categories, clavicipitaceous and non-clavicipitaceous, and four classes (Rodriguez et al., 2009) based on their symbiotic and ecological patterns. Clavicitipaceous endophytes, also called class 1 endophytes, belong to Clavicipitaceae (Hypocreales; Ascomycota) and are restricted to a narrow range of hosts, but they can colonize the whole plant and transmit vertically and horizontally. On the contrary, non-clavicipitaceous endophytes, which comprise Class 2, Class 3, and Class 4, are characterized by colonizing a broad range of hosts, which include both monocot and dicot plants. Class 2 is formed by ascomycetes or basidiomycetes fungi and can be found in any part of the plant and transmitted both vertically and horizontally. Opposed to Class 2, endophytes in Class 3 and Class 4 are restricted to shoots or roots, respectively. Class 3 includes a diverse group of fungi that is transmitted horizontally, whereas Class 4 includes a specific group of sterile fungi also known as dark septate endophytes, which manifest melanized septa (Rodriguez et al., 2009).

In addition, the diversity of microbial symbionts varies on a host plant, and environmental conditions including biogeography, as seen in Kivlin et al. (2017). Yet, endophyte diversity is not the only complex aspect of endophytes, since the relation between the host plant and its fungal endophytes is also intricate (Saikkonen et al., 1998). Although their interaction commonly provides nutrients and stress or competition tolerance to a degree, Schultz & Boyle (2005) hypothesized that there is a continuum of antagonistic interactions and no neutral interactions, and each relationship differs from another. Thus, understanding the nature and particularities of the interactions between endophytes and host plants and how they affect the host could be key to improving agricultural management.

Recent studies have shown that the microbiome has an impact on different aspects of the host plant, such as improving tolerance to drought, heat, or saline

stress, reducing susceptibility to diseases, and increasing vigor (Llorens et al., 2019; Weiß et al., 2016). For instance, species that improve the growth of certain types of grass (*Panicum virgatum* or rod grass) for the production of biofuels or species that protect maize from fungal pathogens have been reported. Moreover, it has also been described that certain species from wild herbs, when transferred to wheat and tomato, are capable of improving the growth of these plants under conditions of heat and salinity stress (Redman et al., 1986; Rodriguez et al., 2008). The mechanisms by which the endophytic microorganisms improve the performance and the resistance of the plants could be divided into direct and indirect mechanisms. The direct mechanisms include compounds that are directly secreted by the endophyte that have a straightforward effect. These mechanisms comprise secretion of antibiotics, lytic enzymes, phytohormones, indolic compounds, or direct competition of niche. On the other hand, indirect mechanisms include plant responses induced by the presence of the endophyte. These mechanisms include the stimulation of Induced systemic resistance (ISR) and Systemic acquired resistance (SAR) or the stimulation of plant secondary metabolites (Fadiji & Babalola, 2020).

The ability and feasibility of improving plant performance and resistance to different stressors using fungal endophytes have fomented the interest in this research area. However, the possibilities of research on different fungi, hosts, stressors, and many other variables give almost endless combinations that can lead to the underestimation of some areas. The rising attention given to microbial symbiosis in the scientific community is reflected in the high number of publications regarding this topic, including several reviews (Gautam & Avasthi, 2019; Pozo et al., 2021; Tan & Zou, 2001) and some meta-analysis (Dastogeer, 2018; Mayerhofer et al., 2013). Yet, many are focused on a specific aspect of symbiosis, such as induction of resistance or production of metabolites, and some don’t focus on the fungal endophytes.

Now, we have at our disposal the data of many types of study about endophytic fungi, but what is the magnitude of each one? What is the relevance of one type of endophyte compared to others? In order to know to what extent a study

category is relevant, we would need a clear description of the whole research field. We hereby introduce a precise interpretation of the data observed in fungal endophytes' studies to demonstrate the state and tendency of the field by analyzing published literature. In this work, the objective was to contrast the main study aspects and find some minor categories of research that are usually overlooked. The following questions were addressed:

- What is the diversity of the studied fungal endophytes and their host plants?

- Which are the potential endophyte effects that are addressed in the studies? Were the results positive?

- What aspects of fungal endophytes are extensively investigated and what ones are scarcely studied?

2. METHODOLOGY

Study design

We conducted a systematic analysis of literature published about the effect of fungal endophytes in plants. The structure of the analysis process was performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

Literature search

Eligibility criteria and search methodology

To obtain the metadata that would allow analyzing the current status of fungal endophyte studies we performed a literature search on the Scopus database in 2021, including all the articles published up to December 2021.

The following terminology was used for the search:

- *Endophyte*
- *Plant* OR *grass*: This term was included to avoid research with no inoculation in plants.

- *Fungi*: Fungus, fungal and variations were included.
- *Stress* OR *tolerance*: To find the purpose of the endophytes.
- NOT *mycorrhiza*: Excluded mycorrhizal fungi to find strict endophytic fungus.

The terminology was looked for in title-abstracts and keywords of articles.

A second search was conducted afterward for growth-promoting endophytes. This time, the keyword for “stress or tolerance” was changed to “growth”.

Other non-vital roles like phytoremediation, fruiting characteristics and such can be of great interest for specific host plants or environment but are not as globally significant as the main roles previously discussed, hence their absence in our literature search.

Study selection and criteria for inclusion

Results from both search queries were merged, and then examined to ensure the relevance of the publications for our topic concerns. In order to achieve equality of the results, references with the following conditions were removed:

(a) studying endophyte presence and identity without further application,
(b) having its focus on the cattle industry,
(c) aiming to particularly identify the mechanism of the host-endophyte relation,
(d) not fulfilling the search requirements due to other unspecific reasons, and
(e) not having its original source available or verifiable.

From the selected manuscripts, the information provided about the fungal endophytes, the host plants, the type of stress used, and the results were included in the final analysis. In addition, highly similar articles such as in same authors, endophyte, and host, were only counted once, including all the results from them.

Data collection

Subsequently, the references were exported to an MS Excel spreadsheet for further analysis. To compare selected articles, the data was broken down into different sets:

(1) The fungal endophytes' species, or at least their genus. DSE, unidentified fungi, or the use of all microbiome of a plant was recorded as such if appropriate.

(2) Original host plants' species, from which the endophytes were isolated. Their condition as wild or cultivated plant was distinguished, and endophytes that were not isolated in the current study had this section unfilled to avoid duplicates.

(3) Study host plants' species, whether the endophyte is inoculated in the same host plant from which it was isolated, or a new host. Their condition as wild or cultivated plant was noted.

(4) Method used to produce endophyte-free plants, if pertinent. This section does not account surface sterilization in, since it's a widespread procedure that occurs in most, if not all, studies.

(5) Tested effects of the endophytes.

(6) Experimental results, whether the endophytes conferred clearly beneficial effects, had some varying impact, had no significant or negative effects on host plant.

When several species were used, they were all registered. Taxonomic synonymy was avoided by assigning only the current taxonomic name, as consulted in NCBI database at the moment of the analysis.

Furthermore, to simplify results, we recorded some data into groups:

(7) Fungal endophytes' division. Ascomycota, Basidiomycota and Mucoromycota. When fungi of different phylum were reported on the same article, it was considered that the article worked with various phyla.

(8) Original host plants' family.

(9) Study plants' family.

(10) Function group of the endophyte. The potential effects were classified into one of these categories: abiotic stress tolerance, biotic stress tolerance, growth promotion, other effects or several of them. When growth promotion

was assessed under stress conditions, only the stress tolerance effect was considered.

Study quality and risk of bias

This meta-analysis aims to perform a qualitative analysis of the currently available literature. In this way, it is necessary to consider that, due to the diversity and complexity of the analyzed studies, comparing experiments with different study parameters is not always feasible. Therefore, for the purpose of this work, the simplest and clearest variables have been compared.

It is also important to address the issue of independence of the analyzed studies since the search results include papers that are part of the same study or research group and only differ on either study parameters or research progression. To avoid inaccuracy caused by counting dependent items as independent ones, a set of papers from the same study or research group was considered as a single independent item with several study parameters.

We are aware of the possible bias committed by considering only those articles that use the word “endophyte” in the title, keywords, or abstract, excluding some potential useful reports. Yet, opposite bias could also happen, since there might be articles that elaborate on endophytic organisms only in the main text, hence remaining out of the current research and analysis. All this said, the only way to correct such bias would be reading all articles with the word “endophyte” in the main text and selecting those that meet the other criteria for inclusion, which effort we considered as unmanageable for the purpose of this work.

3. RESULTS AND DISCUSSION

Beneficial microorganisms play an important role in plant performance and adaptation to adverse conditions. Among them, the interest in fungal endophytes has raised in the last decades due to their ability to produce secondary metabolites that could protect the host plant against herbivory (Bultman & Bell, 2003; Shiba & Sugawara, 2005). More recently, it also has been observed that fungal endophytes could play other roles related to plant protection against stress

conditions (Hossain et al., 2017; Waller et al., 2005), increasing the interest of the researchers in this emerging area.

In order to confirm and analyze the state of fungal endophytes' studies, we performed two literature searches that retrieved around 1138 online references for the first one, covering stress tolerance, and 418 for the second one, focused on growth promotion. After the pertinent reductions described in the methodology section of this paper, we elaborated a database with a sum of 392 papers, containing scientific publications that range from the year 1988 to December 2021.

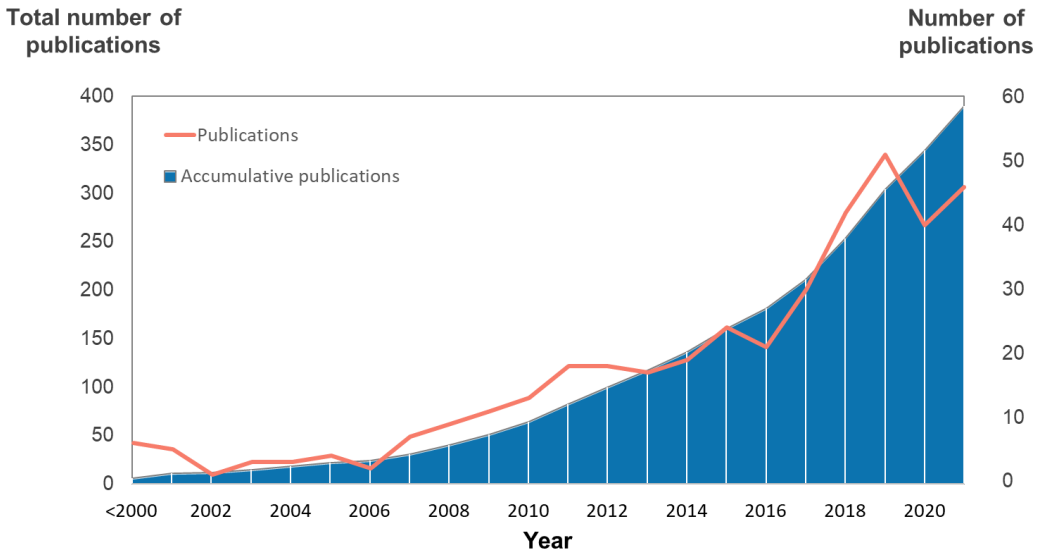

Figure 1. Number and accumulative number of published articles identified in the present literature research until 2021. Right-side axis belongs to graphic line; Left-side axis belongs to graphic area.

The preliminary analysis of the gathered literature proves the rising tendency of this research area, as observed in the graphic slope of figure 1. This figure shows how this study field has been increasing steadily since 2006, and how the number of publications on this topic in the last 5 years is higher than the number of publications before 2017. However, it has been also observed that, in the last year analyzed, the number of publications that fulfill our search criteria has been slightly lower. On the other hand, an enhancement of articles with related topics as the study of plant microbiomes has increased. Thus, this makes clear the

outstanding performance of fungal endophytes as a research field and the ongoing attention given to these microorganisms by the research community.

The literature we examined usually had a common way of working, with experiments arranged following next steps: isolation of endophytes from a host of interest or acquisition from a provider, selection of most relevant endophyte or few endophytes, inoculation in the plant, and evaluation of its effects in growth promotion or against some kind of stress. In the following sections, we show our analysis of the relevant aspects and other details of interest of the reviewed papers.

3.1. Fungal endophytes and their diversity

Studies usually state the fungal strain used in their experiments, either specified by the provider or obtained by identifying the isolates of a host plant. Yet, 21% of the literature did not completely identify the endophyte, leaving the determination at the genus level or potential candidates. This might be caused by several reasons: not finding total coincidence with any species on fungal databases, focusing on the role of the endophyte rather than its identity, etc.

Tested endophytes are categorized at the division level in figure 2, and genera frequency is shown in figure 3(a). Also, though it was outside the scope of the study, root endophytes seemed to be more commonly studied, followed by leaf and stem endophytes. Our analysis showed that the vast majority of the studies are focused on Ascomycota, which turned out to be almost 80% of the database results, while Basidiomycota covered around 12%, and Mucoromycota barely added up to another 1% of the results.

Ascomycota's prevalence is not surprising, since ascomycetes fungi are the commonest in nature. In this category we can find genera such as *Epichloë*, *Penicillium*, *Trichoderma, Fusarium, Aspergillus* and *Alternaria.*

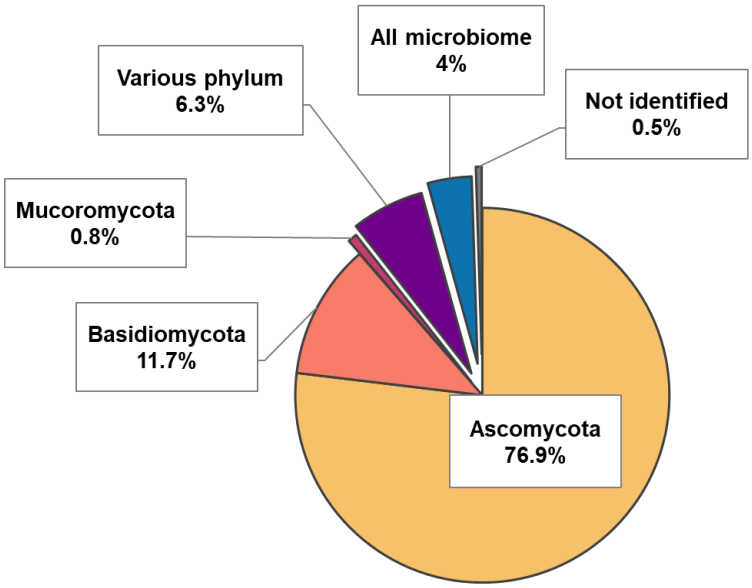

Figure 2. Fungal endophyte phyla recurrence in literature research.

The relevancy of *Epichloë*, also known as *Neotyphodium* or *Acremonium* in past literature for its asexual morphs, goes back to 1988 when it was discovered. It is considered the first reported endophytic fungi, and it is beyond question the most known fungal endophyte genus since it extensively colonizes widespread forage grasses, such as *Lolium spp.* (Schardl et al., 2004). As seen in reviewed articles, studies about *Epichloë* are usually focused on plants as forage or turf, and there are no studies about inoculation of this Class 1 endophyte in non-host plants due to its limited host range. West et al. (1988) observed that endophyte-infected *Lolium arundinaceum* gained tolerance to drought and nematode infection and Elmi et al. (2000) and other posterior works confirmed those premises relating both stresses. *Lolium perenne* as endophyte host has therefore been studied to demonstrate, for instance, tolerance to drought and nitrogen deficit (Ravel et al., 1997), to heavy metals like zinc (Monnet et al., 2001) or predators like rice leaf bug (Shiba & Sugawara, 2005).

Another recurrent genus of Ascomycota endophytes is *Penicillium* for the ability to improve growth and abiotic stress resistance, with species like *P. janthinellum* (Khan et al., 2014) or *P. funiculosum* (Khan et al., 2011). Likewise, *Fusarium* is not only studied for abiotic resistance ability but to confer biotic resistance, with

special regard to pathogenic *F. oxysporum* strains (Nefzi et al., 2019; Ting et al., 2008).

Some of the articles refer to the use of dark septate endophytes (DSE). This definition was used for the first time by Read and Hasel Wandter (1981) to describe a diverse group of ascomycetous endophytes with melanin hyphae that live in roots without causing any visible symptoms. Subsequently, numerous researchers have used this informal term without further classification. Even so, few articles describe the use of DSE and identify the fungi as *Alternaria sp*. or *Penicillium sp*.

In contrast to the diversity of ascomycetous endophytes exposed in the publications, basidiomycetes and mucoromycetes have been less studied according to our literature database. One exception to this is the basidiomycete *Serendipita indica,* previously known as *Piriformospora indica. S. indica* was firstly reported in 1998 (Verma et al., 1998), discovered in desert soil from India, and found to be similar to arbuscular mycorrhizal fungi (AMF), yet, as opposed to them, it was able to grow in axenic culture (Varma et al., 2001). And, unlike *Epichloë,* it has been since widely tested in both monocots and dicots. The most common benefits of this endophyte have been reported to be growth promotion (Bagde et al., 2011; Noora et al., 2017; Rai et al., 2001; Sahay & Varma, 1999; Satheesan et al., 2012), as well as inducing resistance against abiotic stress such as drought (Ahmadvand & Hajinia, 2018; Hosseini et al., 2017; Hussin et al., 2017; Sherameti et al., 2008) and salinity (Abdelaziz et al., 2017; Li et al., 2017; Sharma et al., 2017; Sinclair et al., 2013; Waller et al., 2005). Some studies also report a beneficial role against pathogens (Cosme et al., 2016; Lin et al., 2019), but this field is less studied.

Not a considerable amount of studies worked with multiple endophytes at once. Some articles referred to various endophytes from different phyla (6%), through the application of various potential endophytes or a selection from the microbiome of the host plant. In this case, studies use a consortium of fungal strains, though using a consortium of both bacterial and fungal strains like done by Varkey et al. (2018) was apparently more common. Furthermore, a scarce

number of studies worked with the whole microbiome from a plant (4%). We hypothesize that the scarcity of these types of study is a sign of researchers considering beneficial properties provided by individual endophytes more relevant than their interactions within plant tissue. This, at the same time, is probably due to the complications that involve studying several endophytic species at once since, according to Kivlin et al. (2013), since the interactions depend on not clear conditions, there is the danger of underestimating or overestimating the global effects of a group of endophytes.

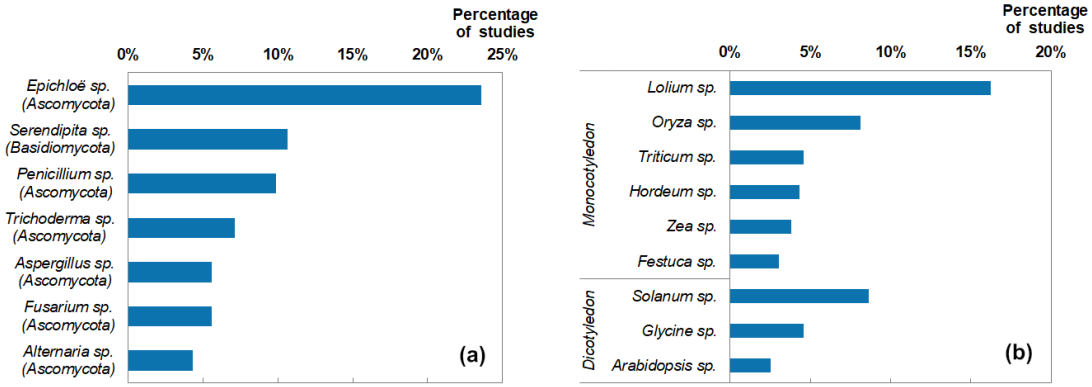

Figure 3. Most common endophyte genera (**a**) and study plant genera (**b**) found in literature research.

3.2. Host plant and their diversity

Results of the analysis regarding endophyte host plants are illustrated in Fig. 4. Poaceae family dominates the studies with half of the literature results (51%). The other half refers mostly to a diversity of host plants, usually of economic relevance, and only a few studies refer their experiments to several plant families.

As seen in figure 3(b), ryegrass (*Lolium sp.)* stands out among the recurrent host plants. This grass genus, which includes *Lolium perenne*, *Lolium arundinaceum* and *Lolium multiflorum,* has been included in more than 16% of the total number of articles. We consider that the main reasons for this might be its wide range, importance in the cattle sector and identity as host of *Epichloë* endophytes. Another well-used monocot in literature is rice (*Oryza sativa*). This is one of the most relevant food crops in the world and is inoculated with non-host endophytes

in order to obtain growth enhancement and abiotic stress tolerance. Other species from the Poaceae family commonly mentioned are barley (*Hordeum vulgare*), maize (*Zea mays*), common wheat (*Triticum aestivum),* and red fescue (*Festuca rubra)* in order of recurrence. All of them are extensively cultivated species, and barley and red fescue, along with some close relatives, are also commonly infected with *Epichloë sp.*

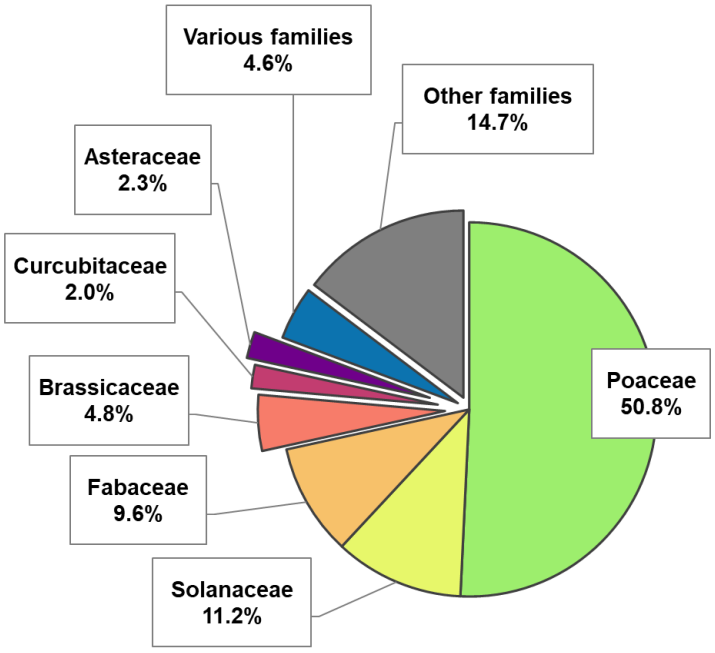

Figure 4. Family of study host plant recurrence in literature research.

Monocots are surely the prevalent plant group of interest, since they are of great economic and cultural relevance and are relatively easy to work with. However, dicot plants as endophyte hosts have yet a lot of unrevealed potential. Around 30% of the studies covered species that belong to families with relevance as food resources such as *Solanaceae*, *Fabaceae, Brassicaceae* and *Cucurbitaceae*. Among these, the most used species are tomato (*Solanum lycopersicum*) and soybean (*Glycine max*), which take part in around 9% and 5% of the articles, respectively. Both species hold great economic value similar to the previously mentioned monocots yet are seldom used as biological models in front of biotic stress.

The remaining studies in the database worked with plants that have significance either as ornamental plants, ecological models, or sources for other goods such as medicine, lumber or resin. In addition to these, a few articles (barely 4%) need to be considered for applying endophytes to several plant species that belong to different families.

Finally, the environment in which plants grow and where the experiments are conducted can also have an impact on the reliability of the results. Although field experiments would be the most realistic approach for the studies (Rai et al., 2001; Zhou et al., 2018), it is difficult to perform this kind of experiments because of the complexity of all the factors that get involved in it. Instead, most publications referred to growth chambers and greenhouses to carry out their experiments. On the opposite, a couple of studies (5%) performed their experiments only *in vitro* bioassays (Dovana et al., 2015; Khan et al., 2017), usually with mutant rice. This last kind of studies has particularly controlled environmental conditions and further experiments would be required to test the effects of the endophytes on the behavior and interactions of the host plant under more realistic conditions.

3.3. Host plant and study plant conditions

Since the original host plant of the studied endophytes may differ from the study's host plant, the information regarding their conditions was recorded (Fig. 5).

Around 30% of the studies referred to host plants from wild origin (Li et al., 2018; Li et al., 2017). We consider studying endophytes from nature-adapted plants of great significance due to their biodiversity and wide-ranging microbiome. Those endophytes are also typically acquired from locations with extreme environmental conditions such as deserts to assess their potential role in adverse conditions. On the contrary, only 18% of study plants were clearly traditional or wild plants, against 53% of the studies that inoculated cultivated species. This confirms that finding endophytes from wild origin to use them as means to improve the behavior of crops is more relevant than discerning their role in a natural environment.

In 52% of the articles, isolating the fungal endophyte strains was an integral part of the study. In the other cases, the strains were either isolated in previous work of the research group or provided by microbial banks or another entity.

On another note, we compared the identity of the host and study plant. In this case, we could see that the fungal endophyte was inoculated to the same host plant species from which it was isolated in at least 48% of the articles, while at least 25% of them used a different species of host plants in the experiments. Inoculation of the same plant species from which the endophyte was isolated is, possibly, an indicator of the interest in exploring the effect of the endophyte on its natural host.

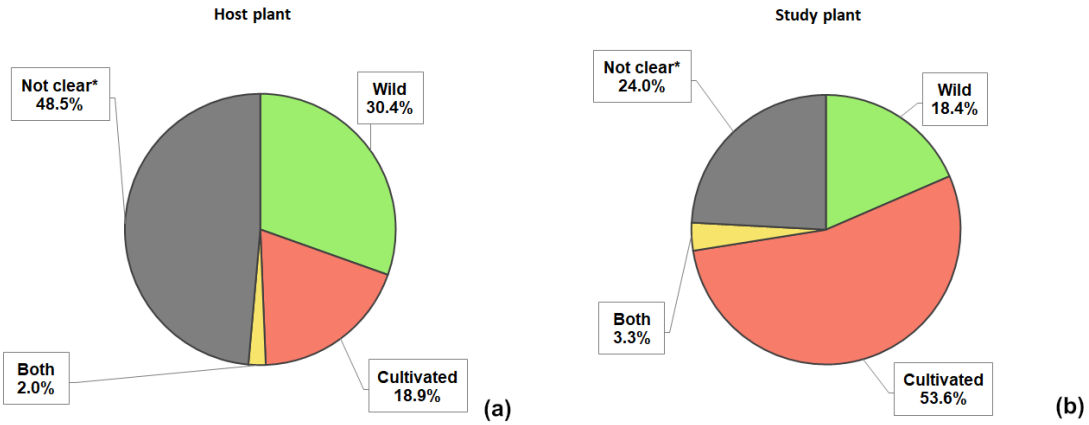

Figure 5. Host plant (**a**) and study plant (**b**) origin in literature research. (*) Not stated or endophyte is whether from soil, previously isolated or provided by a bank.

There also was a tendency to use some methods in order to produce endophyte-free plants, but this seems to be of decreasing importance in the more recent studies. These methods are specified in table 1 and focus on obtaining plants that are cleared up from any microbiota to avoid interference with the endophyte of interest. Among these treatments, systemic fungicide treatment prevailed (11%), especially in the early years of research. However, these practices have been discontinued since, presumably due to the need of considering interaction within the plant microbiome in order to ascertain the effect of the study endophyte. Likewise, heat treatment use has almost disappeared nowadays (4%). In this case, the reason may be the possible negative impact of heat treatment in seed

germination and future plant physiologic aspects and performance. The only method that is still used with the same incidence as before is the individual selection of study material (6%). This is a procedure that usually makes use of microscopy to identify the presence or absence of the study endophyte to classify their plant material. In the end, these treatments are not used anymore on a current basis, and most of the studies (73%) tend to not apply them. This includes the great number of publications where inoculated plants differ from the original host plant of the study endophyte and the other studies that only resort to surface sterilization of the seeds before inoculation.

Table 1. Used method to obtain endophyte-free plants besides surface sterilization.

Recurrence	Method	Description	Potential consequences
13%	Fungicide	Application of chemical compounds such as propiconazole or systemic fungicide like benomyl to seed plants.	Harm to physiologic functions and growth of the plants.
4%	Heat	Application of high temperatures to seed plants, with specific conditions that are previously tested. Temperature is higher than 50ºC, either with hot water or in oven, for a period between an hour and a week.	Physical damage and germination difficulty of seeds, physiologic alteration of plants.
1%	Storage	Infected seeds are stored at room temperature for a long period of time, which sometimes result in a loss of endophyte infection.	Incomplete removal of the fungal endophyte from the seeds.
7%	Selection	Individual selection of seeds, either as an option provided from seed bank or after examination by microscopy.	Error in detection of endophyte strain.
75%	None	There's no method applied to eliminate endophytes from plant besides surface sterilization of the used seeds.	Not considered.

3.4. Role of fungal endophytes in studied plants

Plants are always exposed to multiple environmental factors and many of them can result in biological stress, an adverse condition that inhibits their normal functioning (Jones & Jones, 1989). However, fungal endophytes can also affect

multiple aspects of the host plant's life cycle. It has been extensively reported how fungal endophytes can alter host physiology under stress and confer tolerance to host plants by enhancing activation of the host's defense system (Shankar Naik, 2019; Tidke et al., 2017). The diverse effects that endophytes trigger in their host plants can range from growth improvement to protection against cattle. Thus, the effects are not straightforward and there is a whole range of benefits that could be induced in the plant and play a critical role in their resilience (Mei & Flinn, 2010; Rodriguez et al., 2009; White & Torres, 2010).

In this study, studied effects of fungal endophytes are sorted as seen in figure 6. Resistance against abiotic stress is the most studied condition (57%), followed by growth promotion (20%) and resistance against biotic stress (15%). The remaining articles experimented with several types of plant stress conditions (8%).

In the following sections, we check the specific results for each type of stress, taking into account individual results for the studies.

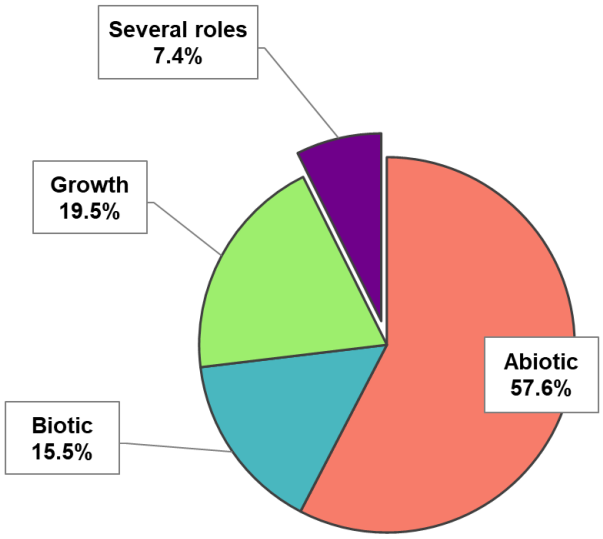

Figure 6. Potential roles of fungal endophytes studied in literature research.

3.4.1. Effect of fungal endophytes under abiotic stress

With the advance of climate change, plants are severely exposed to abiotic stress, both physical and chemical. Abiotic stress is defined, according to Ben-Ari and Lavi (2012), as the negative impact of non-living factors on living organisms in a specific environment. These conditions, such as water deficit, extreme temperature, soil salinity and heavy metal contamination are common adversities that affect crop productivity worldwide.

In this context, endophytes can play a significant role in conferring stress tolerance to host plants by altering water relations, osmolyte production, and synthesis of hormones and reactive oxygen species (ROS) (Shankar Naik, 2019). Thus, we believe that countering the extensive consequences of global warming and contamination is one of the main goals of studying fungal endophytes. The interest in the potential induction of resistance against abiotic stress is reflected in a high number of the analyzed articles in the current review (58%). The specific stress conditions tested in each study are classified and presented in figure 7(a).

Water stress takes part in 34% of the studies, and this being the most studied condition is expected since drought is the most widespread limiting factor for plant productivity (Ahmadvand & Hajinia, 2018; Kane, 2011; Kavroulakis et al., 2018; Kuzhuppillymyal-Prabhakarankutty et al., 2020). Following water stress, heavy metals (Bilal et al., 2018; Ikram et al., 2018) and salinity stress (Radhakrishnan et al., 2015; Z. Wang et al., 2020) were also recurrently studied, with each covering 23% and 20% of the studies about abiotic stress, respectively. These are the commonest source of soil contamination, and both are capable of interfering with vital cellular processes and causing severe injuries to plants (Munns & Tester, 2008; Yadav, 2010).

The remaining articles dealt with temperature stress, nutrient availability, cutting and other minor categories. It is known that plants subjected to extreme temperature stress alter processes that critically affect development and growth (Bokszczanin & Fragkostefanakis, 2013). However, it is surprising there isn't a considerable amount of studies about the stress caused by temperature change,

albeit the importance of its environmental effects. In this way, Acuña-Rodriguez et al. (2020) consider cold stress as a substantial gap in this study field, and according to their analysis, microbial symbionts confer more benefits at low temperature.

3.4.2. Effect of fungal endophytes under biotic stress

Another relevant condition that can affect plants severely is biotic stress. Biotic stress refers to the negative impact caused by other living organisms, usually plant pathogens like insects, nematodes, pathogenic bacteria and fungi or competitive plants or weeds (Gull et al., 2019). Plants respond to this type of stress with defense mechanisms of their immune system, generating chemical compounds such as salicylic acid (SA) or jasmonic acid (JA), producing proteins and enzymes, increasing cell lignification and other morphological or structural barriers (Madani et al., 2018)

In order to confront biotic stress, endophytic symbioses can play an important role, since these associations are reported to affect the performance and behavior of pathogenic organisms (Hartley & Gange, 2009; Shankar Naik, 2019). In this way, entomopathogenic fungi (EPF) are common in terrestrial environments and can be important natural regulators of insect and arachnid populations (Chandler, 2017).

However, the analysis reveals that, compared to abiotic stress, endophyte roles regarding biotic stress are much less studied. Less than 20% of articles studied the role of endophytes for biotic stress resistance. Biotic stressors (Fig. 7b) mainly cover fungal pathogens (Waqas et al., 2015), insects (Cosme et al., 2016) and soil nematodes (Liarzi et al., 2016). Fungi pathogens are present in 41% of studies, while insects add up to 23% and nematodes to 16%. Thus, the main tested effect of fungal endophytes is to confer protection against other fungi, presumably pathogenic, and probably anticipating a competition for ecological niche (Oliva et al., 2021).

Interestingly, the effect of fungal endophytes against bacterial pathogens was rarely evaluated (6%). In spite of that, manuscripts describing the protective effect

of fungal endophytes against bacterial pathogens usually report good control of the disease. For instance, Lin *et al.* (2019) observed that plants treated with *Serendipita indica* and infected with the bacterial pathogen *Ralstonia solanacearum* showed an enhanced response of the Jasmonic acid pathway and the expression of *VSP, PR1* and *PR5* genes.

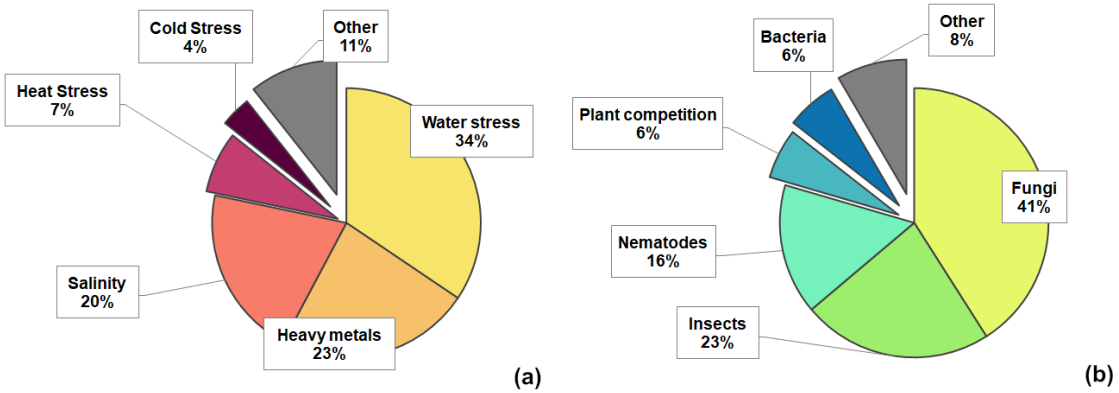

Figure 7. Most studied potential roles of fungal endophytes in front of abiotic (**a**) and biotic (**b**) stresses.

3.4.3. Effect of fungal endophytes in promoting growth

Aside from detrimental abiotic and biotic stress conditions, the growth properties of plants are the main concern in crops. Fungal endophytes, along with bacterial endophytes and mycorrhizae, are associated with the transfer of nutrients and minerals in the host plant (Shankar Naik, 2019), so they are biological treatments that can improve different aspects of the plant life cycle. In this way, Plant Growth Promoting Fungi (PGPF) are known as beneficial fungi in close interaction with plants, inducing an enhancement of the plant performance by alteration of physiological pathways and molecular mechanism regulation. They're also associated with induced systemic resistance (ISR) through the ability to enhance nutrient uptake and phytohormone production (Hossain et al., 2017).

Therefore, fungal endophytes have been extensively studied for their beneficial properties and potential to enhance productivity for crop plants. The literature research conducted in this paper reveals that studies focused on PGPF take near

20% of the fungal endophyte investigation, covering both *in vitro* and in field application on plants. These reports cover their beneficial effects on germination (Vujanovic et al., 2016), nutrient uptake (Khayamim et al., 2011), host plant vigor, biomass (Al-Hosni et al., 2018) and fruit production (Rho et al., 2020), all in accordance with an ecological agriculture approach.

Among the studies that assess growth promotion, 80% of the research was done in absence of pathogens in stable environmental conditions, while the other 20% further studied the effects of the same endophytes when the host plants were exposed to abiotic or biotic stress. The latter scenario is especially useful since it allows comparison of effects both on stable and stress conditions. It should be reminded that the effects on growth promotion only under stress conditions are not accounted for in this section since it has no stable conditions to be compared to.

3.5. Results of efficacy

Research results are decisive to know the real impact of the experimental studies. As expected, most analyzed articles in the current review report beneficial roles of the studied endophytes, irrespective of the magnitude of the effects. We categorized these results as good, neutral or variable. We expected to find a high number of positive results since there is a preference for reporting positive results and keeping the negative ones unpublished (Mehta, 2019).

Around a quarter of the studies agreed that the effects on the host and environmental factors are variable, to the extent that some endophytes could negatively affect the host plant in some way. This potential negative impact is likewise reported in a small number of articles that were not able to provide positive results. This usually occurred in experiments that focus on finding new effects of previously known endophytic fungi, such as *Epichloë* spp., inoculating it on non-host plants and/or under new conditions (Hall et al., 2014; Heineck et al., 2018). It is to be noted that the particular conditions of the experiments can affect the reliability of the study comparisons (Arnold, 2007). For instance, as observed by Singh et al. (Singh et al. 2016) with *Serendipita indica*, growth media

can influence the relationship between the fungal endophytes and their host plant. Yokoya et al. (2017) also showed how habitats with suboptimal conditions have more endophytes that prove to be beneficial to their host plant. Regardless, we consider these results to be key in providing knowledge about research limitations, since they indicate what can be avoided in future studies.

4. CONCLUSIONS AND FUTURE PROSPECTS

This work has analyzed the current structure of fungal endophyte research, focused on the organization and variables of the studies that aim to find beneficial effects of fungal endophytes on a host plant. This field of study has been on the rising trend for 20 years now and has worked mostly on Ascomycota fungi, with ubiquitous and widely known endophytes such as *Epichloë* spp. We here confirmed that only a few fungal species are recurrently studied as endophytes, while there is a broad spectrum of novel, potentially interesting endophytic fungi that have only been sporadically studied. Moreover, less than 10% of the studies deal with several endophytes, implying a shortage of studies regarding the symbioses interaction and the high difficulty attached to them. Furthermore, other analyzed aspects of the study also denote certain gaps. In particular, host plants are still mainly monocots like Lolium spp., leaving fair room to improve on dicots' studies. Wild species are frequently used as a source of fungal endophytes but quite less as study plants. The potential effects of fungal endophytes are most commonly tested on cultivated species (between 51% and 80%) and assessed in front of abiotic stress (around 57%), especially on drought conditions. A relevant abiotic scenario that is surprisingly barely studied is stress caused by temperature changes (11%), especially cold stress. Biotic stress experiments, in turn, only appear in 15% of the literature, and among the biotic factors, fungal pathogens are the most recurrently studied, while other biotic factors like bacterial pathogens are scarcely covered. On another note, resorting to procedures to ensure endophyte-free plants has decreased lately. The majority of the studies use growth chambers or greenhouse experiments, while field studies are very limited. Lastly, we found that more than 25% of the studies reported neutral,

variable, or negative effects from some endophytes, which denotes the difficulty of this experimental research.

The results showed in this work highlight the interest in the field of the use of fungal endophytes to improve the plant performance, but also uncover certain areas are still understudied such as protection against bacterial diseases. Despite that, the use of fungal endophytes is still in its beginning and present some interesting questions for future studies such as: what is the effect of an introduced endophyte in the plant microbiome? Could it affect other functions besides the studied ones? Could different fungal endophytes be combined in a synthetic community to achieve a broad spectrum of protection? Is the observed effect caused by the presence of the endophyte or by its secreted metabolites? If the latter, could it be possible to mimic the effect using endophyte exudates?

Taken together, fungal endophytes and their interaction with their host plant are still an intricate subject, with effects that vary from mutualist to pathogenic depending on several factors. Nonetheless, their relevance as a potential source of growth promotion and stress tolerance is unquestionably recognized, and their beneficial effects on plant physiology are reported across several endophytes and host plants. The results presented here demonstrate the incidence and direction of fungal endophyte research, and also prove the presence of some unexplored subjects in the field, especially regarding dicot plants and against biotic stress.

Data availability statement for Basic Data Sharing Policy

All reviewed articles in the present manuscript are accessible through Scopus® database and checked on 30th December 2021. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CHAPTER 2

Exploiting tomato genotypes to understand heat stress tolerance

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ABSTRACT

Increased temperatures caused by climate change constitute a significant threat to agriculture and food security. The selection of improved crop varieties with greater tolerance to heat stress is crucial for the future of agriculture. To overcome this challenge, four traditional tomato varieties from the Mediterranean Basin and two commercial genotypes were selected to characterize their responses at high temperatures. The screening of phenotypes under heat shock conditions allowed to classify the tomato genotypes as: heat-sensitive: TH-30, ADX2; intermediate: ISR-10 and Ailsa Craig; heat-tolerant: MM and MO-10. These results reveal the intra-genetical variation of heat stress responses, which can be exploited as promising sources of tolerance to climate change conditions. Two different thermotolerance strategies were observed. The MO-10 plants tolerance was based on the control of the leaf cooling mechanism and the rapid activation of H2O2 and ABA signalling pathways. The variety MM displayed a different strategy based on the activation of HSP70 and 90, as well as accumulation of phenolic compounds correlated with early induction of PAL expression. The importance of secondary metabolism in the recovery phase has been also revealed. Understanding the molecular events allowing plants to overcome heat stress constitutes a promising approach for selecting climate resilient tomato varieties.

KEYWORDS: Heat stress, tomato, thermotolerance

1. INTRODUCTION

Heat stress (HS) is one of the most important abiotic stresses that limit crop productivity worldwide (Bita & Gerats, 2013) In the current scenario of global warming, it is expected that temperatures will rise between 2 and 5 °C by the end of the 21st century (Battisti & Naylor, 2009; Masson-Delmotte et al., 2021). The average of global surface temperature, will cause serious damage on growth and development of plants, resulting in a catastrophic reduction of crop productivity (Battisti & Naylor, 2009; Bita & Gerats, 2013; Iizumi et al., 2018; Masson-Delmotte et al., 2021).

In addition to the progressive increase in temperatures, another threat to crop productivity is the increased frequency of extreme weather events such as heat waves, floods or prolonged droughts (Lohani et al., 2020). Therefore, comprehending the response mechanisms of plants to abiotic stress (specially heat stress), the adaptation of seed varieties and the management of crops at high temperatures will be key to the agronomic sustenance (Schlenker & Roberts, 2009). In addition, the increased production required to meet the nutritional needs of a constantly growing world population (9 billion by the year 2050) together with the expected crop losses, is a challenge that can only be solved by agricultural production systems based on the use of crop varieties that are more tolerant to abiotic stress.

The negative effects of increased temperatures on plant growth and productivity have been already described in crops such as wheat, rice, barley, sorghum or maize have already been described (Challinor et al., 2014; Hasanuzzaman et al., 2013; Iizumi et al., 2018). Tomato is considered particularly sensitive to heat stress since, the increase in temperature produces yield losses of up to 28% (Alsamir et al., 2019). The increase of 1 °C up to its optimal growth temperature can greatly impair pollen viability and female fertility, seriously affecting fruit set (Firon et al., 2006). Moreover, it is commonly accepted that the reduction of the life cycle that decreases plant productivity upon heat stress is produced by the impact of this stress on respiration and photosynthesis rates (Barnabas et al., 2008).

Plants as sessile organisms have evolved a series of strategies to ensure survival and reproductive success when confronting heat stress. The ability of plants to survive abrupt temperature increases is known as basal thermotolerance (Mittler et al., 2012). There are some strategies in charge of inducing acclimation mechanisms or to avoid the stress produced by the increase in short-term temperatures, such as the reorientation of the different organs, the manifestations of the lipid composition of the membrane or the increase of transpiration rate to allow leaf cooling (Wahid et al., 2007). It is commonly accepted that transpiration cooling is an important process in thermotolerance. This process is based on keeping the stomata open during high temperature stress, which allows the diffusion of CO_2 through the leaf blade, which allows plants to control leaf temperature (Porch & Hall, 2013; Prasad et al., 2017). A recent study demonstrated that heat tolerant *Common bean* genotypes cool more than heat sensitive genotypes as a result of higher stomatal conductance and enhanced transpiration cooling (Deva et al., 2020).

Plants under the increase of temperature conditions will activate and accumulate heat shock proteins (HSPs), including HSP100, HSP90, HSP70, HSP60, and small HSPs (smHSPs) (Young, 2010) This HSPs are considered crucial molecular chaperones that participate in the thermotolerance responses to maintain the stability of cells (Richter et al., 2010). In addition, heat shock transcription factors (HSFs) are responsible for regulation of HSPs, which control protein quality (Scharf et al., 2012). For example, the overexpression of *heat stress transcription factor A2* of *Oryza sativa* in *Arabidopsis* induced the up-regulation of HSPs what enhance thermotolerance (Nishizawa et al., 2006; Yokotani et al., 2008).

As occurs in other stress situations, heat stress produces secondary stress in plants such as oxidative stress, caused by damage to chlorophylls and the photosynthetic apparatus, which results in an overproduction of reactive oxygen species (ROS) (Shi et al., 2006). Heat stress affects the membrane fluidity, producing increased cytosolic calcium that transduced via respiratory burst oxidase homolog (RBOH) proteins, initiating the ROS burst at the apoplast (Mittler & Blumwald, 2015; Tian et al., 2016). Thus, the increase of both signalling

molecules contributes to activate downstream signalling pathways that regulates HSFs, initiating therefore, heat stress responses (Devireddy et al., 2021). Plants also induced the expression of antioxidant proteins like ascorbate peroxidase (APX), catalase (CAT), or superoxide dismutase (SOD) to counteract the negative effects of excessive ROS under high temperatures (Baxter et al., 2014). The importance of ROS-scavenging enzymes as well as other antioxidant molecules like ascorbic acid in thermotolerance has been largely demonstrated by using knockout mutants what was recently reviewed by (Devireddy et al., 2021).

Plant hormones, including abscisic acid (ABA), brassinosteroids (BRs), cytokinins (CKs), salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), play important roles controlling complex stress-adaptive signalling cascades that triggered heat stress responses (Muñoz-Espinoza et al., 2015; Ozga et al., 2016). The role of ABA and SA in basal thermotolerance has been extensively studied. Exogenous application of ABA 5 μM on tall fescue produced an enhancement of heat tolerance based on the increase of leaf photochemical efficiency and membrane stability (Wang et al., 2017). Likewise, *Arabidopsis* microarray suggests that ABA can induce thermotolerance by inducing the expression of HSFA6b (Huang et al., 2016). Previous studies suggest that SA is primarily involved in promoting the basal thermotolerance by inducing several HSPs (Clarke et al., 2004). However, recent studies showed the relevance of this phytohormone in the thermotolerance mechanisms by regulating the antioxidant defence system and improving photosynthetic efficiency upon heat stress (Rai et al., 2019; Wassie et al., 2020).

In addition to the classical ABA- and SA-mediated responses, tomato plants respond to environmental changes like temperature increase producing significant changes in the phenolics and flavonoids contents (Alhaithloul et al., 2021; Ilahy et al., 2016). These secondary metabolites play a key role in the protection of plants against unfavourable situations "through their antioxidant capacity since they're able to inhibit ROS formation via a range of different mechanisms (Mierziak et al., 2014). Plant phenolics are synthesized through the shikimate/phenylpropanoid pathway from phenylalanine. Tryptophan is another

relevant amino acid because is precursor of the hormone melatonin which is involved in plant growth and development and plays a key role in a wide range of abiotic stresses (Hassan et al., 2022). In the recent years, the importance of this hormone in the plant responses against heat stress is becoming clear, since it is involved in the improvement of photosynthetic efficiency, the regulation of stomatal movement and the synthesis of chlorophyll and RuBisCo activity (Arnao & Ruiz, 2017; Nawaz et al., 2020; Varghese et al., 2019). Thermotolerance is improved by treating tomato and wheat plants with melatonin, which also maintains membrane stability, plant water relations, and increases antioxidant activities (Buttar et al., 2020; Ding et al., 2017; Martinez et al., 2018).

A general response mechanism of plants against heat stress has been stated, but it is well known that these defences vary according to the developmental stage, genotype, species and heat stress experimental conditions (Sakata & Higashitani, 2008; Shanmugam et al., 2013; Sharma et al., 2015). In fact, resistance to heat shock (HS) is genetically diverse (Ayenan et al., 2019; Bineau et al., 2021) and, therefore, it is important to exploit this genetic diversity to elucidate the mechanisms that allow certain genotypes to grow and have greater yield under warm temperatures, being of great interest for breeding programs to select tolerant varieties to heat (Hoshikawa et al., 2021). Landraces, or traditional varieties defined by (Villa et al., 2005) as “a dynamic population of a cultivated plant that has historical origin, distinct identity and lacks formal crop improvement, as well as often being genetically diverse, locally adapted and associated with traditional farming systems”, are one of the most important components of plant genetic resource. Nowadays, tomato landraces are still cultivated for local use and consumption (Lázaro, 2018) The adaptability of landraces to unfavourable environmental situations (Henareh et al., 2015) translates into the possibility of finding new genes or strategies for thermotolerance, which can be used in breeding and selecting climate-resilient plants. In addition to the traits that produce thermotolerance in plants, identifying the mechanisms that help to overcome the damages produced by heats shocks or waves once the stress is release, is a key point for plant breeding in the fight against climate change.

However, these recovery processes are actually poorly understood (Jagadish et al., 2005).

The main objective of this work is the characterization of traditional tomato varieties (or tomato landraces) of different areas of the Mediterranean Basin against heat stress. To accomplish this purpose, four traditional varieties *S. lycopersicum* genotypes: TH-30 (Greece*);* ADX (Spain); ISR-10 (Israel); MO-10 (France) and two commercial genotypes: *Ailsa Craig (*Ailsa*)* and Moneymaker (MM), were screened for heat tolerance. To understand the intra-genetical variation, the main defence signalling pathways activated to counteract the negative effect of increased temperature were evaluated. We provide evidence of the relevance of leaf cooling mechanisms and the regulation of the oxidative burst produced by HS. We also demonstrated that SA, phenolic compounds and melatonin are crucial components to overcome HS. Understanding the molecular events that take place in the different phenotypes will help us get a deeper understanding of plant response to heat stress, which can be fundamental for the maintenance of food production in the near future.

2. RESULTS

2.1. Screening tomato phenotypical variations in thermotolerance

To achieve HS, the temperature of the growth chamber was increased to 42ºC for 6 hours. Plant recovery capacity was subsequently evaluated after two hours under normal temperature conditions. In order to check how HS could affect different tomato genotypes, leaf damage was evaluated at 2, 4, 6 and 6+2 hpHS (hours post *heat shock*) and the results were expressed as the percentage of damaged leaflets of third and fourth true leaves. Leaf damage was measured using a four-level severity scale that determined the damage depending on the rolling of the leaflets: *level 0* (healthy), *level 1* (minor roll), *level 2* (medium roll) and *level 3* (severe roll or dead). At 2hpHS, we observed that TH-30, Ailsa and ADX2 genotypes displayed higher leaf damage than the other tomato genotypes.

Leaf damage was especially important in ADX2 plants, which had more than 10% of their leaflets in *level 2* (Figure 1a). ISR-10, MM and MO-10 genotypes showed less than 50% damaged leaflets (most of them in *level 1*) after 4 hpHS. Moreover, at this time point a significant damage on TH-30, Ailsa and ADX2 leaves was observed, since they exhibited 80, 70 and 50% of damaged leaflets, respectively (Figure 1b). At the climax of stress (6hpHS), clear phenotypic differences were observed among the studied tomato genotypes. TH-30 plants were strongly affected by the increase of temperature, showing 100% of its leaflets damaged (50% in *level 3*). Ailsa and ADX2 genotypes showed more than 70% damaged leaflets. On the opposite, we found that MM and MO-10 genotypes displayed less than 60% damaged leaflets (only 20% in *levels 2* and *3*) showing a tolerant phenotype (Figure 1c).

Once classified the tomato genotypes depending on their thermotolerance, we evaluated their behaviour in the recovery phase. For this purpose, the visual phenotype was evaluated after 2 hours of absence of stress without water added.

Figure 1d shows leaflet damage after 6 hours of heat shock and 2 hours of recovery (6+2 hpHS). ISR-10 plants showed 100% of leaflets in *level 0* (full recovery). Moreover, Ailsa and ADX2 displayed the 80% of their leaflets in *level 0*, an almost full recovery. However, TH-30 plants still revealed to have 50% damaged leaflets at this time point (part of them in *levels 2* and *3*). MM and MO-10 plants displayed 30% damaged leaflets although most of them were in *level 3*. On the other hand, the plants of MO-10 genotype displayed 50% damaged leaflets but most of them were found at *level 1*.

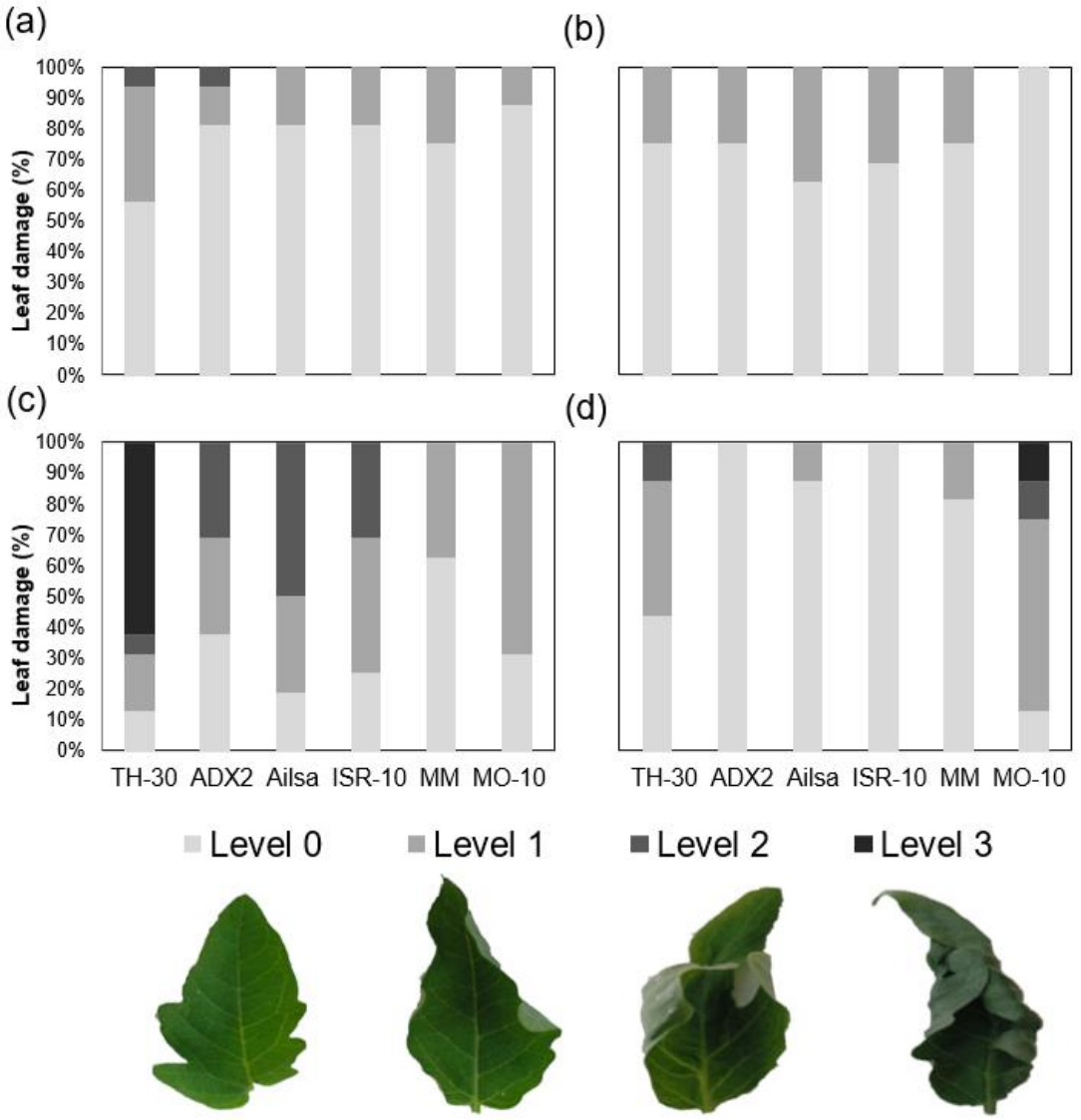
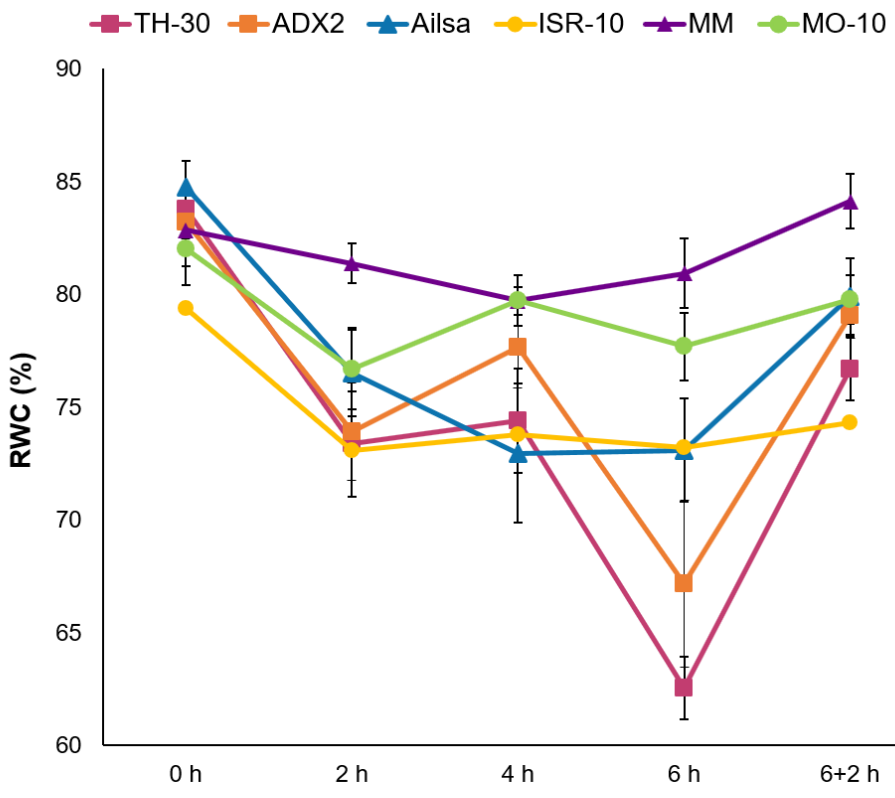


Figure 1. Evaluation of leaf damage provoked by HS on the six tomato genotypes. Leaf damage was measured using a four-level severity scale that identifies the damage depending on the leave roll: *level 0* (healthy), *level 1* (minor roll), *level 2* (medium roll) and *level 3* (severe roll or dead). The photograph shows a representative picture of damage level. Leaf damage produced by the increase of temperature until 42ºC were measured as a percentage of the leaflets with symptoms in relation to the total number of analysed leaves at (**a**) 2 hpHS, (**b**) 4 hpHS, (**c**) 6 hpHS and (**d**) 6+2 hpHS. Data show the average of three independent experiments, with values of 10 seedlings per experiment.

2.2. Effect of time of HS on the relative water content (RWC)

To characterize the response of the different tomato genotypes to high-temperature stress, the relative water content (RWC) of each leaflet was evaluated, since it is known that high temperature results in rapid loss of water that may cause dehydration (Machado & Paulsen, 2001). We observed a HS related RWC reduction on all tomato genotypes, though significant differences between them were detected (Figure 2). At 2 hpHS, it was observed that TH-30, ADX2 and ISR-10 genotypes displayed a clear reduction in RWC that reached as low as 75%. However, MM plants were able to maintain high RWC levels after 2 hours at 42 ºC. At 4 hpHS, plants of MM and MO-10 genotypes displayed a slight reduction of RWC content. However, ISR-10, Ailsa and TH-30 plants showed reduction of 11.8, 12.8 and 11.1 % respectively, comparing with their basal values indicating that the differences in dehydration produced by the increase of external temperatures could be correlated with the different sensitivities of the varieties to HS. The most significant changes associated with the reduction of RWC on tomato leaves was observed at 6 hpHS. MM and MO-10 plants maintained the RWC of their leaves at a similar level to basal ones throughout the HS process. Ailsa and ISR-10, although they displayed an initial loss of water in the first two hours of HS, RWC was unaffected at 4 and 6 hpHS maintaining a value of 73%. Finally, plants of ADX2 and TH-30 genotypes showed at 6hpHS a noticeable reduction in RWC content induced by the HS, which is especially important in TH-30 genotype. In the recovery phase, (6+2 hpH), plants of MM, MO-10 and ISR-10 varieties, which showed less water loss during HS, maintained similar RWC after two hours in the absence of stress. However, genotypes such as Ailsa, TH-30 and ADX2 that displayed a severe decline during the HS process, recovered to values around 77-80%.



	0 h	2 h	4 h	6 h	6+2 h
TH-30	b	a	a	a	ab
ADX2	ab	a	ab	ab	b
Ailsa	b	ab	a	bc	b
ISR-10	a	a	a	bc	a
MM	ab	b	b	d	c
MO-10	ab	ab	b	cd	b

Figure 2. Relative water content (RWC, %) of the six tomato genotypes upon HS. HS was applied on four-weeks-old tomato plants by the increase of 42 ºC in the culture chamber during 6 h and the recovery capacity was subsequently evaluated after 2 h under normal temperature conditions. Leaflets were collected at various time points and the RWC were evaluated. Data show the average of three independent experiments, with values of 10 seedlings per experiment ±SE. Letters in the table indicate statistically significant differences between genotypes at each time point ($P < 0.05$; LSD test).

2.3. Effect of HS on photosynthetic parameters of different tomato genotypes

In order to determine the tolerance or susceptibility of tomato genotypes to HS, different photosynthetic parameters have been analysed (Figure 3). Interestingly, in the absence of HS, the genotypes showed statistically significative differences

regarding photosynthetic parameters (Figure 3a). As expected, all tomato genotypes showed a significant reduction of A through HS, however TH-30 and ADX2 plants displayed a strong reduction of A at 2, 4 and 6 hpHS. Surprisingly, the MO-10 plants showed a slight reduction of A at 2 and 4 hpHS compared with its basal levels, but it showed a significative A decrease at 6 hpHS MM plants displayed a strongly reduced A at 2 hpHS, showing a recovery at 4 hpHS and another marked reduction at 6 hpHS, reaching values of 6 µmol CO_2 m^{-2} s^{-1}. Although at 6+2 hpHS, in the recovery phase, all genotypes displayed lower A when compared to their basal values, statistical differences were seen between the genotypes. MM, MO-10, ISR-10 and Ailsa that showed photosynthetic rates close to 6, while the most affected genotypes, TH-30 and ADX, presented a rate of 0,27 and 0,44 µmol CO_2 m^{-2} s^{-1} respectively.

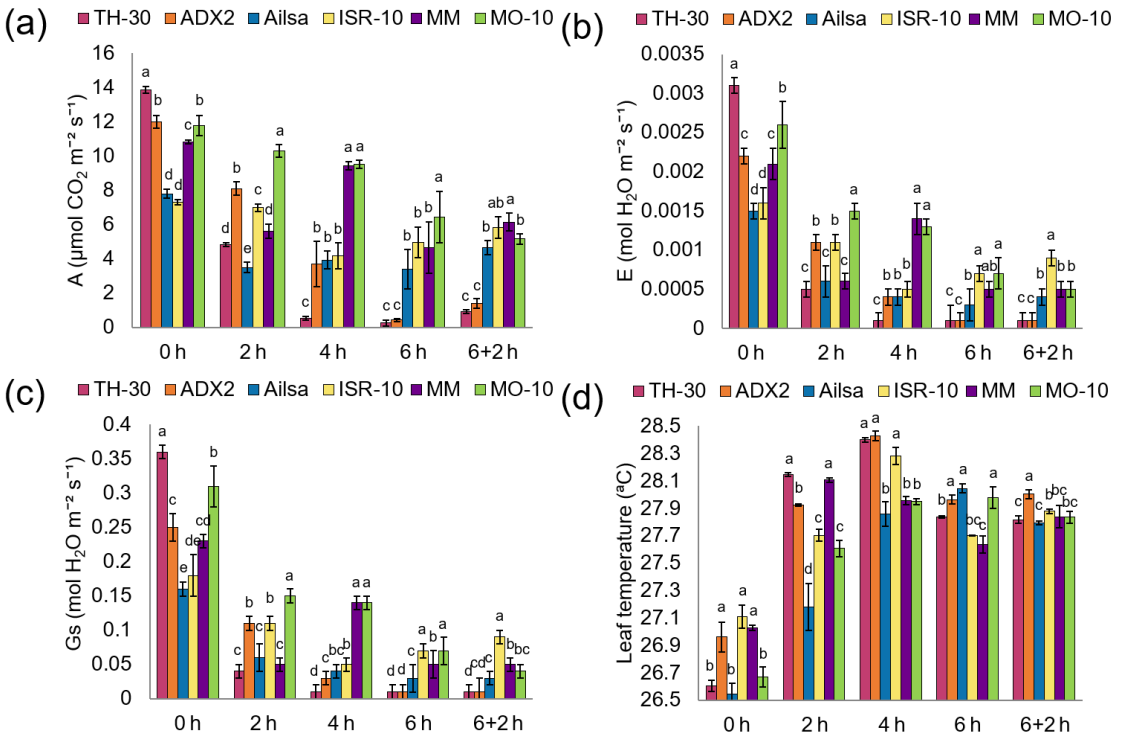


Figure 3. Photosynthetic parameters of the six tomato genotypes during the HS. The stress was applied to four-weeks-old tomato plants by increasing the culture chamber temperature to 42ºC during 6 h, and the recovery capacity was subsequently evaluated after 2 h under normal temperature conditions. The measurements of (**a**) photosynthetic rate (A: µmol CO_2 $m^{-2}s^{-1}$); (**b**) transpiration rate (E: mol H_2O $m^{-2}s^{-1}$); (**c**) stomatal conductance (g_s: mol H_2O $m^{-2}s^{-1}$); (**d**) leaf temperature (Tleaf: ºC), were done at different time points. Data are expressed as mean ± SE from three biological replicates, each replicate consisting of 3 plants with three technical replicates (n=27). Letters indicate statistically significant differences between genotypes at each time point ($P < 0.05$; LSD test).

The transpiration rate (E) and stomatal conductance (g_s) of the tomato genotypes selected for the study are represented at Figure 3b and 3c. The most significant changes on E and g_s occur at 2 hpHS, where all genotypes displayed a decrease of both parameters being this reduction especially important in MM, Ailsa and TH-30 plants. ISR-10 and ADX2 genotypes showed a strong reduction when compared to their basal values, however, these plants maintained higher levels of both parameters than the susceptible genotypes. In the late stage of HS, significant differences were seen in E and g_s, where TH-30 and ADX2 plants showed the lowest values of both parameters, demonstrating the HS damage. In addition, ISR-10, MM and MO-10 genotypes revealed a strong reduction of E and g_s at 6 hpHS compared to their basal values but presented higher values than the most affected genotypes without statistically significant differences among them. Interestingly, no variation in the E and g_s values were found in the recovery phase between genotypes.

On the other hand, leaf temperature (Tleaf, Figure 3d) was evaluated throughout the HS in the six genotypes under study. At 2 hpHS, an increase in the Tleaf in all groups of plants was observed. Still, this increase was especially significant in TH-30, MM and ADX2 plants. Moreover, at 4 hpHS, Tleaf was the highest temperature scored during the HS, reaching values of 28.39, 28.42 and 28.28 ºC in the TR-30, ADX2 and ISR-10 genotypes, respectively. At 6 hpHS all genotypes were able to reduce Tleaf value, being MM plants the ones that displayed the lowest foliar temperature (Figure 3d).

2.4. Analyzing the changes in Fv/Fm and ETR to characterize tomato genotypes as a heat-sensitive or heat-tolerant

The maximum quantum efficiency of Photosystem II (Fv/Fm) is used to quantify damage to Photosystem II. This parameter has been used to evaluate stress response in many plants' species (Bineau et al., 2021). In this study, Fv/Fm ratio has been analysed to evaluate the thermotolerance of diverent tomato varieties. Healthy plants hold Fv/Fm values that range around 0.8, which decreases upon stress. In our experimental procedure, basal Fv/Fm ranged around 0.75+SE. After

2 hpHS, an important reduction of Fv/Fm ratio was observed in ADX2, Ailsa, MM and TH-30 plants compared to their respective basal values, while MO-10 and ISR-10 plants were able to maintain similar basal ratio at this time point. At 6 and 6+2 hpHS, TH-30 and ADX2 genotypes displayed Fv/Fm ratios between 0.4-0,5 demonstrating the serious leaf damage due to increased temperature (Figure 4a).

In addition to Fv/Fm, another stress marker was evaluated. As expected, HS produced in tomato genotypes a reduction of electron transport rate (ETR). However, ETR reduction was statistically significant in TH-30 and ADX2 plants, with reductions of 71 and 49% at 6 hpHS relative to their basal values. On the other hand, MO-10 plants displayed high ETR values at 6 and 6+2 hpHS confirming lower level of cell damage by HS (Figure 4b).

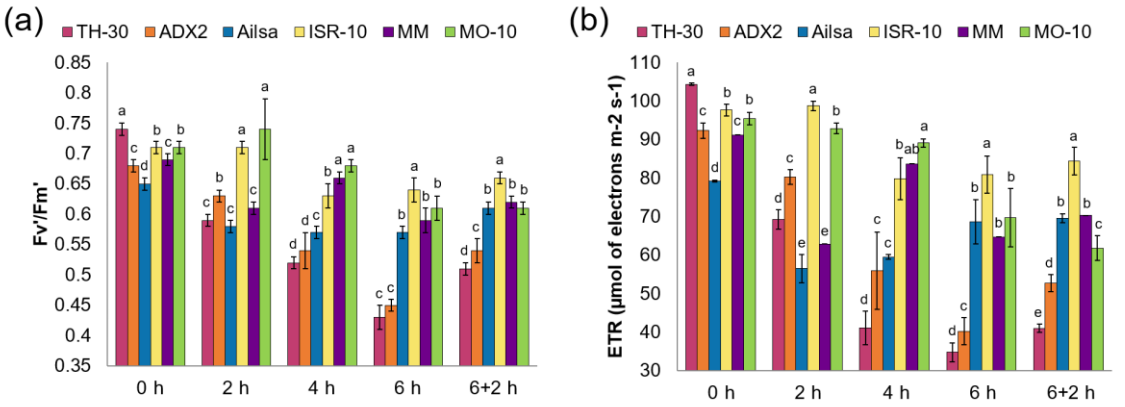


Figure 4. Impact of HS on the photosystems. Tomato plants were grown and the HS was applied as described in Figure 2. The measurements of (**a**) quantum efficiency of photosystem II (Fv/Fm) and (**b**) electron transport rate (ETR, µmol of electrons m^{-2} s^{-1}) were done at different time points. Data are expressed as mean ± SE from three biological replicates, each replicate consisting of 3 plants with three technical replicates (n=27). Letters indicate statistically significant differences between genotypes at each time point ($P < 0.05$; LSD test).

Based on visual damage evaluation, leaf RWC, photosynthetic parameters and parameters related to photosystems damage, a scale of thermotolerance was established considering: thermo-sensitive genotypes TH-30 and ADX2; genotypes with intermediate behaviour, ISR-10 and Ailsa; and thermo-tolerant genotypes, MM and MO-10. To gain further insight into the biochemical and molecular mechanisms related to thermotolerance in tomato plants, two

susceptible (TH-30 and ADX2) and two tolerant (MM and MO-10) genotypes were selected to continue with the characterization.

2.5. Modification of HSP pattern expression among tomato genotype under HS

To determine whether the tomato responses against HS is genotype-dependent, the expression of marker genes for thermotolerance-related pathways was evaluated (Figure 5). First, the expression of *heat shock proteins* was analyzed, specifically *HSP70* and *HSP90* and the transcription factor *HsfA2*, which is involved in their regulation. In all the studied genotypes, an early induction of *HSP70* was observed at 2 hpHS (Figure 5a). Moreover, TH-30 and MM plants had an exponential increase of *HSP70* transcript levels at 2 hpHS. Regarding to the expression of the *HSP90* gene (Figure 5b), a strong induction was observed at 2 hpHS in all genotypes, being this increase strongly marked on MM plants compared to MO-10 genotypes. Interestingly, the genotypes considered tolerants, MM and MO-10, showed a slight induction of this gene in the recovery phase. Figure 5c shows the expression values of *HsfA2*, which is induced in all the varieties at 2 and 6 hpSH, specially in TH-30 plants.

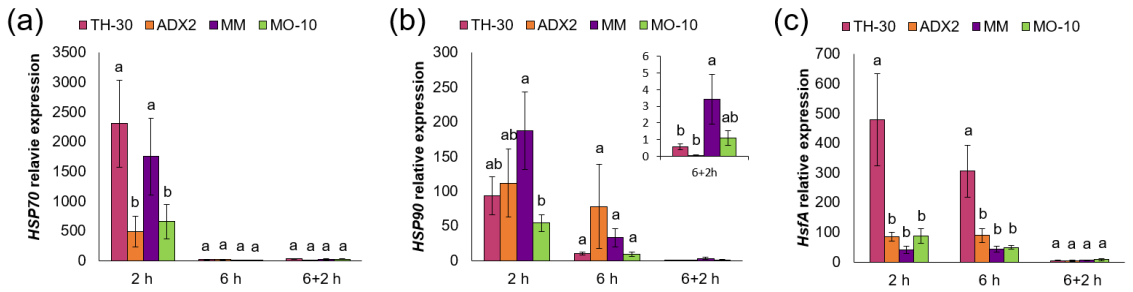


Figure 5. Effect of genotype on the heat responses related genes of tomato plants under HS. Tomato plants were grown and the HS was applied as described in Figure 2. Total RNA was isolated from leaves at different time points, converted cDNA and subjected to qRT-PCR analysis. The expression levels of genes (**a**) *HSP70*, (**b**) *HSP90* and (**c**) *HsfA* were analyzed. The relative expression levels of each gene were normalized to those of *EF1α*. Letters indicate statistically significant differences between genotypes at each time point ($P < 0.05$; LSD test).

2.6. Control of antioxidant machinery is crucial in thermotolerance

In addition to the study of chaperones for characterizing the response of different tomato genotypes to HS, genes related to the oxidative burst produced in plants after a stress situation and the antioxidant genes were analysed (Figure 6). The obtained results of *RbohB* expression (Figure 6a) showed that at early phase of HS, it was activated in MO-10 plants. At 6 hpHS, a strong induction of this gen was exhibited in both MO-10 and MM genotypes, which was maintained in the recovery phase. In general, genes related to oxidative stress were more induced in those genotypes that displayed greater tolerance phenotype against HS. Specifically, MO-10 plants showed an early activation of the *DHAR1* gene when compared to the rest of genotypes. In these plants, a statistically significant increase in *SODct* expression was also observed at 6 hpHS. Moreover, MM plants showed an induction of *CAT* gene at 6 hpHS, which remained in the recovery phase. Hence, control of antioxidant systems seems to have a clear role in tomato thermotolerance.

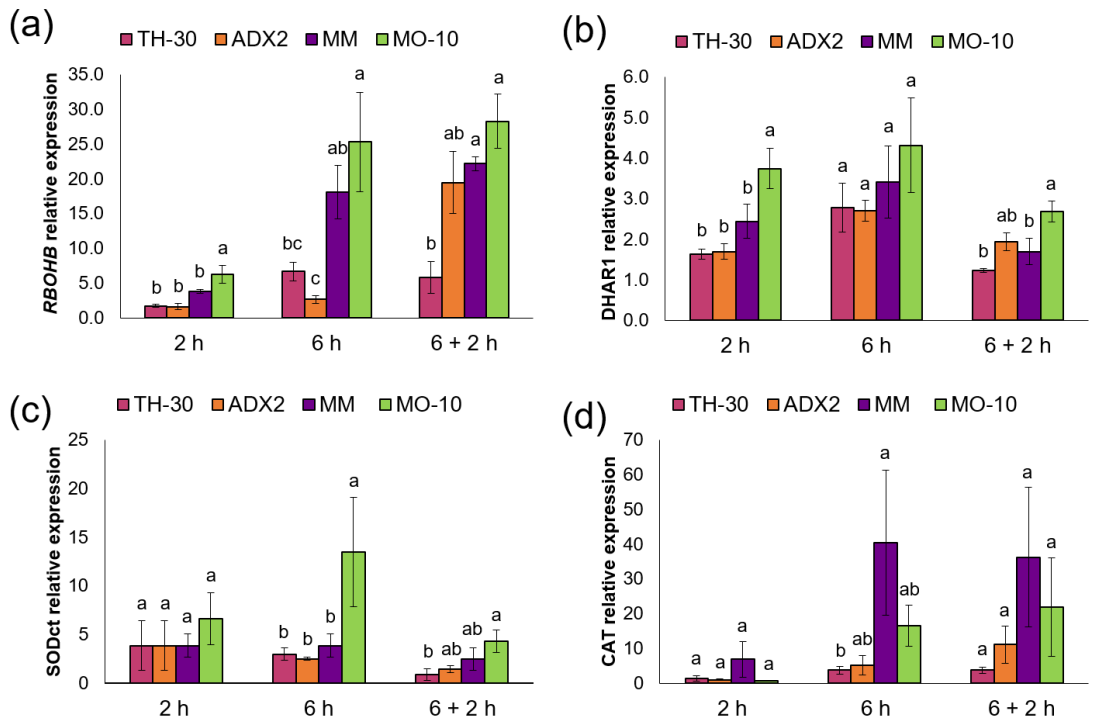


Figure 6. Expression profile of the genes involved in the antioxidant system in the four tomato genotypes upon/under HS. Tomato plants were grown and the HS was applied as described in Figure 2. Total RNA was isolated from leaves at different time points, converted cDNA and subjected to qRT-PCR analysis. The expression levels of genes (**a**) *RbohB*, (**b**) *DHAR1*, (**c**) *SODct* and (**d**) *CAT* were analyzed. The relative expression levels of each gene were normalized to those of *EF1α.* Letters indicate statistically significant differences between genotypes at each time point ($P < 0.05$; LSD test).

2.7. Tolerant genotypes displayed an early activation of ABA-dependent signalling pathways under HS

To establish the molecular mechanisms that underlie the thermotolerance observed in some of the studied genotypes, we analysed the hormonal and metabolite content in tomato leaves under HS (Figure 7). Regarding ABA accumulation, although all genotypes displayed slight changes on ABA content during the HS, MO-10 plants showed statistically significant accumulation of ABA at 2 hpHS. On the other hand, TH-30 plants displayed a higher accumulation of ABA at 6 hpHS and these levels were increased during the recovery phase, reaching values of 850 ng/g FW. To delve into the role of ABA in the tolerance or susceptibility of different tomato genotypes against HS, the expression of the *asr1*

gene has been evaluated (Figure 7b). The increase of temperature induced the transcript levels of *asr1* in MM and MO-10 plants in an early phase of HS. Moreover, at 6 hpHS, although we found an induction of *asr1* gene in all group of plants against HS, the expression was higher in the plants of the tolerant genotypes MM and MO-10. Although HS did not increase the SA levels of any of the tomato genotypes (Fig 7c), a significant accumulation of SA occurs in the recovery phase in all group of plants, where TH-30 and ADX-2 genotypes showed the highest levels of SA.

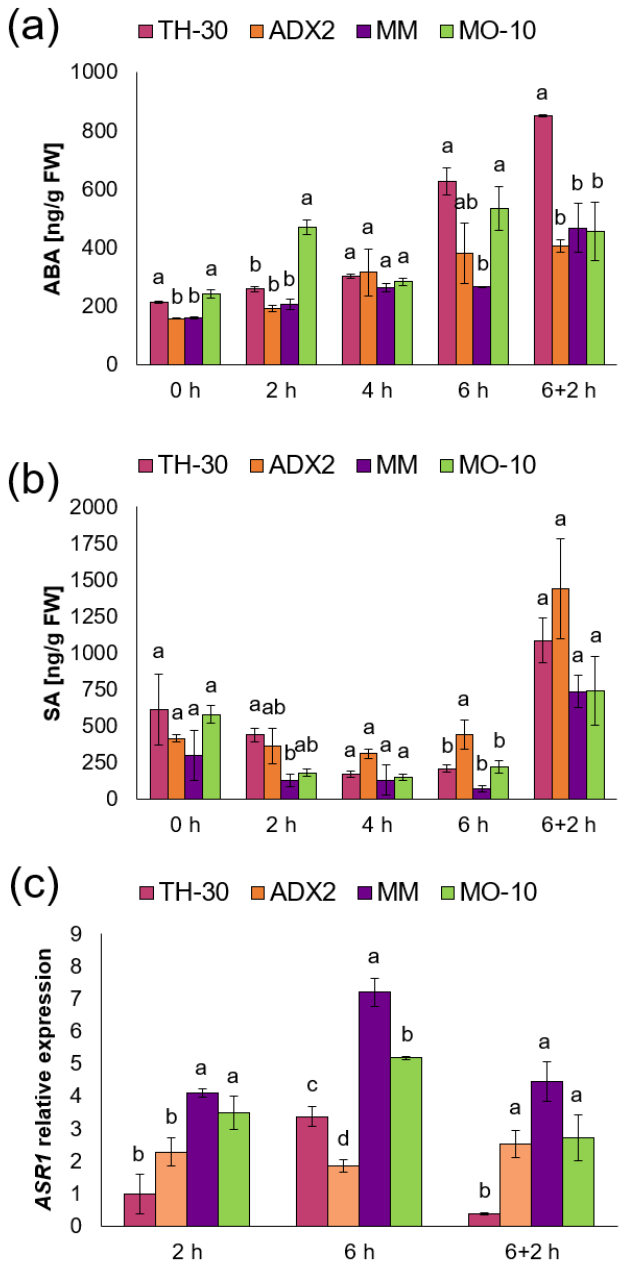


Figure 7. Effect of genotype on the hormonal response of tomato plants under HS. Tomato plants were grown and the HS was applied as described in Figure 2. Leaves were collected at different time points, and (**a**) ABA and (**b**) SA levels were determined using UPLC–MS. The relative levels of (**c**) *asr1* were analyzed and normalized to the *EF1α* gene expression level measured in the same sample. Letters indicate statistically significant differences between genotypes at each time point ($P < 0.05$; LSD test).

2.8. Phenolic compounds and melatonin synthesis seem to be key for the recovery of cells after HS

To clarify the mechanisms involved in plant thermotolerance, the metabolic pathways related with the synthesis of active compounds against abiotic stresses were studied. First, the phenylpropanoid pathway was analyzed in four tomato genotypes at 2, 4, 6 hpHS as well as in the recovery phase. The MM plants, cataloged as a tolerant genotype, displayed large increase of *PAL* transcript accumulation at 2 hpHS (Figure 8a). The activation of *PAL* correlated with an increase in chlorogenic acid (CGA) in these plants at 4 hpHS (Figure 8b). Interestingly, this phenolic compound was differentially accumulated at 6 hpHS in genotypes considered thermo-sensitive (TH-30 and ADX-2). In the recovery phase, susceptible genotypes displayed a great induction of *PAL* expression, which produced an increase of CGA content in plants of TH-30 and ADX2 genotypes. This last genotype showed a significant increase of ferulic acid (FA) at 6+2 hpHS.

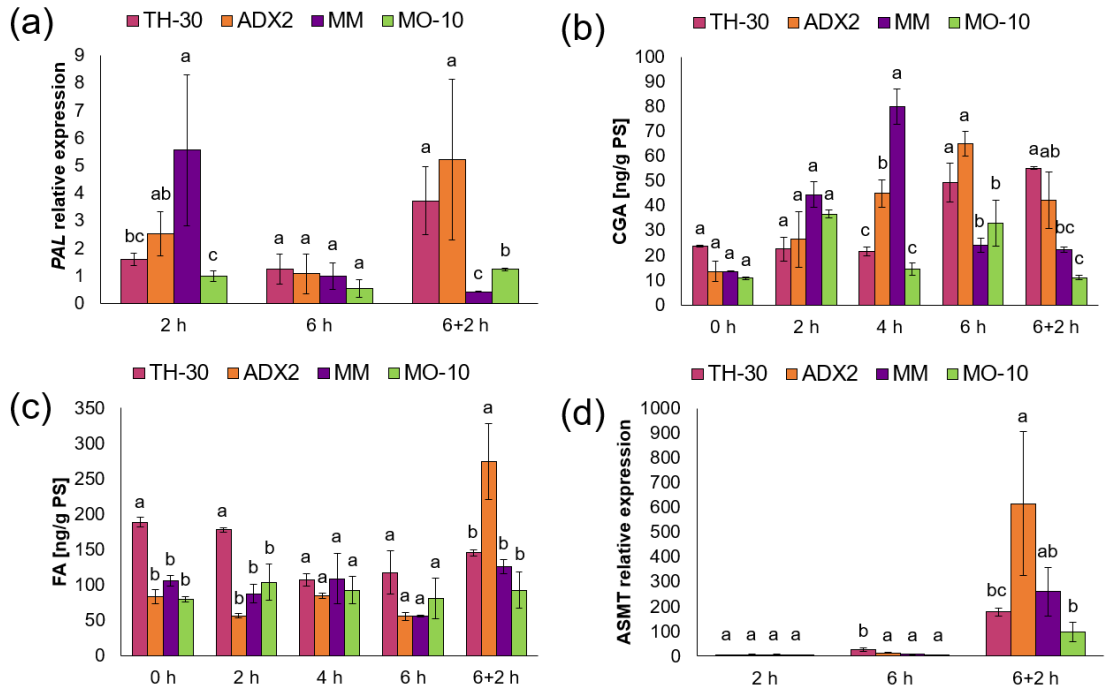


Figure 8. Effect of genotype on the variation of secondary metabolisms related responses of tomato plants under HS. Tomato plants were grown and the HS was applied as described in Figure 2. Leaves were collected at different time points, and (**a**) *PAL* expression, (**b**) CFG and (**c**) FA levels were determined. Moreover, the relative levels of (**d**) *ASMT* were analyzed. The relative expression levels of each gene were normalized

to those of *EF1α.* Letters indicate statistically significant differences between genotypes at each time point (P < 0.05; LSD test).

Finally, the role of melatonin in the plant responses against the temperature rise was evaluated. To do this, we evaluated the expression of *ASMT*, which encodes a protein that catalyzes the last step of the melatonin biosynthetic pathway (Figure 8d). *ASMT* was induced by HS in all tomato genotypes at 2 hpHS. However, at 6 hpHS, TH-30 plants displayed a greater induction of *ASMT* gene compared to the other genotypes. Interestingly, the highest levels of *ASMT* expression were found in the recovery phase without statistically significant changes between the genotypes. Taken together, these results suggest the involvement of phenylpropanoid derived compounds and melatonin in the recovery of tomato plants after a HS situation.

3. DISCUSSION

Increased temperatures caused by climate change are a significant threat to agriculture and food security. Current models predict an increase in the global average temperature between 2 and 5 °C over the next hundred years. Extreme weather events, which include heat waves, will have devastating consequences for crop productivity (Battisti & Naylor, 2009; Bita & Gerats, 2013; Iizumi et al., 2018; Masson-Delmotte et al., 2021). Along with heat stress, extreme drought is the other main abiotic stress directly related to climate change, and both will affect the Mediterranean Basin more severely than other areas of the world. For this reason, the development of varieties with greater tolerance to these extreme weather events is considered a vital need for the future of agriculture (Raza et al., 2019).

To overcome this predicted situation, special attention is being paid to the genetic potential that wild and traditional plants constitute, since they represent a genetic reservoir not yet explored. In this study, we analysed the response of different tomato genotypes to HS from different perspectives with the purpose of finding which set of responses are the key mechanisms to thermotolerance. To do so, we selected four traditional *S. lycopersicum* genotypes, originated from Mediterranean area: TH-30 (Greece*); A*DX2 (Spain); ISR-10 (Israel); MO-10

(France) and two commercial genotypes: *Ailsa Craig* (Ailsa) and *Moneymaker* (MM).

HS was induced by increasing growth chamber temperature to 42ºC for 6 h, simulating the environmental conditions of an extreme summer. In addition, we analysed the plant recovery 2h post-stress what reflects plant thermotolerance. After checking for any damage induced by HS, we found a differential response to this unfavourable situation among genotypes. Specifically, the visual evaluation of foliar damage revealed that the most affected genotypes by the increase in temperature were TH-30 and ADX2, and the most tolerant were MM and MO-10. We also evaluated RWC, which is considered an indicator of water condition of cells and correlated with tolerance or susceptibility to biotic and abiotic stresses (Farooq et al., 2019). Our results are in concordance with this idea since HS produced a reduction of RWC content in the six tomato genotypes. MM and MO-10 displayed a slight change in RWC content, whereas TH-30 and ADX2 plants suffered great dehydration during HS.

Another common effect of heat stress on plants is the impact on photosynthesis, mainly through affecting biochemical reactions (Allakhverdiev et al., 2003). Although a general reduction in A was observed during HS, we found clear differences among genotypes. At 4 hpHS, TH-30 plants displayed a strong reduction of A rate, followed by the ADX-2, Ailsa and ISR-10 genotypes which displayed a strong reduction of A rate compared to their basal values. Nevertheless, MM and MO-10 were able to maintain higher A during the HS stress. The ability to sustain leaf gas exchange under HS has been directly correlated with heat tolerance (Bita & Gerats, 2013). Recently, it has been demonstrated that heat tolerant wheat cultivars could maintain high rates of photosynthesis and stomatal conductance during HS (Sharma et al., 2015).

The literature demonstrates that transpirational cooling is an important mechanism for heat avoidance in food crops (Deva et al., 2020). In fact, the results obtained for transpiration and stomatal closure, leaf Tº and cell damage parameters are probably the core point in the intra-genetical thermotolerance variation obtained in this work. As expected, plants of TH-30, Ailsa and MM

genotypes showed a sudden reduction in transpiration (E) and stomatal conductance (g_s) values during Heat stress. However, no changes of E and gs were observed in the early phase of HS in MO-10 plants when compared to the values obtained in stress absence. These results suggest that the capacity of MO-10 to maintain high levels of E and gs during HS might be the key event related to the tolerance phenotype since these processes contribute to leaf cooling, allowing to keep leaf temperature within the range required to maintain its optimal physiological function (Deva et al., 2020). Therefore, the maintenance of the transpirational cooling in MO-10 plants might regulate the temperature of the leaves, keeping it almost 0.5ºC below the foliar Tº observed in the susceptible genotypes. Moreover, in this work, we have studied the cell damage produced by heat stress (measured based on the reduction in both ETR and FV/Fm parameters) observing that MO-10 plants did not show the cell damage detected at 2 hpHS in the other genotypes. This demonstrates that the genotypes capable of activating the leaf blade cooling process by maintaining E and gs, are also able to reduce the excessive increase in foliar Tº that could avoiding this way damages to the cellular structures. This hypothesis is confirmed by the results obtained in the MM genotype, since at 2hpHS these plants displayed a decrease of E and Gs, that probably reduces the leaf cooling. This Tº increase could produce cell damage as demonstrated by the increment observed for both ETR and FV/Fm parameters. However, at 4 hpHS, MM plants opened stomata and increased the transpiration rate up to 0.0014 mol H_2O $m^{-2}s^{-1}$XX (reaching values observed in MO-10 plants), that correlates with a reduction of leaf Tº, which normalizes the FV/Fm and ETR values.

According to these findings, the mechanism of transpiration cooling is a key event in the response of tomato plants to HS since it allows the maintenance of leaf temperature within optimal ranges that ensure the correct cell function. After the analysis of the visual phenotype, the RWC and the evaluation of the photosynthetic parameters in different tomato genotypes under HS, we established a thermotolerance classification. The obtained results demonstrated that the genetical variation of tomato plants responses against HS exists, which

allowed to characterize the genotypes studied in this work as: heat-sensitive: TH-30, ADX2; Intermediate: ISR-10 and Ailsa; heat-tolerant: MM and MO-10.

To understand the mechanisms activated in tolerant tomato genotypes against HS, a comparative study was performed between heat-sensitive (TH-30 and ADX2) *versus* heat-tolerant (MM and MO-10) genotypes.

The induction of *HSFs* and *HSPs* is considered essential for plant thermotolerance. As expected, all tomato genotypes displayed a strong increase in the expression of *HSP70* and *90*, encoding chaperones related with plant response to heat stress, and the transcription factor *HsfA* involved in their regulation. Despite the rapid and marked increase in these transcripts in all the studied genotypes, surprising differences were detected among them. TH-30 plants showed the highest induction of *HsfA* expression at 2 and 6 hpHS, that did not correlate with an increase of thermotolerance. Interestingly, the highest levels of *HSP70* expression were observed in TH-30 and MM genotypes, which displayed an opposite visual phenotype. The importance of HSP70 in the plant responses against heat stress is noteworthy since its expression correlated with an increase of thermotolerance in *Arabidopsis* (Jackson-Constan & Kenneth, 2001). Interestingly, (Schroda et al., 1999) reported that *Hsp70B* found in the stroma of chloroplasts participates in photo protection and the repairing of photosystem II during and after the photoinhibition. According to these findings, it can be speculated that a higher expression in *HSP70* is correlated with a severe damage on the photosystems in TH-30 and MM plants probably due to a poor control of leaf cooling process that increased leaf temperature. Regarding *HSP90* expression, a strong induction was observed in all genotypes, but the highest level of induction was found in the MM plants. The crucial role of HSP40/HSP70 chaperone machinery in abiotic stress response is a well-known mechanism (Wang et al., 2004). However, *HSP90* expression has been related to direct protection against abiotic stresses almost as a transduction signal (Yamada et al., 2007). For example, the expression of *Glycine max HSP90* in *Arabidopsis* protected plants against heat, salt, and oxidative stress (Xu et al., 2013). The synergic effect of *HSP70* and *HSP90* activation in MM might have an important

role in the tolerant phenotype showed by these plants against HS despite the initial damage produced by a sudden increase of temperature. It can be speculated that *HSP70* activation could be related with the rebuilding of photosynthetic apparatus and that *HSP90* induction could activate mechanisms related to thermotolerance.

To elucidate the mechanisms related to stress tolerance in tomato genotypes, in addition to the expression of *HsfA, HSP70* and *HSP90*, control of leaf cooling by transpiration and water relations, the antioxidant machinery and hormone, and secondary related signalling pathways were analysed. Heat stress produces ROS in plant tissues (Zhao et al., 2018). In our study, an early induction of *RbohB* was found in MO-10 plants. Interestingly, at 6 hpHS a strong induction of this gene was only observed in the thermo-tolerant genotypes (MO-10 and MM). Recent studies provide compelling evidence showing that mutation in brassinosteroid pathways impaired the induction of *Rboh1*, H_2O_2 apoplastic accumulation and thermotolerance, and that exogenous H_2O_2 fully recovered the heat stress tolerance of the mutants (Yin et al., 2018). Although the production of ROS causes damage to cell structures, it can play an important signalling role in stress responses (Mittler, 2017). When ROS increase, one of the main mechanisms of thermotolerance is the upregulation of the antioxidant system (Saleh et al., 2007) to generate cellular homeostasis and fix the injuries produced by HS (Hasanuzzaman et al., 2013). In this work, we demonstrated that MO-10 plants displayed an early induction of the *DHAR1*, which is a gene related to ascorbic acid metabolism. Recently, Alayafi (2020) postulated that ascorbic acid produced priming effect on tomato roots against HS by reducing the oxidative damage as well as increasing key components of thermotolerance. In addition to the accumulation of metabolites with antioxidant capacity, the up-regulation of genes encoding antioxidant enzymes like *Cu/Zn-SOD*, *Mn-SOD* and *GR* by heat priming was effective to induce tolerance in wheat seedlings to subsequent HS (Wang et al., 2014). In this work, a correlation was observed between heat-tolerant genotypes and the activation of antioxidant machinery, given that a marked statistically significant increase was observed in *SODct* and *CAT* genes in MO-10 and MM genotypes respectively, which supports the notion that control of

oxidative burst produced by HS is a key point in thermotolerance. The H_2O_2 accumulation associated to *RbohB* activation could be crucial for the heat-tolerant phenotype of MO-10 plants, since the accumulation of this signalling molecule could be related to the activation of HS response. However, control of the damage associated to the oxidative burst through the accumulation of antioxidant enzymes (only observed in MM and MO-10) is important to reduce oxidative stress.

The metabolic profile of tomato plants from different genotypes against HS, provide a clue to better understand the signalling networks that act in the intra-genetical variation of HS responses. The increase of endogenous ABA concentration after heat stress enhances the antioxidant ability to confer heat tolerance in plants (Hu et al., 2018). In this work, *asr1* expression and ABA content were evaluated to clarify the role of ABA-dependent signalling pathways on the contrasting levels of heat tolerance obtained among tomato genotypes. Heat-tolerant phenotype displayed a great *asr1* activation at 2 and 6 hpHS. Although ABA accumulation is not observed at the studied time points, *asr1* and *RbohB* expression profiles correlate. These results are consistent with those published by (Driedonks et al., 2015), who observed that ABA-treated plants showed a higher accumulation of H_2O_2 that mediated the induction of heat tolerance.

Moreover, phenolic compounds are an important class of plant secondary metabolites which play crucial physiological roles throughout the plant life cycle. The analysis of phenylpropanoid pathway revealed that MM plants displayed an early *PAL* upregulation, which was correlated with a CGA accumulation at 4 hpHS. A recent study postulated that the accumulation of phenolic compounds in *Festuca trachyphylla* subjected to HS was accompanied by enhanced tolerance to the increase of temperature (Wang et al., 2019), and other works concluded that their implication on heat responses relies on their capacity to prevent heat induced oxidative damage (Commisso et al., 2016). According to these findings, early phenylpropanoid pathway activation in MM plants could have an important role in the thermotolerant phenotype, despite the initial damage due to HS.

To better understand the role of the activated pathways throughout HS, we also studied the mechanisms involved in the recovery phase. Although the analysis of the photosynthetic parameters in this phase confirmed the genotype variation in responses, the study of the active pathways after stress provided very interesting information about how plants recover their structures after an adverse situation. Although it is widely known the implication of SA in abiotic stress tolerance (review by Khan *et al.* (2015)), little investigation of the SA-mediated responses in the recovery phase has been done. The widespread SA accumulation at 6+2 hpHS in all the genotypes analysed regardless of their phenotype under HS supports that SA-related pathways might have an important role in ameliorating post-stress heat-induced cellular damage. Foliar application of SA 1 mM on *Cucumis sativa* produced a decrease on electrolyte leakage and oxidative stress, and improved maximum yield of PSII, Fv/Fm, and the quantum yield of the PSII electron transport after both HS and in the recovery phase (Shi et al., 2006). In higher plants, SA is synthesized by two distinct pathways, PAL- and ISC-pathways (Miura & Tada, 2014; Vlot et al., 2009). Interestingly, the highest levels of *PAL* expression at recovery phase occurred in TH-30 and ADX2 plants with a heat-sensitive phenotype. Moreover, the high expression level of *PAL* in TH-30 and ADX2 plants correlates with a high accumulation of phenolic compounds such as CGA and FA at 6 hpHS and in the recovery phase. Although, the positive effect of phenolic compounds in the plants responses against heat stress is well documented (Sharma et al., 2015), our findings support that these secondary metabolites are one of the keys to overcome the damage caused by high temperatures. When we searched the mechanisms activated in the post-stress period, we found that *ASMT*, a gene related with melatonin biosynthetic pathway, was strongly induced in all the tomato genotypes with respect to the HS period. Recent studies have demonstrated that melatonin treatment of tomato seedlings could enhance glucose metabolism, improve photosynthetic efficiency, up-regulate melatonin biosynthesis, and reduce the photoinhibition occurred in the HS (Jahan et al., 2021). Therefore, our data support that the activation of this metabolic pathway is an important event for plant recovery from leaf damage produced under HS conditions.

4. MATERIALS AND METHODS

4.1. Plant Materials and Growth Conditions

Commercial cultivars *Moneymaker* (MM) and *Ailsa craig* (Ailsa) were used as a reference and four traditional tomato varieties, originated from the Mediterranean area, TH-30 (Greece*); A*DX2 (Spain); ISR-10 (Israel); MO-10 (France) were selected for the study. Tomato varieties were obtained from the COMAV-UPV (Institute for the Preservation and Improvement of Valencian Agro-diversity, Universidad Politécnica de Valencia).

Tomato seeds were germinated in vermiculite in a growth chamber under the following environmental conditions: light/dark cycle of 16/8 h, temperature of 26/18 °C, light intensity of 200 µmol m^{-2} s^{-1}, and a relative humidity of 60%. The seeds were irrigated twice a week with distilled water. Ten days after germination and during the three next weeks, tomato genotypes were irrigated twice a week with Hoagland solution (Hoagland & Arnon, 1950) applied at the same proportion.

Four-week-old tomato plants were transferred to another growth chamber to apply HS. In order to confirm that all tomato plants started from the same substrate humidity, 24 hours before the HS, the pots were saturated with Hoagland solution and the excess was removed before the start of the heat shock process. To achieve HS, the temperature of the culture chamber was increased to 42ºC during 6 h and the recovery capacity was subsequently evaluated after 2 h under normal temperature conditions. The phenotype evaluation and the collection of plant material were carried out at 0, 2, 4 and 6 hours post heat shock (2, 4 and 6 hpHS), as well as two hours after the end of the stress in recovery phase (6+2 hpHS).

4.2. Visual damage

Four damage levels were established depending on the leaf roll level: *level 0* (healthy), *level 1* (minor roll), *level 2* (medium roll) and *level 3* (severe roll or dead).

The phenotype was determined by the percentage of leaflets of the third and fourth true leaf with symptoms in relation to the total number of analysed leaflets.

4.3. Relative water content (RWC)

Apical leaflet from the third fully expanded leaf of five plants per cultivar was cut from a plant at 0, 2, 4 6 and 6+2 hpHS. Fresh weight (FW) of the leaflet was immediately measured after cutting. Then, the leaflet was immersed in dd-H_2O and incubated overnight under normal room temperature. The leaflet was taken out, properly dried to remove the water drops from the surface of the leaf and weighed to obtain the turgid weight (TW). Immediately, the leaflet was put in a drying oven for 24 h and weighed to obtain dry weight (DW). Relative water content was calculated as (RWC in %) = [(FW - DW)/(TW - DW)] * 100.

4.4. Photosynthetic parameters

Determinations were carried out *in situ* on the apical part of leaves of the same age belonging to four-week-old tomato plants. During the gas exchange measurements, plants were maintained in the chamber where the HS was applied. The gas exchange analysis was carried out using a portable open system infrared gas analyser (LI-6800 portable photosynthesis system, LI-COR, USA) under ambient CO_2 and humidity. The parameters of interest were: The photosynthetic rate (A, µmol CO2 $m^{-2}s^{-1}$), transpiration rate (E, mol H_2O $m^{-2}s^{-1}$), stomatal conductance (gs, mol H_2O $m^{-2}s^{-1}$), Electron transport rate (ETR, µmol of electrons m^{-2} s^{-1}), quantum efficiency of photosystem II (Fv/Fm) and leaf temperature (Tleaf, ºC). The results were obtained by taking 3 measures per leaf on three different leaves. The experiment was repeated 3 times (n=27).

4.5. Gene Expression

Gene expression by qRT-PCR was performed on the RNA samples extracted from tomato leaves using the Total Quick RNA Cells and Tissues kit (E.Z.N.A. Mini kit; http://omegabiotek.com), according to the manufacturer's instructions. The tomato leaf samples for RNA isolation were collected at 0, 2, 4 6 and 6+2 hpHS.

Highly pure RNA was used for the RT reaction. The RT reaction was performed according to the manufacturer's instructions for the Omniscript Reverse Transcriptase kit (QIAGEN; http://www.qiagen.com/). The primers used for the qRT-PCR are listed in Table 1. As an internal housekeeping control, the expression of EF1α gene was used. At least three independent experiments were performed to confirm the results.

Table 1. Primers used for plant gene expression analyses.

Function	Gene	Primer
Heat shock protein 70	*HSP70*	F 5'- GCATTGCCGGATTAGATGTT -3' R 5'- CATCACCTCCCAAGTGTGTG -3'
Heat shock protein 90	*HSP90*	F 5'- CTTGGATTCGTGAAGGGTGT -3' R 5'- GCCCAGCTTCAAGTTCTTTG -3'
Respiratory burst oxidase B	*RbohB*	F 5'- AGGGAATGATAGAGCGTCG-3' R 5'- CATCGTCATTGGACTTGGC-3'
Dehydroascorbate reductase 1	*DHAR1*	F 5'- AGGTGGCTCTTGGACACTTC-3' R 5'- CTTCAGCCTTGGTTTTCTGG-3'
Heat shock transcription factor A2	*HsFA2*	F 5'- GATCTGGTGCTTGCATTGAA-3' R 5'- TGGGGGTCATCGTTAGTCTC-3'
Catalase	*CAT*	F 5'- TGCATTGAAACCAAATCCAA-3' R 5'- TGTGCTTTCCCCTCTTTGTT-3'
Superoxide dismutase 1	*SODct*	F 5'- GGAAAGGGAGGACATGAGCT-3' R 5'- ACCCCAATTCAAAAGGCGTC-3'
Phenylalanine ammonia-lyase 1	*PAL*	F 5'- CAAGAATTAGATGCCTTAACCAA-3 R 5'- ACTATTCAAAAGGTCCATCAGTTT-3'
Acetylserotonin O-methyltransferase	*ASMT*	F 5'- GCATGGCTGCACTTGTCTTA -3' R 5'- ATGCTCCGGATTGATTTTTG -3'
Elongation factor 1-alpha	*EF1α*	F 5'- GACAGGCGTTCAGGTAAGGA-3' F 5'- GGGTATTCAGCAAAGGTCTC-3

4.5. Chromatographic Analysis

For the hormonal analysis, fresh material was frozen in liquid N, ground and freeze-dried. Fresh tissue (0.5 g) was immediately homogenized in 2.5 mL of ultrapure water, and 100 ng mL^{-1} of a mixture of internal standards [(2H6-ABA (to quantify ABA), 2H4-SA (to quantify SA and propylparaben (to quantify phenolic compounds like ferulic acid (FA) and chlorogenic acid (CGA)), (Sigma–Aldrich)] were added prior to extraction. The samples were centrifuged at 5,000 rpm for 45 min at 4 °C. The supernatant was partitioned against diethylether, dried in a speed vacuum and resuspended in 90:10 H_2O:MeOH (Scalschi et al., 2018). After extraction, a 20 µL aliquot was injected directly into an ultra-high performance liquid chromatography (UPLC) system with an ACQUITY UPLC BEH C18 column (1.7 µm 2.1 × 50 mm) (Waters, Mildford, MA, United States), which was interfaced with a triple quadrupole mass spectrometer (TQD, Waters, Manchester, United Kingdom). Version 4.1 of the MASSLYNX NT software (Micromass) was used to process the quantitative data from the calibration standards and plant samples. The concentrations of hormones and phenolic compounds were determined in each sample by normalizing the chromatographic area for each compound with the fresh weight of the corresponding sample.

4.6. Statistical analysis

Statistical analysis was carried out by one-way ANOVA using the Statgraphics plus software for Windows, V.5 (Statistical Graphycs Corp., Maryland, USA). The means were expressed with a standard error (SE). The comparison was carried out using Fisher's Least Significant Difference (LSD) at 95%. Differences were taken into account only when there were significant at the 5% level. Each experiment was repeated at least three times.

5. CONCLUSION

As a result of climate change, crops are predicted to be exposed to high temperatures more frequently. Hence, it is essential to explore how tomato

genotypes respond to high temperatures to select those that will be better suited for future climate change. In this way, the use of traditional varieties could be a promising approach to understand the complex response to heat stress. After the screening of the response of several tomato landraces against heat stress, we identify that their thermotolerance strategies are very different. MO-10 plants could maintain the photosynthetic parameters without variations during the early stages of HS, which kept the leaf Tº within an optimal range for normal functions without damage on the photosystems. Moreover, we observed an early activation of H_2O_2 and ABA-related signalling pathways together with an induction of SOD levels to control the oxidative burst. However, in MM plants, a sudden and strong reduction of photosynthetic parameters was observed at 2 hpHS, triggering leaf temperature increase and producing severe damage on the photosystems. On the other hand, the synergic activation of *HSP90* and *HSP70* genes, as well as *PAL* induction probably boosted the overcoming stress, showing a recovery of E and g_s parameters that participate in transpirational cooling at 4 hpHS, thus reducing leaf temperature. In this case, to control the HS-mediated oxidative burst, MM plants synthesize FA and induced the synthesis of *CAT*. Moreover, in this work it has been demonstrated that SA-dependent pathways, as well as the synthesis of phenolic compounds and melatonin, are key points in the recovery processes after severe heat stress. Therefore, the characterization of traditional and commercial varieties against HS allows to find heat tolerant genotypes of tomato for their introduction into breeding programs. Moreover, understanding the molecular events related to thermotolerance as well as the mechanisms responsible for cell recovery after stress are key to generate more resistant and resilient crops to extreme environmental episodes.

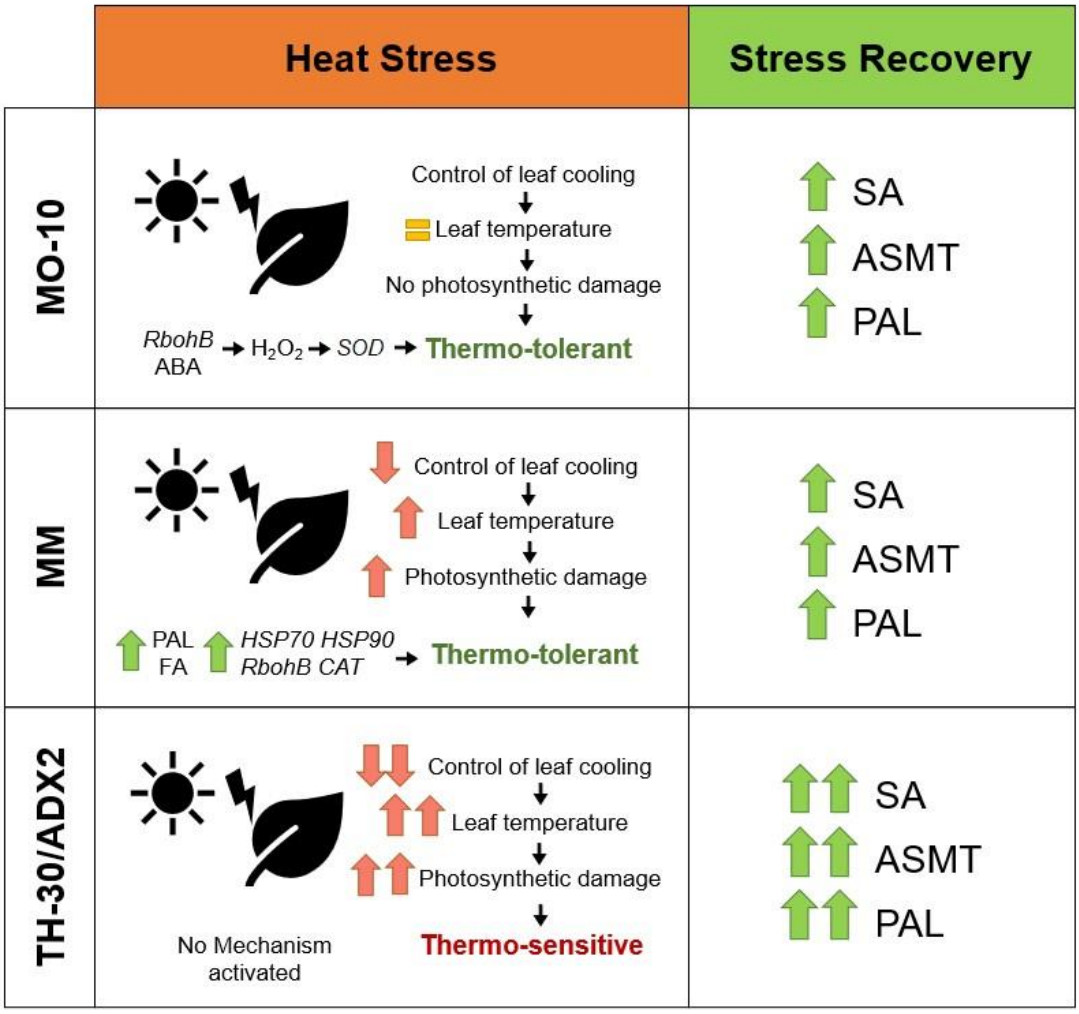


Figure 9. Comparative diagram between the mechanisms activated in MM and MO-10 (thermotolerant genotypes), and TH-30 and ADX2 (thermo-sensitive genotypes) after heat stress.

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CHAPTER 3

Exploring the impact of plant genotype and fungicide treatment on endophytic communities in tomato stems

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ABSTRACT

Plant microbiome composition and structure are reported to be affected by several biotic and abiotic factors, including plant genotype and human manipulation. Given the critical role that the microbiome plays in plant behavior, especially under stress conditions, understanding these influences could be of extreme relevance. In this study, we used Illumina-based sequencing of the 16S and ITS2 regions of rRNA genes to examine the stem microbiome of six tomato (*S. lycopersicum*) genotypes. Identified taxa of endophytes were compared based on the traditional or commercial origin of the tomato genotype. Moreover, we evaluated the impact of a generic fungicide on the tomato microbial communities. Our findings indicate that the microbiome of traditional genotypes harbors significantly more unique taxa and a broader phylogenetic background than commercial genotypes. Additionally, our data analysis revealed that fungicide treatment alters the tomato microbiome by shifting both fungal and bacterial communities. Our study provides evidence of the importance of considering the impact of plant genotype and fungicide use on the plant microbiome. The findings highlight the potential benefits of using traditional genotypes and avoiding fungicides as a rich source of potential beneficial microbiota for enhancing plant health and productivity.

KEYWORDS: *S. lycopersicum*, tomato, traditional genotypes, commercial genotypes, microbiome, fungal communities, bacterial communities, fungicides.

1. INTRODUCTION

Plants harbor communities of microorganisms on and inside their tissues, and the spectrum of relationships with their host can vary from mutualism to commensalism and parasitism (Schlaeppi & Bulgarelli, 2015). The assemblage of microorganisms present in a defined environment or organism is known as the "microbiota" and their study is named under the term "microbiome". Recently, a broader concept of the microbiome encompasses the microbiota as well as the interactions that occur amongst them and with the host. Plant microbiome, or phytobiome, plays a key role in the performance and behavior of the host plant; and its status and diversity vary upon several factors like environmental or host conditions. In this regard, some authors prefer to address the whole concept as the plant holobiont (Vandenkoornhuyse et al., 2015).

The microbiota of any given plant comprises microorganisms from all ranges: beneficial to pathogenic. Nevertheless, it has been demonstrated that plant-associated microbes are usually neutral or beneficial, capable of inducing resistance against several stresses or promoting plant growth (Gouda et al., 2018; Pieterse et al., 2014; Richardson & Simpson, 2011). These microorganisms can improve plant performance through direct or indirect mechanisms. Direct mechanisms include improving the availability and uptake of essential nutrients (Backer et al., 2018), enhancing resistance to biotic stresses by releasing hydrolytic enzymes, antibiotic compounds, or direct competition with pathogenic microorganisms (Trivedi et al., 2016), and mitigating the effects of abiotic stresses such as drought or salinity (Rodriguez & Redman, 2008). On the other hand, the presence of beneficial microorganisms could provide indirect benefits through the enhancement of innate plant defensive responses (Gautam & Avasthi, 2019). Hence, there is an emerging interest to ascertain both the composition of microbial communities and their interactions with the host plant. In this way, understanding the potential effects provided by the presence of certain beneficial microorganisms could represent a means to increase plant fitness under harsh environmental conditions (Ravanbakhsh et al., 2019). The effects on plants include essential functions such as nutrient uptake, flowering time, and biotic and

abiotic protection (Berg et al., 2017; Busby et al., 2017). Due to the relevance of these functions in plant performance, it seems feasible that hosts have developed mechanisms to attract, select and maintain those microbial populations that based on their induction, stimulation, or inhibitory activities could play a role in plant development (Vorholt et al., 2017). Moreover, the plant microbiome is reported to be a source of several secondary metabolites with great bioactivity potential (Aghdam & Brown, 2021). Ultimately, all effects of plant microbiota could be caused by a microorganism, a community, or several of them interacting with each other, therefore the importance of having an exhaustive view of the fungal and bacterial communities within a plant.

Current plant microbiome studies are focused on the establishment of host-associated microbial communities, their structure, and how this microbiome is affecting the host phenotype under certain conditions. This research field has great potential since it could lead to the design of more sustainable agronomic practices based on plant-microbe interactions.

Microbiome composition can be commonly divided into core and satellite microbiome. Core microbiome is usually referred to as the microbial communities that are deeply related to the plant, playing an essential role in the plant holobiont by their presumable association through evolutionary selection. Though other methods are currently being introduced to evaluate core microbiome, the taxonomic approach still prevails as the main tool to learn about microbiome composition (Neu et al., 2021). Core microbiome is therefore determined by the common taxa in samples of the same environment or group depending on the report. In the present study, it is referred to as the common taxa among the different genotypes. In turn, the satellite microbiome is comprised of microorganisms that are present in fewer amounts and are mainly related to the environment or specific habitat (Magurran & Henderson, 2003). These microorganisms, despite being less present, could be responsible for enhancing the traits that lead a plant to adapt to adverse environments. Hence, the loss of these satellite microorganisms could impair plant growth and productivity (Gera Hol et al., 2015).

The composition of the microbiome can also be altered due to other factors besides environmental conditions. The development of modern agriculture was based on the employment of new and more productive varieties that require fewer inputs than traditional cultivars. It has been previously hypothesized that the pressure for the domestication of the plants might impact the symbiotic organisms that are associated with certain crops (Pérez-Jaramillo et al., 2017; Zachow et al., 2014; Zheng et al., 2020). Thus, the human control and manipulation of plant material and growth conditions could have determined the presence or absence of certain microorganisms that would differ in natural conditions. For this reason, there is a possibility of wild plant species and traditional cultivars holding a broader range of microorganisms compared to commercial cultivars.

Similarly, current agronomic practices often include pesticide treatments in order to avoid losses in productivity due to pest attacks. These treatments often include chemicals that may act locally or systemically while affecting all the microorganisms that are in contact with the plant. Despite the fact that these practices are routinely used in common agriculture, their impact on the microbiome of plants and, therefore the impact on the plant performance, has been poorly studied.

In the current work, tomato (*Solanum lycopersicum*) was studied due to its significant relevance as a crop species and we attempted to discern the influence of genotype on the microbiome, as well as the impact of a generic agronomic treatment. This was aimed to understand how microbiome composition and structure can differ and be altered by plant genotype and human actions. For this purpose, several varieties were selected, including both traditional and commercial genotypes. We analyzed the stem microbiome of these genotypes grown under the same controlled conditions. This was done to minimize the presence of the microbial communities that were bound to their original environment and likely transmitted to the plant from the soil and air. We hypothesized that traditional tomato plants, which have been exposed to less selection or manipulation, would hold a richer microbiome than their cultivated counterparts. Likewise, non-treated tomato plants were expected to comprise a

more diverse microbiome than fungicide-treated plants. The abundance and diversity of identified taxa for fungal and bacterial communities would prove helpful to study the similarities and differences between each microbiome and imply the connection and effects between plant behavior and human manipulation.

2. MATERIALS AND METHODS

2.1. Plant material

Six tomato plant (*Solanum lycopersicum* Mill.) genotypes from the Mediterranean area were used in the current study. As traditional genotypes, varieties from Spain (ADX2), Greece (TH-30), Israel (ISR-10) and France (MO-10) were used. As commercial cultivars, Moneymaker (MM) and Ailsa Craig (AIL) cultivars were selected.

The seeds that were used to produce the plant material for this study were obtained from the Institute for the Conservation and Improvement of the Valencian Agrodiversity (COMAV), Polytechnic University of Valencia, Spain. The seeds were stored in aseptic conditions and germinated in individual pots with 12g of vermiculite as substrate, under controlled conditions in a growth chamber (16h photoperiod, 26ºC:17ºC day:night, 80% humidity). These steps were taken to restrict the environmental factors that would affect the tomato microbiome in more open fields. Plantlets were watered twice a week with Hoagland nutritive solution for 4 weeks, until they reached the 5th leaf stage. At that point, fungicide treatment was applied to two traditional varieties (ADX2, TH-30) and two commercial ones (AIL, MM). For this part, only these genotypes were selected to allow a comparison of the effects among similar-sized plants. Five plants of each genotype were randomly selected and treated with combined systemic fungicides (Tebuconazole 10% w/w + dichlofluanide 40% w/w) as recommended by the manufacturer (Folicur, Bayer) while the rest were mock-treated with distilled water. All plant material was collected 48 hours after fungicide treatment when visual differences for treated plants appeared.

Sample preparation was done following the same methodological structure previously described by Sun *et al.* (2020). Briefly, plants had their stems cut, and 1cm long stem segments were surface-sterilized by submerging them in a 4% bleach solution for 1 minute, 70% ethanol for 3 minutes and rinsed with sterilized distilled water.

Afterwards, 200mg of the sample comprised of pieces from each internodal segment were placed in Eppendorf tubes while another 150mg of sample was reserved in Eppendorf tubes and stored in a freezer at -80ºC. The 200mg samples were used for DNA extraction, which followed the CTAB method (Tamari et al., 2013) using DMSO to enhance strand separation. ITS2c and ITS86 primers (Op De Beeck et al., 2014), which melting point is 60ºC, were used for fungal DNA amplification. For bacterial identification, 16S rDNA primers 515FB and 926R (W. Walters et al., 2016) targeting the region V4-V5b were used, with a melting point of 62ºC. Furthermore, agarose gel electrophoresis was performed to check DNA integrity. For comparisons between commercial and traditional varieties, 10 plants were used whereas comparisons between fungicide and control plants were performed with 5 plants per variety.

2.2. Amplicon sequencing

To perform sequencing of amplicons, samples were sent to the Integrated Microbiome Resource (IMR) at Morgan Langille Lab from the Department of Pharmacology in Dalhousie University (Canada). The protocols for the amplicon sequencing, barcoding adaptors and PCR conditions were described previously by Comeau *e*t al. (2017). Amplicon fragments were PCR-amplified from the DNA in duplicate using separate template dilutions (1:1 & 1:10) using the Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific™). A single round of PCR was done using fusion primers that include Illumina adaptors, indices and specific regions, targeting the ITS2 region for fungi analysis and the V4-V5 region of 16S for bacterial analysis. The PCR reactions from the same samples were pooled in one plate, then cleaned up and normalized using the high-throughput Just-a-Plate 96-well Normalization Kit (Charm Biotech™). All samples were then pooled to

make one library which was quantified fluorometrically before sequencing. Amplicon samples were run on Illumina MiSeq using 300+300 bp paired-end V3 chemistry which allows overlap and stitching together of paired amplicon reads into one full-length read of higher quality.

2.3. Bioinformatics analysis

Forward and reverse reads were imported and demultiplexed using the QIIME2 platform (version 2020.2; https://qiime2.org). Sequence quality control and feature table construction were performed using *Deblur workflow*, applying a trimming value of 250 as described by Amir *et al.* (2017). Bacterial samples were analyzed under *Deblur denoise-16S* build-in protocol, whereas quality control and construction for ITS were performed with UNITE Version 8.2 (https://unite.ut.ee/) as reference. The taxonomic composition of the samples was assessed with the classifier feature *classify-consensus-blast* using UNITE version 8.2 as a reference database for ITS and *Greengenes 13_8 99%* operational taxonomic units (OTUs) for 16S with a resolution of 99% (McDonald et al., 2012). Since the DNA was extracted from plant tissue, the presence of plant mitochondria and chloroplast might arise. Hence, a taxa filtration to exclude these terms was performed. For diversity analysis, the table of frequencies was rarefied (subsampled) according to the values obtained from deblur workflow for ITS and 16S respectively. Afterwards, the obtained results were subject to statistical analysis performed by different software packages. Alpha diversity metrics were achieved through the QIIME2 package. The resulting artifacts were imported to R statistical software (R version 4.0.2) using qiime2R package. Principal Coordinates Analysis (PCoA), taxabars, hieratical clusters, and diversity plots were executed with *Microbiotaprocess* ver. 1.2.2 and Vegan ver. 2.6-2 packages. Shannon graphs were made using Tidyverse package ver. 1.3.1. The abundance of taxa was calculated using Phyloseq package ver. 1.34.0 and phylloclades were calculated with coin ver. 1.4-1package.

3. RESULTS

3.1. Distribution and overall comparison of present microbial communities

A total sum of 3,823,640 and 2,709,671 reads were retrieved for ITS and 16S respectively from the analyzed samples of tomato varieties under normal conditions (non-treated) after the filtering for mitochondria and chloroplast. From those, 237 OTUs belong to fungi, from which 232 were successfully aligned to the UNITE reference database. From the 179 OTUs that belonged to bacteria only 57 were successfully aligned. Venn diagrams were made with this data to compare overall taxa between the traditional and commercial varieties of tomato (Fig. 1a) and between each of the traditional ones (Fig. 1b).

From Fig. 1a, it can be observed that traditional tomato plants held 47 unique taxa (sup. table 1) while commercial tomato contained only one. Incidentally, this unique taxon was retrieved as an uncultured Ascomycota. Bacterial communities followed the same pattern with 21 unique taxa in traditional genotypes (sup. table 2) and only one in commercial ones. The unique taxon from commercial genotypes was assigned as an uncultured Rhodobacteria.

The variation among traditional cultivars is described in Fig. 1b. For fungal taxa, ADX2 held the highest number (188) followed by TH-30 (165), MO-10 (163), and ISR-10 (155). Traditional genotypes shared a common taxa core with a sum of 86 taxa which represented around 50% of the fungal communities. Unique taxa were distributed fairly with similar abundance in ADX2 (10), ISR-10 (8), and MO-10 (8) but fewer in TH-30 (3). Opposed to what was observed in fungal endophytic communities (FECs), bacterial endophytic communities (BECs) showed that there were only 11 common taxa among the traditional varieties. This represented nearly 25% of BECs for TH-30, ADX2 and MO-10; while it was close to 40% for ISR-10. This followed the same trend of the total number of bacterial taxa which was highest for TH-30 (47) followed by ADX2 (43), MO-10 (41) and finally ISR-10

(29). It is noteworthy to observe that, in addition to the common taxa, ADX2, MO-10, and TH-30 shared 15 taxa that were absent in ISR-10.

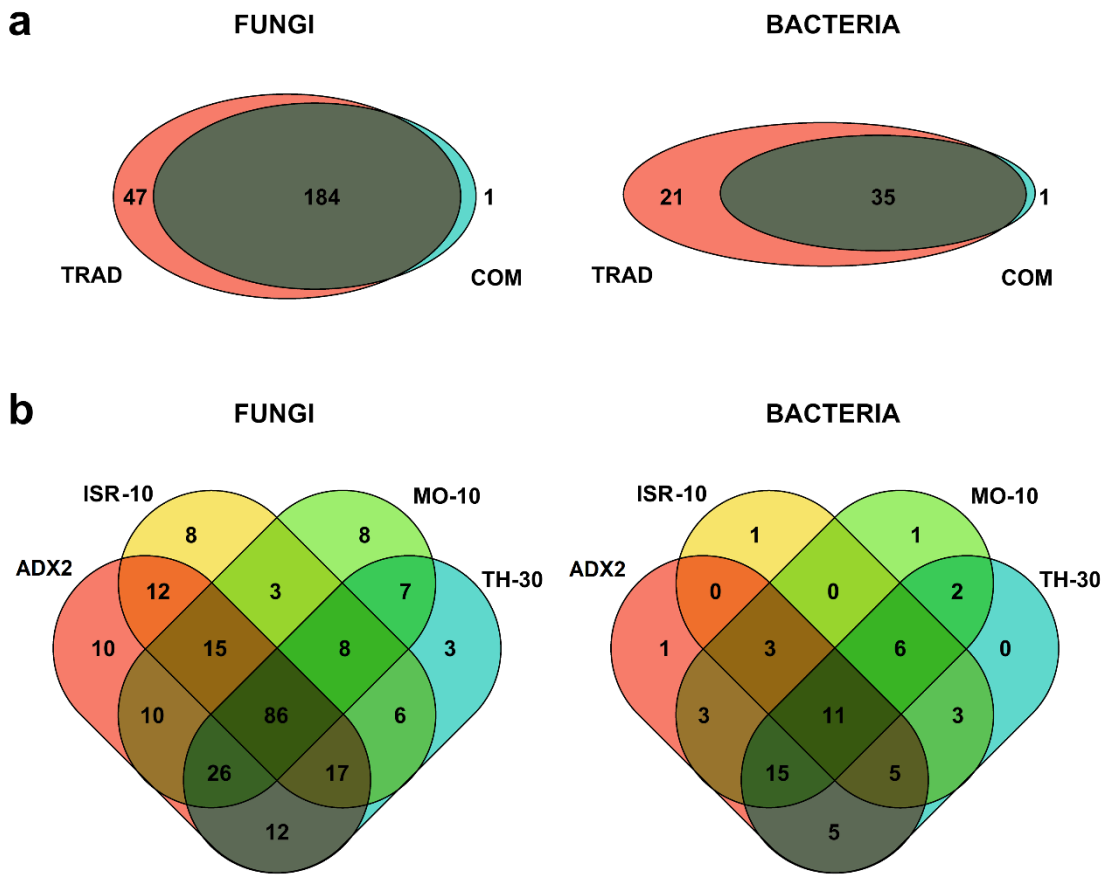

Figure 1. Venn Diagrams of the microbial operational taxonomic units (OTUs) of four traditional varieties (ADX2, TH-30, ISR-10, MO-10) and two commercial cultivars (AIL, MM) of *S. lycopersicum*. Ellipsoidal areas represent the different varieties, with shared OTUs among varieties represented as overlapped areas. (**a**) Overall comparison between traditional and commercial varieties' OTUs for fungal endophyte communities (FECs) and bacterial endophyte communities (BECs). (**b**) Unique and shared OTUs between traditional varieties for fungal and bacterial taxa.

Overall alpha-diversity was represented in rarefaction curves (Fig. 2a, 2c). TH-30 genotype showed the highest values of diversity along with ADX2 for both fungi and bacteria. Commercial AIL reflected similar alpha diversity with ISR-10. The study's sequence depth was sufficient to reach sample saturation. Incidentally, traditional genotypes showed higher bacterial diversity than commercial genotypes and might need more depth of study to fully represent their bacterial communities. PCoA plots (Fig. 2b) followed by Permutational Multivariate Analysis

of Variance (PERMANOVA) supported existent differences between the fungal communities of the traditional and commercial genotypes for fungal communities and for bacterial communities ($p<0.01$).

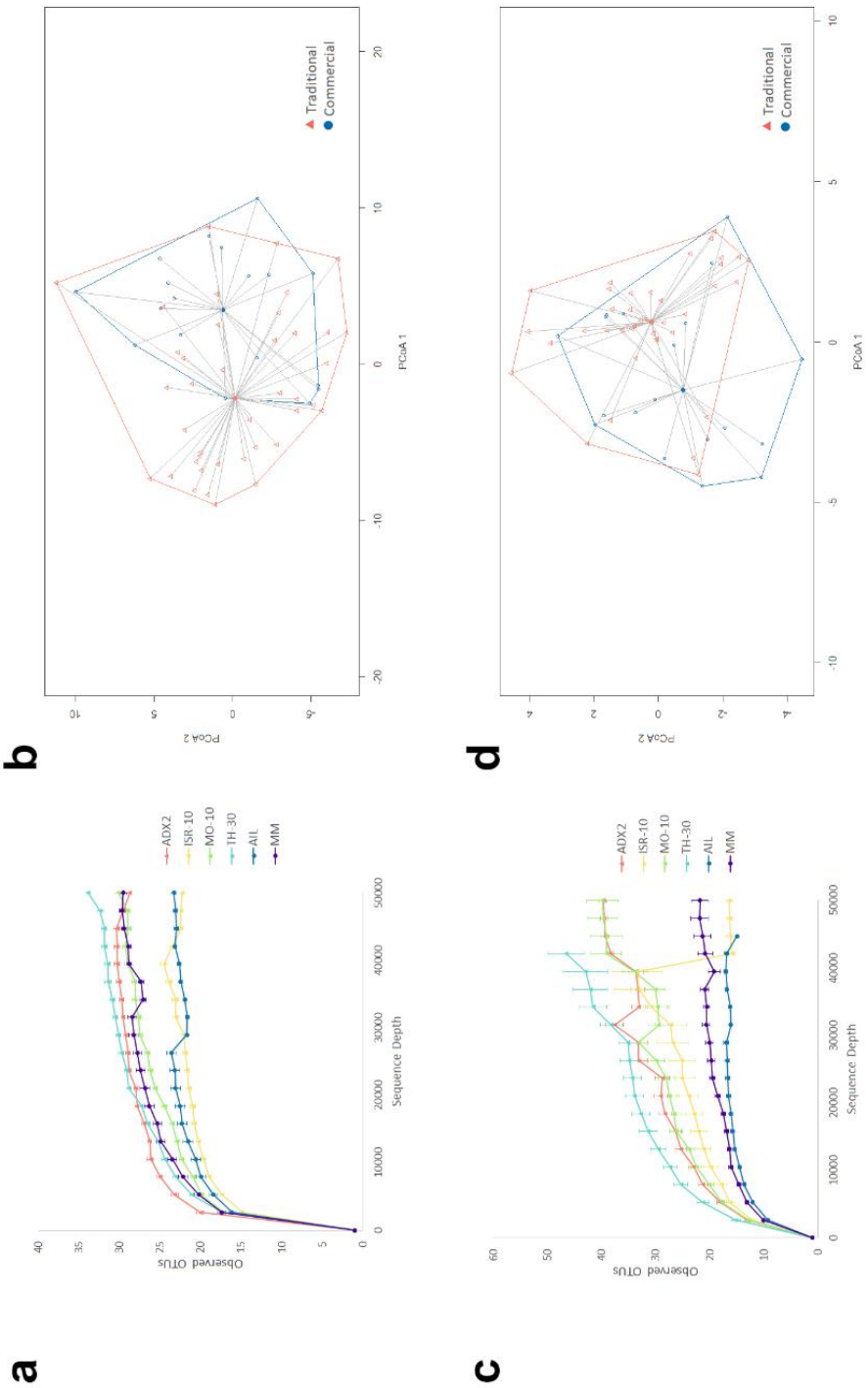

Figure 2. Diversity of fungal (**a, b**) and bacterial (**c, d**) samples according to genotype or genotype origin. (**a, c**) Alpha-diversity of microbial communities for each tomato genotype shown by rarefaction curves based on number of observed OTUs. (**b, d**) Principal Coordinates Analysis (PCoA) plot of microbial communities between traditional and commercial genotypes, with distance matrices based on the Bray-Curtis dissimilarity index and highlighting the clustering of samples according to genotype origin.

3.2. Variation of FECs and BECs among different varieties of tomato

The composition of microbiota was studied and reflected in bar plot graphics (Fig. 3). The difference in abundance was visible between traditional and commercial varieties (Fig. 3a, 3c). Traditional tomato, especially ADX2 and TH-30, harbored more fungal and bacterial communities than commercial genotypes which was more than twice than AIL, which was was statistically significant at $p<0.05$ (sup. table 3).

The most abundant fungal division by far is Ascomycota (Fig. 3a-b). The main fungal communities belonged to the Sordariomycetes, Leotiomycetes and Dothideomycetes classes which constituted more than two thirds of all fungal taxa. Fungal abundance found in commercial genotypes seemed to mainly suffer from a reduction of ascomycetous communities, especially its predominant class Sordariomycetes. Basidiomycota and Mortierellomycota were also apparently abundant though found at a much lower level than Ascomycota. Other classes including Chytridiomycota and Mucoromycota exhibited a relatively low prevalence.

While ascomycetes represented the main FECs, BECs maintained a more balanced diversity (Fig. 3c-d). The bacterial order Flavobacteriales followed by Rhizobiales and Pseudomonadales covered between 50% and 75% of the endophytic microbiota. Bacterial microbiota was also fairly contributed by non-assigned bacterial taxa (NA). Among identified taxa, Flavobacteriales was the most abundant bacterial order across all genotypes. Along with the Sphingobacteriales and Chitinophagales, these communities bring Bacteroidia to be the predominant bacterial class. The distribution of the most dominant classes Alphaproteobacteria, Bacteroidia, and Gammaproteobacteria was similar in the four traditional varieties. Bacteroidia in TH-30 was by far the most abundant bacterial class seen in our study which covered twice as much of each class for the other varieties. Samples of AIL genotype showed the least abundance of bacterial communities and most of the bacterial taxa were not assigned to any

order. In this case, Aphaproteobacteria was the main identified taxa and showed an interesting structure. Unlike the communities found in the other genotypes, Rhodobacterales was surprisingly abundant and Rhizobiales was almost absent in comparison.

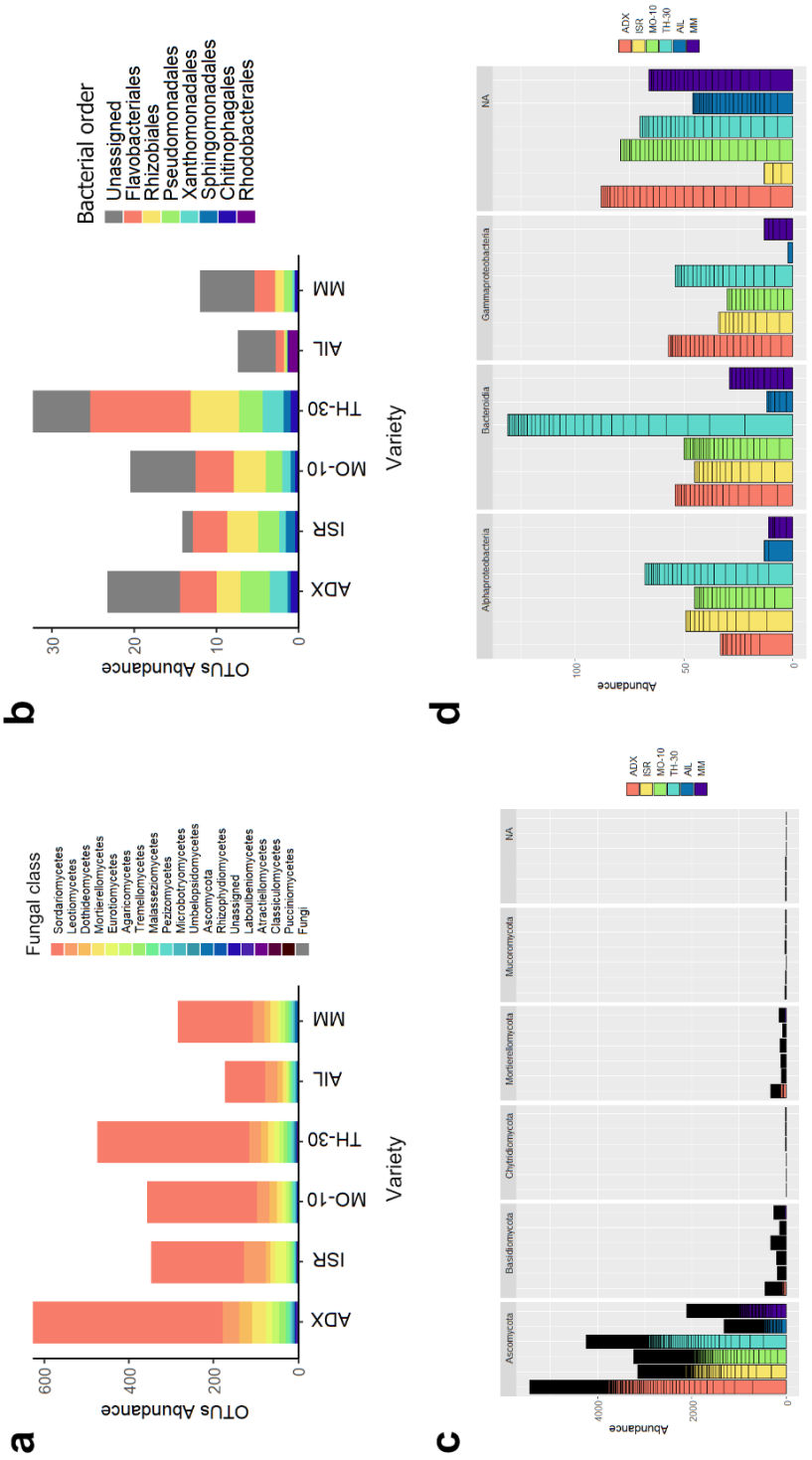

Figure 3. Abundance and structure of FECs and BECs within host for each studied tomato variety. (**a**) Taxonomic composition and structure of FECs expressed at class rank. (**b**) Taxonomic composition and structure of BECs expressed at order rank. (**c**) Distribution and abundance of main taxonomic phyla in FECs: *Ascomycota, Basidiomycota, Chytridiomycota, Mortierellomycota* and *Mucoromycota*. (**d**) Distribution and abundance of main taxonomic classes in BECs: *Alphaproteobacteria, Bacteroidia* and *Gammaproteobacteria*.

The microbiome structure of the studied plants was analyzed with a distinction between genotypes. The hierarchical cluster analysis (HCA) of each sample's OTUs for FECs and BECs (Fig. 4a, 4c) seemed to indicate a distinction between traditional and commercial varieties which was more remarkable for fungal clusters. Phylogenetic dendrograms also revealed that traditional varieties are significantly richer in fungi from Hypocreales and Chaetothyriales orders (Fig.4b). Among them, *Fusarium* (Nectriaceae) and *Exophalia* (Herpotrichiellaceae) were significantly present in the fungal structure of traditional plants. Conversely, only Lasiosphaeriaceae was significantly present in commercial varieties. The bacterial dendrogram was less complex (Fig.4d), yet differences were shown in bacteria composition at phylum and class levels. Traditional varieties showed a high presence of Gammaproteobacteria whereas unassigned OTUs stood out in commercial varieties.

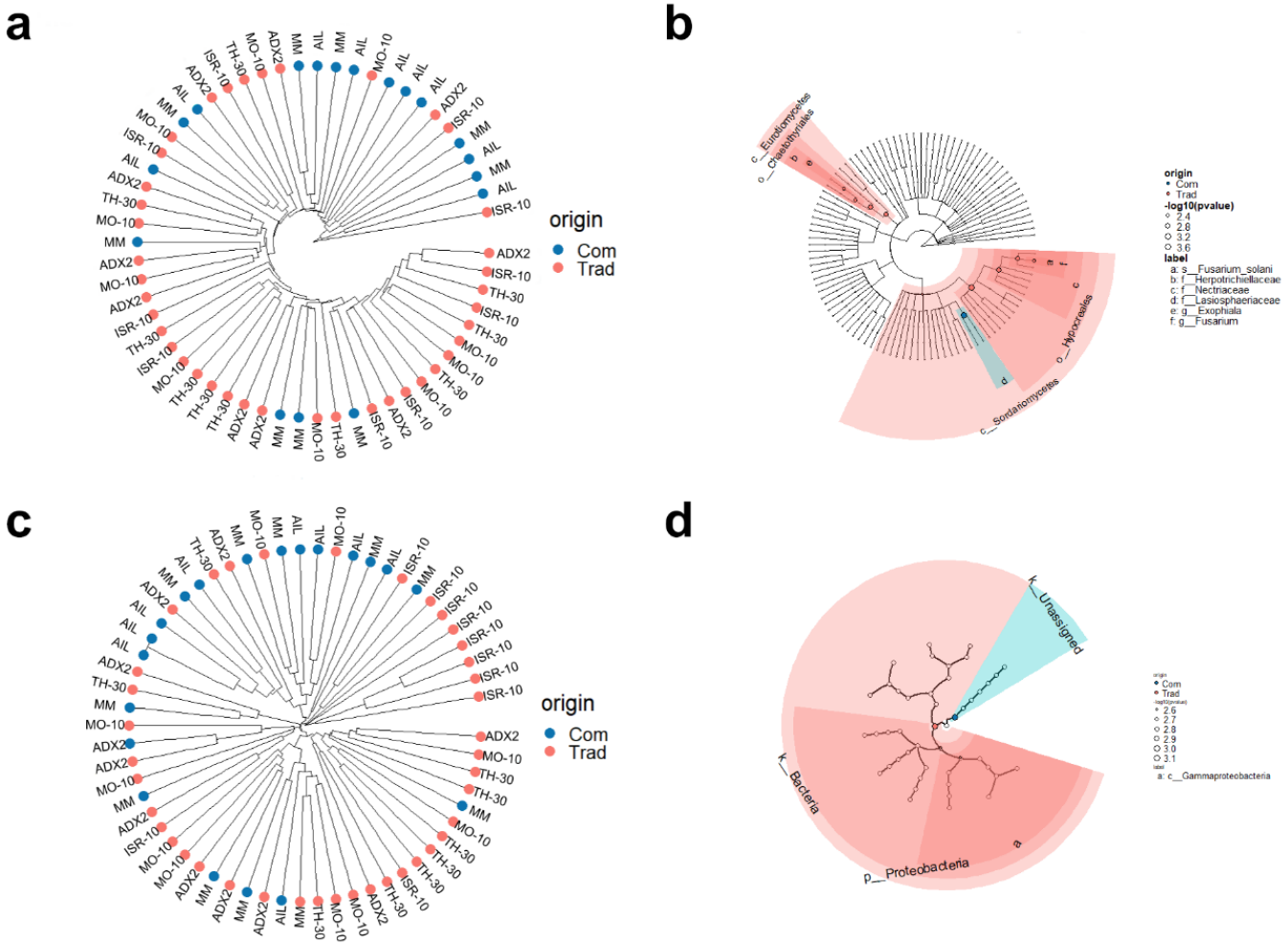

Figure 4. Dendrogram layout for microbiome in *S. lycopersicum*, both traditional and commercial varieties. Euclidean hierarchical cluster of all fungal (**a**) and bacterial (**c**) OTUs. Phylogenetic cladograms of fungal (**b**) and bacterial (**d**) OTUs, with highlighted area representing greater density for OTUs from a particular tomato origin (commercial or traditional).

3.3. Microbial diversity under fungicide treatment

The plant's microbiome can be significantly impacted by the application of fungicides. In this study the use of dichlofluanid and tebuconazole resulted in a shift in the microbial populations compared to control plants (Fig. 5).

The relative abundance of microbial communities suggested changes in the microbiome caused by fungicide treatment (Fig. 5a, 5c). It can be observed that the abundance of the different fungal phyla remained similar in treated and untreated plants (Fig. 5a), indicating that there might not be a change in the number of fungal endophytes but in their composition. Ascomycota remained as the most abundant fungal division, though Leotiomycetes and Dothideomycetes in treated plants seemed to be reduced in favor of Sordariomycetes which gained relative abundance. In contrast, results for bacteria manifested a seeming decrease in the relative abundance of unassigned OTUs from nearly 50% in control plants to almost 25% in fungicide-treated plants (Fig. 5c). Consequently, this reduction led to a corresponding increase in the relative abundance of other major taxonomic orders.

Differences in the microbiome with and without fungicide treatment were also visible when presenting fungal taxa in a cladogram (Fig. 5b, 5d). Dothideomycetes stood out in control plants while Sordariomycetes was the main class in treated plants. FECs were more diverse in control plants with main OTUs assigned to the order of Pleosporales, Helotiales, and Glomerellales. FECs in treated plants were mainly comprised of Sordariomycetes, particularly *Fusarium spp*. In contrast, bacterial microbiota highlighted the density of bacterial OTUs in treated plants.

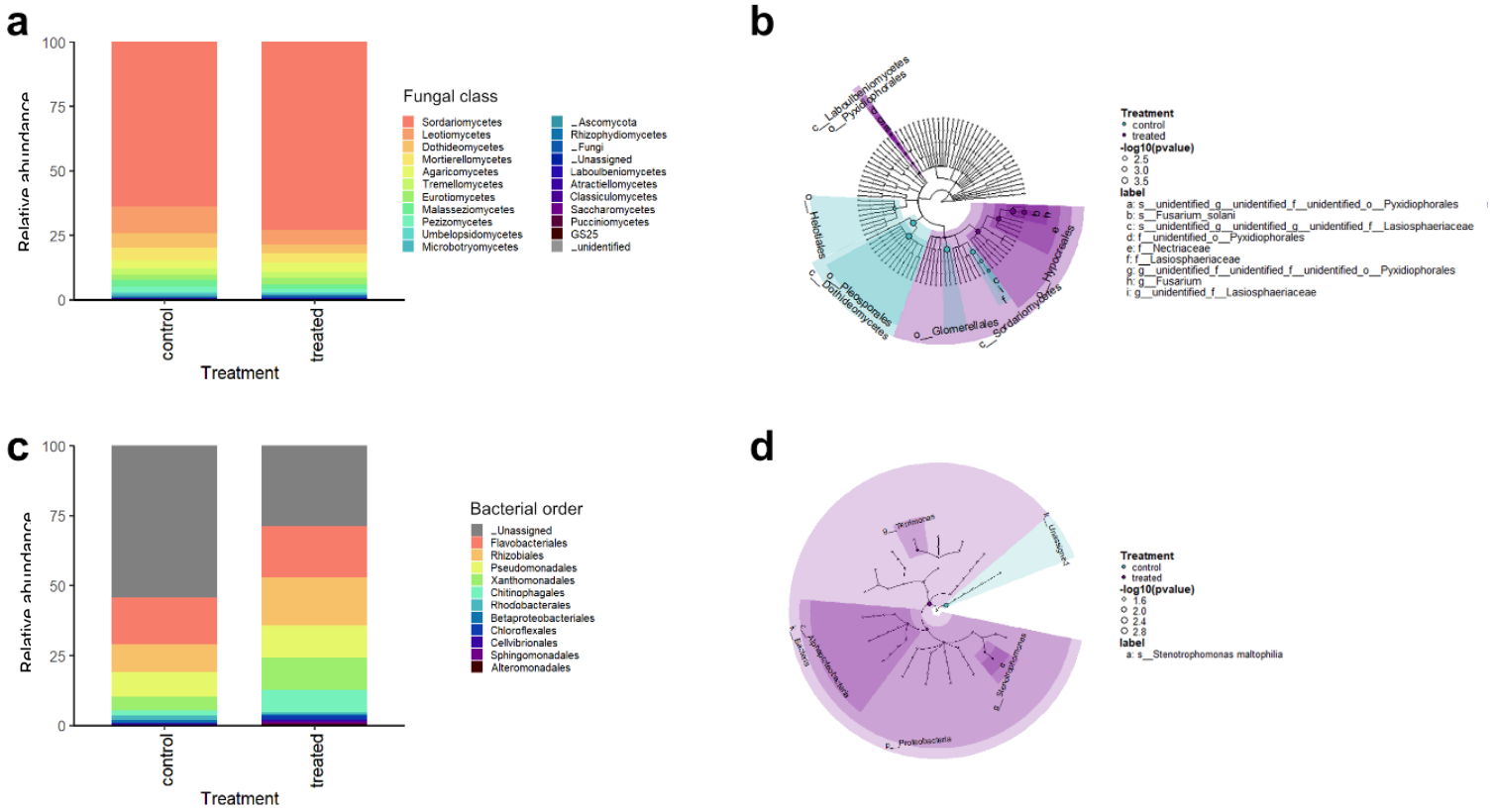

Figure 5. Relative abundance of FECs and BECs within host for control (untreated) and fungicide treated S. lycopersicum, based on 2 traditional varieties (ADX2, TH-30) and 2 commercial cultivars (AIL, MM). (**a**) Taxonomic composition and structure of FECs expressed at class rank. (**b**) Phylogenetic cladogram of fungal OTUs from fungicide treated or non-treated samples. Highlighted area represents greater density for OTUs from one of the conditions. (**c**) Taxonomic composition and structure of BECs expressed at order rank. (**d**) Phylogenetic cladogram of bacterial OTUs from fungicide treated or non-treated samples. Highlighted area represents greater density for OTUs from one of the conditions.

To ascertain the effect of the fungicide in the microbiome, several diversity indexes were calculated (Fig. 6). This revealed treated plants had a significantly smaller fungal diversity according to Shannon, Simpson and J test. On the other hand, fungicide treatment did not show a direct impact on bacterial diversity. Shannon Index's results for each variety were specified in bar plots (Fig. 7). As seen by the sample dispersion, the effect of the fungicide was dependent on tomato genotype. Traditional TH-30 suffered the highest drop in fungal diversity supported by a diminished sample dispersion. Overall, ADX2 and TH-30 had lower sample dispersion when treated while dispersion did not change for commercial genotypes. This was seen in both fungal and bacterial communities regardless of the impact on the overall richness.

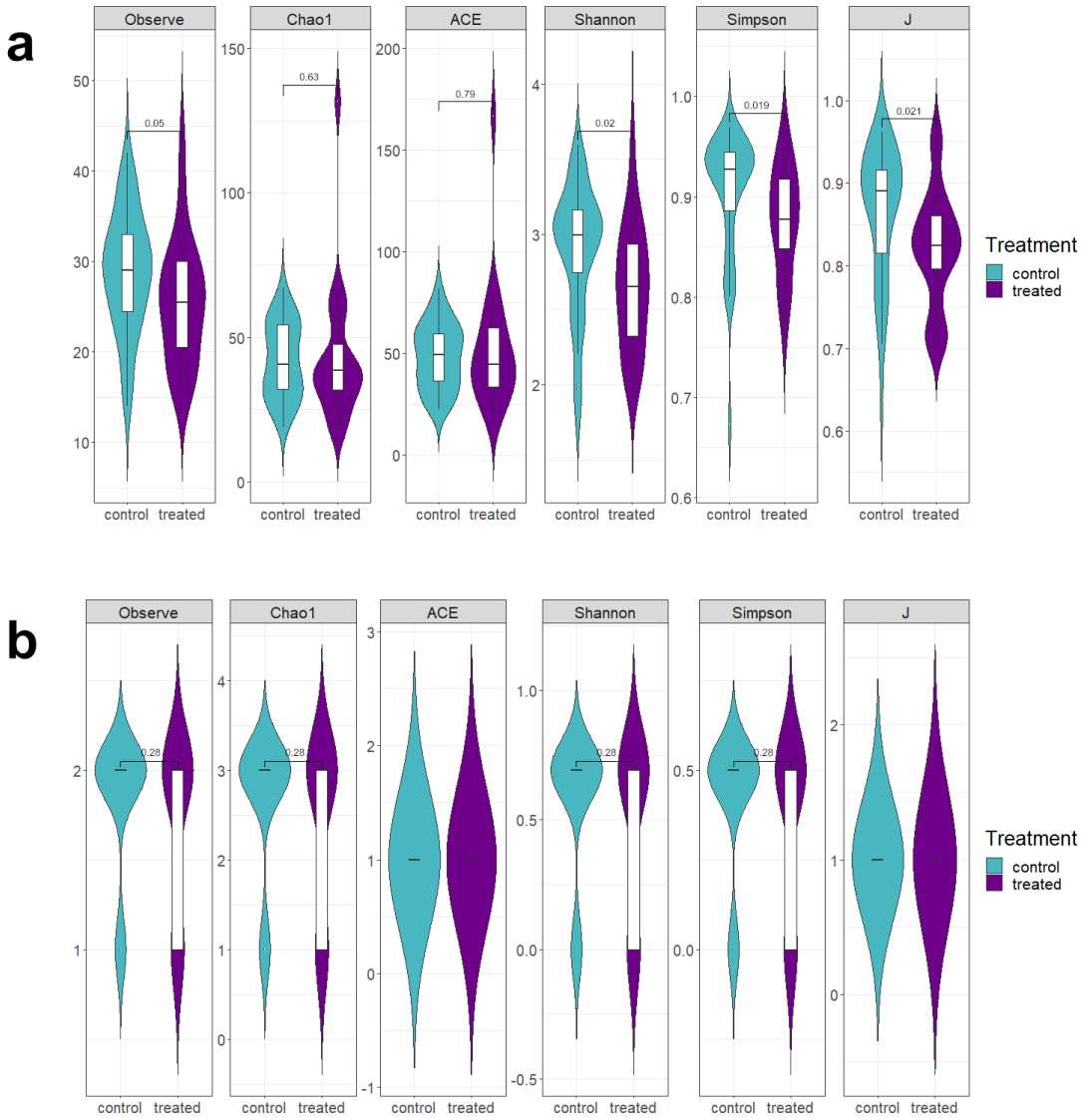

Figure 6. Estimated richness of control versus fungicide treated *S. lycopersicum* by several diversity test for (**a**) fungal communities and (**b**) bacterial communities. Calculated indexes correspond to tests of Observed diversity, Chao1 and ACE richness, Shannon and Simpson diversity and J evenness.

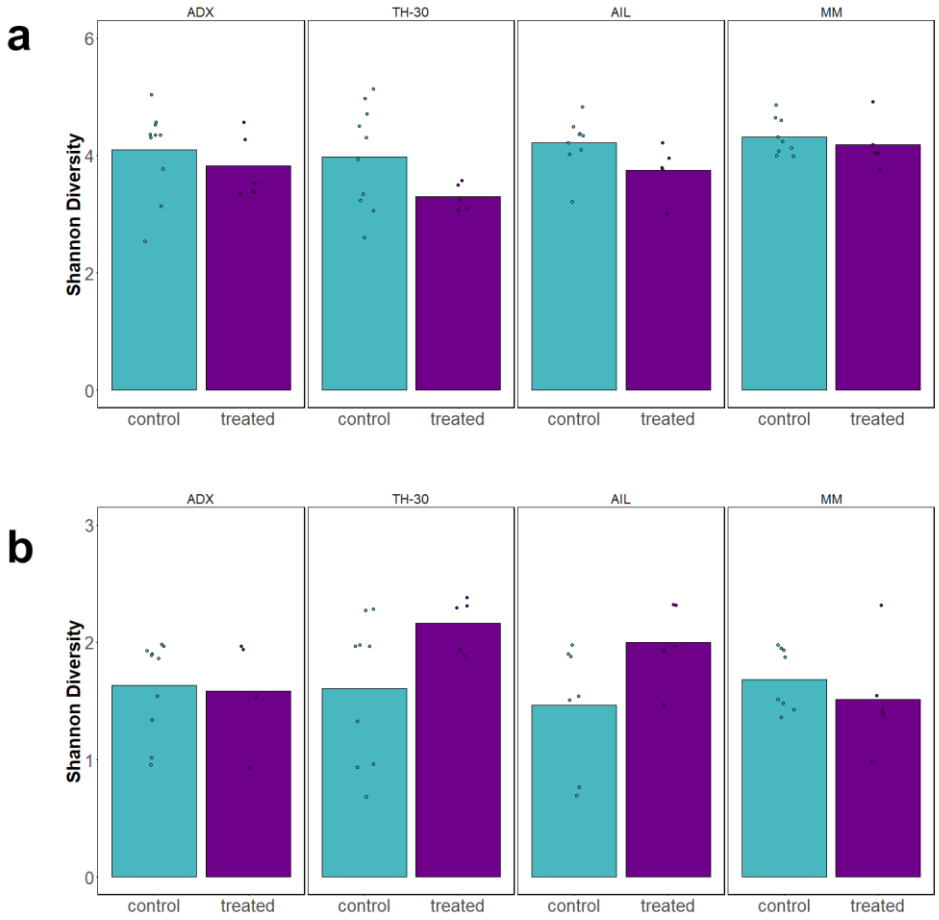

Figure 7. Alpha diversity of control and fungicide treated *S. lycopersicum* for 2 traditional varieties (ADX2, TH-30) and 2 commercial cultivars (AIL, MM), as calculated by Shanon Index, for (**a**) fungal communities and (**b**) bacterial communities. Bar plot represents overall index, while dot dispersion displays each sample values.

4. DISCUSSION

The study of the plant microbiome is gaining importance since it has been reported as an important modulator of plant growth, health, and resistance against biotic and abiotic stresses (Berg et al., 2017; Vandenkoornhuyse et al., 2015). It is also known that the microbiome fingerprint could be unique depending on the plant, soil, growth area, and even plant stage (Brown et al., 2020; Compant et al., 2019; Zachow et al., 2014).

Soil is known to have a strong influence on shaping root and rhizosphere microbiome (Wang et al., 2016). Likewise, aerial parts of the plants are exposed

to several factors that determine the microbial communities and community shifts (Dong et al., 2021; Ishida et al., 2022). Studies have shown that microbiota also changes from outside to inside tissues (Zhang et al., 2021). The relevance of the external and internal factors in shaping the microbial communities is yet a condition that seems to depend on species (Barbosa Lima et al., 2015; Berg & Smalla, 2009)

Genotype has been considered the other main factor and can have an influence and synergistic relation with the effects of soil. Comparisons of several genotypes have been mainly seen for bacterial communities in below ground area. These studies report the potential effect of genotype to select and associate with environmental microorganisms beyond rhizosphere. Therefore, understanding how different tomato genotypes can influence its endophytic microbial communities in aerial parts and how this, in turn, could shape the different genotypes is still an unexplored area.

Contrary to the soil microbiota, endophytic microbiota of aerial parts of the plant has been thought to be rather gained by vertical transmission (Bergna et al., 2018; Frank et al., 2017). Seed microbiota has been previously found to possess a core microbiota that is transmitted vertically (Zhang et al., 2021). Stem and root endophytic communities have been reported to be mainly shared with those in sprouts, indicating the transmission in plant (Wang et al., 2016) and the possibility of studying core microbiota by using stem endophytes based on genotype instead of soil influence.

Tomato, as one of the most relevant crops worldwide, has been the focus of several studies in order to discern the differences in the microbiota. The bacterial microbiota of tomato has been following a long tendency of research on bacterial communities (Bergna et al., 2018; Dong et al., 2019; French et al., 2020; Haq et al., 2021; Zhang et al., 2022), yet mycobiota reports are smaller and more recent (Dong et al., 2021; Kokaeva et al., 2020; Manzotti et al., 2020). Similarly, most microbiome analyses in tomato are focused on the below-ground communities (Chialva et al., 2018; Poli et al., 2016) and phyllosphere (Llontop et al., 2021; Toju et al., 2019) which are reported to be heavily influenced by environmental factors.

To the best of our knowledge, the stem microbiome has only been previously analyzed by Ottesen et al. (2013) with commercial tomato and has been focused on studying epiphytic communities. Here we provide an analysis of endophytic stem microbiota by comparison between several tomato genotypes.

On the other hand, the effects of human practices on the microbiome of cultivated tomato have been rarely studied. Some recent advances have examined the influence of fertilization and farming practices on bacterial root and rhizosphere communities (Caradonia et al., 2019; Haq et al., 2021; Zhang et al., 2022). However, how manipulations with pesticides affect microbiota is still unknown. In this study, it has been demonstrated how the use of a generic fungicide could correlate with significant changes in microbiome composition and structure in tomato plants.

Two aspects need to be taken into consideration in this study. Firstly, the relation between stem and root microbiome previously found in other species (Cregger et al., 2018; Wang et al., 2016) makes it feasible to consider the base-line connection between the stem microbiome in our study and the root microbiome. Secondly, regarding the plant stage, we need to take into account that tomato plants used in our study had not yet entered the flowering stage which could potentially accentuate certain differences between the studied genotypes.

4.1. Overall presence of microbial communities is higher in traditional tomato varieties

According to the obtained results, traditional tomato varieties host a more diverse microbiome than commercial cultivars (Fig. 1, Fig. 2) including several unique microbial taxa that are not found in their commercial counterparts. The Venn Diagram suggests the existence of a potential core microbiota that is shared among all plant genotypes and indicates that satellite microbiota shown in traditional plants is clearly more abundant.

The microbiome contrast among them supports the perception of how human manipulation and selection of crop species can negatively affect their microbial

diversity. Up until now, some studies have observed that the microbiome varies between genotypes of the same plant. As an example, a previous comparison of potato varieties showed that several microorganisms were cultivar-specific (Fitzpatrick et al., 2018; Inceoğlu et al., 2011). In this way, the effects of the cultivar have been observed in the study of bacterial communities of maize, potato, sweet potato, wheat, pea, oat, or barley (Bulgarelli et al., 2015; Marques et al., 2014; Peiffer et al., 2013; Turner et al., 2013). The establishment of a certain community of microorganisms in association with a plant is not arbitrary but it is a process that is highly influenced by the resistance of both the plant and the autochthonous microbial community to avoid the presence of extraneous organisms (Berg et al., 2016). Therefore, the intrinsic characteristics of each species would provoke the association with different core microbiome. We understand this core microbiome as a subset of the total microbiome of a certain plant that is consistently present through different environmental conditions. It has also been suggested that the core microbiome has co-evolved with the plant to provide certain host functions (Singh et al., 2020), for instance, to adapt to environmental changes like the soil quality (Podolich et al., 2015).

In this study, it can be observed that there are almost no exclusive microorganisms from commercial cultivars which suggests that the selection pressure has provoked the loss of the satellite microbiome and maintained only the core microbiome. Furthermore, while all four traditional varieties seem to share a similar core microbiome, their satellite microbiome exposes noticeable differences (Fig. 1b) showing how diverse the microbiome can be within varieties of the same plant species. Among these traditional genotypes, ISR-10 seems to be the one with less microbiota. We suspect this could be related to the warm environment in which this variety grows since biodiversity tends to be higher in milder climates. Our results also showed that more fungal OTUs were detected than bacterial OTUs. In regard to this, this tendency has been seen in other microbiome studies (Wang et al., 2016) and theorized that it was due to the fact that most endophytic bacteria might come from the soil while endophytic fungi would come from aerial spores.

In summary, the stem microbiome analysis supports the idea that microbial taxa can differ by plant genotype as previously seen in other studies (French et al., 2020; Manzotti et al., 2020; Toju et al., 2019). Our fungal results align with root microbiota studied by Manzotti et. al (2020) who reported the influence of plant genotype on root microbiome between CastleMart and UC82B genotypes. Our findings were also in concordance with previous studies of fungal communities in aerial parts of tomato although its abundance differed. For instance, Dong *et al.* (2021) found the main fungi class to correspond to Dothideomycetes in stems, leaves and seeds; contrary to our findings which predominantly identified Sordariomycetes. Bacterial taxa, which has been extensively studied, showed diversity with previous reports in tomato. For instance, we found Bacteroidia to be more predominant in Toju *et al.* (2019) and Llontop *et al.* (2021) which studied leaf microbiome. Sphingomonadales was also seen as a predominant order in leaves of grafted tomato (Toju et al., 2019), yet this was not the case in our study. Our findings also differed in main bacterial communities from the root microbiota seen in French *et al*. (2020) and Haq *et al.* (2021). We might interpret this higher diversity of bacterial communities across different studies to be caused by the different predominance of bacteria in the plant compartments as mentioned before. Lastly, we can't discard the fact that some microorganisms might also be present by chance as a result of stochastic events (Vorholt et al., 2017), yet our results improve the knowledge of genotype influence and could also correlate to a degree to other parts of the plant.

4.2. Traditional tomato microbiota possesses higher diversity and a wider genetic background

Comparing each one of the tomato varieties in our study, we can infer that the microbiota of the four traditional varieties not only exceeds that of the modern, commercial varieties, but also show higher diversity (Fig. 2, Fig. 3, Fig. 4).

Overall, the diversity of fungal and bacterial communities is similar for each tomato variety. Genotypes with a lower number of FECs also showed a lower number of BECs. The only remarkable exceptions happen with the richest

varieties: ADX2 and TH-30. ADX2 contains the most fungal communities while TH-30 possesses more bacterial communities. It is also notable that the AIL variety stands out for hosting notably lesser microbiota than the rest of the varieties and preliminary results showed that the plants of this variety were smaller and less resistant to different stresses than others (data not shown). Although the correlation of plant development and the diversity of the microbiome has not been tested yet, the phenotype differences made us initially expect some peculiarities that were indeed revealed with the microbiome analysis.

Fungal communities are dominated by ascomycetous fungi for all studied genotypes. This is expected because of the ubiquitous nature of this type of fungi and has been described as the most common group in plants ranging from tobacco leaves to native desert plants (Camarena-Pozos et al., 2021; Fuentes et al., 2020; Zhou et al., 2020). Additionally, for all tomato varieties, at least 50% of the identified taxa are classified in the Sordariomycetes class. This seems to reinforce that the manipulation in commercial tomato does not affect the core microbiome, since it is maintained an overall prevalence of ascomycetous fungi, as well as a similar relative abundance is maintained between fungal communities.

As for bacterial communities, while they have a variable abundance depending on the plant genotype, significant number of taxa belonged to the order of Flavobacteriales. A considerable number of taxa is also not assigned to any bacterial order which percentage is especially relevant in commercial tomato.

Incidentally, the higher number of retrieved OTUs from fungi compared to bacteria could be a result of the ways they transmit. Fungal species may require less environmental conditions and tolerate more harsh environments like heat than bacterial species (Cregger et al., 2018) and, along with the high rate of spore dispersion through the air, could lead to more coexistence of fungal species in plant tissue.

4.3. Fungicide causes a clear unbalance in microbial communities

Microbial communities respond to changes in the environment (soil conditions, plant conditions, pathogens…). The combination of tebuconazole and

dichlofluanide used in this study has been vastly used for all kind of agronomic practices. Dichlofluanide prevents a wide range of diseases that include rust, black spot, Botrytis cinerea and mildew by inhibition of spore germination. On the other hand, tebuconazole is used against several pests like bunt, smut, net blotch and powdery mildew by membrane disruption and inhibition of sterol synthesis.

Other studies have reported pesticides to induce changes in the rhizosphere and soil microbiome (Chen et al., 2021; Nettles et al., 2016; Vozniuk et al., 2019; Zhang et al., 2021). In this sense, we believed that treatment with these fungicides could also cause some relevant changes in the stem microbial communities of tomato plants. Our results revealed that the use of fungicides does indeed cause a visible unbalance in the tomato microbiome; in concordance with other studies concerning the effects of pesticides.

The alteration of the microbiome entails a reduction of some communities in spite of others (Fig. 5a, 5c) and the cladograms showed a clear shift (Fig. 5b, 5d). The relative abundance of fungal communities seemed to stay quite similar between control and treated communities at the fungal class level. However, diversity tests indicated that there was indeed a reduction of fungal diversity when treated (Fig. 6, Fig. 7). We consider this an indicator of a change of fungal communities at the species level that is not translated at higher taxonomic levels (class or order). In contrast, bacterial communities suffered a relevant decrease in abundance when treated with fungicide, including a significant reduction of unassigned bacterial taxa. If we reflect on how most studies center on commercial plants, the lack of these unknown bacterial communities could hint at the reason why they are still unidentified. Since commercial cultivars are more susceptible to being treated, and therefore having their microbiome altered, this would translate to the microbiome studies. Yet, the estimated richness of BECs between control and treated plants didn't show any noticeable change. Although the reduction of fungal population could translate to less competition for microbes within the plant tissues.

It is currently known that some bacteria have the ability to degrade pesticides which gives them an advantage against other microorganisms (Alexandrino et al.,

2020; Satapute & Kaliwal, 2016). Han *et al.* (2021) observed that in tebuconazole treated soils bacteria that can degrade this fungicide (*Methylobacterium, Burkholderia, Hyphomicrobium*, and *Dermacoccus*) increase their activity. In our case, it could be plausible that the new available space would be exploited by bacteria which would colonize the plant and unbalance the composition of the microbiome. Nevertheless, more experiments should be arranged in order to clarify this last point.

5. CONCLUSIONS

The relation of plant microbiome in the behavior of the host plant and plant genotype has been previously reported in several crop species (Pérez-Jaramillo et al., 2017; Zachow et al., 2014; Zheng et al., 2020), yet tomato endophytic communities still need further research for its characterization. This is the first study to compare the stem microbiome between tomato genotypes from both traditional and commercial origins. In this way, our results support that traditional tomato genotypes, which have been subject to a lower pressure of manipulation, are not only a richer source of potentially beneficial microbiota but possess higher diversity than commercial genotypes. Traditional genotypes clearly differ in their satellite microbiota too which makes them distinct from each other. The presence of all these endophytes is of great interest since they could influence the behavior and physiological responses of their host plants in front of stress conditions. Furthermore, microbiome composition is not only related to the environment and plant genotype but directly affected by human practices. In the present work, the impact of a generic fungicide on the tomato microbiome was assessed. While its effects were not apparent at first glance, there was a clear shift in microbial communities at lower taxonomic levels, both fungal and bacterial ones, and the estimated richness declined especially for fungi taxa. Ultimately, it is our belief that the advances in the understanding of tomato microbiome and their relationship with host plants will certainly be a valuable resource for better agronomic management in the demanding future.

Acknowledgments

The authors are grateful to the Integrated Microbiome resources and especially to Andre Comeau.

SUPPLEMENTARY DATA

Supplementary Table 1. Unique fungal taxa found in traditional genotypes of tomato (ADX2, ISR-10, MO-10, TH-30) and absent from the commercial genotypes.

OTU Code	Level of identification	Taxa
0045f318dc0fd3f2de3585ffbe0afa86	Order	Pleosporales
036c76d141d3bd2617b2ad9151c729eb	Genus	Solicoccozyma
07b7ab3320258c520cbed2dce1d7b69c	Family	Chaetomiaceae
12cb8995d2df767b7724d4c0daf8da04	Class	Leotiomycetes
15ab12e749d4962e74eaf3ed614b2c76	Genus	Conlarium
1d9d8accc18c3b3a38c5efa8becc4b4b	Family	Mrakiaceae
23feda89f5fb98763f6d48e18f18b966	Order	Sordariales
2dd848ecf666722a63a52b6a2d5e5081	Order	Platygloeales
2e6424ddee42a1112fea9d6256c2d8a1	Genus	Conlarium
303f6a2506fceec435e60af84126da55	Family	Lindgomycetacea
31c1e3dc32d527c4f6e2d706c583762c	Order	Chaetothyriales
38077cdf871e8e5d9237e6e6643274f0	Order	Pyxidiophorales
389c524d14d2ecffa871a7807a5b44ea	Family	Nectriaceae
4c160255d5aa6f9814d4750976a17ab0	Genus	Exophiala
5f1dbc0f54230c66898363ddb63fc3ac	Order	Helotiales
62a3116c2528c3ec56d451c501b61488	Order	Sordariales
669fc5a37db656c92d57c77ead101893	NA	NA
6ba7aa3f835545e09e4058224b03464e	Genus	Holtermanniella
73cb7dcdd2b2390e00a2c34c6da2377e	Genus	Cephalotrichum
7871be11e62c6e95550d2a0d872dd0db	Genus	Talaromyces
7948e1196fcdaf8c294c6a1d477a725f	Order	Myrmecridiales
7d9d5b431d926dd3341432a7d46b095e	Genus	Conlarium
82f80598e589acb4439c4e7c31e226d0	Family	Pyronemataceae
834d6f5c57a1206db9f7b80ddeea9b5d	Order	Atractiellales
877ae4765470686a25384b469c1df46c	Genus	Tetracladium

8f543486245102a8ea206a9e00da208d	Order	Helotiales
91b397b833028a9f9c6eae9bdb70ff0e	Genus	Trichoderma
94ee23c0b3aca4de5e230e4e63b7871e	Family	Nectriaceae
9e0c0c423bcb0bf45efca08d5fef24dc	Family	Nectriaceae
a26427aea532eb05c9adcdf933f7f6f9	Genus	Vishniacozyma
a82a5d11e2b81d348b349f313dd448f7	Family	Cephalothecacea
b33358528cc0ba537cdf033b4cc71297	Family	Chaetomiaceae
b623e6bfaa095875e56fa2ddf9a22725	Genus	Chaetomium
b827bf35c04e3fc39ead6522550c9226	Genus	Ganoderma
bc10eccdc662154b8025567cb26d17cb	Order	Hypocreales
c34cec4dcb7435a69fca3957af06a32f	Genus	Talaromyces
c49f7a4f244d7a4449e816474e776a43	Genus	Mortierella
c601b4fb2f6e0c480a46ecee7006545c	Genus	Malassezia
ce1c3ab791486d37aa0fd0d8245a9f81	Family	Chaetomiaceae
d555184cf5b4c6e93f2328a0a49ec98b	Order	Pleosporales
d67291ddd42ff0e001d8d0bc948f4e06	Class	Leotiomycetes
d9c596f2e04121924d1bd881aca8aa75	Genus	Pseudeurotium
dd0ede03155e66d9d8b64a8c30102774	Genus	Psathyrella
dd5e47c10e1e177074dd02c032a08376	Genus	Neobulgaria
e9bf7f0206d2460def456a59bf2b05b6	Genus	Oidiodendron
ea7a29e405664cd815483822c95514db	Family	Chaetomiaceae
f26cbc239e6f265569e7d13cfcf21cc1	Order	Glomerellales

Supplementary Table 2. Unique bacterial taxa found in traditional genotypes of tomato (ADX2, ISR-10, MO-10, TH-30) and absent from the commercial genotypes.

CODE	Level of identification	Taxa
0023488894785cdf977d7418f3a814a3	Family	Xanthomonadaceae
052a86039ff0342fe42085ae5dd4b638	Genus	Flavobacterium
07844c96db7ea8eb2398ac84b1497274	Genus	Sphingobium
323932cb2b20d0c9a4988e8d6b6879e8	Genus	Pseudomonas
3f9433a957150892c890f88bc72ee86b	NA	NA
42126bea18ba7a86aa818cc97d8d8b2b	Genus	Pseudomonas
56841e6aa76cac1906e71e0b3d42d829	Genus	Sphingobium
56fe271a4c43eb9271fef6c217df9237	Genus	Flavobacterium
6fc988e3494176827d34647fefdb4008	Genus	Pseudomonas
7fdfbd959676e76041ff2433d6ad02ef	NA	NA
86e9ea5991416cca72d8d9bed923f30f	Genus	Pseudomonas
8c5eb2bc5d888417743f4e3bb5808cad	Genus	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
90b85b588583ed7c0e4f0075d065f2e9	NA	NA
a5f2288219ec3855b644e7b6f1aea5ae	Genus	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
a6797a1591489b472579b1cfa5272644	Genus	Sphingobium
b3f28c4a0453df93611a1cc42a18e757	Genus	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
bb4e2266280e4b4a90c0e728674aa2d7	Genus	Flavobacterium
c06a238c1cb3b556f6ca57661fb3fbcc	Genus	Flavobacterium
ce8ee47efd8464dbb514b3e8e1c3034a	Genus	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
d7a396fb3a1d8ce9126a7156fa603433	NA	NA
e085ee738215aadc574a50899bdff517	NA	NA

CHAPTER 4

A new endophytic *Leptobacillium* sp. strain isolated from *S. lycopersicum*: implications and significance

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ABSTRACT

Beneficial endophytes are a promising resource for future agronomic practices, yet research on the use of fungal endophytes in tomato (*Solanum lycopersicum*) is still limited. In the current study, several endophytic fungi were isolated from tomato plants, and a novel potential candidate for improving crop performance was identified. Here we focus on fungal isolate SI27, which was determined to be a novel strain belonging to *Leptobacillium sp*., a species that has not been previously reported as an endophyte of agronomic relevance. The morphological characteristics and growth parameters of the fungal isolate were analyzed under varying conditions and the isolate revealed its capacity for synthesizing siderophores and low levels of IAA. Furthermore, the interaction with the host plant was examined under controlled conditions to prove a symbiotic relationship was established. The experiments in the growth chamber and greenhouse showed no detrimental effects in the studied tomato varieties; however, the influence of the fungal isolate appeared to be dependent on the tomato genotype. Growth parameters suggested an improvement in plant development for genotype TH-30, while ADX2 and MO-10 showed fewer changes. Moreover, chlorophyll and lycopene content in fruits were enhanced. These findings provide a basis for further studies on the potential application of this new isolate to confer stress resistance.

KEYWORDS: *S. lycopersicum*, endophyte, beneficial microorganisms, fungal isolate, *Leptobacillium* sp., fungal characterization, plant growth promotion.

1. INTRODUCTION

Plants are the focus in agronomy but there are many more organisms that are becoming relevant in recent years. Several microbial communities act in close association with plants and their development. The diverse groups of microorganisms interact in multiple ways, potentially making lifelong associations within plant tissues as endophytes. The relevance of fungal and bacterial endophytes is studied not only to know their influence on plant behavior, but to understand the possible outcomes of their relation and potential applications (Alam et al., 2021; Chialva et al., 2022; Gouda et al., 2016; Tidke et al., 2017). Thus, research on plant microbiome is providing new insights to improve agronomical techniques and handle future challenges.

Beneficial interactions between plants and microorganisms are extended in nature, and the symbiotic relationship between plants and their endophytic microorganisms plays an important role in current agriculture. It's been reported that endophytes could see an application in conferring resistance to plants against abiotic or biotic stress, improving nutrient acquisition and growth, as well as being new potential sources for compounds of agronomic or industrial interest (Gautam & Avasthi, 2019; Mei & Flinn, 2010; Segaran & Sathiavelu, 2019). All this constitutes an attractive alternative to already established agricultural techniques. The role of plant endophytes is, therefore, a crucial point in the future to avoid the abuse of agrochemical compounds and reduce the environmental and biological drawbacks those entail (Xu et al., 2021). However, although endophytic fungi are predominant in the plant microbiota, there is still a lack of knowledge of the functions of most fungal communities in plant development and behavior (Brader et al., 2017; Sun et al., 2020).

In recent years, there have been numerous efforts to find new species of endophytic fungi and bacteria that could be of application to improve plant performance. Most known ones are ascomycetes or basidiomycetes and almost 75% of studies about fungal endophytes are covered by species of *Epichloe*, *Serendipita*, *Penicillium*, *Fusarium* and *Trichoderma* (Liu-Xu et al., 2022). These fungal endophytes have been shown to be effective in helping plants against

several challenges. For instance, *Epichloe sp*. is the most studied grass endophyte because it synthesizes alkaloids that can protect plants against herbivory. Furthermore, it has been observed that the inoculation of these fungi can improve plant performance, serving as an alternative method for sustainable agriculture (Kauppinen et al., 2016).

Few studies have addressed the isolation and application of fungal endophytes in tomato plants (Bogner et al., 2016; Kavroulakis et al., 2018; Mahmoud & Narisawa, 2013). These reports demonstrate the potential of studying the microbiota in tomatoes in greater depth. In previous works, we studied the microbiota diversity of several *S. lycopersicum* genotypes denoting the relevance of traditional tomato genotypes as a source of higher microbial diversity compared to commercial cultivars. Along the way, traditional genotypes were found to resist heat stress better than the common commercial varieties (Fernández-Crespo et al., 2022), which could relate to their microbiome structure and composition. Thus, the next goal was to explore the endophytic communities of traditional tomato and find a potential candidate to expand the current knowledge of beneficial endophytes in tomato plants to improve this worldwide relevant crop.

In this work, a single fungal strain was chosen among the isolates from *S. lycopersicum* for further examination. Other non-selected isolates included mostly *Penicillium* sp. and several saprophytic and opportunist fungi according to current data and literature. Since plant-microbial interactions vary and are subject to several factors, it is important to discern whether the isolated fungus is beneficial or not for the host plant, and the conditions to establish a symbiosis. This work covers the isolation of this novel endophyte and the basic characterization and growth pattern, as well as the influence on tomato plant development under controlled conditions to prove their beneficial interaction.

2. MATERIAL AND METHODS

Fungal isolation

Fungal endophytes were isolated from several traditional tomato genotypes that were previously studied and found to be potential rich sources of less known endophytic fungi. These genotypes include red tomato from Thessaloniki (TH-30) and Montfavet (MO-10) and hanging tomato from Alcalà de Xivert (ADX2). Plant material was grown from seeds obtained from the Institute for the Conservation and Improvement of the Valencian Agrodiversity (COMAV), Polytechnic University of Valencia, Spain.

The leaves, stems and roots from 4-week-old plants were taken and surface sterilized by immersion following this sequence: 70% ethanol for 1 minute, 4% sodium hypochlorite solution for 3 minutes and rinsed with sterilized distilled water. Tissue was cut with a sterilized scalpel into segments of small size to fit petri plates of 90cm. Leaf, stem and root segments were placed separately on 10% PDA plates, ensuring tissue was in contact with media, and cultivated at 27ºC. A daily check was done for the detection and isolation of fungal colonies. Purified strains were saved by cryopreservation with glycerol as in (Ofek-Lalzar et al., 2016).

Molecular identification

DNA extraction of the purified strains was done by CTAB method (Tamari et al., 2013) and PCR was performed using universal primers for the internal transcribed spacer (ITS) region. Further PCR was performed targeting the large ribosomal subunit (LSU) and the beta-tubulin (TUB) regions (sup. table 1). DNA integrity was checked by gel agarose electrophoresis and followed by PCR purification. DNA samples were sent to the *Instituto de Biología Molecular y Celular de plantas (ibmcp)* in Valencia, Spain, for amplicon sequencing. Molecular identification was done through the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were assessed. ITS sequences were compared to potential

sequences from the GenBank database at a threshold value of 0,001, following verifications through the UNITE and BOLD databases as recommended by (Raja et al., 2017). A phylogenic tree was built using MEGA v.11 to discern the phylogenetic position of the isolate among the closest relatives stated by previously mentioned databases. ITS sequence data was obtained from the GenBank for *Simplicillium* sp., *Leptobacillium* sp. strains, and *Cladosporium herbarum* type as outgroup. The analysis was performed using the Tamura-Nei model and neighbor jointing (maximum composite likelihood) statistical methods with the application of a bootstrap test of 500 replicates.

Morphological characterization

Pure culture of isolate Sl27 was maintained on Potato Dextrose Agar (PDA) at 27ºC for periods of 4-weeks before transferring it to fresh PDA for subcultures. Perceptible characteristics and morphological traits were noted following indications by Watanabe (2003). Examination of mycelial structure was done using a biological microscope. Tissue from the edges of mycelium were placed on glass slides. Mycelial spheres from culture in liquid media were also examined. Staining with 0.1% Lacto-fucsine 1:1 (Sigma-Aldrich) was applied when appropriate for color contrast (Dhingra & Sinclair, 1995). Immersion oil was applied for examination with 100x objective.

Growth of fungal colonies was also observed by laying conidia on glass slides and incubating at 27ºC for short periods of time (12h, 24h) before microscopical observation. Conidia solution was obtained by sieving 7-day-old liquid culture through a cheese cloth, followed by centrifugation at 5500rpm and resuspension in 0.85% NaCl (Kavroulakis et al., 2018). For the incubation on glass slides, conidia solution was diluted in 10uL of PDB medium.

Physiological characterization

Evaluation of optimal growth conditions

Conditions for optimal fungal growth were assessed. The following parameters were studied:

Light: A light regimen of 16/8 and dark conditions were applied to test the influence of light in fungal growth.

Media: Potato dextrose agar (PDA) and maltose extract agar (MEA) were compared. PDA was prepared from industrial PDA, 200% industrial PDA and natural PDA. Natural PDA was made from boiled white potato supplemented with 20g/L of sucrose, and sucrose only media was used as its control.

Temperature: Optimal media plates were tested at several temperatures, ranging from 15ºC to 40ºC.

pH: Optimal media was adjusted to several pH values between 3 and 7.

Assays were done with fungal plugs of 7mm from 2-week-old mycelia margins and were incubated for 14 days. All tests were performed in triplicate.

Production of compounds of interest

Production of Indole-3-Acetic Acid (IAA) was evaluated by photometry following the commonly used Salkowski colorimetric assay (Gordon & Weber, 1951). Isolate was cultured with and without the presence of tryptophan (1% L-tryptophan) in PDB medium. Culture filtrates were obtained from 7-day old cultures and prepared in three dilution factors (1:1, 1:2; 1:4). Salkowski reagent (*Sigma-Aldrich*) was added, and samples were placed on a shaker in the dark for 20 minutes to ensure color stability. Absorbance was read at 530 nm and concentration was determined using a standard curve prepared with IAA (*Sigma-Aldrich*) stock dilutions.

The effects on phosphorus macronutrient were assessed in media plates. To evaluate the capability of SI27 to solubilize phosphates, insoluble tricalcium phosphates (TCP) were used as the only source of phosphorus (P) in growth media. Isolate SI27 was cultivated on media agar plates made of slightly modified Pikovskaya medium (PVK) (Pikovskaya, 1948), which is vastly used for detecting phosphate-solubilizing microorganisms. After a period of 2 weeks at 27ºC, the appearance of a light halo around the colonies instead of the white color of PVK media would indicate phosphate solubilization.

Capability of siderophore production was detected by colorimetric assay using the Fe(III)-chrome azurol (CAS) complex (*Santa Cruz Biotechnology*). Layered CAS-media agar plates were made as proposed by Andrews et al. (2016) to avoid media toxicity. Plates were prepared with a layer of CAS topped with a layer of PDA, and fungal plug was then placed on the PDA layer. Plates were incubated at 27ºC for 2 weeks to determine whether a change in color occurred in the CAS layer, indicating the reduction of Fe(III) to Fe(II).

Effect of fungal isolate in plant development

Effects of the endophytic isolate were assessed through the original host *plant, S. lycopersicum*. A preliminary test was conducted *in vitro* to assess the viability of the interaction between tomato seedlings and isolate SI27. Development of the plantlet and SI27 was monitored through stereoscopy for two weeks. Afterwards, two experiments under controlled conditions were performed, in the growth chamber and in the greenhouse, and each experiment was conducted three independent times for each location. Tomato plants were evaluated to detect any physiological changes between inoculated and non-inoculated plants.

Plant experiment in growth chamber

The experiment consisted of 3 tomato genotypes (ADX2, TH-30 and MO-10), with 10 biological replicates for each condition and variety. Seeds were surface sterilized with 75% ethanol for 3 minutes, followed by a 4% sodium hypochlorite solution for 1 minute and rinsed with sterile distilled water. Clean seeds were inoculated in a fungal conidia solution for 6 hours in a rotation shaker. The conidia solution was obtained as stated previously, and concentration was ensured to 10^5 spores/mL by counting with a hemocytometer. Then, dry seeds were sown in vermiculite substrate. Plants grew in growth chamber, watered with fixed amounts of Hoagland solution for 4 weeks. The following growth parameters were measured: Root length, root fresh and dry weight, basal diameter, stem length, stem fresh and dry weight, and chlorophyll content.

Plant experiment under greenhouse conditions

Experiment II used 2 tomato genotypes (TH-30, MO-10), with 10 plants for each condition and variety. Seeds were surface sterilized and inoculated as stated in experiment I. Seeds were germinated in growth chamber. After 1 week, plantlets were transferred to greenhouse and placed into individual big pots with a mix of peat and perlite (4:1). Plantlets were re-inoculated with 1mL of conidia solution after acclimation. Periodic measurements were done for aerial growth parameters (length, number of main leaves, flowers and fruits). Plants were grown for 3 months, until fructification. Full ripe fruits were collected and stored at -20ºC to maintain conditions the of the fruit during the recollection period. Root height and weight were measured on the last day.

Fruits were used for lycopene quantification. The analysis was performed by spectrophotometry at 503 nm following the steps in (Anthon & Barrett, 2007). Tomatoes were diced in similar size fragments and then homogenized with a blender (1500W), time-controlled and avoiding high temperatures. Tomato juice was achieved by straining through a fine sieve (0,1mm) and stored in the dark at 4ºC until analysis. 10 replicates were prepared using 100µL of juice for each. The solvent solution was prepared with a 2:1:1 ratio of hexane, ethanol, and acetone. Samples were sealed and incubated in the dark for 1 hour at 150rpm on an orbital shaker. 1mL distilled water was added for phases to separate in the samples before measuring.

Inoculation success rate

Several roots and leaves from both control and inoculated plants were sampled to verify endophyte's presence in inoculated plants. DNA extraction and PCR amplification was performed as previously stated in this work using specific designed primers for SI27. Sample DNA and purified fungal strain DNA were compared to determine whether endophytic SI27 was present in inoculated plant tissue and absent in control plant tissue.

3. RESULTS

3.1. Molecular identification

79 samples were sequenced and identified through BLAST analysis. 8 unique fungi genera with potential were found. Bibliographic support and preliminary testing were applied and isolate SI27 was selected as a result. SI27 shared identity at 99% with several fungal species and uncultured fungi with a query coverage of 98% for ITS sequence. BLAST results for LSU sequence supported similar matches. A GenBank screening was done by comparison with each taxonomic match and followed by verification through the UNITE and BOLD databases. The phylogenic position of the strain was determined within several screening results (Fig. 1). Thus, the fungal isolate was mainly identified as a *Leptobacillium* sp. strain, a genus that was formerly classified in *Simplicillium* sp.

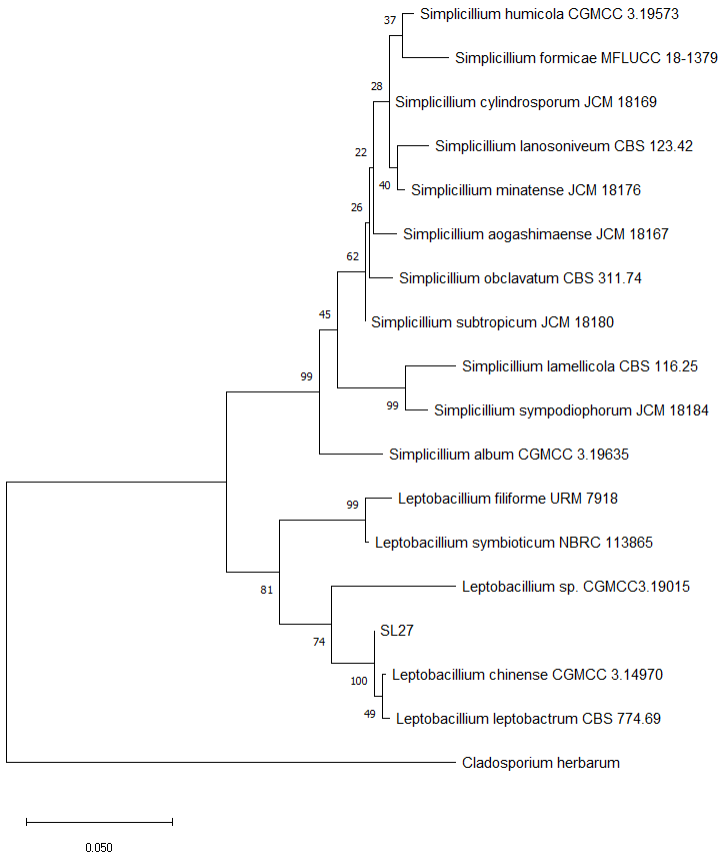


Figure 1. Evolutionary analysis of isolate SI27 seen as phylogenetic position calculated by Maximum Likelihood method (Tamura-Nei model). Taxa clustered together is shown in percentage and branch length is measured in number of substitutions per site.

3.2. Morphological characterization

Fungal colonies grown in PDA plates were defined with a white or cream color mycelium from above and reverse (Fig. 2a). Texture was characterized as dense and hard to break down. Mycelium had a velvety appearance, forming entire margins. In old colonies, margins dissipated and mycelium was more cotton-like from above, while the reverse got darker. This was also the case in the presence of suboptimal conditions such as lack of nutrients. Mycelium growth reached 3 to 4 cm in diameter in a 14-day-old culture. In liquid culture, mycelia turned into white smooth spheres (Fig. 2b). A layer of mycelia might also be formed on the surface of the medium which could indicate its affinity to aerobic metabolism.

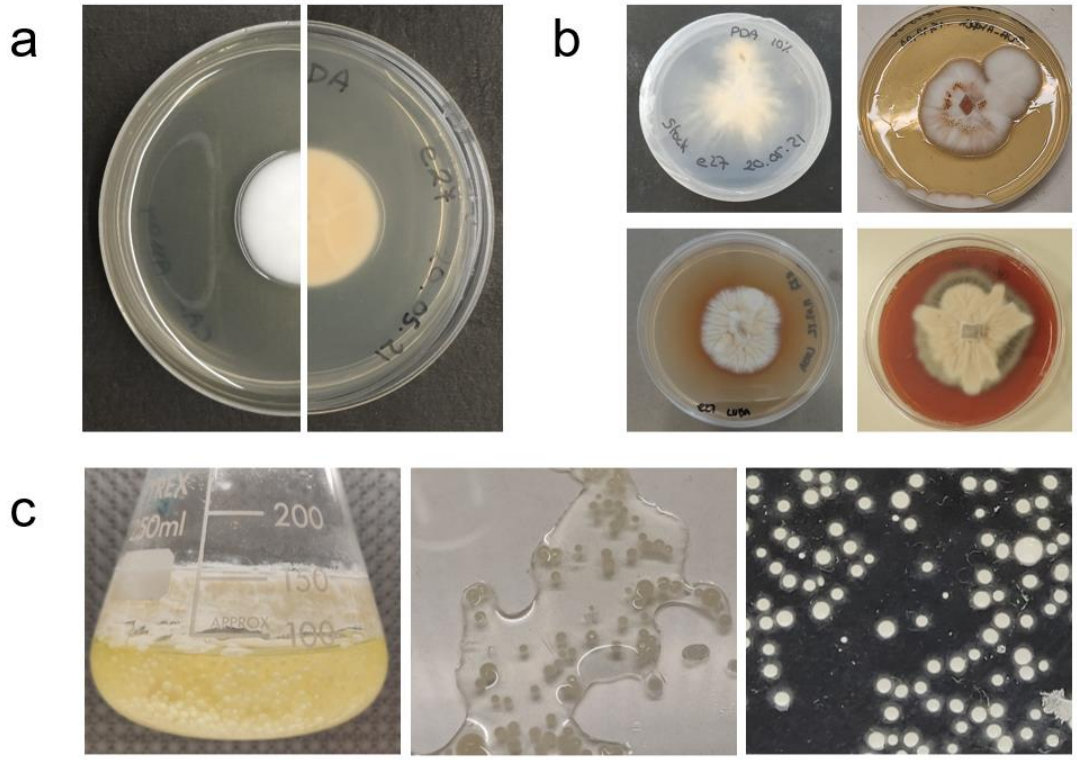


Figure 2. Isolate SI27 as seen in different growth conditions. (**a**) Front and reverse view of culture grown in normal conditions on a PDA plate. (**b**) Observation of certain morphological traits in solid media: mycelium growth in low nutrition conditions (10% PDA), presence of guttation under normal conditions, unusual production of orange pigmentation and unusual strong production of orange pigmentation and darkening of old mycelium. (**c**) Mycelia spheres produced in liquid culture under constant agitation (150rpm): in suspension and over white and black background.

Microscopy observation revealed that the fungal mycelium consisted of apparently coenocytic hyphal threads of usually few ramifications (Fig. 3). When spreading conidia suspension on glass slides, erect conidiophores were easily distinguished. No branching structures were seen in conidiophores and conidia were fusiform to cylindrical looking spores sizing 5-8um while the first conidium had a more ovate-roundish shape.

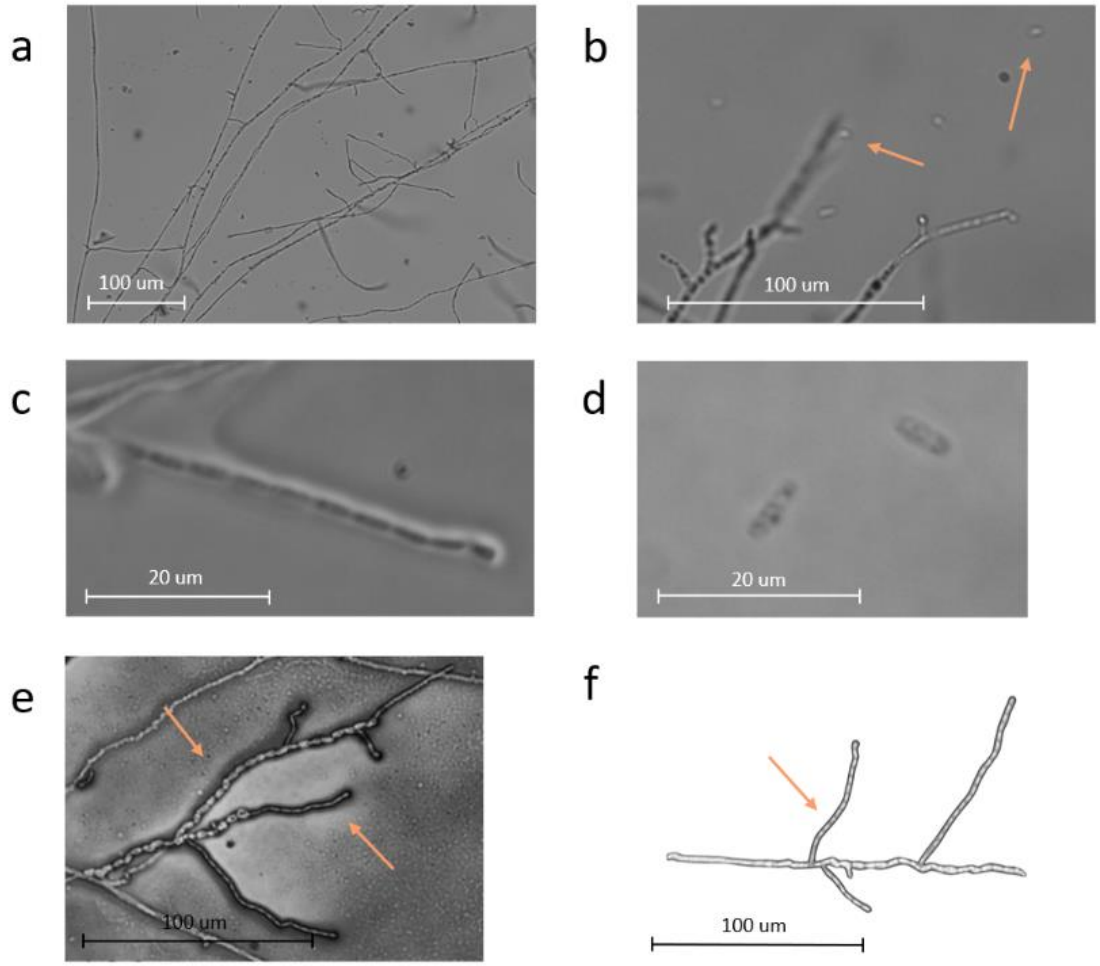


Figure 3. Morphology of isolate SI27 at microscopic level: (**a**) Normal mycelial structure; (**b**) Colony edge detail with presence of conidia; (**c**) Conidiophore detail; (**d**) Conidia; (**e**) Conidiophore structure at double contrast with distinction between phialide and conidial chain; (**f**) Conidiophore representation with long conidial chain.

3.3. Physiological characterization

Evaluation of optimal growth conditions

Light, temperature, pH and medium conditions were assessed for optimal isolate cultures (Fig. 4). While the presence of light had no influence in the diameter of fungal mycelia, media distinction showed apparent differences. Colonies grew better on natural PDA than industrial PDA, and doble industrial PDA showed similar results with natural PDA. For comparison, isolate SI27 was barely able to grow on sucrose media and no dense mycelium was formed. Moreover, fungal isolate was only able to grow between 15ºC and 30ºC. Colonies grown in this range could recover growth speed after returning to an optimal temperature (25ºC), but isolate was not able to survive after incubation at 35ºC. The effect of pH variation followed a normal distribution up to pH 7, with optimal range between 3.5 and 5.

Production of compounds of interest

The capability of isolate SI27 of producing certain compounds (Sup. Fig. 1-2). Fungal colonies were able to start producing exudate droplets after one week when grown between 20ºC and 30ºC. 7-day-old culture exhibited production of IAA in small levels (2ug/mL) which was favored when media was provided with 1% L-tryptophan (5ug/mL). SI27 was also found to produce siderophores by reducing the Fe (III) in CAS-agar plates. Despite the small mycelia size, the reduction halo was seen as early as day 2 of culture growth and full plate at day 10. 2-week-old fungal colonies showed uncertain ability to solubilize phosphates when in presence of TCP. Main SI27 characteristics found can be summed up in Table 1.

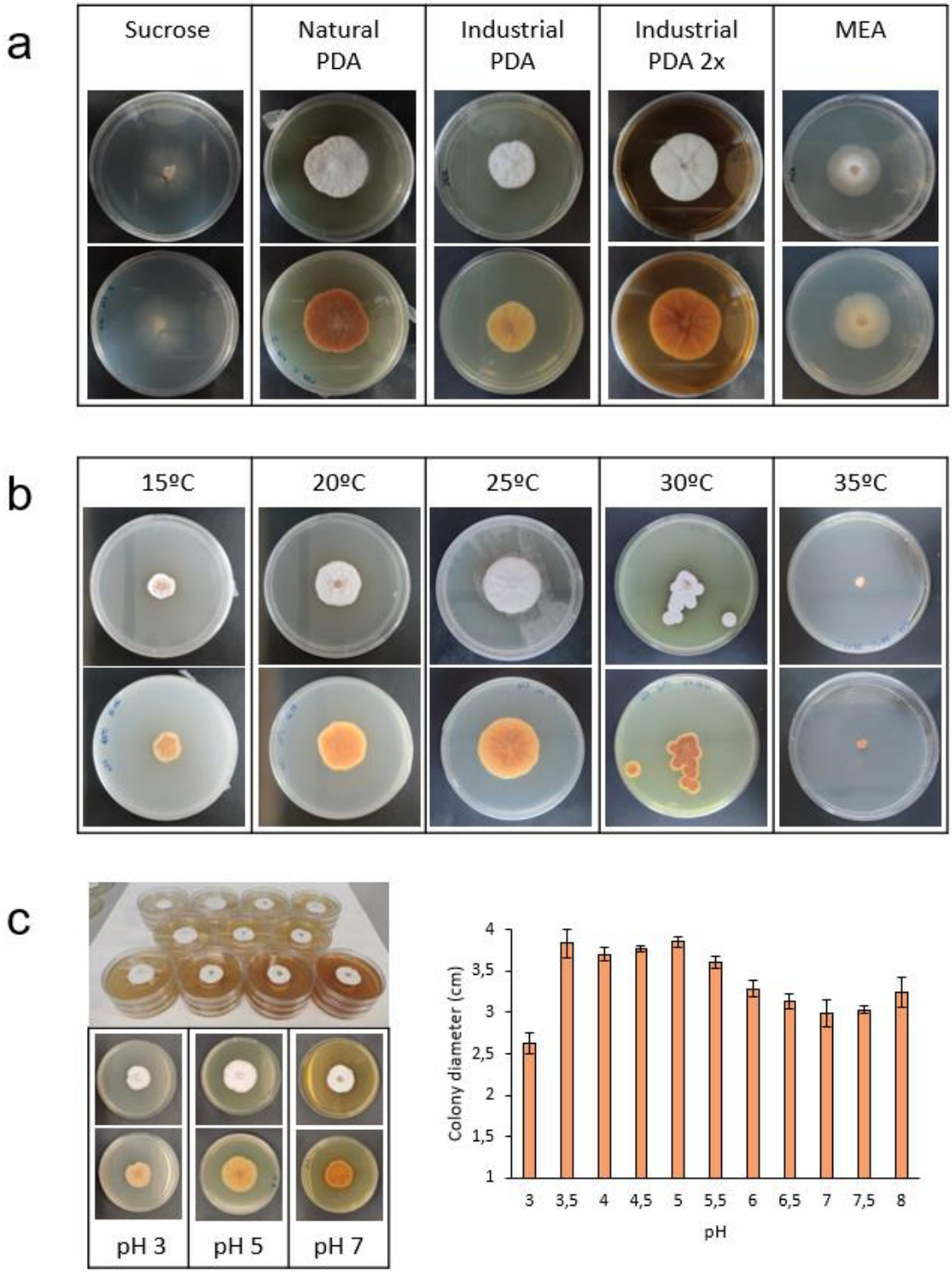


Figure 4. Growth pattern of 2-week-old cultures of isolate SI27 under different conditions: (**a**) Several media: sucrose, natural PDA, PDA, double PDA, MEA; (**b**) Temperature in the range of 15ºC-35ºC; (**c**) Media pH ranging from acidic (3) to slightly alcaline (8).

Table 1. Summary of isolate SI27 growth conditions and characteristics.

Condition	Assessment
Light	No influence
Media	Natural PDA
Temperature (ºC)	25-30
pH	3.5-5
IAA production	Low production
Phosphate solubilization	Unclear
Siderophore production	High production

3.4. Effects of fungal isolate in plant growth

The presence of SI27 did not appear to have any noticeable effect on the growth of the plantlets, nor was there any observed negative interaction between the plant and fungus during the *in vitro* assay.

Growth chamber experiment

The influence of isolate SI27 in seed inoculated plants in the growth chamber was variable and dependent on the studied genotype, yet no negative effects were seen. Results of a representative experiment can be found in Figure 5. Shoot parameters (length, fresh and dry weight) were seen to improve in inoculated plants, though the degree of improvement was not found consistent in all experiments. Nevertheless, chlorophyll content was reliably proven to be significantly better for the TH-30 variety, while it was variable for ADX2 and MO-10 (Fig. 7a).

Greenhouse experiment

Growth parameters for greenhouse experiment were focused on height and number of leaves, flowers and fruits, and differences were more relevant during the cold season (Fig. 6). Number of leaves were counted as new apical sprouts, and number of flowers as flowering buds. Seed inoculation with SI27 improved

growth for 7-week-old tomato plants, especially for the TH-30 variety. Height improvement was constant in time and not focused on a particular stage of growth, while sprouts seemed to experience a spurt on the second week for inoculated plants. Chlorophyll content was also improved for the TH-30 genotype (Fig. 7b) while no differences were seen in MO-10. Fructification stage did not seem to be significantly altered by presence of fungal endophyte. However, fruits of inoculated plants yielded higher levels of lycopene for both tomato varieties.

Inoculation success rate

The presence of the fungal isolate was confirmed in all inoculated roots, while absent in most leaf tissue of inoculated plants. Small presence of SI27 was also found in few of the non-inoculated plants' roots, yet nowhere close to inoculated plants (Sup. Fig. 3).

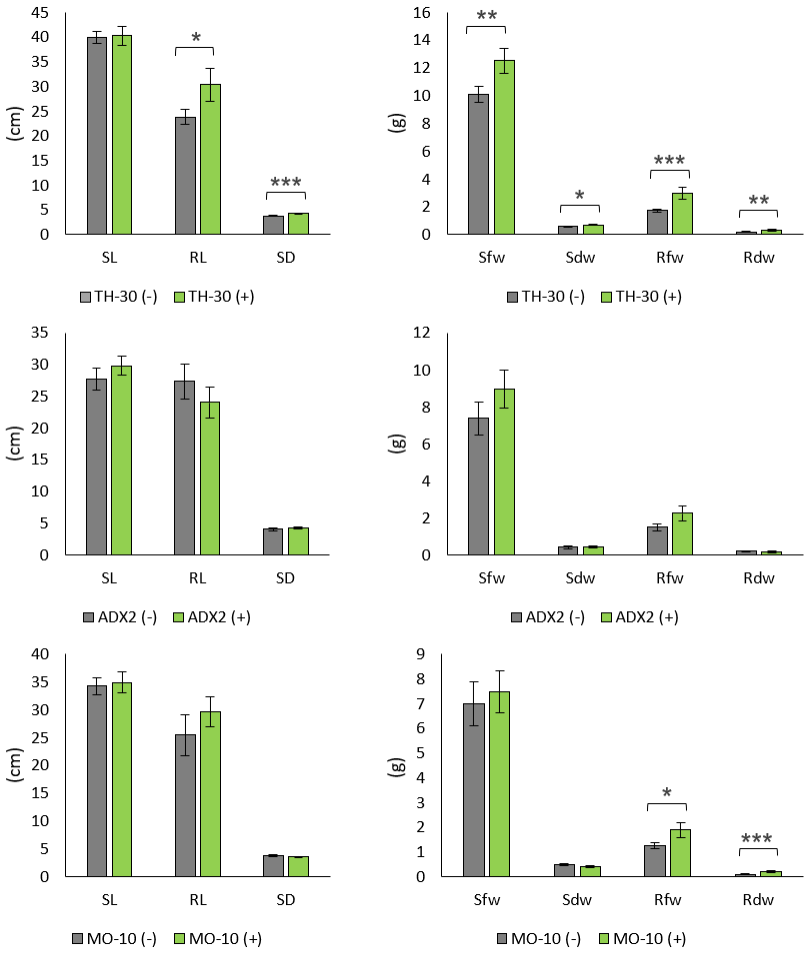


Figure 5. Growth parameters for non-inoculated (-) and inoculated with Sl27 (+) tomato plants of 4 weeks under normal conditions in growth chamber. Study parameters are as follows: SL Shoot Length; RL Root Length; SD Shoot basal diameter; Sfw Shoot fresh weight; Sdw Shoot dry weight; Rfw root fresh weight; Rdw root dry weight. Statistical significance of differences between groups was determined using ANOVA: (*) $p<0.1$, (**) $p<0.05$, (***) $p<0.01$.

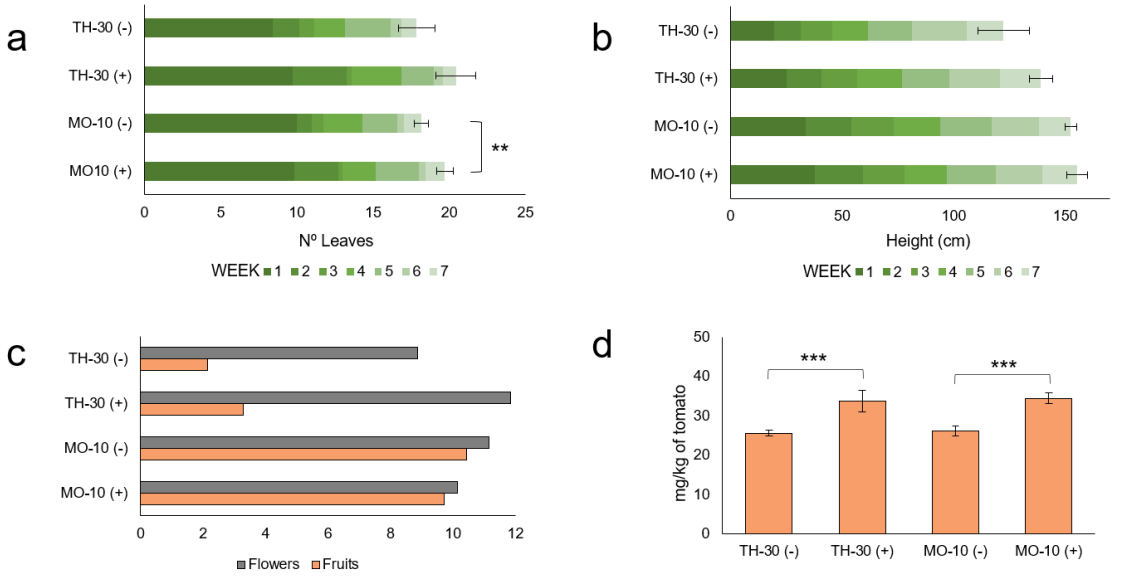


Figure 6. Growth parameters for non-inoculated (-) and inoculated with SI27 (+) tomato plants of 7 weeks under normal conditions in greenhouse. a) Number of main leaves counted as new apical leaves for each time point; b) Plant height increase for each week; c) Average number of identified flowers and fruits for plant; d) Lycopene quantification from tomato juice. Statistical significance of differences between groups was determined using ANOVA: (*) p<0.1, (**) p<0.05, (***) p<0.01.

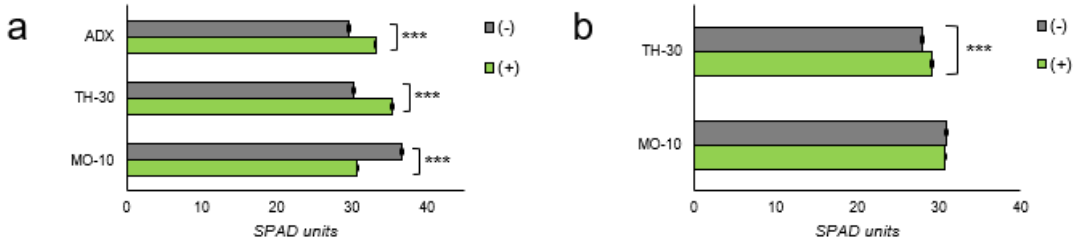


Figure 7. Chlorophyll content of leaves of non-inoculated (-) and inoculated with SI27 (+) tomato plants for (a) growth chamber and (b) greenhouse in a representative experiment. Statistical significance of differences between groups was determined using ANOVA: (*) p<0.1, (**) p<0.05, (***) p<0.01.

4. DISCUSSION

Isolate SI27 belongs to the genus *Leptobacillium*

As mentioned in this work, most of the research about efficacy of endophytic fungi on plants is focused on certain species. Around half of the studies performed in the last 25 years are focused on Poaceae (Liu-Xu et al., 2022). These circumstances make less known endophytic fungi to be poorly studied which provides us of a promising research area to find new alternatives for a sustainable agriculture.

Among the less studied genus, *Leptobacillium* has been gaining interest in the last few years. This genus was described in 2016 including only the single species *Leptobacillium leptobactrum* which was first introduced as *Verticillium leptobactrum*. Posteriorly, several species of *Leptobacillium* have been described from different environments such as walls of paleolithic caves (*L. cavernicola*) or decayed plant tissue (*L. symbioticum*). In the same way, some species previously assigned to the closely related genus *Simplicillium* were re-assigned to the genus *Leptobacillium*, including *L. coffeanum* and *filiforme*, which increased the total number of species included in this genus to 6. Interestingly, some of these species were isolated from healthy plant tissues and demonstrated to have a beneficial effect by emitting volatiles that inhibit pathogen growth (Gomes et al., 2018).

In this work, the BLAST analysis of our fungal strain indicated that closest species were those of the related genres of *Simplicillium and Leptobacillium*. Mycelia of SI27 resembled most to the ones in the current genus of *Leptobacillium* in color and texture, as well as slow-growth pace (1-2cm/week), but further characterization leads to uncertain identification with the species in current knowledge. Structures at the microscopic level, such as solitary conidiophore and microconidia were most similar to *L. leptobactrum*, which was supported by molecular identification and phylogenic tree. Like isolate SI27, *L. leptobactrum* has been reported to be recovered from plant tissue (Zare & Gams, 2016). However, strains of this species were also stated to not be able to grow at 30ºC

and having different optimal conditions than our isolate SI27. Isolate spores were also distinct in size and form and did not correspond to the previously described species (Gomes et al., 2018; Okane et al., 2020; Wei et al., 2019; Zhao et al., 2013). Yet, *Leptobacillium* species seem to be the closest since *Simplicillium* genus is known to be mostly entomogenous (Leplat et al., 2022). To the best of our knowledge, fungal isolate SI27 could be a newfound species inside the *Leptobacillium* genre which we propose to name *L. solani*.

Since endophytic fungi are vastly diverse and can be greatly influenced by environmental factors (Arnold, 2007; Marian et al., 2022; Mengistu, 2020), experimental conditions are expected to be a main factor in the response of fungal behavior and interaction with the host plant. Hence, the need to examine the isolate under different conditions like light, temperature and pH to find the optimal conditions that are favorable for the isolate. The fungal isolate was found to ideally grow at 25ºC on heavy nutrient media (natural PDA or doble industrial PDA) to show the same growth rate compared to other fungal species. SI27 had optimal growth when pH was between 4 to 5, while most plants grow in slightly acidic to neutral pH, which could be relevant for a plant-microorganism interaction to occur (Adejumo & Orole, 2010). The optimal growth conditions for SI27 are common for other fungi, so the slow growth is therefore not associated with suboptimal culture parameters. Since temperature conditions do not differ from the previously reported *Leptobacillium* sp. strains, we hypothesize the growth of SI27 might be related to their endophytic condition or their ability to produce some antimicrobial compounds as reported previously (Mejía et al., 2008), which would complicate the isolation and culturability *in vitro* (Huang, 2020).

Fungal isolate is able to synthesize Indole Acetic Acid (IAA) when supplemented with L-tryptophan. Though the quantification might not seem promising, this ability leads to the possibility for plant growth promotion, taking into account that effects on plant are dependent on several factors including the plant-microorganism interaction and environmental conditions (Nieto-Jacobo et al., 2017). Isolate SI27 is also able to produce siderophores to bind insoluble iron (III) from the environment, which could have potential effects on the host plant in front

of nutrient stress (Chhabra & Dowling, 2017). Though many soil microorganisms have this ability, its beneficial effects as endophytes are mainly reported in bacteria (Maheshwari et al., 2019; Naveed et al., 2020)

Inoculation with SI27 can improve plant development

The study of plant growth-promoting fungi (PGPF) is of special relevance due to their ability to improve plant health and resist plant pathogens. One of the most well studied growth promoting endophytes is *Piriformospora Indica*, a basidiomycete isolated from soil that is able to confer a wide range of beneficial effects in the host plant including growth-promotion and stress resistance (Zuccaro et al., 2011). Dark septate endophytes (DSE) are also frequently reported as growth-promoting species in, for instance, cabbage, chili and tomato (Mahmoud & Narisawa, 2013; Naziya et al., 2019; Usuki & Narisawa, 2007).

To study the interaction between our endophytic fungal strain and tomato plants, as well as the potential for promoting plant growth, we need to evaluate how the isolate SI27 affects host plants in controlled conditions. Before conducting any further studies, it was crucial to confirm that SI27 behaves as an endophyte and does not exert pathogenic effects on plant development. Our hypothesis was that the fungal isolate would exhibit neutral or positive effects on the host plant, based on previous reports about endophytes being isolated and inoculated in the same host species (Mayerhofer et al., 2013). The *in vitro* assay showed no negative effects on plants when in the presence of SI27. Nevertheless, roots would not prioritize contact with fungal mycelia, hinting there was no need for a symbiotic relationship at this point. The effect of fungal endophytes might not be visible in the first stages of plant development, but differences might arise at later stages.

The tomato genotypes that were used in this study were based on results found in Fernández-Crespo et al. (2022). A susceptible (TH-30), a neutral (ADX2) and a resistant (MO-10) genotype against heat stress were selected to evaluate its development in interaction with SI27. The development of the tomato plants that were seed inoculated was either positive or neutral which indicates that the isolated fungus does indeed have a mutual relationship and it is not detrimental

for the host plants. Specifically, inoculation with Sl27 might improve some plant parameters such as chlorophyll content. Lycopene quantification also indicated a significant increase of this carotenoid compound for fruits obtained from inoculated plants which would be of interest for crop management.

No clear promotion of morphological parameters like plant and root length across the plant genotypes and experiments was seen. This is not a surprising result but a common report in many endophytes. For instance, Hoyos et al. (2009) studied several isolates of Trichoderma sp., well-known as biocontrol agents and growth-promoters, and found a variation of results in the abilities to produce growth promoting compounds like auxins and no clear correlation with the growth-promotion seen in plant. More recently, Attia et al. (2022) has studied the effect of several PGPF isolated from rhizosphere soil in conferring promotion to tomato against Fusarium wilt. The improvement of photosynthetic pigments was reported yet plant morphological indicators were also studied with varying effects.

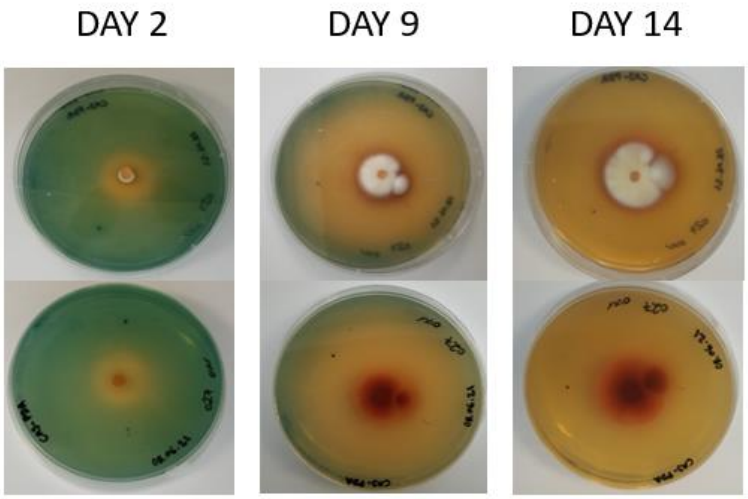
In addition, it is relevant to consider experimental factors that may vary between the growth chamber and the greenhouse experiments, as well as during different seasons in the experimental replicates. As previously discussed by (Mayerhofer et al., 2013), different factors might reflect in the variability of plant responses, such as the pH of the substrate, and influence plant response and interaction with the endophytes.

Thus, in this study, we have demonstrated the endophytic behavior of this novel fungal isolate by determining that no negative effects occurred on plant development while observing its potential beneficial influence on growth. Further studies will lead us to determine specific beneficial functions that this endophyte could confer on tomato, which could provide us with new agronomically relevant practices.

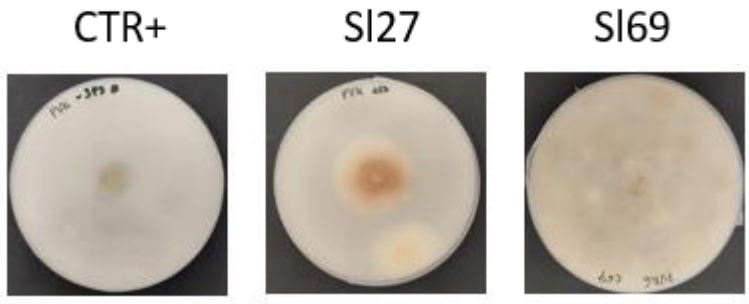
5. CONCLUSIONS

This work examined and characterized a novel endophytic fungal strain isolated from *S.lycopersicum* and studied its potential as a beneficial endophyte. Isolate SI27 has been proven to act as an endophytic microorganism in tomato roots, from which it was originally discovered. The phylogenetic analysis coupled with the morphological characterization did not concur in a clear identification of SI27, though most results suggest it to be closely related to *Leptobacillium leptobactrum*. To the best of our knowledge, this would be the first time this species has been studied as an endophyte of an agronomic important crop. The effects of SI27 on tomato plants under natural conditions were determined to be dependent on plant genotype. Beneficial effects included plant height, chlorophyll content and lycopene content in fruits. The advantages of inoculation were more prominent for the TH-30 variety and no detrimental effects were seen in plants for a period of up to 8 weeks. The potential effects of this isolate remain to be explored in further studies to determine its application for sustainable agronomic practices.

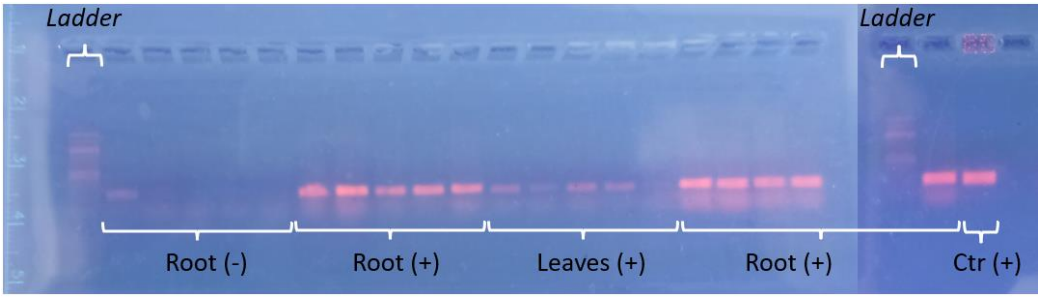
SUPPLEMENTARY DATA



Supplemental Figure 1. Assessment of siderophore production by isolate SI27 over a 2-week culture period indicated by the diameter of the orange halo on CAS-agar media.



Supplemental Figure 2. Assessment of phosphate solubilization by isolate SI27 in a 2-week culture compared to reference cultures of a phosphate-solubilizing bacteria (Ctr+) and isolate SI69 without solubilizing ability (Ctr-).



Supplemental Figure 3. Representative outcomes from DNA gel electrophoresis during the reisolation process of isolate SI27 from tomato plants. DNA extracted from inoculated plant tissue(+) and non-inoculated plant tissue (-) is represented.

Supplemental table 1. Universal primers employed for amplification and identification of fungal isolate DNA and specific primers developed for the reisolation of SI27.

	Sequence (5'-3')	Previously described by
ITS1	TCCGTAGGTGAACCTGCGG	(T. J. White et al., 1990)
ITS4	TCCTCCGCTTATTGATATGC	(T. J. White et al., 1990)
LR0R	ACCCGCTGAACTTAAGC	(Penton et al., 2013)
LR3	CCGTGTTTCAAGACGGG	(Penton et al., 2013)
β-tubulin T12	TAACAACTGCTGGGCCAAGGGTCAC	(O'Donnell & Cigelnik, 1997)
β-tubulin T22	TCTGGATGTTGTTGGGAATCC	(O'Donnell & Cigelnik, 1997)
SI27 (fw)	CGCCGAGGACACTTAAACTC	-
SI27 (rv)	GGGTTGAAATGACGCTCGAA	-

Supplemental table 2. Quantification of indole acetic acid (IAA) production of isolate SI27 by measuring culture filtrate of isolate SI27 after 1-week of incubation.

Dilution factor / Medium	1:1	1:2	1:4
PDB	2.613 ug/mL	1.146 ug/mL	0.429 ug/mL
PDB supplemented with 1% L-Tryptophan	5.126 ug/mL	2.810 ug/mL	0.948 ug/mL

CHAPTER 5

Endophytic *Leptobacillium sp.* isolate confers resistance to tomato plants against P. syringae.

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Manuscript in progress

ABSTRACT

Plant endophytes have been shown to improve crop's performance and resistance against environmental stress factors. This study aimed to evaluate the potential of an endophytic fungal strain, isolated from traditional tomato (*S. lycopersicum*) cultivars, as a biocontrol agent against pathogenic bacteria *Pseudomonas syringae* pv. tomato (Pst) and to investigate the involved mechanisms. To achieve this objective, the *in vitro* effect of fungal exudates was first assessed using culture filtrates (CF). This result showed a significant reduction in Pst growth upon addition of CF in culture media. To confirm the efficacy of the endophytic isolate as a biocontrol agent, we conducted different experiments with tomato var. Ailsa Craig. 4-week-old plants using with two different approaches: the first one based on seed inoculation and the other one using plants treated with culture filtrate. The two groups were infected with Pst. At 72hpi, a phenotype analysis of infected leaves and bacterial colony counting was performed. Both seed-inoculated and CF-treated plants showed a significant reduction in symptoms and infection rates. Transcriptomic analysis targeting stress-related gene regions showed that CF-treated plants did not have a significant difference in gene expression from the control group, yet seed-inoculated plants had a significantly lower expression of PR5, LOX and OPR3 genes post-infection. These results suggest that while both seed inoculation and application of CF help the plant fight Pst infection, the involved mechanisms differ. Furthermore, the effectiveness of CF application led to a metabolomic analysis to detect compounds with potential impact on the pathogenic system.

KEYWORDS: *Solanum lycopersicum,* beneficial fungi, endophytic fungal isolate*, Leptobacillium sp., Pseudomonas syringae,* plant resistance induction*.*

1. INTRODUCTION

Agronomy is facing several threats in this century. These include the consequences of climate change, natural resources shortage, the land scarcity and the increasing demands derived from the ever-increasing population. In this paradigm, agronomical systems also need to deal with diseases which are estimated to cause a reduction up to 40% of crop production (‘Pathogens, Precipitation and Produce Prices’, 2021). Pathogenic microorganisms, as one of the dangers, are also affected by the climate change and must be confronted on a daily basis.

Tomato plants are prone to a range of bacterial diseases, and these infections depend on the plant's health and environmental conditions. Among the bacterial diseases, the ones with the greatest economic impact include black rot (*Xanthomonas vesicatoria*), bacterial speck (*Pseudomonas syringae* pv. tomato), and bacterial canker (*Clavibacter michiganensis* subsp. michiganensis) (Kolomiets et al., 2017; Wang et al., 2018).

In regard to bacteria, we must address the genus *Pseudomonas*. This genus is known to have a long evolutive history and is famous for its high diversity, which includes human pathogens and several wide range plant pathogens (Spiers et al., 2000). In particular, *Pseudomonas syringae* is one of the most common plant diseases worldwide and its capability to infect most important crops make it the most studied plant pathogen. Like most plant pathogens, it can survive as a saprophytic and epiphytic bacterium on leaf surfaces. It only penetrates plant tissue through natural openings (such as stomata) or wounds. Pseudomonas syringae is also able to be propagated by water droplets and survive on seed surface until germination. This pathogenic species is classified in more than 60 pathovars depending on the host specificity (Xin et al., 2018).

The causal agent for the disease in tomato is the *Pseudomonas syringae* pv. tomato (Pst). As one of the main worldwide crops, the production of tomato needs to address this infection for good. Commonly used strategies to avoid this pathogen include chemical treatments, field rotation and using clean plant

material. For over 50 years, the main method of controlling diseases caused by *Pseudomonas syringae* has been the use of bactericides, predominantly copper compounds or other heavy metals, sometimes in combination with fungicides or other pest-control chemicals. Antibiotics and organic bactericides have also been used to a lesser degree. Despite the widespread application of these strategies, they have not proven to be effective in preventing significant crop damage during severe outbreaks. Furthermore, the emergence of copper-resistant pathogenic strains has been observed worldwide posing a serious threat to the continuity of this approach (Bashan, 1997).

Though pesticides can reduce disease caused by these pathogens, they are also known to be detrimental for the environment and human and animal health (Muthu Narayanan et al., 2022). In this context, beneficial microorganisms are one of the most promising alternative resources in agriculture to reduce dependence of chemical inputs and confront the consequences of climate change on agronomic crops. It has been demonstrated that plant beneficial microorganisms, including specific groups of bacteria and fungi, support plant growth and improve productivity. (Rouphael & Colla, 2020; Santos et al., 2019). These beneficial microorganisms play a vital role in maintaining essential agroecological cycles that enrich soil nutrients, enhance crop nutrient uptake, increase plant tolerance to biotic and abiotic stresses, provide biocontrol of pests and diseases and improve water uptake (Lobo et al., 2019). They actively contribute to healthy plant development and growth by secreting hormonal growth regulators that, in turn, are able to induce resistance against phytopathogens (Dakora et al., 2015).

Among them, fungal endophytes have been shown to improve performance of crops and their resistance against stress factors (Attia et al., 2022; Hossain et al., 2017). Fungal endophytes, which are asymptomatic inhabitants of plant tissue, are reported from all parts of plants and can offer a range of benefits to their plant hosts increasing plant fitness over uninhabited counterparts. They can alleviate abiotic and biotic stressors such as drought, salinity, heavy metals and other toxic compounds introduced by the environment, flood, extreme temperatures, predators and pathogens. Endophytes have beneficial biological properties to the

hosts, deterring pathogenic microbes, insects and other herbivores while also providing stimulants for plant growth and development (Busby et al., 2016).

Some endophytes can also induce plant defense mechanisms such as systemic acquired resistance (SAR) or induced systemic resistance (ISR). For example, *Piriformospora indica* can induce a jasmonic acid-dependent defense response in Arabidopsis thaliana by co-inoculation with a pathogen (Stein et al., 2008). Furthermore, some endophytes have been found to have plant growth promoting properties, providing nutrients such as nitrogen, phosphates and iron, as well as facilitating plant growth and development through growth stimulation. Plant growth promoting (PGP) microbes can produce chemical compounds that influence plant growth and development, including plant hormones like indole-3-acetic acid (IAA), gibberellins, cytokinins, and/or 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity (Joseph & Priya, 2011).

Although over one million endophytic species are estimated to exist in 300,000 different plant species, only a small fraction have been isolated and investigated for their roles within the plants they inhabit. Nonetheless, this research area still has many unexplored areas, such as the plant protection against biotic stressors (Liu-Xu et al., 2022).

In previous studies, we isolated a novel fungal endophyte which we named Sl27 (manuscript in press). This isolate was obtained from the roots of a healthy traditional tomato plant (*S. lycopersicum*) and belonged to the genus *Leptobacillium,* which has not yet been reported as a potential beneficial endophyte of plants. In this study, we tested the potential of Sl27 to confer biotic resistance to tomato plants against some plant pathogens, in particular to Pst. This included the study of Sl27 as an endophyte inoculated at seed stage and its capability to produce bioactive metabolites to be used as potential natural compounds against Pst. We initially analyzed the potential antagonism between our isolate and Pst *in vitro* which was done using fungal exudates present in culture filtrates. Since antagonism was found, Sl27 was proven to be able to produce a component to suppress the growth of Pst. We then tested if Sl27 could confer resistance to plants by applying these exudates and by seed inoculation.

Finally, we also studied the potential antimicrobial compounds by analysing the metabolome of the filtrates.

2. MATERIAL AND METHOD

Antagonism test against pathogenic fungal strains

Interaction of isolate SI27 with fungal plant pathogens (*Alternaria* sp., *Botrytis* sp., *Fusarium* sp., *Phytophtora* sp.) was analyzed by co-culturing on PDA plates. Fungal disks of 7mm diameter were taken from the edges of mycelia. A SI27 plug was placed in PDA opposed to a pathogenic fungal plug at 70mm of distance. Mycelia growth and presence of inhibitory halo was examined for 14 days. Fresh PDA plates were used as control.

Biocontrol capability against pathogenic bacteria *P. syringae* in *in vitro* assay

The development of fungi and their interaction with other organisms usually involve the production of secondary metabolites that are released to the environment. In this study, the exudates of isolate SI27 found in liquid media were studied in order to determine the potential future application of antimicrobial compounds to plants. First, determination of a common growth medium for both the bacteria and the fungal endophyte was required; and nutrient broth (NB) was selected as a viable medium. Endophytic isolate was grown with an initial concentration of 10^6 spore solution in 100mL NB and put on a shaking incubator (150rpm 27ºC). After the incubation period, culture was filtered through two layers of cheesecloth and syringe-filtered with a 0,45um membrane filter to remove fungal mycelium. Then, it was filtered with a 0,22um sterile membrane filter for every 5mL to ensure no spores remained.

Several culture filtrates (CF) were prepared, from cultures of 3, 5 and 7 days. Along with isolate SI27, a 5-day-old fungal culture of an isolate of *Alternaria sp.* was also prepared as fungal control. A 10mM $MgSO_4$ solution was used as media

control. Dilutions with NB were made to ensure nutrient media was provided for Pst (1:1, 1:3).

An initial inoculum of 10^5 CFU of *P. syringae* was applied for 200uL medium. For culture details refer to the following section. A microplate photometer (Multiskan™ FC, Thermo Fisher Scientific) was used to assess Pst growth. The microplate was incubated at 27ºC with constant stirring and readings were done at OD = 600nm. The experiment was carried out for a period of 72h, using five replicates. Two previous tests were run to verify the study parameters of this experiment.

Capability of inducing resistance in host plant against *P. syringae* infection

To confirm the biocontrol potential of SI27 in plant, experiments with tomato var. Ailsa Craig were set up. Two approaches of SI27 application were considered: inoculation of seeds and treatment of already grown plants with fungal exudates.

For endophyte inoculation, tomato seeds were surface sterilized (70% ethanol for 3 min, double rinsed with sterile dH_2O) and inoculated by dipping in fungal suspension for 6 hours on a shaking incubator (150rpm 27ºC). Inoculated seeds were then collected and dried at room temperature. Non-inoculated tomato seeds followed the same steps but with NB media as mock. Both seed batches (I-, I+) were sown in vermiculite substrate. Then, they were germinated in a growth chamber at 25ºC and 70% humidity with a 16/8 light/dark regimen. Plants were watered with Hoagland solution for 4 weeks as needed.

For exudate treatment, culture filtrates (CF) were used. These were prepared from fungal cultures of 5 days following the methodology mentioned above in the *in vitro* assay. Treatments were performed 48 h prior to infection. Three methods of treatment were studied, and an optimal method was identified and subsequently used. These methods include infiltration to the first leaves, spraying over the leaves and soil-drenching.

At this point, 4-week-old plants of the same size for each condition were selected and placed inside plastic containers to acclimate before infection. Distinction between inoculated plants (I+) and exudate treated plants (T+) was done. Control plants were mock-inoculated and mock-treated (I-T-).

One day prior to the infection, Pseudomonas syringae pv. tomato DC3000 (Pst) was cultured in solid King's B medium (KB) supplemented with rifampicin (100 µg/ml) and cycloheximide (100 µg/ml) at 27ºC for 24h. Bacterial suspension was prepared to a concentration of $5 \cdot 10^5$ in $MgSO_4$ and 1% Silwet® surfactant was added. For inoculation, the third and four leaves of the plants were soaked in the bacterial suspension for 3 seconds.

At 72hpi, a phenotype analysis was performed and sampling was done for subsequent analysis. The degree of disease symptoms (DS) was calculated based on leaf surface percentage. Disease incidence (DI) was done by counting number of colony-forming-units (CFU/g). This was calculated based on González-Hernández et al. (2018)**.** Briefly, the fresh infected leaves (third and fourth true leaves) were rinsed with sterile dH_2O, crushed in a mortar and diluted with 10mM $MgSO_4$. 20uL of sample dilution was used to grow in KB-agar plates at 27ºC for 24h before CFU counting using a stereo microscope.

Interaction with both inoculation and treatment was studied (I+T+). An accumulative effect was seen and thus the study of inoculation and treatment separately prevailed. The experiments were done in triplicate.

Following transcriptomics were performed targeting biotic stress related gene regions. Gene expression was quantified through qRT-PCR with *PAL, PR1, PR4, LOX, OPR3, CAT, ASR1, and ACO* as target regions. The expression of the tomato EFα gene was used as a reference gene.

For hormonal analysis, an extraction was performed as described by González-Hernández et al. (2018). In brief: fresh tissue samples were frozen in liquid nitrogen, freeze-dried and ground. A 0.5 g of the fresh tissue was homogenized in 1.5 mL of ultrapure water and 100 ng mL^{-1} of a mixture of internal standards (deuterated abscisic acid ([2H6]ABA), deuterated salicylic acid ([2H4]SA),

dihydrojasmonic acid (dhJA) and propylparaben from Sigma–Aldrich were added before extraction. The samples were then centrifuged, and the supernatant was partitioned using diethyl ether, dried and resuspended in a mixture of water and methanol. 20 µL aliquot was injected into an ultra-high performance liquid chromatography (UPLC) with an ACQUITY UPLC BEH C18 column. Data was processed using MASSLYNX NT soft-ware v.4.1 (Micromass).

Screening of secondary metabolites to find bioactive antimicrobial compounds.

In order to study the compounds that Sl27 releases in NB media, an untargeted metabolome analysis was conducted with extracts that correspond to CF obtained from cultures of 3, 5 and 7 days of a Sl27 solution (10^6 spores). The metabolomic process was performed by QTOF-HPLC. Data pre-processing was done using DataBridge and Galaxy analysis platform (Workflow4metabolomics). PCA plots and heatmaps were performed using MetaboAnalyst. Data was normalized by median. Heatmaps of top 150 differential compounds were done using Ward-clustering with group averages and applying T-test and ANOVA. Further analysis was performed using Marvis and Masslynx; and comparison and identification of compounds were done through bases such as Massbank of North America (MoNA), MSSJ Massbank and METLIN™.

3. RESULTS

Antagonism of Sl27 against pathogenic bacteria *Pseudomonas syringae* pv. tomato was studied after observing that no clear antagonism occurred against several pathogenic fungi in dual-culture assays.

Biocontrol capability against pathogenic bacteria *P. syringae* in *in vitro* assay

The *in vitro* assessment revealed effective biocontrol ability of the exudates produced by Sl27 against *P. syringae* (Fig. 1). Differences raised after 4 hours of

microbial growth. At this point, an exponential phase was substantially reduced by the presence of exudates from 7-day-culture. At around 6 hours, the inhibition of all tested Sl27 exudates on Pst growth was significant compared to the control colonies. Presence of older culture exudates was proportional with lower Pst growth with a reduction near 50% OD at the stationary phase at 16h. No growth pattern changes occurred after the 24h incubation period, when bacterial population followed its stationary phase.

Higher volume of CF did exhibit higher inhibition rates but not as high as the ones caused by CF of 7 days. A stable reduction was measured in a dilution 3:1 compared to 1:1 (data not shown) but difference was not significative. Pst was able to grow in 100% CF of 3 and 5 days although less than when provided new NB media. *Alternaria* sp. exudates did not show inhibitory effects on Pst and bacteria population proliferated better in media supplemented with Alternaria exudates for the first 24h. After 24h, no differences were found in relation to control.

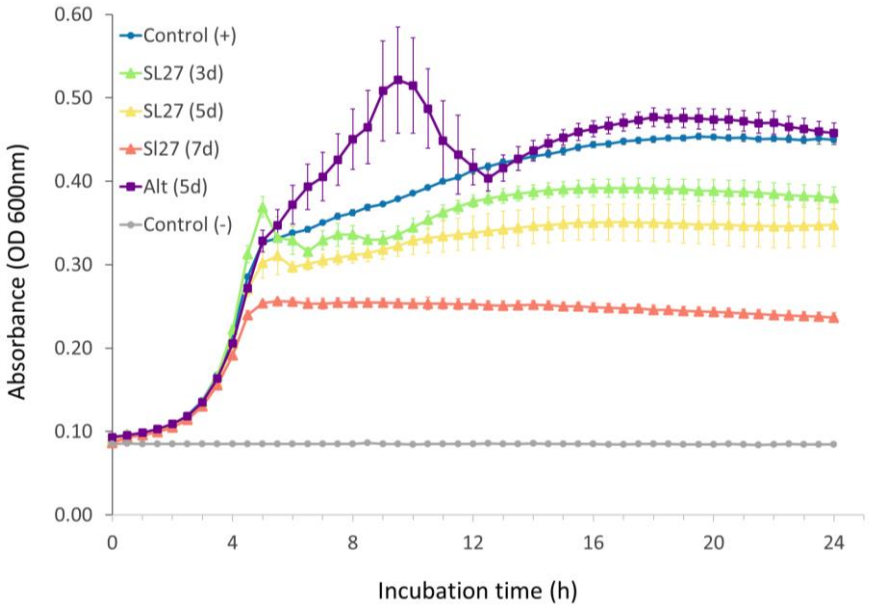

Figure 1. Effect of fungal exudates on *Pseudomonas syringae* pv. Tomato (Pst) *in vitro* growth in a 24h incubation period. Fungal exudates are obtained from culture filtrates (CF) of several cultures of isolate Sl27 (incubation period of 3, 5 and 7 days) are compared to exudates produced by another fungal isolate *Alternaria* sp. (incubation period of 5 days). CF were used in a dilution 1:1 with fresh NB media. $MgSO_4$ was used as control (-) and as control (+) in a dilution 1:1 with NB media. Error bar represents ± SE of the 5 replicates.

Capability of inducing resistance in host plant against *P. syringae* infection

Reduction of Pst infection was seen in both SI27 inoculated (I+) and treated (T+) plants (Fig. 2). A significant reduction of diseased leaf surface and bacteria population was reached for both I+ and T+ ($p \leq 0.05$). I+ was seemingly more effective than T+ at visual levels, but no significant variation was found in the size of bacteria population between them. Treatment by soil-drenching was found to be the most reliable method to apply fungal exudates, showing lower variability than the other two tested methods (data not shown).

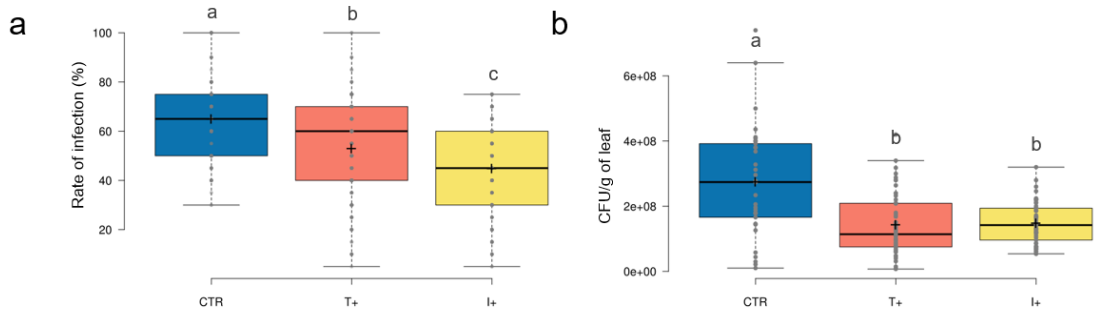

Figure 2. Assessment of Pst disease rate in infected leaves of control (CTR), exudate treated (T+) and SI27 inoculated (I+) tomato plants. (**a**) 72 hours post-infection (hpi) foliar damage index (%) caused by Pst infection calculated as damaged area in the phenotype of inoculated leaves. Data obtained from the average of three independent experiments for control, T+ and I+ conditions. (**b**) Pst bacterial population (CFU/g) after 24h hours incubation in a KB-agar medium from inoculated foliar sample pool of control, T+ and I+ conditions. Error bar represents ± SE of the means of triplicates. Statistical differences according to ANOVA simple and test of Kruskal-Wallis for a 95% probability ($p<0.05$) are indicated by lowercase letters.

Differences appeared in the transcriptomics and hormone analysis. ABA, SA, JA, Ethylene pathways were studied showing distinct patterns for inoculated and exudate treated plants (Fig. 3-4). Expression of defense-related gene markers in T+ plants was mostly similar to control at 72hpi although an induction of JA pathway markers genes *LOX* and *OPR3* took pre-infection. On the other hand, a tendency was seen with I+ plants showing lower genes expression and hormone signalling. Treated and inoculated plants showed lower levels of SA and higher

levels of JA-Ile at 72hpi. This indicated that the higher resistance observed in I+ and T+ plants did not seem to correlate to higher expression of JA and SA signalling pathways at this stage of infection. However, while a significant post-infection increase of SA levels for control plants happened, treated and inoculated plants maintained the same levels pre and post-infection.

Regarding SA pathway, higher *PAL* expression occurred in non-infected I+ and T+ although with no statistical confirmation. A lower basal expression of *PAL* might have affected the susceptibility to the initial bacterial infection. Though SA levels were significantly lower for I+, *PAL* expression was higher 72hpi. SA induced genes *PR1* and *PR5* were expressed post-infection. While no differences were observed in *PR1*, *PR5* was more expressed for T+ than I+.

Treatment with exudates seemed to induce the expression of JA pathway marker genes *LOX* and *OPR3* when no infection was occurring which indicates the exudate treatment influenced normal plant metabolism. *LOX* expression post infection was also significantly lower for I+ which in turn seemed to be in accordance with the lower levels of OPDA. The JA pathway was seen as a key pathway to contrast the effects of both approaches of the experiment.

The *ACO* marker gene was also highly expressed in infected plants, most notably in T+ plants, while I+ returned lower expression in comparison.

Thus, differences between the 2 experimental approaches were apparent in *PR5*, *ACO* and *OPR3* expression post-infection. In all three, gene expression was lower for I+ plants. Yet, they did not differ significantly from control.

No significant results were found in the *ASR1* gene expression, as well as the ABA levels. *CAT* seemed to be more expressed in uninfected I+ and T+ plants, although it was not significant ($p<0.05$).

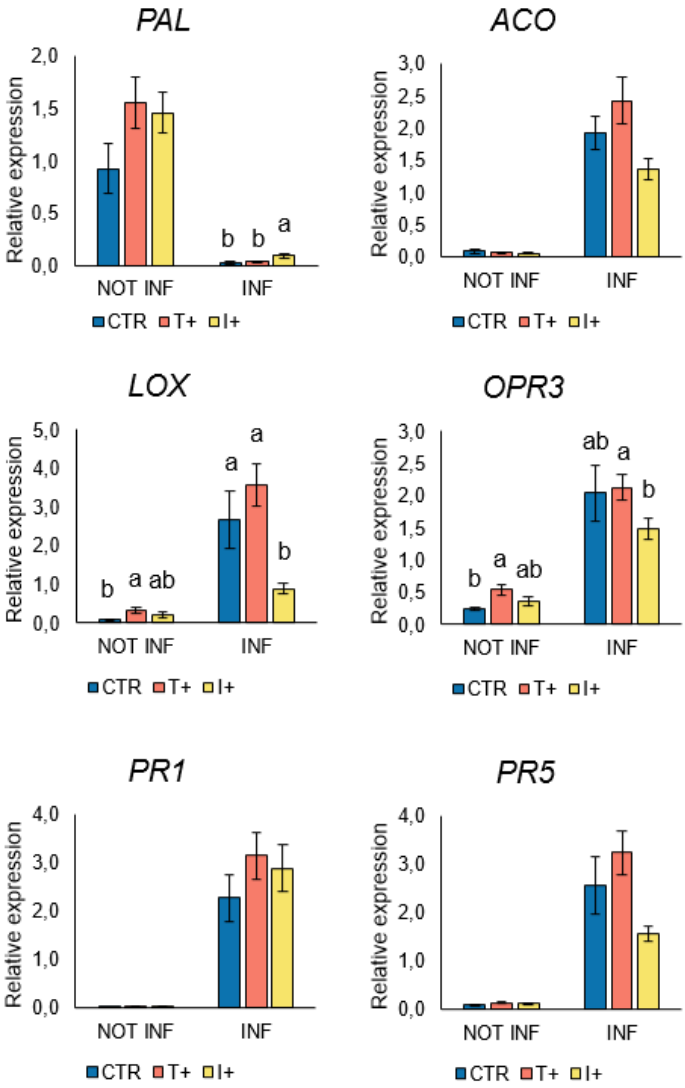

Figure 3. Gene expression analysis of plant defense pathways in infected (INF) and non-inoculated (NOT INF) tomato leaves with Pst. The figure shows the relative expression levels of key genes associated with SA, JA, and ET signaling pathways in control (CTR), exudate-treated (T+) and SI27-inoculated (I+) tomato at 72 hours post-infection (hpi). Specifically, the relative expression levels of the SA biosynthesis gene *PAL* and the SA-induced genes *PR1* and *PR5*, the ET biosynthesis gene *ACO*, and the JA biosynthesis genes *LOX* and *OPR3* are shown. Data is presented as in comparison to *Efα* gene expression. Statistical differences according to ANOVA simple and test of Kruskal-Wallis for a 95% probability ($p<0.05$) are indicated by lowercase letters.

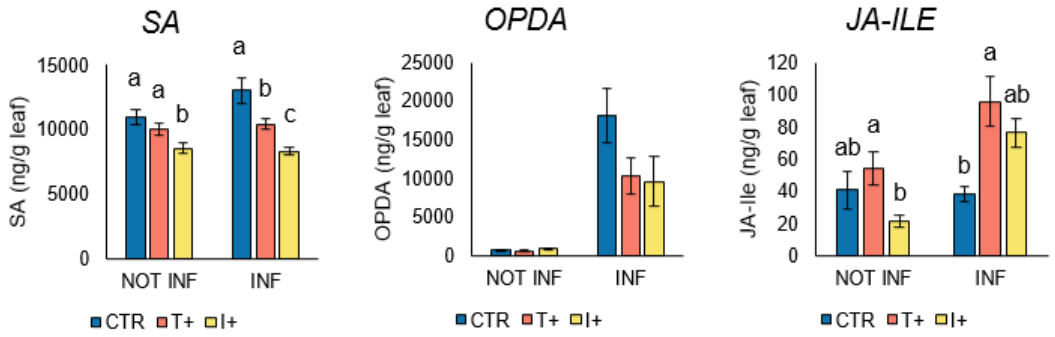

Figure 4. Hormone quantification of SA and JA related hormones in infected (INF) and non-inoculated (NOT INF) tomato leaves with Pst. The figures show the hormonal levels of SA, OPDA and JA-Ile in control (CTR), exudate treated (T+) and SI27 inoculated (I+) tomato at 72 hours post-infection (hpi). Error bar represents ± SE of the means of triplicates. Statistical differences according to ANOVA simple and test of Kruskal-Wallis for a 95% probability ($p<0.05$) are indicated by lowercase letters.

Screening of SI27 secondary metabolites to find bioactive antimicrobial compounds

The untargeted metabolomic analysis can be seen in figure 5. PCAs for both ion modes showed that different culture times implied separate groups of metabolites with cultures of 5 and 7 days having more similar profiles (Fig. 5a). The heatmap hinted at the compounds of interest by its higher presence in CF of 7 days (7D) than in 5 and 3 days (5D, 3D), and absent from control (CTR). The analysis was able to identify a list of 19 compounds that met the criteria for biological activity. However, at this point it was not possible to identify which of these compounds could be the main component responsible for the observed results.

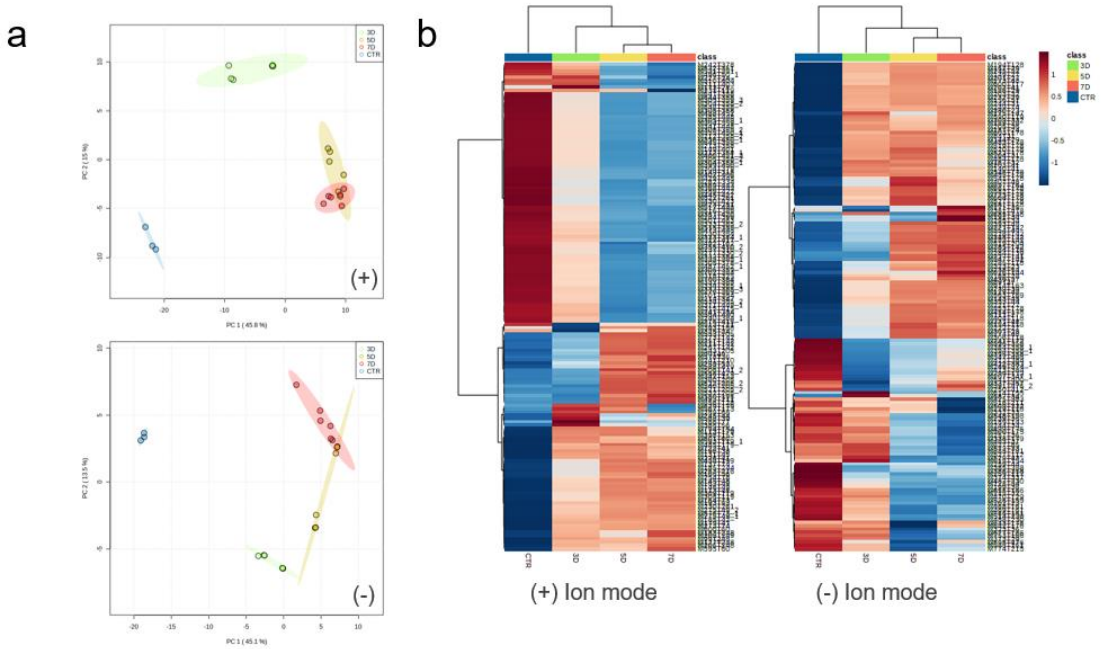

Figure 5. Metabolomic profile of culture filtrates of isolate SI27 with 3, 5 and 7 days of incubation in NB compared to fresh media in both ionization modes. Sample groups are identified as follows: control (green), 3-day-old CF (yellow), 5-day-old CF (orange), 7-day-old CF (red). (**a**) Principal Component Analysis (PCA) plots showing the clustering pattern of samples based on their metabolic profiles (**b**) Heatmaps showing the relative abundance of metabolites in different CF samples.

4. DISCUSSION

In recent years, endophytes have been progressively identified as a resource for plant protection (Arnold et al., 2003; Busby et al., 2016; Segaran & Sathiavelu, 2019). Despite these advances, much is still needed to dilucidate how these could actually improve plant resistance. Here, fungal isolate SI27 was evaluated as a symbiotic endophyte and as a source for antimicrobial compounds against Pst infection in tomato plants.

Influence of Culture Filtrate on Pst Growth: Impact of Culture Time and Nutrient Competition

Previous studies have demonstrated that the exudates of filamentous fungi can provide a rich source of antimicrobial compounds. One of the most potent groups

of antibacterials found in fungal exudates is the peptaibols family, which are short and linear peptides containing non-proteinogenic amino acids. Peptaibols belong to a widely represented group of AMPs and are biosynthesized by non-ribosomal peptide synthetases. These peptides adopt an α-helical conformation and possess an amphipathic nature that enables them to self-aggregate into oligomeric channel assemblies that span across the lipid bilayer (Castagnoli et al., 2018).

The use of fungal exudates has been also used as an alternative strategy to search for new antibiotics to combat antibiotic-resistant bacteria which involves blocking the expression of virulence factors. One example are the polyhydroxyanthraquinones that have been isolated from the exudates of *Penicillium restrictum*. These molecules inhibit a functional accessory gene regulator quorum-sensing system in a clinical isolate of methicillin-resistant Staphylococcus aureus (Figueroa et al., 2014). Another example are destruxins which are cyclic hexadepsipeptides composed of an α-hydroxy acid and five amino acids. Some destruxins possess anti-hepatitis B activity and some of the isolated compounds have revealed inhibitory activity against Zika virus RNA replication (Hutwimmer et al., 2010).

Our results showed that Pst growth was influenced by CF with CF of 7-day-old culture being the most effective. The obtained results on different culture times are likely associated with the stage of the fungal cycle in the cultures which can determine the production of exudates. The possibility of nutrient competition between fungi and bacteria was studied and rejected based on the capability of Pst to grow in *Alternaria* sp. In addition, the capability of Pst to somehow grow in 100% CF of 3 and 5 days indicated that these CF of Sl27 still had available nutrients for Pst growth. Thus, if no inhibitory effect was taking place (like with CF of *Alternaria sp*.), Pst would have a higher growth rate with CF of 3 and 5 days (CF:NB) than in control ($MgSO_4$:NB) Control growth is shown to be sometimes lower than in Alternaria sp. CF dilutions which might be caused by the remaining available nutrient in the fungal culture filtrate. In summary, the relative volume and the culture times were proportional to the inhibitory effect of Pst growth *in vitro*.

Variation of exudate concentration in media from 50% to 75% yielded similar values which indicated that the same inhibitory effect could be provided with the lesser volume of exudates (data not shown).

Comparison of Fungal Inoculation and Treatment with Fungal Exudates on Disease Incidence and Severity in Plants

The use of fungal endophytes for plant protection has been previously studied (Akram et al., 2023). It has been described that endophytes can use various mechanisms to protect host plants such as competition, induction of resistance or antibiosis. During the competition, endophytes create an unsuitable environment for pathogen growth by colonizing in the plant tissues and utilizing available nutrients, therefore preventing pathogen colonization. However, studies suggest that competition as a biocontrol mechanism is usually associated with with production of antimicrobial substances. An example of this is when an endophytic mixture obtained from cacao leaves was applied to the plant's foliage resulting in a significant reduction in *Phytophthora* infection due to competition. Nevertheless, certain strains were discovered to generate active metabolites implying that disease control may not be solely reliant on competition, according to research (Arnold & Herre, 2003).

Another mechanism used by endophytes is the induction of resistance, where endophytic fungi activate plant defense against a pest or a pathogen attack. This mechanism improves the plant defense response against future pathogen attacks and is known as induced systemic resistance or defense priming. Endophytes can also be associated with the upregulation of pathogenicity-related (PR) proteins, which include various enzymes such as chitinase and beta 1,3-glucanase that dissolve invading pathogen cells and strengthen the cell wall boundary to establish the ability to resist the infection and cell demise. For example, *Fusarium solani* was found to activate the *PR*, *PR7*, and *PR5* genes in the roots of tomato plants promoting ISR against the foliar pathogen *Septoria lycopersici* (Kavroulakis et al., 2007). In a different research, non-pathogenic mutant strains of Colletotrichum magna were observed to induce resistance in *Citrullus lanatus* and

Cucumis sativus plants. These strains produced large quantities of peroxidase, phenylalanine ammonia lyase enzyme and lignin deposition which helped protect the plants from infection caused by *C. orbiculare* and *F. oxysporum* (Redman et al., 1999).

To determine the influence of the fungal isolate, seed inoculation (I+) and treatment with fungal exudates (T+) were performed. As a result, disease incidence and severity were significantly reduced by both approaches displaying less disease symptoms on infected leaves.

In the current study, CF were applied by spraying over leaves and by irrigation on plant substrate. The injection method was discarded due to poor results probably caused by the damaging of leaf tissue during the process. While Pst is known to be the causal agent of leaf blackspot, plants responded worse when treated by spray instead of irrigation. Although Pst inoculation took place 48 h post treatment and no visual differences were found between spray-treated and non-spray-treated plants; leaf surface might indeed possess higher humidity due to higroscopy which could favor bacterial mobility and infection. On the other hand, since Sl27 is found to be a root endophyte, we hypothesized that the exudates produced could be easier applied when absorbed by the roots and spread through the xylem.

Incidentally, inoculation with Sl27 seemed to provide better phenotypic results than with the use of exudates. Yet, it is important to remember that in this study the use of CFs was done following a fixed timepoint and concentration. This could imply that the effects might vary if study parameters were changed, and the resistance could be greater. Nevertheless, which of the approaches is considered better will depend on the priorities of the agronomic practices. Isolate Sl27 has been proven to provide plant resistance long time after inoculation, which could be considered as an easier tool that does not involve treatments in grown plants, but application of exudates could be more adaptable based on field requirements.

Inoculation with Sl27 and treatment with Sl27 exudates entail different levels of hormones and gene expression in the plants

In natural environments, plants have mechanisms to cope with different stressors. To protect themselves from these threats, plants have evolved complex immune responses that are regulated by hormonal signaling pathways. The defense against biotic stressors is regulated mainly by salicylic acid (SA) and jasmonic acid (JA). The prevailing model suggests that SA-dependent defense responses control biotrophic pathogens while JA-dependent responses protect against necrotrophic pathogens and insect herbivores. However, the plant's association with certain beneficial symbiotic microorganisms can complement and even promote plant defenses (Pieterse et al., 2012). For instance, recent studies have shown that *Epichloë* symbionts can induce the activation of plant SA and/or JA signaling pathways, thus increasing the levels of resistance of plants against certain attackers including pathogens (Schmid et al., 2017). Therefore, the activation of these plant defensive signaling pathways by beneficial symbionts can have a significant impact on plant fitness and survival in natural environments.

Differences were observed between inoculated and treated plants in the transcriptomic analysis. Salicylic Acid pathway showed that inoculated plants and treated plants had significantly different levels of gene expression, at 72hours post-infection, while not always being different from control. This suggests that the defensive mechanism induced by the inoculation of the endophyte or the treatment with fungal exudates could not be related to the induction of defensive pathways, or at least, the induction of salicylic acid pathway.

Jasmonic acid and Ethylene pathways were also studied as they are essential for Induced systemic resistance (ISR). Again, a tendency was seen with I+ plants which showed lower gene expression than treated plants while the treatment with the culture filtrate has no differences with control plants.

These results seem to indicate that treatment with endophyte exudates and endophyte inoculation have different effects on plant resistance mechanisms to resist Pst infection. While both treatments could effectively reduce the incidence of the bacteria in plant leaves, the treatment with the exudate has no effect on promoting defensive mechanisms. For this reason, it seems plausible that the mode of action of this endophyte would be a combination of induced resistance

and the secretion of antimicrobial compounds. However, the antibiosis produced by fungal secretions in plant is difficult to demonstrate since it would need the verification that the secretions are in contact with the pathogen. This point could be difficult to determine due to the localization of the endophyte inside the plant and the low levels of the produced exudates (Latz et al., 2018).

Potential compounds with antimicrobial capabilities were assessed in an untargeted analysis

Results obtained show that the endophyte SL27 secretes molecules with antimicrobial effects and also suggest that these compounds have a strong effect on the induced plant protection. For this reason, metabolomic profiling was performed to find potential metabolites produced by Sl27 that would intervene in the defense mechanisms against Pst infection. The PCAs showed how CF of Sl27 were grouped differently based on the culture time and these groups were unsurprisingly far away from the control group. Taking into consideration the patterns seen in the *in vitro* assay for biocontrol capabilities of each of the CF, the compounds of interest are absent in control but have higher levels as culture time progresses. Based on this, we were able to reduce the number of metabolites to a list of 19 compounds. However, it was not possible to determine which of them has a bioactive effect.

It has been studied that the diversity of endophytes and their bioactive compounds depends on factors such as plant species, plant parts, and environmental factors, including cultivation history and climate (Mishra et al., 2022). For this reason, the identification of new metabolites using common libraries is rather difficult. Nevertheless, several studies have isolated new metabolites secreted by endophytes showing an antibacterial effect. Similar studies focused on fungal endophytes from leaves of *Indigofera suffruticosa* described the effect of ethyl acetate extract from *Nigrospora sphaerica* as a new biological source of drug candidates (dos Santos et al., 2015). Our next step will be to perform a study of the extract in order to determine which compound is the responsible one for the biological activity.

5. CONCLUSION

An endophytic fungal strain named Sl27 was isolated from tomato in previous studies, and presumedly classified as a *Leptobacillium* sp. The current study has proven the biocontrol capabilities of isolate Sl27 against *Pseudomonas syringae* pv. tomato. Exudates obtained from liquid fungal culture filtrates were able to suppress Pst growth *in vitro* and reduce Pst infection on tomato plants. Likewise, inoculation of tomato seeds with this endophytic fungus was seen to induce resistance against Pst infection on 4-week-old plants. Both approaches for conferring biotic resistance to plant were beneficial, yet they seemed to cause the host plant to respond differently, as showed by the results in the plant transcriptomic and hormonal analysis. This might indicate these approaches induce different mechanisms of defense for the plant to resist Pseudomonas infection, but both are certainly beneficial. Metabolomic analysis of Sl27 from culture filtrates suggested several potential compounds that match the patterns seen in our experiments. Further analysis could lead us to find a fungal metabolite with the potential to be used against biotic stress. Thus, though inoculation with e27 or treatment with e27 exudates clearly help plant resist Pst infection, the mechanisms involved are yet to be disclosed in following studies.

DISCUSSION

The research into the potential uses of beneficial endophytes is of current relevance. However, there is still much to explore to understand the diversity and potential benefits of endophytic fungi and how they can be harnessed for practical applications.

The first approach of this thesis was the study and analysis of the current state of the research field about endophytic fungi (**Chapter 1**). For this, a meta-review was carried out to study the main characteristics of interest in scientific reports. The meta-review differed from a common review in its ability to provide quantifiable data between studies. It also differed from a meta-analysis since it did not analyze the quantifiable results of each study which are often dependent on study parameters and not always comparable among them. We believe that a meta-review was crucial in providing knowledge about research limitations, as they highlight points of interest for future studies.

The meta-review was conducted using a set of specific keywords that are commonly used to identify endophytes. However, we acknowledge that certain studies may not have used the term "endophyte" and instead employed other terms such as "symbiotic fungus." Consequently, it is important to delimit the analysis accordingly. To prevent any biases arising from an overrepresentation of articles of the same study, the weighting of each study was adjusted such a way that only one value is included for a given endophytic fungus, specific function, and host plant.

In this work, exponential growth in the scientific literature on endophytic fungi was observed which demonstrates the significant interest in and broad scope of research in this field. One of the most notable study parameters was the diversity of the species investigated. The analysis revealed that the most extensively studied species, by a large margin, were the ones belonging to the genus *Epichloë* sp., which was the first endophyte discovered in 1988 and is known to colonize herbaceous plants such as *Lolium* sp. These species have been widely studied for a multitude of functions (Ravel et al., 1997; Shiba & Sugawara, 2005; West et al., 1988).

While most of the endophytes studied are ascomycetes, probably due to their usually higher growth rate (Arnold & Lutzoni, 2007) there is one basidiomycetous endophyte that has been of great interest since 1998: *Serendipita indica*, formerly known as *Piriformospora indica*. Initially believed to be a species of arbuscular mycorrhizal fungi (AMF), this endophyte has been extensively studied in both monocots and dicots. Several other well-known ascomycetes, including *Trichoderma*, *Penicillium* and *Fusarium*, have been the focus in various studies. Some researches also included species considered as dark septate endophytes (DSE). Almost 20% of the studies did not identify the fungal strain which was common in literature due to taxonomic identification problems (Porras-Alfaro & Bayman, 2011) where authors resorted to the use of common group names like DSE.

Monocots were the prevalent studied plant group, specially Poaceae plants, probably given their economic and cultural significance and their relatively straightforward study methodology. However, dicot plants as endophyte hosts still hold great potential that has yet to be fully explored.

The origin of the fungal isolates was another aspect that was investigated. At least one-third of the studies isolated the strains from wild species, mostly in natural environments with extreme conditions of study interest (e.g. saline, extreme temperatures). On the other hand, more than half of the studies were carried out on cultivated species including commercial species and commercial mutants. These findings confirm that the discovery of endophytes of wild origin for use in improving crop performance is more relevant than determining their role in a natural environment.

Over half of the analyzed studies focused on investigating the role of endophytes in alleviating abiotic stresses, such as high temperatures, water stress, salinity or heavy metal contamination. This is particularly significant given the current context of climate change and pollution. Studies that focused on growth promotion comprised about 20% of the total while those examining biotic stresses, including damage caused by pathogenic organisms such as bacteria and fungi were less common.

These results underscore the relevance of current endophytic fungal research. Moreover, there is a growing interest in exploring the genetic potential of traditional and wild plants to adapt and resist abiotic stresses. As a result, a study (**Chapter 2**) was conducted to analyze the differences that may arise among tomato genotypes in response to the increasing threat of heat stress.

To perform this study, 6 genotypes of tomato from several points of the Mediterranean area were selected: TH-30 (Greece); ADX2 (Spain); ISR-10 (Israel); MO-10 (France) and two commercial genotypes: Ailsa Craig (Ailsa) and Moneymaker (MM). The plants were submitted to a 42ºC heat stress for 6 hours followed by a recovery period of 2 hours.

We assessed the effects of heat stress by examining the phenotypic changes in leaf curvature and the relative water content (RWC) was used as an indicator of the water condition on leaf. As a result, TH-30 and ADX2 genotypes showed the highest dehydration and damage on its leaves, suggesting an inability to maintain leaf water content and resulting in leaf curvature and damage. Our results also indicated a decline in photosynthetic performance, particularly in the TH-30 and ADX2 genotypes which could be associated with their ability to maintain gas exchange during high-temperature stress and thus their heat tolerance (Bita & Gerats, 2013). Prior research indicated that transpiration cooling, which involves water loss through stomatal closure, is crucial in preventing heat damage in food crops (Deva et al., 2020). Our study supports this by highlighting the importance of transpiration, stomatal closure, leaf temperature (Tleaf) and cell damage parameters in understanding the variation in thermotolerance among plant species and genotypes.

We observed that different plant genotypes within the same species have varying capabilities in coping with heat stress, suggesting the activation of different mechanisms. This variability within and between species provides opportunities for improving crop heat-stress tolerance through genetic means, as previously suggested by Wahid (2007).

Our findings in chapter 1 and chapter 2 have highlighted the relevance of exploring the behavior of different tomato genotypes and the plant's endophytic

communities. Thus, our next step was to study the relation between these by examining the communities of endophytic microorganisms within these genotypes (**Chapter 3**). This was done through a microbiome analysis. According to (Berg et al., 2017; Vandenkoornhuyse et al., 2015), the plant microbiome is a crucial factor in modulating plant growth, health and resistance against biotic and abiotic stresses. Furthermore, investigating plant microbiome might be crucial in discovering new beneficial endophytes.

It is commonly accepted that the primary factor that shapes the microbiome is the soil. However, the genotype of the plant is also an important factor that can influence and interact with soil effects. Nowadays, plant microbiome research is focused on understanding how host-associated microbial communities are established, their structure and how they affect the host phenotype under different conditions.

Tomato microbiome has been previously studied by other authors to analyze the effect of substrate, the differences in the plant compartment and more. However, a study focused on the endophytic characterization based on different traditional and commercial tomato genotypes is missing. For our microbiome analysis, we used the six tomato genotypes studied in chapter 2 and dissected the endophytic microbial communities in them. We focused on stem microbiome because is less dependent on the environmental and soil conditions.

The study showed that traditional tomato genotypes had more unique fungal and bacterial taxa compared to commercial genotypes which hosted almost no unique taxa. PCoA and PERMANOVA analysis confirmed significant differences between the two groups. This raises questions about the shaping of the microbiome through selection and management of commercial cultivars.

Fungal communities were more abundant, possibly due to the higher presence of bacterial communities in root tissue compared to stem tissue. Additionally, the higher number of retrieved OTUs from fungi compared to bacteria could be due to differences in transmission and tolerance to harsh environments. Fungal species, for example, may require less environmental conditions and tolerate

more extreme environments than bacterial species (Cregger et al., 2018) leading to more coexistence of fungal species in plant tissue.

Fungal and bacterial communities were a reflection of each other in relative abundance between genotypes. Not all traditional genotypes were equal with ADX and MO having more microbial communities. The main fungal order observed in all genotypes was Sordariomycetes, followed by Leotidimicetes and Dothideomycetes. Bacteria had less identified taxa, and there was not a significant difference between the genotypes, though we could see the same tendency observed in fungal communities. The most abundant order was Flavobacteriales.

Therefore, the intrinsic characteristics of each plant species may influence the association with different core microbiomes which are consistently present through different environmental conditions. The core microbiome is thought to have co-evolved with the plant to provide certain host functions, such as adapting to environmental changes like soil quality (Podolich et al., 2015; BSingh et al., 2020).

Diversity was more similar but differences between the two groups were also seen in phylogenetic cluster. Likewise, our microbiome analysis has provided results in line with other studies on tomato (Dong et al., 2021; Manzotti et al., 2020), and has helped define the differences between study genotypes.

In addition, to prove the potential of traditional varieties and their probably different behavior, we studied the effect of a common human practice in field: the use of fungicides (a combination of tebuconazole and dichlofluanide) on the microbial communities of two traditional and two commercial tomato genotypes. Our findings showed that the use of fungicides indeed disturbs the tomato microbiome which is consistent with previous studies on the impacts of pesticides (Chen et al., 2021; Nettles et al., 2016; Vozniuk et al., 2019; Zhang et al., 2021). The diversity of the microbial communities, as measured by the Shannon Index, was reduced, and this effect was observed in all genotypes. However, it is likely that this shift in the microbial communities occurred at lower taxonomic levels, as the diversity and abundance at the order level did not change significantly, especially for bacteria, which was not the primary target of the fungicide treatment.

Surprisingly, the use of fungicides increased the richness of bacterial populations in tomato plants possibly due to the reduction in fungal populations, which could result in reduced competition within the plant tissues.

Therefore, we did not only prove the different behavior that different genotypes can have but also the differences between them at a microbial level. All this work has been fundamental to prove traditional tomato plants as a richer source of potential fungal endophytes compared to the commonly used commercial cultivars. Based on this data, we isolated many fungal strains from diverse traditional genotypes. Among them, we found a fungal strain with limited literature background which seemed to act as a neutral or beneficial endophyte. Then, we proceeded to perform a thorough characterization of this fungal isolate (**Chapter 4**).

The fungal strain was identified using ITS sequencing, supported by LSU and TUB sequencing. According to the BLAST analysis, it belonged to the genus *Lepobacillium sp.*, a group of fungal species previously classified as *Verticillium* sp. This genus has gained research interest as entomopathogenic and environmental species, with some reports of isolated strains from plant tissue, but no beneficial endophytic background in tomato.

Our novel isolate, Sl27, exhibited a slow-growing colony of whitish mycelium. At microscopical level it had long hyphal structures, erect single conidiophores, and small conidia which are all consistent with the general morphology of *Leptobacillium* sp. However, exact identification was not possible since its characteristics did not match well with its closest relative *Leptobacillium leptobactrum*. We thus propose this fungal isolate as a separate species, *L. solani*.

The development of the fungal mycelium was characterized under different conditions showing best growth at usual conditions of 25ºC. The isolate demonstrated the ability to produce siderophores and IAA, which could be great indicators of its potential to promote growth and induce resistance against stress, as other studies have reported its correlation. However, the extent of its phosphate solubilization was not clear.

Subsequently, we proceeded to investigate the impact of this endophyte on the host plant using the traditional tomato genotypes TH-30, ADX2 and MO-10. As mentioned earlier, these three genotypes exhibited distinct responses to heat stress, so we wanted to assess the effects of this endophyte on genotypes with diverse characteristics. The interaction between the endophyte and the plants was studied in growth chambers and greenhouses under controlled conditions at varying temperatures. The results indicated an increase in chlorophyll content and lycopene levels, particularly in TH-30. However, the impact on growth parameters such as shoot and root length varied among the tomato genotypes and was not consistently observed. This variability in plant responses has been reported in several studies since the study parameters and specific factors can influence the variability of the plant responses (Mayerhofer et al., 2013).

Isolate Sl27 was confirmed to behave as a root endophytic fungal strain, and although it may not have the best potential to promote plant growth under favorable conditions, its potential may lie in other functions such as inducing resistance against biotic resistance. This was done by studying the behavior of Sl27 against the bacterial plant pathogen *Pseudomonas syringae* pv. t*omato* (Pst) *in vitro* and in plant experiments (**Chapter 5**).

As previous studies have shown, microorganisms are able to produce many compounds with antimicrobial properties (Bérdy, 2005; Newman & Cragg, 2020). In our *in vitro* experiments, we proved that Sl27 can release exudates that inhibit Pst by using fungal culture filtrates (CF). This inhibition was proportionally higher based on the culture times of Sl27 and 7-day-old culture filtrate was the most effective. The experiment was performed with different parameters to ensure Pst inhibition was not due to nutrient scarcity.

Afterwards, in-plant experiments were carried out using Ailsa Craig tomato cultivar, a genotype that has been previously used for assessing interaction with Pst (Belimov et al., 2007; Camañes et al., 2015; González-Hernández et al., 2019). We proved the beneficial effects of Sl27 on disease caused by Pst by seed inoculation (I+) and by application of CF (T+). Previous studies reported that inoculation of plants with endophytes, as well as the application of endophytic

culture filtrates, induces defense mechanisms in the host plant (Zabalgogeazcoa, 2008). In this way, our results showed that in both treatments the incidence and severity were significantly reduced displaying less disease symptoms on infected leaves. However, there were differences in the transcriptomic analysis, with distinct patterns observed in the salicylic acid, jasmonic acid and ethylene pathways between inoculated and treated plants. The results suggest that the mode of action of the endophyte may involve a combination of induced resistance and secretion of antimicrobial compounds. However, demonstrating the antibiosis produced by fungal secretions in planta may be challenging due to the localization of the endophyte inside the plant and low levels of exudates produced.

Our study found that the endophyte SI27 secretes antimicrobial compounds that may play a role in inducing plant protection against Pst infection. Metabolomic profiling was performed to identify potential metabolites produced by SI27 and the results showed distinct patterns based on culture time. However, determining which specific metabolite is responsible for the bioactive effect was challenging due to the diversity of endophytes and their bioactive compounds, as influenced by various factors such as plant species, plant parts and environmental conditions. Despite that, the results suggest that either the isolate or the CF can have an effect on improving plant resistance, fungal endophytes have less frequently been reported to be involved in protection of their hosts via ISR (Hardoim et al., 2015). Nevertheless, it was suggested that some of the compounds that our isolated produced, such as siderophores, can be related with the induction of ISR or the protection against pathogens (Gautam & Avasthi, 2019). However, further studies including extract analysis are needed to identify the compound responsible for the observed biological activity.

In conclusion, this research adds to the expanding literature on fungal endophytes, providing valuable insights into the relationship between tomato genotypes and their microbiome; as well as the differences among various plant cultivars. The characterization of an endophytic isolate underscores the potential of endophytes as a promising source of novel drug candidates. Furthermore, the findings highlight the role of endophytes as an alternative approach for improving and

protecting high-quality crops of agronomic interest such as tomato. Overall, this study enhances our understanding of endophytes and their potential applications in agricultural sciences, offering new avenues for further research and practical applications in crop management and protection.

CONCLUSIONS

CONCLUSIONS

This thesis has examined the correlation between the microbiome and the tomato plant (*Solanum lycopersicum*) genotype, as well as its role in conferring resistance against certain abiotic and biotic stressors. In addition, the isolation, characterization, and application in plant of a novel endophytic fungal strain paves the way for further research in the field of agronomy.

Based on this study, several findings have been inferred which are outlined below:

1. Research of fungal endophytes has greatly progressed in the last decades. According to the performed meta-review, this field has been specially focused on wild grasses and other monocots. In addition, the most studied endophytes were identified (*Epichloë* sp., *Serendipita indica*, *Pennicillium* sp.). Studies focused primarily on their role in improving growth and protection against abiotic stresses (drought, heavy metals, salinity) for cultivated species (rice, wheat, maize). However, there are less studied areas, particularly in the function of endophytic fungi against bacterial pathogens.

2. The screening of six tomato (*S. lycopersicum*) genotypes under heat stress showed different responses in the plants, as reflected by the levels of phenotypic damage and photosynthetic parameters. Transcriptomic analysis revealed diverse thermotolerance strategies in the most resistant genotypes, related to leaf temperature maintenance or the activation of HSP90 and HSP70 genes, as well as SA-dependent pathways during recovery phase. This allowed the characterization of the traditional and commercial varieties and their classification as susceptible or resistant genotypes. These findings can help better understand the mechanisms involved in plant thermotolerance or recovery for crop selection and management.

3. Traditional and commercial tomato genotypes harbor different microbial communities as seen in the analysis of stem microbiome that was conducted in the six tomato genotypes previously mentioned. This demonstrated that traditional genotypes possess greater diversity and a distinct satellite

microbiota compared to commercial ones. These communities could influence the behavior and physiological responses of host plants to stress conditions. In addition, a generic fungicide treatment caused a change in microbial communities and a decrease in richness, especially for fungal taxa.

4. A new endophytic fungal strain has been isolated from the roots of a traditional tomato plant, named Sl27, which seems to be closely related to *Leptobacillium leptobactrum* according to the molecular identification. Sl27 has been morphologically characterized under various conditions and its ability to produce certain compounds of interest has been studied. In addition, its endophytic behavior in tomato plants under controlled conditions has been studied to determine its potential as a beneficial endophyte. Inoculation with Sl27 has shown neutral and positive effects and no harmful effects have been observed for 8 weeks. In particular, the beneficial effects have been more prominent for the TH-30 variety, including an increase in plant height, chlorophyll content and lycopene content in fruits.

5. The capacity of the Sl27 isolate as a biocontrol agent against Pseudomonas syringae pv. Tomato DC3000 (Pst) has been demonstrated. The exudates from fungal culture filtrates were able to suppress Pst growth *in vitro* and reduce Pst infection in 4-week-old tomato plants. Similarly, the inoculation of tomato seeds also induced resistance against Pst. Both approaches were effective but the plant responses in both were different, as reflected in the transcriptomic and hormonal analysis, where inoculated plants suggested lower expression of hormonal signaling pathways (SA, JA, ET) after 72 hours post-infection. Finally, the metabolomic profile of the Sl27 exudates showed diverse compounds of interest. Further analysis of these could lead to the discovery of Sl27 secondary metabolites with antimicrobial capacity.

CONCLUSIONS

La present tesi ha estudiat la relació entre el microbioma y el genotip de plantes de tomaca (*Solanum lycopersicum*), així com el paper que poden tindre en la resistència davant certs estressos abiòtics i biòtics. Amés amés, l'aïllament, caracterització i aplicació en planta d'una nueva cepa fúngica endòfita obri la porta per a futures investigacions d'aplicació agronòmica.

D'aquest treball s'han pogut obtindre unes conclusions principals que són:

1. La investigació de fongs endòfits ha progresat enormement en les últimes dècades. Segons la meta-revisió realitzada, este camp ha estat especialment enfocat en herbàcees silvestres y altres monocotiledònees. Ademés, s'han conegut els endòfits més estudiades (*Epichloë sp., Serendipita indica, Pennicillium sp*.) i cóm els estudis s'han centrat principalment en el seu paper per a la millora del creixement i protecció contra estressos abiòtics (sequia, metalls pessats, salinitat) en espècies cultivades (arròs, blat, dacsa). No obstant, existeixen àrees menys estudiades, particularment en la funció de gongs endòfits front a patògens bacterians.

2. L'estudi de sis genotips de tomaca (*S. lycopersicum*) baix estrés per altes temperaturas va manifestar diferents respostes en les plantes, com indiquen els nivells de dany fenotípic i els paràmetres fotosintètics. L'anàlisi transcriptòmic ha mostrado diverses estratègies de termotolerància en els genotips més resistents, relacionades amb el manteniment de temperatura foliar o la activació dels gens HSP90 i HSP70, així com en rutes dependents del SA durant la fase de recuperació. Tot açò va permetre la caracterització d'aquestes varietats tradicionals i comercials i la seua classificació com genotips susceptibles o resistents. Estos descobriments poden ajudar a compendre millor els mecanismes involucrats en la termotolerància o la recuperació de les plantes per a la selecció i el maneig de cultius.

3. Els genotips tradicionals i comercials de tomaca alberguen diferents comunitats microbianes, tal i com s'ha observat en el microbioma de la tija dels

sis genotips de tomaca prèviament mencionats. S'ha demostrat que els genotips tradicionals tenen una major diversitat i una microbiota satel·lit distinta en comparació amb els comercials. Estes comunitats podrien influenciar en el comportament i les respostes fisiològiques de les plantes hoste davant condicions d'estrés. Ademés, un tractament genèric amb fungicida ha provocat un canvi en les comunitats microbianes i una disminució de la riquesa, especialment per als taxons fúngics.

4. S'ha aïllat una nova cepa fúngica endòfita de les arrels d'una planta de tomaca tradicional, anomenada Sl27, que sembla estar estretament relacionada amb *Leptobacillium leptobactrum* basant-se amb la seua identificació molecular. S'ha caracteritzat morfològicament, front a diverses condicions, i s'ha estudiat la seua capacitat de produir certs compostos d'interés. Ademés, s'ha estudiat el seu comportament endòfit en planta de tomaca en condicions controlades per a determinar el seu potencial com endòfita beneficiosa. La inoculació amb Sl27 ha mostrat efectes neutrals i positius, i no s'han observat efectes perjudicials durant huit setmanes. En particular, els efectes beneficiosos han sigut més prominents per a la variedad TH-30, incloent un augment en la altura de les plantes, el contingut de clorofil·la i el contingut de licopeno en els fruits.

5. S'ha demostrat la capacidad de l'aïllat Sl27 com agent de biocontrol contra *Pseudomonas syringae* pv. Tomato DC3000 (Pst). Els exsudats dels filtrats de cultiu fúngic han pogut suprimir el creixement de Pst *in vitro* i reduir la infecció de Pst en plantes de tomaca de quatre setmanes. De manera similar, la inoculació de llavors de tomaca també ha pogut induir resistència contra Pst. Els dos mètodes han tingut la eficacia esperada, però les respostes de la planta en ambdós han sigut distintes, tal i como es reflecteix en l'anàlisi transcriptòmic i hormonal on les plantes inoculades sugereixen tindre menor expresió de rutes de senyalament hormonal (SA, JA, ET) despés de setantadós hores d'infecció. Finalment, el perfil metabolòmic dels exsudats de Sl27 va mostrar compostos diversos d'interés. Una anàlisi adicional de estos podria conduir a trobar metabolits secundaris de Sl27 amb capacitat antimicrobiana.

CONCLUSIONES

La presente tesis ha estudiado la relación entre el microbioma y el genotipo de plantas de tomate (*Solanum lycopersicum*), así como el papel que pueden tener en la resistencia ante ciertos estreses abióticos y bióticos. Además, el aislamiento, caracterización y aplicación en planta de una nueva cepa fúngica endófita abre la puerta para futuras investigaciones de aplicación agronómica.

De este trabajo se han podido obtener unas conclusiones principales que son:

1. La investigación de hongos endófitos ha progresado enormemente en las últimas décadas. Según la meta-revisión realizada, este campo ha estado especialmente enfocado en herbáceas silvestres y otras monocotiledóneas. Además, se ha conocido las endófitas más estudiadas (*Epichloë sp., Serendipita indica, Pennicillium sp*.) y cómo los estudios se han centrado principalmente en su papel para la mejora del crecimiento y protección contra estreses abióticos (sequía, metales pesados, salinidad) en especies cultivadas (arroz, trigo, maíz). Sin embargo, existen áreas menos estudiadas, particularmente en la función de hongos endófitos frente a patógenos bacterianos.

2. El estudio de seis genotipos de tomate (*S. lycopersicum*) bajo estrés por altas temperaturas manifestó diferentes respuestas en las plantas, como ha reflejado los niveles de daño fenotípico y los parámetros fotosintéticos. El análisis transcriptómico ha mostrado diversas estrategias de termotolerancia en los genotipos más resistentes, relacionadas con el mantenimiento de temperatura foliar o la activación de los genes HSP90 y HSP70, así como en rutas dependientes del SA durante la fase de recuperación. Todo esto permitió la caracterización de estas variedades tradicionales y comerciales y su clasificación como genotipos susceptibles o resistentes. Estos hallazgos pueden ayudar a comprender mejor los mecanismos involucrados en la termotolerancia o la recuperación de las plantas para la selección y el manejo de cultivos.

3. Los genotipos tradicionales y comerciales de tomate albergan diferentes comunidades microbianas, como se ha observado en el microbioma del tallo de los seis genotipos de tomate previamente mencionados. Se ha demostrado que los genotipos tradicionales poseen una mayor diversidad y una microbiota satélite distinta en comparación con los comerciales. Estas comunidades podrían influir en el comportamiento y las respuestas fisiológicas de las plantas huésped ante condiciones de estrés. Además, un tratamiento genérico con fungicida ha provocado un cambio en las comunidades microbianas y una disminución en la riqueza, especialmente para los taxones fúngicos.

4. Se ha aislado una nueva cepa fúngica endófita de las raíces de una planta de tomate tradicional, nombrado Sl27, que parece estar estrechamente relacionada con *Leptobacillium leptobactrum* basándose en su identificación molecular. Se ha caracterizado morfológicamente, frente a diversas condiciones, y se ha estudiado su capacidad de producir ciertos compuestos de interés. Además, se ha estudiado su comportamiento endófito en planta de tomate en condiciones controladas para determinar su potencial como endófita beneficiosa. La inoculación con Sl27 ha mostrado efectos neutrales y positivos, y no se han observado efectos perjudiciales durante 8 semanas. En particular, los efectos beneficiosos han sido más prominentes para la variedad TH-30, incluyendo un aumento en la altura de las plantas, el contenido de clorofila y el contenido de licopeno en los frutos.

5. Se ha demostrado la capacidad del aislado Sl27 como agente de biocontrol contra *Pseudomonas syringae* pv. Tomato DC3000 (Pst). Los exudados de los filtrados de cultivo fúngico han podido suprimir el crecimiento de Pst *in vitro* y reducir la infección de Pst en plantas de tomate de 4 semanas. De manera similar, la inoculación de semillas de tomate también ha podido inducir resistencia contra Pst. Ambos enfoques han sido eficaces, pero las respuestas de la planta en ambos han sido distintas, tal y como se refleja en el análisis transcriptómico y hormonal donde las plantas inoculadas sugieren tener menor expresión de rutas de señalamiento hormonal (SA, JA, ET) tras 72 horas de infección. Finalmente, el perfil metabolómico de los exudados de Sl27

mostró compuestos diversos de interés. Un análisis adicional de éstos podría conducir a encontrar metabolitos secundarios de Sl27 con capacidad antimicrobiana.

结论

本博士论文讨了微生物组与番茄(*S. lycopersicum*)基因型之间的相关性，以及其在对一些非生物和生物应激源的抗性方面的作用。此外，我们分离、表征并应用了一株新的内生真菌菌株，为农学领域的进一步研究铺平了道路。

从这项项研究中可以得出一些主要结论:

1. 近几十年来，真菌内生菌的研究取得了长足进展。根据所进行的元回顾，这个领域特别关注野生禾草和其他单子叶植物。此外，已经确定了最研究的内生菌（*Epichloë sp.*，*Serendipita indica*，*Pennicillium sp.*），研究重点主要集中在它们在促进栽培物种（如水稻、小麦和玉米）生长和抵御非生物胁迫（如干旱、重金属和盐度）方面的作用。然而，还有较少研究的领域，尤其是内生真菌在对抗细菌病原体方面的功能。

2. 对六种不同番茄（*S. lycopersicum*）基因型在热胁迫下的研究表明，植物在热胁迫下的反应存在差异，这反映在表型损伤水平和光合参数上。转录组学分析揭示了最抗性基因型的多种耐热性策略，包括通过叶温维持、HSP90 和 HSP70 基因的激活以及恢复阶段的水杨酸依赖性途径等。这可用于对传统和商业品种进行表征，并将它们分类为易感或抗性基因型。这些发现有助于更好地了解植物的耐热性或作物选择和管理恢复机制。

3. 根据对六种不同番茄基因型茎微生物组成的分析，研究表明传统和商业番茄基因型具有不同的微生物群落。与商业基因型相比，传统基因型拥有更多样化和独特的卫星微生物群，这些群落可能会影响寄主植物对胁迫条件的行为和生理反应。此外，通用杀菌剂处理会导致微生物群落发生变化和丰富度下降，尤其是真菌类群。这些发现对于深入了解植物微生物相互作用的重要性具有重要意义。

4.从传统番茄植物的根部分离出一株新的内生真菌菌株，命名为 SI27，根据分子鉴定，它似乎与 *Leptobacillium leptobactrum* 密切相关。 SI27 已经在各种条件下进行了形态学表征，并且已经研究了它产生某些感兴趣的化合物的能力。 此外，已经研究了其在受控条件下在番茄植物中的内生行为，以确定其作为有益内生菌

的潜力。 真菌接种已显示出中性和积极的影响，并且在 8 周内未观察到有害影响。特别是 TH-30 品种的有益效果更为突出，包括提高株高、叶绿素含量和果实中的番茄红素含量。

5. 已经验证了隔离的 SI27 作为生物防治剂对 *Pseudomonas syringae* pv. tomato DC3000（Pst）的有效性。真菌培养滤液的渗出物能够在体外抑制 Pst 的生长，同时也能够减少 4 周龄番茄植株的 Pst 感染。同样，在番茄种子中接种真菌也能够诱导对 Pst 的抗性。这两种方法都是有效的，但植物的反应不同，如转录组学和激素分析所反映的那样。接种植物表明在感染 72 小时后，激素信号通路（水杨酸、茉莉酸和乙烯）的表达较低。最后，SI27 渗出物的代谢组学特征显示出多种感兴趣的化合物。进一步分析这些化合物可能会发现具有抗菌能力的 SI27 次级代谢产物。

LIST OF ABBREVIATIONS

ABA: Abscisic acid

ACO: 1-aminocyclopropane-1-carboxylic acid oxidase

ADX2: Hanging tomato from Alcalà de Xivert

AIL: Ailsa Craig tomato cultivar

ANOVA: Analysis of Variance

APX: Ascorbate peroxidase

ASMT: Acetylserotonin methyltransferase

ASR: Abscisic acid stress ripening-induced proteins

BECs: Bacterial endophytic communities

BLAST: Basic local alignment search tool

BRs: Brassinosteroids

CAS: Chrome Azurol S

CAT: Catalase

CF: Culture filtrate

CFU: Colony forming units

CGA: chlorogenic acid

CKs: Cytokinins

CTAB: Cetyltrimethylammonium bromide

DHAR1: Dehydroascorbate reductase 1

DMSO: Dimethyl sulfoxide

DSE: Dark septate fungi

DW: Dry weight

E: Transpiration rate

EF: Elongation factor

ET: Ethylene

ETR: Electron transport rate

FA: Ferulic acid

FECs: Fungal endophytic communities

Fv/Fm: Maximum quantum efficiency

FW: Fresh weight

g_s: Stomatal conductance

HCA: Hierarchical cluster analysis

hpHS: Hours post heat shock

hpi: Hours post infection

HPLC: High performance liquid chromatography

HS: Heat stress

HSFs: heat shock transcription factors

HSPs: heat shock proteins

I+: Inoculation with Sl27

IAA: Indole-3-acetic acid

IR: Induced resistance

ISR: Induced systemic resistance

ISR-10: Tomato from Israel

ITS: Internal transcribed spacer

JA: Jasmonic acid

JA-Ile: jasmonyl-isoleucine

KB: King's B medium

LC: Liquid chromatography

LOX: lipoxygenase

LSD Least Significant Difference

LSU: Large ribosomal subunit

MEA: Malt-extract agar

MM: Money Maker tomato cultivar

MO-10: Red tomato from Montfavet

MS: Mass spectrophotometry

NA: Nutrient broth-agar

NB: Nutrient broth

OD: Optical density

OPDA: 12-oxo-phytodienoic acid

OPR3: 12-Oxophytodienoic Acid Reductase 3

OTU: Operational taxonomic unit

OTUs: Operational taxonomic units

PAL: Phenylalanine ammonia lyase

PCA: Principal component analysis

PCoA: Principal coordinate analysis

PCR: Polymerase chain reaction

PDA: Potato dextrose agar

PDB: Potato dextrose broth

PERMANOVA: Permutational Multivariate Analysis of Variance

Plant Growth Promoting Fungi (PGPF)

PR1: Pathogenesis-related protein 1

PR5: Pathogenesis-related protein 5

PSII: Photosystem II

Pst: Pseudomonas syringae pv. tomato

PVK: Pikovskaya medium

qRT-PCR: Real-Time Quantitative Reverse Transcription PCR

QTOF: Quadrupole-time of flight mass spectrometer

Rboh: Respiratory burst oxidase homologues

Rdw: Root dry weight

Rfw: Root fresh weight

RL: Root Length

ROS: Reactive oxygen species

rRNA: Ribosomal RNA

RWC: Relative water content

SA: Salicylic acid

SAR: Systemic acquired resistance

SD: Shoot basal diameter

Sdw: Shoot dry weight

SE: Standard error

Sfw: Shoot fresh weight

SL: Shoot Length

Sl27: Fungal isolate nº27 obtained from Solanum lycopersicum

smHSPs: Small HSPs, low molecular HSPs

SOD: Superoxide dismutase

SODct: Cytoplasmic superoxide dismutase

T+: Treatment with Sl27

TCP: Insoluble tricalcium phosphates

TH-30: Red tomato from Thessaloniki

Tleaf: Leaf temperature

TQD: Triple quadrupole mass spectrometer

TUB: Beta-tubulin

UPLC: Ultra-high performance liquid chromatography

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