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Estrés oxidativo en *Arabidopsis thaliana* en la interacción con *Trichoderma atroviride*

TESIS

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A mis abuelas y a mi madre, por su fortaleza y valentía como mujeres.

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ABREVIACIONES

6-PP – 6-pentil-2H-piran-2-ona

AIA – Ácido indol-3-acético

APx – Ascorbato peroxidasa

CAT – Catalasa

CDPK – "Ca^{2+} Dependent Protein Kinase"

CQ – Centro quiescente

DAB – 3,3' diaminobenzidina

DAMP – Patrones moleculares asociados a daños

Et – Etileno

GST –Glutatión S-transferasa

IAAld –Indol-3-acetaldehído

ICAld – Indol-3-carboxaldehído

IEt – Indol-3-etanol

ISR – Resistencia sistémica inducida

JA – Ácido jasmónico

MAMP – Patrones moleculares asociados a microbios

MAPK – "Mitogen-activated protein kinase"

NOX – oxidasas dependientes de NADPH

PAMP – Patrones moleculares asociados a patógenos

PDA – Agar papa-dextrosa

PRL – Primordios de raíces laterales

PRR – Receptores de reconocimiento de patógenos

RAM – “Root apical meristem”

RBOH – “NADPH oxidase/respiratory burst oxidase homolog”

RL – Raíces laterales

ROS – “Reactive oxygen species”

SA – Ácido salicílico

SAR – Resistencia sistémica adquirida

SM – Metabolitos secundarios

SOD – Superóxido dismutasa

SWEET – “Sugars Will Eventually be Exported Transporter proteins”

VOC – Compuestos orgánicos volátiles

WT – “Wild-type”

ZD – Zona de diferenciación

ZE – Zona de elongación

ZM – Zona meristemática

RESUMEN

Las especies reactivas de oxígeno (ROS) generadas por oxidasas dependientes de NADPH (NOX) funcionan como moléculas de señalización en procesos de defensa y diferenciación en animales, plantas y hongos. Recientemente, se ha descrito que estas enzimas actúan como mediadores en el establecimiento de relaciones benéficas entre plantas y microorganismos, entre los que destacan los hongos filamentosos del género *Trichoderma*, los cuales modulan el crecimiento y la inmunidad vegetal. En este trabajo se muestra que las mutantes Nox (Δ*noxR*, Δ*nox1* y Δ*nox2*) de *T. atroviride* presentan una afectación sobre la capacidad de estimular el crecimiento de *Arabidopsis*. Además, el co-cultivo con la mutante Δ*noxR* provoca una exacerbada respuesta inmune dependiente del ácido jasmónico en las raíces en comparación con la cepa silvestre (WT). De acuerdo con el perfil de expresión génica global realizada en el hongo, existe una importante represión sobre genes relacionados en la degradación de carbohidratos complejos ante la percepción de la planta, lo cual se encuentra ausente en la mutante Δ*noxR*, por lo que el mantenimiento del comportamiento saprófito del hongo compromete la reprogramación transcripcional que permite el equilibrio entre el crecimiento y la defensa.

Por el contrario, el estrés oxidativo durante la colonización de la raíz también influye directamente sobre el desarrollo. La respuesta de plántulas de *Arabidopsis* y mutantes afectadas en genes que codifican para los homólogos de oxidasa de explosión respiratoria (RBOH) bajo el efecto bio-estimulante de *Trichoderma*, indica que la pérdida de función de los genes *RBOHA*, *RBOHD* y *RBHOE*, compromete la ramificación y biomasa inducida por el hongo. De acuerdo con la prueba fluorescente para ROS totales y la tinción de DAB, existe una mayor acumulación de ROS en las puntas de las raíces y sobre los sitios de formación de raíces laterales en plantas inoculadas con *Trichoderma*. La acidificación del sustrato y la emisión del compuesto orgánico volátil 6-pentil-2H-piran-2-ona (6-PP) parecen ser los principales factores por los que el hongo desencadena la acumulación de ROS en las raíces y causa la ramificación de estas. Finalmente,

se reporta que el receptor de tipo cinasa PEPR2 actúa como un regulador río arriba de la actividad enzimática de las RBOH. Estos datos en conjunto demuestran la función de las ROS como mensajeros clave para los procesos de reconocimiento y adecuación del metabolismo en el hongo, así como reguladores de la arquitectura de la raíz durante la interacción con *Trichoderma.*

Palabras clave: *Trichoderma atroviride*, *Arabidopsis thaliana*, especies reactivas de oxígeno, biomasa vegetal, desarrollo radicular.

ABSTRACT

Reactive oxygen species (ROS) generated by NADPH-dependent oxidases (NOX) function as signaling molecules in defense and differentiation processes in animals, plants, and fungi. It has recently been described that these NOX enzymes act as mediators in the establishment of beneficial relationships between plants and microorganisms. *Trichoderma* induces the plant defense system and promotes growth of their hosts. In this study, we report that Nox mutants (Δ*noxR*, Δ*nox1*, and Δ*nox2*) from *T. atroviride* have an affectation in the ability to stimulate the growth of *Arabidopsis* seedlings. Furthermore, co-cultivation with the Δ*noxR* mutant elicits an exacerbated jasmonic acid-dependent immune response in the roots compared to the wild-type (WT) strain. Global gene expression analysis in the filamentous fungi reveals an important repression on genes related to the degradation of complex carbohydrates before the perception of the plant, which is absent in the Δ*noxR* mutant and can be related to the maintenance of the saprophytic behavior of the fungus.

Besides, the oxidative stress caused by root colonization directly influences plant development. The response of wild-type (WT) *Arabidopsis* seedlings and mutants in genes encoding respiratory burst oxidase homologues (RBOH) under the biostimulating effect of *Trichoderma* was evaluated. It was found that the loss of function of the RBOHA, RBOHD and RBHOE genes compromises the root branching in WT plants. The fungus also enhances ROS accumulation in primary root tips, in lateral root formation sites and emerged lateral roots as revealed by total ROS imaging via the fluorescent probe and DAB detection. Acidification of the substrate and emission of the volatile organic compound 6-pentyl-2H-pyran-2-one (6-PP) appears to be major factors by which the fungus triggers ROS accumulation, which accounts for more lateral roots being formed during the root–fungal interaction. Finally, the kinase-like receptor PEPR2 acts as an upstream regulator of RBOH enzymatic activity. Taken together, these data demonstrate the role of ROS as key messengers for recognition processes and metabolic fitness in

the fungus, as well as regulators of root architecture during interaction with *Trichoderma.*

Keywords: *Trichoderma atroviride*, *Arabidopsis thaliana*, reactive oxygen species, plant biomass, root development.

I. INTRODUCCIÓN

En el suelo, las raíces cohabitan con diferentes tipos de microorganismos como bacterias, hongos, actinomicetos, protozoos y algas (Hassani *et al*., 2018). Algunos de estos microorganismos pueden colonizar la superficie de las raíces o crecer dentro de las mismas y causar efectos positivos o negativos sobre el crecimiento, la salud y la productividad de las plantas (Zhalnina *et al*., 2018).

Las raíces secretan substancias que atraen microorganismos benéficos y contribuyen a mantener interacciones simbióticas a través de la liberación de diferentes compuestos, incluyendo azúcares, aminoácidos, ácidos orgánicos, compuestos fenólicos, enzimas, fitohormonas y vitaminas (Pascale *et al*., 2020). Los hongos pertenecientes al género *Trichoderma* se encuentran con frecuencia en la rizosfera, influenciada por la presencia de azúcares y otros exudados (Macías-Rodríguez *et al*., 2018; Villalobos-Escobedo *et al*., 2020; Esparza-Reynoso *et al*., 2021). Esta relación se fortalece mediante el establecimiento de una comunicación química íntima en la que la asimilación de nutrientes y la emisión de compuestos como auxinas, compuestos orgánicos volátiles (VOC) como la 6-pentil-piran-2-ona (6-PP) y péptidos liberados por el hongo mejoran el crecimiento de las raíces y la resistencia sistémica a patógenos (Contreras-Cornejo *et al*., 2009; Garnica-Vergara *et al*., 2016; Villalobos-Escobedo *et al*., 2020; Wang *et al*., 2020).

La producción/emisión de volátiles con efectos sobre el crecimiento de las plantas y sobre la actividad fungistática en *T. atroviride* depende en gran medida de la actividad funcional de las NADPH oxidasas (NOX), enzimas que producen especies reactivas de oxígeno (ROS) en respuesta a estímulos bióticos y abióticos (Cruz-Magalhães *et al.*, 2019). Las NOX fúngicas participan en la formación de conidias, procesos de virulencia e interacciones con las plantas (Segal y Wilson 2018). Hernández-Oñate y col. (2012) identificaron los genes *Nox1* y *Nox2*, que codifican subunidades catalíticas del complejo NOX, así como *NoxR* que codifica una subunidad reguladora. La pérdida de función de *Nox1* y *NoxR* afecta la diferenciación inducida por daño mecánico, mientras que el papel de *Nox2* se

relaciona directamente con la producción de COV con actividad fungistática, incluyendo 6-PP, β-bergamoteno (ST-1) y algunos diterpenos (Hernández-Oñate *et al*., 2012; Cruz-Magalhães *et al.*, 2019). Además, los hongos endófitos y micorrízicos también presentan un estallido oxidativo tras la colonización de raíces, lo que implica un papel directo de los genes *Nox* y ROS fúngicos en el establecimiento de la simbiosis (Abbà *et al*., 2009).

Recientemente, se ha enfatizado el papel que podrían tener las ROS en las plantas como un mecanismo de defensa ante moléculas efectoras producidas por *Trichoderma* como la resistencia sistémica adquirida (SAR) y la resistencia sistémica inducida (ISR) (Ramírez-Valdespino *et al*., 2019; Alfiky *et al*., 2021; González-López *et al*., 2021). Los efectores secretados por microorganismos son percibidos por los receptores de reconocimiento de patógenos (PRR), entre los que destacan los receptores de unión a nucleótidos con repetidos ricos en leucina (NLR), los cuales activan la producción de ROS vía NOX, denominadas como homólogos de oxidasa de explosión respiratoria (RBOH) en las plantas (Hu *et al*., 2020). Las RBOH abarcan un grupo de enzimas unidas a la membrana que tienen homología con el fagocito de mamífero gp91phox (NOX2) que se activan principalmente en respuesta a la entrada rápida de Ca^{2+} o la fosforilación inducida por proteínas cinasas (Chapman *et al.,* 2019; Lee *et al.,* 2020). Aunque la mayoría de las isoformas como RBOHD y RBOHF desempeñan un papel importante en la producción de ROS durante la señalización del estrés abiótico, así como en la respuesta inmunitaria de las plantas; también participan en programas de desarrollo como es la formación del tubo polínico, la elongación de pelos radiculares o la emergencia de raíces laterales (RL) (Otulak-Kozieł *et al*., 2020).

En este estudio se caracterizó el papel de los genes *Nox* de *T. atroviride* y sus homólogos RBOH en *Arabidopsis* durante la interacción. Primeramente, se demostró que *NoxR* es necesario para la ramificación de la raíz y la producción de biomasa por *Trichoderma*, ya que la cepa mutante Δ*noxR* pierde la capacidad manifiesta en la cepa silvestre de inducir la transición de los primordios de raíces laterales a las raíces laterales maduras. Por el contrario, la expresión del factor de

respuesta al ácido jasmónico *JAZ1* se incrementa en las raíces inoculadas con la mutante Δ*noxR*, reforzando así la inmunidad vegetal. Además, se muestra que *T. atroviride* influye sobre la expresión de los genes implicados en el metabolismo de azúcares presentes en los exudados radicales. En la segunda parte del trabajo, se determinó que la producción de ROS mediada por las enzimas RBOH es necesaria para la ramificación de raíces y la producción de biomasa por *T. atroviride* dependiente de los genes *respiratory burst oxidase homologues* (RBOH) *rbohA*, *rbohD* y *rbohE,* cuyos mutantes presentan una disminución de la ramificación de las raíces provocada por la presencia del hongo. Además, se correlaciona la actividad del receptor tipo cinasa PEPR2 con la activación de las enzimas RBOH por la presencia de *Trichoderma*. Con los resultados anteriores, se propone que la percepción del pH ácido o el compuesto volátil 6-PP causan un estallido oxidativo que se observa en las raíces previamente al contacto con el hongo y depende en gran medida de la actividad del complejo NOX.

II. ANTECEDENTES

2.1 *Arabidopsis* como modelo de estudio

Arabidopsis thaliana es una planta herbácea de ciclo anual, nativa de Eurasia y África, que pertenece a la familia *Brassicaceae* en el grupo eudicotiledóneas de las plantas vasculares angiospermas, la cual incluye especies de importancia económica, tanto hortícolas, ornamentales y oleaginosas (Provart *et al*., 2016). Es considerada como el organismo modelo para el estudio de la biología vegetal, debido a que posee una serie de características idóneas para su manipulación y cultivo, como es tener un tamaño pequeño y un ciclo de vida rápido (6-8 semanas) (Woodward y Bartel, 2018). Además, genera una gran cantidad de semillas por cada generación, ya que posee una fecundación autógama y elevada fertilidad (Padole e Ingle, 2017). Cuenta con un genoma diploide relativamente pequeño con alrededor de 125 Mbp y cinco cromosomas, el

cual fue secuenciado desde el año 2000, estimándose que contiene alrededor de 27,000 genes, 4827 pseudogenes y genes de elementos transponibles y 1359 moléculas de ARN no codificantes (Feldmann y Goff, 2014). La disponibilidad de su genoma, junto con el gran acervo de mutantes, herramientas bioinformáticas y líneas con construcciones de genes reporteros dirigidos por diversos promotores, han permitido la elucidación de diversas vías de señalización presentes en la planta, favoreciendo incluso el estudio sobre otras especies eucariotas (Woodward y Bartel, 2018). Su morfología consiste en una roseta densa de hojas basales con margen dentado que, además, presenta tallos erectos poco o nada ramificados de alrededor de 20 a 25 cm, los cuales también poseen hojas más pequeñas que carecen de peciolo y que están cubiertas de tricomas (Fig. 1A). Las flores están conformadas de cuatro sépalos y pétalos, seis estambres y dos carpelos, y su disposición se conforma en una inflorescencia denominada corimbo, estas flores dan origen a frutos en forma de silicua, la cual contiene alrededor de 20 a 30 semillas de color marrón rojizo de forma ovalada (Fig. 1B-E). Asimismo, posee una raíz primaria única de la cual se originan las raíces laterales (Krämer, 2015).

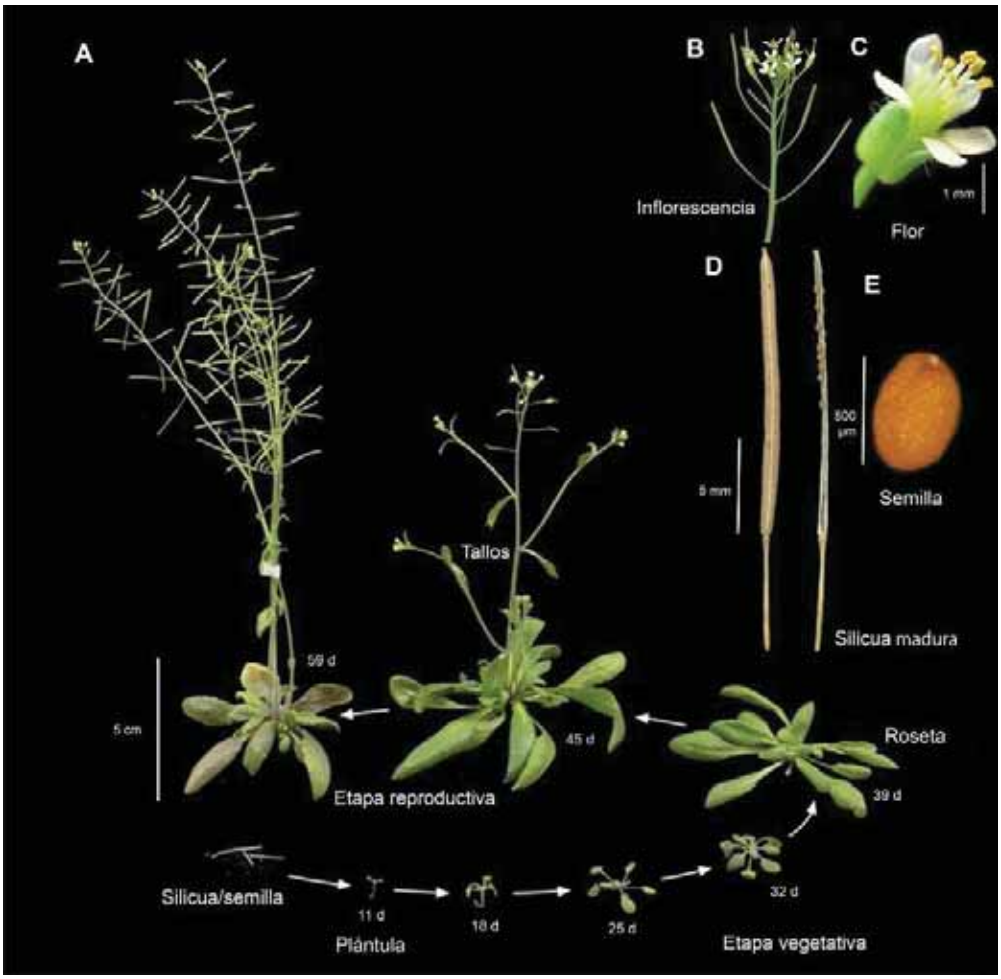

Figura 1. Ciclo de vida de *Arabidopsis*. Desarrollo de *A. thaliana* (ecotipo Columbia-0) desde la semilla hasta la etapa de plántula (11 días), crecimiento vegetativo (39 días) y crecimiento reproductivo (45 días) (A). Fotografías amplificadas de estructuras reproductivas como inflorescencia (B), flor (C), silicuas (D) y semilla (E). Modificado de Krämer (2015).

2.1.1 Sistema radicular de *A. thaliana*

La raíz es un órgano que crece por debajo del suelo, el cual responde a una variedad de estímulos ambientales. No solo brinda soporte estructural a la parte aérea, sino que también lleva a cabo la absorción de agua y nutrientes necesarios para el crecimiento (Nelissen y Gonzalez, 2020). Por lo tanto, la supervivencia general de la planta depende del desarrollo, crecimiento y función apropiados de la raíz.

A diferencia de otros modelos vegetales, la arquitectura y la estructura de la raíz de *Arabidopsis* es muy sencilla. Presenta una raíz primaria de origen embrionario, que mantiene un crecimiento indeterminado y dinámico a lo largo del ciclo de vida (Smith y De Smet, 2012). Su anatomía consiste en un meristemo apical (RAM, por sus siglas en inglés) conformado por un nicho de células iniciales que rodean un par células mitóticamente inactivas, denominadas centro quiescente (CQ) (García-Gómez *et al*., 2021). A partir de estas células iniciales se originan columnas o filas de células que dan origen a tejidos específicos dispuestos en círculos concéntricos como la epidermis, la corteza, la endodermis, el periciclo, el xilema y el floema. No obstante, también se producen células en dirección contraria como es el caso de la capa de tejido llamada cofia, que abarca el extremo distal, cuya función es proteger a la raíz conforme ésta crece a través del suelo (Petricka *et al*., 2012). La raíz primaria se divide en tres zonas consecutivas a lo largo de su eje longitudinal. El área más cercana al CQ corresponde a la zona meristemática (ZM), en donde las células se encuentran en constante división. Estas células cuando pasan por un proceso de expansión celular conforman la zona de elongación (ZE). Posteriormente, entran en la zona de diferenciación (ZD) (Fig. 2A). Morfológicamente, la ZD está marcada por la aparición de pelos radiculares epidérmicos (tricoblastos), la maduración del xilema y la formación de raíces laterales (RL) (Pavelescu *et al*., 2018).

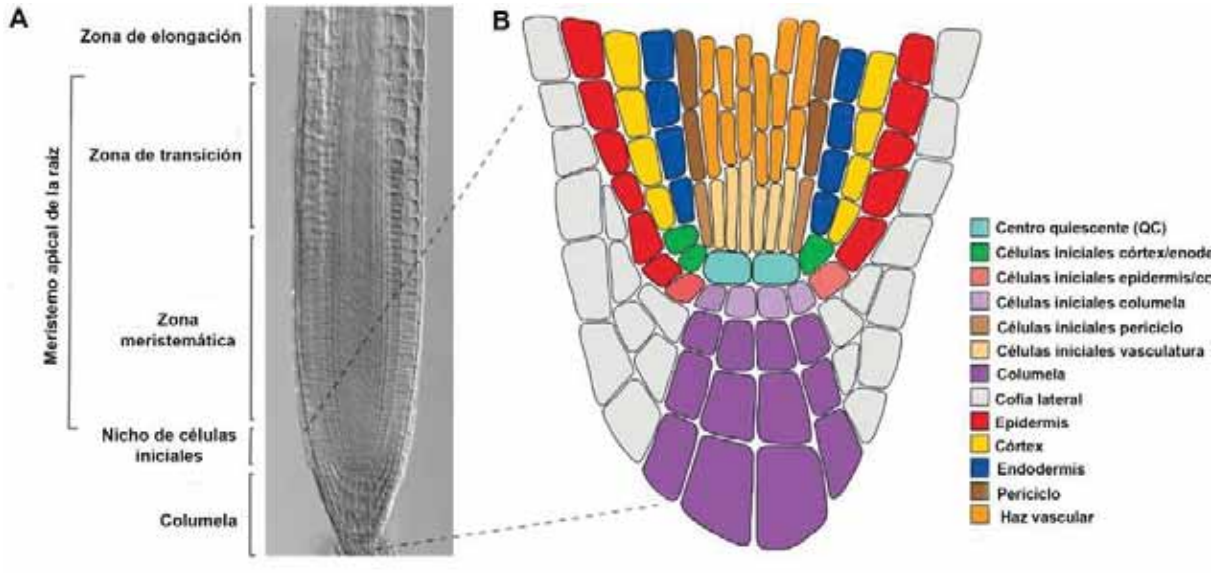

Figura 2. Anatomía y morfología de la punta de la raíz de *Arabidopsis*. (A) Ubicación de las células iniciales, zona de elongación, zona de transición y zona meristemática. (B) Corte longitudinal de la raíz donde se muestran las diferentes zonas y tejidos. Modificado de García-Gómez *et al.* (2021).

Las RL son cruciales para aumentar el área de superficie del sistema radicular y permitir una mayor exploración y captación de nutrientes y agua en ambientes de suelo heterogéneos. Las RL de *Arabidopsis* se originan exclusivamente a partir de células fundadoras del pericicio ubicadas en polos opuestos del xilema (Fig. 3A) (Banda *et al*., 2019). Las raíces laterales se inician cuando las células fundadoras individuales o pares de estas del pericicio se someten a varias rondas de divisiones anticlinales mediante una señalización por auxinas para crear un primordio de raíz lateral (LRP), el cual empieza con una capa de diez células pequeñas de igual longitud (Etapa I) (Du y Scheres, 2018). Posteriormente, las células se dividen periclinalmente, formando una capa interna y otra externa (Etapa II). Después de otras divisiones anticlinales y periclinales se crea un primordio en forma de cúpula (abarcando las Etapas III-VII) que eventualmente emergerá de la raíz parental (Etapa VIII) (Fig. 3B) Después de la emergencia, el meristemo apical de la RL se activa y comienza a crecer (Péret *et al*., 2009).

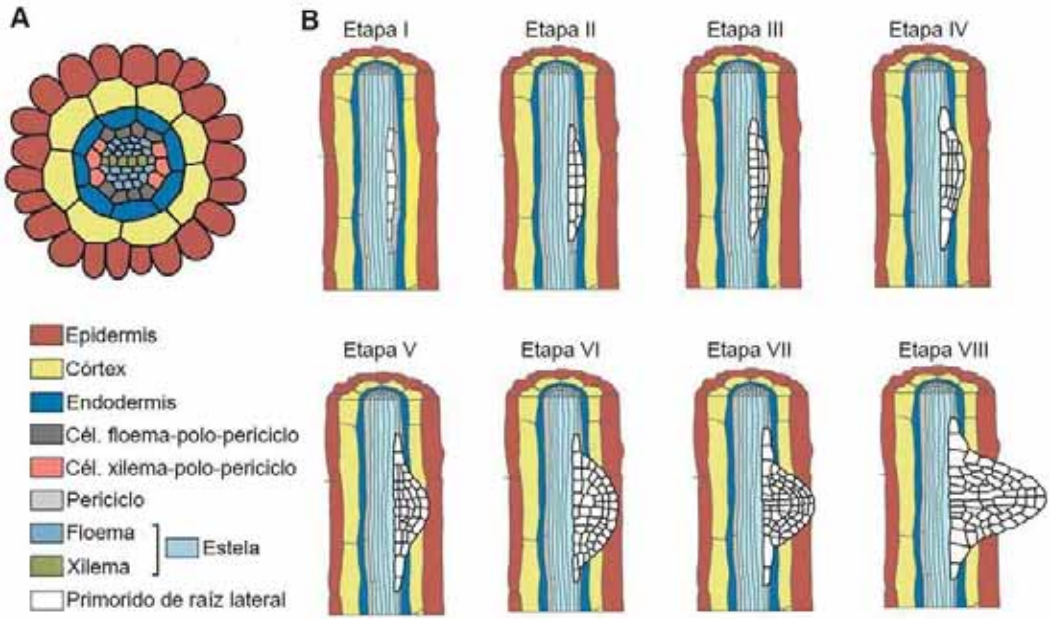

Figura 3. Formación y desarrollo de las raíces laterales en *Arabidopsis*. (A) Representación esquemática de la sección transversal de la zona de diferenciación de la raíz primaria. La raíz tiene una estructura simple compuesta por la estela (pericíclo y vasculatura) rodeada por tres capas unicelulares, endodermis, corteza y epidermis. (B) Las ocho etapas de iniciación y desarrollo de la raíz lateral. Las células fundadoras de la raíz lateral se especifican en el pericíclo del polo del xilema y pasan por ocho etapas de desarrollo para emerger en la superficie de la raíz. Tomado de Jing y Strader (2019).

2.2 Rizósfera

La rizósfera es la región del suelo adyacente a las raíces de las plantas, la cual se ve influenciada física, química y biológicamente por la actividad de los microorganismos (York *et al*., 2016). La rizósfera se compone de tres zonas, endorrizósfera, rizoplano y ectorrizósfera, las cuales están definidas con base en la influencia y proximidad de la raíz (York *et al*., 2016). A través de la liberación de moléculas bioactivas, las raíces pueden modificar la microbiota del suelo (Hu *et al*., 2018). Estos exudados radiculares generalmente comprenden metabolitos primarios como azúcares, aminoácidos y ácidos carboxílicos, así como un conjunto diverso de metabolitos secundarios y compuestos de alto peso molecular como mucílagos y proteínas (Canarini *et al*., 2019). La cantidad y el tipo de

exudados de las raíces están determinadas por la especie, la edad de la planta o por factores externos (Canarini *et al*., 2019). Los exudados radiculares representan una fuente de carbono y nitrógeno para el crecimiento microbiano, no obstante, los compuestos liberados por las raíces pueden actuar como moléculas señalizadoras y atrayentes o como inhibidores o repelentes (Fig. 4) (Hu *et al*., 2018; de la Fuente Cantó *et al*., 2020).

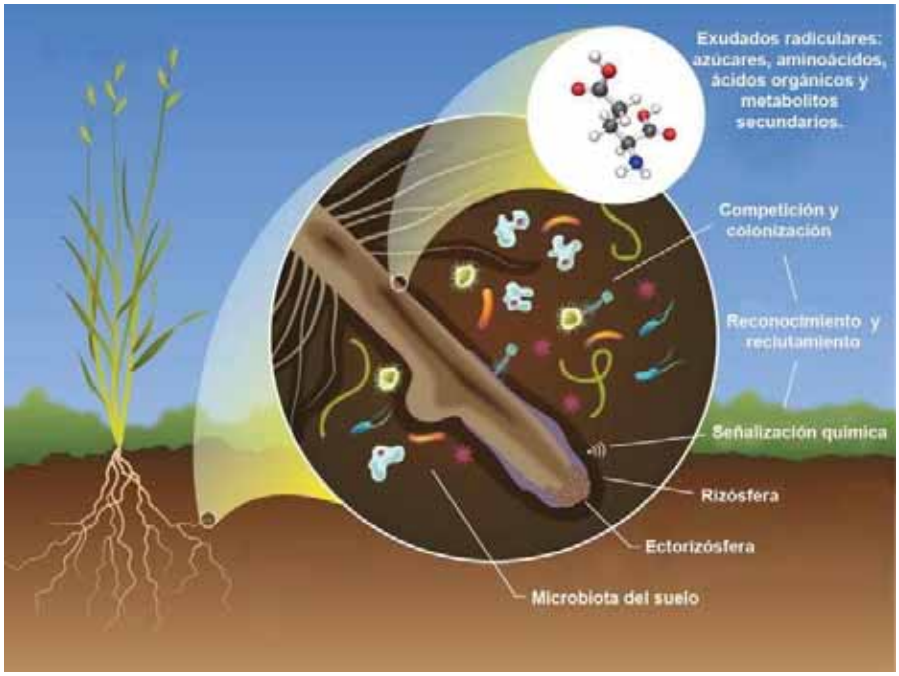

Figura 4. Representación de los componentes y procesos de la rizósfera. La rizósfera es un entorno dinámico de actividad metabólica entre las raíces de las plantas y microorganismos del suelo. Tomado de White *et al.* (2017).

Los microorganismos asociados a la rizósfera no solo perciben e interpretan señales producidas por las plantas, sino que también responden a los producidos por ellos mismos y de otros microorganismos. Además, estos microorganismos liberan diversas moléculas de señalización en la rizósfera que influyen directamente sobre las respuestas quimio-tácticas de la raíz (Venturi y Keel, 2016). En las interacciones benéficas establecidas entre plantas y microorganismos generalmente se promueve el crecimiento y desarrollo vegetal,

se refuerzan las respuestas de defensas contra enfermedades, plagas y factores abióticos, e incluso se mejora la solubilidad de nutrientes. Entre los microorganismos benéficos presentes en la rizósfera se encuentran los hongos micorrízicos, bacterias promotoras del crecimiento vegetal (PGPR) y hongos promotores del crecimiento vegetal (PGPF), por mencionar algunos grupos (Venturi y Keel, 2016).

2.3 El género fúngico *Trichoderma*

Trichoderma es un género de hongos filamentosos, residentes naturales de la rizósfera. Se encuentran distribuidos ampliamente en diversos ecosistemas del planeta como saprófitos facultativos que degradan la materia orgánica en descomposición (Guzmán-Guzmán *et al*., 2019). La mayoría de las especies de *Trichoderma* no tienen una etapa sexual, ya que producen esporas asexuales denominadas conidios (Fig. 5A-B) (Kredics *et al*., 2021). Su clasificación taxonómica corresponde al dominio Eukaryota, reino Fungi, división Ascomycota, subdivisión Pezizomycotina, clase Sordariomycetes, orden Hypocreales y familia *Hypocreaceae*. El género *Hypocrea*/*Trichoderma* incluye más de 375 especies caracterizadas molecular y morfológicamente (Tyśkiewicz *et al*., 2022).

Diversas especies de *Trichoderma* manifiestan un potencial extraordinario para producir enzimas industrialmente importantes o compuestos de interés farmacológico (Kredics *et al*., 2021). Por otra parte, se les reconoce por antagonizar a otros micro-organismos, muy útil en el biocontrol de fitopatógenos (Fig. 5C) (Harman, 2006; Guzmán-Guzmán *et al*., 2019). Ciertas especies actúan como simbiontes oportunistas avirulentos de plantas, ya que pueden crecer como endófitos en las raíces y en algunos casos colonizan partes aéreas, mejorando el crecimiento y desarrollo a través de diferentes mecanismos, entre los que destacan la síntesis de fitohormonas, la solubilización de nutrientes, la ramificación de las raíces, incremento en la capacidad fotosintética y metabolismo de carbono, así como la protección y tolerancia al estrés biótico y abiótico (Contreras-Cornejo *et al*., 2016; Sood *et al*., 2020; Pelagio-Flores *et al*., 2022).

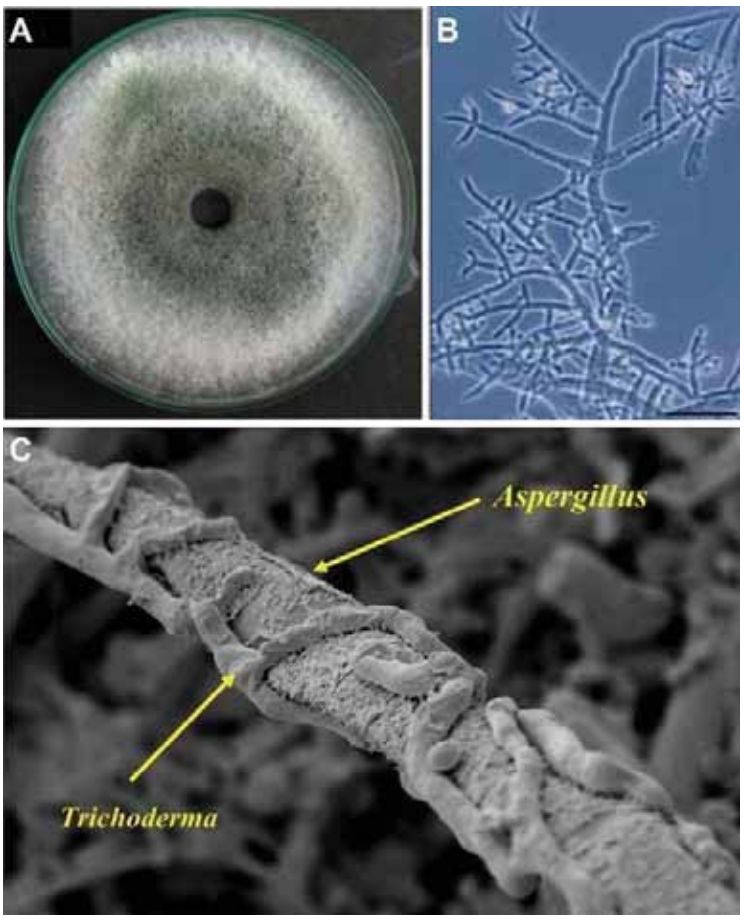

Figura 5. Características morfológicas de *Trichoderma*. (A) Colonia de una especie de *Trichoderma* cultivada en agar papa-dextrosa (PDA), mostrando un abundante micelio aéreo con anillos concéntricos y la presencia de conidios de color verde brillante. (B) Vista microscópica de un conidióforo ramificado. (C) Parasitismo de *Trichoderma* sobre *Aspergillus flavus*. Modificado de Jaklitsch *et al.* (2013) y Kifle *et al.* (2016).

2.4 Interacción *Arabidopsis-Trichoderma*

2.4.1 Regulación del crecimiento vegetal

Algunas especies de *Trichoderma* mejoran el crecimiento de las plantas y modifican la arquitectura de la raíz mediante la producción de fitohormonas y otras moléculas de señalización (Contreras-Cornejo *et al*., 2009; Nieto-Jacobo *et al*. 2017). De acuerdo con el trabajo realizado por Contreras-Cornejo y col. (2009), las

plántulas de *Arabidopsis* crecidas con *Trichoderma virens* Gv29.8 y *T. atroviride* IMI206040 presentaron una mayor formación de raíces laterales y biomasa que las crecidas axénicamente (Fig. 6A-D). Esta estimulación por parte de ambas especies de *Trichoderma* se relacionó con la producción de auxinas por el hongo, por lo que al evaluar la participación de genes involucrados en el transporte o la señalización como *AUX1*, *BIG*, *EIR1* y *AXR1*, se demostró que en *Arabidopsis*, se requiere de la vía de transducción de señales de las auxinas para el incremento de biomasa foliar y radical.

El análisis mediante cromatografía de gases acoplada a espectrometría de masas (GC/MS, por sus siglas en inglés) en *T. virens*, condujo a la identificación de ácido indol-3-acético (AIA) y algunos precursores de esta hormona como el indol-3-acetaldehído (IAAld), indol-3-etanol (IEt) e indol-3-carboxaldehído (ICAld) en el sobrenadante del medio de crecimiento. Estos compuestos indólicos regulan de manera diferencial la expresión génica inducible por auxinas, así como la arquitectura del sistema radicular de *Arabidopsis*, por lo que muestra el importante papel de la señalización de las auxinas en la promoción del crecimiento en las plantas por *Trichoderma*.

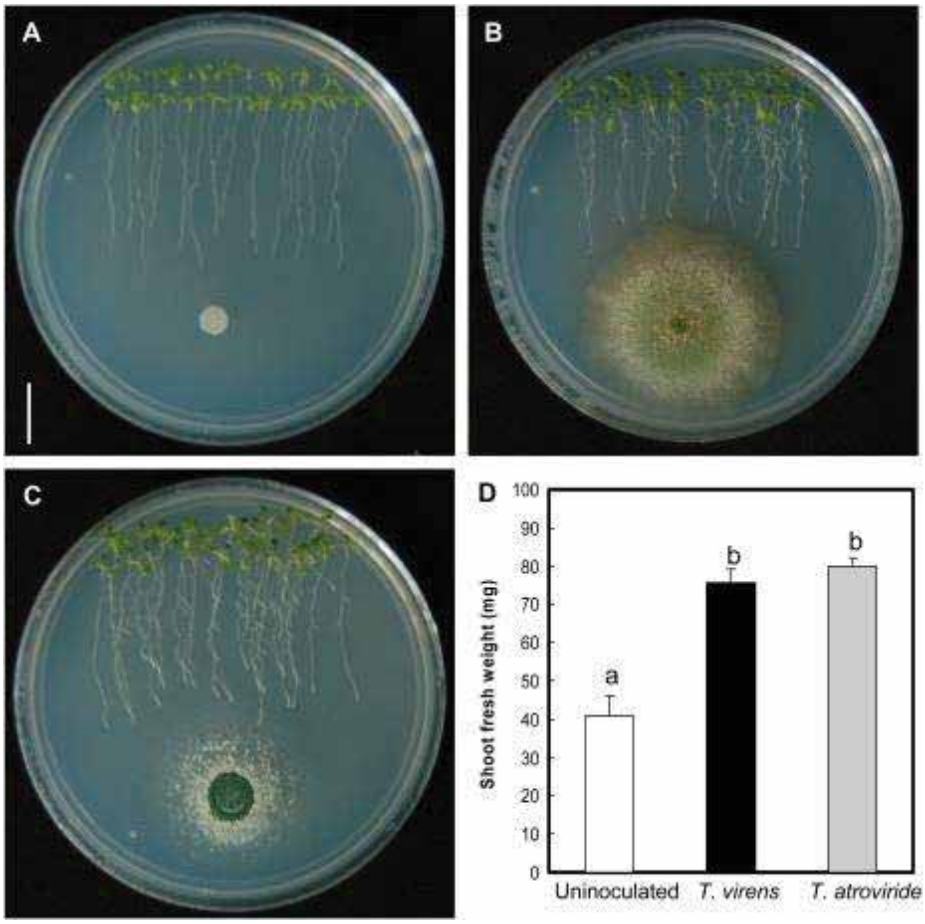

Figura 6. Efecto de *Trichoderma* sobre el crecimiento de *A. thaliana*. A) Plántulas de *Arabidopsis* crecidas en condiciones control. B) Efecto de la inoculación de *T. virens* y C) *T. atroviride*, donde se observa que ambas especies inducen el desarrollo de raíces laterales comparado con el tratamiento control. D) Efecto de *Trichoderma* sobre la acumulación de biomasa foliar. Adaptado de Contreras-Cornejo *et al.* (2009).

Posteriormente, se demostró que *T. atroviride* también produce etileno (ET), una hormona vegetal que en conjunto con el AIA inducen la actividad de MPK6, una proteína cinasa activada por mitógenos que regula el crecimiento de la raíz primaria y la formación de pelos radiculares en *Arabidopsis* (Contreras-Cornejo *et al*., 2015). De acuerdo con el análisis molecular y fenotípico de mutantes relacionados con el ET (*etr1* y *ein2)*, existe una baja inducción en la formación de pelos radiculares, así como una mayor inhibición del crecimiento de la raíz primaria bajo el co-cultivo con *Trichoderma*, demostrando que es necesaria

una señalización cruzada entre MPK6, auxinas y ET en la respuesta del desarrollo de la raíz de *Arabidopsis* ante el co-cultivo con *Trichoderma* (Contreras-Cornejo *et al*., 2015).

La producción de compuestos orgánicos volátiles (COV) es una propiedad ubicua en *Trichoderma* (Estrada-Rivera *et al*., 2019; Guzmán-Guzmán *et al*., 2019; Esparza-Reynoso *et al*., 2021). Estos incluyen alcoholes, hidrocarburos, aldehídos, alcaloides, cetonas, sesquiterpenos, monoterpenos, alcanos, éteres, compuestos heterocíclicos, fenol y benceno (da Silva *et al*., 2021; Nieto-Jacobo *et al*., 2017). La exposición a las mezclas de COV emitidas por *Trichoderma* aumentan la ramificación de raíces y la producción de biomasa en plántulas de *Arabidopsis* (Hung *et al*., 2013; Contreras-Cornejo *et al*., 2014; Esparza-Reynoso *et al*., 2021). También se ha descrito que estas mezclas de volátiles influyen directamente sobre el contenido de clorofila, la eficiencia fotosintética y el metabolismo del carbono en las plantas (da Silva *et al*., 2021; Hung, Lee y Bennett, 2013; Jalali, Zafari y Salari, 2017; Lee *et al*., 2016; Nieto-Jacobo *et al*., 2017; Esparza-Reynoso *et al*., 2021).

Los VOC producidos por *T. atroviride* modifican la expresión de un transportador de sacarosa de larga distancia (*AtSUC2*) y de algunos miembros de transportadores de la familia SWEET (proteínas transportadoras de flujo de salida de mono y disacáridos), contribuyendo en la traslocación de fotosintatos desde las hojas hacia la raíz (Fig. 7a-b) (Esparza-Reynoso *et al*., 2021). Para compensar esta demanda de fotosintatos, los VOC emitidos por *Trichoderma* incrementan la expresión tejido-específico de las isoformas de la proteína sacarosa fosfato sintasa (SPS) en las hojas de las plántulas de *Arabidopsis* (Fig. 7C). Esta modificación sobre la biosíntesis y transporte de azucares por parte de los volátiles favorece a la par el crecimiento y desarrollo de las plantas y el contenido de glucosa y sacarosa de los exudados de raíces (Fig. 7D-F), los cuales eventualmente beneficiarán el crecimiento del hongo (Macías-Rodríguez *et al*., 2018; Esparza-Reynoso *et al*., 2021).

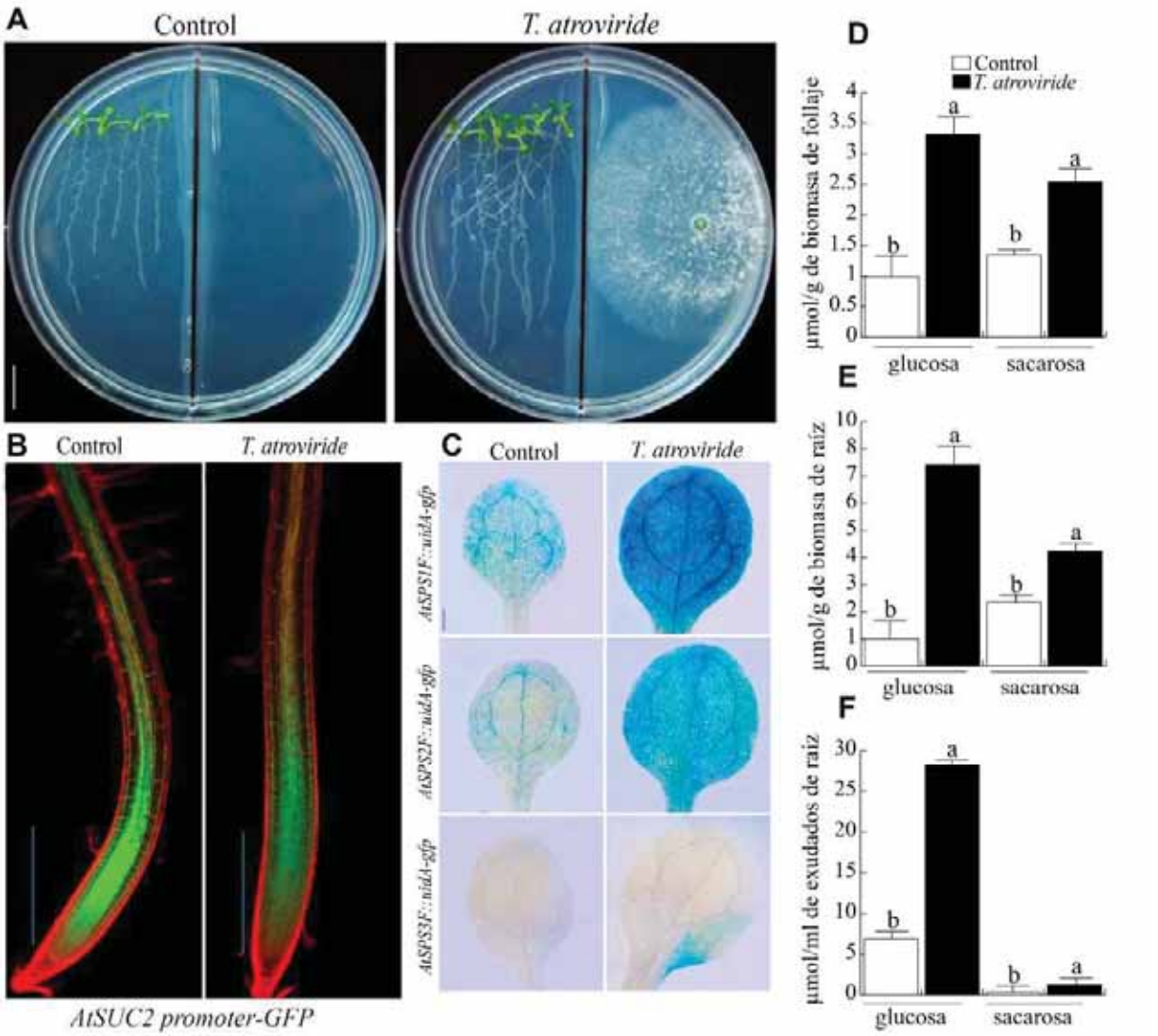

Figura 7. Efecto de los VOCs emitidos por *Trichoderma* sobre el metabolismo y transporte de carbono en *Arabidopsis*. (A) Plántulas de *Arabidopsis* expuestas a los VOCs de *T. atroviride*. (B) Expresión de *AtSUC2-GFP* en el ápice de la raíz primaria. (C) Expresión de las isoformas de las enzimas SPS (*AtSPS1F::uidA-GFP*, *AtSPS2F:: uidA-GFP*, *AtSPS3F::uidA-GFP*) en hojas. (D-F) Contenido de glucosa y sacarosa en tejidos y exudados radiculares de plántulas cultivadas axénicamente o expuestas a los VOCs de *Trichoderma*. Modificado de Esparza-Reynoso *et al.* (2021).

Entre los principales compuestos volátiles sintetizados por *T. atroviride*, la 6-PP posee una actividad similar a las auxinas, ya que promueve el crecimiento de

las plantas (Garnica-Vergara *et al*., 2016; González-Pérez *et al*., 2018; Estrada-Rivera *et al*., 2019). De acuerdo con el trabajo realizado por Garnica-Vergara y col. (2016), la aplicación del compuesto sobre el medio de cultivo mejora la producción de biomasa y la ramificación de las raíces laterales de manera dependiente a la concentración (Fig. 7A-F).

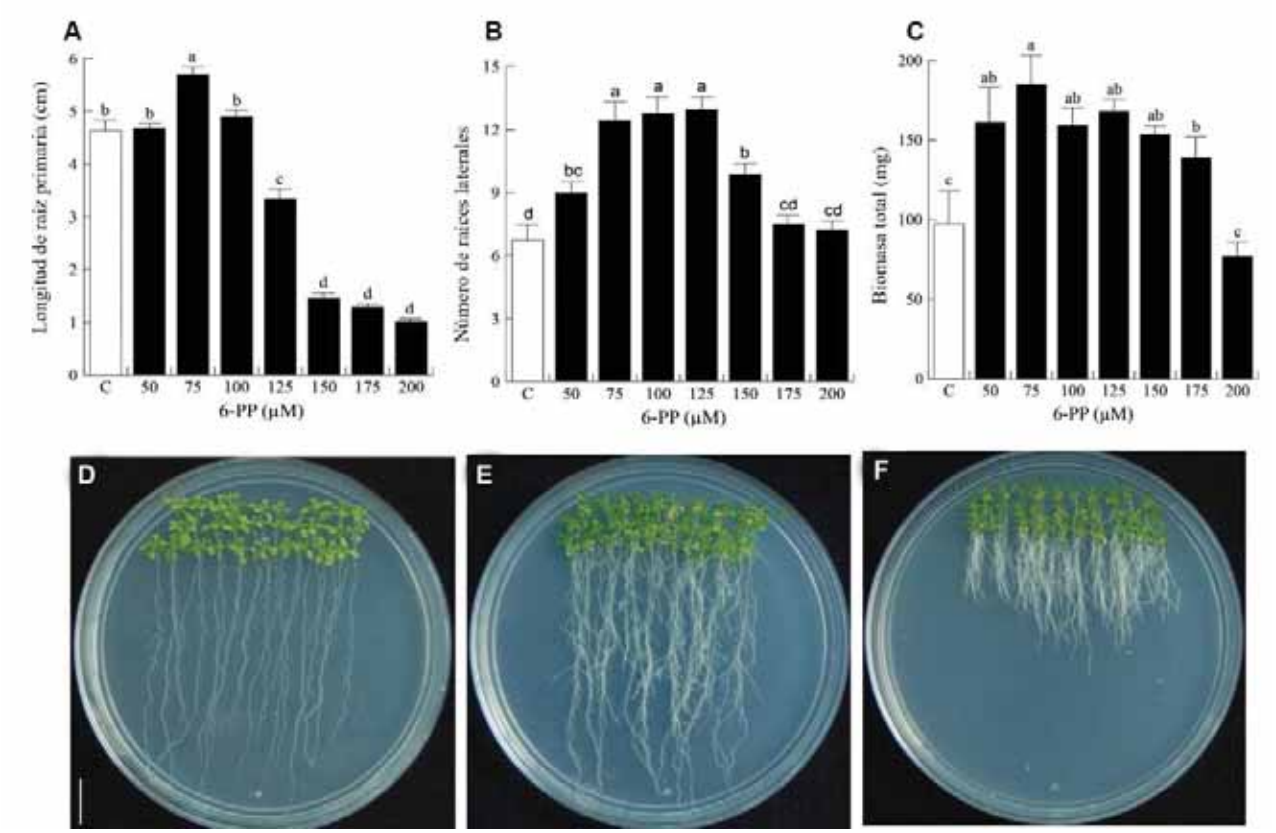

Figura 8. Efecto de la 6-pentil-2H-piran-2-ona (6-PP) en la arquitectura de la raíz de *Arabidopsis*. Plántulas de *Arabidopsis* fueron germinadas y cultivadas durante 12 días con concentraciones crecientes de 6-PP. (A) Longitud de la raíz primaria. (B) Número de raíces laterales emergidas. (C) Biomasa total. (D-F) Fotografías representativas de plántulas cultivadas en medio MS 0.2X suplementado con el solvente (control), 75 y 150 µM de 6-PP. Modificado de Garnica-Vergara *et al.* (2016).

La 6-PP aumenta la respuesta auxínica en los primordios de las raíces laterales al incrementar la expresión de los transportadores de auxinas *PIN1*,

PIN2, *PIN3* y *PIN7*. Finalmente se describió que el efecto del volátil sobre el desarrollo de las RL requiere de la participación de los receptores de auxinas *TIR1*, *AFB2* y *AFB3*, así como de los factores de transcripción *ARF7* y *ARF19*, mientras que para el efecto de este compuesto sobre la raíz primaria es necesaria la participación de un elemento de la vía de señalización del ET denominado ETHYLENE INSENSITIVE 2 (EIN2). Indicando entonces que el volátil modifica la organogénesis de la raíz de *Arabidopsis* al modular tanto la señalización del ET como el transporte de auxinas.

Recientemente, se han descrito otros factores que participan en la regulación del crecimiento de *Trichoderma*, uno de ellos es la acidificación del pH producto del metabolismo y crecimiento fúngico (Pelagio-Flores *et al*., 2017). En tiempos más prolongados de exposición con *T. atroviride* (4 días después de la inoculación) las plántulas de *Arabidopsis* presentan la formación de una estructura de tipo gancho en el ápice de la raíz primaria, la cual obedece una redistribución de auxinas dentro de las células de la columella hacia una cofia lateral provocando la flexión de la punta de la raíz y la subsecuente inhibición del crecimiento (Pelagio-Flores *et al*., 2017). Esta interrupción del crecimiento de la raíz se asocia con la disminución de la división celular y con la pérdida de la integridad y viabilidad celular del meristemo apical de la raíz. Este fenotipo sobre el ápice de la raíz es revertido al amortiguar el medio de cultivo, mostrándose además un efecto más exacerbado de la estimulación del crecimiento de la raíz primaria y la emergencia de raíces laterales en plántulas co-cultivadas con *Trichoderma* (Fig. 8). Aunado a esto, la capacidad de *T. atroviride* para modificar el pH del medio de cultivo fue evidenciado a través de un indicador de pH (azul de bromofenol) suplementado en el medio de cultivo, el cual reveló un color amarillo (pH 3.0) en función del crecimiento de la colonia a través de los días. Además, la sensibilidad del mutante *stop1* a la interacción con *Trichoderma* indica que la acidificación del medio por el hongo es percibida por la raíz a través de una ruta putativa que involucra al factor de transcripción STOP1 para la reconfiguración de la arquitectura de la raíz durante la interacción (Pelagio-Flores *et al*., 2017).

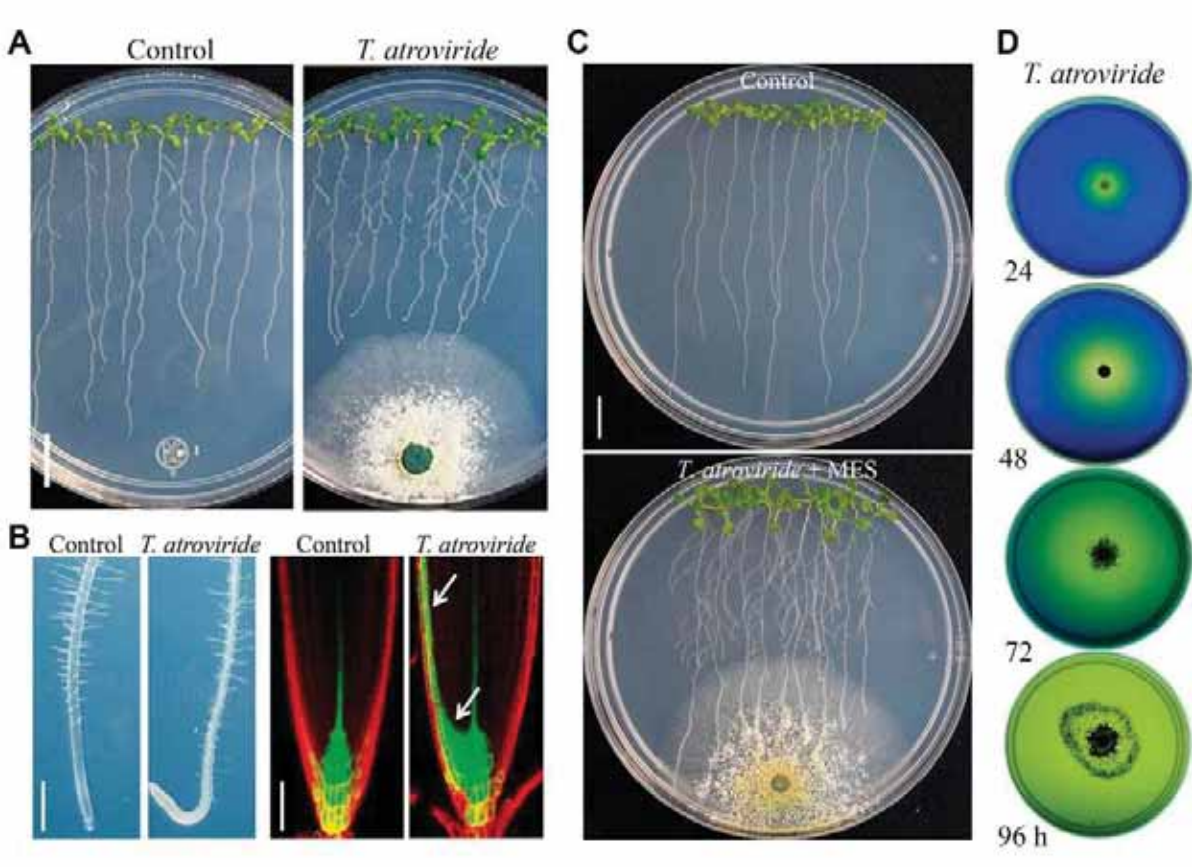

Figura 9. Efecto de la acidificación de *T. atroviride* en la arquitectura de la raíz de *Arabidopsis*. (A) Fotografías representativas de plántulas de *Arabidopsis* cocultivadas con *Trichoderma* durante 4 días. (B) Puntas de raíz de plántulas y expresión de *DR5::GFP*. Las flechas blancas muestran la redistribución de auxinas. (C) Plántulas de *Arabidopsis* cocultivadas con *Trichoderma* en medio MS 0.2X amortiguado con MES al 0.12 %. (D) Acidificación por *T. atroviride* en medio suplementado con el indicador de pH azul de bromofenol por días. Modificado de Pelagio-Flores *et al.* (2017).

2.4.2 Inducción de la respuesta inmunitaria

De manera paralela a la promoción del crecimiento, *Trichoderma* spp. promueve las respuestas de defensa en las plantas (Contreras-Cornejo *et al*., 2016). Como se ha descrito con anterioridad, el co-cultivo *in vitro* de raíces de *Arabidopsis* con *T. virens* y *T. atroviride* estimula el desarrollo de raíces laterales y la producción de biomasa vegetal, sin embargo, la colonización de ambas

especies de *Trichoderma* genera una acumulación de peróxido de hidrógeno (H_2O_2) en los ápices de hojas y raíces, indicando que se activan respuestas de defensa a través de un mecanismo dependiente de ROS (Contreras-Cornejo *et al*., 2011). A la par de la acumulación significativa de H_2O_2, las plantas también presentan incrementos en los niveles endógenos de SA y JA, relacionándose positivamente con la inducción de dos genes inducibles para ambas vías canónicas de defensa (Fig. 9A-D) (Contreras-Cornejo *et al*., 2011). El análisis de la expresión de los marcadores relacionados con la patogénesis *pPr1a:uidA* y *pLox2:uidA* en respuesta a *T. virens* o *T. atroviride* muestran que ambos genes se inducen fuertemente en el follaje conforme aumenta el tiempo de exposición a los hongos, evidenciando que ambas vías de señalización de defensa son activadas al mismo tiempo. La participación simultánea entre el JA y el SA en la inmunidad vegetal inducida por *Trichoderma*, confiere resistencia al daño ocasionado en las hojas por *Botrytis cinerea* (Contreras-Cornejo *et al*., 2011). Además, el co-cultivo con ambas especies de *Trichoderma* también promueve la biosíntesis de compuestos antimicrobianos como la camalexina que, en conjunto con la producción de ROS, refuerzan y favorecen la modificación de las paredes celulares a través de la deposición de lignina y callosa en las células aledañas para limitar el crecimiento del hongo dentro de las primeras capas de células como la epidermis y el córtex, y así permitir la colonización superficial de las raíces (Contreras-Cornejo *et al*., 2011).

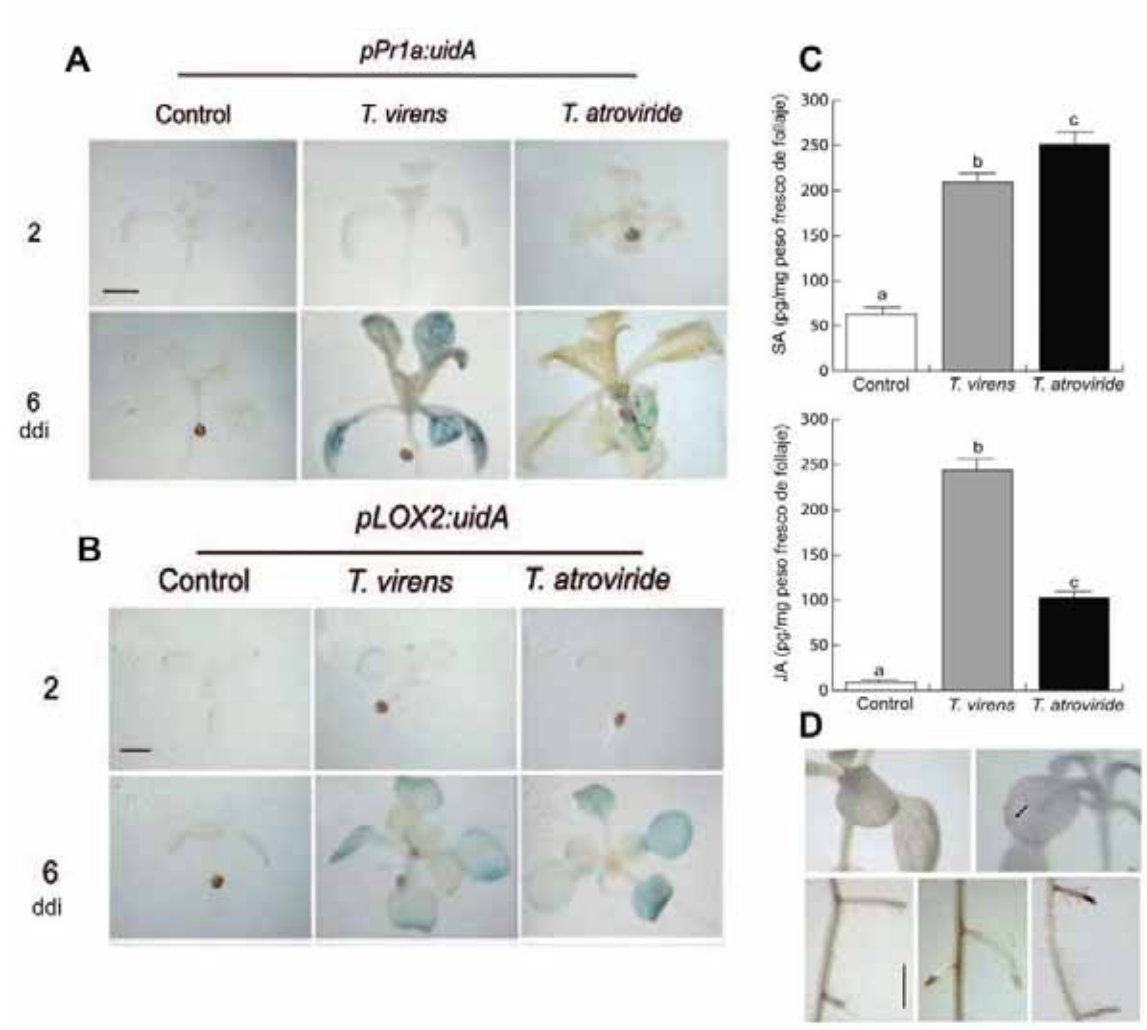

Figura 10. Efecto de *Trichoderma* sobre las respuestas de defensa dependientes de hormonas en *Arabidopsis*. (A-B) Visualización genes inducibles por JA y SA, *pPr1a:uidA* y *pLox2:uidA* en función al tiempo de interacción con *T. virens* y *T. atroviride*. (C) Cuantificación del contenido endógeno de ácido salicílico (SA) y ácido jasmónico (JA) a los 8 días de interacción con ambos hongos. (D) Fotografías representativas de la acumulación de peróxido de hidrogeno (H_2O_2) en los ápices de hojas y raíces de plántulas cultivadas axénicamente (control) o co-cultivadas con *T. atroviride*. Modificado de Contreras-Cornejo *et al.* (2011).

Algunos de los estudios realizados sobre la vía de transducción de señales involucrada en la inducción de resistencia sistémica conferida por *T. atroviride* en *Arabidopsis* muestran que las enzimas relacionadas con la defensa en las plantas, incluidas las peroxidasas, quitinasas, β-1-3-glucanasa (PR-2) son fuertemente

inducidas en las hojas en respuesta a la inoculación con el hongo (Salas-Marina *et al*., 2011). Además, los niveles de expresión de genes relacionados a la vía del JA/ET como *PDF1.2* y *LOX-1* mostraron cambios significativos en su expresión en las raíces y en las hojas. El análisis de la expresión del gen *PAD3* que codifica para la enzima encargada de la síntesis de la camalexina, mostró una regulación positiva en toda la planta después del tratamiento con *T. atroviride* (Salas-Marina *et al*., 2011). No obstante, la colonización de *Trichoderma asperelloides* T203 sobre las raíces de *Arabidopsis* desencadena un rápido aumento en la expresión de factores de transcripción como WRKY18, WRKY40, WRKY60 y WRKY33, los cuales activan las respuestas de la vía de señalización del JA a través de la supresión de los represores JAZ, pero que regulan negativamente la expresión de los genes de defensa *FMO1*, *PAD3* y *CYP71A13* reprimiendo así la señalización del SA (Brotman *et al*., 2013). Esto demuestra la compleja superposición de la ISR y la SAR que genera la colonización de la raíz de las diferentes especies de *Trichoderma*.

Trichoderma establece un diálogo químico con la planta para suprimir las defensas y establecer una asociación mutualista prolongada (Contreras-Cornejo *et al*., 2016). Durante esta interacción, *Trichoderma* libera una mezcla de diversos compuestos como proteínas, ARN pequeños y metabolitos secundarios (SM) como los COV, los cuales actúan como patrones moleculares asociados a microbios (MAMP), patrones moleculares asociados a daños (DAMP), elicitores y/o efectores que pueden ser reconocidos por los receptores y activan la inmunidad basal (Mukherjee, Horwitz y Kenerley, 2012; Hermosa *et al*., 2013; Mendoza-Mendoza *et al*., 2018; Nogueira-López *et al*., 2020). El efector proteico no enzimático producido por *T. virens* conocido como Sm1, así como sus ortólogos presentes en *T. atroviride* y *T. harzianum* Epl1 y Sm2, respectivamente, inducen la producción de ROS, así como la expresión de genes relacionados con respuesta inmunitaria local y sistémica (Djonovic *et al*., 2007; Crutcher *et al*., 2015; Gaderer *et al*., 2015; Salas-Marina *et al*., 2015). Algunas enzimas como las hidrofobinas, glucosidasas, celulasas y proteínas que contienen el dominio CFEM o LysM no sólo participan en la colonización de las raíces, sino que también en la

activación de la ISR que da como resultado una mayor protección al ataque de patógenos (Nogueira-López *et al*., 2020). Por otro lado, algunos metabolitos secundarios sintetizados por *Trichoderma* como terpenoides, peptaiboles, lactonas, policétidos y tricotecenos también han sido reportados como inductores de las respuestas de defensa. El volátil 6-PP producido por *Trichoderma asperellum* IsmT5 incrementa la expresión génica de *PR-1* (proteína relacionada con patógenos e inducida por SA), *VSP2* (gen marcador para JA-ET), y el factor de transcripción *GL3* (involucrado en la formación de tricomas), confiere así resistencia al daño provocado por los hongos fitopatógenos *Alternaria brassicicola* y *B. cinerea* (Fig. 11A-D) (Kottb *et al*., 2015).

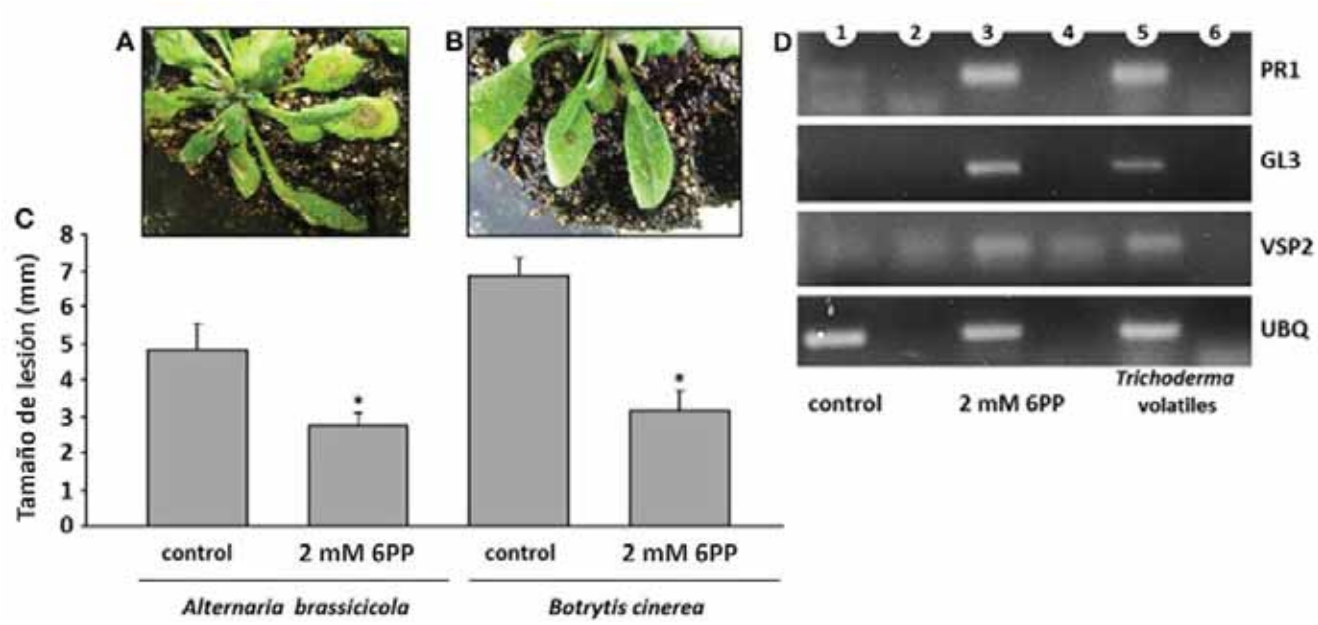

Figura 11. Efecto de la 6-PP en la inmunidad de *Arabidopsis*. En el panel superior (A) se muestran ejemplos representativos de plantas de *A. thaliana* tratadas con una suspensión de esporas de *B. cinerea*: sin exposición previa a 6PP = control; (B): con pre-exposición a 6PP. Cuantificación del diámetro de las lesiones (C). Expresión de genes de defensa mediante RT-PCR: *PR1*, proteína relacionada con la patogénesis 1; *GL3*, factor de transcripción GLABRA 3; *VSP2*, proteína de almacenamiento vegetativo (D). Modificado de Kottb *et al.* (2015).

La producción de ROS en *Trichoderma* ocurre principalmente a través del complejo NADPH oxidasa (NOX). Dichas proteínas participan en la señalización que da lugar al desarrollo del hongo, así como en el establecimiento de las interacciones simbióticas con las plantas (Cruz-Magalhães *et al*., 2019). En *T. atroviride*, el complejo enzimático de la NADPH oxidasa está compuesto por las subunidades reclutadora, reguladora y catalítica codificadas por los genes *Nox1, Nox2* y *NoxR*, respetivamente. La generación de las mutantes disfuncionales para estos genes sirvieron para elucidar la participación de las ROS en el proceso de regeneración y diferenciación de las hifas ante daño mecánico (Hernandez-Oñate *et al*., 2012). En el trabajo realizado por Cruz-Magalhães y col. (2019), se realizó una comparación entre las cepas knock-out Δ*nox1*, Δ*noxR* y Δ*nox2* y la cepa WT en cuanto a su capacidad para crecer y producir conidias en diferentes condiciones de estrés. Las cepas mutantes Δ*nox1* y Δ*noxR* redujeron significativamente la actividad antagónica de *T. atroviride* contra *Rhizoctonia solani* y *Sclerotinia sclerotiorum* en ensayos de confrontación directa, contrariamente a Δ*nox2*, que mostró una actividad similar a la WT. Además, las mutantes Δ*nox1*, Δ*noxR* y Δ*nox2* mostraron diferencias cuantitativas en la emisión de varios compuestos orgánicos volátiles (COV) y la promoción del crecimiento de *Arabidopsis*. El aumento en la biomasa de raíces y brotes inducido por los COV de *T. atroviride* fue reducido en las mutantes Δ*noxR* y Δ*nox1*, a diferencia de Δ*nox2*, lo cual correlacionó con la sobre-producción de la 6-PP y 6-pent-1-enil-2H-piran-2-ona (6PP-2) en la mutante.

Se ha reportado que la emisión de volátiles por parte de *Trichoderma* también influye sobre la resistencia a estrés abiótico en *Arabidopsis*. Plantas expuestas durante 2 semanas a los COV de *Trichoderma koningii*, mostraron menos acumulación de H_2O_2 bajo estrés salino en comparación con el control. Este resultado refleja el posible papel de los COV en la protección de las plantas contra el daño oxidativo bajo estrés salino (Jalali *et al.* 2017). De hecho, algunos de los cambios observados en el proteoma de *Trichoderma* muestran que existe la secreción de proteínas antioxidantes para contrarrestar el estallido oxidativo inducido por la activación temprana de las respuestas de defensas dependientes

de ROS (Alfiky y Weisskopf, 2021). Entre los mecanismos descritos en *Trichoderma* para la mitigación del estrés biótico y abiótico, se incluye la producción de enzimas antioxidantes como la catalasa (CAT), superóxido dismutasa (SOD), ascorbato peroxidasa (APx) o glutatión S-transferasa (GST), las cuales reducen la acumulación de ROS que pueden afectar la viabilidad de las células. Las plantas inoculadas con *Trichoderma* presentan acumulaciones transitorias y ondulantes de ROS debido precisamente a la regulación de enzimas antioxidantes y a la participación de la señalización del SA (Morán-Diez *et al*., 2021).

2.5 Metabolismo de ROS en plantas

Las ROS como el anión superóxido (O_2^-), el H_2O_2, el radical hidroxilo (·OH) y el oxígeno singlete ($^1O_2$) influyen sobre una amplia gama de procesos biológicos involucrados en el desarrollo y crecimiento de las plantas, así como en la adaptación al estrés biótico y/o abiótico (Hu *et al*., 2020). Las ROS son consideradas como subproductos del metabolismo aeróbico y son generados en diferentes compartimentos celulares como cloroplastos, mitocondrias y peroxisomas (Fig.12) (Huang *et al*., 2019). No obstante, la generación de ROS en el apoplasto juega un papel esencial en la interacción planta-microorganismo, ya que son necesarias para la activación de las respuestas de defensa (Lee *et al*., 2020). La producción apoplásica de ROS depende en su mayoría de la actividad de las enzimas NADPH oxidasas (NOXs), las cuales son homólogas a la subunidad catalítica (gp91phox) de los fagocitos de mamíferos y son denominadas como RBOH (por sus siglas en inglés) en plantas (Lee *et al*., 2020; Castro *et al*., 2021).

Las enzimas RBOH son proteínas integrales de la membrana plasmática compuestas por seis dominios transmembranales que soportan dos grupos hemo, dominios hidrofílicos C-terminal FAD y NADPH y dos dominios N-terminales de unión al calcio (EF-hand). El NADPH actúa como un donante de electrones citosólico que reduce al oxígeno molecular (O_2) para la generación del O_2^-. El

radical O_2^- puede dismutarse espontáneamente a H_2O_2 o por acción de la SOD, para continuar su eventual transformación a agua y dioxígeno mediante la actividad de las peroxidasas y CAT (Lee *et al*., 2020; Castro *et al*., 2021). Estas enzimas forman parte del sistema antioxidante enzimático que funcionan de forma sinérgica con el sistema no enzimático (glutatión, ácido ascórbico y flavonoides) para neutralizar los radicales libres y el $^1O_2$, y evitar que las células entran en el estado oxidativo que resulte en daño o muerte celular (Huang *et al*., 2019). Hasta la fecha, se han identificado y caracterizado alrededor de 150 miembros de proteínas de la familia NOXs/RBOHs en varias especies de plantas y específicamente se han reportado 10 genes para las isoformas RBOH (A–J) para el genoma de *Arabidopsis* (Waszczak *et al*., 2018; Hu *et al*., 2020).

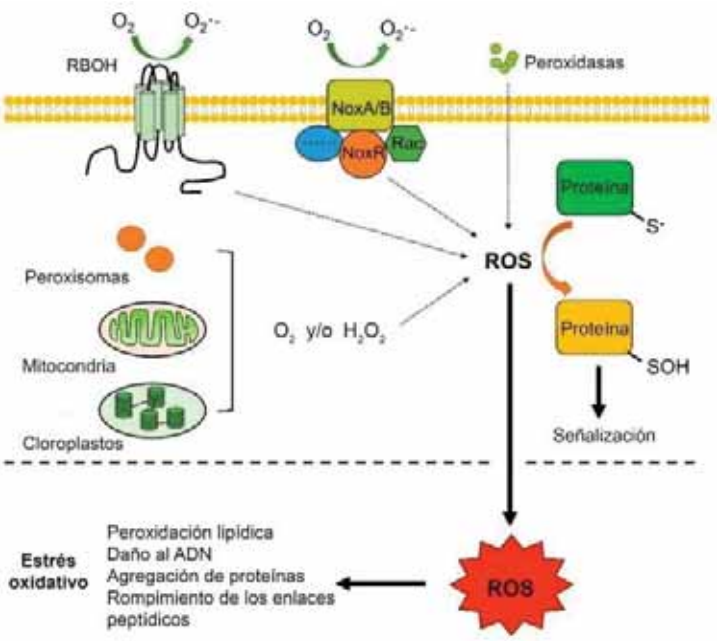

Figura 12. Sitios de generación de especies reactivas de oxígeno (ROS). Las ROS son producidas por homólogos de oxidasa de explosión respiratoria (RBOH), mitocondrias, cloroplastos, peroxisomas y peroxidasas residentes en la pared celular (PER). La acumulación posterior de H_2O_2 puede oxidar los residuos de cisteína en las proteínas, afectar sus funciones y estados redox y regular las vías de señalización relacionadas. El exceso de ROS puede provocar estrés oxidativo, lo que puede causar oxidación de lípidos, daño en el ADN, carboxilación de proteínas y lesiones en otros componentes celulares. Adaptado de Wang *et al.* (2019).

2.5.1 Mecanismos de regulación de las enzimas RBOH

La actividad de las RBOH puede ser regulada a nivel transcripcional, ya que se han identificado diversos elementos reguladores en *cis* los cuales se encuentran distribuidos aleatoriamente en las secuencias de los promotores de los genes RBOH (Waszczak *et al*., 2018). Se ha descrito que el elemento GCN4 puede desempeñar un papel en el crecimiento y desarrollo reproductivo, mientras que el elemento TATCCAT/C puede funcionar en condiciones de inanición. Por su parte, otros elementos distribuidos en los genes de la familia NOX/RBOH como los motivos G-Box y ABRE (elementos sensibles al ácido abscísico, ABA), TGA-element (elemento sensible a auxinas), ERE y GCC-box (elementos sensibles al ET), GARE, P-box y TATC (elementos sensibles a giberelinas, GA), CGTCA y TGACG (elementos sensibles a JA) y TCA (elemento sensible a SA) también están involucrados en la regulación por señalización hormonal, respuesta a luz y otros procesos biológicos, como la senescencia de las hojas y el desarrollo de semillas (Hu *et al*., 2020). Además, el elemento W-box es específico de los genes RBOH, el cual funciona principalmente ante las respuestas a patógenos ya que interactúa con los factores transcripcionales WRKY dependiente de la cascada de señalización de las MAPK (Hu *et al*., 2020). Sin embargo, la actividad de las enzimas RBOH también se puede regular a nivel postraduccional, ya que se ha demostrado que la fosforilación de la región N-terminal en residuos conservados a través de distintas cinasas afecta la actividad de estas enzimas. Se ha descrito que múltiples miembros de la subfamilia VII de los receptores de tipo quinasas citoplasmáticas RLK (por sus siglas en inglés) influyen directamente sobre la actividad de las RBOHs durante procesos de inmunidad desencadenada por patrones moleculares asociados a patógenos (PAMP) (Lee *et al*., 2020).

En *Arabidopsis*, el receptor transmembranal de tipo cinasa, FLAGELLIN SENSITIVE 2 (FLS2) posee un dominio extracelular de repeticiones ricas en leucina (LRR) que sirve para el reconocimiento de la flagelina bacteriana (flg22). Este péptido de 22 aminoácidos desencadena la formación de un complejo entre su receptor FLS2 y el co-receptor BAK1 (BRASSINOSTEROID INSENSITIVE 1-

associated receptor kinase 1), el cual desencadena una serie de eventos de transfosforilación entre varias cinasas intracelulares asociadas, entre ellas BIK1 (KINASA1 INDUCIDA POR BOTRYTIS). BIK1 interactúa con AtRBOHD y fosforila directamente su dominio N-terminal en la S39, S343 y S347 para su activación (Lee *et al*., 2020). Por su parte, la cinasa SERINE-THREONINE KINASE1 (SIK1) (también conocida como MAP4K3) también influye sobre la producción de ROS, ya sea por su interacción con BIK1 o por su fosforilación directa sobre AtRBOHD en la S347 (Wang *et al*., 2020).

Las plantas también liberan moléculas especializadas, conocidas como patrones moleculares asociados al daño (DAMP) para desencadenar las respuestas de defensas (Lee *et al*., 2020). En *Arabidopsis*, se ha descrito que los péptidos inductores de señales (Peps) al ser percibidos por los receptores de tipo cinasa PEPR1 y PEPR2, provocan una fuerte producción de ROS de manera similar a la señalización de flg22-FLS2. Incluso se ha descrito la participación de BIK1 que funciona río abajo de la señalización de Pep1-PEPR al interactuar físicamente con PEPR y RBOHD/F para regular la producción de ROS y la inhibición del crecimiento de la raíz (Jing *et al*., 2020). No obstante, la enzima RBOHD también es activada a través de los cambios conformacionales que originan las fluctuaciones de los niveles de Ca^{2+} citosólico sobre el motivo EF-hand del extremo N-terminal de la enzima, así como las fosforilaciones provocadas por las proteínas cinasas dependiente de Ca_{2+} CDPK (por sus siglas en inglés) (Lee *et al*., 2020). Recientemente, un estudio encontró que un RLK rico en cisteína (CRK2) fosforila el extremo C de *AtRBOHD* en la S611, S703 y S862 y regula positivamente la producción de ROS inducida por flg22 y la defensa contra *Pseudomonas syringae* pv. tomato DC3000 (Fig. 13) (Wang *et al*., 2020). Además, en el extremo C-terminal de AtRBOHD, PBL13, una proteína cinasa de serina/treonina también lleva a cabo una fosforilación en S862 que conduce a la regulación negativa de la producción e inmunidad de ROS desencadenada por PAMP. Curiosamente, la ubiquitina ligasa E3 (PIRE) que interactúa con PBL13 en el extremo C-terminal de *AtRBOHD* regula positiva o negativamente la activación de esta para su degradación (Wang *et al*., 2020).

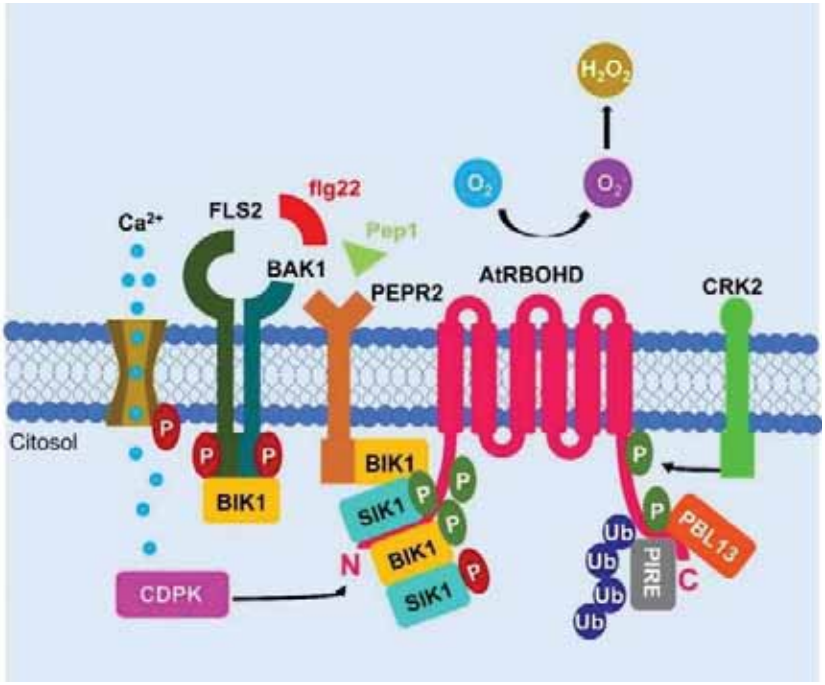

Figura 13. Regulación de las enzimas RBOH para la generación de ROS extracelulares en plantas. Fosforilación y ubiquitinación en la producción de ROS mediada por AtRBOHD en *Arabidopsis*. Formación de los complejos FLS2-BAK1 y Pep1-PEPR tras la percepción de la flagelina (flg22) y el péptido Pep1, los cuales fosforilan a BIK1, lo que conduce a la disociación de esta cinasa de ambos complejos y fosforila el extremo N-terminal de AtRBOHD para aumentar la producción de ROS. Por otro lado, SIK1 no solo interactúa y fosforila BIK1 para mejorar su actividad, sino que también interactúa directamente y fosforila el extremo N-terminal de AtRBOHD. La activación dependiente de Ca^{2+} de las CDPKs también conduce a la fosforilación de AtRBOHD. El extremo C-terminal de AtRBOHD también es fosforilado por CRK2, además de influir sobre su degradación a través de la ubiquitina ligasa E3 (PIRE). Modificado de Wang *et al.* (2020).

Por lo anterior, se considera que la producción de ROS por parte de RBOHD se relaciona estrechamente con la deposición de callosa, la lignificación de la pared celular, el cierre de estomas y la SAR (Castro *et al*., 2021). Mientras que la isoforma RBOHF es parcialmente redundante con su homólogo RBOHD debido a que también participa en las respuestas inmunes de las plantas, parece no ser inducible en respuesta a los tratamientos con MAMP o patógenos (Waszczak *et al*., 2018). Sin embargo, las ROS producidas por las RBOH también son consideradas como moléculas de señalización que participan sobre procesos

del desarrollo como la germinación y la floración, el desarrollo del meristemo apical de la raíz y el meristemo apical del brote, la formación de los pelos radiculares y tubos polínicos, el crecimiento de las hojas y la emergencia de las RL (Choudhary *et al*., 2019). La expresión y actividad de las isoformas *AtRbohH* y *AtRbohJ* en la punta del tubo polínico correlacionan positivamente con el proceso de elongación de este órgano, e incluso se ha demostrado que *AtRbohC/hair root2* (*hrd2*) también juega un papel en la regulación del crecimiento normal del pelo radicular (Hu *et al*., 2020). Por otra parte, la actividad de *AtRbohE* es fundamental para la formación y emergencia de los PRL, mientras que las isoformas *AtRbohD* y *AtRbohF* modulan negativamente el desarrollo de las raíces laterales (Waszczak *et al*., 2018; Hu *et al*., 2020; Castro *et al*., 2021).

2.5.2 Papel de las ROS en el desarrollo de raíces laterales

En conjunto con la señalización por auxinas, las ROS participan en procesos del desarrollo de las raíces, como en la diferenciación del xilema, el gravitropismo, la formación de raíces adventicias, así como en la emergencia y alargamiento de RL. De acuerdo con el trabajo de Orman-Ligeza y col. (2016), la exposición de plántulas de *Arabidopsis* a concentraciones crecientes de H_2O_2 conduce a una represión de la tasa de crecimiento de la raíz primaria, que a la par incrementa significativamente la densidad y la longitud de las RL (Fig. 14A). Un análisis puntual sobre la formación de primordios de RL mostró que las ROS facilitan los eventos de desarrollo temprano que conducen a la formación de primordios. Sin embargo, este efecto del H_2O_2 sobre la formación de RL se compromete si el transporte polar de auxinas mediada por transportadores PIN y los módulos de señalización de auxinas dependientes de *iaa28*, *arf7arf19* y *slr* se encuentran afectados (Fig. 14B). Además, los autores mostraron que existe una acumulación especifica de ROS extracelulares en la lámina media de las células que recubren a los PRL, la cual correlaciona con el patrón de expresión de diversos genes RBOH dentro de los mismos PRL y sobre las células de la endodermis, córtex y epidermis subyacentes a estos, involucrándose posiblemente

sobre la remodelación de la pared celular de estas células (Fig. 14C). Dichos genes RBOH son inducibles por la aplicación exógena de auxinas, la cual también incrementa los niveles de ROS en las raíces, lo que sugiere que la producción de ROS ocurre río abajo de la transducción de señales mediada por las auxinas para acelerar los primeros pasos de la formación de RL (Manzano *et al*., 2014; Orman-Ligeza *et al*., 2016; Huang *et al*., 2019). Finalmente, el interrumpir (o mejorar) la expresión de RBOH desacelera (o acelera) el desarrollo y la emergencia de raíces. Por lo tanto, la producción de ROS mediada por RBOH parece facilitar el crecimiento de las RL al promover la remodelación de las paredes celulares de las células adyacentes.

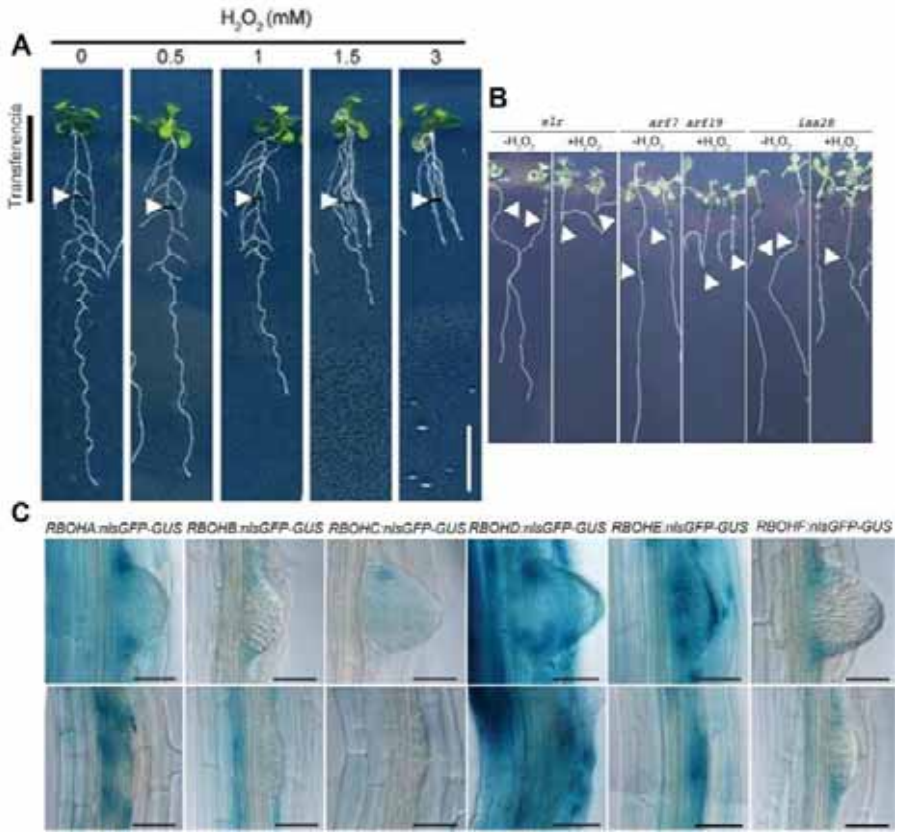

Figura 14. Efecto del H_2O_2 sobre el desarrollo de raíces en *Arabidopsis*. (A) Crecimiento y desarrollo de plántulas de siete días de edad transferidas a medios con concentraciones crecientes de H_2O_2. Las puntas de flecha blancas indican la región de la punta de la raíz en el momento de la transferencia. (B) El efecto del H_2O_2 en el fenotipo de las mutantes *slr*, *arf7*, *arf19* e *iaa28*. (C) Patrón de expresión de genes *RBOH* durante el desarrollo de la raíz. Tomado de Orman-Ligeza *et al.,* (2016).

III. JUSTIFICACIÓN

En los organismos eucariotas, las NADPH oxidasas (NOX) generan especies reactivas de oxígeno (ROS) como parte de sus funciones fisiológicas. Sin embargo, también se ha descrito que la generación de ROS extracelulares controla una amplia gama de procesos biológicos como el crecimiento, el desarrollo y las respuestas a estímulos bióticos y/o abióticos, así como también el proceso de patogénesis o simbiosis. *Trichoderma* es un género de hongos simbiontes que promueven el crecimiento, desarrollo y fortalece la inmunidad de las plantas a través de una señalización por auxinas, ácido jasmónico y etileno. Existe un mecanismo de regulación entre estas hormonas vegetales y la producción de especies reactivas de oxígeno, por lo que dilucidar la participación de estos mensajeros celulares y los sitios en los que actúan sobre la regulación de las respuestas de crecimiento y defensa por *Trichoderma* en *Arabidopsis* quedan por esclarecerse.

IV. HIPÓTESIS

T. atroviride regula el crecimiento, desarrollo y la inmunidad de *Arabidopsis* a través de un mecanismo dependiente de especies reactivas de oxígeno.

V. OBJETIVOS

5.1 Objetivo general

Determinar la participación de las especies reactivas de oxígeno como moléculas de señalización en la interacción entre *Arabidopsis thaliana* y *Trichoderma atroviride*.

5.2 Objetivos específicos

1. Evaluar la participación las especies reactivas de oxígeno generadas por el complejo NADPH oxidasa de *T. atroviride* en la regulación de las respuestas de crecimiento y defensa en *Arabidopsis*.
2. Caracterizar el papel de las especies reactivas de oxígeno generadas por las enzimas RBOH de *Arabidopsis* sobre el crecimiento de la raíz en interacción con *T. atroviride*.
3. Identificar los factores que desencadenan el estrés oxidativo como respuesta temprana en *Arabidopsis* en la interacción con *T. atroviride*.

VI. RESULTADOS

Los principales resultados obtenidos durante la realización de este proyecto de tesis se presentan en los siguientes capítulos:

Capítulo 1: The fungal NADPH oxidase is an essential element for the molecular dialog between *Trichoderma* and *Arabidopsis*. (2020) *The Plant Journal* 103 (6), 2178-2192.

Capítulo 2: *Trichoderma atroviride* triggers reactive oxygen species production in *Arabidopsis* roots and requires RBOH family members and PEPR2 for plant biomass production and reconfiguration of root architecture. (2022)

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The fungal NADPH oxidase is an essential element for the molecular dialog between Trichoderma and Arabidopsis

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SUMMARY

Members of the fungal genus Trichoderma stimulate growth and reinforce plant immunity. Nevertheless, how fungal signaling elements mediate the establishment of a successful Trichoderma–plant interaction is largely unknown. In this work, we analyzed growth, root architecture and defense in an Arabidopsis–Trichoderma co-cultivation system, including the wild-type (WT) strain of the fungus and mutants affected in NADPH oxidase. Global gene expression profiles were assessed in both the plant and the fungus during the establishment of the interaction. *Trichoderma atroviride* WT improved root branching and growth of seedling as previously reported. This effect diminished in co-cultivation with the Δ*nox1*, Δ*nox2* and Δ*noxR* null mutants. The data gathered of the Arabidopsis interaction with the Δ*noxR* strain showed that the seedlings had a heightened immune response linked to jasmonic acid in roots and shoots. In the fungus, we observed repression of genes involved in complex carbohydrate degradation in the presence of the plant before contact. However, in the absence of NoxR, such repression was lost, apparently due to a poor ability to adequately utilize simple carbon sources such as sucrose, a typical plant exudate. Our results unveiled the critical role played by the Trichoderma NoxR in the establishment of a fine-tuned communication between the plant and the fungus even before physical contact. In this dialog, the fungus appears to respond to the plant by adjusting its metabolism, while in the plant, fungal perception determines a delicate growth–defense balance.

Keywords: reactive oxygen species, plant immunity, plant growth promotion, Trichoderma, Arabidopsis, transcriptome.

INTRODUCTION

Fungal–plant interactions may lead to different outcomes, from pathogenesis to symbiosis. Root-associated Trichoderma species provide several benefits to the host, strengthening immunity, improving root absorptive potential and conferring protection to environmental stress. Thus, recent research has concentrated on how this mutualistic interaction could be used to enhance crop productivity (Harman *et al.*, 2004; Brotman *et al.*, 2013; Contreras-Cornejo *et al.*, 2014a; López-Bucio *et al.*, 2015).

Various stages and signaling mechanisms have been established for the Trichoderma–plant interaction using *Arabidopsis thaliana* as a model, highlighting the extraordinary complexity of the fungal–plant recognition process. Early perception of fungal-emitted volatile organic compounds (VOCs) triggers defense-related responses, such as accumulation of hydrogen peroxide, anthocyanin, camalexin and heightened expression of defense-related genes, while the most abundant VOC, 6-pentyl-α-pyrone, modulates growth via activation of auxin transport in roots (Velázquez-Robledo *et al.*, 2011; Contreras-Cornejo *et al.*, 2011, 2015; Kottb *et al.*, 2015; Garnica-Vergara *et al.*, 2016). Moreover, Arabidopsis response to Trichoderma volatiles includes resistance against the airborne phytopathogenic fungi *Botrytis cinerea* and *Alternaria brassicicola*, and even salt stress (Contreras-Cornejo *et al.*, 2011, 2014b; Kottb

et al., 2015; Jalali *et al.*, 2017). Trichoderma species also release a wide variety of diffusible natural products, including indole-3-acetic acid and auxin precursors, and strongly acidify the rhizosphere. In such molecular, cross-kingdom dialog hydrophobins have been shown to play a role in plant root colonization, and it has been proposed that other secreted proteins act like effectors, allowing the establishment of the interaction (Guzmán-Guzmán *et al.*, 2017; Mendoza-Mendoza *et al.*, 2018). Together, these traits modulate root architecture, stimulating primary root growth, formation of lateral roots and root hairs, and determine its biofertilizer and probiotic capabilities (Contreras-Cornejo *et al.*, 2009; Hermosa *et al.*, 2012; Nieto-Jacobo *et al.*, 2017; Pelagio-Flores *et al.*, 2017; Mendoza-Mendoza *et al.*, 2018).

Root-derived sucrose attracts Trichoderma to the rhizosphere and favors a long-lasting symbiosis, apparently without causing damage to the roots but, instead, leads to an increased photosynthetic capacity. Moreover, sucrose availability and fungal hydrolysis of sugar via an invertase (*tvinv*) are necessary for strengthening leaf immunity (Vargas *et al.*, 2009). In contrast, during its life as a saprotroph, the fungus relies on the degradation of organic matter for growth via the release of degradative enzymes, including cellulases, xylanases and peptidases (Crivelente-Horta *et al.*, 2018; Ramírez-Valdespino *et al.*, 2019).

The sessile lifestyle of plants relies on the coordination of adaptive traits to simultaneous stresses, including pathogen challenges, nutrient starvation, as well as exposure to toxins and contaminants, which compromise growth. Growth defense tradeoffs imply the efficient acquisition and prioritization of the use of resources, tightly linked to changes on biotic or abiotic interactions (Huot *et al.*, 2014; Hacquard *et al.*, 2016). Under such situations, reactive oxygen species (ROS) production, which results from the normal aerobic metabolism, controls important developmental processes and cross-kingdom relationships (Hernández-Oñate *et al.*, 2012; Pelagio-Flores *et al.*, 2016; Segal and Wilson, 2018). NADPH oxidases (Nox) are considered the most important enzymes in the production of ROS and, therefore, mutation of the corresponding genes affects growth, virulence and host interactions (Segal and Wilson, 2018). Hernández-Oñate *et al.* (2012) identified two genes (*nox1* and *nox2*) encoding Nox catalytic subunits in *Trichoderma atroviride*, and *noxR* encoding a regulatory subunit. In response to injury, the Δ*nox1* and Δ*noxR* mutants did not conidiate, which correlated with null production of superoxide and H_2O_2, and differential expression of proteins involved in calcium signaling, oxylipin biosynthesis and ROS scavenging systems. In this regard, fungi acting as plant endophytes or taking part in mycorrhizal interactions undergo an oxidative burst upon root colonization, implying a direct role of fungal Nox proteins and ROS in the establishment of symbiosis (Abbá *et al.*, 2009). Although increased production of antioxidant compounds and antioxidant enzymes has been shown to occur in plants during biotic interactions (Foyer and Shigeoka, 2011; Wrzaczek *et al.*, 2013), less attention has been paid to understanding the roles of ROS-producing enzymes in their fungal partners.

This study aimed to characterize the possible role of *T. atroviride* Nox genes during interaction with Arabidopsis. Although Nox1 and NoxR are necessary for conidiation upon injury (Hernández-Oñate *et al.*, 2012), whether these enzymes are required to stimulate growth or immunity in a plant host is unknown. Here, we show that NoxR and Nox1 are necessary for fungal-induced root branching and biomass production in Arabidopsis as Δ*noxR* mutants fail to strongly induce the transition from lateral root primordia (LRP) into mature lateral roots. In contrast, *JAZ1* expression and disease response to *B. cinerea* reveal that Δ*noxR* strongly reinforces jasmonic acid (JA)-dependent plant immunity. Importantly, we show that *T. atroviride* adjusts the expression of genes involved in metabolism in response to the presence of Arabidopsis, likely to avoid causing damage to the plant and take advantage of the simple carbon sources available in root exudates. We provide evidence indicating that this Trichoderma plant dialog is broken when the Nox complex is dysfunctional. Thus, the *T. atroviride* Nox complex orchestrates growth defense tradeoffs in a plant host, and is necessary for the establishment of the mutualistic interaction between *T. atroviride* and *A. thaliana*.

RESULTS

Trichoderma atroviride NADPH oxidase is required for fungal-induced lateral root formation and biomass production

Early response of plants to Trichoderma involves the reconfiguration of the root system architecture via changes in branching and epidermal cell differentiation patterns, which directly impact on biomass, and nutrient and water absorptive potential (Contreras-Cornejo *et al.*, 2009). To determine the role of *T. atroviride* Nox genes in plant development, we assessed the effects of the wild-type (WT) strain, and the Δ*nox1*, Δ*nox2* and Δ*noxR* mutants on primary root growth, lateral root formation and density, and root biomass in Arabidopsis seedlings co-cultivated with fungal colonies *in vitro*. While growth of the primary root was only slightly influenced by co-cultivation with the fungus (Figure 1a i), both lateral root number and density increased by threefold in seedlings co-cultivated with the *T. atroviride* WT strain, which correlated with a twofold increase in root biomass when compared with axenically grown seedlings. In plants co-cultivated with the Δ*nox1*, Δ*nox2* and Δ*noxR* strains, the increased root branching and biomass production provoked by the WT clearly

diminished, particularly in response to the Δ*noxR* mutant, in which the NADPH regulatory subunit is defective (Figure 1a–i), indicating the importance of NADPH oxidases for the interaction with plants.

NoxR is required for *Trichoderma atroviride*-induced lateral root formation and maturation

Root branching requires LRP to be formed from the parent root and lateral roots to achieve active growth (Malamy and Benfey, 1997). The Δ*noxR* strain showed the strongest defects in the stimulation of lateral root formation and biomass production and it should, in principle, be required for the activity of both catalytic subunits. Therefore, we used only this strain to investigate if delayed lateral root formation in response to Δ*noxR* could be caused by the failure to promote the transition of LRP into mature lateral roots and correlate such developmental transitions with auxin-induced gene expression. The stages of LRP formation were analyzed in *DR5:GUS* Arabidopsis seedlings, which report auxin-inducible expression, exposed to either the *T. atroviride* WT or the Δ*noxR* mutant. We found slight effects of the WT strain in increasing LRP at stages I–V (Figure S1a). By contrast, the number of primordia at stages VI and VII, as well as the number of emerged lateral roots (ELR; Figure S1a), and total LRP strongly increased (Figure S1b), indicating a strong influence of the fungus in the maturation of root primordia. On the other hand, the Δ*noxR* mutant failed to promote the initiation of LRP, and showed a weaker induction of primordia maturation than the WT (Figure S1a,b). Detailed analysis of *DR5:GUS* expression at all developmental stages during LRP formation indicated that the WT strain increases the auxin response at most stages analyzed, whereas Δ*noxR* failed to sustain an increased auxin response (Figure S1c). We conclude that the lateral root formation program elicited by *T. atroviride* in Arabidopsis requires NoxR.

The transcriptional response of Arabidopsis is exacerbated during the interaction with *Trichoderma atroviride* Δ*noxR* mutant

To evaluate the transcriptional changes underlying the molecular responses of Arabidopsis root during the interaction with Trichoderma WT and Δ*noxR*, we performed a differential gene expression analysis at 3 and 5 days post-inoculation (dpi). This analysis showed that at 3 dpi, when there is still no contact between the hyphae and the plant, a total of 429 genes were differentially expressed, 230 upregulated and 199 downregulated, as a response to the WT strain (Table S1). The number of differentially expressed genes increased at 5 dpi, when the root system

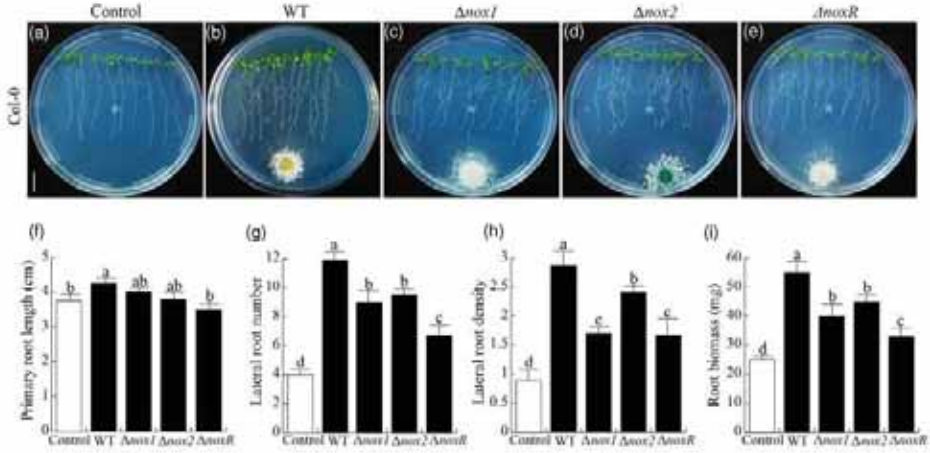

Figure 1. Effect of wild-type (WT) *Trichoderma atroviride* and the NADPH oxidases mutant strains on Arabidopsis root architecture.
Four day-old Arabidopsis seedlings were inoculated with *T. atroviride* WT and the Δ*nox1*, Δ*nox2* and Δ*noxR* mutants at 5 cm from the root tip and allowed to grow for 4 additional days.
(a–e) Representative photographs of seedlings co-cultivated with the Trichoderma strains.
(f) Length of primary root.
(g) Lateral root number.
(h) Lateral root density (number of emerged lateral roots (ELR) cm^{-1}).
(i) Root biomass. Bars show the means ± SE. Different letters indicate significant statistical differences ($P < 0.05$; $n = 30$). Scale bar: 1 cm. Similar results were obtained in three independent repetitions of the experiment.

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is already covered by the fungal colony (contact stage), showing 1277 genes upregulated and 1348 downregulated (Table S2). Interestingly, even before contact with the root (3 dpi), the Δ*noxR* mutant promotes a stronger change in the plant gene expression profile than the WT with 390 genes upregulated and 267 downregulated (Table S3). This trend is more marked at 5 dpi, where the mutant provoked induction of 1586 and repression of 1881 genes (Figure 2a; Table S4). Furthermore, a detailed analysis of the gene expression profile showed that many of the induced genes are expressed at a higher level in response to the Δ*noxR* strain than in response to the WT (Figure 2b–d), which is more evident at 3 dpi (Figure 2b). At 3 dpi, when Arabidopsis is interacting with the Δ*noxR* strain, 79 plant genes are induced and 59 repressed (Figure 2c; Table S5), while at 5 dpi, 81 genes increased and 82 decreased their expression, as compared with the set of Arabidopsis genes responding to the WT strain (Figure 2d; Table S6). Venn diagrams show the number of plant genes specifically repressed and induced in response to the Δ*noxR* strain, both at 3 and 5 dpi, as well as those responding at both stages of the interaction (Figure 2e,f).

The Δ*noxR* mutant enhances to a greater extent the plant systemic response to damage

Functional category enrichment analysis of the differentially expressed genes made evident that before contact with the plant (3 dpi) the *T. atroviride noxR* mutant elicits a strong immune response in the seedlings, switching on processes such as response to chitin, bacterial pathogens and herbivores, and metabolism of indole-containing compounds (Table S7). These categories are clearly enriched when the roots are colonized (5 dpi) by both the *T. atroviride* WT and Δ*noxR* strains (Figure S2), but the response was exacerbated in the interaction with the Δ*noxR* mutant (Figures 3a and S3). Interestingly, genes encoding elements of the JA pathway, such as PAD3, JAZ1, JAZ6 and LOX1, increased their expression level to a higher level in response to the Δ*noxR* mutant than to the WT strain at 5 dpi (Figure 3b). These results indicate that Arabidopsis

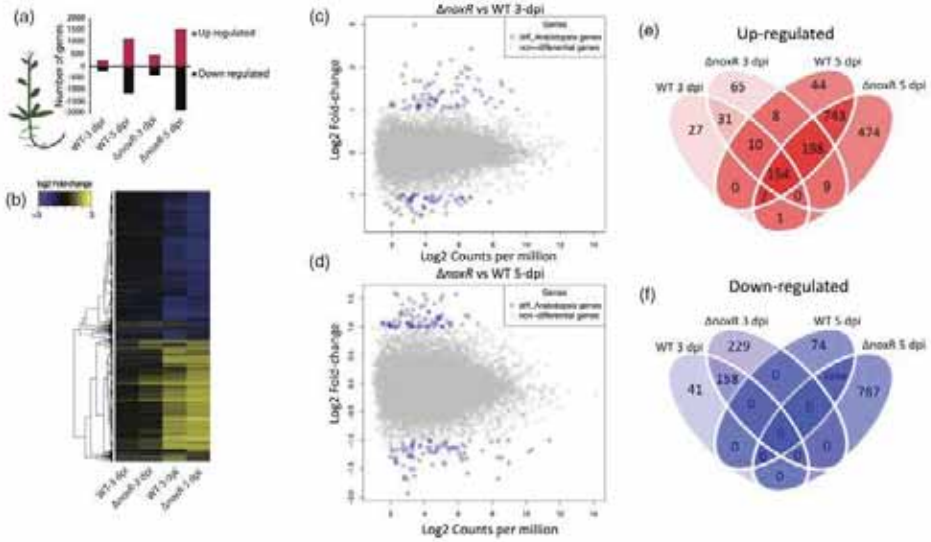

Figure 2. Transcriptomic profile of the Arabidopsis root during the interaction with *Trichoderma atroviride*.
Number of Arabidopsis genes differentially expressed in the interaction with the *T. atroviride* wild type (WT) and Δ*noxR* strains at 3 and 5 days post inoculation (dpi), plants growing in MS medium without the fungus were used as controls (FDR < 0.05, fold-change >1) (a). Heatmap of the genes differentially expressed in the plant when interacting with the WT and the Δ*noxR* mutant, compared with the non-inoculated control plants (b).
Comparison of the Arabidopsis gene expression profile during the interaction with the Δ*noxR* and the WT strains, the blue dots represent differentially expressed genes at 3 dpi (c) and 5 dpi (d).
Venn diagrams showing the number of genes shared by the four comparisons or that are unique to each contrast; the red diagram shows the upregulated genes (e) and the blue diagram the downregulated genes (f).

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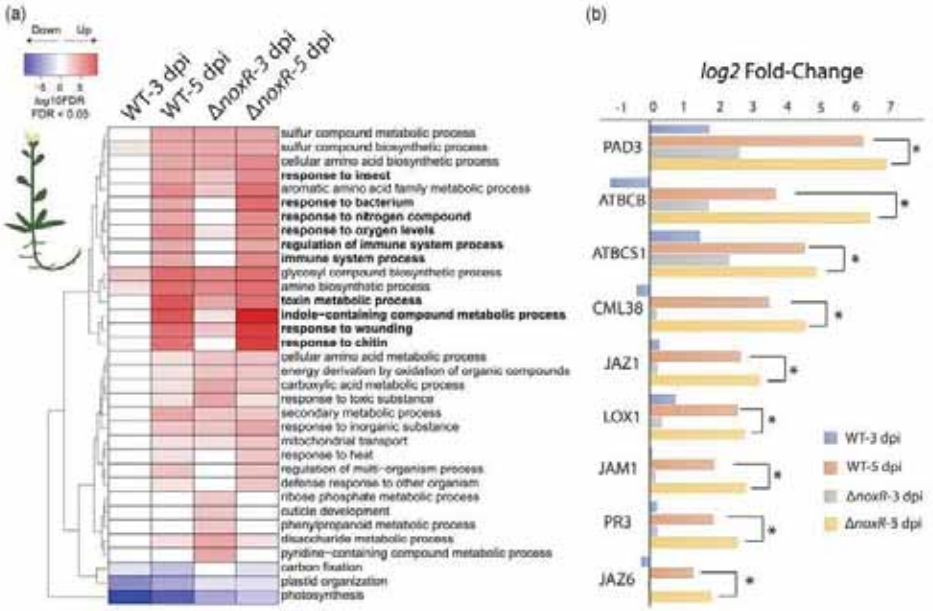

Figure 3. Functional analysis of Arabidopsis response to Trichoderma.
Category enrichment of Biological Process in differentially expressed genes (FDR < 0.05). Each vertical block contains the up- and downregulated categories in the heatmap. Color intensity represents the significance in logarithm base 10 of the FDR per each category (a).
Counts per million of stress response genes in each evaluated condition (b) (*FDR < 0.05).

displays a strong transcriptional response to the presence of the fungus even before contact.

Trichoderma atroviride Δ*noxR* increases JAZ1 protein levels in roots and shoots

Trichoderma atroviride strengthens plant immunity, and the underlying transcription dynamics is undulating (Contreras-Cornejo *et al.*, 2011; Velázquez-Robledo *et al.*, 2011; Moore *et al.*, 2011; Hermosa *et al.*, 2012; Rubio *et al.*, 2014; Medeiros *et al.*, 2017). As part of this response, *JAZ1* protein levels increase in leaf and root cells (Grunewald *et al.*, 2009). To know if *T. atroviride* could induce JAZ1, *JAZ1-GFP* expression pattern was compared in Arabidopsis seedlings grown under axenic conditions, treated with JA or co-cultivated with *T. atroviride* WT or the Δ*noxR* mutant. In agreement with a previous study (Grunewald *et al.*, 2009), *JAZ1-GFP* was detected in nuclear bodies of the stele in response to JA treatment (Figure 4a,b). Seedlings inoculated with *T. atroviride* WT had a greater number of cells with nuclei expressing *GFP* in the vascular bundle at the differentiation zone of the primary root, a response clearly exacerbated in plants co-cultivated with the Δ*noxR* mutant (Figure 4a,b). On the other hand, analysis of *JAZ1:GUS* expression in cotyledons further indicated its strong induction by both the WT and Δ*noxR* mutant, although this occurred earlier in plants interacting with the mutant strain (Figure 4c). These data are consistent with the transcriptomics analysis, and indicate that NoxR critically mediates the host defense response.

Trichoderma metabolic response to Arabidopsis relies on *noxR*

To understand how the fungus reacts to the presence of living plants growing in close proximity, an analysis of the changes in gene expression was performed for the *T. atroviride* WT and the Δ*noxR* mutant during the interaction with Arabidopsis. Interestingly, many genes changed their expression in response to the plant in the WT strain, even before contact (400 up- and 872 downregulated). This result indicates that even before physical contact there is a two-way communication between the partners (Table S8). Once the hyphae contact roots (5 dpi), Trichoderma seems to return to its basal state of gene expression (Figure 5a; Table S9). Surprisingly, the Δ*noxR* mutant showed only

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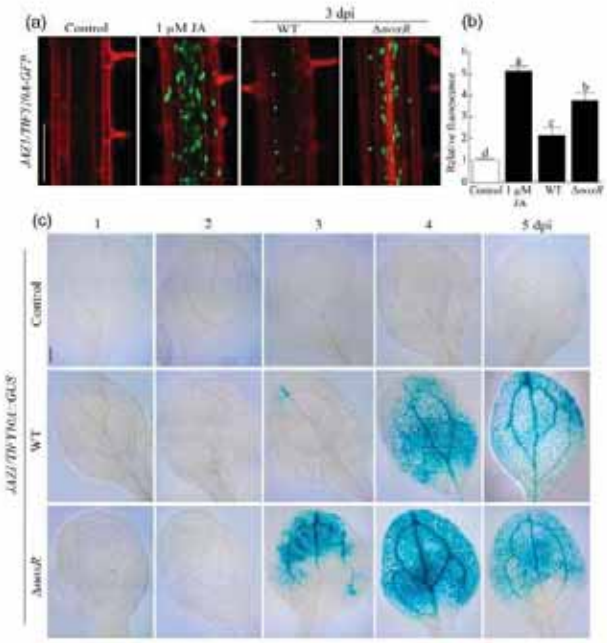

Figure 4. *Trichoderma atroviride* Δ*noxR* upregulates JAZ1 expression in Arabidopsis roots and leaves.

Transgenic Arabidopsis seedlings harboring the *JAZ1/TFY10A-GFP* gene construct were co-cultivated with *T. atroviride* wild-type (WT) and the Δ*noxR* mutant for 5 days. Expression of JAZ1 in the primary root after 3 days of co-cultivation (a).

The graph illustrates differences in expression, assessed as relative fluorescence intensity (b).

Representative micrographs of leaves expressing *JAZ1/TFY10A::GUS* at the indicated day post-inoculation (dpi) with the *T. atroviride* WT strain or the Δ*noxR* mutant, and the corresponding controls without fungus (c).

Values shown represent the means for 8 seedlings ± SD. Different letters indicate means that are statistically different ($P < 0.05$).

minor changes in gene expression profile before and during the interaction with the plant, with very similar changes in the total of differentially expressed genes at 3 and 5 dpi (130 and 187 differentially expressed genes, respectively; Figure 5a; Tables S10 and S11). Detailed gene expression profile showed that a group of genes is clearly repressed in the WT strain, while their expression remains unaltered in the Δ*noxR* mutant (Figure 5b; Tables S12 and S13). It is noteworthy that several genes related to carbon and nitrogen metabolism, such as carbohydrate-binding proteins (ID: 283182 and ID: 28839), a glycoside hydrolase (ID: 48371), cellulases (ID: 314392 and ID: 84753) and sugar transporters (ID: 143414 and ID: 40863) are repressed in the presence of the plant at 3 dpi in the WT strain, but remain active in the Δ*noxR* mutant, independently of the interaction time evaluated (Figure 5c). These results suggest that Δ*noxR* is affected in plant recognition, which would explain their reduced transcriptional response.

Trichoderma atroviride Nox mediates perception and response to molecular signals emitted by plants

The observation that genes involved in nitrogen and carbon nutrition are repressed in the WT strain, but not in the Δ*noxR* mutant, suggests an alteration in the fungus–plant dialog. Fungal degradative enzymes are a hallmark during the saprophytic life cycle of Trichoderma, thus we evaluated the expression of all genes annotated as glycoside hydrolases and peptidases in the *T. atroviride* WT and the Δ*noxR* mutant 3 dpi. Interestingly, a Wilcoxon test marks a tendency to induction ($P < 0.05$) in the Δ*noxR* mutant for both groups of genes (Figure 6a). A bar-plot of genes with greater change in both peptidases and glycoside hydrolases show their repression in the WT strain before contact with the plant, which never happens in the mutant background (Figure 6b). An interesting observation was that in the absence of sucrose and the presence of cellulose, both the WT and Δ*noxR* mutant strains seem to take advantage of this complex source of carbon in a similar way; however, in the presence of sucrose, the WT strain grows faster, while the Δ*noxR* mutant does not seem to use this carbon source efficiently (Figure S4).

The analysis of functional categories revealed that the processes of cellular response to stimulus, cell cycle, DNA metabolism and cell communication are enriched at 3 dpi of the interaction with Arabidopsis in the WT strain of Trichoderma (Table S14); however, these processes are less active in the Δ*noxR* mutant (Figure 6c). Individual analysis of the genes contained in the categories of cell communication, and DNA metabolic process (Figure 6d), revealed that a series of genes directly involved in environmental

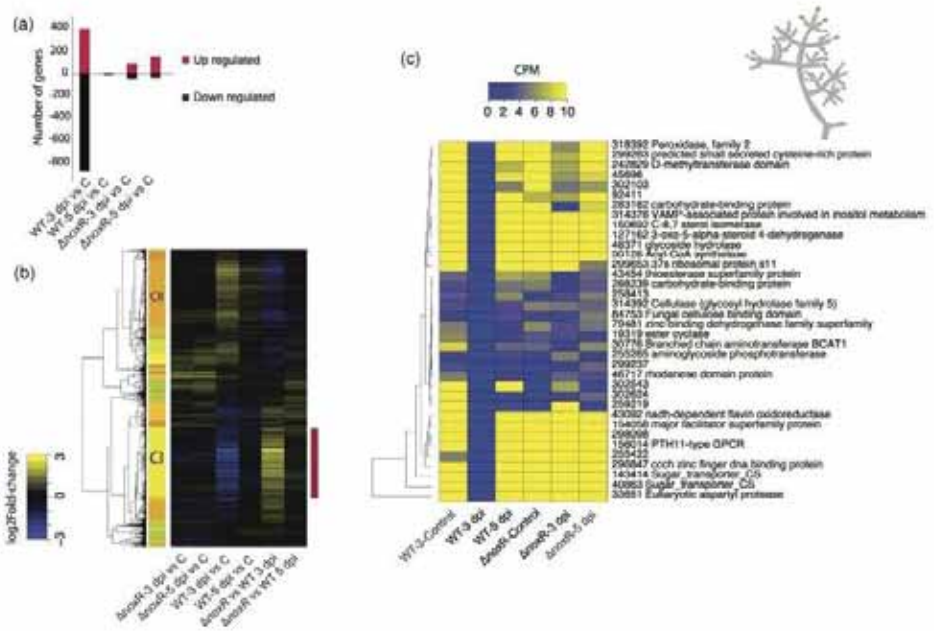

Figure 6. Transcriptomic profile of *Trichoderma atroviride* during the interaction with *Arabidopsis thaliana*.
Number of differentially expressed genes in Trichoderma when interacting with the plant, both in the wild-type (WT) and Δ*noxR* strains, at 3 and 5 days post-inoculation (dpi), as control was used fungus growing in MS medium without the presence of the plant (FDR < 0.05, fold-change > 1) (a).
The heatmap shows the genes differentially expressed in the WT and Δ*noxR* strains of Trichoderma interacting with the plant (b).
The heatmap shows the counts per million of the genes belonging to cluster 1, which is highlighted with a brown bar (c).

signal perception, such as sensory transduction histidine kinase (ID: 133533), signal transducer activity (ID: 312727) and phospholipase C (ID: 147071), among others, are not responsive in the Δ*noxR*, while genes involved in stress response and initiation of DNA replication such as MCM5 (ID: 31705), exonuclease-1 (ID: 319288) and pob-3 (ID: 305357) are activated at 3 dpi in the WT strain. However, this activation is not observed in the Δ*noxR* mutant at any stage of the interaction. This supports the notion that NoxR is necessary to perceive the plant even before contact that in turn can trigger DNA replication processes and activation of the cell cycle, which enables root colonization by the fungus.

Trichoderma atroviride WT and Δ*noxR* mutants induce plant resistance to *Botrytis cinerea*

Trichoderma activates defense mechanisms that lead to *B. cinerea* resistance in plants (Contreras-Cornejo *et al.*, 2011). *JAZ1* upregulation by Δ*noxR* suggested that a strong JA response can be activated in leaves following interaction of roots with this mutant. Next, we compared the responses of 15-day-old Arabidopsis plants grown axenically or co-cultivated with the *T. atroviride* WT strain or the Δ*noxR* mutant to infection by *B. cinerea*, which causes the spread of necrotic lesions. The effects of inoculating *B. cinerea* conidia on disease symptoms were evaluated 3 and 5 days after pathogen inoculation, considering the percentage of plants showing necrosis, lesion diameter and leaf colonization. In plants grown without Trichoderma, *B. cinerea* caused necrotic lesions in about 75% of plants by 3 days, and reached 100% at day 5, at this later stage, the diameter of the lesions on leaf surfaces correlated with the spread of *B. cinerea* (Figure 7a–c). In contrast, WT or Δ*noxR* decreased the percentage of symptomatic plants at the two times assayed (Figure 7a), and reduced the size of lesions as well as the spread of *B. cinerea* (Figure 7b). These data show that the protection conferred by the WT strain against *B. cinerea* in Arabidopsis was not compromised by NoxR loss-of-function. Instead, a decrease in the damage by the infection was

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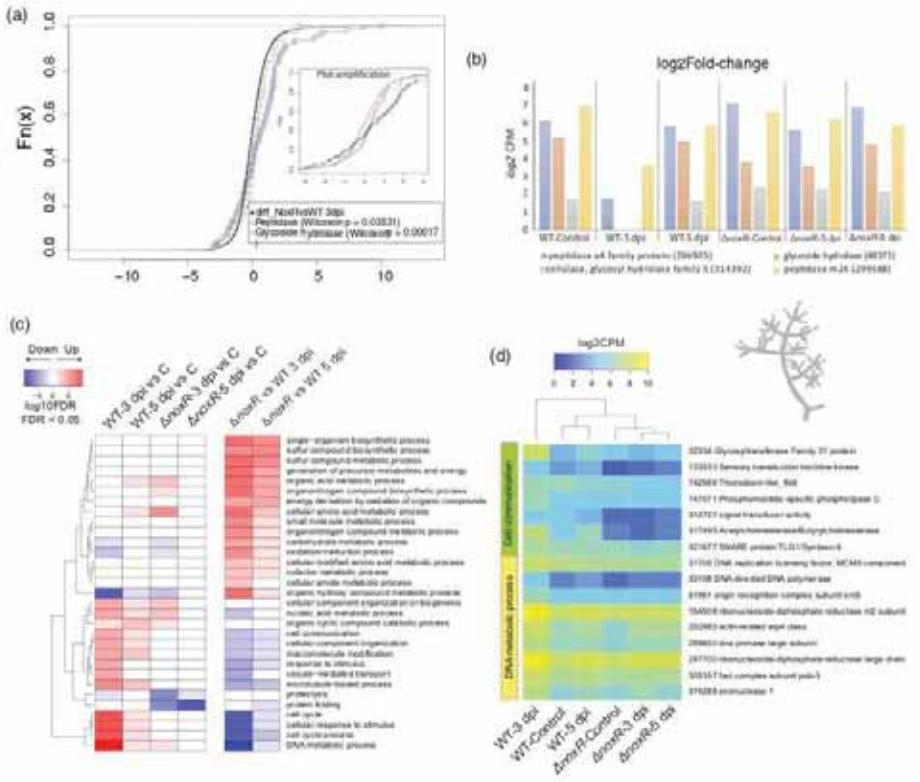

Figure 6. NoxR affects the fungal ability to sense and respond to plants.
Cumulative probability curve of the expression profile of all peptidase (32) and glycoside hydrolase (105) genes in the comparison wild-type (WT) versus Δ*noxR* at 3 dpi, the '*x*'-axis shows the log2-fold-change, the black curve represents the expression profile of all the genes (9326). A zoom-in of the plot allows distinguishing the tendency of the genes from the distribution of the rest of the genes in the comparison Δ*noxR* versus WT at 3 dpi (a).
A Wilcoxon test was performed ($P < 0.05$). Counts per million of the genes encoding peptidases and glycoside hydrolases showing higher overexpression in the Δ*noxR* mutant (b).
The expression data were not filtered by fold-change or FDR. Category enrichment of Biological Process in differentially expressed genes. Each vertical block contains the up- and downregulated categories in the heatmap. Color intensity represents the significance in logarithm base 10 of the FDR per each category (FDR < 0.05) (c).
Counts per million of the differential genes contained in cell communication and DNA metabolic process GOs (d).

observed in the Arabidopsis leaves when it grew in the presence of the Δ*noxR* mutant (Figure 7c), as the proportion of leaves without damage observed is larger (class I) and the proportion of class III leaves tends to be smaller in comparison with the WT strain, while there is no apparent difference in classes II and IV.

DISCUSSION

Trichoderma species are free-living fungi with versatile metabolism that interact with plants via an intricate chemical communication (Ramírez-Valdespino *et al.*, 2019). Root colonization by Trichoderma does not only reconfigure root development and boost plant growth, but also reinforces the defense systems involving JA, ethylene (ET) and salicylic acid (SA), which results in an improved capability to resist pathogen attack and herbivory (Hermosa *et al.*, 2012; Rubio *et al.*, 2014; Leonetti *et al.*, 2017; Poveda *et al.*, 2019). The plant defense response is expensive, such that during pathogen challenges a decrease in growth and biomass accumulation is commonly observed (Kang and

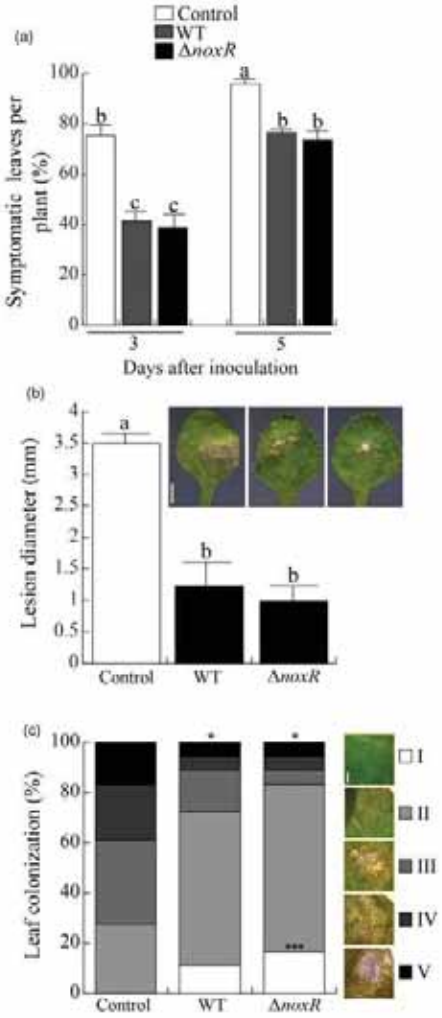

Figure 7. Trichoderma wild-type (WT) and Δ*noxR* mutant strains increase disease resistance against *Botrytis cinerea* in *Arabidopsis thaliana*.
Seven-day-old grown Arabidopsis seedlings co-cultivated 3 days with both *Trichoderma atroviride* strains *in vitro* were transferred in a vessel for plant tissue culture containing 0.2 × MS medium and further inoculated with a 5 μl droplet of *B. cinerea* (1×10^5 spores ml^{-1}) on leaf surfaces.
(a) The percentage of leaves with necrotic symptoms at 3 and 5 days after inoculation was determined by counting the number of droplets that produced lesions and representative inoculated leaves were imaged.
(b) Size of expanding lesions was measured 3 days after pathogen inoculation. Error bars represent the mean ± SD of 4 inoculated leaves from 12 different plants per Trichoderma strains-treatment. Different letters represent means statistically different ($P < 0.05$).
(c) Disease resistance in seedlings inoculated with Trichoderma strains is shown as the percentage of leaves in each resistance class. An asterisk indicates statistically significant differences with the control without Trichoderma, and the three asterisks statistically significant differences in the percentage of leaves in the different classes between the treatments with the different strains (Fisher's Exact Test, $P < 0.001$). The experiment was repeated twice with similar results. Scale bars: 1 and 100 μm, respectively.

Singh, 2000; Rivas-San Vicente and Plasencia, 2011; Wasternack and Hause, 2013). Thus, Trichoderma elicits growth and development while activating a strong immune response in the plant (Hermosa *et al.*, 2013). Although such a phenomenon has attracted considerable interest due to its potential agricultural applications, our knowledge on the molecular mechanisms underlying growth–defense tradeoffs is still limited.

Our data show that the communication established between *T. atroviride* and Arabidopsis is broken by mutation of genes encoding NADPH oxidase proteins in the fungus, specifically Nox1 and the regulatory subunit NoxR, which are affected in the production and accumulation of ROS (Hernández-Oñate *et al.*, 2012). The fact that fewer lateral roots were observed in Arabidopsis seedlings co-cultivated with the Δ*noxR*/Δ*nox1* mutants compared with the WT suggests that the Nox complex and/or the generation of ROS through this complex are necessary for stimulating the emergence of primordia and their transition into a new branching structure. Coincidentally, the Δ*noxR* and Δ*nox1* mutants were less effective for phytostimulation, which may be due to a reduced capacity to elicit the root absorptive potential. In this regard, it has been shown that ROS treatment increases lateral root (LR) number via the activation of LR pre-branch sites and LRP outgrowth (Orman-Ligeza *et al.*, 2016). Thus, the reduced auxin response in LRP and weak promotion of LRP maturation elicited by the Δ*noxR* mutant in Arabidopsis seedlings may be due to a reduced ROS signaling, affecting the Trichoderma–root communication.

Plants coordinate gene expression and metabolic networks for adaptation. Transcription is the first and often limiting process to convert genetic information into the proteins that allow readjustment of the phenotype. Transcriptional control of enzyme-encoding genes also plays a major role in constructing the metabolic networks necessary to respond to environmental stimuli and to undergo a change in a developmental program (Gaudinier *et al.*, 2015). To clarify the molecular signatures underlying the plant responses to Trichoderma involving the Nox complex, a transcriptomic analysis was performed comparing the impact of the WT and Δ*noxR* strains on the gene expression profile of Arabidopsis. Surprisingly, the Δ*noxR* mutant gave rise to changes in the pattern of expression of

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a greater number of genes than the WT, before and during contact of hyphae with roots, indicating that ROS production by the fungus is highly relevant for communication with the plant. Importantly, the altered response caused by the Δ*noxR* mutant resulted in stronger activation of functions related to responses to bacteria and insects, wounding and reinforcement of the host immune system even before contact of roots with the mutants. In this regard, a previous study showed that the plant transcriptomic response to *Trichoderma parareesei* for plants fluctuated, and the JA-dependent response can be upregulated at a given point of the interaction but downregulated at earlier stages (Rubio *et al.*, 2014).

Genes involved in phytoalexin biosynthesis, and the JA and SA signaling pathways showed increased expression in response to *T. atroviride* that was exacerbated when the plant interacted with the Δ*noxR* mutant. One of these genes, namely *PAD3*, participates in the biosynthesis of camalexin, an antifungal compound that protects *A. thaliana* from infection by *A. brassicicola* (Zhou *et al.*, 1999). In accordance with previous studies, this result suggests that Trichoderma inoculation primes plants to counteract infection by pathogens, which is further stimulated by the mutant strain Δ*noxR*. The *AtBCB* gene is induced before root contact with Δ*noxR*, a gene that when overexpressed in Arabidopsis drives over-production of lignin in whole roots, which correlates with resistance to colonization by fungi (Ezaki *et al.*, 2005). A transcriptional co-regulation mechanism integrating energy allowance required for growth, defense and lignin biosynthesis, which involves readjusting resource allocation to different pathways, was recently proposed (Xie *et al.*, 2018). On the other hand, the *AtBCS1* and *CML38* genes, likely involved in tolerance to drought stress and hypoxia, are also overexpressed in plants in the presence of the Δ*noxR* mutant (Zhang *et al.*, 2014; Lokdarshi *et al.*, 2016), suggesting that plants exposed to this strain may perform better when grown under adverse environmental conditions.

Particularly relevant was the finding that systemic acquired resistance genes such as *JAZ1*, *JAZ6*, *JAM1*, *LOX1* and *PR3* involved in the JA pathway were induced in plants in the presence of the Δ*noxR* mutant. JAZ proteins interact with and repress the activity of various transcription factors, including the JA master regulator MYC2 (Guo *et al.*, 2018). The JAZ1/TIFY10A construct is expressed in nuclei, and is induced by JA and auxins (Grunewald *et al.*, 2009). The increase in the number of nuclei expressing JAZ1 at the inner cell layers of the primary root indicates that more cells are reacting to Trichoderma-released metabolites (i.e. auxin) or plant-produced JA, which may be locally produced or systemically transported from the shoot. In addition, when evaluating this response with the *JAZ1:GUS* marker line in leaves at different times of interaction, the Δ*noxR* mutant elicited an earlier activation of the jasmonate pathway. The reduced effect of the Δ*noxR* mutant in root branching may be explained by an over-activation of the JA response, which may lead to carbon deprivation or reduced energy resources in the LRP for outgrowth (Guo *et al.*, 2018).

The absence of ROS as signaling molecules in the Δ*noxR* mutant may stimulate the release of metabolites or enzymes such as cellulases (glycoside-hydrolases), which induce the activation of the stress pathways. For instance, the cellulases Thph1 and Thph2 of *Trichoderma harzianum* induced the jasmonate/ET signaling pathway in maize and improved their ability to contend with *Fusarium graminearum* infection, whereas mutants in these genes failed to trigger this protective effect in the plant (Saravanakumar *et al.*, 2016, 2018). Similarly, the *noxR* mutant conferred slightly higher resistance to Botrytis. These results point to the overstimulation of defense systems, which is inversely correlated with the capability of *T. atroviride* to promote lateral root formation and plant biomass accumulation.

Auxin signaling directly influences the root branching process induced by Trichoderma, which is disturbed in Arabidopsis during interaction with the Δ*noxR* mutant. Several studies support a model to explain the balance between growth defense tradeoffs based on mutually antagonistic activation between auxin and JA signaling (Hermosa *et al.*, 2013; Medeiros *et al.*, 2017). Both hormonal pathways control root morphogenesis in Arabidopsis (Cai *et al.*, 2014; Huang *et al.*, 2017). Moreover, changes of endogenous JA levels affect the formation of lateral roots and influence auxin homeostasis via auxin biosynthetic genes like *ANTHRANILATE SYNTHASE α1* (*ASA1*) and some YUCCA gene family members (*YUCCA8* and *YUCCA9*; Sun *et al.*, 2009; Hentrich *et al.*, 2013). Besides, jasmonates influence auxin transport by a downregulation of the auxin efflux carrier PIN-FORMED 2 (PIN2) abundance (Sun *et al.*, 2011). This shows the important role of Trichoderma NoxR-generated ROS in the complexity of the hormonal network orchestrating growth suppression and defense activation in plants.

Recently, it was reported that Trichoderma–Arabidopsis communication via VOCs is affected by the mutation of *noxR* and *nox1* in the fungus (Cruz-Magalhães *et al.*, 2019), apparently due to defective production of secondary metabolites. However, little is known about the transcriptional changes underlying fungal metabolism. To assess how the fungi sense plants, comparisons of the transcriptional response of the Trichoderma WT and Δ*noxR* strains during the interaction with Arabidopsis were done. Strikingly, while the WT strain can perceive the presence of Arabidopsis even before contact and changes its gene expression profile, the Δ*noxR* mutant does not react to the presence of the plant, indicating that mutation of *noxR* affects the fungal ability to sense plants. Before root contact, in the WT strain, genes encoding peroxidases,

cellulases, sugar transporters and in general carbohydrate-related genes are repressed. In full agreement with our results, it was recently described that during the interaction of *Trichoderma virens* with maize, there is global repression of genes encoding cell-wall-degrading enzymes (Malinich *et al.*, 2019). In our view, these observations suggest that suppression of metabolic processes related to the degradation of complex carbohydrates in the fungus is necessary to establish a beneficial interaction with the plant, likely as a result of the adaptation of the fungus to the availability of simple carbohydrates such as sucrose provided by the roots (Macías-Rodríguez *et al.*, 2018). An analysis of the use of cellulose and sucrose as sole carbon sources or in combination showed that the mutant strain Δ*noxR* does not use sucrose efficiently, but the mutant and the WT strain appear to have the same capacity to consume cellulose. An independent study showed that *noxR* mutants have a reduced capacity to utilize sucrose as a sole carbon source to sustain growth (Cruz-Magalhães *et al.*, 2019), suggesting their inability to undergo this adaptation. This could explain why, even in the presence of plant exudates, the Δ*noxR* mutant does not turn off genes encoding proteins that enable degradation of complex carbon sources, which can have deleterious effects on the cell wall of the roots or on the chemical dialog established before physical hyphae root contact. Thus, modulation of degrading enzymes occurs in a ROS-dependent manner, possibly to avoid overstimulation of the defense response pathways in roots. Modulation of degrading enzymes has also been observed during oomycete parasitism by Trichoderma. Overexpression of Nox1 in *T. harzianum* led to upregulation of genes encoding protease, cellulase and chitinase activities when confronted with *Pythium ultimum* (Montero-Barrientos *et al.*, 2011). It was also of great interest to observe that cell cycle processes and DNA metabolism were activated only in the WT strain, likely due to the availability of simple sugars and/or secondary metabolites present in root exudates, resulting in chemiotropic growth of hyphae and improved colonization.

To perceive environmental stimuli, eukaryotes use transmembrane receptors such as G-protein-coupled receptors (GPCRs). It has been shown that GPCRs participate in decoding the signals that activate the production of ROS through NADPH oxidases. GPCRs act via tyrosine phosphorylation of proteins such as Janus kinases, which participate in the initiation of signaling for basic cellular programs (Pelletier *et al.*, 2003). Rho family GTPases are regulators of cell migration, cell–cell adhesion, and cell matrix adhesion remodeling the cytoskeleton. ROS have been shown to directly activate RhoA, which induces cytoskeletal rearrangements (Aghajanian *et al.*, 2009). In *T. atroviride*, the Gpr1 receptor participates in the sensing of host signals and activation of mycoparasitic host attack (Omann *et al.*, 2012). These evidences allow us to suggest that perception of plant exudates may be carried out via GPCRs, which downstream activate the Nox protein complex leading to the production of ROS, which in turn can activate signaling pathways such as Rho GTPases to give rise to changes in metabolism, cytoskeletal remodeling and cell cycle activation, processes that allow interaction with the plant in a controlled manner.

In *Epichloë festucae*, disruption of genes encoding the NADPH oxidase complex components NoxA, NoxR and a small GTPase RacA compromise its ability to maintain the mutualistic symbiotic association with *Lolium perenne* (Becker *et al.*, 2016; Kayano *et al.*, 2018). Mutations in these genes lead to the growth of hyperbranched hyphae in the intercellular spaces of leaves, which causes a severely stunted host plant phenotype, premature senescence and enhanced expression of defense genes, symptoms like those caused by pathogenic fungi (Scott *et al.*, 2012). Therefore, in Trichoderma and Epichloë, ROS production mediated by the Nox complex is essential for the establishment of symbiotic associations with plants.

Taken collectively, our data indicate that ROS produced by Trichoderma play a major role in the inter-kingdom communication with plants. The Trichoderma–plant communication begins before physical contact, leading to the activation of signaling processes in both organisms that allow the successful establishment of their mutualistic relationship. In this dialog, the fungus appears to respond to nutrients released by roots that lead to overall metabolic adjustments. At the other end, the plant establishes a delicate growth/defense balance in response to Trichoderma. When this balance is broken due to a defective communication, the plant establishes a stronger defense response, with significant costs for both organisms.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Plant materials used in the experiments reported here were the *A. thaliana* WT (Col-0) ecotype and derived lines *DR5::uidA* (Ulmasov *et al.*, 1997) and *JAZ1/TIFY10A GFP* (Grunewald *et al.*, 2009). Surface disinfection of seeds was carried out by soaking them in 95% (v/v) ethanol and 20% (v/v) bleach for 5 and 7 min, respectively. The seeds were then washed five times with sterilized, distilled water, and sown on Petri dishes containing MS 0.2× medium (Murashige and Skoog, 1962) using MS Basal salts mixture (Sigma, https://www.sigmaaldrich.com). The medium was supplemented with sucrose 0.6% (w/v) and solidified with agar 1% (w/v; micropropagation grade; PhytoTechnology Laboratories, https://www.phytotechlab.com) at pH 7. The Petri dishes were included into a Percival AR-95L (https://www.percival-scientific.com) cabinet, with controlled environment (16 h of light/8 h of darkness, 300 μmol m^{-2} sec^{-1} light and temperature of 22°C).

Fungal growth and plant inoculation experiments

The WT strain of *T. atroviride* (IMI 206040) and the Δ*nox1*, Δ*nox2* and Δ*noxR* mutant strains used in the experiments reported here (Hernández-Oñate *et al.*, 2012) were grown on potato dextrose

agar (PDA; Difco [Becton Dickinson], https://www.bd.com) at 28°C for 5 days and conidia harvested using sterile distilled water. Conidia (1×10^6) were inoculated at the 5 cm of the main root tips of 4 day-old Arabidopsis seedlings and the interaction allowed to proceed using 0.2× MS medium solidified with agar. Every plate included 10 Arabidopsis seedlings, and the placement of all plates inside the chamber (Percival AR95L) followed a randomized design. Analysis of plant growth was performed at day 4 in the interaction.

Growth analysis

The growth of primary roots was quantified using a ruler, while all mature roots emergen from the parent one were visually counted at a 10× objective of a Leica (https://www.leica-microsystems.com/) MZ6 stereomicroscope. The density of lateral roots was estimated dividing the number of lateral roots by the length of the primary root for each seedling. Statistical analyses were done using the STATISTICA 10.0 program (Dell StatSoft, https://www.statsoft.cz). Statistically significant differences in root traits were determined via univariate and multivariate analyses with Tukey's *post hoc* tests.

Determination of the developmental stages of lateral root primordia

Quantification of the developmental transitions in LRP was done 8 days after germination in cleared seedlings, and the developmental stages considered were those reported by Malamy and Benfey (1997).

Propidium iodide staining and GFP fluorescence quantification

Quantification of GFP fluorescence was performed via confocal microscopy in transgenic Arabidopsis seedlings that were stained with propidium iodide (PI; 10 mg ml^{-1}) for 1 min, rinsed in water and mounted in 50% (v/v) glycerol on microscope slides. Detection of PI red fluorescence and GFP emission were done on an Olympus (https://www.olympus-global.com), FV1200 confocal laser scanning microscope with an argon blue laser with an excitation line from 488 to 568 nm and an emission window from 585 to 610 nm, and the two images merged. Eight micrographs from each treatment were taken at the root vasculature, from the differentiation zone of the root, and green pixels quantified by using the IMAGEJ software (http://rsbweb.nih.gov/ij/). Each micrograph yielded an arbitrary unit value (A.U. = green pixels μm^2), and the means were graphed starting with control, where A.U. are equal to 1, and thus the means from the different treatments represent changes in fluorescence relative to 1.

Histochemical analysis of GUS expression

Analysis of β-glucuronidase (GUS) expression was performed in seedlings incubated in a solution composed of 0.1% X-Gluc (5-bromo-4-chlorium-3-indolyl, β-D-glucuronide) in phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 0.1 M; pH 7), 10 mM EDTA, 0.1% (v/v) Triton X-100 with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide for 12 h at 37°C. After this time, seedlings were processed to eliminate pigments and clarify the inner tissues following the protocol of Malamy and Benfey (1997). The seedlings were mounted on slides in 50% glycerol solution to make semi-permanent preparations, and photographs were taken from 10 seedlings of each treatment using a Leica DFC450C microscope with Nomarski optics.

Bioassays for Trichoderma-induced resistance against *Botrytis cinerea*

Pathogenesis assays were done as reported (Contreras-Cornejo *et al.*, 2011; Weiberg *et al.*, 2013). Reinforcement of immunity by *T. atroviride* WT and Δ*noxR* mutant strain against *B. cinerea* was assessed in 7-day-old Arabidopsis seedlings co-cultivated for 3 days with Trichoderma. After co-cultivation with the fungus and before the colonization of the roots, seedlings were placed in a vessel for plant tissue culture containing 30 ml^{-1} of medium 0.2× MS. Previously, *B. cinerea* was grown on agar PDA medium (PhytoTechnology) for 7–12 days at 22°C in darkness, then conidia were harvested using sterile distilled water. Five-microliter drops of a conidial suspension (5×10^5 conidia ml^{-1}) were placed on the surface of leaves, and the disease symptoms monitored 3 and 5 days after inoculation in a dissecting microscope (Leica MZ6). Necrotic lesions were measured with the IMAGEJ software (https://rsbweb.nih.gov/ij/). Growth of *B. cinerea* was analyzed on leaves stained with trypan blue, and photographed using a Leica DFC450C microscope. Colonization levels are expressed as the percentage of leaf colonization by mycelium, and were classified as class I: no pathogen growth or 0% of hyphal colonization; class II: 1–25% of hyphal colonization; class III: 26–50% of hyphal colonization; class IV: 51–75% of hyphal colonization plus conidia; and class V: 100% of hyphal colonization with presence of conidia. Differences in resistance class distributions between treatments were analyzed by Fisher's exact test using XLSTAT software for MICROSOFT EXCEL (Microsoft, https://www.microsoft.com).

Trichoderma–Arabidopsis transcriptomics

To prepare the RNAseq libraries of the Trichoderma–Arabidopsis interactions, after germinating and growing for 4 days, 20 *A. thaliana* seedlings per plate were inoculated with *T. atroviride* and co-cultivated for 3 or 5 days, as indicated. The WT and Δ*noxR* mutant strains were used in this experiment.

RNA extraction from Arabidopsis

For 2 days, Arabidopsis seeds were subjected to stratification at 4°C in darkness, sown and incubated in a growth chamber at 24°C, under a 16 h light (200 μmol m^{-2} sec^{-1})/8 h dark photoperiod. After four days, 1×10^6 conidia of the respective strains were inoculated 5 cm away from seedlings. Plants were collected at 3 and 5 dpi, and excised with the help of a scalpel at the base of the stem (neck) to separate the shoots from the roots. Samples were frozen in liquid nitrogen and stored at −80°C. Three replicates of this experiment were carried out. As a control, Arabidopsis roots without interaction with any Trichoderma strain were collected. RNA was then extracted using TRIZOL (INVITROGEN, https://www.thermofisher.com/invitrogen).

RNA extraction from *Trichoderma atroviride*

The RNA of the fungus was extracted from the mycelium closest to the root of Arabidopsis at 3 and 5 dpi when there is already contact between the fungus and the plant. As a control of this experiment, the fungus was grown without the presence of Arabidopsis at 5 dpi. RNA from *T. atroviride* mycelium was also extracted with TRIZOL (INVITROGEN).

RNA-seq and differential expression analysis

cDNA synthesis was performed according to the TruSeq® RNA Sample Preparation v2 protocol and sequenced in an Illumina

HiSeq 2000 platform, in a 2 × 100 format. Read quality was analyzed using the FastQC software, executed as part of a pipeline programmed in Perl. Reads were then mapped to the predicted transcripts of the *T. atroviride* genome v2 (http://genome.jgi.doe.gov/Triat2/Triat2.home.html). For *A. thaliana*, we used version 10 of the genome obtained from TAIR for read mapping (https://www.arabidopsis.org/). The Salmon software was used to map paired end library mode (Patro *et al.*, 2017). The count tables output of Salmon was used to construct an expression matrix with all conditions evaluated for both Trichoderma and Arabidopsis using the statistical package R. Isoforms of the Arabidopsis analysis were removed using *tximport*.

On average, 10 million high-quality reads per library were obtained. The RNAseq data analyzed and discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE138203 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138203). Differential gene expression analysis was performed as described by Medina Castellanos *et al.* (2018).

Genome annotation and GO-enrichment analysis

Annotation of the *T. atroviride* transcriptome was performed as described by Carreras Villaseñor *et al.*, (2013). The GO.db 2.10.1 package from Bioconductor was also used to obtain the ancestors of each GO. The complete genome annotation was inherited to the differentially expressed genes. For Arabidopsis, we used the annotation obtained from TAIR. Enrichment analyses were performed as previously described (Medina Castellanos *et al.*, 2018).

Growth in different concentrations of cellulose and sucrose

The growth of the Δ*noxR* and WT strains was evaluated in different concentrations of cellulose (1.2, 2.4%), sucrose (1.2, 2.4%) or a combination of both carbon sources (0.6, 1.2, 2.4, 3.6%) were evaluated. 1 × 10^5 conidia were inoculated in Vogel's Minimal Medium and the diameter of the colony measured 60 h after inoculation.

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AUTHOR CONTRIBUTIONS

JMVE and SER designed and performed experiments, collected, and interpreted data; RPF, LFRH and FLR provided technical support; JLB and AHE designed experiments, contributed to data interpretation and applied for funding. JMVE, SER, JLB and AHE wrote the manuscript. All authors revised and approved the submission.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA ACCESSIBILITY

The fastq files of the transcriptomic experiments generated in this work were deposited in the NCBI GEO (GSE138203).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Effect of *T. atroviride* WT and Δ*noxR* mutant strains on LRP development and auxin response. *DR5:GUS* seedlings were germinated and grown for 4 days on 0.2× MS medium, then were inoculated with 1 × 10^6 spores at the opposite side of the plates and LRP number and density and GUS expression analyzed at 4 days of interaction. (a) Number of LRP and ELR per plant at the indicated stage of development. (b) Total LRPs per plant. Stage I: LRP initiation (in the longitudinal plane, approximately 8–10 'short' pericycle cells are formed). Stage II: the LRP is divided into two layers by a periclinal division. Stage III: the outer layer of the primordium divides periclinally, generating a three-layered primordium. Stage IV: an LRP with four cell layers. Stage V: the LRP is midway through the parent cortex. Stage VI: the LRP has passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII: the LRP appears to be just about to emerge from the parent root. (c) Representative micrographs of the expression of *DR5:GUS* at each stage of LRP formation. Bars represent means ± SE for 12 GUS-stained seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. Scale bar: 100 μm. The experiment was repeated twice with similar results.

Figure S2. Root colonization by *T. atroviride* strains. The figure shows confocal microscopy images at 5 dpi of the control plant without fungus and in interaction with the WT and the Δ*noxR* strains, as indicated. Roots were stained with PI at a concentration of 0.01 mM and *T. atroviride* hyphae with WGA Alexa Fluor 488 diluted in 1 × PBS buffer.

Figure S3. Pairwise functional analysis of Arabidopsis response to Trichoderma. Category enrichment of Biological Process in differentially expressed genes (*FDR < 0.05; **FDR < 0.01). Each vertical block contains the up- and downregulated categories in the heatmap. Color intensity represents the significance in logarithm base 10 of the FDR per category. Counts per million of stress response genes in each evaluated condition.

Figure S4. WT and Nox strains growing in cellulose or sucrose as a sole carbon source. Colony diameter of the WT, Δ*nox1*, Δ*nox2* and Δ*noxR* strains grown in minimal medium, using as source of carbon sucrose or cellulose in a standard and a high concentration (1.2 and 2.4%). Significant differences were obtained with a Tukey test (*$P < 0.01$). The error bar shows the standard deviation (a). WT and Δ*noxR* strains growing in sucrose and cellulose combination (1:1) (b). Colony diameter of the fungal strains in centimeters, grown in the sucrose/cellulose combination. All paired comparisons are significantly different ($P < 0.01$). The error bar indicates standard deviation (c).

Table S1. Differentially expressed genes of Arabidopsis when interacting 3 days with *T. atroviride* compared with non-inoculated plants.

Table S2. Differentially expressed genes of Arabidopsis when interacting 5 days with *T. atroviride* compared with non-inoculated plants.

Table S3. Differentially expressed genes of Arabidopsis when interacting 3 days with Δ*noxR* mutant of *T. atroviride* compared with non inoculated plants.

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Table S4. Differentially expressed genes of Arabidopsis when interacting 5 days with Δ*noxR* mutant of *T. atroviride* compared with non-inoculated plants.

Table S5. Differentially expressed genes in the comparison of Arabidopsis inoculated with Δ*noxR* versus WT after 3 dpi.

Table S6. Differentially expressed genes in the comparison of Arabidopsis inoculated with Δ*noxR* versus WT after 5 dpi.

Table S7. Gene ontology enrichment analysis in the different contrasts performed in Arabidopsis.

Table S8. Differentially expressed genes of WT-Trichoderma strain when interacting 3 days with Arabidopsis compared with the fungus that grows in MS medium without the plant.

Table S9. Differentially expressed genes of WT-Trichoderma strain when interacting 5 days with Arabidopsis compared with the fungus that grows in MS medium without the plant.

Table S10. Differentially expressed genes of Δ*noxR* Trichoderma strain when interacting 3 days with Arabidopsis compared with the fungus that grows in MS medium without the plant.

Table S11. Differentially expressed genes of Δ*noxR*-Trichoderma strain when interacting 5 days with Arabidopsis compared with the fungus that grows in MS medium without the plant.

Table S12. Differentially expressed genes of Δ*noxR*-Trichoderma strain compared with WT-Trichoderma when these interacting 3 days with Arabidopsis.

Table S13. Differentially expressed genes of Δ*noxR*-Trichoderma strain compared with WT-Trichoderma when these interacting 5 days with Arabidopsis.

Table S14. Gene ontology enrichment analysis in the different contrasts performed in Trichoderma.

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Trichoderma atroviride **triggers reactive oxygen species production in** ***Arabidopsis*** **roots and requires RBOH family members and PEPR2 for plant biomass production and reconfiguration of root architecture**

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Abstract

Oxidative regulation of plant growth and development is a hallmark during the interactions with microorganisms, but the molecular mechanisms that drive reactive oxygen production (ROS) to reconfigure root morphogenesis remain unknown. In this report, through comparing the biostimulant effect of the plant beneficial fungus *Trichoderma atroviride* in *Arabidopsis* WT seedlings and mutants defective in genes that encode *RESPIRATORY BURST OXIDASE HOMOLOGS* (*RBOH*), it could be found that disruption of *RBOHA*, *RBOHD*, and *RBHOE*, impairs root and shoot fresh weight and the root branching capacity enhanced by the fungus in the WT *in vitro*. These effects correlated with altered growth responses in *pepr2* mutant and the *Trichoderma*-induced expression of *PEPR2*, suggesting that act as an upstream modulator of RBOH enzymatic activity. *T. troviride* enhances ROS accumulation in primary root tips, in lateral root formation sites and emerged lateral roots as revealed by total ROS imaging via the fluorescent probe, DAB detection and *Hyper* sensor. Acidification of the substrate and emission of the volatile organic compound 6-pentyl-2H-pyran-2-one (6-PP) appears to be major factors by which the fungus triggers ROS accumulation, which accounts for more lateral roots being formed during the root–fungal interaction. These data shed light on the roles of ROS as messengers for plant growth and root architectural changes during the interaction with a symbiotic fungus.

Keywords: *Trichoderma atroviride*, *Arabidopsis thaliana*, reactive oxygen species, plant biomass, root development.

INTRODUCTION

Root plants cohabit with a myriad of soils microbes in a complex manner through chemical communication within the rhizosphere (Nath et al. 2016). Some of these microbes, which mostly belong to bacteria and fungi, can grow inside or outside of plant tissues and result in neutral or beneficial symbioses or harmful diseases that directly impact plant life cycle and overall fitness (Hassani et al. 2018). Furthermore, plants are known to effects a selective pressure on the microbial community through root exudation of metabolites to recruit beneficial microbes, such as endophytes, mycorrhizal fungi, and plant growth-promoting rhizobacteria (PGPR) (Pascale et al. 2020). *Trichoderma* is a genus of soil-dwelling filamentous fungi that can recognize root-derived exudates to colonize the root surface, and exert a direct effect on plant-growth and development, providing increased nutrient absorption capabilities and protection against pathogens or abiotic stress (Villalobos-Escobedo et al. 2020; Esparza-Reynoso et al. 2021). Recent knowledge advancement in *Trichoderma*–plant interplay reveals that an alteration in the coordinate exchanges of chemical signals between the plant and the fungus impairs the fine-tuned communication between both partners (Villalobos-Escobedo et al. 2020; Alfiky et al. 2021). Indeed, the fungal NADPH oxidase-mediated reactive oxygen species (ROS) production in *T. atroviride* is involved in the fungal ability to perceive plants, which in turn triggers an adjustment in carbon metabolism to assimilate simple forms of sugars released by plant roots (Villalobos-Escobedo et al. 2020). On the other hand, ROS production in the plant host is generated as a consequence of aerobic metabolism or in reply against biotic and abiotic stresses (Choudhary et al. 2017; Huang et al. 2019). Previous studies have clearly

shown that an oxidative burst in plants takes place as an early response to *Trichoderma* inoculation, as well during root colonization, activating afterward an antioxidant mechanism which confers tolerance the oxidative stress caused by pathogens or environment, as well to regulate the salicylic acid-dependent defense responses (Nogueira-Lopez et al. 2018; Nawrocka et al. 2019; Alfiky et al. 2021). Besides, *Trichoderma*-induced ROS production is closely associated with defense responses that trigger the fungus-produced effector molecules during interplay with plants (Ramírez-Valdespino et al. 2019). Several reports demonstrate that *Trichoderma* spp. secrete a plethora of effectors and secondary metabolites which act as elicitors in the simultaneous activation of systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Ramírez-Valdespino et al. 2019; Alfiky et al. 2021; González-López et al. 2021). *Trichoderma*-secreted effectors are perceived by intracellular immune receptors named nucleotide-binding leucine-rich repeat receptors (NLRs) leading a ROS accumulation, callose deposition, biosynthesis of antimicrobial metabolites, and the production of the plant defense phytohormones (salicylic acid, jasmonate and ethylene) (González-López et al. 2021). NADPH oxidases (NOXs), also called respiratory burst oxidase homologues (RBOHs) in plants, generate superoxide anions ($O2^-$) in the apoplast which are rapidly dismutated to hydrogen peroxide (H_2O_2) (Hu et al. 2020). RBOHs encompass a group of membrane-bound enzymes that have homology to the mammalian phagocyte gp91phox (NOX2) that are mainly activated in response to the rapid influx of Ca^{2+} or intracellular protein kinases-induced phosphorylation (Chapman et al. 2019; Lee et al. 2020). Besides, the *A. thaliana* genome encodes ten Rboh genes that are known or predicted to control a wide range of environmental as well as developmental responses (Chapman et al. 2019). Previous reports have demonstrated that RBOHD and RBOHF play an important role in ROS production during abiotic stress signaling as well in

plant immune response, however, the site-specific production of ROS through these enzymes is necessary for the proper lateral root (LR) development (Otulak-Kozieł et al. 2020).

Here, we show that RBOHs enzyme-mediated ROS production is necessary for root branching and biomass production elicited by *T. atroviride* in plants. Furthermore, *Trichoderma* induce a specific accumulation of H_2O_2 at different sites within the root tip, which indicates the possible involvement of peroxidases in the adaptative response to eventual root colonization. In addition, ROS production in roots before *Trichoderma* colonization seems to be attributed to the signaling that triggers the perception of acidic pH or 6-PP, however, both stimuli trigger an increased accumulation of ROS leading to inhibition of root cell elongation and root growth. On the other hand, *Tichoderma* induce the expression of the receptor PEPR2, which modulates ROS levels upstream RBOHs enzyme activity, besides, an alteration of the signaling that triggers the receptor impairs *Trichoderma*-induced phytostimulation in plants.

MATERIALS AND METHODS

Plant material and growth conditions

A. thaliana ecotype Columbia (Col-0) was used as wild type (WT) plant throughout the study. The mutant lines *RbohA* (SALK_047391), *RbohD* (SALK_044865) and *RbohE* (SALK_030395) were obtained from the Salk Institute for Biological Studies (La Jolla,

California, US), while the mutant lines *pepr2* (Yamaguchi et al. 2010) and *clv2-3* (Kayes et al. 1998), the *pepr2cvl2* double mutant line (Gutiérrez-Alanís et al. 2017) and transgenic line *pPEPR2::GUS* (Wu et al. 2016) were kindly provided by Dr. Luis Herrera Estrella (CINVESTAV-Irapuato). *Arabidopsis* seedlings transformed with the yellow fluorescent protein-based redox biosensor HyPer employed to screen the intracellular H_2O_2 levels (Hernández-Barrera et al. 2015;) was kindly given by Dr. Luis Cárdenas (IBT-UNAM). All seeds from lines used for analysis were surface sterilized using 95% (v/v) ethanol and 20% (v/v) bleach for 5 and 7 min, respectively, followed by five washes with distilled and sterilized water. Then, were stratified for two days at 4°C, and grown on Petri dishes containing 0.2x Murashige and Skoog (1962) medium (MS Basal salts mixture, Catalogue No. M5524 Sigma), 0.6% sucrose (w/v) 1% agar (w/v) (Micropropagation grade, Catalogue No. A111 PhytoTechnology Laboratories); pH 7.0, 5.5 or 4.5. Petri dishes were placed vertically (at an angle of 65°) in a plant growth chamber (Percival AR-95L) at 22°C under continuous light conditions (300 µmol m^2 s^{-1}) and photoperiod (16 h of light/8 h of darkness).

Fungal growth and inoculum preparation

Trichoderma atroviride strain IMI206040 used to perfom plant-fungus interaction assays was provided by Dr. Alfredo Herrera-Estrella (Centro de Investigación y de Estudios Avanzados del IPN, Mexico). Four days after germination, seedlings were inoculated with a spore suspension adjusted to 1 x 10^6 spores at 5 cm from the root tip and incubated for

additional four days to evaluate the plant responses to *Trichoderma*. The fungal growth trial and spore harvest were performed according to Pelagio-Flores et al. (2017).

Effect of 6-PP on ROS-mediated *Arabidopsis* growth

Ten *Arabidopsis* seedlings were germinated and grown in each Petri dish containing 0.2x MS medium supplemented with micromolar concentrations (75 and 150 μM) of 6-PP (Sigma-Aldrich), which were prepared according to Garnica-Vergara et al. (2016). Ten days after germination, determination of the dose-response effect of 6-PP in plant growth compared to ROS accumulation was performed.

Analysis of plant traits

The length of primary roots was measured with a graduated ruler, while the LR length was measured using the IMAGEJ software (http://rsbweb.nih.gov/ij/). The quantification of total LRPs was determined by counting all mature roots that emerged from the primary root using a stereomicroscope (Leica MZ6). Lateral root density was scored as the LR number per centimeter of primary root and was calculated by dividing the number of LR by the primary root length for each seedling. Fresh weights of shoots or roots were determined using an analytical balance. Petri dish pictures were taken using a digital camera (Nikon D5600, Japan).

ROS detection

The production of intracellular ROS was assayed using the oxidation-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA). *Arabidopsis* seedlings were incubated with 10 uM of H2DCF-DA (Invitrogen™) in Trizma® hydrochloride buffer solution at 10 mM (pH 7.4) for 60 min in darkness and rinsed and mounted with fresh buffer solution on microscope slides. The 2′,7′-dichlorofluorescein (DCF) fluorescence was detected through excitation and emission wavelength of 485 nm and 500-535 nm using a confocal laser scanning microscope (Olympus FV1200). Fluorescence from at least 8 treated seedlings was measured by calculating the green pixels in a determined area of each image using IMAGEJ software (http://rsbweb.nih.gov/ij/). Means of the relative fluorescence of each treatment were normalized according to the pixel values from the control condition.

PI staining and YFP detection

To evaluate the H_2O_2 accumulation in roots through the genetically encoded biosensor HyPer probe, transgenic *Arabidopsis* seedlings harboring the Hyper biosensor which consists of a circularly permuted yellow fluorescent protein (cpYFP) fusioned to H_2O_2-sensitive regulatory domain of *OxyR* (*E. coli* transcription factor), were soaked in propidium iodide (PI) solution (10 mg ml^{-1} for 1 min), rinsed, mounted in 50% (v/v)

glycerol on microscope slides and then recorded by Olympus FV1200 confocal microscope. The detection of H_2O_2-induced change in the excitation wavelength of cpYFP required a filter wheel to switch between the excitation wavelength of the H_2O_2-independent signal (440 nm) and H_2O_2-dependent signal (495 nm) (Belousov et al. 2006), while the emission spectra of PI was detected using a 568 nm excitation line and an emission window of 585 to 610 nm, and all emission spectra were recorded through an emission filter at 550/20 nm to obtain a final image merged. Quantification of the relative fluorescence intensity was performed as mentioned above.

Histochemical analysis

For the analysis of the expression of the *PEPR2* gene, transgenic *Arabidopsis* seedlings that express the uidA reporter gene driven by *PEPR2* promoter were incubated in a GUS reaction buffer (0.5 mg mL-1 5-bromo-4-chloro-3-indolyl-β-d-glucuronide in 100 mM sodium phosphate, pH 7) in darkness for 12 h at 37°C. After staining, seedlings were cleared and mounted according to the protocol of Malamy and Benfey (1997). The processed roots were placed on glass slides and analyzed by Nomarski's differential interference contrast (DIC) microscopy. The photographs shown are representative of the experiment conducted with 8 biological replicates.

The production of H_2O_2 in *Arabidopsis* seedlings co-cultivated with *Trichoderma* was also determined via DAB staining. DAB is oxidized by H_2O_2 forming a dark brown precipitate staining. For histochemical analysis of DAB staining, *Arabidopsis* seedlings were

immersed in 1 mg/ml solution of 3,3′-diaminobenzidine (DAB; Sigma), incubated 2 h, fixed and cleared with 70% ethanol solution (v/v) and mounted on glass slides and then observed using DIC microscopy. For each treatment at least 6 plants were analyzed.

Data analysis

The data were analyzed through univariate and multivariate analyses (ANOVA) followed by Tukey's post hoc tests using statistical software STATISTICA 10.0 program (Dell StatSoft, Austin, Texas, USA). All experiments were repeated twice or three times. Different letters were used to indicate means that differ significantly ($P < 0.05$).

RESULTS

NADPH oxidases RBOHA, RBOHD and RBOHE are required for *Trichoderma*-induced plant growth promotion

T. atroviride induce the expression of genes involved in defense response and promote plant growth synergistically (Villalobos-Escobedo et al. 2020). However, little is known about how RBOHs-dependent ROS production regulates plant development and stress responses. To further explore the mechanism of *Trichoderma*-induced ROS metabolism we compared the biostimulant effect of *T. atroviride* in *Arabidopsis* WT (Col-0) seedlings and

mutants defective in Rboh genes (*RbohA*, *RbohD* and *RbohE*). WT and mutant seedlings were grown for 4 d after germination and then were inoculated with *T. atroviride* at 5 cm from the root tip. After 4 days of co-cultivation *in vitro*, the WT seedlings inoculated with *T. atroviride* showed a clear phytostimulation concerning un-inoculated plants, the results revealed that primary root growth was slightly affected by co-cultivation with the fungus, whereas the number and length of LR increased by fourfold and threefold respectively, compared to axenically grown seedlings. This response correlated with a less than twofold difference in all biomass quantifications (Fig. 1a-d and Suppl. Fig. S1). In contrast, the increased root branching and biomass production provoked by *Trichoderma* diminished in the Rboh mutants, particularly the *RbohE* mutant plants showed an impressive reduction of the root system with regard to the *RbohA* and *RbohD* mutants (Fig. 1a-d and Suppl. Fig. S1). These data show the critical role of RBOH-dependent ROS synthesis in growth promotion by *T. atroviride* in *Arabidopsis*.

T. atroviride strongly induced ROS production in roots

An oxidative burst is a critical event upon plant colonization by *Trichoderma*, and this reaction is activated by the rapid production of ROS in the apoplast, which is involved in plant response signaling against plant pathogens (Saravanakumar, 2016; Chen et al. 2019). To visualize the ROS signal in roots, we used the fluorescent cell-permeable probe H2DCF-DA through confocal microscopy. To relate the activity of RBOHs enzymes in the production of ROS as an early response to *Trichoderma* inoculation, we used the

fluorescent cell-permeable probe H2DCF-DA to visualize the ROS signal in roots through confocal microscopy. *Arabidopsis* seedlings were spot-inoculated 4 d after germination at 5 cm below the root tip and allowed to grow for 4 days. Plants grown without fungus presence showed basal ROS levels which were generated as by-products of metabolism, whereas roots of *Trichoderma*-inoculated seedlings displayed a stronger ROS production in the root (Fig. 2a-f). The relative fluorescence intensity indicated that *Trichoderma*-mediated ROS accumulation was more increased at the cells surrounding the LRP than epidermal cell layer of LR emerged, however, the ROS levels at primary root were two-and-one-half times more than those obtained in the control condition (Fig. 2g-i). Additionally, the fungus caused a similar ROS accumulation at the meristem and elongation zone of LR (Suppl. Fig. S2). The results clearly showed that *Trichoderma* triggers a ROS generation on the root as an early response before root colonization.

T. atroviride mainly induced H_2O_2 production in roots

H_2O_2 is a relatively long-lived ROS molecule that participates in several plant developmental processes and defense responses (Huang et al., 2019). To know the changes in the spatial distribution of H_2O_2 in the primary root, we evaluate the DAB-mediated tissue staining in roots of inoculated and non-inoculated seedlings with *Trichoderma*. Tiny brown staining in the root apex of uninoculated plants was observed, however, *Trichoderma*-inoculation triggers stronger staining in the stele of the maturation zone (Fig. 3a-b). To gain better resolution of the cellular localization of H_2O_2 at the root tip, we assess the activity of

YFP-based biosensor HyPer. A basal H_2O_2 accumulation in the columella and lateral root cap cells was observed, and this pattern of H_2O_2 production was increased and extended to the cells of the epidermis and cortex, showing a correlation with the relative fluorescence intensity (Fig. 3c-e). These data indicate that *Trichoderma* inoculation causes specific H_2O_2-dependent oxidative burst to regulate root growth processes.

Low pH reconfigures *Arabidopsis* root system architecture

The acidification induced by *Trichoderma* can regulate the root system architecture plasticity, determining primary root growth, LR formation and root meristem viability (Pelagio-Flores et al., 2017). To assess whether acidic pH could trigger root branching in *Arabidopsis* seedlings, we germinated and grown seedlings for 8 d on agar plates containing MS 0.2x medium adjusted to pH 7.0, 5.5 and 4.5. *Arabidopsis* root growth was affected by acidic pH of 5.5 and 4.5, decreasing by nearly 40% and 50%, respectively, and showing an alteration in gravitropism response (Fig. 4a-d). Interestingly, at pH 5.5, the LR formation was five times larger than seedlings grown at pH 7.0, however, this stimulus was repressed when plants were grown under medium adjusted to pH 4.5, which also affected the root biomass production (Fig. 4e-g). These data show that low pH comparably alters root growth to *Trichoderma* effect.

Low pH increases ROS production in roots

To assess how the low pH can be the factor that triggers *Trichoderma*-dependent ROS production and/or root branching in *Arabidopsis*, we evaluated the intracellular ROS accumulation in seedlings grown on 0.2x MS agar medium adjusted to pH 4.5, 5.5 and 7.0 through H2DCF-DA staining. The acidic pH (5.5 and 4.5) induced an apoplastic ROS accumulation at the maturation zone of the primary root, and specifically at pH 4.5, plants displayed an exacerbated quantity on the tissue layers of epidermis, cortex, endodermis and vasculature, spreading even within LRP (Fig. 5a-c). Besides, plants growing under pH 5.5 and 4.5 also exhibited a higher ROS accumulation at the meristematic zone of the root tip, showing a significant increase in ROS levels from the columella and lateral root cap until the elongating epidermal cells, including over the root stem cell niche (Fig. 5d-f). These disturbances of intracellular ROS levels were confirmed through the quantification of fluorescence in each zone (Fig. 5g-h). Moreover, it was appreciated that LR of seedlings grown at pH 4.5 displayed a shortening of the meristematic zone and similar increased ROS accumulation to the pattern observed in the primary root tip, suggesting that increases in ROS production compromise the proliferation/differentiation in LR meristems (Suppl. Fig. S3). These data indicate that acidic pH is partially involved in the oxidative burst driven by *Trichoderma*.

6-PP induces ROS production in a concentration-dependent manner in roots

T. atroviride-derived 6-PP is a volatile organic compound with an auxin-like effect that stimulates plant growth in a dose-dependent manner (Garnica-Vergara et al., 2016; Carillo

et al., 2020). To evaluate whether ROS production can be influenced by 6-PP, *Arabidopsis* seedlings were germinated and grown on 0.2x MS medium supplemented with 75 and 150 μM of 6-PP. At 10 days after germination, seedlings were stained with H2DCF-DA for detection of ROS in the primary root tip by confocal microscopy. As expected, we found that total ROS increased in plants treated with 6-PP (Fig. 6a-c). Quantification of fluorescence confirms that 6-PP provokes a ROS accumulation which is dependent on the concentration of fungal compound supplied in the growth medium (Fig. 6d-e). Interestingly, when plants were grown under 150 μM, a reduction in the width of the root tip and a shortening of the meristematic and elongation zone were observed. Thus, we suggest that the inhibitory effect of 150 μM of 6-PP in primary root growth is caused by ROS overproduction, affecting cell division and expansion processes. These data show that 6-PP reconfigures the root architecture via ROS production.

T. atroviride induces expression of the NLR receptor PEPR2

Early extracellular ROS production by RBOHs oxidases is triggered by specific pathogen effectors through RLKs-dependent signaling (Huang et al. 2019; Kimura et al., 2020). To test whether the PEPR2 receptor may or not participate in the *Trichoderma*-regulated ROS production, we analyzed the expression of *pPEPR2::GUS* (Yamaguchi et al. 2010) in transgenic *Arabidopsis* seedlings grown in co-culture with *T. atrovirde*. Histochemical staining of transgenic *pPEPR2::GUS* seedlings revealed that a basal expression of *PEPR2* is located at the leaf veins (Fig. 7a-b). In contrast, the expression of *pPEPR2::GUS* was

increased in leaf tissue and extended to the stele of the differentiation zone of the primary root of *Trichoderma*-inoculated seedlings (Fig. 7c-d). This result shows that *PEPR2* expression is induced by *Trichoderma* in the early stages of the interaction.

PEPR2 receptor is necessary for *Trichoderma*-induced root architectural alterations

To further define the particular role of PEPR2 perception in the *Arabidopsis* developmental responses to *Trichoderma* inoculation, we evaluated the primary root growth, LR formation and biomass production of *Arabidopsis* seedlings WT and single mutants *pepr2* and *clv2-3*, and the *pepr2clv2* double mutant grown in co-culture with *T. atroviride* for 4 days. Interestingly, we found that *pepr2* showed insensitivity to the promotion of LR branching to *Trichoderma* compared with WT seedlings, while *clv2-3* single mutant and *pepr2clv2* double mutant displayed clear phytostimulation triggered by the fungal inoculation, allowed plants to produce similar root biomass like WT seedlings (Fig. 8a-d). In addition, the *pepr2* mutant also showed a low production of shoot biomass, while the *clv2-3* mutant exhibited a greater shoot development than WT plants, possibly attributed to the mutant phenotype (long-hypocotyl phenotype), however, the growth enhancement was compromised in the double mutant, indicating that loss of function of *pepr2* impairs the plant development conferred by mutation of the *clv2-3* gene, and hence the phytostimulation response to *Trichoderma* (Suppl. Fig. S4). These data show that *T. atroviride* requires PEPR2 to trigger plant growth and development.

DISCUSSION

Some species of the genus *Trichoderma* positively influence plant health and productivity by stimulating plant growth and development and suppressing plant diseases caused by microbial pathogens (Guzmán-Guzmán et al. 2019; Alfiky and Weisskopf, 2021). The versatile mechanisms employed by *Trichoderma* to promote plant growth include synthesis of phytohormones (mainly IAA-related indoles), solubilization of soil nutrients, increased uptake and translocation of nutrients, enhanced tolerance to abiotic stress, improved sucrose metabolism and photosynthetic capability, and production of secondary metabolites with plant growth-promoting activity and antibiotic properties (Guzmán-Guzmám et al., 2019; Ramírez-Valdespino et al. 2019; Khan et al. 2020; Esparza-Reynoso et al. 2021; Harman et al. 2021; Vinale and Sivasithamparam et al., 2021). Among *Trichoderma*-derived volatile organic compounds, the unsaturated lactone denominates 6-PP has been cataloged as one of the most important VOC to the establishment of *Trichoderma*–plant beneficial interaction (Garnica-Vergara et al. 2016; Estrada-Rivera et al. 2019; Guzmán-Guzmám et al., 2019). The perception of 6-PP induces JA/ET-dependent plant defense responses and simultaneously triggers a complex reconfiguration of root architecture implying crosstalk between ethylene and auxin, however, the fungal volatile also can regulate plant growth and development by regulating sucrose transport in the phloem (Kottb et al. 2015; Garnica-Vergara et al. 2016; Esparza-Reynoso et al. 2021). Besides, plants can sense the substrate acidification generated by *Trichoderma* mycelial growth and this stimulus is the main cause of root tip bending and following root stoppage observed as an early response to *Trichoderna* (Pelagio-Flores et al. 2016). Villalobos-Escobedo et al.

(2020) reported that ROS produced by fungal NADPH oxidase is involved in the recognition of the plant by *T. atroviride*. Indeed, the alteration in ROS production via NoxR compromises the adjustment of saprophytic behavior stimulated by fungal perception of sugars secreted from roots, generating a stronger plant defense response. This could imply that host-produced oxidative burst also influences the plant growth and immune responses triggered by *Trichoderma*.

Recently it has been reported that ROS act as secondary messengers in regulating important developmental processes, such as root-hair formation, primary root elongation and LR formation (Chapman et al. 2019). According to Orman-Ligeza et al. (2016), the ROS generation by RBOH enzymes facilitates cell wall remodeling of overlying cell layers for LR outgrowth and emergence. To investigate the role of RBOH-mediated ROS production in plant growth promotion effects of T*richoderma*, *Arabidopsis* wild-type (Col-0) seedlings and Rboh-deficient mutants (*RbohA*, *RbohD* and *RbohE*) were subjected to co-culture with *T. atroviride* for 4 days. Our results showed that *T. atroviride* promoted root and shoot biomass production and increased root branching in WT seedlings compared to non-inoculated plants, however, the mutations in either tested RBOH enzymes caused a decrease in plant growth-promoting activity of *Trichoderma*. Collectively, our data suggest that ROS production via RBOHA, RBOHD and RBOHE triggered by *Trichoderma* positively modulate LR formation in plants. Interestingly, the *RbohE* mutant displayed reduced LR branching and elongation, indicating that functional loss of RBOHE compromises the plant growth promotion triggered by *Trichoderma*. It has been described that *Arabidopsis RbohE* mutant exhibits a reduced number of emerged LR due to delayed development of lateral root primordia (LRP) (Chapman et al. 2019). This RBOHE-

mediated LR development is related to its specific pattern of expression in the cells overlying/surrounding the LRP and auxin-inducible expression (Chapman et al. 2019; Eljebbawi et al. 2021). *RbohA* also shows a similar auxin-induced transcriptional response like *RbohE*, however, its spatiotemporal expression pattern is situated at both LRP and maturation zone of primary root (stele and endodermis), suggesting that RBOHA participates in different biological processes and not only in the ROS-facilitated LRP emergence (Orman-Ligeza et al. 2016; Chapman et al. 2019). Neuser et al. (2019) revealed that RBHOA-catalyzed ROS contributes to cell expansion during leaf growth and also confers resistance against *Pseudomonas syringae* through transcriptional co-regulation by the growth-related transcription factor HOMOLOG OF BEE2 INTERACTING WITH IBH 1 (HBI1). In contrast, *RbohD* expression is induced by the plant's defense response to biotic and abiotic stresses mediated by MPK1 and ERF74, respectively (Morales et al. 2016; Yao et al. 2017; Escudero et al. 2019; Lee et al. 2020). Furthermore, the C-terminal tail of RBOHE can be phosphorylated on serine residues by different protein kinases such as PBL13, CPKs, BIK1, and SIK1 which regulate its NADPH oxidase activity (Lee et al. 2020). However, *AtrbohD* and *AtrbohF* have been reported as negative regulators of laterla root formation (Otulak-Kozieł et al. 2020; Mase and Tsukagoshi, 2021). Li et al. (2015) showed that the double mutants *atrbohD1/F1* and *atrbohD2/F2* exhibited a higher LR density in comparison to the wild-type plants or the single mutants, indicating that both genes have functional redundancy in the auxin-regulated LR organogenesis. Thus, we assume that *RbohA*, *RbohE* and *RbohD* are downstream target genes of the auxin-dependent growth programs and/or and defense response signaling pathway (ethylene-MAPK) elicited by *Trichoderma* in plants.

Colonization of plant roots by *Trichoderma* induces a local and systemic oxidative burst throughout the plant, since an increased O_2^- and H_2O_2 accumulation at both sites colonized and distal parts of the plant has been detected (Contreras-Cornejo et al. 2011; Nawrocka et al. 2019; Xu et al. 2020; González-López et al. 2021). To correlate the RBOH enzymes activity in the oxidative stress response triggered by *T. atroviride*, we evaluate the ROS generation in roots using fluorescence-based probes and DAB staining. According to the H2DCF-DA assay, the intracellular ROS levels were higher in the roots of plants inoculated with *T. atroviride* compared with noninoculated plants, showing a higher ROS accumulation at the root apex of primary and LR. Besides, the H_2O_2-responsive expression of Hyper and the DAB staining revealed that *Trichoderma* triggers a specific H_20_2 accumulation at the columella root cap and maturation zone of the primary root, especially in the inner tissues making up the stele. This stele-specific accumulation of H_2O_2 is consistent with the high transcript levels of *RbohF*, which in turn can be induced by salinity or ACC treatment, suggesting an involvement of this isoform in *Tichoderma*-mediated oxidative stress in roots (Jiang et al. 2012; 2013; Chapman et al. 2019). Furthermore, this accumulation of H_2O_2 in stele may indicate the activity of class III peroxidases which trigger a lignification of the xylem under salt stress, indicating a possible adaptative response to *Trichoderma* root colonization (Herandez et al. 2010). In contrast, the accumulation of O_2^- and H_2O_2 at the root tip observed in plants inoculated with *Trichoderma*, could influence the balance of cellular proliferation and differentiation processes at the root apex. The exogenous application of H_2O_2 in *Arabidopsis* seedlings has been reported to stimulate LR development and primary root shortening by modulating the length and cell division events of the root meristem (Su et al. 2016; Orman-Ligeza et al. 2016). Moreover, it has been reported that H_2O_2 affect the directional transport of auxin

through changes in the expression of auxin carriers (mainly PIN efflux carriers), however, exogenous auxin application increases ROS levels in the root tip too, indicating the existence of cross-talk between ROS and auxin signaling to control root system architecture (Ivanchenko et al. 2013; Orman-Ligeza et al. 2016; Su et al. 2016; Velada et al. 2020).

The acidification generated by *Trichoderma* growth on media induces a redistribution of auxins within the root apex that originates a reorientation of the root growth and subsequent formation of the hook, followed by root meristem exhaustion (Pelagio-Flores et al. 2017). The gravity-dependent root bending involves an auxin redistribution within the root tip that triggers ROS generation (Eljebbawi et al. 2021). Therefore, we hypothesize that the accumulation of auxins induced by *Trichoderma* acidification at the root tips causes a decreased primary root growth via ROS overproduction. To test this hypothesis, we analyzed the impact of acidic pH on the architecture of the *Arabidopsis* root system. According to the results obtained, the plants grown at acidic pH (5.5 and 4.5) displayed altered root gravitropism and shorter primary root length. Subsequently, we observed that low pH values induce strong intracellular ROS accumulation in the whole root tissues. Such results fit very well to those previously reported, which mention that exposure to low-pH stress causes root growth and development inhibition and excessive accumulation of ROS, such as superoxide radicals and H_2O_2 in roots apex (Koyama et al. 2001; Zhang et al. 2015; Long et al. 2019; Graças et al. 2020). Moreover, Lager et al. (2010) have determined that pH sensing by the plant triggers regulation of gene expression that resembles the transcriptional response provoked by auxin or pathogen defense signaling. They also assume that perception external pH may act as an underlying signal to the cellular responses of auxin and pathogens which lead to apoplastic acidification and alkalinization,

respectively. Under this assumption, we suggest that low pH-dependent accumulation of ROS functions as a downstream component in auxin-mediated signal transduction.

On the other hand, the evaluation of the inhibitory effect of 6-PP on primary root growth at higher concentrations was associated with an increased accumulation of ROS in root tips. Notably, Garnica-Vergara et al. (2016) reported that ETHYLENE INSENSITIVE 2 (EIN2) is a key component in plant response to 6-PP, evidenced by insensitivity to primary root growth inhibition in ethylene-insensitive *ein2* mutants. Inhibition of root growth and superoxide anion accumulation in roots are typical effects of ethylene or its precursor ACC (Lv et al. 2018), thereby we suppose that 6-PP regulates primary root elongation via ethylene-dependent regulation of ROS homeostasis. Intriguingly, it has been reported that EIN2 is required for the exacerbated oxidative stress and root growth repression caused by plant-pathogen effectors such as bacterial flagellin (flg22) and pyocyanin, however, loss-of-function of EIN2 enhances the generation of ROS under salinity stress too, indicating the involvement of ethylene/ROS crosstalk in activation of stress responses and defense pathways (Mersmann et al. 2010; Lin et al. 2012; Beck et al. 2014; Ortiz-Castro et al. 2014).

Pathogen-associated molecular pattern (PAMP)-triggered immune responses induce a transient apoplastic ROS through the enzymatic activity of the RBOHs mediated by host surface receptor proteins called pattern-recognition receptors (PRRs) (Hu et al. 2020; Jing et al. 2020). It has been reported that recognition of flg22 by FLS2 receptor (a leucine-rich repeat-receptor kinase) triggers the activation of the membrane-localized ser/thr protein kinase BIK1, which directly phosphorylates the NADPH oxidase RBOHD to induce an extracellular oxidative burst in response to the pathogen (Kadota et al. 2014; Li et al. 2014;

Noman et al., 2019). To elucidate the possible involvement of PEP1 RECEPTOR 2 (PEPR2) in the oxidative stress triggered by *Trichoderma*, we evaluated the expression patterns of *PEPR2* using *Arabidopsis* transgenic line *pPEPR2::GUS*. We also analyzed the root architectural responses in wild-type Col-0 plants compared to the single mutants of *pepr2* and *clv2-3* and double mutant *pepr2clv2* to determine the participation of CLAVATA 2 (CVL2) and PEPR2 in regulating the plant growth mediated by *Trichoderma*. Our results showed that *PEPR2* expression patterns in leaves and the maturation zone of the primary root (stele) increased in plants inoculated with *Trichoderma*. Consistently, mutation of *pepr2* significantly affected the reconfiguration of root architecture and phytostimulation elicited by *Trichoderma*, denoting that PEPR2 can be a potential upstream regulator of RBOH-dependent ROS synthesis. As previously reported, PEPR2 is a receptor-like kinases that binds to Pep1 and Pep2 peptides to regulate root immunity through ROS production triggered by BIK1-mediated phosphorylation of RBOHD (Zixu et al., 2013; Jing et al. 2020). Yamaguchi et al. (2010) have indicated that *PEPR1* and *PEPR2* gene expression is induced by exogenous application of methyl jasmonate (MeJA), Pep peptides, microbial compounds and mechanical wounding, indicating that PEPRs receptors are mainly involved in the regulation of plant immune responses. However, the *PEPR2* expression is also induced in response to Pi starvation, and under this phosphate-deficient condition binds to CLE14 peptide to drive the root meristem differentiation (Gutiérrez-Alanís et al. 2017). Furthermore, the calcium sensor protein CALMODULIN-LIKE-38 (CML38) interacts with PEPR2 to inhibit primary root growth under nitrate deprivation, evidencing that PEPR2 receptor regulates the root growth in response to nutrient deficiency (Song et al. 2021). Besides, it has been reported that loss-of-function of *PEPR2* compromises the ROS production in root during pathogen infection (Jing et al. 2020).

Therefore, we suggest that reduced root branching and biomass production of *pepr2* mutant plants inoculated with *Trichoderma* seems to be attributed to the reduced capacity to produce ROS conferred by loss of PEPR2 function. This assumption would imply that early *Trichoderma* perception through the PEPR2 receptor is strongly linked to the plant growth mediated by RBOHs-catalyzed ROS production.

Taken together, our data reveal that plants upon perceiving *Trichoderma*, trigger an intricate RBOH-mediated ROS production via PEPR2 which seems to be an adaptive defense response that impacts plant growth and developmental processes. This trait of *T. atroviride* open the possibility to use it in agricultural production to overcome the limitations to crop production brought by abiotic stress and for better plant health and protection

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

SER, AAR and JLB designed and performed experiments and interpreted data; RPF and AAR provided technical support and analyzed data. SER and JLB wrote the manuscript. All authors revised and approved the submission.

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SUPPORTING INFORMATION

Supplementary figure 1. Effect of *T. atroviride* on biomass production of *Arabidopsis rboh* mutant plants. Four day-old WT *Arabidopsis* seedlings and mutants lacking of functional isoforms of *RbohA*, *RbohE* and *RbohD* enzymes were inoculated with *Trichoderma* at 5 cm from the root tip. After 4 days of co-culture, representative photographs of seedlings co-cultivated with *T. atroviride* were taken (a-h), and shoot fresh weight (i) and total fresh weight (j) were recorded. Bars show the means ± SD. Different letters indicate significant statistical differences ($P < 0.05$; $n = 15$). Scale bar: 1 cm. Similar results were obtained from three independent repetitions.

Supplementary figure 2. ROS production in LR of plants inoculated with *T. atroviride*. Representative micrographs of the detection of endogenous ROS levels in LR (a-b). The graphs shown represent the means of relative fluorescence from meristematic and elongation zone for 8 seedlings ± SD. Different letters indicate significant statistical differences ($P < 0.05$). Scale bar: 100 μm. The experiment was repeated two times, and similar results were obtained.

Supplementary figure 3. Effect of low pH on ROS production in LR. Representative micrographs of the detection of endogenous ROS levels in LR(a-b). The graphs shown represent the means of relative fluorescence from meristematic and elongation zone for 8 seedlings ± SD. Different letters indicate significant statistical differences ($P < 0.05$). Scale bar: 100 μm. The experiment was repeated two times, and similar results were obtained.

Supplementary figure 4. Effects of *T. atroviride* inoculation on biomass production in wild-type *Arabidopsis* (Col-0) and RLKs-related mutants. Four day-old WT col-0 seedlings, *pepr2* and *clv2-3* single mutants and *pepr2clv2* double mutant were inoculated with *T. atroviride* at 5 cm from the root tip. After 4 days of co-cultivation, representative photographs of seedlings were taken (a-h), and shoot fresh weight (i) and total fresh weight (j) were recorded. Bars show the means ± SD. Different letters indicate significant statistical differences ($P < 0.05$; $n = 15$). Scale bar: 1 cm. Similar results were obtained from three independent repetitions.

FIGURE LEGENDS

Figure 1. Effect of *Trichoderma* on the root system architecture of *Arabidopsis Rboh* mutant plants. Four day-old WT *Arabidopsis* seedlings and mutants lacking of functional isoforms of *RbohA*, *RbohE* and *RbohD* enzymes were inoculated with *Trichoderma* at 5 cm from the root tip and allowed to grow for 4 additional days. Primary root length (a), lateral root number (b), lateral root length (c) and root fresh weight (d) were recorded. The values

shown represent the means of 15 ± SD. Different letters indicate means that are statistically different ($P < 0.05$). The experiment was repeated three times with similar results.

Figure 2. Analysis of ROS production in plants inoculated with *T. atroviride*. Visualization of intracellular ROS was registered using the oxidation-sensitive fluorescent probe H2DCF-DA and confocal microscopy. Representative micrographs of the detection of endogenous ROS levels in LRP and primary roots tips (a-f). The graphs shown represent the means for 8 seedlings ± SD. Different letters indicate significant statistical differences ($P < 0.05$). Scale bar: 100 μm. The experiment was repeated two times, and similar results were obtained.

Figure 3. Effect of *T. atroviride* on the H_2O_2 distribution in the primary root tip of *A. thaliana*. Visualization of intracellular H_2O_2 in roots was recorded through DAB staining and the YFP-based genetically encoded biosensor Hyper. Micrographs of root tips showing DAB staining and HyPer fluorescence (a-d). The bar graph illustrates differences in expression, assessed as relative fluorescence intensity present in the root tip (g). Scale bar: 100 μm. The values shown represent the means for 8 seedlings ± SD. Different letters indicate means that are statistically different ($P < 0.05$).

Figure 4. Effect of acidic pH on root system architecture in *Arabidopsis* seedlings. To evaluate whether low pH could trigger root branching in *Arabidopsis* seedlings, we germinated and grown seedlings for 8 d on agar plates containing MS 0.2x medium

adjusted to 7, 5.5 and 4.5 of pH. Representative photographs show the root system architecture of plants grown on media with acidic pH (a-c). Primary root length (d), lateral root number (e), lateral root density (g) and root fresh weight (h) were recorded. The values shown represent the means of 30 seedlings ± SD. Different letters indicate means that are statistically different ($P < 0.05$). The experiment was repeated three times with similar results.

Figure 5. Effect of acidic pH on ROS production in *Arabidopsis* roots. Representative micrographs of the detection of endogenous ROS levels in primary roots tips and maturation zone of the primary root through the fluorescent probe H2DCF-DA (a-f). The graphs show the means of relative fluorescent from meristematic and elongation zone of 8 seedlings ± SD (g-h). Different letters indicate significant statistical differences ($P < 0.05$). Scale bar: 100 μm. The experiment was repeated two times, and similar results were obtained.

Figure 6. 6-PP regulates ROS production in *Arabidopsis* roots. Seedlings were germinated and grown on 0.2x MS medium supplemented with 75 and 150 μM of 6-PP. At 10 days after germination, detection of endogenous ROS levels was performed using H2DCF-DA probe. Representative micrographs show the increased ROS accumulation in root tips (a-c). The graphs represent the mean values of the relative fluorescent from meristematic and elongation zone for 8 seedlings ± SD. Different letters indicate significant statistical differences ($P < 0.05$). Scale bar: 100 μm.

Figure 7. Effect of *Trichoderma* inoculation in *PEPR2* expression of *Arabidopsis* seedlings. Four-day-old transgenic *Arabidopsis* seedlings harboring the *pPEPR2::GUS* gene construct were inoculated with *T. atroviride* at 5 cm from the root tip. After 4 days of co-cultivation, GUS activity driven by the *PEPR2* promoter was recorded by DIC microscopy. Representative micrographs show the expression of *PEPR2* in leaves (a-b) and the differentiation zone of the primary root (c-d). Scale bars: 1 mm and 100 μm, respectively.

Figure 8. Effect of *T. atroviride* inoculation on root architecture in WT *Arabidopsis* (Col-0) seedlings and RLKs-related mutants. Four day-old WT col-0 seedlings, *pepr2* and *clv2-3* single mutants and *pepr2clv2* double mutant were inoculated with *T. atroviride* at 5 cm from the root tip and allowed to grow for 4 additional days. Length of primary root (a), lateral root number (b), lateral root density [number of emerged lateral roots (ELR) cm^{-1}] (c) and root biomass (d) were recorded. Bars show the means ± SE. Different letters indicate significant statistical differences ($P < 0.05$; $n = 15$). Similar results were obtained in three independent repetitions of the experiment.

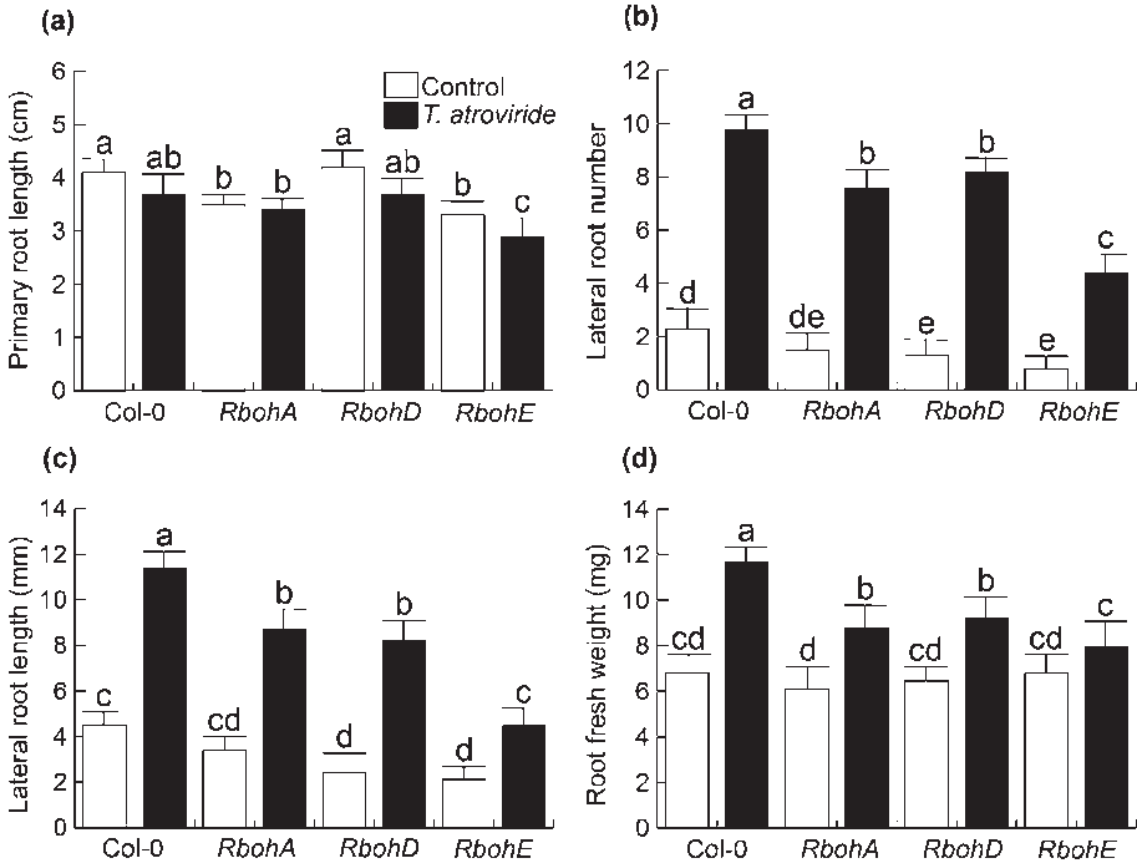
(a)
Primary root length (cm)
Control
T. atroviride
a
ab
b
b
a
ab
b
c
Col-0
RbohA
RbohD
RbohE
(b)
Lateral root number
d
a
de
b
e
b
e
c
(c)
Lateral root length (mm)
c
a
cd
b
d
b
d
c
(d)
Root fresh weight (mg)
cd
a
d
b
cd
b
cd
c

Figure 1

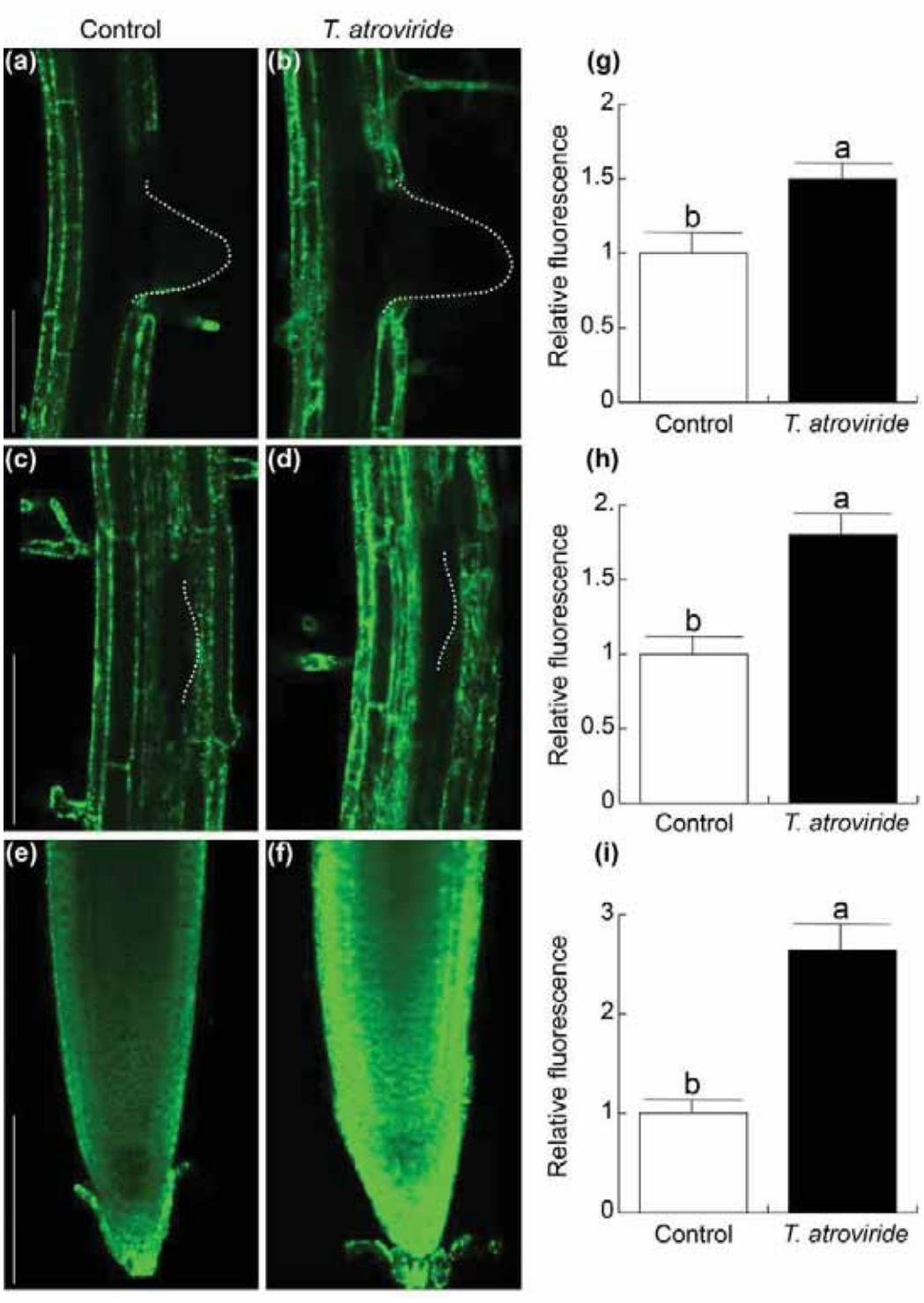
Control
T. atroviride
(a)
(b)
(c)
(d)
(e)
(f)
(g)
Relative fluorescence
2
1.5
1
0.5
0
a
b
Control
T. atroviride
(h)
Relative fluorescence
2.
1.5
1
0.5
0
a
b
Control
T. atroviride
(i)
Relative fluorescence
3
2
1
0
a
b
Control
T. atroviride

Figure 2.

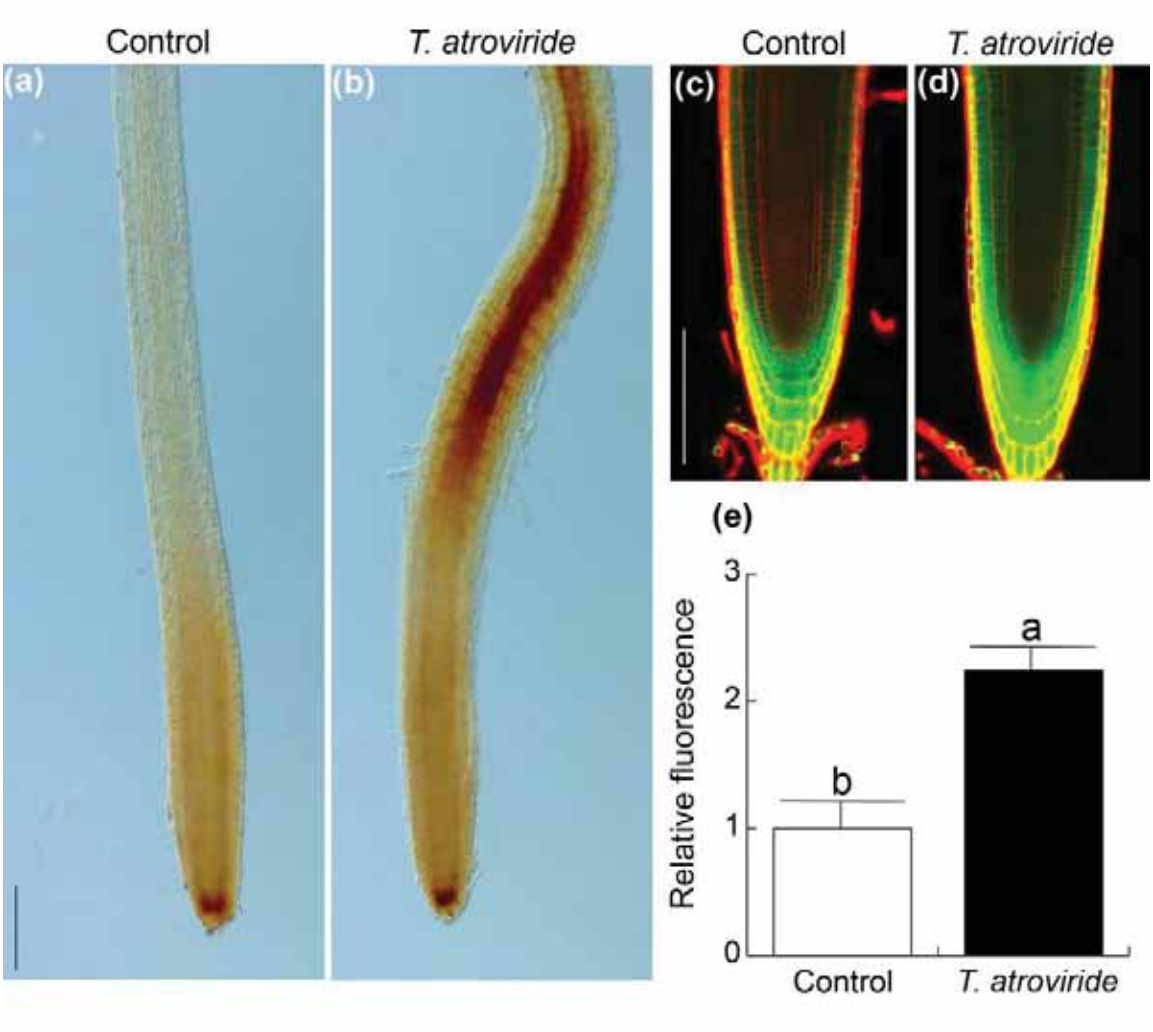
Control
T. atroviride
Control
T. atroviride
(a)
(b)
(c)
(d)
(e)
Relative fluorescence
3
2
1
0
a
b
Control
T. atroviride

Figure 3

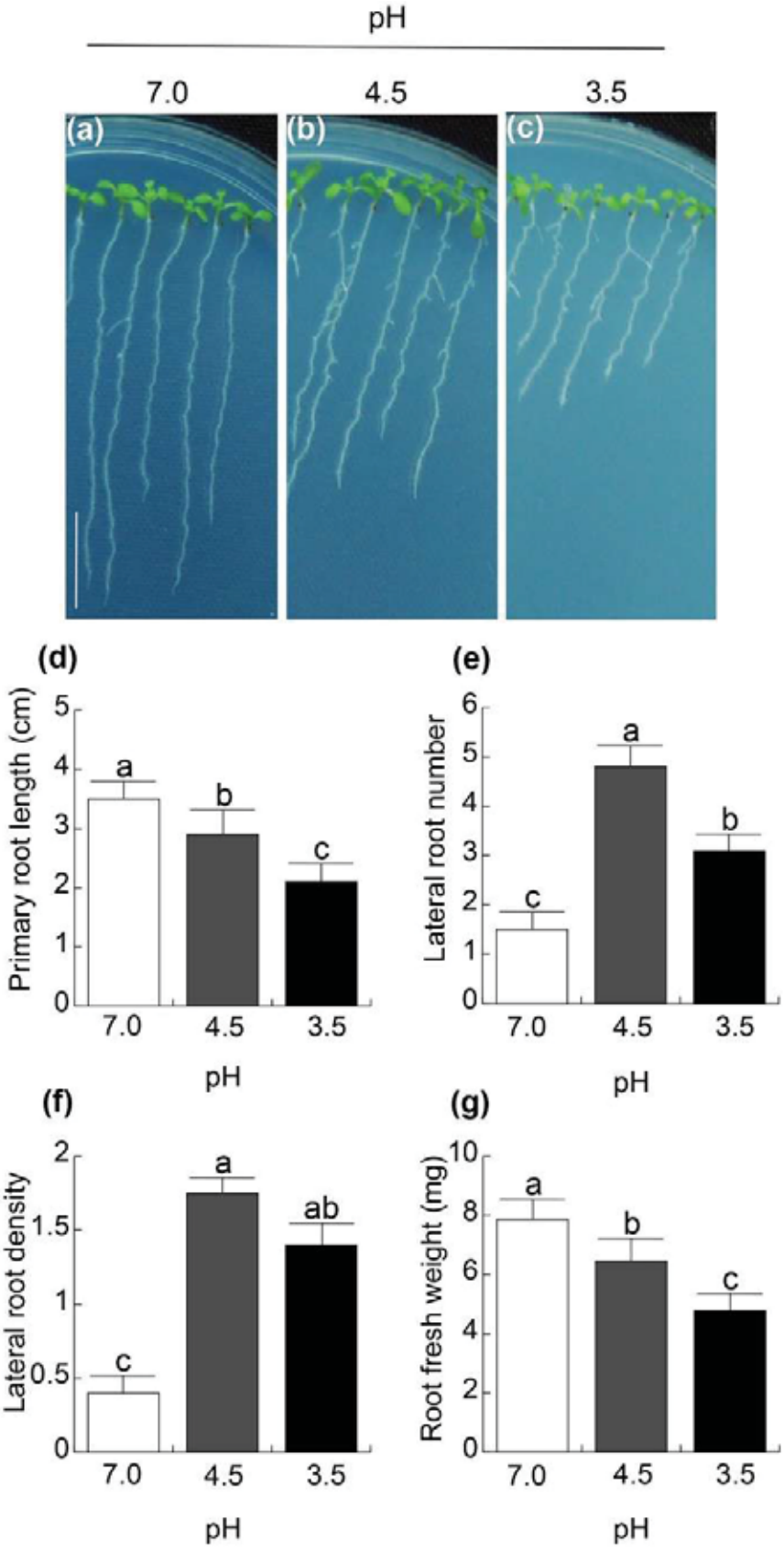
pH
7.0
4.5
3.5
(a)
(b)
(c)
(d)
Primary root length (cm)
a
b
c
7.0
4.5
3.5
pH
(e)
Lateral root number
a
b
c
7.0
4.5
3.5
pH
(f)
Lateral root density
a
ab
c
7.0
4.5
3.5
pH
(g)
Root fresh weight (mg)
a
b
c
7.0
4.5
3.5
pH

Figure 4

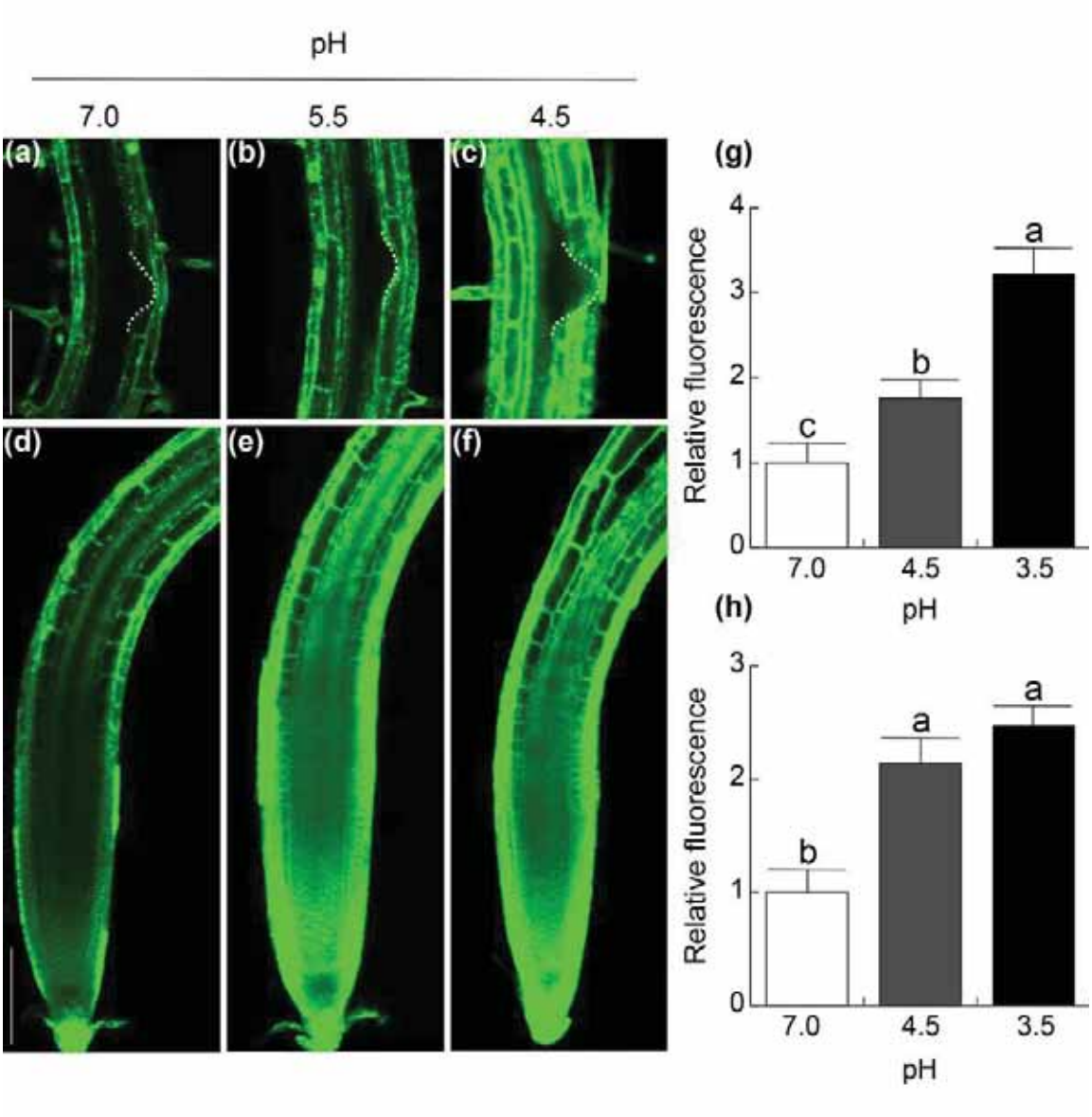

Figure 5

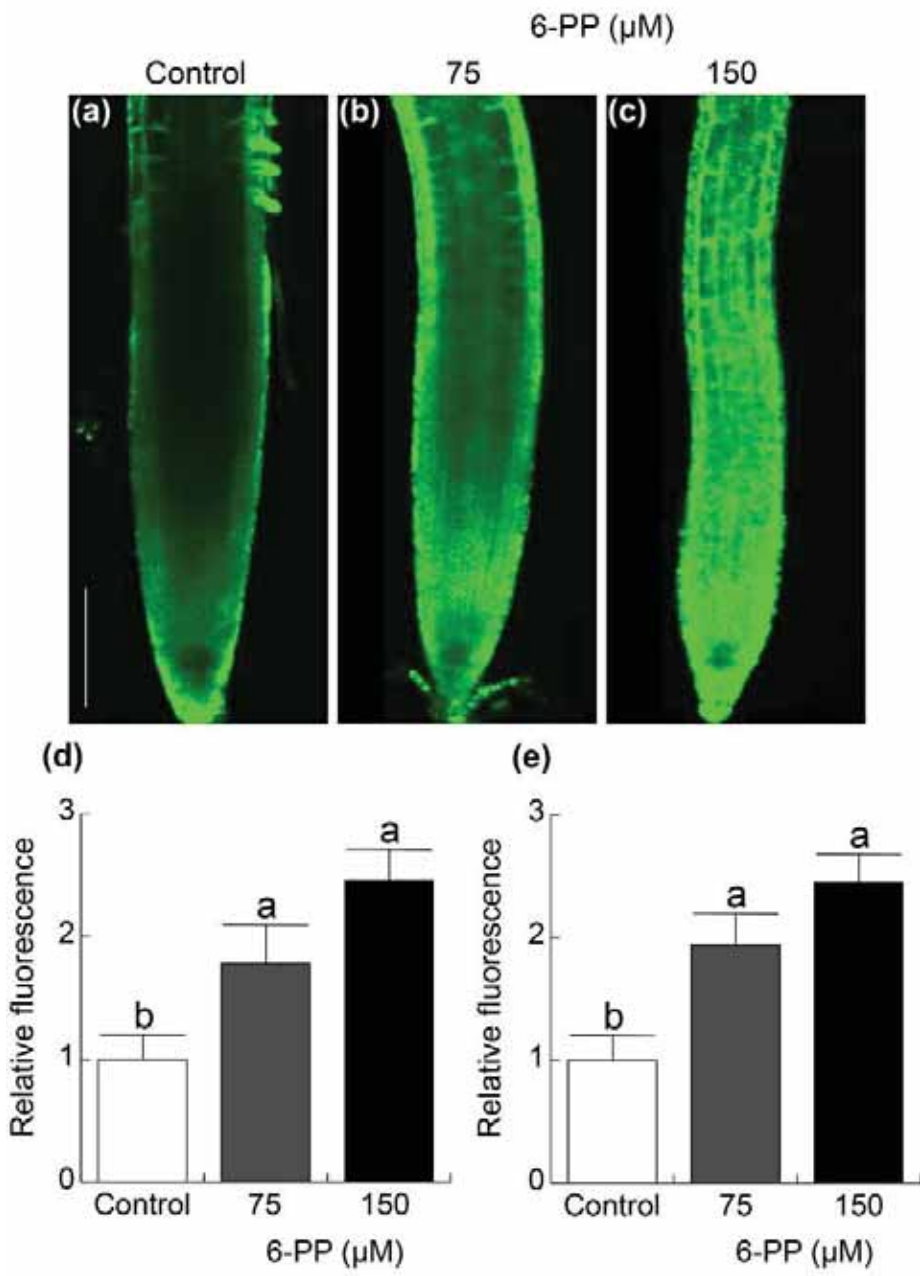
6-PP (μM)
Control
75
150
(a)
(b)
(c)
(d)
(e)
Relative fluorescence
3
2
1
0
a
a
b
Control
75
150
6-PP (μM)

Figure 6

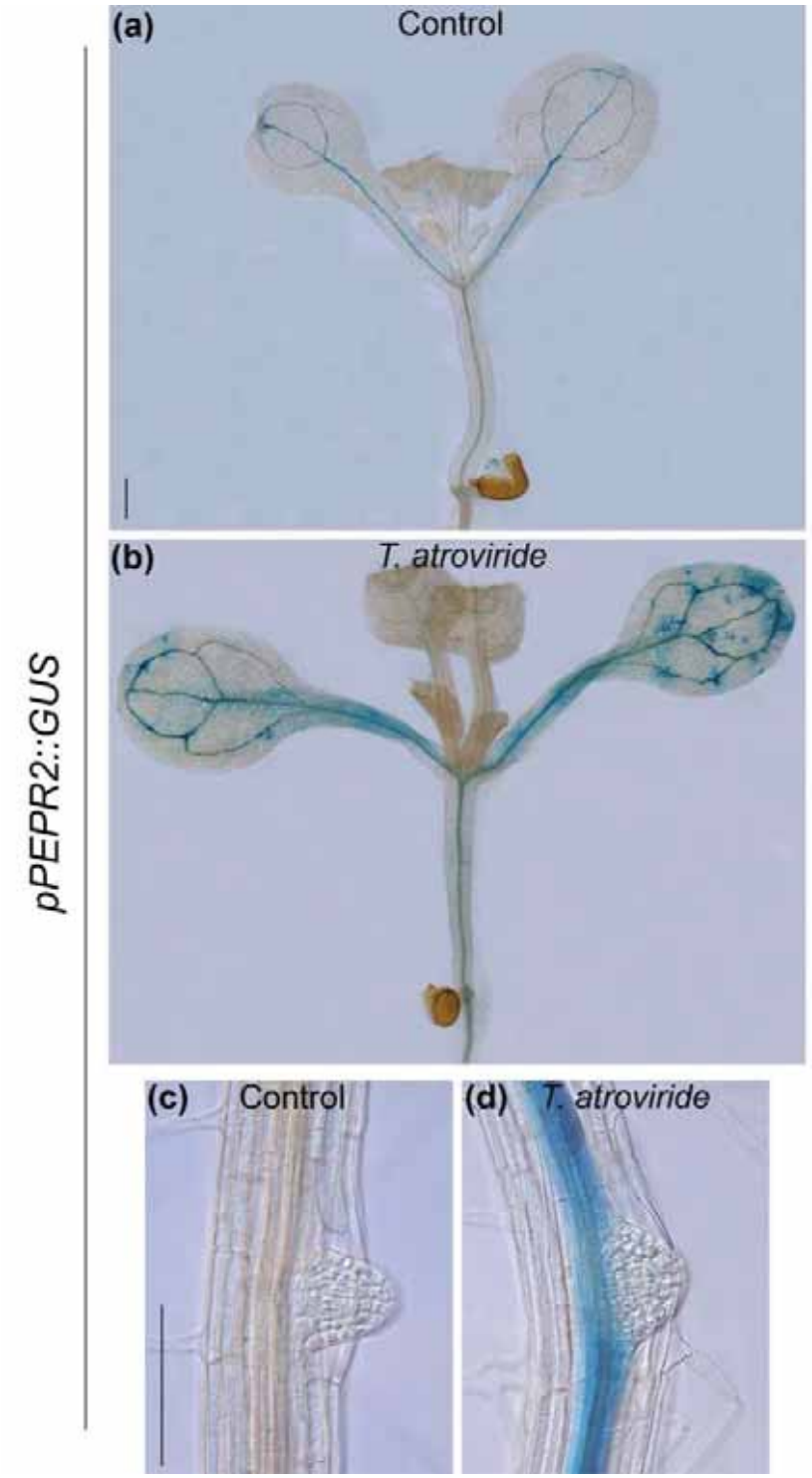

Figure 7

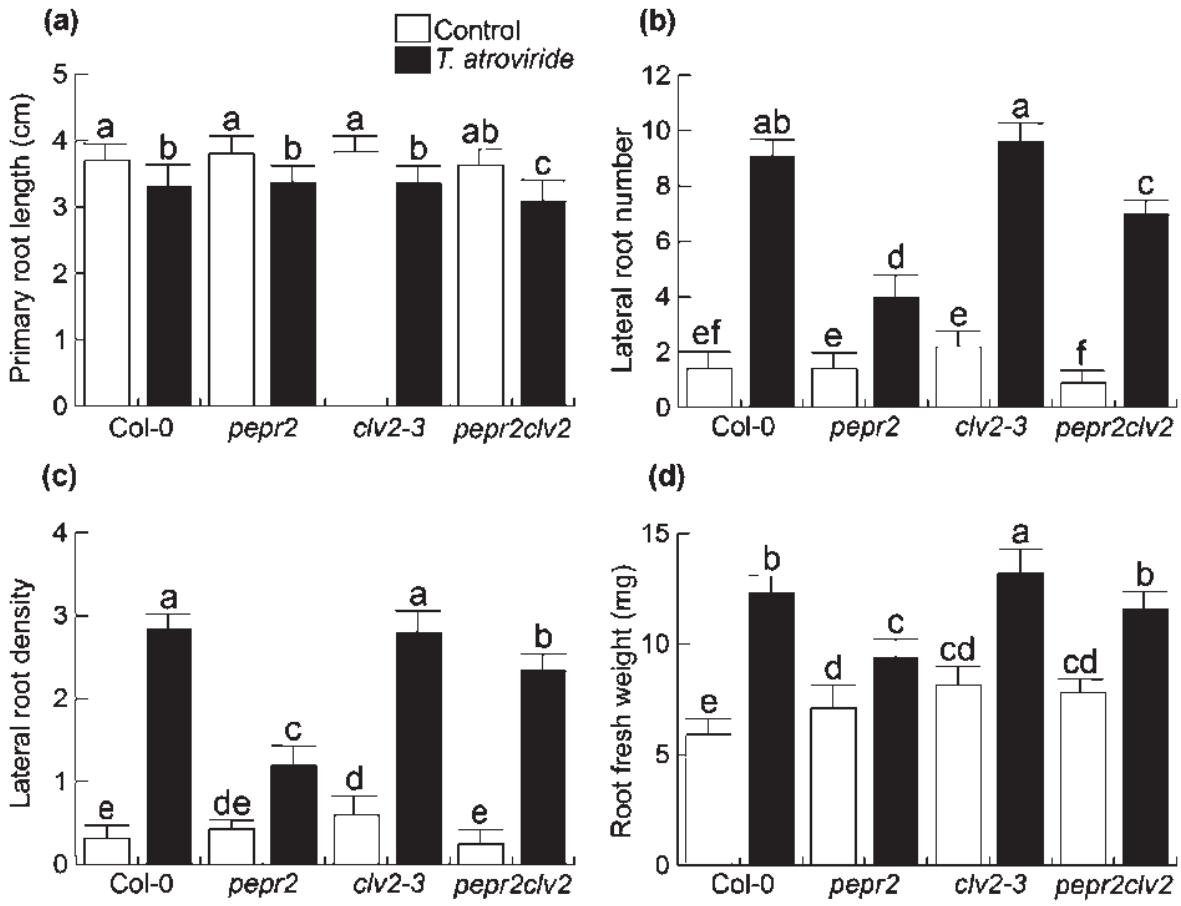
(a)
Control
T. atroviride
Primary root length (cm)
0
1
2
3
4
5
a
b
a
b
a
b
ab
c
Col-0
pepr2
clv2-3
pepr2clv2
(b)
Lateral root number
0
2
4
6
8
10
12
ef
ab
e
d
e
a
f
c
Col-0
pepr2
clv2-3
pepr2clv2
(c)
Lateral root density
0
1
2
3
4
e
a
de
c
d
a
e
b
Col-0
pepr2
clv2-3
pepr2clv2
(d)
Root fresh weight (mg)
0
5
10
15
e
b
d
c
cd
a
cd
b
Col-0
pepr2
clv2-3
pepr2clv2

Figure 8

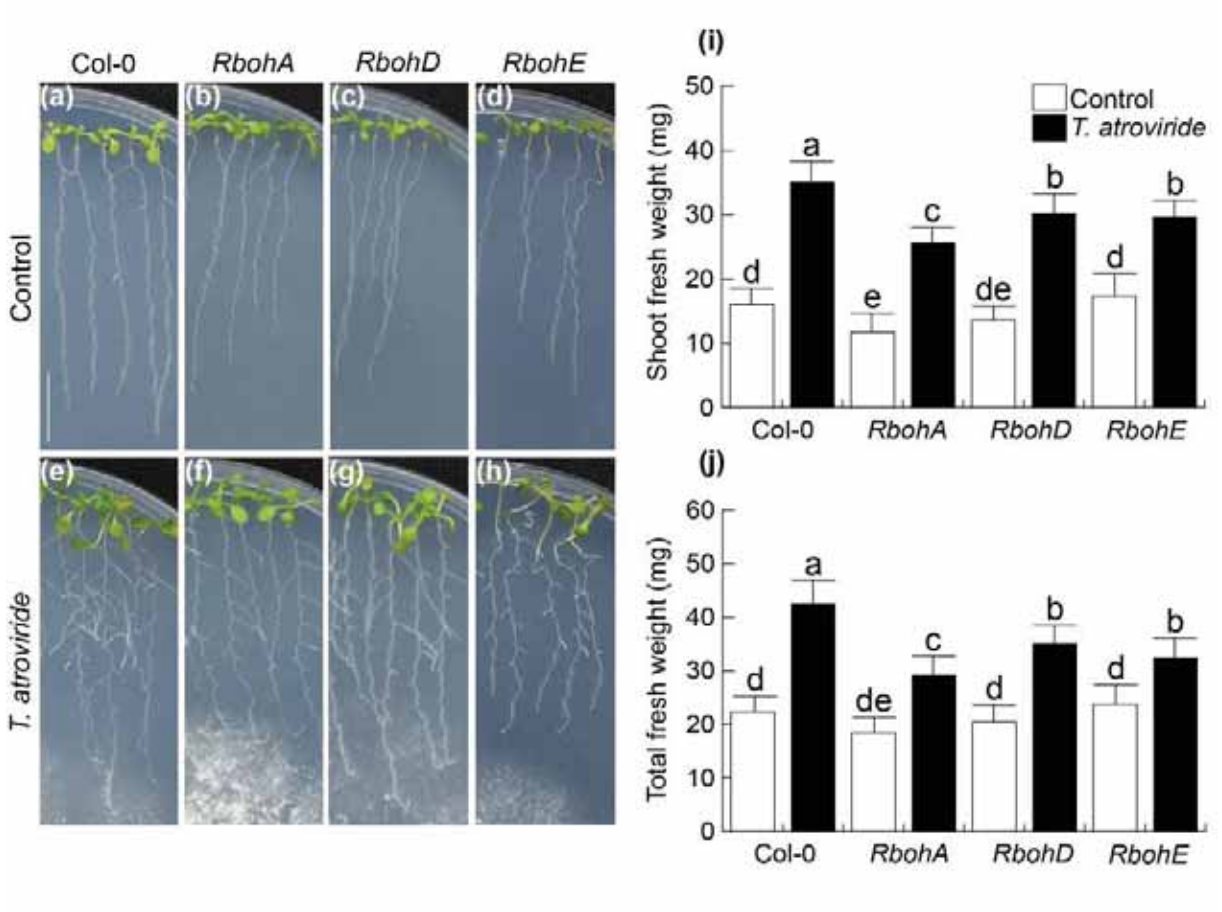
Col-0
RbohA
RbohD
RbohE
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
Control
T. atroviride
(i)
Shoot fresh weight (mg)
Control
T. atroviride
Col-0
RbohA
RbohD
RbohE
(j)
Total fresh weight (mg)

Figure S1

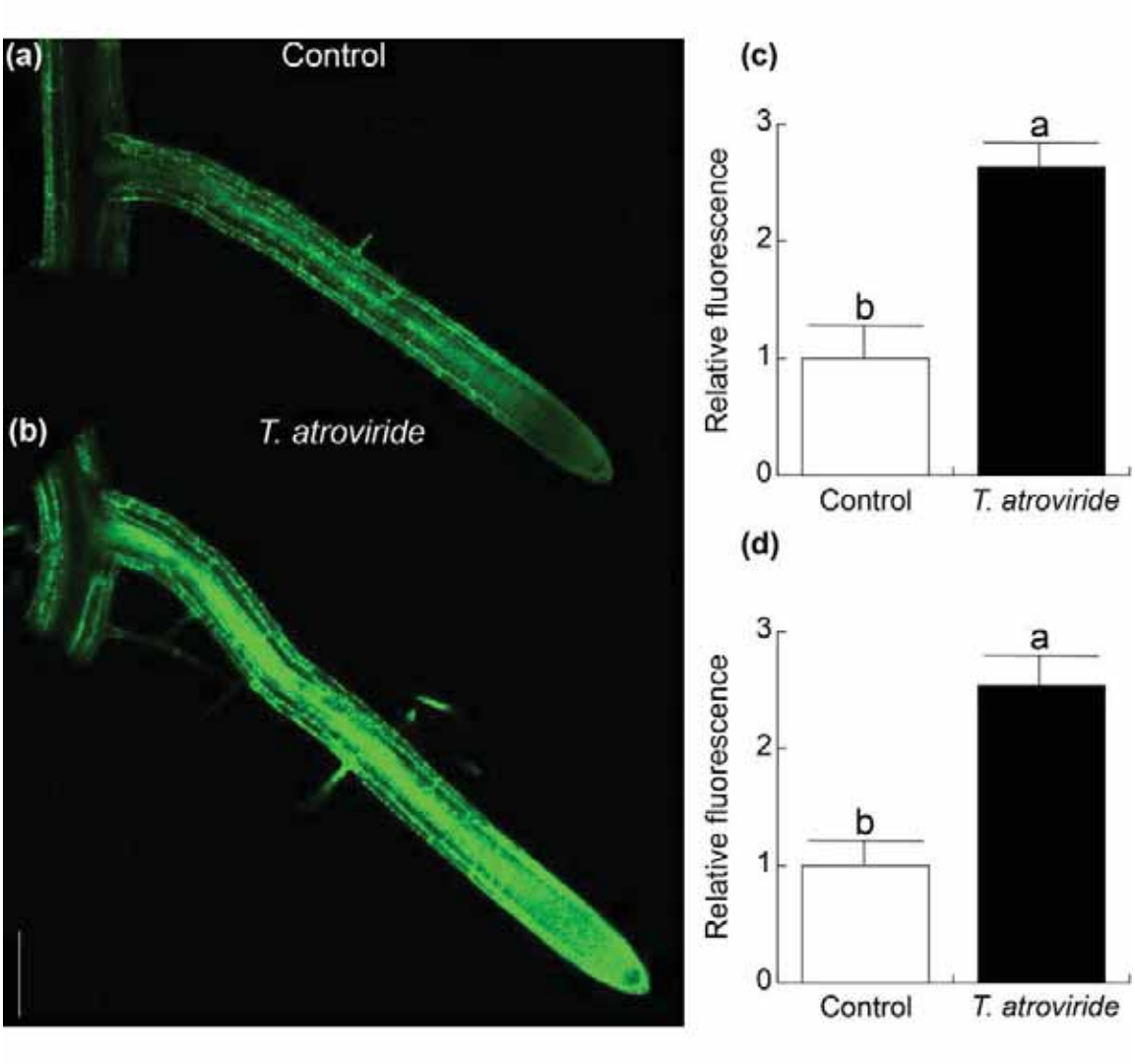
(a)
Control
(b)
T. atroviride
(c)
Relative fluorescence
3
2
1
0
a
b
Control
T. atroviride
(d)
Relative fluorescence
3
2
1
0
a
b
Control
T. atroviride

Figure S2

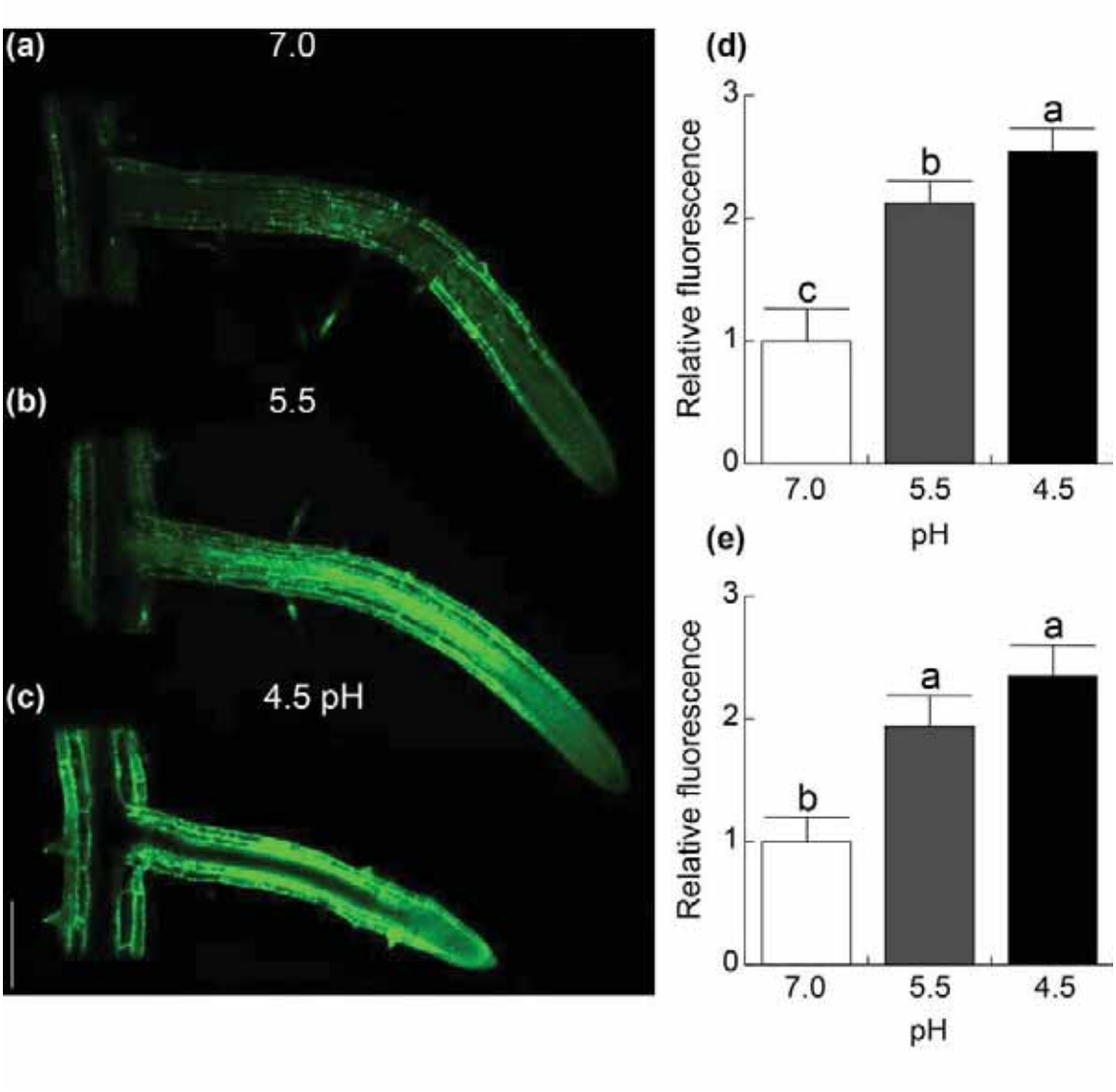
(a)
7.0
(b)
5.5
(c)
4.5 pH
(d)
Relative fluorescence
3
2
1
0
c
b
a
7.0
5.5
4.5
pH
(e)
Relative fluorescence
3
2
1
0
b
a
a
7.0
5.5
4.5
pH

Figure S3

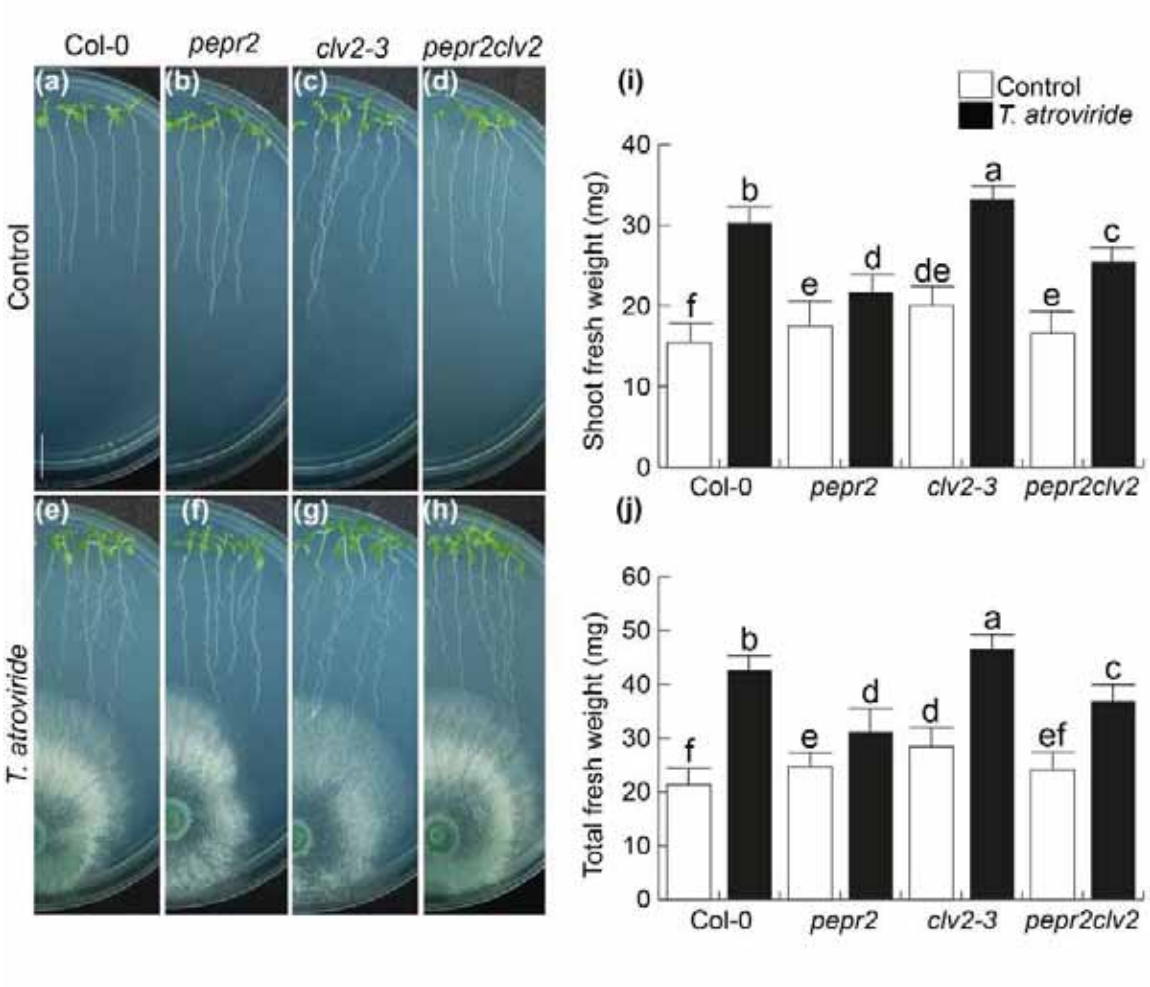
Col-0
pepr2
clv2-3
pepr2clv2
(a)
(b)
(c)
(d)
Control
(e)
(f)
(g)
(h)
T. atroviride
(i)
Control
T. atroviride
Shoot fresh weight (mg)
40
30
20
10
0
f
b
e
d
de
a
e
c
Col-0
pepr2
clv2-3
pepr2clv2
(j)
Total fresh weight (mg)
60
50
40
30
20
10
0
f
b
e
d
d
a
ef
c
Col-0
pepr2
clv2-3
pepr2clv2

Figure S4

VII. DISCUSIÓN Y CONCLUSIONES

Los mecanismos que subyacen a la simbiosis entre las especies de hongos del género *Trichoderma* y las plantas han sido ampliamente investigados en los últimos años (Tyśkiewicz *et al*., 2022). Estos hongos poseen una variedad de atributos para su uso como bioestimulantes en la agricultura (López-Bucio *et al*., 2015; Doni *et al*., 2017; Alfiky *et al*., 2021). Además de mejorar el desarrollo de la arquitectura de la raíz con el subsecuente incremento de la adquisición de agua y nutrientes, *Trichoderma* refuerza la inmunidad mediante la inducción de las hormonas canónicas de defensa (JA, SA y Et), lo cual ayudan directa o indirectamente a las plantas a resistir diversos tipos de estrés (Contreras-Cornejo *et al*., 2011; 2015; Martínez-Medina, Appels y van Wees, 2017; Wang *et al*., 2020).

Los resultados de este trabajo muestran que la comunicación establecida entre *T. atroviride* y *Arabidopsis* se pierde por la mutación de los genes que codifican para las proteínas NADPH oxidasas en el hongo, específicamente la subunidad reguladora NoxR, ya que se observa una disminución en la formación de raíces laterales en plántulas de *Arabidopsis* co-cultivadas con la mutante Δ*noxR* en comparación con cepa silvestre. Esto sugiere que la producción de ROS mediante el complejo NOX es necesaria en *T. atroviride* para estimular la aparición de primordios a partir de la raíz principal. Para comprender a detalle la respuesta de las plantas a *Trichoderma* y la señalización que involucra el complejo NOX, se realizó un análisis transcriptómico comparando el impacto de las cepas WT y la mutante Δ*noxR* en el perfil de expresión génica de *Arabidopsis*. Interesantemente, la mutante Δ*noxR* dio lugar a cambios en el patrón de expresión de un mayor número de genes que la cepa WT, antes y durante el contacto de las hifas con las raíces. Además, la respuesta causada por la mutante Δ*noxR* resultó en una activación más fuerte de las funciones relacionadas con las respuestas a bacterias e insectos, heridas y refuerzo del sistema inmunológico del hospedante antes del contacto de las raíces en comparación con la cepa WT. Algunos de los genes que presentaron mayor cambio de expresión en presencia de la mutante Δ*noxR* correspondieron a aquellos relacionados a la tolerancia al estrés por sequía e

hipoxia (*AtBCS1* y *CML38*), biosíntesis de camalexina y lignina (*PAD3* y *AtBCB*) y resistencia adquirida sistémica por JA (*JAZ1*, *JAZ6*, *JAM1*, *LOX1* y *PR3*), sugiriendo que las plantas expuestas a esta cepa podrían responder mejor a las condiciones ambientales adversas y patógenos. Estos resultados correlacionaron positivamente con la expresión de *JAZ1/TIFY10A*, la cual se observa que se induce fuertemente por efecto de la mutante Δ*noxR* en los núcleos de las células de las capas internas de la raíz primaria y hojas, indicando que la planta reacciona a *Trichoderma* mayormente en defensa, por lo que el efecto represor del mutante Δ*noxR* en la ramificación de la raíz podría explicarse por la sobre activación de la respuesta al JA (Guo *et al*., 2018).

La ramificación de la raíz de *Arabidopsis* inducido por *Trichoderma* depende en gran medida por una señalización de auxinas, por lo que podría estar comprometida esta respuesta en la interacción con el mutante Δ*noxR*. Varios estudios respaldan un modelo para explicar el equilibrio entre las compensaciones de crecimiento y defensa basadas en la señalización de auxina-JA (Hermosa *et al*., 2013; Medeiros *et al.*, 2017). Ambas vías hormonales controlan la formación y el desarrollo de raíces en *Arabidopsis* (Cai *et al*., 2014; Huang *et al*., 2017). Sin embargo, los cambios en los niveles endógenos de JA afectan la formación de raíces laterales e influyen en la homeostasis de las auxinas a través de la modulación de genes biosintéticos como *ANTHRANILATE SYNTHASE α1* (*ASA1*), algunos miembros de la familia de genes *YUCCA* (*YUCCA8* y *YUCCA9*) y transportadores de eflujo de auxinas PIN-FORMED 2 (PIN2) (Sun *et al*., 2009; 2011; Hentrich *et al*., 2013). Esto muestra el importante papel de las ROS generadas mediadas por NoxR en *Trichoderma* en la compleja red hormonal que dirige la supresión del crecimiento y la activación de las defensas en las plantas.

Recientemente, se ha informado que la comunicación de *Trichoderma-Arabidopsis* a través de VOC se ve afectada por la mutación de *noxR* y *nox1* en el hongo (Cruz-Magalhães *et al*., 2019). Sin embargo, se sabe poco sobre los cambios transcripcionales que subyacen al metabolismo fúngico. Para evaluar cómo los hongos detectan las plantas, se realizaron comparaciones de la

respuesta transcripcional de las cepas *Trichoderma* WT y Δ*noxR* durante la interacción con *Arabidopsis*. Se obtuvo como resultado que la cepa WT puede percibir la presencia de las plantas antes del contacto, modificando su perfil de expresión génica. Antes del contacto con la raíz, en la cepa WT, los genes que codifican peroxidasas, celulasas, transportadores de azúcar y, en general, genes relacionados con carbohidratos están reprimidos. Esto coincide con lo descrito por Malinich y col. (2019), quienes describen que durante la interacción de maíz con *T. virens*, se produce una represión global de los genes que codifican las enzimas que degradan la pared celular. Estas observaciones sugieren que la supresión de los procesos metabólicos relacionados con la degradación de los carbohidratos complejos en el hongo es necesaria para establecer una interacción beneficiosa con la planta, probablemente como resultado de la adaptación del hongo a la disponibilidad de carbohidratos simples como sacarosa proporcionada por las raíces (Macías-Rodríguez *et al*., 2018). Esto se refuerza con el análisis de la capacidad de crecimiento de la cepa WT y la mutante Δ*noxR* en celulosa y sacarosa como fuentes de carbono, en el que se muestra que ambas cepas utilizan de manera eficiente la celulosa, pero no de la sacarosa por la cepa mutante. Por lo tanto, la modulación de maquinaria enzimática dependiente de ROS durante la interacción de *Trichoderma* con las plantas ocurre posiblemente para evitar la sobre-estimulación de las vías de respuesta de defensa en las raíces.

También se observaron cambios sobre procesos del ciclo celular y el metabolismo del ADN en la cepa WT ante la presencia de las plantas, sobre todo en receptores acoplados a proteína G (GPCR), los cuales inducen la producción de ROS a través de los sistemas NADPH oxidasa como mecanismo de señalización en respuestas biológicas, como desarrollo, diferenciación y proliferación celular (Pelletier *et al*., 2003). En el caso del hongo simbionte *Epichloë festucae*, la interrupción de los genes que codifican los componentes del complejo NOX oxidasa *NoxA*, *NoxR* y una pequeña GTPasa (*RacA*) comprometen su capacidad para mantener la asociación simbiótica mutualista con *Lolium perenne* (Becker *et al*., 2016; Kayano *et al*., 2018). Las mutaciones en estos genes

conducen al crecimiento de hifas hiper-ramificadas en los espacios intercelulares de las hojas, lo que provoca la senescencia temprana de las plantas hospedantes (Scott *et al.*, 2012), por lo que asumimos que la producción de ROS mediada por el complejo Nox en los hongos es fundamental para el establecimiento de asociaciones simbióticas con las plantas.

Las plantas activan respuestas inmunitarias, incluida la producción de ROS durante la interacción con microorganismos (Chapman *et al*., 2019; Eljebbawi *et al*., 2021). Se ha reportado previamente que la colonización de las raíces por *Trichoderma* induce un estallido oxidativo local y sistémico en las plantas, debido a la observación de acumulaciones de O_2^- y H_2O_2 sobre los tejidos colonizados y partes distales como hojas (Contreras-Cornejo *et al*., 2011; Nawrocka *et al*., 2019; Xu *et al*., 2020; González-López *et al*., 2021). Para determinar el estatus del estrés oxidativo que provoca *T. atroviride* previo a la colonización de las raíces, se evaluó la generación de ROS en las raíces a través de la visualización de la sonda fluorescente H2DCF-DA y la tinción de DAB. De acuerdo con los resultados, los niveles intracelulares de ROS totales fueron más altos en las raíces de las plantas inoculadas con *T. atroviride* en comparación con las plantas no inoculadas. Además, la exposición a *Trichoderma* provoca una acumulación específica de H_2O_2 en la cofia de la raíz y sobre la zona de maduración de la raíz primaria, especialmente en los tejidos internos que forman la estela. Esta acumulación delimitada de H_2O_2 en el cilindro vascular es consistente con los transcritos del gen *RbohF*, inducidos por salinidad o suplementación de ácido carboxílico 1–amino–ciclo–propano (ACC). Esto sugiere la participación de las enzimas NOX, conocidas como homólogas de la explosión oxidasa respiratoria (*respiratory burst oxidase homologues*-RBOH) en el estrés oxidativo mediado por *Trichoderma* en las raíces, y que puede influir sobre los procesos de proliferación y diferenciación celular.

La aplicación exógena de H_2O_2 en plántulas de *Arabidopsis* estimula el desarrollo de RL y el acortamiento de la raíz primaria mediante la modulación de los eventos de división celular (Su *et al*., 2016; Orman-Ligeza *et al*., 2016). De

acuerdo con Orman-Ligeza y col. (2016), la generación de ROS por las enzimas RBOH facilita la remodelación de la pared celular de las capas celulares subyacentes de los PRL, para su posterior emergencia. Para investigar el papel de la producción de ROS mediada por RBOH en los efectos de promoción del crecimiento vegetal de *Trichoderma*, se co-cultivaron plántulas de *Arabidopsis* (Col-0) y mutantes afectadas en las enzimas RBOH (*rbohA*, *rbohD* y *rbohE*) con *T. atroviride*. Los resultados mostraron que *T. atroviride* promueve la producción de biomasa en raíces y brotes, y aumenta la ramificación de raíces en las plántulas silvestres en comparación con plantas no inoculadas; sin embargo, las mutaciones en cualquiera de las enzimas RBOH causaron una disminución en la bio-estimulación. Estos resultados sugieren que la producción de ROS a través de RBOHA, RBOHD y RBOHE son determinantes para la ramificación de las raíces por *Trichoderma*. Se ha reportado que la mutante *rbohE* muestra una baja formación y elongación de RL, debido a que la pérdida funcional de *RBOHE* compromete fuertemente la emergencia de RL (Chapman *et a*l., 2019). Este desarrollo de RL mediado por RBOHE está relacionado con su patrón específico de expresión en las células que recubren los PRL y su expresión inducible por auxinas (Chapman *et al*., 2019; Eljebbawi *et al*., 2021). Por su parte, *RBOHA* también presenta una expresión inducible por auxinas, sin embargo, su patrón de expresión tejido-específico no se limita en los PRL, sino que también se presenta sobre la zona de maduración de la raíz primaria (estela y endodermis), sugiriendo que RBOHA participa en procesos biológicos relacionados a defensa (Orman-Ligeza *et al*., 2016; Chapman *et al*., 2019; Neuser *et al*., 2019). De manera similar, la expresión de *RbohD* es inducible por estrés biótico y abiótico mediante una señalización que implica la participación de MPK1 y ERF74 (Morales *et al*., 2016; Yao *et al*., 2017; Escudero *et* al., 2019; Lee *et al.,* 2020). Por lo que se plantea que *RbohA*, *RbohE* y *RbohD* son genes diana de los programas de crecimiento dependientes de auxinas y/o la vía de señalización de respuesta de defensa (Et-MAPK) provocada por *Trichoderma* en las plantas.

El H_2O_2 afecta el transporte direccional de las auxinas a través de cambios en la expresión de los transportadores de eflujo de la familia PIN (PIN-FORMED),

sin embargo, la aplicación de auxinas de manera exógena también aumenta los niveles de ROS en la punta de la raíz, lo que indica la relación entre la generación de ROS y la señalización de auxinas para controlar la arquitectura de las raíces (Ivanchenko *et al*., 2013; Orman-Ligeza *et al*., 2016; Su *et al*., 2016; Velada *et al*., 2020). La acidificación del medio causada por el crecimiento de *Trichoderma* en los medios de cultivo induce una redistribución de auxinas dentro del ápice de la raíz que origina una reorientación del crecimiento de la raíz y la posterior formación del gancho apical seguido del agotamiento del meristemo (Pelagio-Flores *et al*., 2017). Este fenotipo de flexión de la raíz se relaciona con alteraciones de la gravedad, ya que también implica una redistribución de auxinas dentro de la punta de la raíz que precisamente provoca una acumulación puntual de ROS (Eljebbawi *et al*., 2021). Por lo que planteamos que posiblemente la acumulación de auxinas causada por la acidificación de *Trichoderma* provoca una disminución del crecimiento de la raíz primaria a través de una sobreproducción de ROS. Para corroborar dicho planteamiento, se analizó el impacto del pH ácido sobre la arquitectura del sistema radicular de *Arabidopsis*. De acuerdo con los resultados obtenidos, las plantas cultivadas a pH ácido (5.5 y 4.5) presentaron una alteración del gravitropismo, además de reprimir el crecimiento de la raíz primaria. Aunado a esto, el pH ácido también induce una fuerte acumulación intracelular de ROS en el ápice de la raíz primaria. Tales resultados coinciden con los reportados previamente que demuestran que el estrés por pH ácido causa una acumulación excesiva de superóxido y H_2O_2 en el ápice de las raíces que conlleva a la inhibición del crecimiento de las raíces (Koyama *et al*., 2001; Zhang *et al*., 2015; Long *et al*., 2019; Graças *et al*., 2020).

La evaluación del efecto inhibitorio de la 6-PP sobre el crecimiento de la raíz primaria a concentraciones elevadas también se asocia con una acumulación de ROS en las puntas de las raíces. Garnica-Vergara y col. (2016) reportaron que EIN2 es un componente clave en la respuesta de la planta al volátil 6-PP, ya que las mutantes *ein2* presentan insensibilidad a la inhibición del crecimiento de la raíz primaria causada por concentraciones elevadas de 6-PP. La inhibición del crecimiento de la raíz y la acumulación de O_2^- en las raíces son efectos típicos del

Et o de su precursor el ACC (Lv *et al*., 2018), por lo que se consideraría que la 6-PP regula el alargamiento de la raíz primaria a través de la regulación de la homeostasis de ROS dependiente del Et. EIN2 es un elemento necesario para el estrés oxidativo y la represión del crecimiento de raíces causado por efectores de patógenos como la flagelina bacteriana (flg22) y la piocianina, indicando un entrecruzamiento entre el Et y las ROS en la activación de las respuestas de defensa (Mersmann *et al*., 2010; Lin *et al*., 2013; Ortiz-Castro *et al*., 2014). Las respuestas inmunitarias desencadenadas por los PAMP inducen una producción de ROS apoplástica transitoria a través de la activación de las RBOH mediante la señalización de receptores de reconocimiento de patrones (PRR) (Hu *et al*., 2020; Jing *et al*., 2020). El reconocimiento de flg22 por el receptor FLS2 (una cinasa receptora repetida rica en leucina desencadena la activación de la cinasa ser/thr BIK1, la cual fosforila directamente a RBOHD para inducir un estallido oxidativo extracelular (Kadota *et al*., 2014; Li *et al*., 2014; Noman *et al*., 2019). Para dilucidar la posible participación del RECEPTOR 2 DE PEP1 (PEPR2) en el estrés oxidativo desencadenado por *Trichoderma*, se evaluó el patrón de expresión de *PEPR2*, el cual mostró ser inducible por la presencia de *Trichoderma* tanto en las hojas como en la zona de maduración de la raíz primaria (estela). Asimismo, el análisis de la arquitectura de la raíz en plantas normales en comparación con las mutantes simples *pepr2*, demostraron que la mutación de *pepr2* afecta la reconfiguración de la arquitectura de la raíz y la bio-estimulación provocada por el hongo. Estos resultaos plantean que el receptor PEPR2 puede ser un regulador río arriba de la actividad de las enzimas RBOH.

Finalmente, proponemos que la acidificación del sustrato y la emisión de moléculas volátiles y difusibles por parte del hongo, actúan como señales específicas que influyen indirectamente sobre la reconfiguración de la arquitectura de la raíz, el desarrollo en general de la planta y las respuestas de defensa. Un desequilibro en la producción de ROS mediante el complejo NADPH oxidasa tanto en el hongo como en las plantas afectan el establecimiento de la simbiosis al comprometer el desarrollo e inmunidad de las plantas.

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IX. ANEXOS

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ORIGINAL ARTICLE

WILEY

Trichoderma atroviride-emitted volatiles improve growth of *Arabidopsis* seedlings through modulation of sucrose transport and metabolism

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Abstract

Plants host a diverse microbiome and differentially react to the fungal species living as endophytes or around their roots through emission of volatiles. Here, using divided Petri plates for *Arabidopsis*-*T. atroviride* co-cultivation, we show that fungal volatiles increase endogenous sugar levels in shoots, roots and root exudates, which improve *Arabidopsis* root growth and branching and strengthen the symbiosis. Tissue-specific expression of three sucrose phosphate synthase-encoding genes (*AtSPS1F*, *AtSPS2F* and *AtSPS3F*), and AtSUC2 and SWEET transporters revealed that the gene expression signatures differ from those of the fungal pathogens *Fusarium oxysporum* and *Alternaria alternata* and that AtSUC2 is largely repressed either by increasing carbon availability or by perception of the fungal volatile 6-pentyl-2H-pyran-2-one. Our data point to *Trichoderma* volatiles as chemical signatures for sugar biosynthesis and exudation and unveil specific modulation of a critical, long-distance sucrose transporter in the plant.

KEYWORDS
Arabidopsis, root exudates, sucrose, sugar transporters, *Trichoderma*, volatile compounds

1 | INTRODUCTION

Plants are engaged in a myriad of interactions with microorganisms through an exquisite and complex chemical communication. Roots release a wide variety of metabolites including sugars, amino acids and organic acids, which influence the microbial composition of their surroundings, trigger chemotactic responses and determine successful colonization (Biniyamin, Nadeem, Khan, & Anjum, 2019; Canarini, Kaiser, Merchant, Richter, & Wanek, 2019; Vives-Peris, de Ollas, Gómez-Cadenas, & Pérez-Clemente, 2020).

The genus *Trichoderma* includes opportunistic avirulent fungal species with a versatile lifestyle, which rely on plant-derived sugars for fast growth and root colonization. They also thrive as decomposers of decaying plant residues or parasitize other fungi when nutrients are limiting (Ramírez-Valdespino, Casas-Flores, & Olmedo-Monfil, 2019; Vargas, Crutcher, & Kenerley, 2011; Vargas, Mandawe, & Kenerley, 2009; Villalobos-Escobedo et al., 2020). Upon root colonization, *Trichoderma* influences shoot performance through long distance, systemic signalling relying on oxylipins that strengthen photosynthesis, sugar homeostasis and exudation patterns

(Coppola et al., 2019; Doni et al., 2019; Macías-Rodríguez, Guzmán-Gómez, García-Juárez, & Contreras-Cornejo, 2018; Wang, Borrego, Kenerley, & Kolomiets, 2020). Further, *Trichoderma* effectively antagonizes soil-borne pathogens through mycoparasitism, antibiosis and competition for nutrients and space (Zin & Badaluddin, 2020). Enhancement of the defensive capacity of their plant hosts towards pathogen invasion and herbivore attack is also an important hallmark of these fungi (Guzmán-Guzmán, Porras-Troncoso, Olmedo-Monfil, & Herrera-Estrella, 2019; Vinale & Sivasithamparam, 2020).

Plant growth promotion elicited by *Trichoderma* has been attributed to their capacity to solubilize nutrients through acidification of the rhizosphere, the production of auxins and secondary metabolites as well as the emission of blends of volatiles (Estrada-Rivera et al., 2019; Guzmán-Guzmán et al., 2019; López-Bucio, Pelagio-Flores, & Herrera-Estrella, 2015). Fungal blends include volatile organic compounds (VOCs), inorganic volatiles and CO_2; this later is a main reactant for photosynthesis and may contribute to growth promotion in autotrophic organisms (Effmert, Kalderás, Warnke, & Piechulla, 2012; García-Gómez et al., 2019; Kai & Piechulla, 2009; Piechulla & Schnitzler, 2016). Volatiles act as elicitors of defence and developmental processes, and are considered important cues during plant-fungus recognition due to their highly diffusible properties (Piechulla, Lemfack, & Kai, 2017; Vinale & Sivasithamparam, 2020). VOCs emitted by *Trichoderma* include lactones, ketones, alcohols, mono- and sesquiterpenes, esters and aldehydes from different metabolic origin and selective bioactivity (da Silva et al., 2020; Nieto-Jacobo et al., 2017). For instance, the main VOC from *T. atroviride*, namely 6-pentyl-2H-pyran-2-one (6-PP) changes root organogenesis modulating both ethylene signalling and auxin transport (Estrada-Rivera et al., 2019; Garnica-Vergara et al., 2016; Kottb, Gigolashvili, Großkinsky, & Piechulla, 2015; Zin & Badaluddin, 2020). Overall, the fungal volatile blends may affect chlorophyll content, photosynthetic efficiency and hence carbon metabolism (da Silva et al., 2020; Hung & Lee, 2013; Jalali, Zafari, & Salari, 2017; Lee, Yap, Behringer, Hung, & Bennett, 2016; Nieto-Jacobo et al., 2017; Wonglom, Ito, & Sunpapao, 2020). However, the molecular mechanisms mediating these responses are unknown.

Photosynthetic efficiency closely depends on the sunlight absorbed by chlorophyll (Chl), which is converted into chemical energy needed to reduce carbon dioxide into glucose (López-García et al., 2020). Sucrose-6-phosphate-synthase (SPS) enzymes catalyse the reaction between UDP-glucose and fructose-6-phosphate to form sucrose-6-phosphate, which is rapidly hydrolysed by sucrose phosphate phosphatase (SPP) (Stein & Granot, 2019; Yoon, Cho, Tun, Jeon, & An, 2020). The translocation of sucrose from source to sink organs requires phloem loading through proton-coupled transporters or carriers (SUTs/SUCs), which load sucrose into sieve element/companion cell (SE/CC) complexes (Hennion et al., 2019; Julius, Leach, Tran, Mertz, & Braun, 2017). Once unloaded into sink tissues, sucrose can be cleaved by sucrose synthase (SuSy) or cytosolic invertases to generate glucose and fructose (Stein & Granot, 2019; Yoon et al., 2020). The distribution of these sugars at the sub-cellular level requires transport across membranes via monosaccharide transporters (MST) and sugar efflux carriers SWEETs (abbreviation of *Sugars Will Eventually be Exported Transporter*) (Chen, 2014; Jeena, Kumar, & Shukla, 2019). The latter protein family has been tightly involved in carbon supply to symbiotic microbes and plant pathogens (Chandran, 2015).

In the interaction of arbuscular mycorrhizal (AM) fungi with roots, an enhanced carbohydrate metabolism and CO_2 assimilation rises hexose levels in roots to ensure the fungal and root carbon demand (Boldt et al., 2011). Besides, the increased sucrose transport from leaves to roots induced by AM fungi has been attributed to the up-regulation of SUT and SWEET transporters (Boldt et al., 2011; Manck-Götzenberger & Requena, 2016). The transporters mediating carbon allocation within the plant to potentially support *Trichoderma* growth still remain to be identified.

Here, we show that *Arabidopsis* growth promotion by volatiles from *Trichoderma atroviride* depends on long-distance transport of sucrose from leaves to roots likely through the sucrose transporter AtSUC2 and drives enrichment of this disaccharide in root exudates. The interaction up-regulates the expression of sucrose phosphate synthase (SPS) isoforms, and affects mRNA levels of *AtSUC2* and some *Arabidopsis SWEET* genes that are different from those induced by phytopathogenic fungi.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Arabidopsis thaliana wild-type Columbia-0 (Col-0), and the transgenic lines *AtSUC2-GFP* (Imlau, Truernit, & Sauer, 1999), *AtSPS1F::uidA-GFP*, *AtSPS2F::uidA-GFP* and *AtSPS3F::uidA-GFP* (Solis-Guzmán et al., 2017) were used. Seeds were disinfected using 95% (vol/vol) ethanol and 20% (vol/vol) bleach for 5 and 7 min, respectively, and washed five times with distilled, sterilized water. Later, seeds were stratified at 4°C for 2 days and germinated and grown on side 1 of divided Petri plates containing 0.2x Murashige and Skoog (1962) medium (MS Basal salts mixture, Catalogue No. M5524 Sigma) supplemented with varied sucrose concentrations and agar (1% wt/vol) as solidifying agent (Micropropagation grade, Catalogue No. A111 PhytoTechnology Laboratories) at pH 7. Finally, plates were placed into a plant growth chamber (Percival AR-95 L), with a photoperiod of 16 hr of light/8 hr of darkness, the light intensity of 300 μmol m^{-2} s^{-1} and a temperature of 22°C.

2.2 | Fungal growth and plant inoculation experiments

Trichoderma atroviride strain IMI 206040 was used in these studies. The fungus was propagated on potato dextrose agar (PDA) in darkness for 5 days at 28°C. The conidial suspension was obtained scraping the surface of the fungal colony with sterile distilled water. The inoculum was adjusted to 1×10^6 spores and was placed at the

opposite half (side 2) of the divided plates containing agar-solidified 0.2x MS medium with 4-day-old *Arabidopsis* seedlings. The plates were sealed with a single row of plastic wrap and after 4 days of co-cultivation, determination of plant growth and gene expression analyses was performed.

2.3 | Sucrose treatments to shoots

To mimic the effect of sucrose as a leaf-derived signal, seedlings were grown on 0.2x MS medium plates for 4 days as described above, and then were transferred to fresh plates with the shoot placed over agar patches of 0.2x MS medium enriched with 0 to 9.6% of sucrose and solidified with 1% wt/vol phytagar and allowed to grow for 4 days. Drops of 100 µl of melted medium supplied with agar and enriched with sucrose were laid over sterilized Parafilm strips to prevent contact of the shoot with the rest of the medium (MacGregor, Deak, Ingram, & Malamy, 2008; Raya-González et al., 2017).

2.4 | 6-PP analysis

Arabidopsis seedlings were germinated and grown on Petri plates containing agar-solidified 0.2x MS medium supplied with micromolar concentrations (0, 50, 100 and 150 µM) of 6-PP (Sigma-Aldrich) dissolved in ethanol. For the control condition, ethanol was added in a volume equal to the highest 6-PP concentration applied. Petri plates containing 10 seeds were placed into the above-mentioned growth chamber for 12 days to enable growth.

2.5 | Analysis of plant traits

The length of primary roots was measured using a ruler, and the lateral root formation was determined by counting all mature roots that emerged from the primary root using a stereomicroscope (Leica MZ6). The lateral root density was calculated by dividing the lateral root number by the primary root length for each seedling. Fresh weights of shoots or roots were determined using an analytical scale.

2.6 | Propidium iodide staining and GFP fluorescence quantification

Visualization and quantitation of GFP fluorescence were performed using a confocal laser-scanning microscope (Olympus, FV1200). Transgenic *Arabidopsis* seedlings were stained with propidium iodide (PI) for 1 min, rinsed in water and mounted in 50% (vol/vol) glycerol on microscope slides. Red fluorescence of PI and GFP emission were detected through the multi-argon laser with an excitation line from 488 to 568 nm and an emission window from 585 to 610 nm, after which the two images were merged to produce the ending image. The green pixels from six micrographs were quantified through IMAGEJ software (http://rsbweb.nih.gov/ij/). The arbitrary unit value was obtained (A.U. = green pixels μm^2) for each micrograph. A.U. from control conditions was given a value of 1.

2.7 | Histochemical analysis

Arabidopsis seedlings were incubated at 37°C in a GUS reaction buffer (0.5 mg ml 5-bromo-4-chloro-3-indolyl-β-d-glucuronide in 100 mM sodium phosphate, pH 7) in darkness. Stained seedlings were cleared and fixed according to Malamy and Benfey (1997). The seedlings were incubated in a solution 0.24 N HCl in 20% methanol (vol/vol) for 1 hr at 62°C. The solution was substituted by 7% NaOH (wt/vol) in 60% ethanol (vol/vol) for 20 min at room temperature. Later, plants were dehydrated with ethanol treatments at 40, 20 and 10% (vol/vol) for a 20 min, and fixed in 50% glycerol (vol/vol). The processed roots were placed on glass slides and analysed by differential interference contrast microscopy (DIC). For each marker line and treatment, at least eight transgenic plants were analysed.

2.8 | Extraction of soluble sugars from plant tissues and root exudates

Shoot and roots tissues were excised by using a sterilized scalpel and then were ground using liquid nitrogen. One hundred mg of each sample was homogenized with 500 µl of ethanol at 80% (vol/vol) and incubated at 65°C with agitation at 1200 rpm for 8 hr. After centrifugation, the supernatant was collected and mixed with 200 µl of dichloromethane. For quantification in root exudates, *Arabidopsis* seedlings were transferred on 24-well cell culture plates that contained 1 ml of deionized water and then placed in darkness for 24 hr (25 plants per well). An aliquot of 0.5 ml of the sample was subjected to lyophilization and later resuspended in 500 µl of ethyl acetate. All the samples were transferred to a conical reaction vial and dried under nitrogen gas.

2.9 | Derivatization of sugars for GC–MS analyses

Sugars were converted to their aldonitrile per-acetylated derivatives. Thus, samples were resuspended in 1.5 ml of a hydroxylamine chloride-pyridine solution (53 mg 3 ml), sonicated for 30 min and heated for 1 hr at 85°C. After cooling, 500 µl of pyridine and 1 ml of acetic anhydrous were added to each vial, and samples were heated again for 25 min at 85°C. Then, samples were placed into assay tubes with 1 ml of chloroform and then two washes with deionized water were carried out. Finally, the organic phase was circulated through columns packed with anhydrous Na_2SO_4. Derived samples were dried under nitrogen gas and then resuspended with 25 µl of chloroform to be injected.

2.10 | Quantification of soluble sugars by GC–MS

GC–MS analyses were performed in a gas chromatograph (Agilent 6,850 Series II; Agilent, Foster City, CA, USA), equipped with an Agilent MS detector model 5,973 and HP-5 MS capillary column (5% [wt/vol] phenyl methyl polysiloxane, 30 m X 0.25 mm I.D., a film thickness of 0.25 μm). Operating conditions were: 1 ml/min helium as the carrier gas, detector temperature of 300°C, and injector temperature of 250°C. The column was held for 3 min at 150°C and programmed at 6°C min to a final temperature of 270°C for 15 min. Carbohydrates were identified by the use of a combination of NIST 2.0 mass spectra database search and deconvolution software (AMDIS v.2.0). Derived D-(+)-Glucose ≥99.5% (Sigma Aldrich) and sucrose (Zulka) were used as an external standard of soluble sugars for quantification, correlating the injected concentration (1 μg/μl) with the peak area of the eluted compound.

2.11 | RNA isolation and RT-qPCR analysis

Total RNA from *Arabidopsis* seedlings was isolated using TRIzol reagent (Invitrogen, USA) following the manufacturer's specifications. The RNA was quantified using a NanoDrop™ 2000 spectrophotometer and its integrity was checked by 1% (wt/vol) agarose gel electrophoresis. cDNA was synthesized from 1 μg of total DNA-free RNA per sample using the reverse transcription reaction mix from ImProm-II Reverse Transcription System (Promega, USA) following the manufacturer's recommendations. The RT-qPCR was performed in a 7,500 Real-Time PCR System (Applied Biosystems, USA). The PCR reaction mix contained 10 μl SYBR Green Master Mix (Applied Biosystems), 0.15 μl forward and reverse primers (20 μM), 2 μl cDNA, and 7.7 μl nuclease-free water. The reaction conditions included a holding state at 95°C for 10 min; a cycling stage of 40 cycles at 95°C for 15 s and 60°C for 1 min; and a melting curve stage at 95°C for 15 s and 60°C 1 min. The gene-specific primers used in RT-qPCR for *Arabidopsis SWEET* genes were listed in Table S1 (Chen et al., 2010). The amplification efficiency was calculated using a calibration curve with a serially diluted cDNA and the following formula: $E = (10^{[-1/m]}) * 100$, where m is the slope curve of Ct versus the logarithmic concentration (Table S2). Expression levels were calculated relative to *Arabidopsis* β-actin according to the method described by Pfaffl (2001) and López-Coria et al. (2019).

2.12 | Data analysis

For most experiments, the overall data were statistically analysed using STATISTICA 10.0 programme (Dell StatSoft, Austin, Texas, USA). Statistically significant differences in plant traits were determined through univariate and multivariate analyses with Tukey's post hoc tests. Different letters were used to indicate means that differ significantly ($p < .05$). For the RT-qPCR analysis, student-*t* test was performed to determine significant changes. p value less than .05 was taken as statistically significant.

3 | RESULTS

3.1 | *T. atroviride* volatiles improve growth and root development of *Arabidopsis* seedlings in media with contrasting sucrose supplements

Exposure to volatiles emitted by *Trichoderma* has an overall biostimulant effect in *Arabidopsis* (Contreras-Cornejo, Macías-Rodríguez, Herrera-Estrella, & López-Bucio, 2014; Estrada-Rivera et al., 2019; González-Pérez et al., 2018; Hung & Lee, 2013; Jalali et al., 2017; Lee et al., 2016; Nieto-Jacobo et al., 2017). However, little is known about how biosynthesis and re-allocation of photosynthates during the interaction support plant biomass accumulation. To gather information on this topic, the effect of volatiles emitted by *T. atroviride* in *Arabidopsis* root growth and biomass production was assessed on divided Petri plates in which side 1 (harbouring the plants) included agar-solidified 0.2x MS media supplied with 0 or 0.6% sucrose. Four days after germination, the seedlings were inoculated with *T. atroviride* in the opposite side (side 2) of the Petri dishes that contained agar-solidified 0.2x MS media supplied with 0.6% sucrose to allow volatile-mediated recognition. The lack of sucrose in the growth medium of side 1 of the plate caused roots to grow slowly and hence reduced biomass production in *Arabidopsis* seedlings. However, plants exposed to *Trichoderma* volatiles exhibited increased primary root lengths, root branching and total biomass production irrespective of the availability of sucrose (Figure 1a–e). The greatest plant growth-promoting effect by the fungus was observed when sucrose was available since the primary root significantly increased and both lateral root number and density boosted by more than threefold and twofold respectively, which in turn strongly correlated with the increased biomass production compared with the axenically grown seedlings (Figure 1b–e). These results indicate that the seedlings growing with available carbon resources are much more responsive to *Trichoderma* volatiles, which can boost plant growth and development as the concentration of sucrose in the media rises.

3.2 | *T. atroviride* volatiles strongly induce expression of the sucrose transporter *AtSUC2* under sucrose deprivation

In *Arabidopsis*, the source-to-sink sucrose transport is mainly mediated by AtSUC2, a H^+/sucrose symporter that support apoplastic sucrose loading into the phloem (Durand et al., 2018). To assess the possible contribution of AtSUC2 in plant growth and developmental programmes elicited by *Trichoderma* volatiles, the expression pattern driven by the *AtSUC2* promoter was assessed using a fusion with GFP in volatile-exposed *Arabidopsis* seedlings either in sucrose-deprived medium or in medium supplemented with 0.6% sucrose. The expression of *AtSUC2 promoter-GFP* was localized in CCs of the phloem of mature roots and root tips, and in this latter region correlates with the zone of symplastic unloading, that is, the meristem (Figure 2a). *T. atroviride* volatiles strongly increased *AtSUC2-GFP* expression in the phloem poles and root meristem of seedlings grown in a

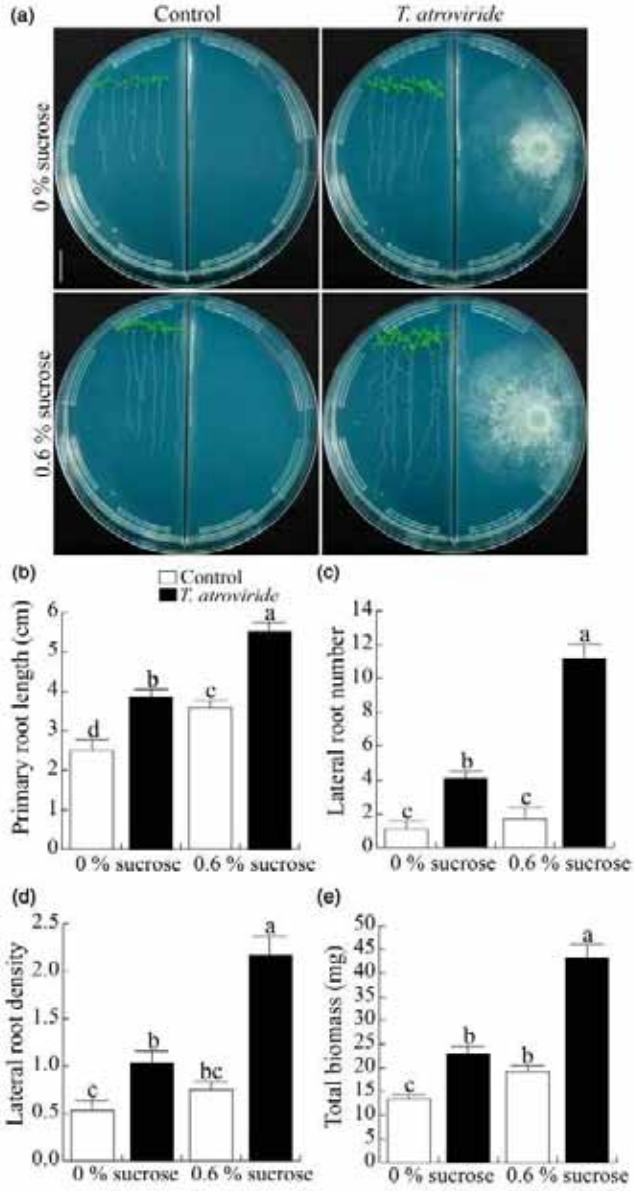

FIGURE 1 Effect of volatiles from *T. atroviride* on biomass production and root architecture of *Arabidopsis*. (a) Representative photographs of 8-day-old *Arabidopsis* seedlings grown on 0.2x MS media supplied with 0 and 0.6% sucrose and exposed to *Trichoderma* VOCs using a split-plate assay. (b) Length of the primary root, (c) lateral root number, (d) lateral root density (number of emerged lateral roots (ELR) cm) and (e) total fresh weight were recorded. Bars show the *M* ± SD. Different letters indicate significant statistical differences ($p < .05$; $n = 18$). Scale bar: 1 cm. Similar results were obtained from three independent repetitions [Colour figure can be viewed at wileyonlinelibrary.com]

sucrose-deprived medium (Figure 2b). On the other hand, volatile-exposed seedlings grown in a medium supplemented with 0.6% sucrose showed a reduced transcription of *AtSUC2* compared with non-inoculated plants (Figure 2c,d). Interestingly, *AtSUC2* levels were much higher in plants exposed to the volatiles in the absence of sucrose than under any other condition, suggesting that AtSUC2-mediated sucrose transport is increased in this condition to maintain and ensure the growth of the root apical meristem

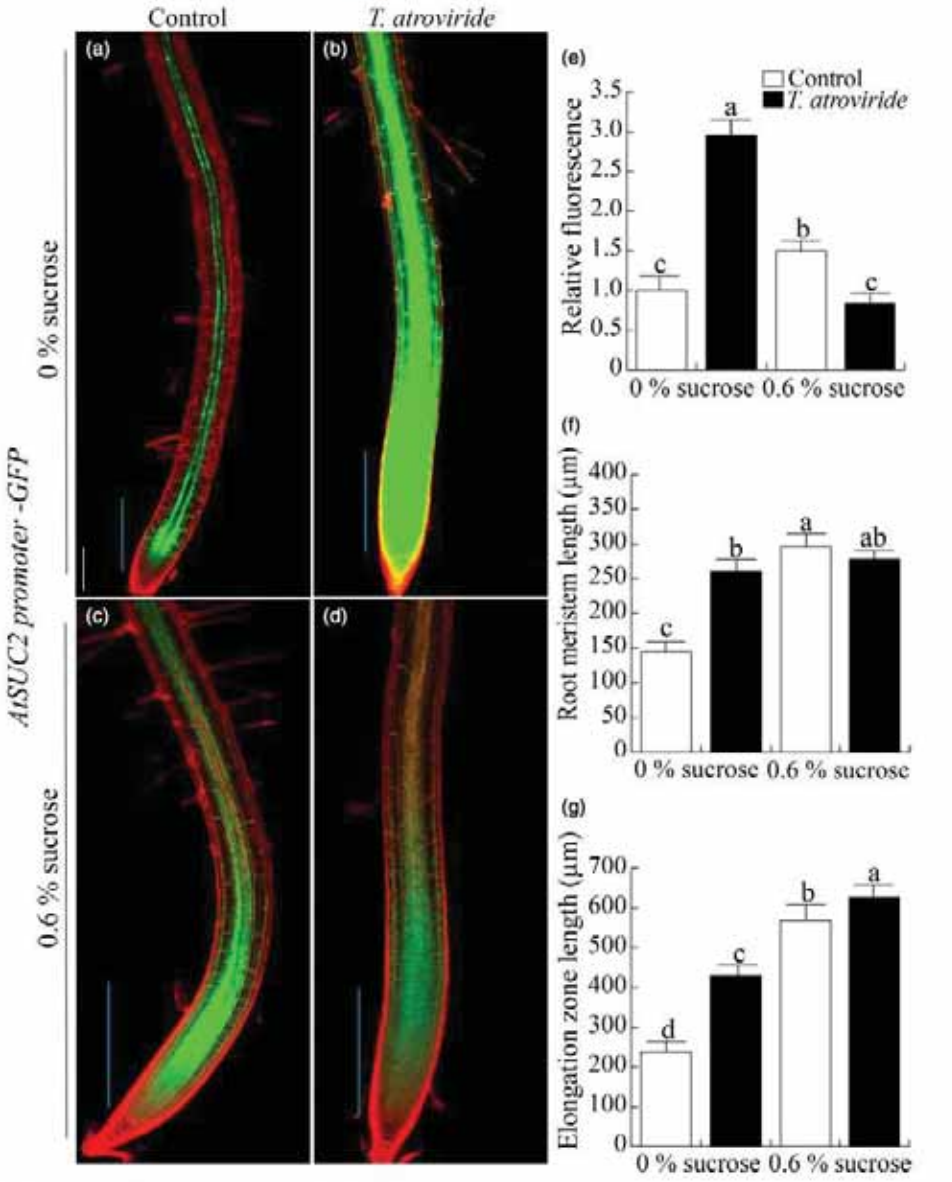

FIGURE 2 Effect of *T. atroviride* volatiles on *AtSUC2-GFP* gene expression and root meristem size. Transgenic *Arabidopsis* seedlings expressing *AtSUC2-GFP* were germinated and grown on agar-solidified 0.2x MS media with or without sucrose for 4 days and subsequently, *Trichoderma* was inoculated at 2 cm from the border on the opposite side of the plate. At 4 days of interaction, GFP detection was performed by confocal microscopy. Representative micrographs of roots expressing *AtSUC2* promoter (a–d). The meristem zone is marked with a blue line. Scale bar: 100 µm. (b) Bars graphs show differences in expression, assessed as relative fluorescence intensity, (c) meristem length (from the quiescent centre to the start of the elongation zone) and (d) mean length of cortical cells. Values shown represent the *M* ± *SD* (*n* = 6). Different letters indicate significant statistical differences ($p < .05$). The experiment was repeated twice with similar results [Colour figure can be viewed at wileyonlinelibrary.com]

(Figures 2a–e and S1). The roots of seedlings grown in medium lacking sucrose and co-cultivated with the fungus showed nearly 80% increase in both meristem and elongation zone lengths and meristem cell number, and these regions were comparable to those of seedlings supplemented with 0.6% sucrose (Figures 2f,g and S1). These data correlated *AtSUC2* expression with growth zones in the *Arabidopsis* primary root, which are modulated by the presence of the fungus at the opposite side of the Petri plate.

3.3 | 6-pentyl-2H-pyran-2-one (6-PP) represses *AtSUC2* expression in the primary root tip

6-PP is the most abundant, bioactive volatile produced by *T. atroviride* during co-culture with *Arabidopsis* seedlings (Garnica-Vergara et al., 2016). To evaluate if the *AtSUC2* gene could be influenced by 6-PP, *AtSUC2-GFP* seedlings were germinated and grown on 0.2x MS medium supplemented with 0.6% sucrose and increasing concentrations (0, 50, 100 and 150 μM) of 6-PP. After 10 days, a dose-dependent growth was observed, at concentrations of 50 and 100 μM the growth of roots and shoots of seedlings and root branching increased (Figure 3a–c). In contrast, the concentration of 150 μM decreased primary root growth but strongly promoted lateral root development (Figure 3d). Interestingly, 6-PP application reduced GFP fluorescence in the vascular bundle (Figure 3e–h) and root tip (Figure 3i–l). Quantitation of relative GFP florescence in vascular bundle (*m*) and root tip (*n*) clearly shows the link between the reconfiguration of root architecture mediated by 6-PP and repression of *AtSUC2* in primary root meristem.

3.4 | *AtSUC2* expression in root tips is repressed by locally supplying the shoot with sucrose

Sucrose is the main transport form of photoassimilates and a leaf-derived signal that promotes root growth (Kircher & Schopfer, 2012; Raya-González et al., 2017). To further determine how *Trichoderma* volatiles influence leaf-derived sucrose to promote root development, we analysed the effects of locally applied sucrose to shoots in *AtSUC2* expression. *AtSUC2-GFP* seedlings were germinated and grown on 0.2x MS medium for 4 days and then were transferred to fresh medium with only the shoot being placed over agar-solidified patches of 0.2x MS medium enriched with 0, 0.6, 1.2, 2.4, 4.8 and 9.6% sucrose. After four additional days, *AtSUC2*-driven GFP expression and plant growth were analysed. Shoot supplied sucrose strongly stimulated primary root growth, lateral root formation and biomass production (Figure S2). Confocal microscopy analysis showed that sucrose treatments greater than 0.6% decreased *AtSUC2* expression in the major veins of cotyledons (Figure 4a–f,m), and in root tips (Figure 4g–l,n). These data imply a sucrose regulation loop, where its high availability to the shoot efficiently triggers shoot and root growth and at the same time reduces *AtSUC2* expression in a comparable manner to *Trichoderma* volatiles.

3.5 | *T. atroviride* volatiles increase sucrose and glucose content in plant tissues and root exudates

Photosynthetically produced sucrose from leaves is transported towards sink organs such as roots to support cell division and elongation. Besides, some proportion of this carbon resource is secreted from roots into the rhizosphere (Hennion et al., 2019). Therefore, it was determined whether the changes in the expression of *AtSUC2-GFP* could be related to the endogenous sucrose and glucose content in shoots, roots and root exudates via gas chromatography–mass spectrometry (GS–MS) analyses in *Arabidopsis* seedlings grown axenically in divided Petri plates or being exposed to *Trichoderma* volatiles. The glucose and sucrose content clearly increased in shoots, roots and root exudates in the seedlings influenced by the fungal volatiles when compared with non-inoculated plants (Figure 5a–c and S3). These data show that *T. atroviride* volatiles increase the endogenous content of sucrose and glucose in plants and also enrich root exudates with these important carbon resources for microbial growth.

3.6 | *Trichoderma* volatiles and sucrose influence the expression of SPS genes in shoots and roots of *Arabidopsis* seedlings

The enzyme SPS catalyses the conversion of fructose-6-phosphate (Fru6P) and UDP-glucose into sucrose-6-phosphate, which is subsequently dephosphorylated by the SPP (Anur, Mufithah, Sawitri, Sakakibara, & Sugiharto, 2020). The *Arabidopsis* genome encodes four SPS isoforms (AtSPS1-4; Solis-Guzmán et al., 2017). In this work, AtSPS1, AtSPS2 and AtSPS3 expression patterns in roots were analysed to determine their possible role in the growth promotion programme elicited by *Trichoderma* volatiles, since AtSPS4 is only expressed in siliques and flowers (Solis-Guzmán et al., 2017). The expression patterns of *AtSPS1F::uidA-GFP*, *AtSPS2F::uidA-GFP*, *AtSPS3F::uidA-GFP* gene constructs were compared in *Arabidopsis* seedlings grown under axenic conditions, treated with sucrose or exposed to *Trichoderma* volatiles. The exogenous sucrose supply and volatiles from *T. atroviride* increased the SPS-driven GUS activity when compared with axenically grown seedlings (Figure 6a). On the other hand, the analysis of GFP fluorescence in roots showed that *AtSPS1F* and *AtSPS2F* expressions in the columella of primary roots could be induced only by sucrose (Figure 6b–e). Interestingly, sucrose supply and the exposure of seedlings to *Trichoderma* volatiles provoked an increase in *AtSPS3F* expression in developing lateral root primordia (Figure 6f,g). These results highlight the possible role of the three SPS isoforms during the *Trichoderma* phytostimulation programme.

3.7 | *T. atroviride* volatiles affect the expression of genes encoding *SWEET* sugar transporters

SWEET genes encode sugar transporters involved in plant growth and development, abiotic stress tolerance, responses to pathogen

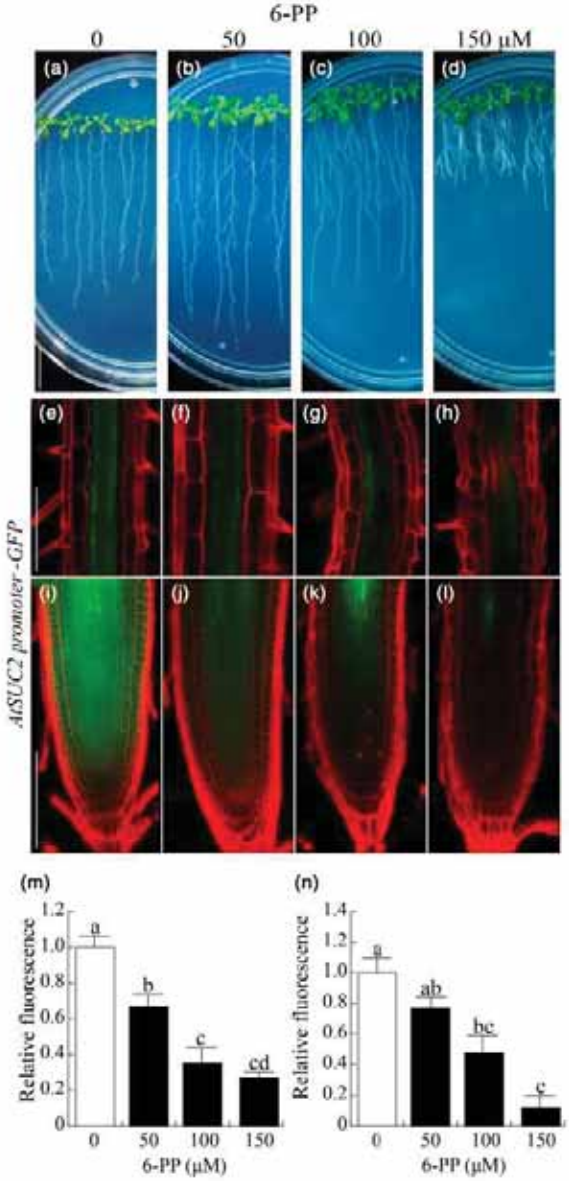

FIGURE 3 Effect of 6-PP on growth and *AtSUC2-GFP* expression in *Arabidopsis* seedlings. Transgenic seedlings expressing *AtSUC2-GFP* were grown in 0.2x MS medium supplemented with the solvent or 50, 100 and 150 μM 6-PP. 8 days after germination, seedlings were stained with PI and analysed by confocal microscopy. Representative photographs show the effects of 6-PP on root-system architecture and overall growth of seedlings (a–d). Micrographs of the vascular bundle at the differentiation zone of the primary root (e-h) and root apex (i–l) expressing *AtSUC2-GFP*. Bar graphs illustrate differences assessed as relative fluorescence intensity in vascular bundle (m) and root apex (n), respectively. Values shown represent the means for 6 seedlings ± *SD*. Different letters indicate significant statistical differences ($p < .05$). Scale bar: 1 cm and 100 μm, respectively. The experiment was repeated two times with comparable results [Colour figure can be viewed at wileyonlinelibrary.com]

infection or symbiosis events (Chandran, 2015). To examine whether the volatiles from *T. atroviride* may affect the sugar efflux and distribution mediated by plant *SWEET* transporters, we assessed the expression of *AtSWEET2*, *AtSWEET4*, *AtSWEET11*, *AtSWEET12*, *AtSWEET13*, *AtSWEET14*, *AtSWEET16* and *AtSWEET17* by RT-qPCR. Increased expression levels of *AtSWEET4* and *AtSWEET16* were observed in plants exposed to *Trichoderma* volatiles; however, substantial repression was found for *AtSWEET11*, *AtSWEET12* and *AtSWEET17*

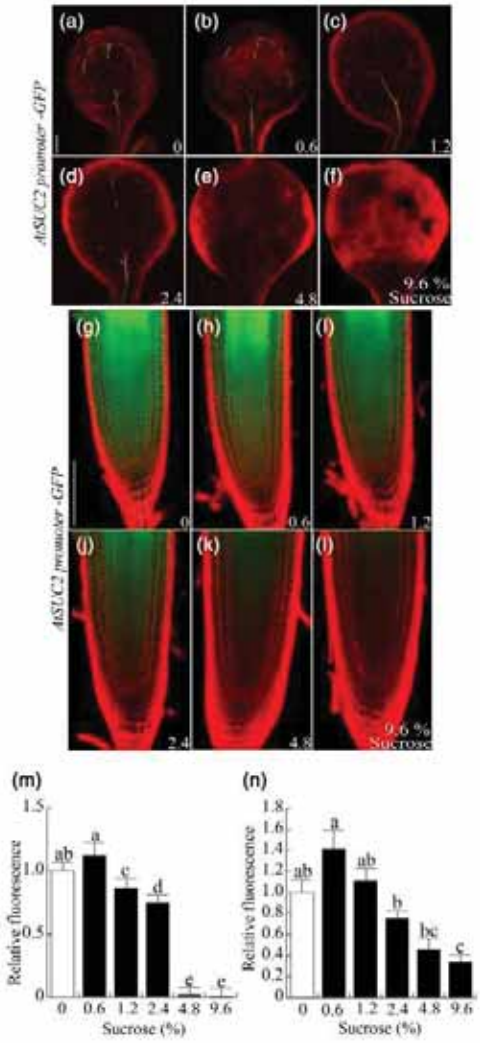

FIGURE 4 Effect of sucrose on *AtSUC2-GFP* expression in *Arabidopsis* leaves and roots. Four-day-old transgenic seedlings expressing AtSUC2-GFP were transferred to fresh plates with the shoot placed over an agar drop of 0.2x MS medium enriched with 0 to 9.6% sucrose and allowed to grow for 4 days. The expression of *AtSUC2-GFP* in leaves (a–f) and root tips (g–l) in the indicated sucrose concentrations is shown. Bar graphs illustrate differences in expression in leaves (m) and root tips (n) assessed as relative fluorescence intensity. Scale bars: 100 μm (leaves) and 200 μm (roots), respectively. Values shown represent the means for 8 seedlings ± *SD*. Different letters indicate means that are statistically different ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

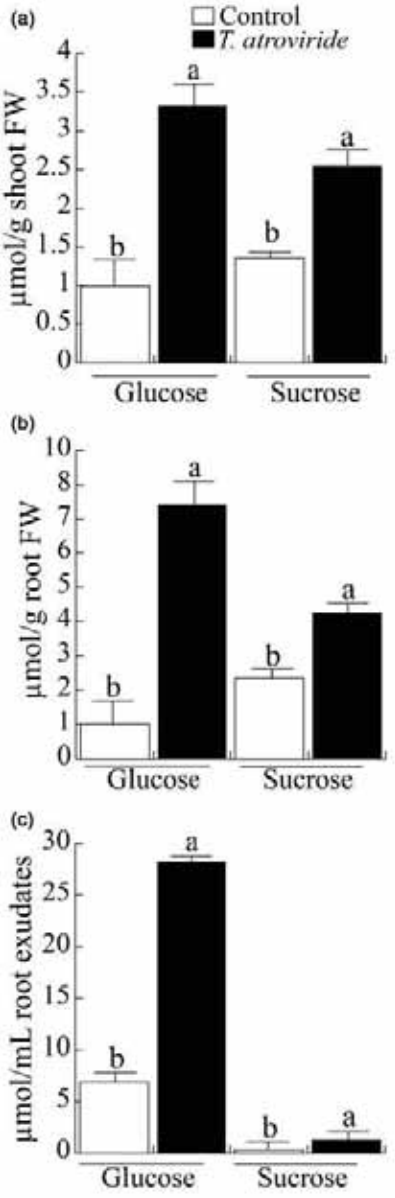

FIGURE 5 Effect of *T. atroviride* volatiles on the endogenous content of sucrose and glucose in *Arabidopsis* tissues and root exudates. (a–c) Glucose and sucrose amount in tissues and root exudates in axenically grown seedlings or exposed to *Trichoderma* volatiles. Values are the *M* ± *SE* of three replicates. FW, fresh weight. Different letters above the bars indicate a significant difference ($p < .05$)

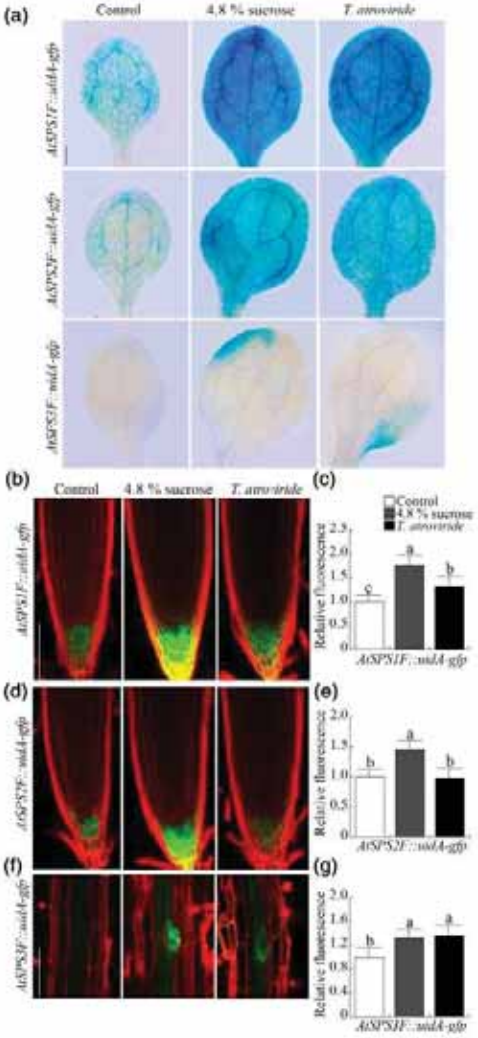

FIGURE 6 Effect of *Trichoderma* volatiles on expression of genes encoding SPS enzymes in *Arabidopsis* leaves and roots. Transgenic *Arabidopsis* seedlings harbouring the *AtSPS1F::uidA-GFP*, *AtSPS2F::uidA-GFP*, *AtSPS3F::uidA-GFP* gene constructs were transferred to agar-solidified patches of media enriched with 4.8% of sucrose or co-cultivated with *T. atroviride* for 4 days. Representative micrographs show the expression of each SPS isoforms in leaves (a), root tips (b–e) and vascular bundle (f, g) according to their respective expression domains. Scale bars in (a) 200 μm and (b, f) 100 μm. The graphs illustrate differences in expression, assessed as relative fluorescence intensity (c, e, g). Values shown represent the means for 8 seedlings ± SD. Different letters indicate means that are statistically different ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

(Figure 7a–h). To further explore how these genes respond to volatiles from fungi with contrasting lifestyles, we compared the mRNA levels of the same *SWEET* genes in plants exposed to volatiles released by two common phytopathogenic fungi, *Fusarium oxysporum* and *Alternaria alternata*. The volatile blends from both fungal pathogens had a stimulating effect on plant growth and biomass production (Figure S4). Besides, *A. alternata* increased the transcript level of *AtSWEET11*, while *AtSWEET13* expression was enhanced only by *F. oxysporum*; however, the volatiles of both pathogens induced the expressions of *AtSWEET16* and *AtSWEET17* at different intensity (Figure S4). Taken together, these results show the differential effects of beneficial and pathogenic fungi on the expression of *SWEET* genes in *Arabidopsis*.

4 | DISCUSSION

In the last decade, accumulating information has unveiled the critical role of the volatiles from *Trichoderma* spp. for plant growth and immunity (Hung & Lee, 2013; Jalali et al., 2017; Kottb et al., 2015; Lee et al., 2016; Nieto-Jacobo et al., 2017). These chemicals are diverse and their blends depend on the fungal species, substrate composition and environmental conditions (Cruz-Magalhaes et al., 2019; Estrada-Rivera et al., 2019; González-Pérez et al., 2018; Guo et al., 2019; Wonglom et al., 2020). Garnica-Vergara et al. (2016) reported that the production of 6-PP by *T. atroviride* directly influences plant biomass production and root architecture via modulation of ethylene signalling and auxin transport. Several studies have evidenced the commonalities between auxin biosynthesis and distribution in roots and the contents of soluble sugars including sucrose and glucose, which promote root branching (Kircher & Schopfer, 2012; Raya-González et al., 2017). *Trichoderma*-emitted volatiles may not only affect sucrose distribution and metabolism within the plant; they rather represent a plant-fungus recognition mechanism important for a long-lasting symbiosis.

Our results showed that *Arabidopsis* seedlings grown in a sucrose-free medium develop short primary roots and less lateral roots that *T. atroviride* co-cultivated plants under the same conditions. Besides, the fungus strongly enhanced *AtSUC2-GFP* expression, which improved root growth, the size of root meristems and cell elongation zones. Since the interaction was designed to occur using divided Petri plates, whose divisions restrict the passage of diffusible compounds, we conclude that the fungal volatiles being released influence carbon resources to be redistributed through AtSUC2.

Carbon dioxide (CO_2) is released upon respiratory metabolism of animals and microorganisms and is the main substrate for photosynthesis. Although the importance of CO_2 as part of the *T. atroviride* volatiles in triggering plant growth seems to be obvious, unveiling its specific role in root architectural and gene expression adjustments deserves attention. A recent study demonstrated that the changes in the transcriptome of *Arabidopsis* plants cultivated in vitro and exposed to high CO_2 did not modify SWEET, SPS and SUC2 gene expression (García-Gómez et al., 2019), which is in marked contrast to our data.

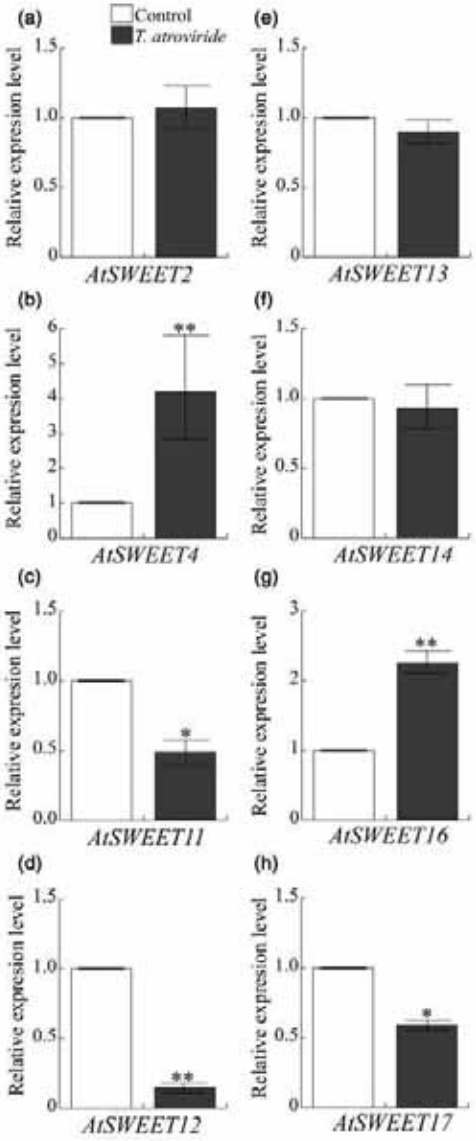

FIGURE 7 Effect of *Trichoderma* volatiles on expression of AtSWEET genes. Transcript levels of *AtSWEET2*, *AtSWEET4*, *AtSWEET11*, *AtSWEET12*, *AtSWEET13*, *AtSWEET14*, *AtSWEET16* and *AtSWEET17* in *Arabidopsis* seedlings after exposure to *Trichoderma* volatiles (a–h). The transcript levels were monitored by real-time reverse transcription-polymerase chain reaction (RT-qPCR). Actin2 was used as internal control. Data are the *M* ± *SD* from three biological replicates for each treatment. Asterisks indicate significant differences between control and inoculated treatment (Student's *t* test, $^*p < .05$ $^{**}p < .01$)

Indeed, application of CO_2 did not lead to significant promotion of root growth or lateral root formation, in contrast to what was observed in plants co-cultivated with *Trichoderma atroviride* or treated with its main volatile 6-PP (Garnica-Vergara et al., 2016). Thus, it is unlikely that either root architecture remodelling or the changes in gene expression reported in this work are due to respiratory CO_2 being accumulated into the divided plate, but instead to other volatiles such as 6-PP. Noteworthy, plants grown with sucrose supplements had reduced *AtSUC2-GFP* expression in roots particularly when co-cultivated with *T. atroviride* indicating that a single compound or a combination of volatiles represses sucrose transport through a positive regulation loop directly involving AtSUC2. The natural candidate is 6-PP since its application to the growth medium reduces *AtSUC2* expression in root tips in a dose-dependent manner. Thus, 6-PP production by the fungus may be highly relevant for photoassimilate translocation from shoot to root.

The transcriptional repression of AtSUC2 caused by *Trichoderma* VOCs appears to be tightly regulated by sucrose availability in the root. In other words, the volatiles stimulate the expression of AtSUC2 when there is low availability of sucrose in the root caused by the lack of this sugar in the culture medium. This can be attributed to the fact that the volatiles trigger the translocation of sugars towards the root for their subsequent release. In contrast, when there is sugar available into the medium or the shoots accumulate this disaccharide through photosynthesis, the expression of the gene reaches a sufficient level that efficiently supports plant growth; however, when plants are exposed to *Trichoderma* volatiles, the sucrose availability increases in roots where it represses the expression of its own transporter, possibly due to a negative signalling loop.

Sucrose loading into the phloem from source leaves is a key step of photoassimilate partitioning among sinks, and this process depends largely on the function of SUTs/SUCs (Durand et al., 2018). Consistently, overexpression of SUTs results in enhanced phloem loading and carbon partitioning into sink organs (Xu, Chen, Yunjuan, Chen, & Liesche, 2018; Yadav, Ayre, & Bush, 2015). SUT genes have sugar-responsive *cis*-elements in their promoters responsible of sucrose responsiveness, and even factors that trigger sucrose biosynthesis, such as carbon dioxide (CO_2) availability regulate their expression (Duan et al., 2014; Vaughn, Harrington, & Bush, 2002). Increased phloem loading appears to decrease SUC2 protein stability, which depends on phosphorylation and an ubiquitin-dependent turnover rate (Xu et al., 2020). Therefore, it seems that decreased AtSUC2 expression controlled at the transcriptional level by *Trichoderma* volatiles, and more specifically by 6-PP, may be attributed to sucrose accumulation in source tissues through an increased sucrose phloem loading. On the other hand, if transcript reduction is translated to a less AtSUC2 protein at the plasma membrane of root cells, this could lead to a reduced capacity of the root to control the sucrose concentration within the cell and then sucrose could be released to the apoplastic space by the efflux transporters such as the SWEETs; then, *T. atroviride* could use that carbon resource.

To understand how *T. atroviride* volatiles may affect the endogenous sugar content of plants, sucrose and glucose levels were quantified in shoots, roots and root exudates. The concentration of both

sugars increased in plants exposed to volatiles emitted by *T. atroviride*. This may account to the important functions that soluble sugars play when plants face challenges imposed by abiotic stresses such as salinity or drought, acting as osmolytes to avoid water loss (Contreras-Cornejo et al., 2021; Coppola et al., 2019; Elkelish, Alhaithloul, Qari, Soliman, & Hasanuzzaman, 2019; Pociecha & Dziurka, 2015; Yu, Wang, Zhang, Wang, & Liu, 2020; Zhang, Gan, & Xu, 2016). The accumulation of sugars induced by *T. harzianum* T22 in maize and tomato plants has been related to the up-regulation of enzymes involved in carbohydrate metabolism and photosynthesis (Coppola et al., 2019; De Palma et al., 2019; Shoresh & Harman, 2008a, 2008b). *Trichoderma* can also influence the quality and yield of fruits, according to Mei et al. (2019) inoculation of cucumber plants with *T. asperellum* S25, *T. harzianum* 610 and *T. pseudokoningii* 886 increased the average fruit weight and the content of soluble sugars. Macías-Rodríguez et al. (2018) indicated that carbohydrate root exudation from tomato seedlings varies in response to *T. atroviride* and these exudates may act in a positive chemotactic response of the fungal hyphae, which direct their growth towards roots and that subsequent colonization induces higher sucrose release of the host plant. From this information, we hypothesize that *Trichoderma* volatiles act as info-chemicals that elicit sucrose and glucose accumulation in plants and their release by roots before root colonization, leading the fungus to repress the expression of their potent degradative enzymes that are the hallmark of its saprotrophic phase. This mechanism could be employed by the fungus to establish proper mutualistic interactions and to have access to carbon supplements from their plant hosts.

T. virens utilizes sucrose for growth by expressing a sucrose transporter (TvSut) and an intracellular invertase (TvInv) to take up and hydrolyse the sucrose from the rhizosphere (Vargas et al., 2009, 2011). According to Vargas, Laughlin, and Kenerley (2013), *T. atroviride* has an intracellular invertase (Triat51014) and two putative sucrose transporters (Triat226844 and Triat83012) to be able to use plant-derived carbon. Recently, Villalobos-Escobedo et al. (2020) demonstrated that *T. atroviride* represses its genes encoding enzymes involved in complex carbohydrate degradation prior to root colonization of *Arabidopsis*, suggesting that its saprophytic behaviour changes to acquire and use the simple sugars available in root exudates.

Sucrose biosynthesis and accumulation in plants depends mainly on SPS activity (Anur et al., 2020). Histochemical GUS staining and GFP expression driven by *AtSPS1F*, *AtSPS2F* and *AtSPS3F* promoters showed that these genes were up-regulated by *T. atroviride* volatiles and sucrose, although differential and overlapping expression patterns in leaves and roots were observed. The induced SPS expression by *Trichoderma* volatiles mainly on leaves fits well with the sucrose and glucose content in plants, indicating that *Trichoderma* promotes sucrose biosynthesis in *Arabidopsis* through enhancing SPS activity. Indeed, these results are consistent with those of Pociecha and Dziurka (2015), who reported that *T. harzianum* induces a higher sugar reallocation towards the roots and triggers a greater activity of SPS in leaves of winter rye (*Secale cereale* L.).

Regarding other metabolic attributes of plants whose roots are colonized by *Trichoderma*, Elkelish et al. (2019) showed that *T. harzianum* improves the activity of SuS in tomato plants grown in waterlogged soils, which means that *Trichoderma* can help to better adapt their host to this abiotic stress via sugar metabolism. On the other hand, transgenic plants overexpressing SPS genes show increased growth and development, which correlate with higher sucrose synthesis and content of soluble sugars (Anur et al., 2020; Maloney, Park, Unda, & Mansfield, 2015). Thus, SPS activity plays an important role in carbon partitioning leading to sucrose accumulation and starch production. The SPS enzymes are activated by light and osmotic stress due to phosphorylation in serine residues, as well as allosteric activation by glucose-6-phosphate; moreover, they can be regulated at the transcriptional level at specific developmental stages (Volkert et al., 2014; Yonekura et al., 2013) and under conditions of osmotic stress (Solís-Guzmán et al., 2017). Our results support the notion that the metabolites produced by a probiotic fungus can trigger sucrose synthesis/metabolism via regulation of all three SPS isoforms in *Arabidopsis*, both in leaves and in roots, and the changes in expression underlies its plant probiotic attributes.

A higher concentration of chlorophyll is associated with enhanced photosynthesis rate (Cardona, Shao, & Nixon, 2018). Different endophytic *Trichoderma* spp. up-regulate genes encoding photosynthesis-related proteins especially under stressful conditions (Harman, Doni, Khadka, & Uphoff, 2021; Vargas et al., 2009), which can take place even before colonization (Kotth et al., 2015; Lee et al., 2016; Lee, Behringer, Hung, & Bennett, 2019; Nieto-Jacobo et al., 2017). It may be possible that the fungal volatiles directly influence chlorophyll synthesis/degradation. These data concur with the up-regulation of genes related to chloroplast and thylakoid membranes by *T. asperellum* SL2 inoculation described by Doni et al. (2019).

Leaf-produced sugars are translocated to sink organs, mainly roots, which largely depend on these carbohydrates to support growth and branching, which are energetically demanding and improve symbiosis events. SWEET proteins play an important role not only in carbon partitioning in plant relations with AM fungi but also with pathogenic organisms (Chen et al., 2010; Manck-Götzenberger & Requena, 2016). Gene expression analysis via real-time qPCR indicated that *T. atroviride* volatiles induced the expression of *AtSWEET4* and *AtSWEET16*, which encode hexose and sucrose transporters. AtSWEET4 is a plasma membrane hexose transporter, mainly expressed at the stele of roots and veins of leaves. AtSWEET4 overexpressing plants have higher plant size and higher resistance to freezing temperatures and when challenged with *Pseudomonas syringae pv. phaseolicola* NPS3121, they were resistant to the infection for an extended period (Liu, Zhang, Yang, Tian, & Li, 2016). AtSWEET16 is mainly a vacuolar hexose and sucrose transporter, that is expressed at vascular tissues. Plants overexpressing AtSWEET16 also showed higher tolerance to freezing temperatures, and this trait is related to variation in sugar concentration within the plant (Klemens et al., 2013). The expression pattern of AtSWEET4 and AtSWEET16 and the known participation on different stresses suggest that the proteins might regulate the sugar allocation on the whole plant during *Trichoderma* interaction. Nevertheless, the expressions of *AtSWEET11*, *AtSWEET12* and *AtSWEET17* slightly decreased in plants exposed to *Trichoderma* volatiles. SWEET11 and SWEET12 transporters

are responsible for sucrose efflux from the mesophyll cells to the phloem apoplast (Eom et al., 2015), whereas the vacuolar transporter AtSWEET17 facilitates bi-directional fructose transport across tonoplast to control cytosolic fructose levels (Chardon et al., 2013; Guo et al., 2014; Klemens et al., 2013). According to previous reports, biotic or abiotic stresses such as pathogen invasion or drought increase the transcript levels of AtSWEET11 and AtSWEET12 genes to facilitate the local distribution of sugars from the leaves towards the roots (Durand et al., 2016; Walerowski et al., 2018). However, it has been reported that colonization of AM fungus *Rhizophagus irregularis* on potato roots triggers downregulation of some SWEET genes belong to the clade III. For instance, the expressions of *SWEET10d* and *SWEET12c* were repressed during the entire inoculation period, while *SWEET1g*, *SWEET10a*, *SWEET10b*, *SWEET10c*, *SWEET11a*, *SWEET12f* and *SWEET17c* showed specific repression at 6 weeks post-inoculation (Manck-Götzenberger & Requena, 2016). Additionally, VOCs emitted by *F. oxysporum* and *A. alternata* altered the expression of *AtSWEET11*, *AtSWEET13*, *AtSWEET16* and *AtSWEET17* genes. Comparable results have been reported from other studies that used diverse fungal pathogens, which can promote the expression patterns of several *SWEETs* genes belonging to clades I and III upon infection (Chen et al., 2010, 2015; Li et al., 2017, 2018). In our research, the volatiles emitted by pathogenic fungi also enhanced plant growth and accelerated development. *A. alternata* and *F. oxysporum* behave similarly to *Trichoderma* since their volatile blends stimulate plant growth. This is consistent with several reports showing that fungal pathogens promote plant growth through a cross-talk between auxin, cytokinin and sugar signalling (Bitas et al., 2015; Moisan et al., 2019; Sánchez-López et al., 2016). However, our results show that volatiles from *T. atroviride* or pathogenic fungi provoke comparable biological responses in plants through different molecular mechanisms involving SWEET genes.

In conclusion, our results provide novel insight into how plant–*Trichoderma* interactions are established since volatile compounds released by *T. atroviride* can adjust physiological and metabolic responses in plants to warrant access to carbon resources before colonization (Figure 8). We have further identified SUC2 as a key element in the sucrose transport mediated by *Trichoderma*, as well as some SWEET transporters in the sugar allocation pathway within the plant. Besides, the expression of different isoforms of SPS (SPS1, SPS2 and SPS3), which are enzymes involved in sucrose biosynthesis were found to correlate with biostimulation of *Trichoderma*. This comprehensive knowledge could be exploited to enhance crop productivity, and to better equipe crops to adapt to environmental stress.

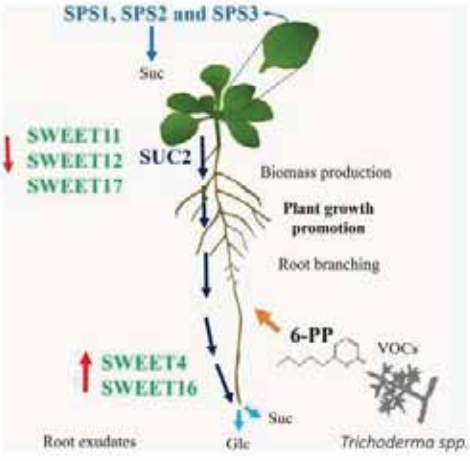

FIGURE 8 *Arabidopsis* response to *T. atroviride* and the regulation in sucrose metabolism and transport. *T. atroviride* induces lateral root proliferation and enhances biomass production via volatiles emission. Sucrose biosynthesis is induced in plants upon *Trichoderma* co-cultivation. The volatiles may affect sucrose transport via sucrose transporter SUC2 and SWEET sugar efflux carriers to trigger root growth and sugar exudation, which ultimately attract *Trichoderma* to the rhizosphere [Colour figure can be viewed at wileyonlinelibrary.com]

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Sarai Esparza-Reynoso and José López-Bucio designed and performed experiments and interpreted data; Miguel Martínez-Trujillo, Ramón Pelagio-Flores, Lourdes Iveth Macías-Rodríguez, Alfredo Herrera-Estrella, León Francisco Ruíz-Herrera, Montserrat López-Corla and Sobeida Sánchez-Nieto provided technical support and analysed data; Sarai Esparza-Reynoso and José López-Bucio wrote the manuscript. All authors revised and approved the submission.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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ORIGINAL PAPER

Nitrogen availability determines plant growth promotion and the induction of root branching by the probiotic fungus *Trichoderma atroviride* in *Arabidopsis* seedlings

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Abstract
Plant growth-promoting fungi are integral components of the root microbiome that help the host resist biotic and abiotic stress while improving nutrient acquisition. *Trichoderma atroviride* is a common inhabitant of the rhizosphere, which establishes a perdurable symbiosis with plants through the emission of volatiles, diffusible compounds, and robust colonization. Currently, little is known on how the environment influences the *Trichoderma*–plant interaction. In this report, we assessed plant growth and root architectural reconfiguration of *Arabidopsis* seedlings grown in physical contact with *T. atroviride* under contrasting nitrate and ammonium availability. The shoot and root biomass accumulation and lateral root formation triggered by the fungus required high nitrogen supplements and involved nitrate reduction via AtNIA1 and NIA2. Ammonium supplementation did not restore biomass production boosted by *T. atroviride* in *nia1nia2* double mutant, but instead fungal inoculation increased nitric oxide accumulation in *Arabidopsis* primary root tips depending upon nitrate supplements. N deprived seedlings were largely resistant to the effects of nitric oxide donor SNP triggering lateral root formation. *T. atroviride* enhanced expression of *CHL1:GUS* in root tips, particularly under high N supplements and required an intact CHL1 nitrate transporter to promote lateral root formation in *Arabidopsis* seedlings. These data imply that the developmental programs strengthened by *Trichoderma* and the underlying growth promotion in plants are dependent upon adequate nitrate nutrition and may involve nitric oxide as a second messenger.

Keywords *Trichoderma* · Root architecture · Lateral roots · Nitric oxide · Nitrate reduction · Nitrate transporters

Introduction

In natural and agricultural ecosystems, plants are assisted by microorganisms to survive and thrive. Fungi are the principal decomposers of decaying leaves, stems, and roots through the release of pectinases and cellulases and thus play critical roles in carbon recycling (Frąc et al. 2018; Naranjo and Gabaldón 2019).

Fungi are critical components of the plant microbiome, for instance, about 85% of plant species are colonized by vesicular arbuscular mycorrhizal fungi, which spread into roots and promotes nutrient uptake (mainly phosphate) through a better exploration of the substrate by the hyphae and improved transport from soil to roots (Kobae 2019). In the past two decades, it has been increasingly evident that more fungal species, along with mycorrhizae behave as probiotic microorganisms, including members of the *Trichoderma* genus. In contrast to mycorrhizal fungi, *Trichoderma* parasitizes other fungi, including phytopathogens, which justifies their strong potential for use in biocontrol (Benitez et al. 2004; López-Bucio et al. 2015; Sood et al. 2020).

Trichoderma may not only compete for space with potentially deleterious microbes at the rhizosphere, several species have been found as plant endophytes, which

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may explain their very interesting probiotic attributes that include an enhanced growth of vegetative and reproductive plant organs, promotion of root branching through the release of volatiles, such as 6-penthyl-pyran-2-one (6PP), indole-3-acetic acid (IAA, auxin), auxin precursors, secondary metabolites as well as improved adaptation to both biotic and abiotic challenges (Contreras-Cornejo et al. 2009; Garnica-Vergara et al. 2016; Tseng et al. 2020). Moreover, *Trichoderma* induces the accumulation of polyamines in *Arabidopsis*, which participate in development and response to stress (Salazar-Badillo et al. 2015).

Until recently, it was a mystery how *Trichoderma* could trigger root branching due to its release of highly active enzymes, particularly those with cellulolytic activities that may damage living roots (Horta et al. 2018). Nevertheless, it appears that the fungus can change its gene expression and metabolic requirements upon sensing fungal exudates, mainly sugars. When *T. atroviride* perceives sucrose, the genes encoding cellulases and pectinases are down-regulated and instead proteins involved in glycolysis are over-represented to enable use of simple carbohydrates as nutritional resources (Villalobos-Escobedo et al. 2020; Esparza-Reynoso et al. 2021).

Plants require macro- and micronutrients to complete their life cycles adequately. These minerals are taken directly from the soil in water solution (Wang et al. 2020). Nitrogen is by far the most limiting macronutrient that is required in high amounts to support biomass production. The main form by which roots acquire nitrogen is nitrate (NO_3^-), which is reduced to nitrite and then to ammonium (NH_4^+) for assimilation into amino acids (Wang et al. 2012; Chamizo-Ampudia et al. 2017; Hou et al. 2021). Both NO_3^- and NH_4^+ can be internalized into roots by specific membrane transporters for direct use in intensely demanding zones, such as meristems or long distance distribution to leaves, flowers and fruits (Noguero and Lacombe 2016; Dechorgnat et al. 2019).

Plant roots acquire NO_3^- through membrane transporters that belong into four different families, NPF, NRT2, CLC, and SLAC/SLAH (Li et al. 2007; O'Brien et al. 2016; Hachiya and Sakakibara 2017). The *Arabidopsis* CHL1 (AtNRT1.1/NPF6.3), is a dual-affinity nitrate transporter and sensor, or transceptor (Wang et al. 1998; Liu et al. 1999; Guo et al. 2001; Ho et al. 2009). NRT1.1 influences not only N uptake but also the expression of genes encoding proteins for nitrate assimilation, re-configurates root growth and branching, relieves seed dormancy and influences nitrate/ammonium balance, which rely on the structure of the protein and its phosphorylation status (Bouguyon et al. 2015).

Nitrate reduction is catalyzed by nitrate reductase and nitrite reductase, respectively. In *Arabidopsis*, two homodimers of the nitrate reductase (NIA1 and NIA2) have been reported, which play an important role in plant growth and development (Li et al. 2007; Park et al. 2011). These enzymes release large amounts of nitric oxide (NO), a diffusible, reactive gas influencing root architecture, and the expression of genes for the incorporation of ammonium into amino acids (Méndez-Bravo et al. 2010; Fernandez-Marcos et al. 2011; Dolch et al. 2017; Chamizo-Ampudia et al. 2017). Thus, nitrate assimilation through its impact on nutrition or following NO reactions may affect plant growth and productivity in several ways, but how plant associated fungi modulate this pathway remains to be characterized.

In this report, through in-depth analysis of the interaction between *T. atroviride* and *Arabidopsis* seedlings under contrasting N sources and availabilities, we show the strong dependence of nitrate availability and reduction through the activity of the NIA1 and NIA2 enzymes for plants to mount a successful symbiosis. The fungus triggered nitric oxide accumulation in roots and induced the expression of the CHL1 nitrate transceptor and N availability appears to change the root sensitivity to react to nitric oxide donor sodium nitropruside (SNP). Moreover, a comparison of the growth and developmental responses of the WT and two mutant lines defective at the CHL1 locus to fungal inoculation, suggests the requirement of the encoded protein to manifest a full phytostimulation. Collectively, our data provide the basis to manage both plant growth and development and the *Trichoderma*–plant symbiosis through N nutrition.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) and the mutants *chl1-5* and *chl1-12* (Tsay et al. 1993), and *nia1nia2* (Wilkinson and Crawford 1993), and the transgenic line expressing *CHL1-GUS*, were used to evaluate the effect of nitrogen source on plant growth-promotion by *T. atroviride*. Seeds were disinfected by immersion in 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min, and washed with sterile distilled water. The seeds were stratified for 3 d at 4 °C and then were germinated and grown on Petri plates containing a Murashige and Skoog (1962) modified medium supplemented with KNO_3 or NH_4NO_3 at low (10 µM), mild (100 µM), sufficient (1 mM) or high concentrations (10 mM) of both salts. The media contained 0.3 mM $CaCl_2$, 0.3 mM $MgSO_4 7H_2O$, 0.25 mM KH_2PO_4, 50 µM $FeSO_4 7H_2O$, 20 µM $Na_2EDTA 2H_2O$, 20 µM H_3BO_3, 18 µM $MnSO_4 H_2O$, 10 µM $ZnSO_4 7H_2O$, 0.9 µM KI, 0.2 µM $Na_2MoO_4 2H_2O$, 0.02 µM $CuSO_4 5H_2O$ and 0.02 µM $CoCl_2 6H_2O$. All media were supplemented with sucrose (0.6%; w/v) and solidified with 1% agar (w/v;

micropropagation grade; PhytoTechnology). The pH was adjusted to 7 before autoclaving at 121 °C for 20 min. Petri plates were placed vertically in a plant growth chamber (Percival Scientific AR-95L) with a photoperiod of 16 h of light, 8 h of darkness, the light intensity of 300 µmol m^2 s^{-1}, the temperature of 22 °C and 60–70% relative humidity. For nitric oxide donor sodium nitroprusside (SNP) experiment, *Arabidopsis* seeds were disinfected and germinated on modified MS medium as described above, and 4 day after germination, the seedlings were transferred to fresh media supplied with micromolar concentrations of SNP (0, 5, 10 and 15 µM). Plates containing 10 seedlings each were placed back into the growth chamber for 6 day to enable further growth and assessment.

Fungal growth and plant inoculation experiments

T. atroviride strain IMI 206040 was kindly provided by Dr. Alfredo Herrera-Estrella (Centro de Investigación y de Estudios Avanzados del IPN, México). The fungus was cultured in Petri plates with potato dextrose agar (PDA) (DIFCO Laboratories, USA) in darkness at 28 ± 1 °C for 5 day, after which conidia were harvested by gently scraping the mycelium surface and suspending the fungal mass in sterile distilled water. Briefly, the suspension was filtered to remove mycelium and agar debris. The conidial inoculum was adjusted to 1×10^6 spores and then was placed at 5 cm distance from the primary root tip of 4 day-old *Arabidopsis* seedlings grown on agar plates containing a modified MS medium. The plates were sealed with plastic wrap and were placed randomly into the growth chamber. After 4 day of co-cultivation, determination of plant growth and gene expression analyses were performed.

Confocal imaging and detection of nitric oxide

Nitric oxide levels in roots were imaged using 4,5-diaminofluorescein diacetate (DAF-2DA) fluorescent probe. *Arabidopsis* seedlings were incubated in 300 µL of a detection buffer containing 0.1 M Tris–HCl (pH 7.4) and 10 µM DAF-2DA (Sigma, USA) for 1 h in darkness and then washed three times with fresh buffer. The fluorescence emission of DAF-2 T (triazolofluorescein) was visualized using a confocal laser scanning microscope (model BX50; Olympus, Japan), upon excitation line from 488 to 568 nm with an argon blue laser and an emission window from 585 to 610 nm. The fluorescence intensity of the root tip for each treatment was measured from six micrographs using IMAGEJ software (http://rsbweb.nih.gov/ij/). The means of arbitrary unit values (A.U. = green pixels μm^2) obtained from each micrograph were graphed, showing the fluorescence changes of treatments in relation to the normalized control value to 1.

Histochemical analysis

Arabidopsis seedlings were immersed in GUS staining buffer (0.5 mg mL 5-bromo-4-chloro-3-indolyl-β-d-glucuronide in 100 mM sodium phosphate, pH 7), and incubated for 6 h at 37 °C in darkness. Stained seedlings were cleared and fixed according to Malamy and Benfey (1997) and then were mounted in 50% glycerol solution (v/v) onto glass slides to make semi-permanent preparations. *GUS* expression was monitored by Nomarski differential interference contrast (DIC) microscopy (Leica DMR microscope). For the transgenic line *CHL1-GUS*, at least eight plants by treatment were analyzed and the expression patterns were further confirmed in two subsequent repetitions of the experiment.

Analysis of growth and statistical analysis

The lengths of primary roots of seedlings grown over vertically-inclined Petri plates were measured employing a ruler and the lateral root number was determined by counting all lateral roots that emerged from the primary root tip to the root/stem transition using a stereomicroscope (Leica MZ6). The lateral root density was calculated by dividing the lateral root number by the primary root length for each seedling. The fresh weight of plants was determined with an analytical scale (Ohaus Corp.). Images of the plates were recorded using a digital camera (Nikon D5600, Japan). For all experiments, the overall data were statistically analyzed in the SPSS software version 10 (Statistical Package for the Social Sciences). Univariate and multivariate analyses with Tukey's post hoc test were used for testing differences in growth and root development responses in wild-type and mutant seedlings. Values followed by different letters are significantly different ($P \leq 0.05$).

Results

Nitrate reduction is critical for plant growth promotion by *T. atroviride*

The requirement of nitrate for plant growth promotion by *Trichoderma* has not been previously assessed. To investigate whether nitrate availability could affect the production of plant biomass and root growth and branching, and to examine the dependence of the nitrate reductases NIA1 and NIA2 in these nutritional responses, experiments were conducted to compare the response of *Arabidopsis* WT (Col-0) seedlings and *nia1nia2* double mutant. The seedlings were germinated and grown on modified 0.2×MS medium supplied with increasing concentrations of KNO_3, and four days after germination were inoculated with *T. atroviride* 5 cm from the root tip. In WT Columbia-0 (Col-0) seedlings,

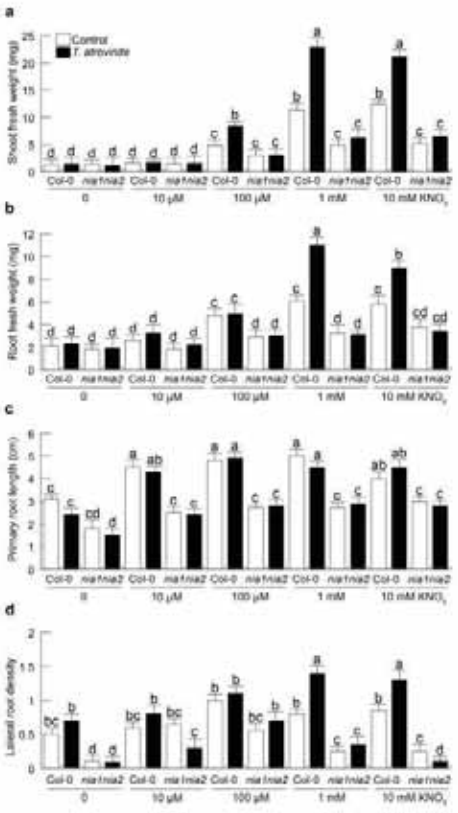

Fig. 1 Effect of nitrate availability and reduction via AtNIA1 and NIA2 in *Arabidopsis* growth promotion and root architecture by *Trichoderma atroviride*. Shoot fresh weight (**a**), root fresh weight (**b**), primary root length (**c**) and lateral root density (**d**) were quantified in *Arabidopsis* seedlings grown in media supplemented with 0, 10, 100, 1000, and 10,000 µM KNO_3 after four days of plant-fungal co-cultivation. Bars show the means ± SD. Different letters indicate significant statistical differences ($P < 0.05$; $n = 15$). Similar results were obtained in three independent repetitions of the experiment

nitrate supplements increased shoot and root biomass and promoted primary root growth and branching, being the maximum response observed at 1 mM potassium nitrate (Fig. 1a–d). In contrast, nitrate provision in the *nia1nia2* double mutants, neither improves shoot and root biomass production, nor enhances primary root growth or branching (Fig. 1a–d).

In WT seedlings inoculated with *T. atroviride*, a clear phytostimulation effect could be observed at 100 µM KNO_3, or greater nitrate concentrations, and shoot and root fresh weight, root growth and lateral root density were almost doubled by the fungus when compared to un-inoculated seedlings at 1 mM KNO_3 (Fig. 1a–d). Noteworthy, all four plant growth parameters already stimulated by *T. atroviride* in the WT, were not influenced in the *nia1nia2* double mutant irrespective of nitrate availability, which attained compromised growth compared to the Col-0 seedlings at all nitrate KNO_3 concentrations assayed (Fig. 1a–d). These data show the critical role of nitrate availability and its reduction by nitrate reductases for growth promotion by *T. atroviride* in *Arabidopsis*.

Ammonium supplements dynamically orchestrate plant responses to *T. atroviride*

Reduction of nitrate to ammonium is necessary for amino acid biosynthesis in plant cells. To know if ammonium availability is necessary to bypass the nitrogen (N) deficiency symptoms manifested by *nia1nia2 Arabidopsis* mutant when nitrate is applied as the only N source to achieve a strong growth response upon fungal interaction, the effect of NH_4NO_3 as a readily available NH_4 source in WT (Col-0) and *nia1nia2* mutant seedlings was assessed in axenic media or in media where a colony of *T. atroviride* was inoculated in the vicinity of growing plant roots.

As expected, following the results of the previous experiment, supply of NH_4NO_3 strongly promoted shoot and root biomass production and lateral root density in WT, axenically grown seedlings and in seedlings co-cultivated with *T. atroviride* with the greatest phytostimulation observed at 1 mM NH_4NO_3 in the presence of the fungal colony (Fig. 2a–d; Fig. S1). Also, in *nia1nia2* mutant, an increasing NH_4 availability promoted by three-fold shoot and root biomass, but interestingly, the effect of the fungus on both these traits remained negligible. Indeed, in the mutants, the lateral root density remained low even under the highest concentration applied (10 mM), indicating that ammonium supplements only partially restore the growth and development, but not the strong fungal phytostimulation, and cannot fully complement the lack of NIA1 and NIA2 enzymes.

Trichoderma atroviride triggers nitric oxide accumulation in *Arabidopsis* primary root tips depending upon nitrate supplements

Nitric oxide is an important signaling molecule that accumulates in plant cells following the reactions catalyzed by the nitrate reductases. The detection of endogenous NO was performed using 4,5-diaminofluorescein diacetate in primary root tips of *Arabidopsis* WT seedlings inoculated or not with *T. atroviride* on modified 0.2×MS medium supplied with 1 mM (Fig. 3a–c) or 10 µM (Fig. 3d–f) KNO_3.

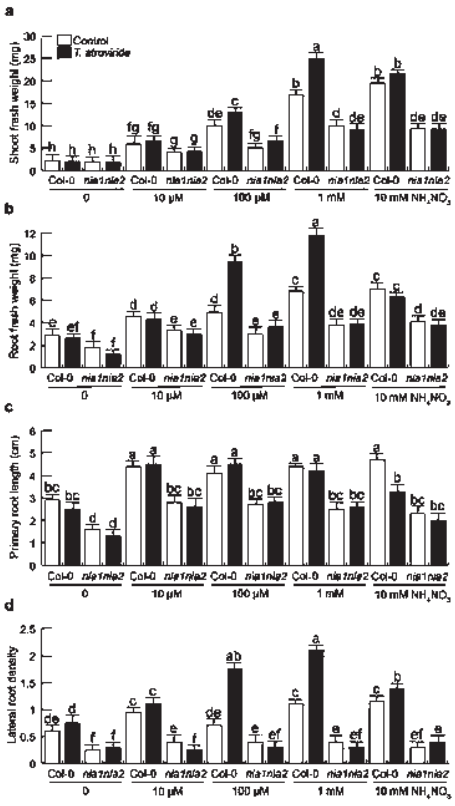

Fig. 2 Effect of ammonium availability on biomass production and root architecture triggered by *Trichoderma atroviride* in WT and *nia1nia2* double mutant. Four-day-old *Arabidopsis* WT (Col-0) and *nia1nia2* seedlings grown on modified MS medium supplied with 0, 10, 100, 1000, and 10,000 µM ammonium nitrate were inoculated with *T. atroviride*. After 4 days of inoculation the shoot fresh weight (**a**), root fresh weight (**b**), primary root length (**c**) and lateral root density (**d**) were quantified. Bars show the means ± SD. Different letters indicate statistically significant differences ($P < 0.05$; $n = 15$). The experiment was repeated three times with similar results

The differences in green fluorescence and quantification of relative fluorescence intensity observed in confocal microscopy images obtained from *Arabidopsis* primary roots tips (Fig. 3a–f), clearly indicate that *T. atroviride* strongly induces NO production, particularly at high (1 mM) nitrate availability. These data imply that nitrate reduction and NO accumulation are integral to root cells responses during acclimation to fungal symbiosis.

Nitric oxide donor SNP enhances root branching and root biomass production at high but not low nitrogen availability

To assess whether NO donor SNP could trigger root branching in *Arabidopsis* seedlings growing under contrasting nitrogen availability, WT (Col-0) seedlings were grown for 6 d on agar plates containing 0.2 × MS medium provided with 10 µM or 1 mM final concentrations of NH_4NO_3, which ensures readily available ammonium and nitrate sources and supplied with micro-molar concentrations (0, 5, 10 and 15 µM) of SNP. Representative photographs of plant development and quantification of the effects of treatments on primary root length, lateral root density, and root biomass made clear that SNP was actively inducing lateral root density and root biomass at 1 mM but not at 10 µM NH_4NO_3 (Fig. S2). Regarding the growth of primary roots, SNP had a growth repressing effect at a dose-dependent manner irrespective of the N treatment applied (Fig. S2). These results imply that the seedlings experiencing low N availability are less sensitive to the root branching induced by SNP.

Trichoderma atroviride induces the expression of the nitrate transceptor CHL1 in *Arabidopsis* primary roots having available N resources

The *Arabidopsis* CHL1 (AtNRT1.1/NPF6.3), is a dual-affinity nitrate transporter and sensor involved in plant growth and development. The critical requirement of nitrogen in *Arabidopsis* to mount a strong response to *T. atroviride* implies that the N uptake efficiency in roots may be modified during the symbiosis. Next, we explored whether the expression of the CHL1 gene could be modulated in *Arabidopsis* seedlings grown in media supplemented with contrasting availability of either KNO_3 or NH_4NO_3. Transgenic *Arabidopsis* seedlings expressing *CHL1-GUS* were germinated and grown on agar-solidified 0.2 × MS medium supplied with 10 µM or 1 mM concentrations of salts for 4 days after germination and subsequently, were inoculated with *Trichoderma*. At 4 days of co-cultivation, histochemical staining for GUS activity was recorded using DIC microscopy. Representative micrographs of primary root tips expressing CHL1 gene show that nitrogen supplements critically influence both root tip structure and the expression of CHL1 (Fig. 4a–h). In these experiments, supply of either 1 mM KNO_3 or NH_4NO_3 and *T. atroviride* causes a synergistic effect, making the root tips wider and increasing the expression domain of the GUS reporter driven by the CHL1 promoter, as revealed by blue staining at the root tip (Fig. 4a–h). These data suggest that the expression of CHL1 is enhanced by both nitrogen availability and fungal inoculation.

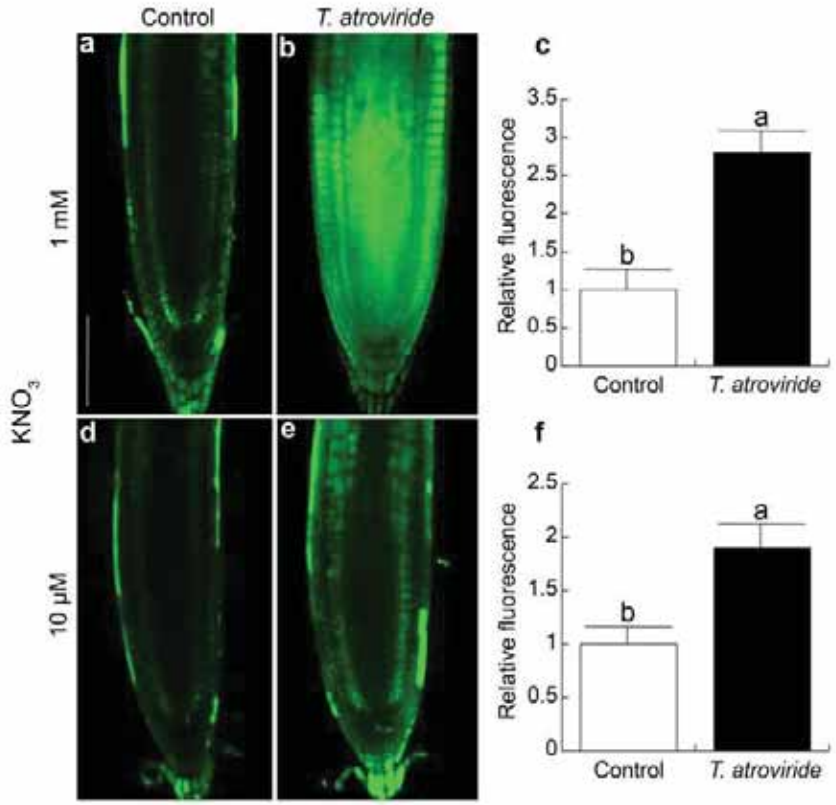

Fig. 3 Effect of *Trichoderma atroviride* on nitric oxide accumulation in *Arabidopsis* primary root tips depending upon nitrate supplements. Representative micrographs show endogenous NO detection with 4,5-diaminofluorescein diacetate in primary root tips of *Arabidopsis* WT seedlings grown and inoculated with *T. atroviride* on MS 0.2 × medium supplied with 1 mM (**a**, **b**) or 10 µM (**d**–**e**) of potassium nitrate (scale bar 100 µm). The graphs illustrate differences in relative fluorescence intensity obtained from primary roots tips from six independent seedlings (**c**, **f**). Different letters indicate means that are statistically different ($P < 0.05$). These analyses were repeated twice with similar results

Trichoderma-induced root branching and root biomass enhancement in response to nitrogen supplementation partially involves the nitrate transporter CHL1

To assess if CHL1 nitrate transporter could play a direct role in *Trichoderma*-mediated biomass production and/or root branching in *Arabidopsis*, the growth of WT seedlings was compared to that of two independent alleles of CHL1, namely *chl1-5* and *chl1-12*. The WT and corresponding mutant plants were grown on agar-solidified 0.2 × MS medium supplied with either 10 µM or 1 mM concentrations of NH_4NO_3. Seedlings were inoculated or not with *T. atroviride* and allowed to grow for 4 additional days to measure root biomass, primary root length and lateral root density. The fungus did not promote any of these traits in the WT or any of the mutants at 10 µM NH_4NO_3 (Fig. 5a–d). In contrast, supplying 1 mM NH_4NO_3 to the medium promoted shoot and root biomass accumulation in the WT, and the fungus had a synergistic effect (Fig. 5a, b). The growth of the primary root was not substantially modified by the N treatment or the fungal interaction in any of the plant genotypes tested (Fig. 5c). In contrast, lateral root density was greatly stimulated in the WT by *T. atroviride* specifically at

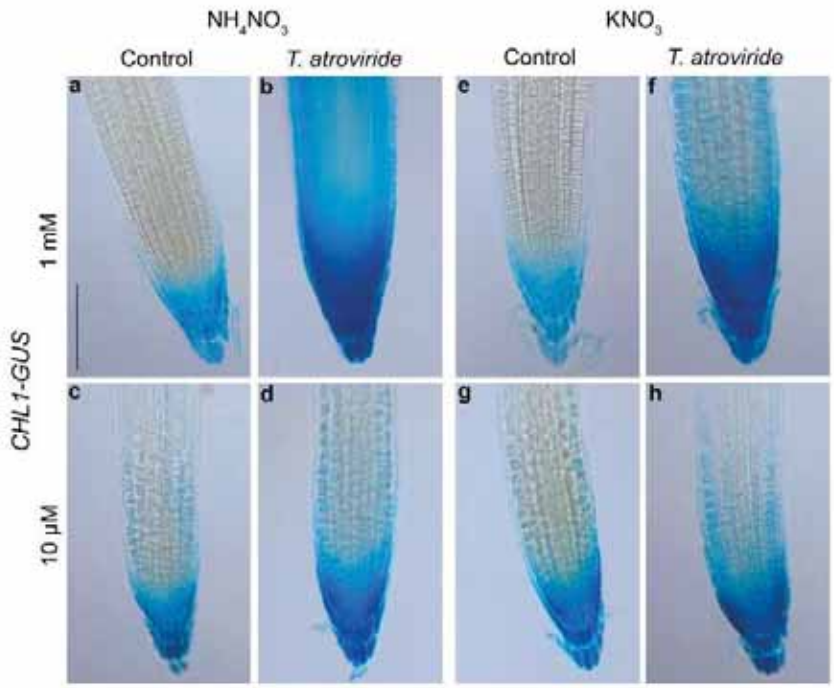

Fig. 4 Effects of contrasting nitrogen supplements and *Trichoderma atroviride* inoculation on *CHL1-GUS* expression in *Arabidopsis* primary root tips. Representative images of transgenic *Arabidopsis* seedlings expressing *CHL1-GUS* supplied with 10 µM or 1 mM KNO_3 or NH_4NO_3 and inoculated or not with *T. atroviride*. Representative photographs were taken from six independent seedlings analyzed after 4 days of fungal co-cultivation using DIC microscopy. Scale bar: 100 µm. Similar results were obtained in three independent repetitions of the experiment

1 mM NH_4NO_3 and to a significantly, lesser extend in the *chl1-5* and *chl1-12* mutants (Fig. 5d). These data indicate that CHL1 is partially involved in the N uptake network driven by *Trichoderma*.

Discussion

Plant evolution and the conquest of land were assisted by fungal symbiosis (Remy et al. 1994). The vesicular arbuscular fungi inhabit the roots of more than 85% of terrestrial species and integrally contribute to host nutrition, protection and resistance to abiotic challenges (Luginbuehl and Oldroyd 2017). Species of the *Trichoderma* genus have received increasing attention because of their wide distribution in ecosystems and the relations established with plants living as rhizospheric and endophytic species (López-Bucio et al. 2015; Sharma et al. 2017).

The *Trichoderma*–plant recognition is complex and involves detection of structural components of cells, the release of bioactive volatiles and plant hormones, such as indole-3-acetic acid (auxin) and its precursors, as well as peptides and secondary metabolites secreted by the fungi, the root in turn provides carbon-rich exudates that influence the fungal metabolism and change their attributes to use raw plant materials or sugars to obtain energy (Villalobos-Escobedo et al. 2020; Esparza-Reynoso et al. 2021). Until now, however, it still remains unclear how the nutritional status contributes to the production of plant biomass and the changes in root growth and branching driven by *Trichoderma*.

Among major macronutrients that plants require to support photosynthesis, metabolism and the making up of macromolecules, nitrogen (N) is at the center of agricultural applications owing to its essential role in amino acid and nucleic acid biosynthesis, which largely influences plant

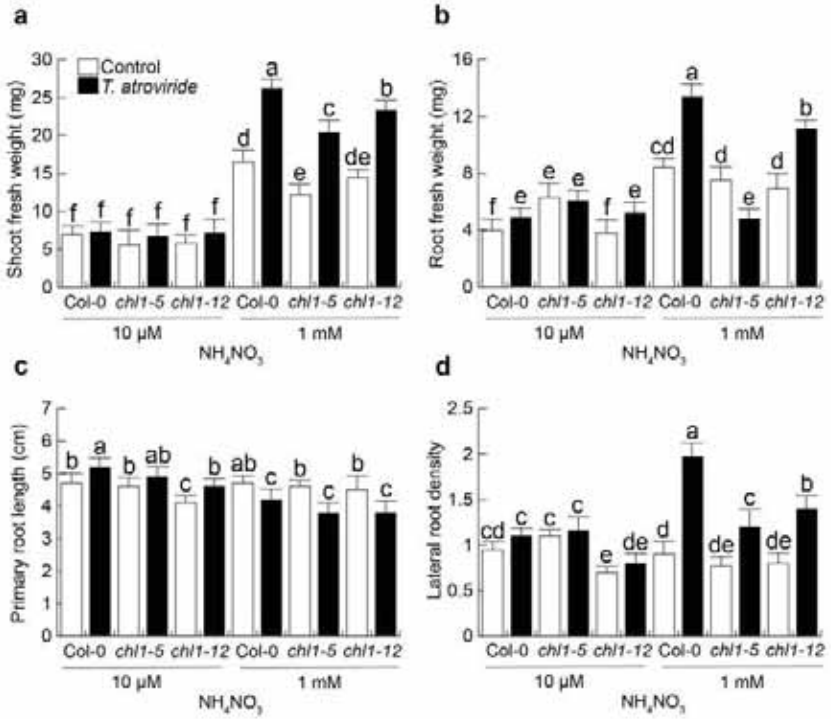

Fig. 5 Effect of *Trichoderma atroviride* in plant biomass and root architecture in response to nitrogen supplements in the WT and mutants defective in the CHL1 nitrate transporter. 4 day-old *A. thaliana* WT seedlings and *chl1-5* and *chl1-12* mutant plants were grown on agar-solidified MS medium supplied with 10 µM or 1 mM concentrations of ammonium nitrate. Subsequently, seedlings were inoculated with *T. atroviride* and allowed to grow for 4 additional days. Effect of co-cultivation on shoot fresh weight (**a**), root fresh weight (**b**), primary root length (**c**) and lateral root density (**d**). Bars show the means ± SD. Different letters indicate significant statistical differences ($P < 0.05$; $n = 15$). Similar results were obtained from three independent repetitions

nutrition and productivity (Gojon et al. 2017). The main ions by which N is available are nitrate and ammonium, commonly accessed by farmers as KNO_3 or NH_4NO_3 salts and sold as fertilizers. NO_3^- needs to be reduced to NH_4^+ for assimilation by cells, and in *Arabidopsis*, two isoforms of the nitrate reductases encoded by the NIA1 and NIA2 genes have been described to catalyze nitrate to nitrite reduction, and during this process, a large amount of nitric oxide is released (Dolch et al. 2017; Chamizo-Ampudia et al. 2017). To analyze the dependence of N nutrition on the successfulness of the *Arabidopsis–Trichoderma* interaction and its impact on plant growth and development, a modified Petri plate system was used in this research, where the level of KNO_3 or NH_4NO_3 along with other essential nutrients could be adjusted. Five treatments of each salt were applied to agar-solidified medium that served as a substrate for testing the *T. atroviride–Arabidopsis* interaction (0, 10 µM, 100 µM, 1 mM and 10 mM), and shoot and root biomass, primary root growth, and lateral root density were considered as important traits that could be influenced either by the imposed N level or by the influence of the fungus on the plants. Through this experimental design, we showed the requirement of high nitrate or ammonium supplements to drive strong shoot and root biomass accumulation and to promote lateral root formation by *T. atroviride*. Indeed, the fungal-mediated phytostimulation program in media with either KNO_3 or NH_4NO_3 as N sources was largely dependent on the genes encoding the two nitrate reductases in *Arabidopsis*, namely AtNIA1 and NIA2, since the *nia1nia2* double mutants grew poorly and developed a weak root system

in contrast to the robust, highly branched root system of the WT seedlings inoculated with the fungus manifested specifically at high N treatments.

Recently, the culture medium (MS or PDA) was found to impact the emission of VOCs by *Trichoderma*, and this in turn impacts in the development of *Arabidopsis*, in particular the nutrient-rich media (PDA) with *T. virens* had the greatest impact on the growth of *Arabidopsis* (González-Pérez et al. 2018). This behavior was also observed in our research, where the higher the concentration of nitrogen in the culture medium, a greater promotion of plant growth was observed when interacting with *Trichoderma atroviride*.

We hypothesized that the poor growth of the *nia1nia2* mutants in medium with nitrate supplements could be normalized by providing high (1 mM or 10 mM) NH_4NO_3 doses, since the provision of ammonium should complement the trouble of lacking the enzymatic capability to reduce nitrate in the mutants. This was not the case, and the *nia1nia2* still manifested reduced biomass production and poor root development even with high $NH4^+$ availability and the presence of the fungus. Our data are consistent with a previous study where *Arabidopsis* mutants unable to reduce nitrate due to the lack of nitrate reductase activity were unable to use NO_3^- or $NH4^+$ as the sole nitrogen sources and instead required large amounts (2.5 mM) of ammonium succinate to grow properly (Wang et al. 2004). From these observations, we speculated that NO could be an interesting candidate and one missing factor that is not substituted by ammonium supplementation only, and this critical reactive nitrogen species that acts in plant signaling could be required for the growth and developmental recovery of plants. Indeed, the NIA1 and NIA2 enzymes control endogenous NO homeostasis in both root and shoot systems of *Arabidopsis* and critically modulate root system architecture (Méndez-Bravo et al. 2010; Fernandez-Marcos et al. 2011; Dolch et al. 2017; Chamizo-Ampudia et al. 2017).

The detection of nitric oxide using 4,5-diaminofluorescein diacetate in primary root tips of *Arabidopsis* WT seedlings inoculated or not with *T. atroviride* showed that the fungus strongly induces NO production specifically at high (1 mM) nitrate availability, which implies that not only nitrate reduction, but also NO accumulation is integral to the root cell response during plant acclimation to a fungal symbiosis. An experiment to compare the sensitivity of *Arabidopsis* roots grown under contrasting NH_4NO_3 supply to NO donor sodium nitroprusside (SNP) further showed that release of nitric oxide strongly promotes lateral root formation in high but not low nitrogen availability. The nutritional and biotic factors that regulate the NO homeostasis are important for both fundamental and applied research, because of the proven functions of this second messenger as a key signaling molecule for root growth and lateral root formation (Méndez-Bravo et al. 2010; Fernandez-Marcos et al. 2011). We propose that during the interaction of roots with *Trichoderma*, both nitrate acquisition/reduction are stimulated and this in turn boost NO endogenous levels that may account for the formation of highly branched root systems, as reported in *Arabidopsis* and horticultural species (Correa-Aragunde et al. 2004; Méndez-Bravo et al. 2010; Sun et al. 2021).

The efficiency of roots to improve the N status of the plant is determined by several interdependent factors that include sensing of inorganic and organic N sources, the regulatory properties of the NO_3^- or $NH4^+$ transporters that contribute to the acquisition of the respective ions from the soil, the expression levels of these transporters in growth zones, and importantly, the overall extension of the root system and its branches. The influence of *Trichoderma* on the expression of nitrate transporters involved in uptake and sensing of this nutrient in *Arabidopsis* roots, to the best of our knowledge, had not been previously assessed. Our analysis of the transcriptional regulation of the CHL1 transceptor by means of evaluating the expression of the GUS reporter gene driven by the CHL1 promoter enabled characterization of the effects of KNO_3 or NH_4NO_3 on root tip structure and gene expression. In *Arabidopsis* root tips, 1 mM KNO_3 or NH_4NO_3 treatments and *T. atroviride* had a synergistic effect, making the root tips wider and increasing the expression domain of the CHL1 promoter. These data suggest that the expression of CHL1 is enhanced by both nitrogen availability and fungal inoculation.

The comparison of growth of WT seedlings with mutants defective on two independent CHL1 alleles, *chl1-5* and *chl1-12* unveiled slightly, albeit statistically significant differences in lateral root density, which was greatly stimulated in the WT by *T. atroviride* specifically at 1 mM NH_4NO_3 and to a lesser extend in the *chl1-5* and *chl1-12* mutants. Interestingly, the *chl1-5* and *chl1-12* mutants described in this report gave some phenotypic differences when compared to each other and to the WT. These differences can be explained because CHL1 is known to have many different roles in the plant that are dependent or independent of the activation of the nitrate reductases NIA1 and NIA2. It is rather possible that the *chl1* phenotype might not only be due to a lack of nitrate transport but also to a defect in nitrate reduction, which might explain why *chl1* mutant alleles and the double *nia1nia2* mutant might have similar phenotypes. Also, since CHL1 can also be an auxin transporter, one could argue that auxin-like molecules could impact plant growth and nitric oxide production, especially during interaction with *T. atroviride*. The differences between both alleles can be explained because the *chl1-5* phenotype is a result of the deletion of either the At1g12090 or the At1g12110 gene (Muños et al. 2004), whereas the *chl1-12* mutant is only defective at the At1g12110 locus. Although more site-directed mutations are needed to distinguish the transceptor and nitrate transport function of CHL1 that are relevant

to understand its contribution during a plant-fungal symbiosis, our data suggest that the underlying phytostimulation relies upon adequate nitrate nutrition and may involve nitric oxide as a second messenger.

CHL1 is partially involved in the N uptake network driven by *Trichoderma* potentially involved in lateral root formation. Currently, the fungal molecular patterns or signaling molecules involved in this regulation remain unknown and it deserves further attention with the aim to manage the symbiosis and formulate new products for applications in agriculture. In this regard, Fiorentino et al. (2018) demonstrated that formulations of *Trichoderma* are effective at improving N acquisition, yield, and nutritional quality of two leafy vegetables, Iceberg lettuce (*Lactuca sativa* L.) and rocket (*Eruca sativa* Mill.) in greenhouse experiments. Whether *Trichoderma* plant growth promotion relies on a suitable N nutrition in horticultural and crop species awaits further investigation.

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Author contributions SER, RPF and JLB. Conceived and designed the experiments; SER, RPF. Performed experiments; SER, RPF and JLB. Analyzed the data; JLB. Contributed reagents/materials/analysis tools; SER, JLB. Wrote the manuscript. All authors read and approved the final draft.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have no conflicts of interest to declare.

Ethical approval Not applicable.

Consent to participate All authors have reviewed and approved the submission.

Consent to publication Not applicable.

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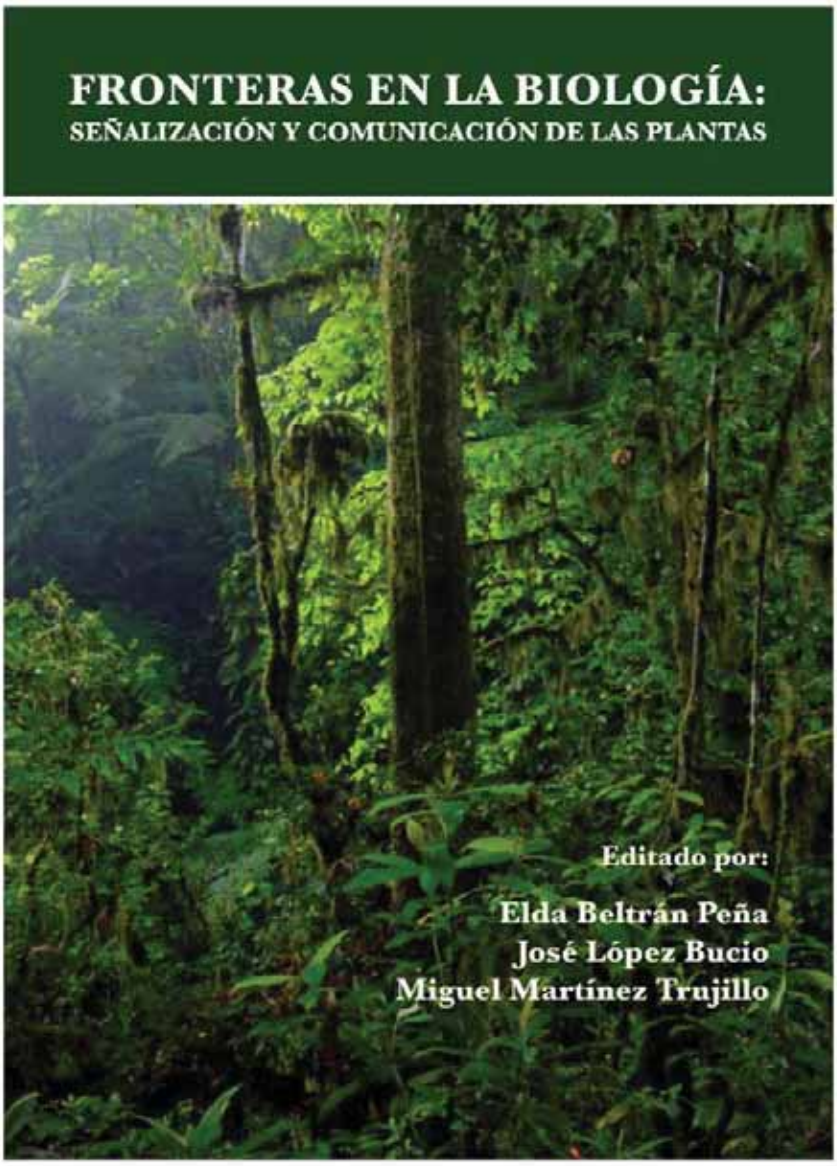
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X.

... Los tres primeros capítulos de este libro presentan una revisión actualizada de las vías de señalización de las principales fitohormonas, haciendo un énfasis especial en las auxinas, ya que éstas participan prácticamente en la regulación de todos los procesos del desarrollo vegetal, desde la embriogénesis hasta la senescencia. Es tal su importancia, que en el capítulo tres se analiza su antagonismo con las citocininas para regular el mantenimiento de los meristemos de la raíz y de los brotes apicales, y permitir el crecimiento y desarrollo continuo de las plantas.

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MORELIA, MICHOACÁN, MÉXICO. AÑO 2018

FRONTERAS EN LA BIOLOGÍA: SEÑALIZACIÓN Y COMUNICACIÓN DE LAS PLANTAS

INSTITUTO DE INVESTIGACIONES QUÍMICO BIOLÓGICAS

FACULTAD DE BIOLOGÍA

UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

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Fidel Anguiano Rodríguez

Cuidado del Contenido Editorial
Ma. Elena Mellado Rojas

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Prefacio

La respuesta de las plantas a los estímulos ambientales, debida en gran medida a la activación de las cascadas de señalización y al cruce de señales entre ellas para regular el adecuado desarrollo vegetal, es la temática del libro **"Fronteras en la biología: señalización y comunicación de las plantas",** correspondiente al tercer volumen de la serie **Fronteras en la biología del desarrollo de las plantas.** La biología vegetal ha tenido varias etapas importantes, iniciando con la nutrición y el transporte, la fotosíntesis y otros procesos del metabolismo, con avances generales de los efectos de algunos compuestos conocidos como hormonas vegetales. No obstante, hubo que esperar al desarrollo de la biología molecular para integrar estos conocimientos con la información genética y su expresión, entre ellos el mecanismo molecular de acción de las hormonas: la percepción de señales endógenas o exógenas, la transmisión de estas señales y su transformación en otras, formando rutas o vías de señalización o transducción que culminan en la modificación de un proceso, siendo común la modulación de la expresión de genes específicos. Estos mecanismos hormonales, entre otros, permiten que haya una comunicación molecular entre las diferentes estructuras de una planta y su ambiente abiótico y biótico, lo que la mantiene en constante alerta para responder y adaptarse a las condiciones existentes, ya que por su naturaleza sésil la planta "está obligada" a vivir en el sitio donde germinó y se estableció. Los tres primeros capítulos de este libro presentan una revisión actualizada de las vías de señalización de las principales fitohormonas, haciendo un énfasis especial en las auxinas, ya que éstas participan prácticamente en la regulación de todos los procesos del desarrollo vegetal, desde la embriogénesis hasta la senescencia. Es tal su importancia, que en el capítulo tres se analiza su antagonismo con las citocininas para regular el mantenimiento de los meristemos de la raíz y de los brotes apicales, y permitir el crecimiento y desarrollo continuo de las plantas. No podría dejar de analizarse la vía de señalización del regulador maestro TOR, originalmente encontrada en levaduras y animales donde integra las señales de energía, disponibilidad de nutrientes y factores de crecimiento que permiten el desarrollo; en las plantas cada vez se reporta más su participación en dicha integración. En los últimos capítulos se revisan los mecanismos moleculares que participan en el transporte de azúcares, en la deficiencia del hierro, y los involucrados en la tolerancia a los metales pesados. La edición de este libro, al igual que los dos anteriores de la serie **"Fronteras en la biología del desarrollo de las plantas"** es uno de los objetivos del curso de "Biología del desarrollo vegetal" que se imparte a los estudiantes de posgrado del Instituto de Investigaciones Químico Biológicas de la UMSNH. Finalmente, agradecemos a todos los autores su esfuerzo por presentar de manera actualizada la información existente en las diferentes áreas analizadas y que cumple con el propósito de divulgar los avances científicos e introducir a los estudiantes en este ámbito.

Elda Beltrán Peña
José López Bucio
Miguel Martínez Trujillo

Morelia, septiembre de 2018

Contenido

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Mecanismo molecular del transporte de azúcares en las plantas durante su interacción con hongos

Saraí Esparza Reynoso, José López Bucio y Elda Beltrán Peña

Las plantas son organismos autótrofos capaces de producir los hidratos de carbono que necesitan para su crecimiento y desarrollo. La sacarosa es el principal azúcar que se moviliza a través del floema desde los órganos fotosintéticos hacia los tejidos no fotosintéticos denominados demandantes. En las plantas, los transportadores de sacarosa y monosacáridos intervienen en el desplazamiento de azúcares a larga distancia por lo que son componentes clave en la partición de carbono. Sin embargo, cuando existen interacciones entre plantas y hongos, dependiendo del tipo de asociación con el organismo, las células colonizadas de las plantas se convierten en órganos de demanda y, el mecanismo de transporte de los azúcares será el que regule el metabolismo de carbono en las plantas. En esta revisión se describe el papel esencial de los transportadores de azúcares para la distribución de los carbohidratos dentro de las células vegetales durante su interacción con los hongos.

Introducción

El transporte de larga distancia o traslocación de fotoasimilados a partir de tejidos fuente a tejidos de demanda se produce a través de los haces vasculares del floema. La sacarosa sintetizada como producto principal de la fotosíntesis en las células del mesófilo de las hojas, se mueve a través de los tubos de los elementos cribosos para ser descargada al floema. De ahí, se distribuye a diferentes tejidos demandantes como los brotes apicales, las raíces y los tejidos de almacenamiento para suministrarles el carbono que requieren para su desarrollo (Chang *et al.*, 2004; Sauer, 2007; Ruan, 2014). Tanto el destino como el mecanismo de transporte de la sacarosa dependen de la especie, la etapa de desarrollo y el tipo de tejido de la planta (Rolland *et al.*, 2006). Este proceso inicia con la síntesis del gliceraldehído-3-fosfato (GAP) durante la fase oscura de la fotosíntesis o Ciclo de Calvin, que se lleva a cabo en el estroma de los tilacoides y donde se produce la transformación del CO_2 a carbohidratos utilizando la energía química y el poder reductor obtenidos en la fase luminosa. El GAP se exporta al citosol mediante un sistema traslocador de fosfato que realiza un antiporte con fosfato orgánico (Pi). Una vez en el citosol el GAP a través de la inter-conversión por la triosa fosfato isomerasa (TPI) da lugar a la síntesis de fructosa-1,6-bifosfato, la cual es desfosforilada por la fructosa bifosfatasa para la obtención de fructosa-6-P (Rolland *et al.*, 2006). Todo este proceso culmina con la unión de una molécula de fructosa-6-P con la UDP-glucosa por acción de la sacarosa fosfato sintasa (SPS) para dar origen a la sacarosa-P, para la obtención final de la sacarosa, la desfosforilación la lleva a cabo la sacarosa fosfato fosfatasa (SPP). Sin embargo, el GAP que no se exporta al citoplasma puede convertirse en ADP-glucosa para la síntesis de almidón dentro del cloroplasto durante el día, para su posterior degradación en la noche a glucosa o maltosa. Esta glucosa liberada en el citoplasma es fosforilada por la hexocinasa para generar glucosa-6-P, que puede ser convertida a fructosa-6-P por la glucosa-6-P isomerasa y entrar así a la vía de biosíntesis de sacarosa y posteriormente ser exportada a las pozas de demanda (**Fig. 1**). Existen dos formas de carga y descarga de sacarosa del floema: en la ruta simplástica, los plasmodesmos y el gradiente de concentración de los azúcares determinan la magnitud y la dirección del flujo de fotosintatos, mientras que la vía apoplástica se caracteriza porque la carga y descarga de los azúcares contenidos en el espacio intercelular del floema

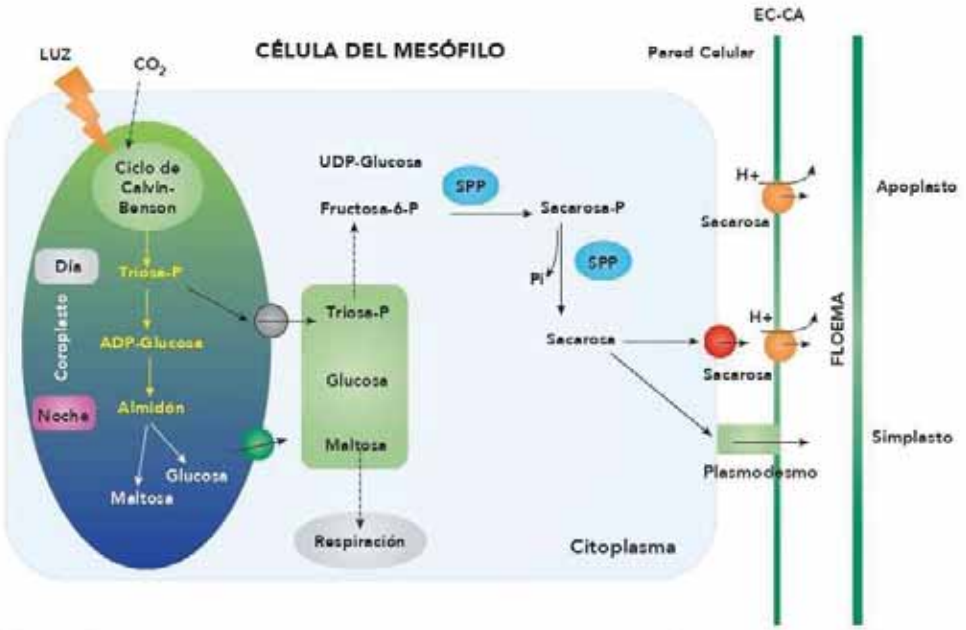

Figura 1. Biosíntesis de sacarosa. La triosa fosfato GAP se obtiene a partir de la fotosíntesis y es exportada al citoplasma para la formación de fructosa-6-fosfato. Esta última por medio de la sacarosa fosfato sintasa (SPS) se une a la UDP-glucosa para la síntesis de la sacarosa. Este metabolito puede distribuirse a través del floema para su transporte a diferentes órganos consumidores, o incluso para su exudación por la raíz (Modificado de Ruan, 2014).

se realiza a través de proteínas transmembranales denominadas transportadores de azúcares (Oparka y Cruz, 2000; Chacón y Martínez, 2007; Lemoine *et al.*, 2013; Ruan, 2014). Además, como la sacarosa puede ser hidrolizada por las invertasas de la pared celular, citosol y vacuola, existen transportadores de hexosas ubicados en la membrana plasmática o tonoplasto que permiten la entrada de glucosa y fructosa. Ambos monosacáridos influyen en procesos metabólicos y de almacenamiento como la biosíntesis de almidón, de proteínas y de celulosa, entre otros. Los niveles de abundancia y el flujo de los azúcares, pueden ser percibidos por medio de diversos sensores como la hexocinasa (HXK), la cual desencadena una vía de señalización que regula la expresión génica de acuerdo al estatus energético de la célula (**Fig. 2**). En consecuencia, la sacarosa, glucosa y fructosa, actúan como moléculas de señalización que regulan la expresión de genes y juegan un papel fundamental en el desarrollo de las plantas (Williams *et al.*, 2000; Ruan, 2014). Alternativamente, los azúcares se pueden movilizar a pozas no propias de la planta, por ejemplo la colonización de plantas por organismos heterotróficos mutualistas o patógenos representa un componente más en la demanda de los azúcares. Sin embargo, los mecanismos de transporte y los transportadores implicados en la partición de carbono entre la planta y el colonizador son escasamente conocidos. Lo único que se ha reportado es que en la interfase generada por los hongos, los transportadores de membrana controlan la captación, el intercambio y la competencia por los azúcares. Existen numerosas familias de transportadores de azúcares en las plantas, los cuales se han clasificado de acuerdo a su

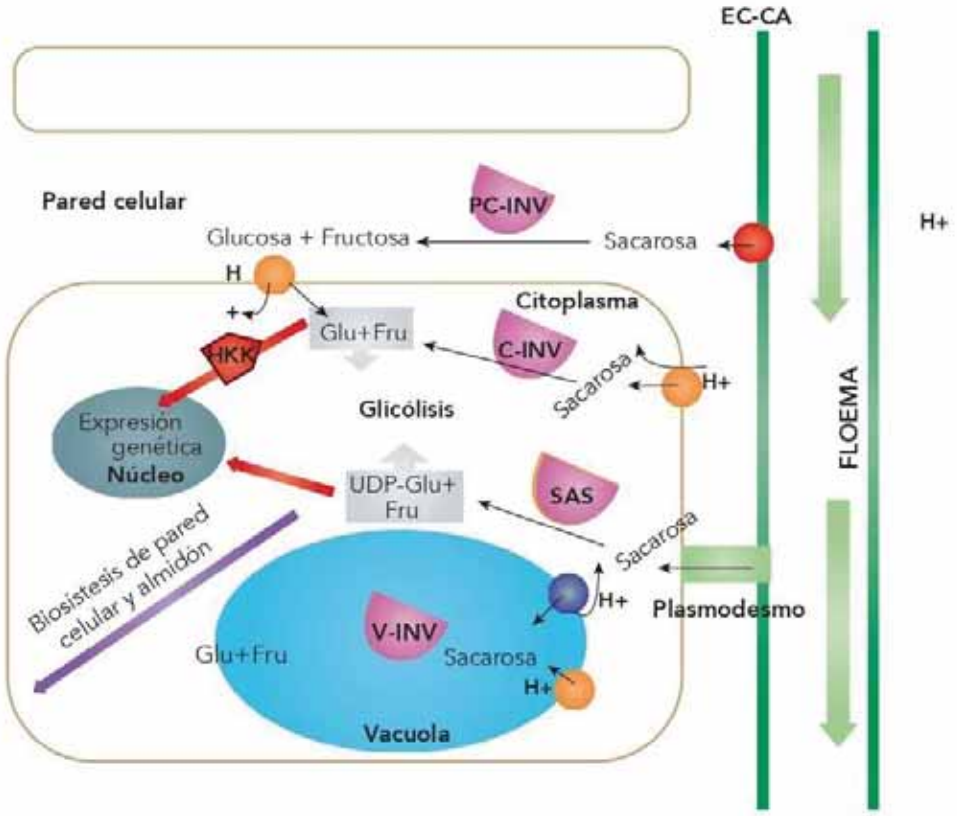

Figura 2. La descarga, transporte y metabolismo de sacarosa en las células. La sacarosa es descargada del floema vía apoplasto o simplasto. Dentro del citosol esta puede ser hidrolizada por la invertasa de pared celular (PC-WIN), por una invertasa citoplasmática (C-INV) en el apoplasto y dentro de la vacuola por la invertasa vacuolar (V-INV). Los productos generados se destinan como fuente de energía, bloques de construcción o moléculas de señalización. (Modificado de Ruan, 2014).

capacidad de flujo de salida y localización subcelular. A continuación se describen las características principales de los transportadores de azúcares.

1. Transportadores de sacarosa tipo SUT y de monosacáridos MST presentes en plantas

La secuenciación del genoma de la dicotiledónea *Arabidopsis thaliana* y la monocotiledónea *Oryza sativa*, permitió determinar que los transportadores de hexosas y de sacarosa pertenecen a grandes familias multigénicas. Los transportadores de sacarosa (SUT) y de monosacáridos (MST) son miembros de una superfamilia que comparten una estructura común que consiste de 12 dominios transmembranales conectados por bucles hidrófilicos, los cuales funcionan en el simporte de H^+/azúcar (**Fig. 3**A) (Lemoine *et al.*, 2013). Todos los SUT en la vía **simplástica** y en la carga y descarga del floema durante el transporte a larga distancia, tienen un importante papel en la movilización de la sacarosa célula a célula (Zhou *et al.*, 2007). Las proteínas SUT son codificadas por una pequeña familia de genes que agrupa cinco genes en arroz y nueve en *A. thaliana* (Aoki *et al.*, 2003; Lalonde *et al.*, 2004; Sauer, 2007; Doidy *et al.*, 2012; Reinders *et al.*, 2012). El análisis más reciente de los genomas de algunas monocotiledóneas como el sorgo, el maíz y el

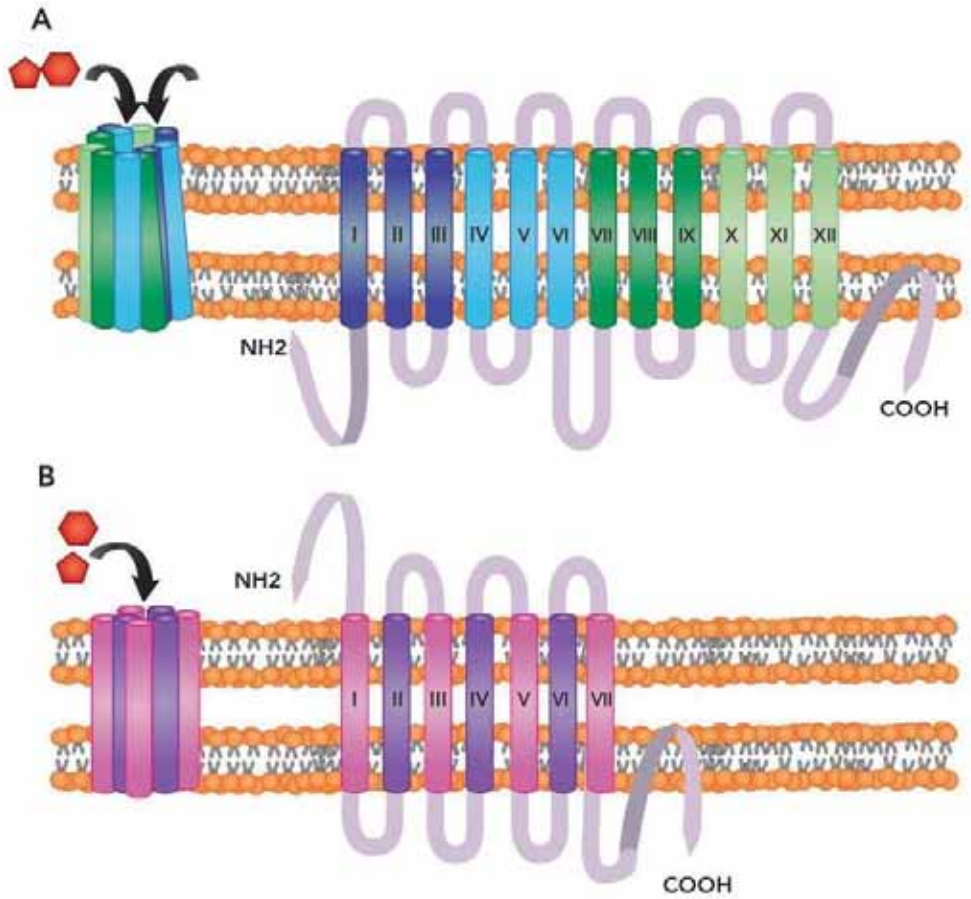

Figura 3. Modelo estructural de los transportadores de azúcares. (A) El transportador de tipo simporte de sacarosa/H^+ presenta sus grupos C- y N- terminal intracelularmente, posee doce hélices que atraviesan la membrana y un bucle hidrófilo entre las hélices 6 y 7. (B) Los transportadores SWEET de tipo uniporte con siete dominios transmembranales y difusionales bidireccionalmente (no sólo catalizan el flujo de salida de los azúcares, sino también su absorción). Los SWEET son transportadores de baja afinidad para la glucosa y la sacarosa.

Brachypodium spp (Braun y Slewinski, 2009), permitió la clasificación tanto en monocotiledóneas como en dicotiledóneas de los transportadores SUTs en cinco clados diferentes (Lemoine *et al.*, 2013). Debido a la capacidad de interacción proteína-proteína de los transportadores SUT1 de *Solanum tuberosum* (StSUT1), se han sugerido diversos mecanismos de regulación postraduccional para estos transportadores. Algunos estudios mostraron que el movimiento vesicular del StSUT1 que establece tanto su orientación subcelular como el reciclaje endosomal de la membrana plasmática se produce de manera dependiente de la actina (uno de los componentes del citoesqueleto). Krügel y Kühn (2013) mediante experimentos de co-inmunoprecitación reportaron que algunas proteínas interactuaban con StSUT1 para su correcta secreción a la membrana plasmática. Por ejemplo, la proteína heterotrimérica Sec61 participa en el transporte de proteínas y se localiza en el retículo endoplásmico (ER) y la Sec34 regula el tráfico vesicular del retículo endoplásmico (ER) al aparato de Golgi. También se ha descrito que cuando SUC6 de *A. thaliana* es fosforilada, interactúa directamente con una proteína 14-3-3χ, afectando de esta manera su localización subcelular. En plantas transgénicas de papa se ha observado una reducción de la expresión de los genes *SUT* cuando los niveles de almidón incrementan drásticamente y durante la interacción de StSUT1 con la adeniltransferasa glucosa-1-fosfatato (subunidad grande de la ADP-glucosa pirofosforilasa AGPasa) una enzima clave en la síntesis de almidón (Bürkle *et al.*, 1998; Hackel *et al.*, 2006; Krügel y Kühn, 2013). Li *et al.*, 2012, demostraron en *Malus domestica* que SUT1 (transportador perteneciente a la subfamilia SUT4) interactúa con las proteínas pequeñas Cyb5 ancladas a la membrana y que participan en reacciones oxidativas. Además, estos investigadores reportaron en *A. thaliana* la interacción de AtSUT4 con cinco diferentes miembros de la familia Cyb5, lo que sugiere que dichos complejos podrían estar involucrados en la sensibilidad y la señalización de la sacarosa. Por otra parte, como una gran cantidad de los transportadores de monosacáridos estudiados se expresan preferencialmente en las membranas plasmáticas de las células de los tejidos demandantes, se ha propuesto que ellos serían los responsables de incorporar a la glucosa y a la fructosa resultante de la hidrólisis de la sacarosa (Tanner y Caspari, 1996). En *A. thaliana* se han identificado 53 transportadores de hexosas (MST), 58 en *Medicago truncatula*, 59 en *Vitis vinifera* y 65 en *O. sativa* (Doidy *et al.*, 2012; Lemoine *et al.*, 2013). Los MST según su especificidad por el sustrato, se clasifican en siete subfamilias o clados entre los cuales se encuentra el transportador poliol/monosacárido (PMT) y el de inositol (INT). Los miembros de la subfamilia PMT, AtPMT1 y AtPMT2 que actúan durante la carga y descarga del floema en *A. thaliana*, presentan una mayor afinidad por la xilosa y la fructosa y se expresan durante el desarrollo de los tejidos de demanda (Doidy *et al.*, 2012). También los transportadores se han clasificado en base a su localización subcelular, como el de glucosa vacuolar (VGT), de la membrana de la vacuola -tonoplasto- (TMT) y el transportador plastídico de glucosa (pGlcT) (Doidy *et al.*, 2012). Los MST se localizan en la membrana plasmática y actúan en el simporte de H^+/hexosa, y muestran amplia especificidad de sustrato, como el AtSTP9 específico de glucosa y AtSTP14 de galactosa. Después de la captación en el citoplasma celular, los azúcares en exceso se almacenan en la vacuola, la cual funciona como un almacén a largo plazo. El almacenamiento vacuolar y su removilización posterior implica un transporte en membrana, clave para el mantenimiento del metabolismo celular, la osmoregulación y la adaptación a las condiciones ambientales (Neuhaus, 2007). Los transportadores VGTs y TMTs se localizan en el tonoplasto de las vacuolas, y se ha reportado que AtVGT1 y AtTMT funcionan como un simporte de H^+/glucosa en *A. thaliana* (Doidy *et al.*, 2012). Como la capacidad de volver a movilizar los carbohidratos es tan importante como su captación y almacenamiento, una serie de transportadores de eflujo han sido identificados en la membrana del cloroplasto, tales como los pGlcTs, transportadores de flujo de salida de la glucosa que proviene de la ruptura del almidón en el cloroplasto (Doidy *et al.*, 2012).

2. Función de los transportadores tipo SWEET

Las proteínas SWEET transportan bi-direccionalmente los azúcares a través de la membrana plasmática o del tonoplasto y participan en una amplia gama de procesos fisiológicos que implican el flujo de salida de sacarosa del floema (Chen *et al.*, 2010, 2012; Chardon *et al.*, 2013; Klemens *et al.*, 2013., Guo *et al.*, 2014; Lin *et al.*, 2014). Los SWEET a diferencia de los SUT, presentan siete dominios transmembranales (**Fig. 3B**) y son codificados por una familia multigénica de 17 miembros en *A. thaliana* y 21 en *O. sativa* (Chen, 2014). Los miembros de la familia SWEET de acuerdo con Eom *et al.*, 2015, se clasifican en cuatro clados filogenéticos de la siguiente manera: I, SWEET 1–3; II, SWEET 4–8; III, SWEET 9–15 y clado IV, SWEET 16–17. A su vez, todos ellos se dividen de acuerdo al transporte específico de sacarosa (Clado I y II) y sólo la fructosa pertenece al clado IV (Chen *et al.*, 2012; Lin *et al.*, 2014; Eom *et al.*, 2015). Durante la carga del floema, los SWEET permiten la salida de sacarosa de las

células del floema para su movilización al complejo de elementos cribosos y células acompañantes (EC-CA) y su posterior carga al floema a través de una proteína SUT. Se ha observado en las líneas reporteras *AtSWEET11* y *AtSWEET12* de *A. thaliana* que estos transportadores se localizan en las hojas y en el floema. Específicamente *AtSWEET11-GFP* en las células del floema, restringe el flujo de sacarosa en la interfase entre el parénquima del floema y el complejo de EC-CA (Chen *et al.*, 2012; Eom *et al.*, 2015). Al observar la expresión de AtSWEET1 en las hojas durante la etapa de senescencia, se ha sugerido que dicha proteína tendría un papel importante en la removilización de la galactosa, fructosa y glucosa. También se ha propuesto que los transportadores SWEET pueden contribuir en la transferencia de asimilados de la cubierta seminal durante el desarrollo del embrión como AtSWEET11, AtSWEET12 y AtSWEET15, involucrados en el eflujo de sacarosa de los tejidos maternos de la semilla durante el llenado de las semillas (Chen *et al.*, 2015ab). Otros estudios han sugerido que AtSWEET9 es esencial en la secreción de sacarosa para la producción de néctar (Lin *et al.*, 2014). Mientras que el transportador de glucosa AtSWEET8, localizado en el tapete de los granos de polen participa durante el crecimiento del grano (Guan *et al.*, 2008). Por otra parte, AtSWEET13 y AtSWEET14 son necesarios para el desarrollo de las anteras y las semillas, además de relacionarse con respuestas fisiológicas reguladas por ácido giberélico. La expresión de ambos también ocurre en la vasculatura de las plantas (Kanno *et. al.*, 2016). Por otra parte, AtSWEET17 y AtSWEET16 actúan como transportadores de fructosa en las membranas del tonoplasto, determinando su contenido en las vacuolas de células de hoja y raíz (Chardon *et al.*, 2013; Guo *et al.*, 2014; Hedrich *et al.*, 2015), mientras que AtSWEET4 juega un papel importante en el transporte de azúcares en tejidos axiales durante el crecimiento y desarrollo de la planta, y su expresión se reportó en la vasculatura de las raíces y nervaduras de hojas y flores (Liu *et al.*, 2016). Finalmente, AtSWEET2 actúa en el mecanismo de defensa contra patógenos, en la restricción de azúcares de las células epidérmicas de la raíz durante la infección por *Pythium irregulare* (Chen *et al.*, 2015b). Un estudio reciente, realizado con homólogos de genes *SWEET* en bacterias (SemiSWEET proteínas pequeñas que contienen tres dominios transmembranales y se ensamblan en un tetrámero), sugiere que dichos transportadores pueden formar homo-oligómeros para permitir el paso de sacarosa (Xuan *et al.*, 2013). Por otra parte, existen diversos reportes del secuestro de los transportadores SWEET por agentes patógenos, posiblemente para obtener azúcares para su crecimiento (Chen *et al.*, 2012; Chong *et al.*, 2014).

3. Mecanismos de los trasportadores de azúcares durante la interacción mutualista planta-microorganismos y planta-patógenos

Los microorganismos de acuerdo a su estilo de vida pueden ser separados en dos grupos: mutualista (micorrizas) y patógenos biotróficos o necrotróficos (Newton *et al.*, 2010). Aunque los microorganismos presentan diferentes maneras de colonización, ellos han desarrollado estrategias sofisticadas para evitar o suprimir las defensas de las plantas y desviar los azúcares de la planta hospedera para su crecimiento. Estos microorganismos al colonizar las plantas interfieren en el equilibrio de los órganos fuente-demanda, modificando el mecanismo de transporte de azúcares y la partición de carbono en la planta completa (Biemelt y Sonnewald, 2006). A continuación se describen los mecanismos más recientes sobre la interacción planta-hongo.

3.1. Mecanismo del transporte de azúcares hacia los microorganismos simbiontes

Algunos de los microorganismos simbiontes más representativos son las micorrizas, cuya interacción con la planta implica una cooperación estable debido a que además de estimular el metabolismo y la actividad fotosintética de la planta, los hongos micorrízicos proporcionan un mayor acceso a los nutrientes que no están directamente disponibles para las raíces de la planta (Bago *et al.*, 2000, Selosse *et al.*, 2006). Como recompensa, la planta redirige entre el 4 y 25% de sus fotoasimilados hacia las raíces colonizadas por su socio fúngico (Graham, 2000; Högberg y Högberg, 2002; Hobbie, 2006). Se demostró que altos niveles de glucosa y fructosa fueron absorbidos por el micelio extrarradical, mediante un análisis con marcado isotópico y espectroscopía de resonancia magnética nuclear en las raíces colonizadas por micorrizas arbusculares (MA), (Casieri *et al.*, 2013). Estas micorrizas a través de una diferenciación dicotómica ramificada de la hifa, denominada arbúsculos colonizan a las células corticales de las raíces de las plantas, creando una interfase entre estas células y las corticales. Dicha interfase se considera como el lugar donde se lleva a cabo la transferencia de los azúcares (Sun y Xu, 2009). A través de este proceso, se inducen cambios en la transcripción del hospedero que permiten reprogramar la partición de carbono en toda la planta. Por otro lado, las denominadas ectomicorrizas (ECM) no penetran en el interior de las células corticales, sino que envuelven a las raíces afectadas formando la llamada red de Harting, desde la cual crecen hifas hacia dentro y fuera. Por lo tanto, es comúnmente aceptado que el intercambio entre

simbiontes micorrízicos ocurre entre la interfase arbuscular y las hifas intercelulares (Helber *et al.*, 2011). Aunque se han identificado algunas proteínas de transporte durante este proceso, los mecanismos que subyacen al transporte y división de los azúcares son desconocidos (**Fig. 4**). Doidy *et al.*, 2012, reportaron que cuando se tiene mayor demanda de sacarosa en las raíces colonizadas, las proteínas de carga del floema como SUT1 podrían participar en la descarga de la sacarosa hacia las células corticales colonizadas por las micorrizas. También a través de importadores de SUT se recupera sacarosa hacia las células vegetales, compitiendo con la absorción de las hifas extrarradicales. Los niveles más altos de trascripción de los transportadores de sacarosa (SUT) y acumulación de sacarosa y monosacáridos en las pozas, se observaron en las raíces micorrizadas de *Solanum lycopersicum* y *Trifolium repens*, lo que indica un aumento del movimiento de sacarosa desde los órganos fuente (Wright *et al.*, 1998; Boldt *et al.*, 2011). Interesantemente, se observó la sobreexpresión de *StSUT1* en *S. tuberosum* durante la interacción con *Rhizophagus irregularis* (Gabriel-Neumann *et al.*, 2011). Otra evidencia sobre la regulación transcripcional de los genes implicados en el transporte de sacarosa fue reportada en la interacción del tomate con *Rectipilus fasciculatus* (Tejeda-Sartorius *et al.*, 2008). Recientemente, Manck-Götzenberger y Requena

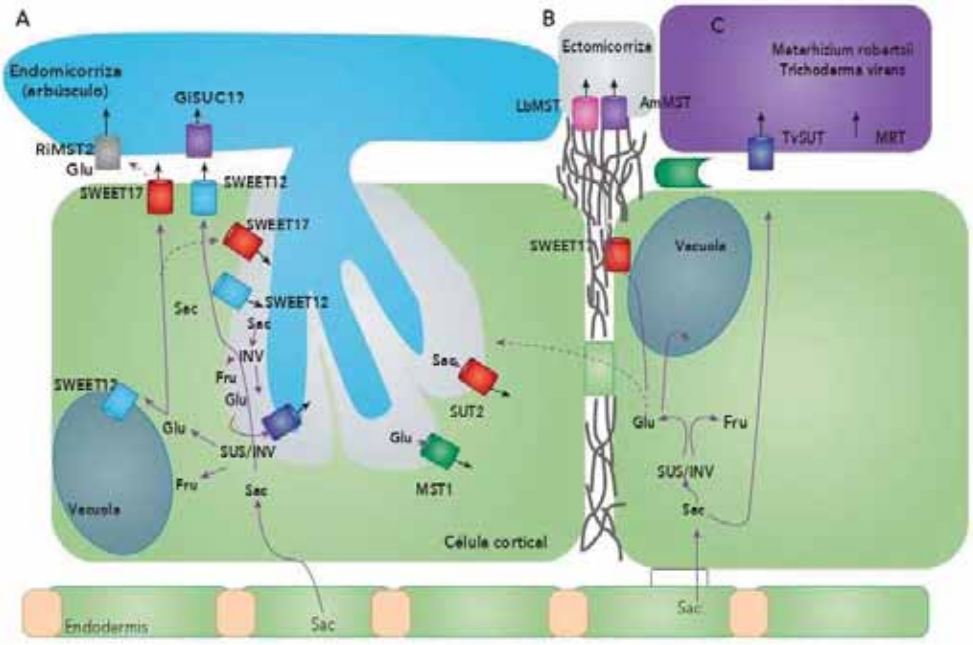

Figura 4. Modelo de la partición del azúcar durante la simbiosis de las micorrizas arbusculares, ectomicorrizas y simbiontes. (A) Los hongos AM penetran en la célula cortical de la planta hospedera a partir de la diferenciación de hifas ramificadas llamadas arbúsculos. La sacarosa que se suministra a las células corticales desde la endodermis puede ser catalizada por la sacarosa sintasa (SUS) o la invertasa citoplásmica (cINV) a glucosa (Glc) y fructosa (Fru). Para mantener el gradiente de concentración favorable, las hexosas pueden ser trasladadas a la vacuola por medio de los transportadores SWEETs localizados en el tonoplasto. Alternativamente, las hexosas en *S. tuberosum*, también son exportadas al apoplasto por StSWEET17. Mientras que la exportación directa de sacarosa al espacio periarbuscular, se lleva a cabo por StSWET12, donde también es hidrolizada por una invertasa de la pared celular. Una vez liberadas la glucosa y fructosa, estas son absorbidas por el hongo a través del transportador de monosacáridos RiMST2 o reabsorbidas por transportadores propios de la célula de la planta como MST1 (en *M. truncatula*) o SUT2 (en *S. lycopersicum*). Las células vecinas también contribuyen a la nutrición del arbúsculo al proporcionales azúcares simplásticamente. (B) Cuando las hifas de las ectomicorrizas colonizan la superficie de la raíz crean la red de Hartig, que forma una interfase de flujo de nutrientes entre ambos socios. Se han reportado dos tipos de transportadores MST para *Laccaria bicolor* y *Amanita muscaria*, los cuales transportan Glu. (C) Para el caso del hongo simbiótico, *T. virens* y el entomopatógeno *M. robertsii*, la expresión de los transportadores y la invertasa TvSUT,MRT, TvInv les permite incorporar e hidrolizar la sacarosa ofertada por la planta. (Modificado de Manck-Götzenberger y Requena, 2016).

(2016) mostraron durante la colonización de *S. tuberosum* con *R. irregularis* una modificación transcripcional de 22 de los 35 miembros de genes de la familia SWEET, además de la re-localización de su expresión en las células colonizadas, lo que sugiere una regulación de los SWEET en la interfase biotrófica con el hongo. Se han descrito diversos tipos de transportadores en los hongos, tal es el caso de MST2 expresado por la micorriza *Glomus spp*, que transporta monosacáridos con alta afinidad durante el establecimiento de la simbiosis con las plantas (Helber *et al*., 2011; Doidy *et al*., 2012). Del mismo modo, el hongo micorrízico *Geosiphon pyriformis* durante la interacción simbiótica con las raíces de las plantas, expresa un transportador de hexosa con mayor afinidad por la glucosa (seguido de manosa, galactosa y fructosa) implicado en la simbiosis mutualista del hongo y la raíz (Schüßler *et al*., 2006). Un estudio transcriptómico realizado en *Populus tremuloides* durante la interacción con la ectomicorriza *Laccaria bicolor* mostró una estimulación general del metabolismo de los carbohidratos, en particular un aumento en la expresión de los genes asociados con el metabolismo del almidón y sacarosa, así como de los transportadores de azúcares (Larsen *et al*., 2011). Otras investigaciones han demostrado que la utilización de la sacarosa por *Amanita muscaria* y *Hebeloma crustuliniforme*, depende en gran medida de las invertasas de la pared celular (CwINV) de las plantas (Salzer y Hager, 1991). De manera similar, Chen y Hampp (1993) mostraron en protoplastos de *A. muscaria* que la sacarosa y el manitol no fueron absorbidos, lo que confirma la participación de las CwINV. Los resultados anteriores, sugieren que los monosacáridos son la forma en que las ECM asimilan el carbono liberado por la planta, a pesar de no expresar invertasas propias (Schaarschmidt *et al*., 2006). También existen reportes sobre la colonización de las raíces por parte de hongos benéficos del género Trichoderma, capaces de degradar y exportar la sacarosa exudada por las plantas. Vargas *et al.* (2009) demostraron mediante la expresión de una invertasa intracelular (*TvINV*), que la sacarosa proveniente de la planta de maíz está intrínsecamente relacionada con la colonización de la raíz por *Trichoderma virens*, es decir, la actividad sucrolítica de las células fúngicas afecta la partición de carbono e incrementa la tasa de fotosíntesis en las hojas. Posteriormente, el mismo grupo de trabajo identificó en el genoma de *T. virens* la secuencia de un transportador de sacarosa similar al reportado en plantas: *Gpb2 8* (*TvSUT*). El transportador TvSUT actúa como un simporte de sacarosa altamente específico que se induce en las primeras etapas de la colonización de la raíz y sólo bajo el establecimiento con las plantas, debido a que su expresión tiene un efecto perjudicial sobre el crecimiento fúngico (Vargas *et al*., 2011). De igual forma, el hongo mutualista y patógeno de insectos *Metarhizium robertsii*, expresa un transportador de rafinosa y sacarosa (MRT), único para los hongos filamentosos ascomicetos y basidiomicetos, que es esencial para el crecimiento en oligosacáridos heterólogos, proporcionando así una explicación para el establecimiento del hongo y su competencia con otros microorganismos de la rizósfera (Fang y St. Leger, 2010).

3.2. Mecanismo del transporte de azúcares empleado por los patógenos

Los hongos patógenos en términos generales pueden dividirse en dos grupos: biotróficos y necrotróficos (Glazebrook, 2005; Jones y Dangle, 2006). Los primeros son parásitos que han desarrollado un mecanismo para crecer dentro de las células de la planta evitando la defensa de las mismas (Mendgen y Hahn, 2002), lo que significa que son capaces de difundirse rápidamente en todo el tejido vegetal y al mismo tiempo, desvían los nutrientes de la planta para alimentarse y crecer a expensas de ella. Por el contrario, los necrotróficos utilizan toxinas y enzimas para matar y degradar a las células vegetales (Oliver e Ipcho, 2004). Estos estilos de nutrición son altamente distintivos entre los hongos, y a pesar de que las plantas han desarrollado mecanismos de defensa para hacer frente a dichos patógenos, éstos pueden presentar ambos tipos de nutrición, es decir pasan de un crecimiento biotrófico hasta causar la muerte de las células vegetales (Oliver e Ipcho, 2004; Wilson y Talbot, 2009). Un hongo patógeno para crecer debe asegurarse una fuente de carbono orgánico a partir de la planta, sin embargo no se conoce la dinámica del crecimiento del hongo invasor en el tejido vegetal. Los hongos son organismos osmotróficos, lo que significa que proliferan en un sustrato mediante la secreción de enzimas extracelulares que degradan polímeros como la celulosa, lignina, proteínas, y lípidos para obtener azúcares simples, aminoácidos y ácidos grasos. Dichos monómeros son absorbidos por las hifas de los hongos a través de transportadores localizados en la membrana plasmática (Mendgen y Hahn, 2002; Tunlid y Talbot, 2002).

3.3. ¿Cómo los patógenos biotróficos adquieren nutrientes de las células hospederas de la planta?

Para entender el proceso de adquisición de nutrientes, es esencial conocer el mecanismo por el cual los hongos patógenos entran en el tejido vegetal. Un gran número de especies de hongos patógenos de plantas, desarrollaron células especializadas llamadas apresorios, que rompen la cutícula de las plantas y así

entran a las células epidérmicas. Como estas últimas células no pueden romperse, el hongo es invaginado y crece dentro en el espacio entre la membrana plasmática y la pared celular de la célula vegetal (apoplasto) a través de la formación de haustorios. Este proceso permite la generación de una interfase especializada donde se secuestran los nutrientes de las células del hospedero a través de los transportadores de hexosa (HXT1) expresados en los haustorios (Hall y Williams, 2000; Voegele *et al.*, 2001; Mendgen y Hahn, 2002; Panstruga, 2003; Wilson y Talbot, 2009; Voegele y Mendgen, 2011). Las investigaciones en *Ustilago maydis*, un patógeno biotrófico que causa la enfermedad denominada carbón en maíz proporcionó un avance significativo en la comprensión del mecanismo empleado para adquirir azúcares a partir de la planta (Wahl *et al.*, 2010). En este estudio, los autores identificaron un transportador de sacarosa codificado por el gen *SRT1* y localizado en la membrana plasmática, que participa en la virulencia del hongo. El transportador SRT1 posee un valor de K_m bajo, 26± 4.3 µM, lo que indica que el hongo es capaz de absorber más eficientemente la sacarosa extracelular en la interfase durante la competencia con otros transportadores de sacarosa de la familia SUC de la planta como ZmSUT1 (Wahl *et al.*, 2010; Doidy *et al.*, 2012; Morkumas y Ratajczak, 2014). Además, también se ha establecido que la sacarosa puede ser utilizada directamente por los patógenos sin necesidad de llevar a cabo una partición extracelular por las invertasas secretadas por el hongo. Se observó que el transportador SRT1 se expresa específicamente durante la invasión de los tejidos de las plantas por *U. maydis*, y que este efecto no se presenta, aún en presencia de sacarosa como fuente de carbono cuando el hongo se cultiva lejos de una planta. Lo anterior sugiere que la expresión de *SRT1* se induce por señales procedentes de la planta (Doidy *et al.*, 2012; Morkumas y Ratajczak, 2014). Como se mencionó anteriormente, en los tejidos infectados la demanda de carbono por parte de los hongos crea una competencia adicional con las pozas propias de la planta. Además, existe una controversia acerca de qué tipo de azúcar es el empleado por los microorganismos, debido a que a pesar de ser la sacarosa la forma en que se transportan los fotoasimilados en el floema, la glucosa parece ser el principal carbohidrato importado desde el hospedero hacia el parásito. Se ha reportado que la sacarosa localizada en el apoplasto es hidrolizada por las invertasas de la pared celular (cwINV) y, existen diversos estudios en diferentes especies de plantas, que muestran un aumento de la actividad de las invertasas en respuesta a los hongos (Roitsch *et al.*, 2003; Kocal *et al.*, 2008; Siemens *et al.*, 2011). Este incremento de la actividad de la cwINV en los tejidos infectados constituye una importante fuerza impulsora de la descarga de azúcar, debido a que la infección ocasiona un incremento general de los niveles de glucosa apoplástica (Hall y Williams, 2000). Aunque se han identificado varios genes de plantas que codifican para invertasas, su expresión correlaciona con el aumento de la actividad propia de la cwINV (Fotopoulos *et al.*, 2003; Hayes *et al.*, 2010). Se ha reportado que cuando el hongo *Uromyces fabae* interacciona con *Vicia faba*, se incrementa la actividad de la Uf-INV1 (Voegele *et al.*, 2006). Un caso similar fue reportado durante la infección de *Vitis vinifera* con *Botrytis cinerea* (Ruiz y Ruffner, 2002). Ambas interacciones tuvieron como consecuencia la acumulación de hexosas en el apoplasto, debido a la estimulación en la expresión de los transportadores de hexosas de los hongos y de las plantas (Wright *et al.*, 1995; Clark y Hall, 1998). Otros estudios en hongos patógenos han sugerido un papel esencial de los transportadores de hexosas durante la infección de la planta. El hongo que causa la roya del frijol, *U. fabae*, expresa en el haustorio de las hifas un transportador tipo simporte de H^+ con especificidad para la glucosa (Voegele *et al.*, 2001). Además se han caracterizado en el patógeno hemibiotrófico *Colletotrichum graminicola*, cinco transportadores de hexosas (CgHXT1-5), que poseen gran especificidad por el sustrato. Los genes *CgHXT* son expresados diferencialmente durante todas las fases de infección, desde su comportamiento biotrófico hasta necrotrófico (Lingner *et al.*, 2011). También se ha reportado en *B. cinerea* un transportador de fructosa de alta afinidad (BcFRT1). No obstante, el gran número de reportes sobre los transportadores de hexosas, el mecanismo de absorción de sacarosa de forma directa es probablemente una estrategia por parte de los hongos para prevenir las respuestas de defensa de las plantas que se desencadenan por la liberación de glucosa a partir de la hidrólisis de la sacarosa (Ehness *et al.*, 1997). Por esta razón, el hospedero presenta una estrategia de defensa indirecta que consiste en privar al patógeno de su fuente de carbono limitando la disponibilidad del azúcar en la interfase. Varias investigaciones han descrito después de la estimulación por patógenos biotróficos y necrotróficos, un aumento en la capacidad de recuperación de la glucosa por los tejidos del hospedero (Fotopoulos *et al.*, 2003; Azevedo *et al.*, 2006). Algunos transportadores MST de la planta están implicados en la reabsorción de azúcar después de la infección (Büttner, 2010; Slewinski, 2011). Ejemplo de ello es la regulación de la cwINV AtβFRUCT1 y del transportador de hexosa AtSTP4 en hojas de *A. thaliana* infectadas con *Erysiphe cichoracearum* que correlaciona con el aumento de la actividad de la invertasa y la reabsorción de glucosa (Truernit *et al.*, 1996; Fotopoulos *et al.*, 2003). Lo antes

descrito sugiere una coordinación funcional de los STP y cwINVs en el suministro de hexosas para las pozas. Por otro lado, la inducción del transportador STP4 por *Blumeria graminis*, también estimuló la del homólogo AtSTP4 en hojas de trigo infestadas (Sutton *et al.*, 2007). En hojas de maíz infestadas por el hongo *C. graminicola*, también se observó una mayor expresión para el transportador de sacarosa (Vargas *et al.*, 2012). Mientras que en hojas de vid infestadas por *Erysiphe necator* y *Plasmopara viticola* fueron inducidos numerosos transportadores de hexosas, en *V. vinifera* la expresión de *VvHT5* fue estimulada en respuesta a heridas. De acuerdo a los autores, lo anterior sugiere un papel de dichos transportadores en la respuesta de la planta al estrés (Hayes *et al.*, 2010). El transportador VvHT5 presentó mayor similitud con AtSTP13 y ambos mostraron alta afinidad para la glucosa (K_m=89 mM y K_m=74 mM, respectivamente) (Hayes *et al.*, 2007; Afoufa-Bastien *et al.*, 2010). En el caso de los genes *SWEET*, su expresión también se induce durante la

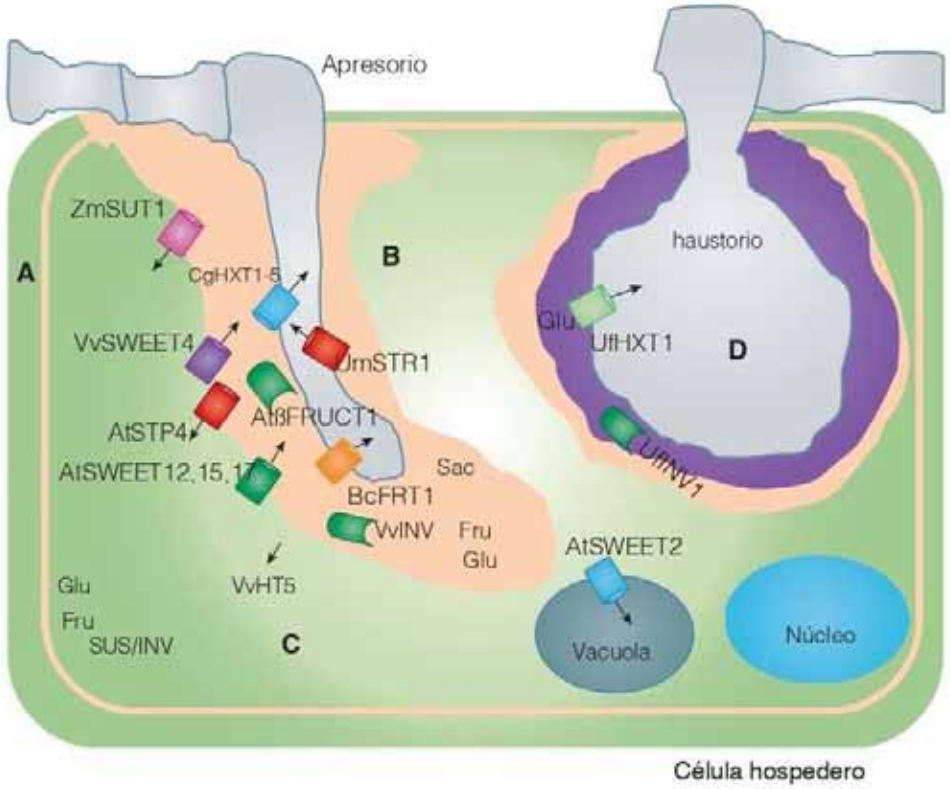

Figura 5. Modelo de la partición del azúcar durante la interacción con biotróficos patógenos. (A) *B. cinerea* expresa el transportador de fructosa BcFRT1, el cual contribuye a la germinación de las esporas durante la infección en *V. vinifera*. Se ha reportado en *A. thaliana*, una inducción significativa regulada por ROS del transportador de glucosa VvSWEET4 en la membrana plasmática, así como de los transportadores AtSWEET2, AtSWEET15 y AtSWEET17 lo que incrementa la susceptibilidad de las plantas al patógeno, al ser estos transportadores empleados por el hongo para la adquisición de azúcares. (B) En *U. maydis* se identificó al transportador de sacarosa SRT1, el cual compite con ZmSUT1 por la sacarosa en la interfase. Mientras que *C. graminicola* expresa diversos transportadores de hexosa, galactosa y xilosa (CgHXT1-5) determinantes durante su corta fase biotrófica a necrotrófica. (C) El transportador de hexosa en *V. vinifera* VvHT5 fue altamente inducido junto con la invertasa de la pared celular VvcwINV durante la infección de *E. necator* y *P. viticola*. Un efecto similar fue observado en la interacción de *A. thaliana* y *E. cichoracearum*, donde la inducción de la expresión del gen *AtSTP4* y el de la invertasa de pared celular *AtβFruct1*, aumentaron sustancialmente durante la infección por este patógeno. (D) El hongo biótrofo *U. fuliginea* expresa un transportador de hexosa en la membrana plasmática haustorial, sugiriendo que este transportador puede tener un papel fundamental en la transferencia de la glucosa disponible, ya que los niveles de este azúcar aumenta considerablemente por la actividad de la invertasa producida por el mismo hongo.

invasión de hongos patógenos (Chen *et al*., 2010). La infección en *A. thaliana* con *Golovinomyces cichoracearum*, induce la expresión de *AtSWEET12*, mientras que la de *B. cinerea* estimula la de *AtSWEET4*, *AtSWEET15* y *AtSWEET17*. Esta regulación diferencial sugiere que cada patógeno tiene su propio mecanismo específicamente adaptado para secuestrar los carbohidratos de su hospedero (Slewinski, 2011; Talbot, 2012). Otro mecanismo para la restricción de los azúcares durante la competencia con los patógenos lo describe Chen *et al*., 2015b, durante la infección de *Pythium irregulare* en *A. thaliana*, donde la expresión de *AtSWEET2* (transportador que contribuye a la secreción de glucosa) localizado específicamente en el tonoplasto de las vacuolas de las células epidérmicas del ápice de la raíz restringe la salida de carbono de las raíces, secuestrando así a la glucosa dentro de la vacuola. Todos los mecanismos antes mencionados se representan en la **figura 5**.

Conclusiones

Se ha generado un conocimiento considerable sobre los genes que codifican para los transportadores de azúcares involucrados en las interacciones planta-hongos, gracias a las recientes aportaciones sobre los genomas y análisis del transcriptoma de plantas y hongos. A pesar de dichos avances, escasamente conocemos los mecanismos del flujo e intercambio de nutrientes en las interfases generadas entre las células hospederas colonizadas por microorganismos mutualistas y patógenos. Como se mencionó anteriormente, los transportadores de azúcares son elementos clave para la regulación de este proceso, por lo que la comprensión de los cambios que conlleva la partición del carbono en la planta puede en un futuro proporcionar herramientas para incrementar la productividad de los cultivos, al controlar el reclutamiento de hongos benéficos y limitar la disponibilidad de azúcares para evitar infecciones de hongos patógenos.

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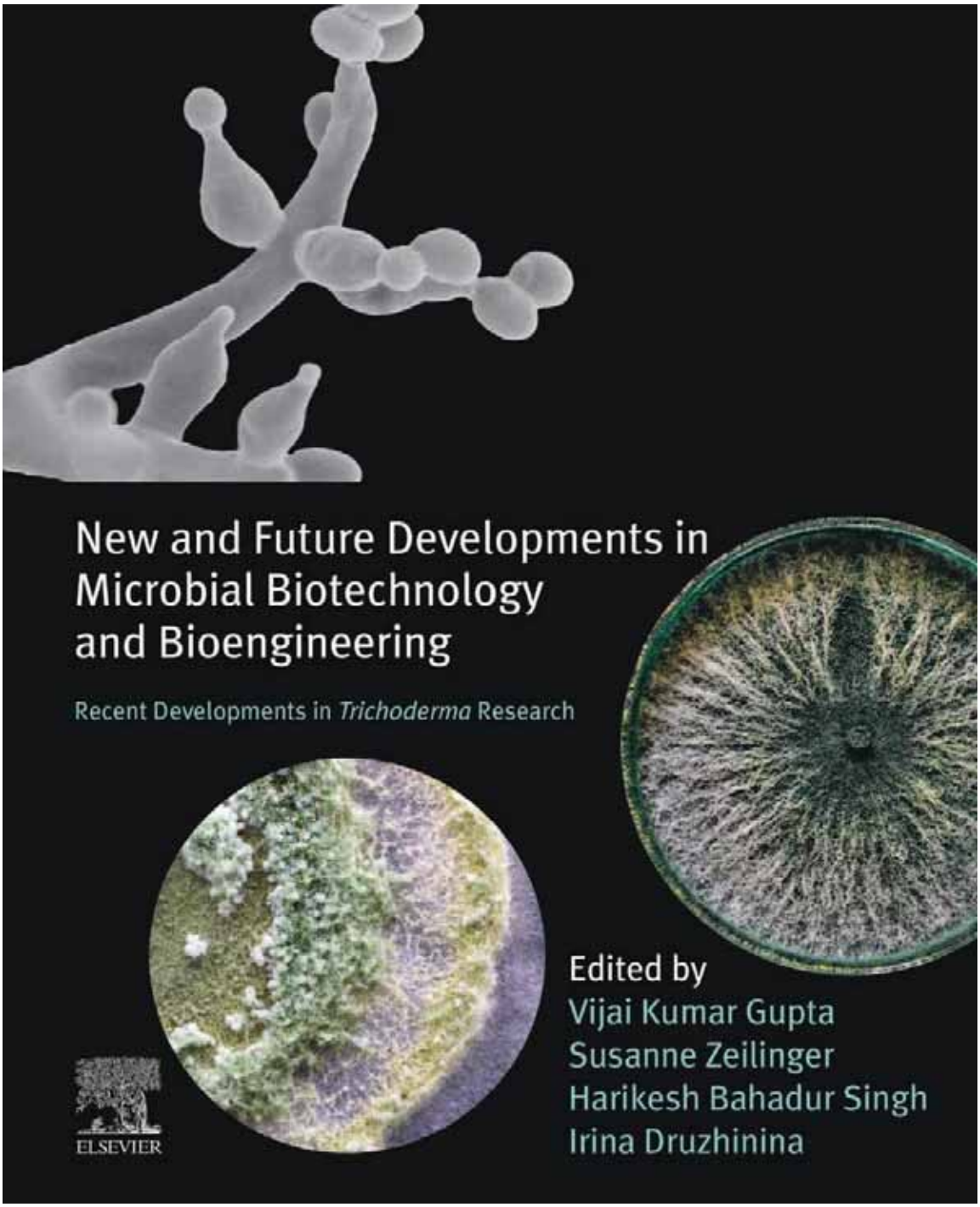
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CHAPTER

3

Mechanism of plant immunity triggered by *Trichoderma*

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OUTLINE

Introduction

The mechanisms underlying the symbiotic interaction between *Trichoderma* and plants have been widely investigated in recent years. These fungi possess a range of attributes that make them very promising crop biostimulants from the greenhouse to field applications (López-Bucio et al., 2015; Doni et al., 2017; Diánez et al., 2018). Along with their critical influence in the modulation of root architecture, which helps plants to better acquire water and fertilizers, *Trichoderma* reinforces immunity via inducing priming depending on the canonical hormones jasmonic acid (JA), salicylic acid (SA), and ethylene (Et) and improves biotic and abiotic stress resistance (Contreras-Cornejo et al., 2011, 2014a, 2015b; Martínez-Medina et al., 2017b; Fiorentino et al., 2018).

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The mutual fungal–plant perception starts with the acidification of the substrate and the emission of volatiles and diffusible molecules by *Trichoderma*, which triggers changes in host metabolism such that an array of lipids, phenolics, carbohydrates, amino acids, and secondary metabolites are secreted into the rhizosphere, which act as microbial inducers (Nieto-Jacobo et al., 2017; Pelagio-Flores et al., 2017; González-Pérez et al., 2018; Macías-Rodríguez et al., 2018; Lucini et al., 2019).

Although early studies with *Trichoderma* spp. highlighted their biocontrol properties, recent data point to a more critical influence of these fungi on phytostimulation and bioprotection, but how these two mechanisms are linked still remains unknown. The sessile lifestyle of plants demands efficient responses and adaptive traits to be activated against simultaneous stresses, including pathogen challenges, nutrient starvation, and exposure to toxins and contaminants, which compromise growth. Growth–defense trade-offs are fine-tuned depending on environmental factors or biotic interactions, and this should be of tremendous agricultural relevance. In this chapter, we focus on the molecular aspects of *Trichoderma*–plant interaction and their impact on orchestrating plant immunity.

Trichoderma root colonization

During root colonization, *Trichoderma* surrounds the epidermal cell layer and forms appressorium-like structures to penetrate internal tissues where it spread through the apoplast (Mukherjee et al., 2012; Yedidia et al., 1999; Chacón et al., 2007; Shoresh et al., 2010; Mukherjee et al., 2012). Attachment to the root epidermis by the hyphae occurs via hydrophobin and hydrophobin-like proteins, which act as adhesive agents (Seidl-Seiboth et al., 2011; Mendoza-Mendoza et al., 2018). A hydrophobin-like protein, TasHyd1 from *Trichoderma asperellum*, is involved in cucumber root colonization (Viterbo and Chet, 2006). Similarly, *Trichoderma harzianum* QID74, a cysteine-rich cell wall protein, is necessary for tomato root attachment and colonization (Samolski et al., 2012). An analysis of the secretomes of *Trichoderma atroviride* and *Trichoderma virens* resulted in the identification and characterization of a class II hydrophobin TVHYDII1 with a role in root colonization of *Arabidopsis* by *T. virens* (Guzmán-Guzmán et al., 2017).

Penetration of the hyphae into the root requires the secretion of cell wall degrading enzymes and other proteins that inactivate the antimicrobial peptides, prevent deleterious effects of reactive oxygen species (ROS), and inhibit plant proteases (Viterbo et al., 2004; Brotman et al., 2008; Martínez-Medina et al., 2016). Furthermore, an expansin-like protein termed swollenin (TasSwo) from *T. asperellum* and cerato-platanin family proteins including Sm1 remarkably increase root colonization efficiency, trigger ROS production, and activate defense-related genes in the plant host (Djonović et al., 2006; Brotman et al., 2008; Baccelli, 2014).

Recent evidence indicates that acidification of the substrate is important during plant–fungus interaction. Soil pH is a major factor for plant growth distribution in ecosystems, and therefore the acidification induced by *Trichoderma* modulates several growth and development responses in *Arabidopsis* seedlings, indicating that root sensing of pH mediates the interaction of *Trichoderma* with plants (Pelagio-Flores et al., 2017). The notion that plant defense could be regulated in response to the soil acidification induced by *Trichoderma* is

supported by the fact that genes involved in responses to pathogens and defense-associated hormones, such as a SA, change in *Arabidopsis* seedlings as a response to low pH (Lager et al., 2010). Global gene expression analysis in response to low pH in wheat seedlings revealed the induction of 16 genes belonging to the WRKY transcription factor family, most of which contain MeJA response elements, as well as genes of SA, JA, and Et-related pathways (Hu et al., 2018).

Recent studies have focused on the identification of possible effector-like proteins among the predicted secretomes of *Trichoderma* spp. using bioinformatics tools (Lamdan et al., 2015; Mendoza-Mendoza et al., 2018; Nogueira-Lopez et al., 2018). Proteins related to pathogenicity or virulence factors are considered as possible effectors, but some putative glycosyl hydrolases and small secreted cysteine-rich proteins have important roles in early fungal–plant interaction (Lamdan et al., 2015; Nogueira-Lopez et al., 2018; Mendoza-Mendoza et al., 2018).

Plant defense triggered by *Trichoderma*

Most fungi are saprophyte organisms that feed on dead or decaying plant material, as such they possess a battery of degradative enzymes, which makes them metabolically diverse and enables their adaptation to many habitats and ecological niches (Harman et al., 2004; López-Bucio et al., 2015). At the rhizosphere, *Trichoderma* establishes a chemical dialog with the plant that mostly leads to symbiosis, and as a result, the structural and biochemical properties of plant cells can be locally or systemically modified upon perception of structural components or secreted metabolites including small volatiles and diffusible compounds, which target root epidermal cells before physical contact (Kottb et al., 2015; Garnica-Vergara et al., 2016). These elicitors can be recognized as microbe-associated molecular patterns (MAMPs) by membrane receptors of plants called pattern recognition receptors that activate a MAMP-triggered immunity signal transduction cascade (Newman et al., 2013; Bigeard et al., 2015).

Later on, the perception of structural components of fungal cell walls or its derivatives may help prepare their hosts for the mutualistic relationship (Sánchez-Vallet et al., 2015). Upon interaction between mycelium and root epidermal cells, the host response can be strengthened by plant cell wall–derived molecules known as damage-associated molecular patterns (DAMPs), released as a result of fungal enzymatic activity (Hermosa et al., 2013; Bisen et al., 2016; Martínez-Medina et al., 2016).

Trichoderma elicitors can also activate the so-called effector-triggered immunity (Jones and Dangl, 2006; Boller and He, 2009) that leads to generation of ion fluxes, the production of ROS, nitric oxide (NO), and Et, callose deposition, and phytoalexin production (Ahuja et al., 2012; Newman et al., 2013). The preparation of plant organs to pathogen attack is a positive response to several *Trichoderma* species through the priming reaction (Conrath et al., 2015), which leads to induced systemic resistance (ISR) and/or systemic acquired resistance (SAR) and subsequent expression of genes encoding pathogenesis-related proteins and defensins (PDFs) (Korolev et al., 2008; Velázquez-Robledo et al., 2011; Mathys et al., 2012; Contreras-Cornejo et al., 2011, 2014c).

Trichoderma elicitors and priming

Chemically induced priming may be integral to the defenses elicited since a wide range of bioactive metabolites are secreted by *Trichoderma* spp. during their interaction with roots. Peptaibols are linear peptides synthesized by nonribosomal peptide synthetases, classified according to their chain lengths (Szekeres et al., 2005). These are mainly known due to antifungal and antibacterial properties and also trigger defense responses in plants (Bisen et al., 2016). Alamethicin, one of the most representative peptaibols produced by *Trichoderma*, induces both endogenous JA and SA levels in lima bean leaves (Engelberth et al., 2001).

Application of two synthetic 18 amino acid peptaibol isoforms from *T. virens* Gv29-8 to cucumber plants conferred systemic protection against the pathogenic bacteria *Pseudomonas syringae* pv. *lachrymans*, whereas mutation of *Tex1* gene, a protein product that is involved in the biosynthesis of peptaibols, renders less protection to the pathogen (Viterbo et al., 2007). Supplementation of low concentrations of alamethicin to *Arabidopsis* seedlings triggered callose deposition, production of ROS and phenolic compounds, and the upregulation of some defense-related genes (Rippa et al., 2010), while trichokonins from *Trichoderma pseudokoningii* strain SMF2 boosted defenses against the tobacco mosaic virus (Luo et al., 2010) and *Pectobacterium carotovorum* subsp. *carotovorum* in Chinese cabbage (Li et al., 2014). Recently, trichokonin IV, a major constituent of the group of TKs peptaibols, induced systemic resistance against *Botrytis cinerea* and at the same time promoted growth in moth orchid (Zhao et al., 2018). Interestingly, in a similar way to peptaibols, harzianic acid, a secondary metabolite produced by *T. harzianum*, protected tomato plants to the pathogenic fungi *Rhizoctonia solani*, by inducing the chemical defenses (Manganiello et al., 2018), and harzianolide mediated tomato systemic resistance against *Sclerotinia sclerotiorum* through the involvement of several SA and JA/Et-related genes (Cai et al., 2013). Very recently, an intracellular iron storage siderophore from *T. virens* was related to root colonization of maize seedlings and the induction of ISR (Mukherjee et al., 2018).

Several volatile metabolites from *Trichoderma* have been identified through chemical screens, but their roles during the plant–fungus interaction have been little studied. One of the most abundant VOCs emitted by *Trichoderma* is 6-pentyl-2H-pyran-2-one (6-PP), a metabolite with dual functions on plant growth and defense (Vinale et al., 2008; Kottb et al., 2015; Garnica-Vergara et al., 2016). El-Hasan and Buchenauer (2009) showed that activities of peroxidase, polyphenol oxidase, and β-1,3-glucanase defense-related enzymes were enhanced by 6-PP application to maize seedlings, which correlated with control of blight disease caused by *Fusarium moniliforme*. Similarly, the symptoms caused by the infection with *B. cinerea* and *Alternaria brassicicola* in *Arabidopsis thaliana* were reduced when plants were preexposed to 6-PP, which increased expression of defense-related genes (Kottb et al., 2015). In addition, the production of trichothecenes by *Trichoderma arundinaceum* triggers defense mechanisms in response to infection with *B. cinerea* faster and at higher levels (Malmierca et al., 2012, 2015).

Different enzymes from *Trichoderma* spp. such as cellulases (Thph1 and Thph2), xylanases (Xyn2/Eix), and endopolygalacturonases (ThPG1, TvPG1 and TvPG2) function as potent elicitors of ISR directly or through the host perception of plant cell wall fragments (DAMPs)

(Martinez et al., 2001; Rotblat et al., 2002; Ron and Avni, 2004; Morán-Diez et al., 2009; Benedetti et al., 2015; Saravanakumar et al., 2016; Baroncelli et al., 2016; Sarrocco et al., 2017) (Table 3.1).

Trichoderma auxins may suppress root immunity

One of the most representative and ubiquitously distributed plants' hormones is the auxin indole-3-acetic acid (IAA). *Trichoderma* species produce IAA and its precursors indole-3-acetaldehyde, and indole-3-ethanol, whose levels increase following application of tryptophan (Contreras-Cornejo et al., 2009; Hoyos-Carvajal et al., 2009; Shi et al., 2016; Nieto-Jacobo et al., 2017).

Trichoderma auxins activate mitogen-activated protein kinase (MAPK)–mediated signaling transduction pathways (Ahuja et al., 2012; Contreras-Cornejo et al., 2015a; Hake and Romeis, 2018). Additionally, *Trichoderma* triggers activation of MAPKs through MAMPs/DAMPs that

TABLE 3.1 MAMPs from *Trichoderma* spp.

MAMPs	Plant responses	References
Peptaibols (alamethicin, trichokonins, and 18mer or 20mer peptaibols)	Elicitation of systemic defense and JA and SA biosynthesis	Engelberth et al. (2001), Viterbo et al. (2007), Luo et al. (2010), Rippa et al. (2010)
Cellulases	Induce defenses through the activation of the SA and ET signaling pathways	Martinez et al. (2001), Saravanakumar et al. (2016)
Xylanases (Xyn2/Eix)	Induce ET production and plant defense response	Rotblat et al. (2002)
Cerato-platanins Sm1/Epl1	Induce expression of defense responses and resistance against pathogens	Djonović et al. (2006), Seidl et al. (2006), Gaderer et al. (2015), Gomes et al. (2015)
Swollenin (TasSwo)	Expression of local defense responses and protection against pathogens	Brotman et al. (2008)
Secondary metabolites (trichothecenes, 6-PP, harzianolide, and harzianopyridone)	Activate plant defense mechanisms	Vinale et al. (2008), Malmierca et al. (2012, 2015), Cai et al. (2013).
Endopolygalacturonases (ThPG1, TvPG1 and TvPG2)	Induce ISR-like defense	Morán-Diez et al. (2009), Benedetti et al. (2015), Sarrocco et al. (2017)
Avr4 and Avr9 homologs	Induce hypersensitive response and other defense-related reactions	Harman et al. (2004), Marra et al. (2006)
LysM protein TAL6	Mediates plant defense responses and fungal growth	Seidl-Seiboth et al. (2013)

induce downstream defense responses (Vos et al., 2015). The *Arabidopsis* MAPKs MPK3 and MPK6 and the MAPK-kinase encoding gene (MKK4) are among the activated proteins (Shoresh et al., 2006; Mathys et al., 2012; Contreras-Cornejo et al., 2015a). The mitogen-activated protein kinase 6 (MPK6) is a critical signaling protein that acts as a node in plant growth and defense (Contreras-Cornejo et al., 2015a; Hake and Romeis, 2018).

Auxins may act as a reciprocal signaling molecule during *Trichoderma*–plant interactions because of their bioactivity in modulating cellular responses in organisms from different kingdoms and lifestyles (Spaepen and Vanderleyden, 2011; Kunkel and Harper, 2018). Available evidence points to an antagonistic role of auxins in plant defense because IAA biosynthesis and signaling promote an increased susceptibility to some phytopathogens. Navarro et al. (2006) found that flg22, a peptide that elicits resistance of *Arabidopsis* to *P. syringae*, induces a plant microRNA that interferes with auxin signaling via repressing the expression of the auxin receptors TIR1, AFB2, and AFB3 with concomitant restriction of *P. syringae* growth.

In a recent report, McClerklin et al. (2018) showed that *P. syringae* DC3000 produces IAA from indole-3-acetaldehyde and that disruption of indole-3-acetaldehyde dehydrogenase, which catalyzes its conversion to IAA leads, to reduced auxin biosynthesis by the pathogen, which correlated with decreased virulence in *Arabidopsis*. Thus, auxins should be considered as candidate molecules that suppress plant defenses during interactions with microbes of different lifestyles and may help *Trichoderma* during root colonization likely via suppression of SA-mediated defenses (Wang et al., 2007; Mutka et al., 2013).

Priming and crop protection

The term "priming" was coined to define a plant physiological state that responds faster and/or stronger to pathogen attack and involves SAR, whereby local pathogen damage primes SA-dependent defenses in distal tissues (Conrath et al., 2015). Interactions with rhizosphere microbes, such as beneficial bacteria and fungi, which normally act as probotics, can also elicit the so-called ISR, which involves JA and Et biosynthesis and signaling (Verhagen et al., 2004; Van der Ent et al., 2009; Van Wees et al., 2008). Application of microbes or their derived metabolites that induce priming is increasingly considered a promising option for integrated pest and disease management (Gamir et al., 2012; Conrath et al., 2015; Luna et al., 2015).

Several reports indicate that in model plants and crops *Trichoderma* species induce priming through multiple mechanisms, regulated at least in part by bioactive metabolites that cross talk with the host defense hormonal pathways. Concomitantly, the SA, JA, and Et pathways mediate the protection conferred by *Trichoderma* to pathogen challenge in *Arabidopsis* (Mathys et al., 2012), grape (Perazzolli et al., 2012), tomato (Leonetti et al., 2017; Martínez-Medina et al., 2013, 2017a,b; Jogaiah et al., 2018), and melon (Martínez-Medina et al., 2014; Diánez et al., 2018) (Table 3.2).

TABLE 3.2 *Trichoderma*-mediated defense responses in plants against different types of pathogens.

Trichoderma strain	Plant host	Target pathogen	Genes/Protein	References
Trichoderma harzianum	Cucumber	*Phytophthora capsici*	Chitinase and peroxidase enzyme	Yedidia et al. (1999)
T. harzianum	Tomato	*Botrytis cinerea*	JA responsive genes like *multicystatin* (MC), *proteinase inhibitor II* (*PI II*), *prosystemin* (*PS*)	Martinez-Medina et al. (2013)
T. harzianum	Grapevine	*Plasmopara viticola*	Several biotic/abiotic stress response– and defense-related proteins	Palmieri et al. (2012)
T. harzianum	Sunflower	*Rhizoctonia solani*	*PAL*, *polyphenol oxidase (PPO)*, *peroxidases (PO)*, *cinnamyl alcohol dehydrogenase (CAD)*, *PR-2* and *PR-3* genes	Singh et al. (2014)
T. harzianum Rifai T39	*Arabidopsis*	*B. cinerea*	SA, JA/ET-impaired mutants	Korolev et al. (2008)
T. harzianum MUCL 29707	Potato	*R. solani*	*PR1*, *PR2*, *PAL*, *Lox*, and *GST1* genes	Gallou et al. (2009)
T. harzianum T-aloe	Soybean	*Sclerotinia sclerotiorum*	Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) enzymes and *PR1*, *PR2*, and *PR3* genes	Zhang et al. (2016)
T. harzianum Ths97	Olive trees	*Fusarium solani*	Diverse genes involved in an oxidative burst, PR-proteins, phenylpropanoid pathway, and hormonal status	Amira et al. (2017)
T. harzianum Tr6 with *Pseudomonas* sp. *Ps14*	Cucumber, *Arabidopsis*	*B. cinerea*	*β-1,3-glucanase*, *PAL1*, *CHIT1*, *LOX1*, *PR1*, *PDF1.2*	Aliradeh et al. (2013)
T. harzianum TH12	Oilseed rape	*S. sclerotiorum (Lib)*	*Allene oxide cyclase 3 (AOC3)*, *PDF1.2*, *ethylene response factor 2 (ERF2)*, *PR-1*, *transcription factor 5 (TGA5)*, and *transcription factor 6 (TG6)*	Alkooranee et al. (2017)
T. harzianum T-22	Corn	*Colletotrichum graminicola*	β-1,3-glucanase, exochitinase, and endochitinase enzymes	Harman et al. (2004)
T. harzianum T22	Tomato	*Macrosiphum euphorbiae* (Thomas) *Aphidius ervi* (Haliday)	Several SA/JA-related genes	Coppola et al. (2017)
T. harzianum T-22	Tomato	*Cucumber mosaic virus* (CMV)	*Cu/Zn superoxide dismutase (Cu/Zn-SOD)*, *Mn superoxide dismutase (Mn-SOD)*, *CAT*, *APX*, and *pathogenesis-related coronative insensitive 1 (COI-1)*	Vitti et al. (2015)
T. harzianum isolates	Pistachio	*Verticillium dahliae*	PO, PAL enzymes, and total phenols	Fotoohiyan et al. (2017)

(*Continued*)

TABLE 3.2 *Trichoderma*-mediated defense responses in plants against different types of pathogens.—cont'd

Trichoderma strain	Plant host	Target pathogen	Genes/Protein	References
T. harzianum Rifai strain T32	Oil palm	*Ganoderma boninense* PER71	Several genes involved in MeJA/MeSA/ET biosynthesis, secondary metabolites, ROS scavenging, cell wall metabolism, and detoxification of phytotoxic compounds	Ho et al. (2016, 2018)
T. harzianum T-78	Tomato	*Meloidogyne incognita*	*PI II, MC, PR1a,* and *PR-P6*	Martínez-Medina et al. (2017b)
Trichoderma asperellum T-203	Cucumber	*Pseudomonas syringae pv. lachrymans*	*PAL* and *hydroperoxide lyase (HPL)*	Yedidia et al. (2003)
T. asperellum T-203	Cucumber	*P. syringae pv. lachrymans*	Several proteins involved in ROS scavenging, stress response, isoprenoid, and ethylene biosynthesis.	Segarra et al. (2007)
T. asperellum T-34	*Arabidopsis*	*P. syringae pv. tomato, Hyaloperonospora parasitica, Plectosphaerella cucumerina*	*LOX2* gene and transcription factor *MYB72*	Segarra et al. (2009)
T. asperellum T-34	Cucumber	*P. syringae pv. lachrymans*	*LOX1, PAL1, ETR1, CTR1, chitinase 1 (Chit1), β-1,3-glucanase,* and *peroxidase*	Shoresh et al. (2005)
T. asperellum SKT-1	*Arabidopsis*	*P. syringae pv. tomato*	*PR-1, PR-2, PR-3, PR-4, PR-5, PDF1.2a,* and *vegetative storage protein 1 (AtVsp1)* genes	Yoshioka et al. (2012)
Trichoderma virens	Maize	*C. graminicola*	*PR1, PR5, PAL, allene oxide synthase (AOS), 12-oxophytodienoate reductase 2/7/8 (OPR2/OPR7/OPR8), fatty acid hydroperoxide lyase, chloroplastic (HPL),* and *LOX10*	Djonović et al. (2007)
T. virens	Tomato	*Fusarium oxysporum f.* sp. *lycopersici*	*PDF1, PR1a*	Jogaiah et al. (2018)
T. virens TRS106	Tomato	*R. solani*	Peroxidase (GPX), syringaldazine peroxidase (SPX), and PAL enzymes	Malolepsza et al. (2017)
Trichoderma hamatum 382	Cucumber, tomato	*Xanthomonas euvesicatoria*	*Extension-like proteins, PR5, osmotin-like protein, fibrillarin, phosphate induced,* and *transcription factor MYB*	Alfano et al. (2007)
T. hamatum T382	*Arabidopsis*	*B. cinerea*	Several JA synthesis/response genes and production of secondary metabolite–related genes	Mathys et al. (2012)
T. hamatum UoM	Pearl millet	*Sclerospora graminicola*	*PAL, peroxidase (POX), β-1,3-glucanase, PPO, PR1, PR5, DAHP synthase, chorismate mutase, isochorismate synthase, chorismate synthase,* and *shikimate kinase*	Siddaiah et al. (2017)

TABLE 3.2 *Trichoderma*-mediated defense responses in plants against different types of pathogens.—cont'd

Trichoderma strain	Plant host	Target pathogen	Genes/Protein	References
Trichoderma atroviride	Tomato	*B. cinerea*	*PR1b1, PR-P2, PINI, PINII, TomLoxA, and TomLoxC*	Tucci et al. (2011)
T. atroviride	*Arabidopsis*	*P. syringae pv.* tomato	*PR2 (SlGLUA), PR5 (SlPR-5), alpha-dioxygenase (Sla-DOX1), chitinase (SlCH19), cell wall protein (SlTLRP),* and *peroxidase (SlCEVII6)*	Salas-Marina et al. (2015)
T. atroviride, T. virens	*Arabidopsis*	*B. cinerea*	*LOX2, PR-1*	Contreras-Cornejo et al. (2011, 2014b)
T. atroviride T11	Tomato	*Meloidogyne javanica*	*LOX1, PR1, peroxidase (TPX1), NADPH Oxidase (LERBOH1), class II chitin synthase (LECHS2), MYC2, NPR1, ERF1,* and *ARF1*	de Medeiros et al. (2017)
T. atroviride TRS25	Cucumber	*R. solani*	Guaiacol peroxidase (GPX), SPX, PAL, PPO enzymes, and defense-related genes (*PR1, PR4, PR5, PR12*)	Nawrocka et al. (2018)
T. atroviride TRS25	Cucumber	*Pseudoperonospora cubensis* (Berkeley and Curtis) Rostovzev	PAL, GPX, and APX enzymes	Szczech et al. (2017)
Trichoderma arundinaceum	Tomato	*B. cinerea, R. solani*	*PR1b1, PR-P2, PINI, PINII* and *TomLoxA*	Malmierca et al. (2012)
Trichoderma citrinoviride PC87	Ginseng	*B. cinerea, Cylindrocarpon destructans*	*PR2, PR4, PR5, PR10*	Park et al. (2018)
Trichoderma spp.	Hot pepper	*P. capsici*	Several defense-related genes	Bae et al. (2011)
Trichoderma spp.	Chili pepper	*Colletotrichum capsici*	PAL, PO, PPO, and SOD enzymes	Saxena et al. (2015)
Trichoderma spp.	Pearl millet	*S. graminicola* (Sacc.) Schroet.	POX, LOX protein	Nandini et al. (2017)
Trichoderma spp.	Tomato	*Fusarium oxysporum L* sp. *lycopersici*	SOD, APX, GPX, and CAT enzymes	Zehra et al. (2017)

Conclusion

Significant progress has been achieved in the past 5 years toward understanding the mechanisms of plant immunity triggered by *Trichoderma*, which reveals the tight connection between plant nutrition, rhizosphere pH, and priming. A major finding was the finding that these fungi strongly acidify the pH of the growth substrate (Pelagio-Flores et al., 2017), and since the rhizosphere is a natural niche where they live and proliferate, it is tempting

to speculate that the overall properties of the soil–root interface can be modified upon robust fungal colonization of roots. pH in most soils around the world is indeed acid (below 4.5) or alkaline (8.0 or higher), and thus overall nutrient availability may be tightly correlated with fungal spread and plant fitness. Noteworthy, *T. asperellum* strain T34 increased the Fe concentration in leaves of lupin plants grown on calcareous soil as well as the activities of peroxidase and catalase activities, which help ROS detoxification (de Santiago et al., 2009). The recent report that volatile compounds from *T. asperellum* and *T. harzianum* not only trigger Fe uptake

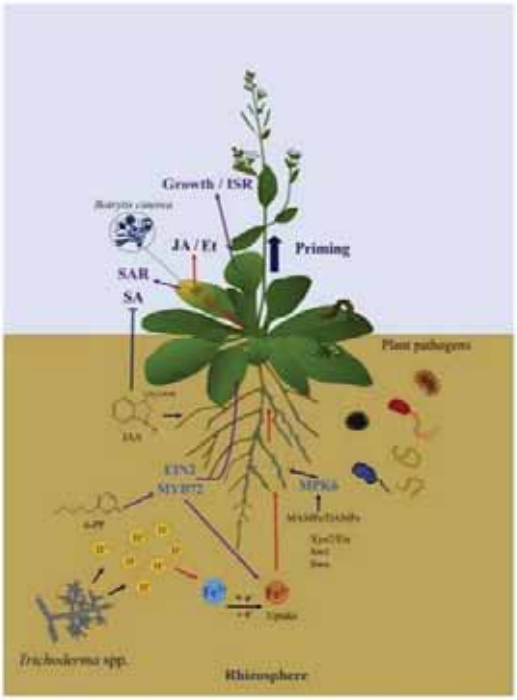

FIGURE 3.1 **Model for *Trichoderma*-induced plant immunity.** *Trichoderma* spp. acidify the pH of the rhizosphere, and this may increase Fe uptake, particularly in alkaline calcareous soils where this micronutrient is limiting, which correlates with elicitation of JA-dependent priming (*red arrows* [*dark gray arrows* in print version]). Volatiles from *Trichoderma* induce the transcription factor MYB72, which plays a dual role in the onset of ISR and the activation of Fe uptake responses and triggers JA-dependent defenses in leaves to enhanced resistance against *Botrytis cinerea* (*purple arrows* [*light gray arrows* in print version]). Fungal-released auxinic compounds, including indole-3-acetic acid (IAA), suppress SA-related genes, and this may enable robust root colonization. MAMPs and *Trichoderma* secondary metabolites like Xyn2/Eix, Sm1, Swo, peptaibols, and harzianolide act as elicitors that after recognition by plant receptors can activate MAPK-mediated signaling transduction pathways and triggers defense responses effective against broad-spectrum of plant pathogens (*blue arrows* [*black arrows* in print version]).

in *Arabidopsis* and tomato but also prime the shoot system for JA-dependent defenses and provide resistance against *B. cinerea* demonstrates the correlation between elicited Fe deficiency responses and shoot immunity in different plant species (Martínez-Medina et al., 2017a).

Auxins, MAMPs, and/or fungal effectors from *Trichoderma* comprise a large group of molecules that fine-tune root colonization, and 6-PP, a major volatile from fungal blends, triggers changes in root architecture and overall plant immunity (Kottb et al., 2015; Garnica-Vergara et al., 2016). The growth and defense trade-offs elicited by *Trichoderma* in crops can be explained via the interactions of effectors and elicitors with host hormonal pathways mostly involving SA, JA, and Et, for which important genes have been identified such as *EIN2*, *MPK6*, and *MYB72* (Contreras-Cornejo et al., 2011, 2015a; Garnica-Vergara et al., 2016; Martínez-Medina et al., 2017a). These genes represent promising targets toward molecular manipulation of the cellular responses and root fungal colonization under more specific contexts and biotechnological applications. Overall, as part of the natural root microbiomes or applied as bioinoculants in the field, members of the *Trichoderma* genus are important probiotics to improve plant defense and crop adaptability to a wide range of biotic challenges (Fig. 3.1).

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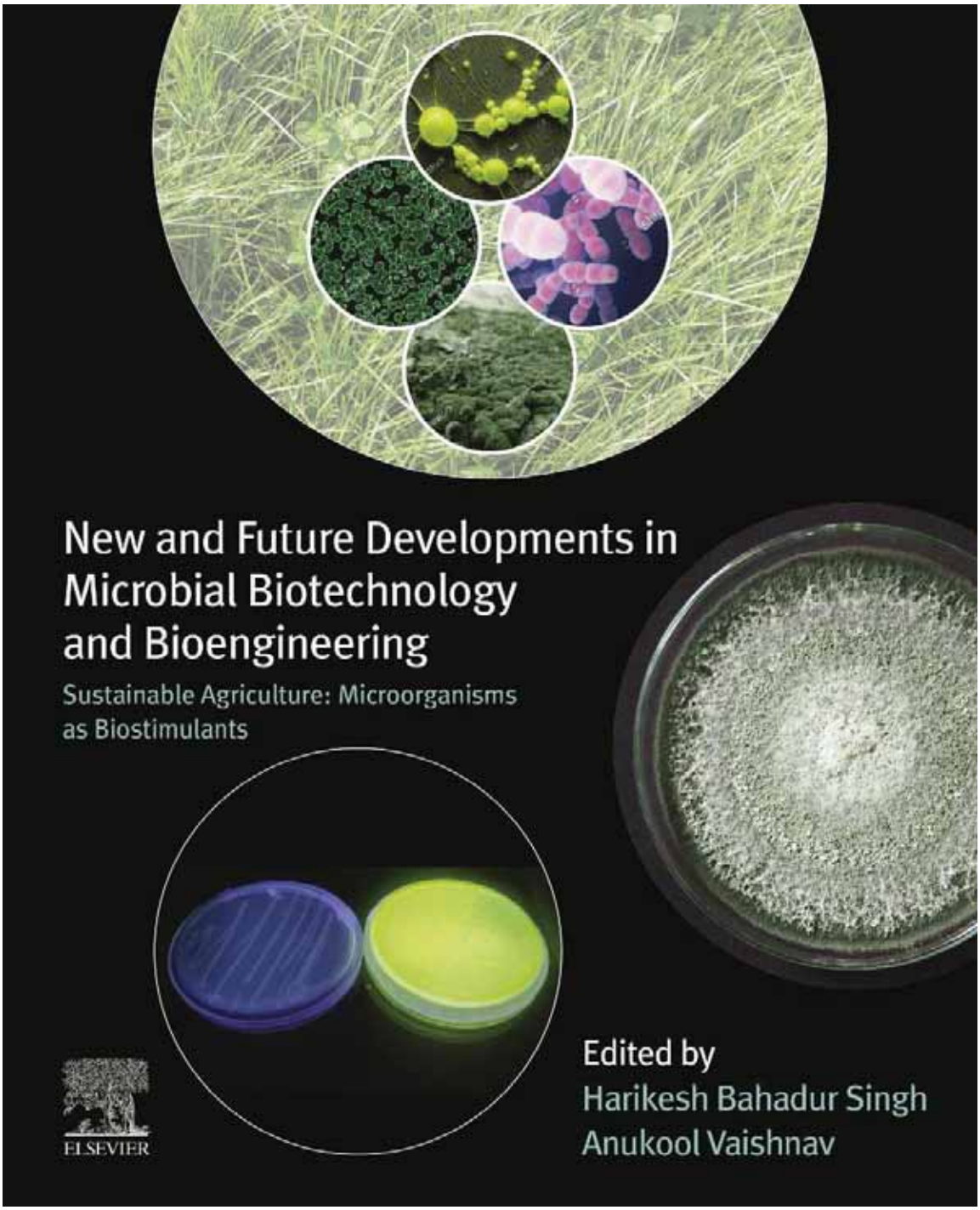
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Contents

CHAPTER

2

Exploiting biostimulant properties of *Trichoderma* for sustainable plant production

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2.1 Introduction

Trichoderma is a fungal genus comprising many species that play an important ecological role in carbon recycling, due to their ability to secrete a variety of enzymes that include cellulases, quitinases, and phosphatases, which enables adaptation to terrestrial and water environments (Ferrigo et al., 2020; Poveda et al., 2020; Wang and Zhuang, 2020). Different species have been isolated from dead wood, organic soil and also parasitizing other fungi, illustrating their versatility to spread in variable ecological niches (Kubicek et al., 2019). Genome comparisons from more than 15 *Trichoderma* species including *T. reesei*, *T. virens*, *T. atroviride*, *T. longibrachiatum*, and *T. asperellum*, revealed their metabolic diversification likely starting from a mycoparasite style towards a more generalist one, which likely occurred by the cretaceous period, around 65 million years ago (Schmoll et al., 2016; Kubicek et al., 2019).

Several studies revealed the long-lasting symbiotic relationships of *Trichoderma* with plant roots, first being attracted to the rhizosphere by sucrose or other simple sugars. This interaction is further strengthened by an intimate chemical communication that includes fungal secretion of plant hormones, secondary metabolites and soil acidification (Fig. 2.1). *Trichoderma* released compounds that improve plant traits are summarized in Table 2.1 according to very

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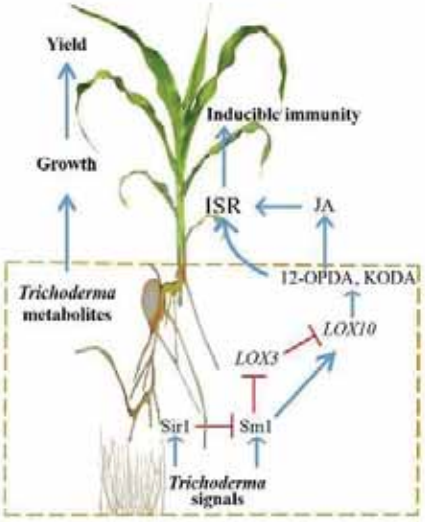

FIG. 2.1 *Trichoderma*-induced systemic resistance involves jasmonic acid and other oxylipins that are translocated from root to shoot. Fungal secondary metabolites, volatiles and peptides are perceived by roots to establish a long lasting symbiosis. Sm1 is an elicitor secreted by Trichoderma that is negatively regulated by Sir1, this node triggers the biosynthesis of 12-oxo-phytodienoic acid (OPDA) and α-ketol of octadecadienoic acid (KODA), two LOX-10-derived oxylipins, which enhance protection to pathogens and pests through JA-dependent and independent signaling mechanisms.

TABLE 2.1 Major factors involved in the *Trichoderma*-plant dialogue and their agricultural applications.

Compounds produced	Plant response
6-PP Indole-3-acetic acid Auxins Protons	Plant growth, development and defense
Ergosterol Squalene Sm1 peptide	Reinforcement of plant immunity
Methyl-salicylate Z-3-exen-1-ol β-caryophyllene 1-octen-3-ol and 6PP	Inducible defense against predators and pests
Organic acids Tricholignan Alkaline phosphatase	Nutrient solubilization and uptake by roots
Antioxidants	Induced abiotic stress tolerance

recent references (López-Bucio et al., 2015; Guzmán-Guzmán et al., 2019; Zhou et al., 2019; Hoermayer et al., 2020; Canher et al., 2020; Saad et al., 2020; Villalobos-Escobedo et al., 2020).

To face global climate changes, soil degradation and the appearance of novel phytopathogens, discovery, characterization and application of probiotic microbial species with multilevel properties are urgent needs for farmers to maintain safe their crops (Saad et al., 2020). In this regard, *Trichoderma* represents a very promising option given their ecological, physiological and molecular attributes.

2.2 *Trichoderma* metabolism: from decomposers to plant growth promoters

More than 60 natural isolates of *Trichoderma*, mutants and over expressing strains have been characterized in the last five years, which makes clear the sophisticated metabolic versatility of this genus. In a recent study, Wang and Zhuang (2020) evaluated the nutritional attributes of five *Trichoderma* species (*T. hengshanicum*, *T. bomiense*, *T. rosulatum*, *T. crystallinum* and *T. fructicola*), which have contrasting production profiles of chitinase and cellulase, differences in phosphate solubilizing capacity and salt tolerance. Their conidia were inoculated in micro plates containing medium supplemented with 95 different carbon compounds and fungal growth was assessed. The five *Trichoderma* strains could metabolize 11 common compounds, whereas a low efficiency to grow on complex carbohydrates was reported for saline-alkaline-tolerant species, which mostly rely on simple sugars for nutrition. In contrast, the species with highest cellulolytic, chitinolytic and phosphate-solubilizing activities could use more diverse carbon resources such as cellulose and hemicellulose and thus may play a decomposer role in ecosystems.

Cruz-Magalhães et al. (2019) tested the growth of WT and NADPH oxidase mutant strains *ΔnoxR*, *Δnox1*, and *Δnox2* of *T. atroviride* under distinct carbon supplements. The biomass decreased in all three mutants in sucrose and d-fructose, which indicates a failure to efficiently metabolize these simple sugars commonly present in root exudates. This report opened the possibility that the NADPH oxidase could influence metabolic reprograming in *T. atroviride*, which could be important to mount an efficient rhizosphere interaction with plants. To answer this question, Villalobos-Escobedo et al. (2020) allowed WT, *ΔnoxR*, *Δnox1*, and *Δnox2* fungal colonies to interact with *Arabidopsis* roots in vivo and investigated both the fungal and plant molecular response at 3 days (early time) and five days (later time), where the hyphae was approaching the root tip, or upon their spread over the entire root system, respectively. The authors found that in the presence of root exudates, the WT strain shows a transcriptomic repression of degradative enzymes, and instead use sugars and simple carbohydrates for nutrition, whereas in *ΔnoxR* the early repression of genes for carbohydrate degradation was missing. Thus, the fungal metabolism shifts from a cellulolytic one to a symbiotic one as a response to root exudates, and this process requires NAPH oxidase.

2.3 *Trichoderma*-plant chemical dialogue

Trichoderma species emit blends of biactive compounds, including plant hormones, secondary metabolites and small peptides (Salwan et al., 2019). The molecular composition of the blends depends on several factors including the fungal species, nutrient availability and

the presence of other microorganism and plants and their biological activities are just begun to be clarified (Lee et al., 2016; González-Pérez et al., 2018; Vinale and Sivasithamparam, 2020).

2.3.1 *Trichoderma* released compounds in plant growth promotion

Trichoderma releases compounds with auxin activity, specifically, *T. virens* secretes indole-3-acetic acid (IAA), which accumulates in fungal cultures upon supplementation of tryptophan (Contreras-Cornejo et al., 2009; Nieto-Jacobo et al., 2017). Additionally, it was established that *T. asperellum* produces auxins that enhance the endogenous auxin pool in plant tissues, inducing auxin signaling and thus resulting in improved growth (Wang et al., 2020).

Evaluation of the effect of 26 volatiles produced by *Trichoderma* on *Arabidopsis* seed germination and growth, identified nine compounds that improve plant growth including 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-heptanone, octanoic acid, (*S*)-limonene, (*D*)-limonene, 2-heptylfuran and 1-decene (Lee et al. 2019). 1-decene, an alkene compound with interesting growth promoting effects influenced the auxin signaling pathway, while repressing defense, stress and disease response genes, which enables fungal root colonization (Lee et al. 2019). Similarly, it could be determined the function of the *Trichoderma* metabolites harzianic acid (HA), hydrophobin1 and 6-pentyl-2H-pyran-2-one (6-PP) individually or in mixture in soybean plants (Marra et al., 2019). All compounds improved overall plant growth when applied in greenhouse conditions, whereas in the field 6-PP was the best in improving plant growth and yield, HA had a middle effect and hydrophobin failed to improve growth (Marra et al., 2019). 6-PP is perhaps the most widely investigated fungal metabolite. Its production by *T. atroviride* increases during plant interaction and changes *Arabidopsis* root architecture through ethylene and auxin-dependent pathways (Garnica-Vergara et al., 2016).

2.4 *Trichoderma*-induced resistance to plant pathogens

Coordination of multiple, hormone-mediated mechanisms related to the canonical JA/ET and SA signaling pathways leads to a faster and stronger transcriptomic, proteomic and metabolomic signatures in both wild and crop species to increase resistance against phytopathogens (Yuan et al., 2019). This immune reaction accounts for ecological fitness, as demonstrated by tripartite interactions, where *Trichoderma*-root colonization above ground influences leaf performance under attack.

2.4.1 Salicylic acid-mediated interactions

Trichoderma can elicit the salicylic acid (SA)-mediated systemic acquired resistance (SAR) pathway, which enables plants to defend better against attack by fungal pathogens (Martínez-Medina et al., 2017a). Studies of differential gene and protein expression revealed that cucumber plants change their hormonal metabolism in response to *T. longibrachiatum* H9, and genes of hormonal biosynthesis and signaling were up-regulated including those related to SA and their corresponding encoded proteins were accumulated in the plants (Yuan et al., 2019).

Trichoderma produces ergosterol and squalene during plant interaction. The ratio of production of both compounds results in differential fungal root colonization and SA dependent signaling. SA produced by plants prevents the spread of the fungus in vascular and aerial tissues as in the *Arabidopsis* mutants impaired in SA biosynthesis in which *Trichoderma* colonization spreads toward inner tissues including the vasculature and triggers eventual plant collapse (Alonso-Ramírez et al., 2014; Martínez-Medina et al., 2017b). Tomato plants treated with ergosterol have induced or reduced expression of PR-P2 and PR1b1 respectively, two genes related to the SA signaling pathway (Lindo et al., 2020). Increased production of squalene in *Trichoderma* triggers the ability to colonize tomato roots but also increase the expression of SA-related genes, whereas impaired production of squalene and ergosterol in *Trichoderma* compromises colonization of tomato roots (Malmierca et al., 2015). *T. parareesei* mutant in *Tparo7* gene that encodes a chorismate mutase was affected in its capacity to colonize roots of tomato, and showed negative effects to plant growth, which correlated with a higher production of SA (Pérez et al., 2015). These data suggest that the root sensing of the fungi allows root colonization and at the same time controls their spread into inner tissues.

2.4.2 Jasmonic acid and other oxylipins

During early root colonization, recognition of microbial elicitors/effectors by plant receptors mainly triggers induced systemic resistance (ISR) through jasmonic acid (JA) biosynthesis and signaling (Djonovic et al., 2007; Zhang et al., 2017; Gupta and Bar, 2020; Chakraborty et al., 2020).

The gene expression patterns responsible of the protection conferred depend on the amount of inoculum, the fungal and plant species and growth conditions. Moreover, the jasmonic acid peak appears to be undulating with time, showing differential profiles at early or late times during the interaction (Contreras-Cornejo et al., 2011; Martínez-Medina et al., 2013; Rubio et al., 2014). *Trichoderma*-released ergosterol and squalene both induce SA and JA signaling (Lindo et al., 2020). A global transcriptomic analysis of *T. longibrachiatum* H9-induced ISR in cucumber against *Botrytis cinerea* through the activation of genes encoding proteins for abiotic stress tolerance, secondary metabolism and phytohormone-dependent defense (Yuan et al., 2019).

It was initially demonstrated that the ISR induced in plants in response to the *Trichoderma* interaction depended on a small peptide called Sm1 and JA-ethylene signaling (Djonovic et al., 2007). Recent studies further indicate that it also elicits biosynthesis of the oxylipins 12-oxo-phytodienoic acid (OPDA) and α-ketol of octadecadienoic acid (KODA), compounds that mediate the ISR in maize against leaf pathogens (Fig. 2.1; Wang et al., 2020a, b). The maize lipoxygenase LOX-10 regulates synthesis of OPDA and KODA, accordingly *lox10* mutants did not manifest ISR triggered by *T. virens* when challenged with *Colletotrichum graminicola*, but instead displayed induced systemic susceptibility, suggesting that LOX10 plays a positive role in *Trichoderma*-induced ISR (Fig. 2.1). *Trichoderma gamsii* also induces the expression of *LOX10*, as well as the *ALLENE OXIDE SYNTHASE* and *HYDROPEROXIDE LYASE* genes (Galletti et al., 2020).

2.4.3 Biocontrol of aphids, nematodes and other pests

The biological control attributed to *Trichoderma* goes beyond microbial bio-control and includes antagonism to insects and nematodes (Martínez-Medina et al., 2017a; Poveda et al., 2020). *T. asperellum*, *T. atroviride* and *T. harzianum* inhibit feeding and oviposition of *Thrips*

tabaci in onion plants (Muvea et al., 2014). Control of aphid infestation in tomato by *T. harzianum* T22 occurs through recruitment of parasitoids and implies an up-regulation on genes involved in metabolic pathways leading to the production of methyl-salicylate, Z-3-exen-1-ol and β-caryophyllene (Coppola et al., 2017), a group of inducible volatile compounds that act as attractants of parasitic wasps (Wei and Kang, 2011; Alquézar et al., 2017). Tomato plants in interaction with *Trichoderma* show up-regulation of defense genes, protective enzimes and production of VOCs that helps to counteract *Macrosiphum euphorbiae* and *Spodoptera littoralis* (Coppola et al., 2019a). The regulation of plant gene expression and defense responses was described during *Trichoderma*-tomato-*Macrosiphum euphorbiae* multitrophic interaction, resulting in induction of direct and indirect related defense genes (Coppola et al., 2019b).

Showed the multi-trophic interplay between endophytic fungal–plant symbiosis and their repercussion on above ground insect communities. In their study, an analysis was carried out to understand how the inoculation of *T. harzianum* in the corn rhizosphere affects the beneficial arthropod and pest insects present in leaves under field conditions. The results showed that *Trichoderma* decreased the presence of members of the *Aphididae* family and increased the total population of its predators (Family *Forficulidae*). Levels of sucrose, nitrogen, jasmonic acid, ascorbic acid, linoleic and linolenic acid were positively correlated with the fluctuations of chewing herbivores observed and the (Z)−3-hexen-1-ol production. The control of *S. frugiperda* via larval feeding inhibition and elevation in parasitism rate of female wasps (*Campoletis sonorensis*) could be induced by the volatile compounds from *T. atroviride* 1-octen-3-ol and 6-PP (Contreras-Cornejo et al., 2018a). *Trichoderma* commercial formulates show induced resistance against *Meloidogyne incognita* in tomato (Pocurull et al., 2020).

The above described studies are of high ecological relevance and provide the basis of a more sustainable agriculture, reduce the application of insecticides, and minimize their adverse environmental impacts on food production.

2.5 *Trichoderma* and plant nutrition

2.5.1 Major nutritional needs of crops

Sustained crop productivity is tightly linked to the availability of three major mineral nutrients, nitrate, phosphate, and iron, which are largely taken up by roots. Towards obtaining an adequate supplement of these minerals and to meet their cellular demands, the root system has evolved molecular, morphological and ecological adaptations. Upon sensing nutrient deprivation by the root tips, the formation of new branching structures is stimulated, which enhances soil exploration. Symbioses events with both bacteria (i.e. *Rhizhobium*) and fungi (mycorrhizae) help support nutrient acquisition to promote growth and photosynthesis (López-Bucio et al., 2003; Ruiz-Herrera et al., 2015).

Soil fertility largely depends upon pH, solubilization of phosphate and biological fixation of nitrogen. Phosphate availability is a major problem in both alkaline and acid soils, whereas iron concentrations are high in acid soils, but limiting in alkaline soils. Nitrate is also very problematic worldwide because microorganisms decompose it (Gojon, 2017; Neina et al., 2019). To satisfy nutrient crop needs, farmers need to add organic materials or chemical

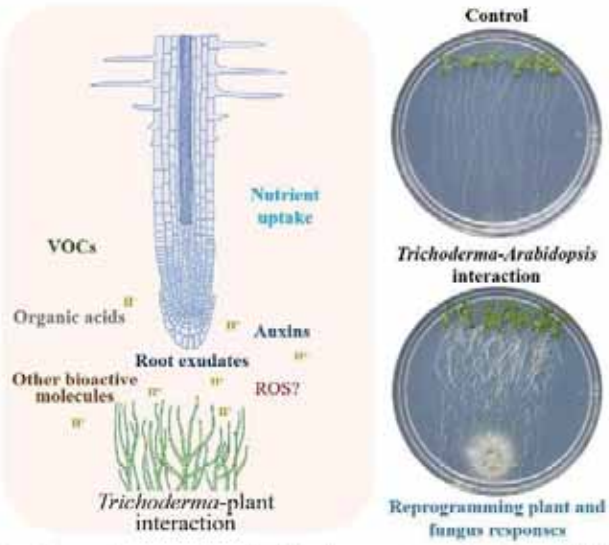

FIG. 2.2 Overview of the mechanisms employed by *Trichoderma* spp. to promote plant growth, induce plant defense and improve crop yield. *Trichoderma* species produce auxins (i.e. indole-3-acetic acid, IAA), which re-configurate root-system architecture as well as diverse volatiles and secondary metabolites such as 6-pentyl-α-pyrone (6-PP), that triggers root branching, increases defense responses and shows antimicrobial properties. A major effect of *Trichoderma* in the rhizosphere is the acidification of the pH and in this manner, phosphate, iron and other nutrients are solubilized and taken up by roots. The probiotic attributes of *T. atroviride* have been investigated in detail using the model plant *Arabidopsis thaliana* in which *T. atroviride* promotes root and shoot biomass production through both hormonal and nutritional mechanisms. Recently, fungal reactive oxygen species emerged as critical players in fungal induction of lateral root formation and orchestrating the balance between growth and defense in the plant host.

fertilizers to their fields, which are often expensive and may cause contamination problems, particularly after long term usage. Due to its versatile metabolism, *Trichoderma* is a very promising bio-fertilizer agent and its capability to solubilize nutrients makes them readily available for uptake by roots (Fig. 2.2).

2.5.2 Phosphate nutrition

The strong acidification capacity by *Trichoderma atroviride* accounts to better phosphate and iron acquisition from alkaline soils (Pelagio-Flores et al., 2017). *T. harzianum* SQR-T037 promoted overall micronutrient (Fe, Mn, Cu and Zn) uptake under non-optimal nutrient supplementation in tomato both in soil and in hydroponic conditions, and this accounts for enhanced biomass production. SQR-T037 solubilized phytate, an organic phosphate source,

as well as Fe and Zn. Fungal secretion of organic acids, including lactic acid, citric acid, tartaric acid and succinic acid could be confirmed by chromatographic means in this report (Li et al., 2015).

A screening of 33 *Trichoderma* isolates (NBRI-PR1–NBRI-PR33) was performed by Tandon et al. (2020), which demonstrated the P-solubilizing properties of the NBRI-PR5 strain corresponding to *T. koningiopsis*, complementary analysis showed the production of oxalic acid, which solubilizes sparingly soluble calcium phosphate under alkaline conditions. Noteworthy, as a response to drought *T. koningiopsis* accumulated poly-phosphate and secreted alkaline phosphatase, an enzyme that enables the use of organic phosphate sources (Tandon, et al., 2020).

In a recent study, Bononi et al. (2020) isolated phosphate-solubilizing *Trichoderma* strains from acid soils in the Amazon rainforest and tested their effects in soybean plants under a gradient of rock phosphate, a poorly soluble phosphate source, and triple superphosphate, a highly soluble chemical fertilizer. The capacity of the isolated strains to produce organic acids was correlated with their phosphate solubilizing properties and accounted for a gain in soybean growth ranging from 2.1 percent to 41.1 percent, and boosted P uptake-up to 141 percent. Efficient P usage by *Trichoderma* goes beyond inorganic phosphate resources.

2.5.3 Nitrate use efficiency

The impact of *Trichoderma*-based biostimulants to improve nitrate nutrition under contrasting fertilization in greenhouse and field conditions in lettuce (*Lactuca sativa* L.), rocket (*Eruca sativa* Mill.) and maize was assessed recently (Fiorentino et al., 2018; Vera-Nuñez et al., 2019). *T. virens* (GV41), *T. harzianum* (T22), and 23 *T. harzianum* isolates from maize fields, showed beneficial effects in yield, nutritional characteristics, N- uptake and mineral composition for each crop analyzed. *T. virens* GV41 increased N-use efficiency of lettuce, and favored N-uptake of both lettuce and rocket, particularly when grown under low N availability (Fiorentino et al., 2018). The biomass of maize plants and 15N-uptake directly correlated with the capacity of *Trichoderma* spp. to colonize the rhizosphere (Vera-Nuñez et al., 2019).

2.5.4 Iron acquisition

Low iron availability is frequent in alkaline-calcareous soils, where competition for Fe between roots and microbial populations influence symbiotic or pathogenic relationships. A number of iron-scavenging siderophores have been found to be effective in bacterial and fungal Fe nutrition and in disease suppression in plants. *T. harzianum* T-22 produces tricholignan A, a redox-active ortho-hydroquinone, which reduces Fe (III) and may be part of the chemical mechanisms that adapt plants to grow in Fe-deficient soils (Chen et al., 2019).

2.5.5 Better usage of organic nutriments

A combination of *T. harzianum* with cow and horse manure compost, which provides organic phosphate, triggered a synergic effect in maize plants in biomass production, nutrient content, and photosynthetic activity. In contrast, combinational application of the inoculum with phosphate fertilizer caused a reduction of growth and nitrate content

(Vinci et al., 2018). Metabolomics profiling indicates that the levels of sugars, amino acids and organic acids increase in maize leaves under organic treatments, while stress signatures could be appreciated for treatments with chemical fertilizer only, which failed to support the optimal growth of plants. Here, the molecular and growth responses to combined treatments of either organic or chemical nutriments with *T. harzianum* point to organic fertilization as a very desirable strategy to incorporate its formulations into the field for optimal plant production.

2.6 Soil acidification in *Trichoderma*-plant interactions

The major confirmed traits by which the fungal hyphae influence nutrient dynamics at the rhizosphere include acidification by extrusion of protons either by roots or by the fungal hyphae, which may be directly linked to organic acid and auxin exudation (Fig. 2.2). López-Coria et al. (2016) showed that *T. asperellum* seed treatment enhanced the growth of maize seedlings, IAA production and root acidification. Noteworthy, *T. asperellum* increased the plasma membrane *H*+-ATPase activity in roots and shoots, which pumps protons out of cells and is sensitive to vanadate.

Pelagio-Flores et al. (2017) investigated the effect of *T. atroviride* IMI 206,040 acidification of the medium for *Arabidopsis* growth and development. *Trichoderma* acidification caused changes in auxin redistribution within root tips, interfering with gravitropism and ultimately blocking growth, which could be reversed by buffering the medium. Lateral root formation was a process stimulated very early during the plant-fungus interaction, which could be explained by either an auxin-inducible program or as a stress response to an acidic pH. Application of vanadate interfered with fungal acidification, indicating the involvement of an *H*+-ATPase activity in the membrane transport activity, likely influencing auxin and proton extrusion by the hyphae. These results may help explain how root sensing of pH mediates the interaction of *Trichoderma* with plants. Further research is needed to understand how hormonal and nutritional responses influence fungal-root symbioses.

2.7 Salt stress tolerance mediated by *Trichoderma*

2.7.1 Plant adaptive responses to salinity

Most soils around the world are considered marginal for agriculture since their physicochemical characteristics and nutrient contents fail to support plant growth. Salt content is a major stressing factor for roots because it causes dehydration. Similarly, alkalinity decreases crop productivity due to a high concentration of calcium and carbonate that imposes a strong osmotic pressure. Such adverse conditions may cause root and shoot dysfunction and in consequence, impaired photosynthesis, reproductive development and grain production (van Zelm et al., 2020). Accumulation of osmolites such proline, glycine betaine and polyols inside cells is an important strategy to mitigate negative osmotic pressures and avoid water loss. Moreover, induction of the antioxidant enzymes catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD) and production of glutathione and ascorbic acid,

compounds that detoxify reactive oxygen species (ROS) help plants to decrease oxidative signaling as well as to protect proteins and nucleic acids from damage (Foyer, 2018).

2.7.2 *Trichoderma* improves plant adaptation to salt stress

Endophytic and rhizospheric species of *Trichoderma* are important partners of plants in order to grow in salty and alkaline soils. These fungi have been isolated from such environments and their application to seeds or different substrates clearly improves plant stress adaptation (Gupta et al., 2020). In wheat, seed inoculation of five isolates of *T. harzianum* improved germination and enabled adaptation to salinity of seedlings, which produced longer shoots and roots and accumulated more proline and phenolic compounds (Rawat et al., 2011). Treatment of maize seeds with *T. lixii* ID11D enhanced fresh and dry weights of root and shoots and decreased lipid peroxidation and the maize plants accumulated more protein, proline, chlorophyll, and carotenoids under varied NaCl doses (Pehlivan et al., 2017). *Arabidopsis* and cucumber plants that were primed with *T. asperelloides* T203 resisted better salt treatment, and have an enhanced osmo-protection and less oxidative stress in roots by the production of ascorbic acid, an effective antioxidant (Brotman et al., 2013).

T. atroviride IMI 206,040 promoted lateral root formation and root hair development in *Arabidopsis* grown under salt concentrations that strongly repressed root branching and thus compromised water and nutrient uptake. In this case, sustained root organogenesis was explained by the activity of fungal-released auxin that promotes lateral root initiation and growth as well as better osmotic stress resistance, since the root inoculated seedlings showed higher levels of abscisic acid, l-proline, and ascorbic acid, and enhanced elimination of sodium through root exudates (Contreras-Cornejo et al., 2014). A sodium excluding mechanisms was also induced in *Brassica juncea* L. plants by *T. harzianum* that mitigates salt stress (Ahmad et al., 2015). The fungus reinforced the enzymatic antioxidant system, improved proline accumulation and the nutritional status, having an overall probiotic effect on plant height, root length and dry weight.

In maize seedlings grown in pots containing a highly alkaline (pH 9.30) soil, application of *T. asperellum* showed an increase in K^+ and Ca^{2+} contents, and a decrease in Na^+ concentration. The maize root system was reinforced by increasing the fungal inoculum in a manner that the roots were thicker, more branched and had higher biomass, while the accumulation of osmolytes, antioxidant enzymes, and antioxidant compounds, helped to decrease reactive oxygen species (Fu et al., 2017). *T. longibrachiatum* T6 promoted growth and tolerance to NaCl in wheat and diminished the stress both by inhibiting the expression of genes encoding ethylene biosynthetic enzymes and increasing ACC deaminase activity, which is involved in ethylene catabolism (Zhang et al., 2019). In this manner, the levels of the plant hormone ethylene, which triggers growth arrest upon sensing stress, decreases to a level that permits sustained growth.

2.8 Conclusions and future prospects

The contributions of *Trichoderma* to develop new agricultural applications and to support current field management are very promising. This assumption is based on the finding that fungi and plants communicate through a fine-tuned chemical mechanism involving root

exudates and fungal metabolites (Williams and de Vries, 2020). During the molecular dialogue established, plants attract *Trichoderma* to their roots via releasing energy rich nutrients and exudates in a highly selective manner (Macías-Rodríguez et al., 2018; Lombardi et al., 2018). The root epidermis is able to perceive the hyphae at some distance, because the fungus strongly acidifies the medium. Fungal perception of root exudates down-regulates the genes encoding enzymes involved in the degradation of cellulose and other complex carbon resources, and up-regulates genes that enable the use and acquisition of sugars (Villalobos Escobedo et al., 2020).

The properties of *Trichoderma* as a biofertilizer are very encouraging. Noteworthy, the release of organic acids and Fe-chelating molecules by fungal hyphae helps in plant nutrient acquisition and the application of *Trichoderma* species as biostimulants are opening new promising avenues for an organic agriculture in many crop species (Chen et al., 2019). The identification of mobile oxylipins other than jasmonic acid in orchestrating the *Trichoderma*-induced plant defense reaction (Wang et al., 2020a), which may boost the protection against root nematodes and foliar pathogens raises new questions into how the balance in growth and defense is achieved upon plant perception of fungal metabolites.

Recent knowledge on the roles of reactive oxygen species produced by fungal and plant NADPH oxidases helps to understand the mechanism of root branching, an important and highly desirable root trait for improving soil exploration, nutrient and water acquisition (Villalobos Escobedo et al., 2020). Many genes and proteins from the fungi and plant hosts are increasingly being discovered, which can be managed via transgenic and mutational approaches in order to change their phenotypes and strengthen the symbiosis.

Translational biology approaches aimed at applying basic knowledge from the lab to the field are very desirable and in this regard, a major goal has been the development of *Trichoderma* inoculants for enhanced crop nutritional quality. Moreover, ongoing studies towards discovery and characterization of bioactive metabolites from plants and fungi are expected to contribute to the formulation of natural biostimulants, highly needed to decrease the use of synthetic pesticides that affect pollinators and enter the food chain. The *Trichoderma* era is just in its infancy.

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Deciphering the *Trichoderma*-plant dialog, importance of root exudates

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Plants as primary producers of ecosystems host a myriad of microbial species, which rely on carbon-rich, root-exuded substances for their nutrition, including sugars, organic acids, and amino acids. A large part of the fungal and bacterial microbiome remains neutral, but a few species may establish pathogenic or symbiotic relationships that ultimately influence plant fitness, adaptation, and productivity (1). A major goal towards sustainable crop management is to identify and characterize microbial species with probiotic traits, from which it may be possible to identify highly active substances to develop the new generation of pesticides, biostimulants, and defense elicitors for the growing market. In this scenario, the fungal genus *Trichoderma* is attaining increasing importance since it comprises more than 200 reported species with versatile metabolism and well-adapted properties to proliferate in soil and water environments (2).

Research from the last two decades increased our understanding of the beneficial effects of *Trichoderma* to plants regarding 1) root branching and absorptive potential, 2) usage of organic amendments and fertilizers, 3) growth and development, and 4) adaptation to abiotic and biotic challenges. In summary, many species including *T. virens*, *T. atroviride* and *T. longibrachiatum* may help plants to survive better and enhance productivity in a safe and eco-friendly manner. The impact of these fungi has been assessed under field conditions, which correlated with yield increases in important cereal, fruit and vegetable crops, including maize, wheat, soybean, tomato, grape and lettuce (2).

In recent years, a major question has been how *Trichoderma* adjusts its metabolism according to the highly variable ecological niches and nutritional resources encountered. Apparently, secretion of potent enzymes such as cellulases, chitinases, and peptidases, are the hallmark that enables exploitation of dead wood and decaying leaf, root and stem materials and underscores its ability to parasitize phytopathogen fungi. However, it appears that the repression of genes encoding fungal degradative enzymes enables root colonization by *Trichoderma* and thus the fungus recognizes healthy roots by means of their exudation profiles (3).

Decoding the *Trichoderma* chemical message

Trichoderma is a biofactory of organic substances and releases volatiles, plant hormones, secondary metabolites and small peptides whose molecular composition depends on several factors including the fungal species, nutrient availability and the interaction with microorganism and plants. These info-chemicals can be perceived by roots through free diffusion within the soil and organic matter and during physical contact between the hyphae with the root epidermis, or at later stages, where the fungus spreads to inner cortical cells (4-6).

The first apparent change in the rhizosphere as a consequence of *Trichoderma* presence is the pH acidification (7). It may explain its highly efficient performance to solubilize sparingly soluble phosphates that accounts for a better plant nutrition. As the fungus grows, volatile emissions are thought to sensitize roots and enable long distance root-fungal recognition. 6-pentyl-2H-pyran-2-one (6-PP) is the main volatile from *T. atroviride* blends, which triggers root branching in *Arabidopsis* via

changes in auxin and ethylene transport and response, respectively (4). 1-decene has been found to repress defense, stress and disease response genes, which facilitates fungal spread in root tissues (6). *T. virens* and *T. asperellum* releases auxins, a class of phytohormones with roles in plant growth and immunity that may be directly related to their biostimulant properties (8, 9).

Physical recognition may trigger further reactions in both the fungal and plant partners. Chitin, a major constituent of fungal cell walls has long been considered an elicitor that triggers defensive reactions in plants. Other molecules such as small peptides as well as membrane or cytoplasmic fungal proteins may further alert roots to be prepared for the interaction in order to avoid deleterious effects, making it much more competitive (10). Through the proliferation of lateral and adventitious roots, plants exploit better the mineral and water resources and are more resistant to abiotic stress, and these processes are effectively induced by *Trichoderma*.

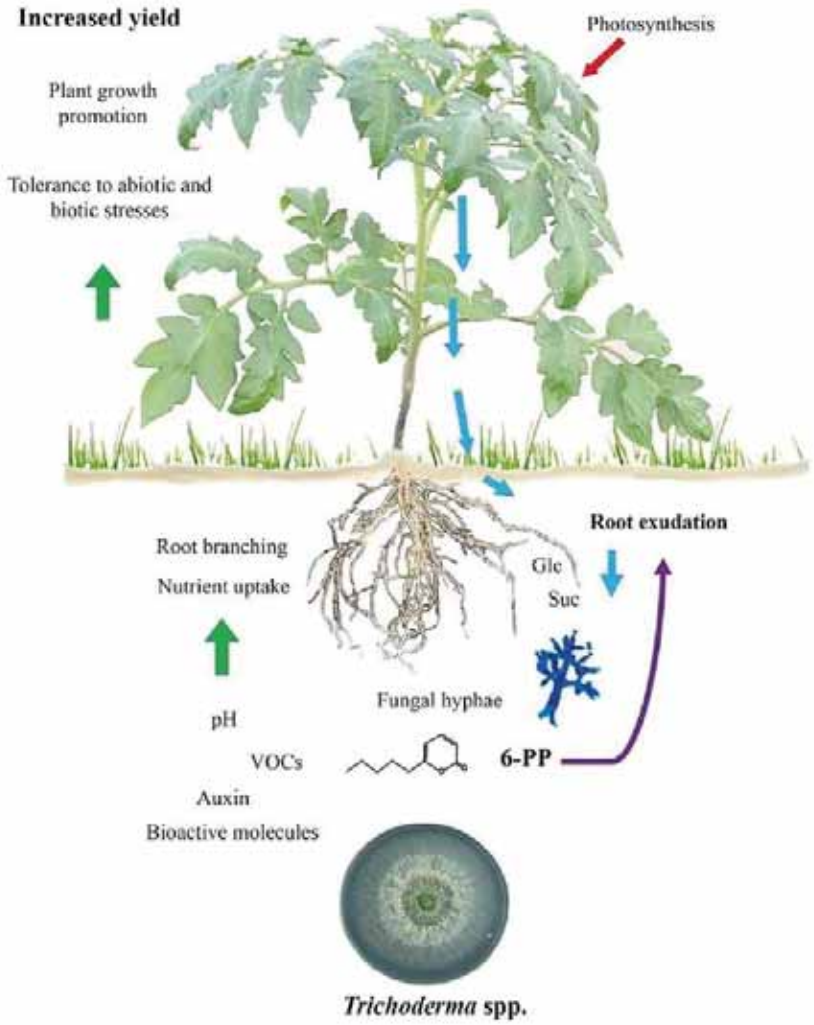

Figure 1.

Trichoderma-induced plant growth and defense depend on root exudates

Growth/defense tradeoffs in plants are modulated during biotic interactions. This ensures developmental transitions to flowering to proceed and warrant seed production. Since *Trichoderma* triggers both plant immunity and growth promotion, it is difficult to ascertain if these programs that are inherently costly may be linked. Moreover, it is possible that fungal-colonized roots could have an enhanced metabolism to support the energetic demand of the symbiosis. Carbohydrate exudation by roots from tomato seedlings has been found to increase in response to *T. atroviride* and sugars may act in a positive chemotactic response to attract the hyphae (11) as reported in figure 1. Consistently, the fungus expresses an intracellular invertase (Triat51014) and two putative sucrose transporters (Triat226844 and Triat83012) to use plant-derived carbon. On the other hand, *T. virens* takes up sucrose via a sucrose transporter and hydrolizes it through an intracellular invertase, which enables rapid growth by the energy provided by this disaccharide (12).

Molecular evidence helps explain how *Trichoderma* changes its metabolic signatures upon detection of root exudates. Villalobos-Escobedo et al. (2020) went further to demonstrate that the expression of genes encoding enzymes involved in complex carbohydrate degradation such as cellulose or chitin prior to root colonization is compromised in *T. atroviride* mutants defective on NADPH oxidase, an enzyme directly involved in the production of reactive oxygen species. Mutation of the corresponding genes not only affects lateral root formation and biomass production in the plant host, but also affects the defense reactions elicited and the fungal saprophytic behavior to acquire and use the simple sugars available in root exudates (3).

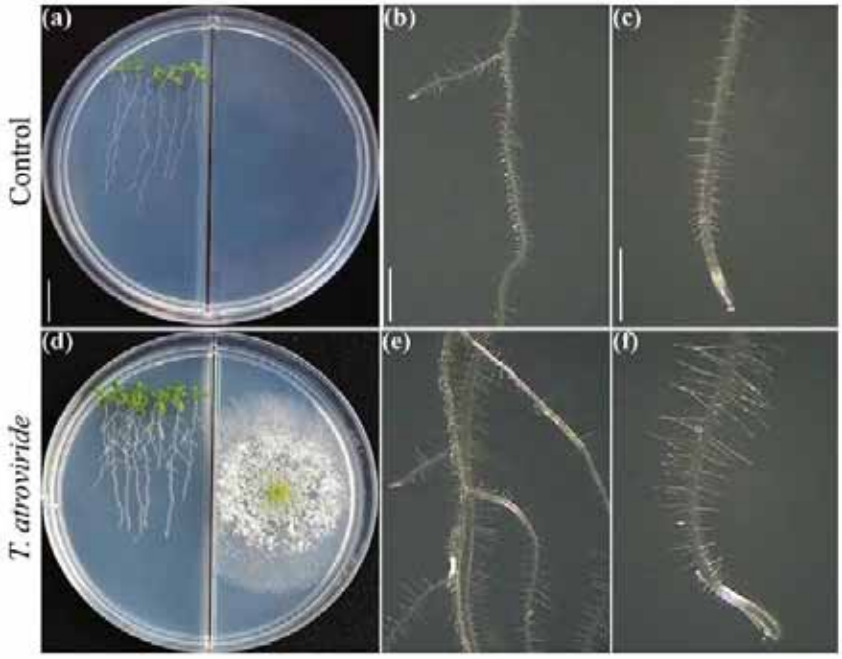

Figure 2.

It has been largely discussed whether the plant traits underlying high biomass production in interactions with microbes rely essentially on emitted carbon dioxide. Certainly, carbon dioxide is a major reactant for photosynthesis and should account for biomass enhancement. Nevertheless tight communication depends on the molecular composition of volatile blends. By testing plant growth and development in divided Petri plates to assess the fungal-plant interaction via volatiles (Fig. 2), we have unveiled the critical role of 6-PP for plant biomass production directly influencing hormonal responses, and shoot-root long distance transport of sugars (Esparza-Reynoso et al. submitted). It supports the notion that a regulatory loop for sugar distribution depending upon photosynthesis, the carbon status of the shoot and the perception of fungal metabolites are critical for mitosis in root meristems. Interestingly, plant growth promoting fungi including *Trichoderma* strains and *Serendipita indica* and *S. williamsii* strongly promote photosynthesis by means of no single compounds, but through mixtures of volatiles (13, 14). Feedback inhibition of carbon dioxide fixation due to elevated sugar levels apparently fails to occur in leaves exposed to fungal volatiles, possibly due to a hormonal imbalance. Plants are not alone, they rely on their fungal symbionts to survive and thrive. Time has come to translate the current knowledge into the implementation of *Trichoderma* for field applications.

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