UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

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ANÁLISIS DE LA EXPRESIÓN DE LOS GENES MARCADORES DE LAS VÍAS DE RESPUESTA A ESTRÉS BIÓTICO Y DEFICIENCIA DE HIERRO MEDIADA POR LA DIMETILHEXADECILAMINA EN PLANTAS DE *Medicago truncatula*

TESIS

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Resumen

Las plantas interactúan tanto con factores bióticos como abióticos a través de mecanismos moleculares y bioquímicos. Ante la presencia de microorganismos o insectos patógenos, las plantas activan diferentes vías de defensa, en las cuales participan fitohormonas como el ácido salicílico (AS), el ácido jasmónico (AJ), el etileno (ET), entre otras. La escasa disponibilidad de nutrientes en el suelo, también genera cambios en las plantas, tal es el caso de la deficiencia de hierro (Fe) durante la cual se activan genes involucrados en la reducción y en el transporte de este elemento. Adicionalmente, existen bacterias benéficas conocidas como rizobacterias promotoras del crecimiento vegetal (PGPR, por sus siglas en inglés de *Plant Growth Promoting Rhizobacteria*) que favorecen la resistencia al estrés biótico y al abiótico mediante distintos procesos, incluyendo la emisión de compuestos orgánicos volátiles (COV).

En el presente trabajo evaluamos el efecto del COV dimetilhexadecilamina (DMHDA) producido por la PGPR *Arthrobacter agilis* UMCV2 sobre el crecimiento de *Medicago truncatula y* en la expresión de los genes de defensa *MtWRKY70*, *MtNPR4*, *MtMYC2* y *MtDef2.1* y los genes de respuesta a deficiencia de Fe *MtbHLH38*, *MtbHLH39*, *MtFIT* y *MtFRO3*. A diferencia del AS y el AJ que activan las vías de SAR e ISR respectivamente, la DMDHA aumentó la expresión de los genes correspondientes a ambas vías. Sin embargo, la inducción es ligera para el caso de los genes *MtWRKY70*, *MtNPR4*, *MtMYC2* y marcada para el gen *MtDef2.1*. Además, la DMHDA promovió el crecimiento de *M. truncatula* e indujo la expresión de los genes involucrados en la toma de Fe, principalmente de los factores de transcripción *MtbHLH38* y *MtbHLH39*; estos resultados contrastan con aquellos obtenidos para el caso del AS y AJ, puesto que estas fitohormonas inhibieron la expresión de los genes de respuesta a deficiencia de hierro y disminuyeron el crecimiento. El aumento en la expresión de los genes de defensa correlacionó con una mayor resistencia a los patógenos *Botrytis cinerea* y *Pseudomonas syringae* en plantas tratadas con DMDHA. En conclusión la DMHDA activa la resistencia a estrés biótico y abiótico a través de una vía alterna al AS y AJ.

Palabras clave: Patógenos, hierro, expresión, defensa.

Abstract

Plants interact with both biotic and abiotic factors through different molecular and biochemical mechanisms. In the presence of pathogenic microorganisms or insects, plants activate different defense pathways with the participation of phytohormones, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), among others.. The scarce availability of nutrients in the soil also generates changes, such is the case of iron (Fe) deficiency during which genes involved in the reduction and transport of this element are activated. In addition, beneficial bacteria, known as Plant Growth Promoting Rhizobacteria (PGPR), promote resistance to biotic and abiotic stress in plants through different processes, including the emission of volatile organic compounds (VOC).

In the present work we evaluate the effect of the VOC dimethyl hexadecylamine (DMHDA) produced by the PGPR *Arthrobacter agilis* UMCV2 on the growth of *Medicago truncatula* and on the expression of the defense genes *MtWRKY70*, *MtNPR4*, *MtMYC2* and *MtDef2.1* and the genes *MtbHLH38*, *MtbHLH39*, *MtFIT* and *MtFRO3* which respond to Fe deficiency. We observed that unlike AS and AJ that activate the SAR and ISR pathways, respectively, DMDHA increased the expression of genes corresponding to both pathways. However, induction is minimal for the genes *MtWRKY70*, *MtNPR4*, and *MtMYC2*; and marked for *MtDef2.1*. Furthermore, DMHDA promoted *M. truncatula* growth and induced the expression of the genes involved in the uptake of Fe, mainly of the *MtbHLH38* and *MtbHLH39* transcription factors; these results contrast with SA and JA, since these phytohormones inhibited the expression of iron deficiency response genes and decreased plant growth. The increase in the expression of defense genes correlated with greater resistance to the pathogens *Botrytis cinerea* and *Pseudomonas syringae* in plants treated with DMDHA. In conclusion, DMHDA activates biotic and abiotic stress resistance through an alternate pathway to SA and JA.

Keywords: Pathogens, iron, expression, defense.

1. Introducción

Las plantas viven en ambientes complejos, en los cuales están expuestas a un amplio rango de factores bióticos. Algunas de las interacciones que ahí se establecen pueden ser benéficas, debido a que se llevan a cabo con hongos o bacterias que promueven el crecimiento vegetal y protegen contra patógenos e insectos herbívoros (Pieterse et al., 2014b). Existen patógenos que son parásitos obligados y que presentan un desarrollo biotrófico donde se mantiene vivas a las células vegetales (Coates y Beynon, 2010). Otros utilizan una estrategia necrotrófica en la que estos se multiplican en el tejido vegetal muerto (Amselem et al., 2011). Algunos patógenos inician su proceso de infección como biótrofos y después cambian a necrótrofos, por lo tanto son conocidos como hemibiótrofos (O' Connell et al., 2012).

Las plantas han desarrollado mecanismos de defensa sofisticados, cuyo enfoque radica en minimizar el éxito de los organismos patógenos. El sistema de defensa inmune innato detecta la presencia de atacantes y activa redes de señalización complejas que conducen a la defensa inducida. Esta requiere de la señalización por fitohormonas (e.g. ácido salicílico, ácido jasmónico y etileno). En este proceso también ocurre la acumulación de especies reactivas de oxígeno (ROS), el fortalecimiento de la pared celular, la deposición de callosa y la expresión de genes de defensa (Nürnberger et al., 2004).

Los procesos de inmunidad están influenciados por la nutrición y el estado metabólico (Datnoff et al, 2007). Diversos reportes señalan la función del hierro (Fe) en la interacción planta-microorganismo (Lemanceau et al., 2009).

El Fe es un elemento esencial, funciona como cofactor para la actividad de diversas proteínas involucradas en procesos celulares esenciales (Pierre y Fontecave, 1999). La relación entre la respuesta de defensa y la deficiencia de Fe, es compleja y depende de varios factores, como el genotipo, el tipo de patógeno y la intensidad y duración de la deficiencia. En algunos casos las plantas son más tolerantes a los patógenos bajo condiciones de deficiencia de hierro probablemente debido a que

los patógenos requieren una cantidad adecuada de hierro para desarrollar virulencia (Kieu et al., 2012; López-Berges et al., 2013). En otros casos, las plantas son más susceptible a los patógenos en condiciones de deficiencia de Fe (Verbon et al., 2017).

Los sideróforos producidos por las rizobacterias promotoras del crecimiento vegetal (PGPR por sus siglas en inglés) pueden inhibir el crecimiento de los patógenos mediante la deprivación de Fe (Verbon et al., 2017). Adicionalmente, estos microorganismos pueden promover la toma de Fe y otros nutrientes a través de la producción de compuestos que acidifican la rizosfera y solubilizan Fe (e.g. ácidos orgánicos) o mediante cambios en la fisiología y arquitectura de la raíz (Orozco-Mosqueda et al., 2013).

Las deficiencia de Fe también incrementa la expresión de genes de defensa como "*Pathogenesis related 1*" (*PR1*) y "*Plant defensin 1.2"* (Def 2.1), de respuesta a patógenos biótrofos y necrótrofos, respectivamente (Koen et al., 2014). Por otra parte existen patógenos que afectan la respuesta a deficiencia de Fe, mediante aumentos en la expresión de genes como "*Natural Resistance-Associated Macrophage Protein 3*" (*NRAMP3*), "*Iron Regulated Transporter*" *IRT1* y "*Ferric Reductase Oxygenase 2* " *FRO2* (Segond et al., 2009) mientras que las PGPR son capaces de activar ambas vías de respuesta (Zhou et al., 2016; Velázquez-Becerra et al., 2011; Montejano-Ramírez et al., 2018).

Las respuestas de defensa y toma de Fe también pueden ser activadas por compuestos orgánicos volátiles (COV) producidos por microorganismos (Martínez-Medina et al., 2017). En nuestro grupo de trabajo se ha demostrado que los COV producidos por la PGPR *Arthrobacter agilis* UMCV2 activan tanto la vía de defensa como la respuesta a deficiencia de hierro (Hernández-Calderón et al., 2018). Dentro de estos COV sobresale la dimetilhexadecilamina (DMHDA) (Velázquez-Becerra et al., 2011) debido a su capacidad para aumentar la expresión de genes involucrados en la síntesis de ácido jasmónico, fitohormona esencial para la activación de defensa ante patógenos necrótrofos (Raya-González et al., 2017). Sin embargo, se desconoce el efecto de la DMHDA sobre los genes de respuesta a patógenos biótrofos, necrótrofos y aquellos involucrados en la toma de Fe y la relación entre

estas dos vías, por lo cual en el presente trabajo se analizará la expresión de los genes de dichas vías en plantas de *Medicago truncatula* crecidas en deficiencia de Fe y tratadas con DMHDA.

2. Antecedentes

2.1 El estrés en las plantas

El estrés hace referencia a las condiciones adversas que afectan el crecimiento, desarrollo y productividad. Durante este fenómeno se dispara un amplio rango de respuestas como cambios en la expresión de genes, en el metabolismo celular, en la disminución en la productividad de los cultivos, etc. Dicho proceso generalmente refleja cambios repentinos en las condiciones ambientales, sin embargo, existen especies tolerantes a esta condición, las cuales logran aclimatarse después de determinado tiempo. El estrés puede ser dividido en biótico y abiótico (Verna et al., 2013).

2.1.1 Estrés biótico

El estrés biótico ocurre ante el ataque organismos denominados patógenos, dentro de los cuales se encuentran virus, bacterias, hongos, nematodos, insectos y arácnidos. Estos agentes privan al hospedero de sus nutrientes llegando a ocasionar la muerte (Gull et al., 2019). De acuerdo con el mecanismo de ataque, los patógenos se clasifican en biótrofos, hembiótrofos y necrótrofos.

Los patógenos biótrofos, como los mohos o especies del género *Peronospora* necesitan células vivas para obtener nutrientes, mientras que los necrótrofos como *Botrytis cinerea* o *Cochliobolus heterostrophus*, dañan el tejido vegetal a través de toxinas y enzimas degradadoras de la pared celular. Por su parte, los hemibiótrofos, se definen como aquellos que tienen un periodo inicial de biotrofía seguido de uno necrotrófico (Perfect y Green, 2001). *Magnaporte grisea*, *Mycospherella graminicola*, *Phytophthora infestans* y *Pseudomonas syringae* pertenecen a este grupo de patógenos. Para hacer frente a estos organismos, las plantas han

desarrollado mecanismos de defensa que se expresan de manera constitutiva o que se inducen ante el reconocimiento del patógeno (Glazebrook, 2005).

2.1.1.1 Mecanismos de defensa en las plantas

El sistema de reconocimiento de patógenos en las plantas está basado en dos componentes: Inmunidad Disparada por Patrones (PTI, por sus siglas en inglés) e Inmunidad Disparada por Efectores (ETI, por sus siglas en inglés) (Jones y Dang, 2006.

La respuesta PTI (**Fig. 1**), inicia después de la percepción de la infección bacteriana o infestación de insectos, para lo cual las plantas poseen Receptores de Reconocimiento de Patrones (PRRs, por sus siglas en inglés). Estos PRRs reconocen moléculas provenientes de los organismos atacantes (Cook et al., 2015). Mediante la detección de estructuras altamente conservadas en los microbios, llamadas Patrones Moleculares Asociados a Microbios (MAMPs, por sus siglas en inglés), se pueden reconocer una amplia variedad de patógenos. De manera similar, los elicitores presentes en la saliva de insectos funcionan como Patrones Moleculares Asociados a Herbívoros. Por su parte, la degradación enzimática del tejido vegetal por microbios o insectos genera elicitores endógenos llamados Patrones Moleculares Asociados a Daño (Ferrari et al., 2013; Savatin et al., 2014; Acevedo et al, 2015). La PTI ocasiona, por ejemplo, el cierre de estomas, así como el refuerzo de la pared celular en el sitio de la infección (Melotto et al., 2006; Underwood, 2012). Esta respuesta puede causar la muerte celular, debido a la liberación de especies reactivas de oxígeno (ROS) (Felix et al., 1999). También ocurre la biosíntesis de la fitohormona etileno y la activación de cascadas de señalización de Proteínas Cinasas Activadas por Mitógenos (MAPK, por sus siglas en inglés), estos eventos regulan la producción de proteínas de defensa así como de metabolitos (Boller y Felix, 2009; Wu y Baldwin, 2010).

La ETI (**Fig. 1**) es una respuesta de defensa más específica que se activa a través de proteínas de resistencia (R). Existen patógenos capaces de evadir PTI mediante la producción de efectores específicos, en un fenómeno conocido como Susceptibilidad Disparada por Efectores (ETS, por sus siglas en inglés) (Pel y

Pieterse, 2013). ETI se caracteriza por una producción rápida de etileno (ET), además de la muerte celular programada en el sitio de la infección que previene ingresos posteriores del patógeno invasor (Broekgaarden et al., 2011). Después del reconocimiento del patógeno se activa una red señalización celular regulada por fitohormonas que activa la producción de proteínas de defensa y metabolitos. Además del ET, existen otras fitohormonas implicadas en esta red regulatoria, como el ácido jasmónico (AJ) y ácido salicílico (AS). Sus interacciones antagonistas o sinérgicas subyacen un extenso potencial regulatorio que permite la activación de defensas específicas (Pieterse et al., 2012).

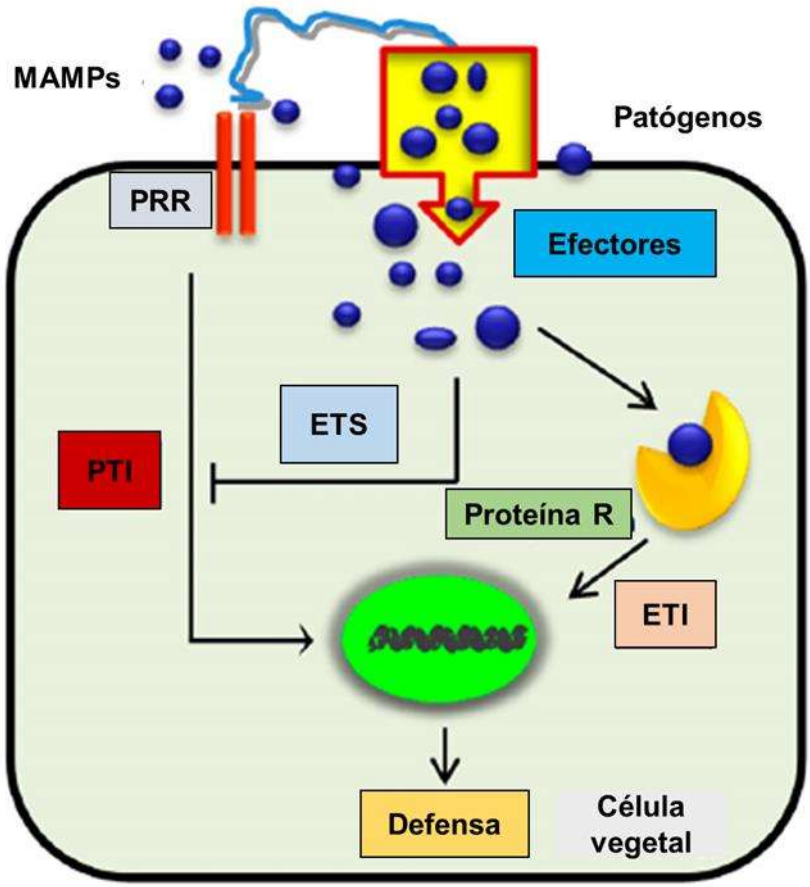

Figura 1. Interacción entre patógenos y plantas. Las moléculas MAMPs son detectadas por proteínas PRR, lo cual dispara PTI. Algunos patógenos inhiben PTI mediante efectores en un proceso conocido como ETS. Las plantas responden a través de la proteína R que reconoce al efector y activa ETI. Modificado de Kazan y Lyons, 2014.

2.1.1.2 Respuestas de defensa mediadas por fitohormonas

Aunque la mayoría de las fitohormonas se han relacionado con la inmunidad en las plantas, destacan el AJ, el AS y el ET(Shigenaga y Argueso, 2016).

El AS, es un compuesto fenólico involucrado en varios procesos que incluyen crecimiento, floración y senescencia además de la respuesta inmune (Raskin, 1992; Vlot et al., 2009; Dempsey et al., 2011). El AS, se sintetiza del corismato, que es el producto final de la vía del shikimato, a través de dos vías biosintéticas. El aminoácido L-fenilalanina, puede ser convertido en ácido salicílico mediante el intermediario benzoato o a través del ácido cumárico, siguiendo una serie de reacciones enzimáticas catalizadas por la enzima Fenilalanina Amonio Liasa (FAL).

El corismato también es convertido en AS vía isocorismato, en un proceso de dos pasos que implica la participación de las enzimas Isocorismato Sintasa (ICS) e Isocorismato Piruvato Liasa (IPL) (Verberne et al., 2000; Wildermuth et al., 2001).

El incremento en los niveles de AS (**Fig. 2**), dispara la respuesta de defensa denominada Respuesta Sistémica Adquirida (SAR) que se activa después de PTI y ETI (Mishina y Zeier, 2007). SAR se define como un fenómeno en el cual las partes no infectadas generan resistencia en respuesta a una infección localizada en otra parte (Ross, 1961). Adicionalmente, durante SAR ocurre la activación de los genes "*Pathogenesis Related Protein* " (*PR*), muchos de los cuales codifican proteínas PR con actividad antimicrobiana. Dentro de los genes *PR*, el mejor caracterizado es *PR1*, el cual es comúnmente usado como marcador de SAR (Van Loon et al., 2006).

Para la iniciación de SAR en órganos distales, se requiere de una cascada de señalización a larga distancia en tejidos vasculares, en la cual la proteína de transferencia de lípidos "*Defective in Induced Resistance1*" (DIR1) actúa como chaperona para la señal móvil de SAR (Maldonado et al., 2002).

Diversos estudios genéticos y bioquímicos proponen varios metabolitos involucrados en la señal a larga distancia de SAR, incluyendo al éster metílico de ácido salicílico (MeSA), diterpenoide dehidroabietinal (DA), glicerol 3-fosfato (G3P), ácido azelaico (AzA) y el ácido pipecólico (Pip). En los tejidos sistémicos, el inicio de SAR requiere la función de "*Flavin-Dependent Monooxygenase 1*" (FMO1), posiblemente para transducir o amplificar las señales a larga distancia originadas desde las hojas primarias (Mishina y Zeier, 2006). Posteriormente, la señalización de SAR, está controlada por la proteína regulada por redox "*Nonexpressor of PR Genes1*" (NPR1), que tras la activación por AS funciona como coactivador transcripcional de un gran conjunto de genes *PR*. En células no inducidas, NPR1 se encuentra secuestrado en el citoplasma como un oligómero a través de enlaces disulfuro intermoleculares (Pajerowska-Mukhtar et al., 2014).

Los cambios en el estado redox celular, inducidos por SA facilitan la monomerización de NPR1, y posteriormente se transloca en el núcleo. NPR1 interactúa con miembros de la familia de factores de transcripción TGA que, junto con los factores de transcripción WRKY18/38/62, se unen a los promotores de los

genes de defensa de respuesta a AS. El funcionamiento adecuado de NPR1 requiere que la proteína sea degradada por el proteasoma, posiblemente para permitir que nuevas proteínas NPR1 reinicien el ciclo de transcripción (Spoel et al., 2009).

WRKY70 es otro de los factores involucrados en la respuesta SAR. La expresión de WRKY70 es activada por AS y reprimida por AJ, sin embargo es independiente de NPR1. Mediante análisis de epistasis se demostró que WRKY70 se encuentra abajo de NPR1 en la vía de señalización dependiente de AS. La modulación de los niveles de transcripción de WRKY70 en líneas transgénicas sobreexpresoras aumenta la resistencia a patógenos virulentos y da como resultado la expresión constitutiva de genes relacionados con la patogénesis inducida por AS. Por el contrario, la supresión antisentido de WRKY70 activa genes de respuesta a AJ. Por lo anterior se sugiere que WRKY70 actúa como un activador de genes inducidos por AS y un represor de genes sensibles a AJ, integrando señales de estas vías mutuamente antagónicas (Li et al, 2004).

Asimismo los parálogos de NPR1, nombrados NPR3 y NPR4 funcionan como receptores de AS que se unen a este con diferente afinidad (Fu et al., 2012). Se demostró que NPR3 y NPR4 funcionan como adaptadores de la ubiquitina de la ligasa E3, "*Cullin 3*" (CUL3) para mediar la degradación de NPR1, regulando su estabilidad y actividad. NPR3 ocasiona la degradación de NPR1 a altos niveles de AS, lo que da como resultado la muerte celular programada durante ETI. En niveles más bajos de SA, como durante PTI o en tejidos distales con respuesta SAR activada, NPR4 estabiliza a NPR1, lo que resulta en la activación de la expresión del gen *PR*. Simultáneamente, NPR1 actúa como un receptor SA, lo que resulta en un cambio conformacional de la proteína que desvela el dominio de activación transcripcional de NPR1 requerido para la activación del gen *PR* (Wu et al., 2012).

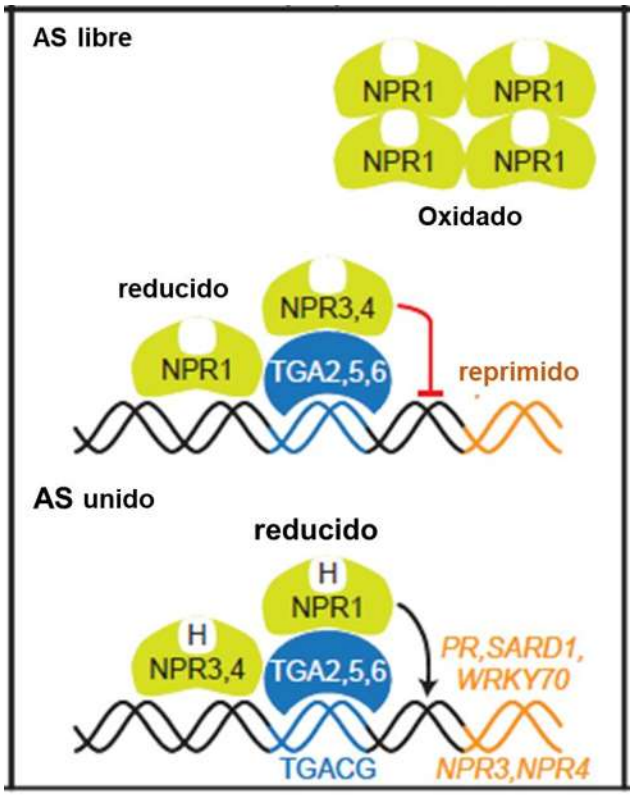

Figura 2. Señalización del ácido salicílico. Mecanismo de regulación transcripcional en ausencia (libre) o presencia (unido) de AS (H). En ausencia de AS, NPR1 se encuentra oxidado en el citoplasma y formando heterodímeros. En presencia de AS, NPR1 se monomeriza y viaja al núcleo, donde interactúa con factores de transcripción tipo TGA para activar la expresión de genes dependientes de AS. NPR3 y NPR4 también funciona como receptores de AS, con diferente afinidad. Adaptado de Waadt, (2020).

La biosíntesis de JA es un proceso complejo mediado por una serie de enzimas. En la mayoría de las plantas, el ácido graso insaturado ácido α-linolénico (18:3) es el precursor de la biosíntesis de JA, aunque algunas plantas pueden utilizar un ácido graso de 16 carbonos. Inicialmente, el proceso de biosíntesis ocurre en el plastidio, donde la enzima 13-lipoxigenasa (LOX) convierte el precursor de 18 carbonos en ácido 13 (S) -hidroperoxi-octadecatrienoico (13-HODE). El 13-HODE es luego catalizado por la aleno óxido sintasa (AOS) y aleno óxido ciclasa (AOC) para formar ácido (+)-12-oxo-fitodienoico (OPDA). La biosíntesis que comienza con el ácido graso de 16 carbonos tiene un conjunto similar de reacciones, produciendo dinor-OPDA (dnOPDA). Luego, OPDA se importa al peroxisoma a través de un

transportador ABC conocido como PXA1, "*Comatose*" o PED3. En el peroxisoma, OPDA es reducido por OPDA reductasa 3 (OPR3) y activado por OPC-8:0 CoA Ligasa 1 (OPCL1). El paso final es la β-oxidación, que elimina carbonos del lado carboxilo de la cadena para producir el ácido jasmónico compuesto de 12 carbonos. La β-oxidación se produce a través de tres enzimas: acil-CoA oxidasa (ACX), una proteína multifuncional con actividad 2-trans-enoil-CoA hidratasa y L-3-hidroxiacil-CoA deshidrogenasa, y 3-cetoa-cil-CoA tiolasa (KAT) (Li et al., 2005).

Una vez que el AJ es sintetizado, este es transferido al citoplasma, y se metaboliza en diferentes estructuras, incluídas metil jasmonato (MeAJ), AJ-isoleucina (AJ-Ile), cis-jasmonio, jasmonyl-1-aminocyclopropano-1-carboxylic acid (AJ-ACC) o ácido 12-hidroxijasmónico (12-OH-JA) (Yan et al., 2016). AJ-Ile es percibido por la proteína nuclear "*F-box CORONATINE INSENSITIVE 1*" (COI1) (**Fig. 3**), la cual en presencia de AJ-Ile se une al represor transcripcional "*JASMONATE-ZIM DOMAIN*" (JAZ) el cual es sometido a una degradación proteosomal mediada por SCF^{COI1}. JAZ funciona en complejo multi-protéico en conjunto con los co-represores "*NOVEL INTERACTOR OF JAZ*" (NINJA) y TPL, MYC2-MYC5 y otros factores de transcripción. La subunidad del complejo mediador MED25 que une factores de transcripción y estos a su vez a la RNA polimerasa II, compite con JAZ por la interacción con COI1 y MYC2. AJ-Ile modula la degradación de JAZ y por lo tanto, promueve las respuestas transcripcionales dependientes de la interacción de MYC2-MED25 (Chini et al., 2016; Howe et al., 2018).

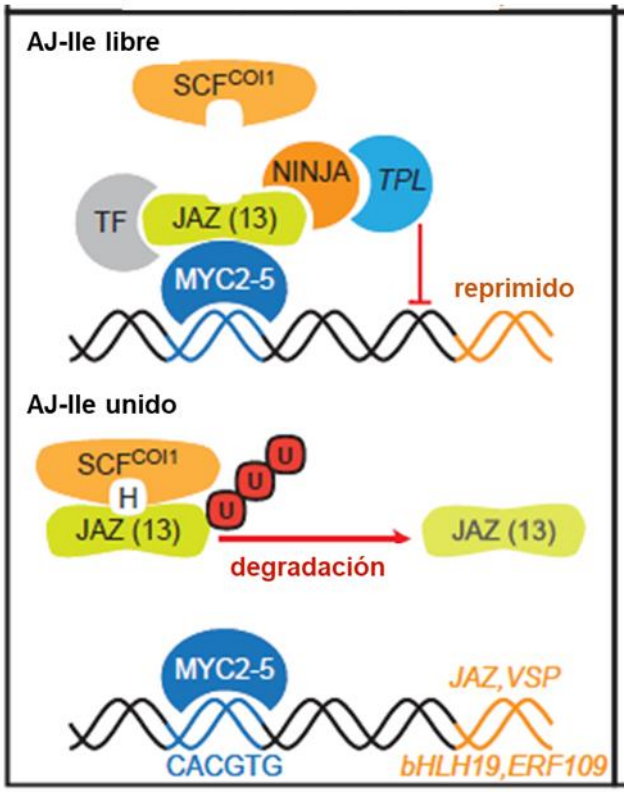

Figura 3. Señalización del ácido jasmónico. Mecanismo de regulación transcripcional en ausencia (libre) o presencia (unido) de AJ-Ile (H). En ausencia de AJ-Ile, los factores de transcripción MYC2-5 se encuentran reprimidos por el complejo multiproteico formado JAZ, NINJA y TPL. Una vez que AJ-Ile es percibido por COI1, JAZ es degradado, permitiendo así la expresión de genes de respuesta a AJ mediada por MYC2-5. Adaptado de Waadt (2020).

Junto con AS, las fitohormonas AJ y ET también son reguladores importantes del sistema inmunológico de las plantas (Thomma et al., 2001). Mediante el uso de mutantes de *Arabidopsis* alteradas en la señalización de AJ o ET, se demostró que estos son elementos centrales en la regulación de la llamada Resistencia Sistémica Inducida (ISR) mediada por rizobacterias (Pieterse et al., 1996). Las Mutantes de señalización de AJ: *jar*1, *jin1* y *coi1* y diversas mutantes de señalización de ET, incluido *etr1, ein2*, *ein3* y *eir1* fueron defectuosas en la respuesta ante *Pseudomonas fluorescens* WCS417r-ISR (Knoester et al., 1999; Pieterse et al., 1998; Pozo et al., 2008). Los resultados fueron similares para otras PGPR, como *Serratia marcescens* 90–166, *P. protegens* CHA0 y *P. fluorescens* Q2-87 y hongos

promotores del crecimiento vegetal (PGPF), como *Penicillium sp.* GP16-2, *Trichoderma harzianum T39* y *P. indica* (Ahn et al., 2007; Hossain et al., 2008; Iavicoli et al., 2003; Korolev et al., 2008; Ryu et al., 2004; Stein et al., 2008; Weller et al., 2012). Lo mismo sucede con otras especies de plantas, como el tomate y el arroz (De Vleesschauwer et al., 2008; Hase et al., 2008; Van der Ent et al., 2009; Yan et al., 2002), apoyando la noción de que el AJ y el ET son esenciales en la regulación de la inmunidad sistémica independiente de AS conferida por microbios benéficos.

En *Arabidopsis*, la vía de señalización de AJ comprende dos ramas separadas: la denominada rama ERF, regulada por los factores de transcripción ERF1 y ORA59 (Lorenzo et al., 2003; McGrath et al., 2005; Pré et al., 2008), y la denominada rama MYC, regulada por los factores de transcripción MYC2, 3 y 4 (Fernández-Calvo et al., 2011). El ET facilita la rama ERF para activar la defensa relacionada genes, como "*Plant Defensin 1.2*" (*PDF1.2*), conduciendo a una respuesta eficaz contra patógenos necrotróficos. La rama MYC, por otro lado, es activada por ácido abscícico (ABA) e induce la expresión de genes relacionados con la defensa, como "*Vegetative Storage Protein 2*" (*VSP2*) en respuesta a insectos masticadores (Lorenzo et al., 2004; Vos et al., 2013).

2.1.2 Estrés abiótico

Debido a su estilo de vida sésil, las plantas están continuamente expuestas varios tipos de estrés ambiental, que Incluyen la sequía, salinidad, calor, frío, congelación, disponibilidad de nutrientes, luz, ozono (O3) (Wang et al., 2003; Chaves y Oliveira, 2004; Agarwal y Grover, 2006; Nakashima y Yamaguchi- Shinozaki, 2006; Hirel et al., 2007; Bailey-Serres y Voesenek, 2008).

Dentro del estrés causado por la disponibilidad de nutrientes, se encuentra la deficiencia de Fe, la cual está ampliamente distribuida, principalmente en suelos calcáreos (aproximadamente un tercio de las tierras cultivadas) que son abundantes en regiones áridas y semiáridas (Briat et al., 2015). Para hacer frente a la deficiencia de Fe, las plantas desarrollan respuestas morfológicas y fisiológicas, principalmente en sus raíces, destinadas a facilitar su adquisición (Kobayashi y Nishizawa, 2012).

2.1.2.1 La deficiencia de hierro en plantas

Para enfrentar los problemas por deficiencia de Fe, las plantas emplean dos estrategias enfocadas a la absorción de dicho metal desde la rizosfera. La estrategia I (**Fig. 4**) es utilizada por las plantas dicotiledóneas y monocotiledóneas no gramíneas, la cual se basa en la acidificación de la rizosfera mediante la liberación de protones a través de la enzima ATPasa AHA2, con la finalidad de aumentar la solubilidad del hierro (Römheld y Marschner., 1986a). Durante la estrategia I participan elementos adicionales, como la secreción de compuestos fenólicos, carboxilatos y flavonoides a partir de la proteína "*Pleiotropic Drug Resistance* 9" (PDR9) para quelar Fe(III) (Rodríguez-Celma et al., 2013). Finalmente, El Fe (III) libre o quelado es reducido a Fe (II) por acción de la enzima férrico quelato reductasa, codificada por el gen "*Ferric Reductase Oxidase* 2" (*FRO2*) (Robinson et al*.,* 1999) para posteriormente, ser internalizado a las células de la raíz a través de la proteína transportadora "Iron Regulated Transporter 1" (IRT1) (Eide et al*.,* 1996). Los genes *FRO* e *IRT* son regulados por el factor de transcripción "*Fer-Like Iron deficiency-induced Transcripcion factor"* (*FIT*) (Bauer et al., 2007). *FIT* es un ortólogo funcional de *FER*, factor de transcripición tipo "*Basic Helix-Loop-Helix"* (bHLH) e interacciona con otros factores de transcripción *bHLH* adicionales (*bHLH38/39/100/101*), para ser funcional (Giehl et al., 2009). A su vez, la expresión de los factores de transcripción *bHLH38/39/100/101 e*s regulada por *bHLH34/104/105*, los cuales forman homodímeros o heterodímeros (Li et al*.*, 2016). Adicionalmente, *bHLH104/105*, interaccionan con *POPEYE (PYE)* otro factor bHLH, involucrado en la regulación positiva de la respuesta a deficiencia por hierro. Un elemento adicional en la respuesta a deficiencia por Fe es BRUTUS (BTS), una ubiquitina ligasa E3 putativa que regula de manera negativa la respuesta a deficiencia por hierro (Long et al*.*, 2010). Además de *AHA2*, *FRO2* e *IRT1*, *FIT* también regula la expresión de los genes *MYB72* y *MYB10* que codifican para factores de transcripción. Juntos, MYB72 y MYB10 inducen la producción de nicotianamina (NA), que es un compuesto quelante de hierro regulando positivamente el gen de la "*NA sinthase*" (*NAS4*) (Palmer et al., 2013). La NA es

importante para la supervivencia de las plantas en suelos alcalinos. Se cree que la NA participa en la distribución de hierro a través del transportador "*Yellow Stripe-Like*" (*YSL2*), pero los mecanismos moleculares implicados son en gran parte desconocidos (Connorton et al., 2017).

Recientemente se identificó al factor de transcripción bHLH121, el cual interactúa con bHLH34/104/105/115 y actúa como un activador transcripcional de los genes clave *bHLH38*, *bHLH39*, *bHLH100*, *bHLH101*, *PYE* y *BTS*. Por otra parte, bHLH121 es necesario para la activación de *FIT* a través de un mecanismo indirecto en respuesta a deficiencia de Fe. El gen *bHLH121* se expresa en toda la planta y su expresión no está afectada por la disponibilidad de Fe. Contrastantemente, la disponibilidad de Fe afecta la localización celular de la proteína bHLH121 en las raíces (Gao et al., 2020).

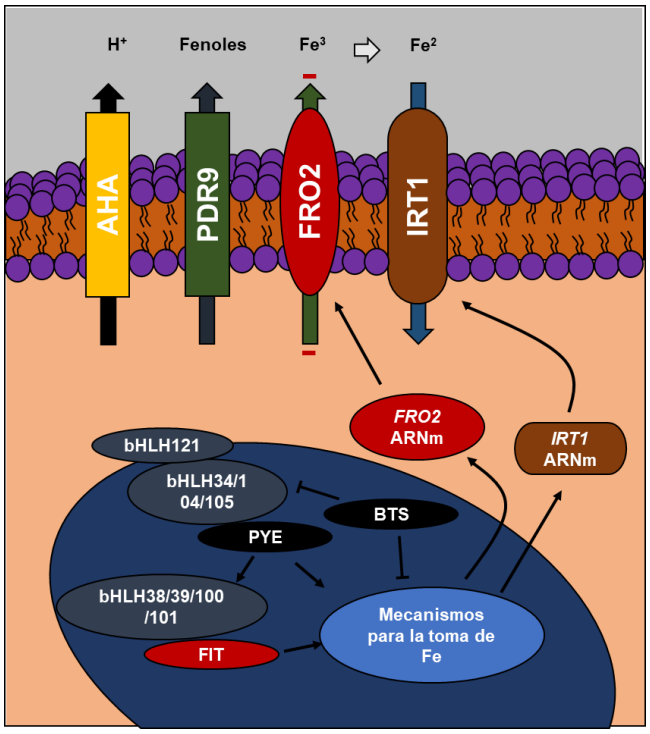

Figura 4. Estrategia I para la adquisición de Fe. Las plantas estrategia I activan la secreción de protones a través de AHA, para la acidificación de la rizosfera. Este mecanismo va acompañado de la liberación de fenoles quelantes de Fe. El Fe subsiguiente, libre o quelado es reducido por la férrico quelato reductasa FRO2 e internalizado por IRT1. Este mecanismo está regulado por los factores de transcripción FIT, PYE, bHLH34/ 38/39/100/101/104/105/ 121 y la ubiquitina ligasa BTS. Elaborado con información de Gao et al., (2020).

Por su parte, las monocotiledóneas gramíneas, emplean la estrategia II (**Fig. 5**) para la absorción de Fe, basada en la quelación de dicho metal. Durante esta estrategia, se liberan fitosideróforos (PS), principalmente, el ácido mugineico (MA), que quela el Fe(III) y es secretado por medio del transportador TOM1 (Römheld y Marschner., 1986b; Nozoye et al, 2011). El complejo Fe(III)-PS es internalizado por el transportador "*Yellow Stripe 1*" (YS1) (Curie et al., 2001).

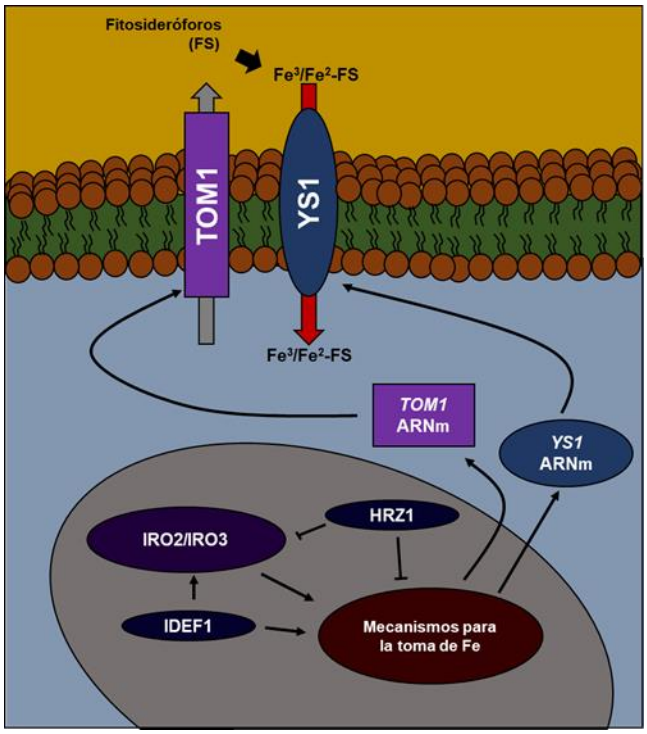

Figura 5. Estrategia II para la adquisición de Fe. En las plantas estrategia II, hay una secreción de fitosideróforos a través de TOM1, que quelan el Fe. Posteriormente, el Fe quelado es internalizado por YS1. El mecanismo de la estrategia II está regulado por los factores de transcripción IRO2/IRO3, IDEF1 y la ubiquitina ligasa HRZ1. Elaborado con información de Nozoye et al, (2011) y Kobayashi et al., (2013).

La respuesta a deficiencia por hierro en monocotiledóneas está controlada por el factor de transcripción IRO2 que regula la expresión de los genes involucrados en la síntesis y transporte de PS (Ogo et al., 2006). Adicionalmente, los factores de transcripción *Iron Deficiency binding Factor* 1 y 2 (IDEF1 y 2) (Kobayashi et al., 2003), regulan la expresión de *IRO2* y de varios genes involucrados en la absorción de hierro: *Yellow Stripe Like 15* (*YSL15*)*, Yellow Stripe Like 2* (*YSL2*) *Iron Regulated Transporter* (*IRT1*)*, Nicotianamine Sintase 2* (*NAS1*)*, Nicotianamine Sintase 2* (*NAS2*) *y Nicotianamine Sintase 3* (*NAS3*) (Kobayashi et al., 2009). La regulación negativa de la respuesta a la deficiencia por hierro en monocotiledóneas, es llevada a cabo por un gen homólogo de *BTS*, denominado *HRZ1*, el cual inhibe la expresión de *IRO2* e *IRO3* (Kobayashi et al., 2013).

2.2 Las moléculas producidas durante la defensa facilitan la toma de hierro

Durante el proceso de defensa, las plantas producen diversos metabolitos secundarios, los cuales derivan principalmente de las vías de isoprenoides, fenilpropanoides, alcaloides o ácidos grasos (Dixon, 2001). Dentro de las moléculas antimicrobianas, los compuestos fenólicos son los más comunes. Las briofitas producen polifenoles y flavonoides, sin embargo, las plantas vasculares tienen la producción más amplia de dichos compuestos (Harbone, 1980). La acumulación de compuestos fenólicos en el tejido de las plantas, se debe a un aumento en la actividad de las enzimas fenilalanina amonio liasa (PAL) (Dixon, 2002), Chalcona sintasa (CHS), entre otras. La actividad de la enzima fosfoenolpiruvato carboxilasa, también aumenta, por lo cual hay un cambio en la producción de sacarosa para favorecer el establecimiento de la defensa (Andersen, 2003). Los compuestos fenólicos confieren varias respuestas fisiológicas a las plantas, para asegurar la sobrevivencia y adaptación a cambios ambientales (Latanzzio, 2009).

Ante la presencia de patógenos, la producción de compuestos fenólicos aumenta. En raíces de chícharo (*Pisum sativum*) infectadas con el hongo *Fusarium oxysporum* se observó una acumulación de fenoles en la pared celular y espacios intracelulares del hospedero, así como en la superficie e incluso dentro de las hifas del patógeno invasor (Benhamou et al., 1996). Adicionalmente, la inoculación de *Serratia marcescens* NBRI1231, una bacteria PGPR, en plantas de Betel (*Piper betle*) infectadas con *Phytophthora nicotianae*, aumentó el contenido de fenoles, principalmente de los ácidos gálico, protocatechuico, clorogénico, cafeico, ferúlico, y elágico (Lavania et al., 2006). Por otra parte, se demostró que los fenoles derivados de plantas, inhiben *per se*, el crecimiento de diferentes especies del género *Pectobacterium*: *Pectobacterium carotovorum*, *P. brasiliensis*, *P. atrosepticum* y *P. aroidearum* desde un 20 a un 100% (Joshi et al., 2015).

En la respuesta a deficiencia por Fe, las plantas estrategia I liberan compuestos fenólicos a través de la proteína PDR9 lo que resulta en la quelación de dicho metal (Rodríguez-Celma et al., 2013). La deficiencia de este nutriente en *Arabidopsis thaliana*, aumenta la expresión de los genes que participan en la síntesis y secreción de compuestos fenólicos pertenecientes a la cumarina, además de la expresión del

gen *PDR9.* En las mutantes *pdr9*, disminuye el crecimiento de las plantas en deficiencia de Fe, además de bajar la expresión de *IRT1* y *FRO2* (Fourcroy et al., 2013). En el caso de la estrategia II, la proteína "*Phenolics Efflux Zero*" (PEZ1) que se localiza en la membrana plasmática y está involucrada en el eflujo de los ácidos protocatechuico (PCA) y cafeico, ambos quelantes de hierro, también se induce por ausencia de hierro (Ishimaru et al., 2010). Las plantas transgénicas con sobreexpresión de *PEZ1* crecen mejor en suelos con pH alto y una baja disponibilidad de dicho metal que las plantas silvestres (Ishimaru et al*.,* 2011). Adicionalmente, el efecto antimicrobiano del PCA ha sido evaluado. El crecimiento de *Bacillus thuringiensis kurstaki* ATCC es menor en deficiencia por hierro, además, durante esta fase estacionaria, se expresa el gen *asbF*, involucrado en la síntesis de PCA por la bacteria. Por lo anterior, se propone que durante la fase de crecimiento de esta bacteria en el suelo, la liberación de este compuesto secuestra al metal previamente mencionado e inhibe el crecimiento microbiano (Williams et al., 2012). Por otra parte, el PCA proveniente de *Veronica montana* L. tuvo efecto antimicrobiano contra *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Micrococcus flavus*, *Listeria monocytogenes*, *Enterobacter cloacae* y *Eschericihia coli* (Stojković et al., 2013). Finalmente, el PCA aislado de *Paenibacillus elgii* HOA73 tuvo un potente efecto antifúngico contra *Botrytis cinerea* y *Rhizoctonia solani* (Nguyen *et al*., 2014). Por su parte, el ácido cafeico inhibe el crecimiento de bacterias como *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescens* y *Staphylococcus aureu*s, hongos como *Aspergillus niger*, *Candida albicans* y *Trichophyton rubrum* (Fu et al., 2010) y tiene un efecto antiviral sobre virus como, hepatitis C (Shen et al., 2013) e influenza A (Utsunomiya et al., 2014).

2.3 Las fitohormonas favorecen la respuesta a deficiencia por hierro

Las fitohormonas son reguladores esenciales para el crecimiento y desarrollo de las plantas, así como de su respuesta al ambiente (Waadt, 2020). Dentro de las fitohormonas relacionadas al desarrollo y crecimiento de las plantas, se encuentran las auxinas (Eyster, 1943), el ácido giberélico (Phinney, 1956), los brasinosteroides (Braun y Wild, 1984) y las citocininas (Kennell, 1960). A pesar de que su efecto

principal es sobre el crecimiento, dichas fitohormonas también modulan la respuesta de defensa (Kazan y Manners, 2009; Navarro et al, 2008; Nakashita et al., 2003; Walters y McRoberts, 2006). Sin embargo, las fitohormonas involucradas principalmente en las vías de resistencia sistémica son el ácido jasmónico, el etileno (Penninckx et al., 1996), el ácido salicílico (Gaffney et al., 1993) y el ABA (Ton et al., 2009).

Las fitohormonas también regulan la toma de Fe. La deficiencia de Fe aumenta la síntesis de auxinas en *Arabidopsis*, lo anterior incrementa la expresión de los genes *FIT* y *FRO2.* Adicionalmente, la aplicación experimental de auxinas estimula la transcripción de dichos genes (Chen et al., 2010). Por otra parte, la producción de ET en plantas de pepino (*Cucumis sativus L*), tomate (*Lycopersicon esculentum* Mill.) y chícharo (*Pisum sativum* L.) crecidas en deficiencia de Fe fue mayor, que en condiciones control (Romera et al., 1999). El tratamiento de *Arabidopsis* y tomate con 1-aminociclopropano-1-carboxylato (un precursor de etileno), aumenta la expresión de *FIT, FRO* e *IRT*. La aplicación de inhibidores de etileno crecidas en Fe limitante, reprime la expresión de estos genes (Lucena et al., 2006). Posteriormente, se demostró que la deficiencia de hierro, aumenta la expresión de los genes *AtSAM1*, *AtSAM2*, *AtACS4*, *AtACS6*, *AtACS9*, *AtACO1*, y *AtACO2*, involucrados en la síntesis de etileno y los genes *AtETR1*, *AtCTR1*, *AtEIN2*, *AtEIN3*, *AtEIL1* y *AtEIL3*, de la señalización de etileno (García et al., 2010).

Respecto a la función del AJ en el establecimiento de la respuesta a deficiencia por hierro, Maurer et al., (2011), proponen a esta fitohormona como un regulador negativo, debido a que plantas de *Arabidopsis* crecidas en deficiencia por Fe y tratadas con 100 µM de metil jasmonato, presentan una inhibición en la expresión de los genes *AtFRO2*, *AtFIT* y *AtIRT1*. Finalmente, el AS aumentó la expresión de los genes *AtbHLH38* y *AtbHLH39*, que son clave para el establecimiento de la respuesta a deficiencia por Fe (Kang et al., 2003). La adición de AS también aumenta el contenido de clorofila en plantas de cacahuate (*Arachis hypogae*) crecidas tanto en condiciones de suficiencia y deficiencia de hierro (Kong et al., 2014).

Por su parte en plantas estrategia II, la producción de etileno en raíces de arroz crecidas en deficiencia de Fe, aumentó y el tratamiento con ACC confirió tolerancia a la deficiencia de dicho metal. Adicionalmente, se demostró que la expresión de *OsIRO2, OsNAS1, OsNAS2, OsYSL15* y *OsIRT1* aumentó, demostrando que el etileno está involucrado en la regulación positiva de sistemas para la absorción de hierro (Wu et al., 2011). Al utilizar plantas de arroz crecidas en condiciones de deficiencia por Fe, Kobayashi et al, (2016), mostraron que varios genes inducidos por AJ también son regulados negativamente por las ubiquitina ligasas "*Hemerythrin motif-containing really interesting new gene (RING)-and zinc-finger protein 1/2*" (OsHRZ1/2) y regulados positivamente por los factores de transcripción IDEF1. Adicionalmente, observaron un incremento en el contenido de JA en plantas transgénicas silenciadas en la expresión de Os*HRZ1* (*iHRZ1)* en condiciones de suficiencia de Fe y que, en plantas no transgénicas, la deficiencia de hierro *per se,* aumentó la síntesis de dicha fitohormona. Por lo anterior, se propone que en estas condiciones, el AJ induce la expresión de *IDEF1*, el cual a su vez aumenta la síntesis de éste y por lo tanto, se incrementa la expresión de los genes involucrados en la toma y translocación de Fe.

2.4 La deficiencia de hierro induce la expresión de los genes de defensa

Adicional a la producción de compuestos antimicrobianos durante la respuesta a deficiencia por Fe (Rodríguez-Celma et al., 2013) que facilitan la toma de este metal y el establecimiento de la defensa; se ha demostrado que en condiciones de deficiencia por Fe, aumenta la expresión de los genes relacionados a defensa. Las plantas de *Arabidopsis*, tratadas con el siderófo bacteriano, crisobactina, mostraron inducción en la expresión de los genes *AtPR1* y *AtPAD4*, involucrados en la respuesta de defensa. Lo anterior, se debe al estatus de deficiencia por Fe, causado por acción del sideróforo (Dellagi et al., 2009). Adicionalmente, se demostró que la inoculación de *Dickeya dadantii* en conjunto con la deficiencia de Fe, aumenta la expresión del gen de defensa *AtPR1* y disminuye la expresión de los genes *Pectato liasa* (*PelA, PelB*, *PelC* y *PelD*) involucrados en el desarrollo de los síntomas de infección. La deficiencia de hierro, también redujo los síntomas de infección

causados por el hongo *Botrytis cinerea* (Kieu et al., 2012) e indujo la expresión de los genes *AtPR1* y *AtPDF1.2* (Koen et al., 2014). Posteriormente, se observó la inducción del gen *MYB72* tanto en condiciones de deficiencia de Fe como en presencia de compuestos orgánicos volátiles producidos por la bacteria *Pseudomonas simiae* WCS417. El factor de transcripción MYB72 regula tanto el establecimiento de la ISR, como la síntesis y secreción de compuestos fenólicos para facilitar la quelación y movilización de hierro durante condiciones deficientes de dicho metal (Zamioudis et al., 2015). Finalmente, la deficiencia de Fe, conduce a la inducción de la expresión de los genes *MPK3/MPK6*, cuyas proteínas participan en la fosforilación de las enzimas ACS2/ACS6, involucradas en la vía de síntesis del etileno; fitohormona clave para el establecimiento de la ISR (Ye et al., 2015).

En el caso de las plantas estrategía II se ha observado que plantas de trigo, tratadas con Fe (III) e infectadas con *Blumeria graminis* f. sp. *tritici* (*Bgt*), mostraron un aumento en la expresión de los genes *PR1a* y *PR1b*, debido a que la presencia del patógeno ocasiona el agotamiento del hierro intracelular de la planta, lo cual causa deficiencia de dicho metal. Los resultados son similares al agregar deferoxamina, un quelante de hierro (Liu et al., 2007). Por otra parte, en plantas de arroz, la expresión del gen *OSRMC* inducido por AJ, aumenta en deficiencia de Fe (Yang *et al*., 2013) y diez de 35 genes que participan en la vía de síntesis de dicha fitohormona, también se inducen en este tipo de estrés (Kobayashi et al., 2016).

2.5 Los microbios benéficos activan las vías de defensa y toma de hierro

La homeostasis de hierro está afectada no sólo por la infección de patógenos, sino también por la colonización de microbiota benéfica específica proveniente del suelo. Entre esta se encuentran las rizobacterias y hongos que promueven el crecimiento y que a su vez desencadenan la ISR. (Lugtenberg et al., 2009; Pieterse et al., 2014). Existe una conexión entre la homeostasis del hierro y la defensa. La activación de la ISR contra *Fusarium* en plantas de rábano, mediada por *Pseudomonas spp.* fue más eficaz en condiciones de bajo contenido de Fe (Leeman et al., 1996). Entre los PGPFs que estimulan tanto la absorción de hierro como la resistencia a

enfermedades, se encuentran *Piriformospora indica* y *Trichoderma spp.*, (Harman et al., 2004; Peskan-Berghofer et al., 2004).

Adicionalmente, existen PGPRs capaces de activar tanto la vía de SAR, ISR y la respuesta a deficiencia de hierro, tal es el caso de *Paenibacillus polymyxa* BFKC01. Esta bacteria Induce al *FIT1*, regulando así la expresión de *IRT1* y *FRO2*. Además de activar ISR mediante un aumento en la transcripción de *MYB72* y *PDF1.2*; y SAR, a través de la inducción de *PR1* y *PR2*. La inoculación de *P. polymyxa* BFKC01 también dispara la producción de compuestos fenólicos que funcionan como antimicrobianos y solubilizan hierro.

Algunos microbios activan las vías de defensa y toma de hierro, mediante la producción de COV, por ejemplo, los hongos *Trichoderma asperellum* y *T. harzianum*, cuyos COV aumentaron la expresión de los genes *bHLH38*, *bHLH39*, *FRO2* y *PDF1.2* en *A. thaliana* (Martínez-Medina et al., 2017)

En nuestro grupo de trabajo se cuenta con *Arthrobacter agilis* UMCV2, una PGPR aislada de la rizosfera de maíz (*Zea mays*) y que produce COV (Valencia-Cantero et al., 2007; Velázquez-Becerra et al., 2011).

2.6 *Arthrobacter agilis* UMCV2

Los miembros del género *Arthrobacter* (Conn y Dimmick 1947; Skerman et al., 1980) son bacterias Gram-positivas, muestran bacilos en crecimiento exponencial y cocos en su fase estacionaria, capaces de crecer tanto en condiciones aeróbicas como anaeróbicas y pertenecen al filo *Actinobacteria* (Garrity y Holt, 2001).

A. agilis presenta un ADN con un contenido de GC de 67-69%, es una bacteria quimioorganotrófica, saprofítica, con metabolismo respiratorio aerobio, no forma esporas, es móvil debido a la presencia de uno o tres flagelos, aunque existen cepas que no son móviles, su crecimiento óptimo ocurre entre 20 y 30°C, el cual no se observa a los 37°C. Su hábitat va desde agua y suelo hasta piel humana. Es susceptible a la penicilina, estreptomicina, cloranfenicol, tetraciclina, eritromicina, novobiocina, ampicilina, carbenicilina y gentamicina. Es resisten a las lisozimas (Koch et al., 1995).

Diferentes especies de *Arthrobacter* han sido implicadas en la promoción del crecimiento de las plantas (Manzanera et al., 2015), la producción de enzimas de importación industrial (Kallimanis et al., 2011; Kiran et al., 2015), como xeroprotector (Manzanera et al., 2014; SantaCruz-Calvo et al., 2013). Estos informes sugieren que las especies de *Arthrobacter* albergan genes para codificar enzimas que pueden ser útiles en la industria, la agricultura y la biotecnología.

Adicionalmente, existen reportes de cepas psicotrópicas de *A. agilis*, como L77, la cual es una PGPR aislada del lago subglacial Pangong, utilizando diluciones seriales en tripticasa. Esta bacteria produce encimas que son activas en frío, además de exopolisacáridos y compuestos anticongelantes (Sing et al., 2016).

A. agilis UMCV2 es una bacteria aislada de la rizosfera de maíz, de un suelo ligeramente ácido, la cual se identificó mediante la secuenciación del gen 16s rRNA. Esta bacteria estimula el crecimiento vegetal mediante el incremento de la biodisponibilidad de hierro (Valencia-Cantero et al., 2007).

La inoculación de *A. agilis* UMCV2 en *Pinus devoniana*, promueve el crecimiento en la primera etapa de desarrollo (primeros 65 días) incrementando la talla de la parte aérea y la proliferación de raíces laterales, además incrementa el índice de germinación. En condiciones de invernadero, se presentan resultados similares, mejorando la velocidad de crecimiento (Montejo-Mayo et al., 2016; Valencia-Cantero et al., 2015). Al igual que muchas PGPRs, *A. agilis* UMCV2 produce diversos COV, dentro de los cuales la dimetilhexadecilamina (DMHDA) es el principal compuesto bioactivo (Velazquez-Becerra et al., 2011).

2.7 Bioactividad de la dimetilhexadecilamina

La PGR *A. agilis* UMCV2 produce diversos COV, los cuales fueron identificados por microextracción en fase sólida-cromatografía de gases-espectrometría de masas (MEFS-CG-EM). En el análisis de MEFS-CG-EM, se encontró 2,5-dimetil-pirazina, ácido acético, DMHDA, quinolina, 3-metil y 2,4-bis(1,1-dimetil)-fenol. La dimetilhexadecilamina fue de particular interés debido a su similitud estructural al grupo de acil-L-homoserina-lactonas (AHL) y alcamidas que promueven el crecimiento vegetal (Velázquez-Becerra et al., 2011).

Se ha observado que la DMHDA tiene diversos efectos benéficos. A una concentración de 8 µM, aumenta la biomasa y la longitud del tallo de la leguminosa *Medicago sativa* (Velázquez-Becerra et al., 2011). En gramíneas como *Sorghum bicolor* crecidas en deficiencia de Fe, la DMHDA aumentó la longitud y biomasa de brote y raíz, además de incrementar la expresión del gen *SbFRO* (Castulo-Rubio et al., 2015). En el caso de *P. devoniana* la DMHDA incrementó el crecimiento un 62% a los 126 días, desde la primera medición realizada a los 21 días, lo anterior en comparación con un 22% observado en plantas control. También hubo una estimulación de 90% en el grosor de la parte media del tallo, en comparación con un incremento de 25% en las plantas control.

Interesantemente, en plantas de *A. thaliana*, la DHMDA inhibe el crecimiento de la raíz primaria afectando la división y la elongación celular, pero promovió la formación de raíces laterales y pelos radiculares (Raya-González et al., 2017). Esta represión en el crecimiento, es antagonizada por la kinetina y correlación con una inhibición en la expresión de los genes marcadores *ARR5::GUS* y *TCS::GFP* relacionados con citocinina en las raíces primarias de *Arabidopsis* (Vázquez-Chimalhua et al., 2019).

Por otra parte, la DMHDA protege a las plantas ante la presencia de patógenos necrótrofos como *B. cinerea*, hemibiótrofos *como Phytophthora cinnamomi* y xilótrofos como *Hipocrea* o *fusarium* (Velázquez-Becerra et al., 2013; Orozco-Mosqueda et al., 2015). Este efecto en parte se explica debido a la inducción mediada por DMHDA del gen *LOX2*, el cual participa en la biosíntesis de AJ (Raya-González et al., 2017).

2.8. Efecto de *Athrobacter agilis* UMCV2 y la DMHDA sobre *Medicago truncatula*.

M. truncatula es una especie anual, importante en el sur de Australia: proporciona alimento para el ganado, beneficia la fertilidad del suelo a través de la fijación de nitrógeno. Esta leguminosa es de tamaño pequeño, tiempo de generación corto y presenta autofertilización, lo cual le confiere ventajas para ser un modelo biológico. Es una planta diploide, tiene un genoma relativamente pequeño, con un total de

500-550 Mpb, por lo que es ampliamente utilizada en estudios de genómica funcional y estructural (Bell et al., 2000).

Existen diferentes ecotipos de *M. truncatula* con variaciones genéticas amplias (Ellwood et al., 2006). Entre los ecotipos de *M. truncatula*, jemalong A17 se ha sido usada para el proyecto de secuenciación del genoma (Choi et al., 2004; Young et al., 2005), mientras que el ecotipo R108, es usado para la transformación genética, debido a su regeneración superior *in vitro* (Hoffmann et al., 1997). Los reportes demuestran que A17 y R108 difieren fenotípicamente (Schnurr et al., 2007; Bolinge et al., 2010), en su respuesta a estrés abiótico (de Lorenzo et al., 2007) y estrés biótico (Salzer et al., 2004; Gaige et al., 2012).

En nuestro grupo de trabajo se demostró que *A. agilis* UMCV2 es una PGPR endófita de la planta *M. truncatula* jemalong A17 (Aviles-Garcia et al., 2016) y que su inoculación en dichas plantas crecidas en deficiencia de Fe y en condiciones de invernadero, activa la maquinara involucrada en la adquisición del metal mencionado, debido a la inducción en la expresión de los genes *MtFRO* (*MtFRO1, MtFRO2, MtFRO3, MtFRO4* y *MtFRO5*). Adicionalmente, la presencia de la bacteria aumentó la expresión de los genes *MtPR1* y *MtDef2.1*, involucrados en las vías de defensa SAR e ISR, respectivamente (Montejano-Ramírez et al., 2018).

Tanto la DMHDA como el conjunto de COV producidos por *A. agilis* UMCV2, indujeron la estrategia I para la adquisición de hierro en plantas de *M. truncatula* crecidas en cultivo *in vitro* y en condiciones de deficiencia de Fe. Lo anterior debido a un aumento en la actividad Fe-reductasa, la acidificación del medio rizosférico y en el contenido de clorofila (Orozco-Mosqueda et al., 2013). Sin embargo, en *M. truncatula* se desconoce si la inducción en los genes de las vías de defensa por la inoculación de *A. agilis* UMCV2, es debido a la bioactividad de la DMHDA. Con la finalidad de esclarecer este fenómeno, en el presente trabajo, *M. truncatula* será nuestro modelo de estudio.

2.9 Descripción de *Medicago truncatula*

M. truncatula se compone de un eje principal que se puede organizar ya sea en una roseta o como un eje alargado, y ramas de diferentes órdenes. La Morfología y

arquitectura de la planta, varía entre los genotipos y son dependientes del ambiente y sus condiciones (Aitken, 1955).

Cada eje en una planta se compone de fitómeros. Un fitómero se compone de un entrenudo, una hoja y un meristemo axilar (Lecoeur, 2005). La germinación de *M. truncatula* es epigea (los cotiledones se expanden sobre el suelo). Una vez que los cotiledones emergen, las hojas que se producen por lo general se organizan en una roseta que es el eje principal de la planta. El acomodo de las hojas es en filotaxia alternada (Moreau, 2006).

La rama primaria se forma de la axila de la primera hoja desarrollada del eje principal (la hoja unifoliada). Después, aparecen sucesivamente las otras ramas primarias, a partir de la axila de la que se desarrollan. Puesto que cada rama primaria se desarrolla de la axila de una hoja del eje principal, la disposición de las ramas primarias en la planta es parecida a la filotaxia del eje. Las ramas secundarias y terciarias generalmente se desarrollan en la axila de las hojas de las ramas primarias y secundarias respectivamente (Moreau, 2006).

M. truncatula muestra un crecimiento indeterminado, dando lugar a un período prolongado de producción de flores y semillas después de desarrollar el meristemo de la inflorescencia primaria. La planta es autógama y las semillas son autofertilizadas de manera eficiente en ausencia de polinización de insectos (Gallardo et al., 2006).

Posee pequeños racimos de inflorescencias, los cuales llevan de 1 a 5 flores amarillas de 5-8 mm de longitud. Las flores contienen 10 óvulos y únicamente abren después de que su polen ha fertilizado sus óvulos. La semilla es de color marrón claro arriñonada. Las flores poseen un “mecanismo tripping”, que es una característica floral compartida en todo el género *Medicago* (Lesins y Lesins, 2012).

3. Justificación

En *Medicago truncatula* la expresión de los genes *MtPR1* y *MtDEF2.1*, aumenta en deficiencia de hierro. Además cuando las plantas son crecidas en dichas condiciones e inoculadas con la bacteria productora de DMHDA, *A. agilis* UMCV2 se observa un efecto sinérgico sobre estos genes. Por otra parte, se sabe que la DMHDA estimula tanto la vía de síntesis del ácido jasmónico como la activación de genes de respuesta a deficiencia de hierro, sin embargo, se desconoce si el efecto que tiene esta molécula sobre la vía de AJ, es suficiente para inducir la expresión de los genes de resistencia sistémica y si a la par, se activan tanto las vías defensa como la deficiencia de hierro.

4. Hipótesis

La DMHDA induce la expresión conjunta de las vías de señalización de resistencia a estrés biótico y deficiencia de hierro e incrementa la resistencia al ataque de patógenos en *M. truncatula*.

5. Objetivo general

Analizar la expresión de los genes marcadores de las vías de respuestas a estrés biótico y deficiencia de hierro en plantas de *M. truncatula* tratadas con DMHDA y si se incrementa la resistencia al ataque de patógenos.

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5.1 Objetivos particulares

1.- Identificar los elementos genéticos de las vías de defensa y respuesta a deficiencia de hierro en *M. truncatula*

2.- Analizar el efecto de la DMHDA, ácido jasmónico y ácido salicílico sobre genes marcadores de las vías de respuesta a deficiencia de hierro, resistencia sistémica inducida y resistencia sistémica adquirida en *M. truncatula*

3.- Analizar el efecto de la DMHDA en la resistencia de plantas de *M. truncatula* a *Pseudomonas syringae* y *Botrytis cinerea.*

6. Resultados

6.1. Capítulo 1. Bacterial Compound N,N-Dimethylhexadecylamine Modulates Expression of Iron Deficiency and Defense Response Genes in *Medicago truncatula* Independently of the Jasmonic Acid Pathway.

En el presente capítulo, se muestra el artículo correspondiente a la investigación realizada al presente proyecto, el cual fue publicado en la revista *Plants* que cuenta con un factor de impacto de 2.632.

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Article

Bacterial Compound *N,N*-Dimethylhexadecylamine Modulates Expression of Iron Deficiency and Defense Response Genes in *Medicago truncatula* Independently of the Jasmonic Acid Pathway

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Abstract: Plants face a variety of biotic and abiotic stresses including attack by microbial phytopathogens and nutrient deficiencies. Some bacterial volatile organic compounds (VOCs) activate defense and iron-deficiency responses in plants. To establish a relationship between defense and iron deficiency through VOCs, we identified key genes in the defense and iron-deprivation responses of the legume model *Medicago truncatula* and evaluated the effect of the rhizobacterial VOC *N,N*-dimethylhexadecylamine (DMHDA) on the gene expression in these pathways by RT-qPCR. DMHDA increased *M. truncatula* growth 1.5-fold under both iron-sufficient and iron-deficient conditions compared with untreated plants, whereas salicylic acid and jasmonic acid decreased growth. Iron-deficiency induced iron uptake and defense gene expression. Moreover, the effect was greater in combination with DMHDA. Salicylic acid, *Pseudomonas syringae*, jasmonic acid, and *Botrytis cinerea* had inhibitory effects on growth and iron response gene expression but activated defense genes. Taken together, our results showed that the VOC DMHDA activates defense and iron-deprivation pathways while inducing a growth promoting effect unlike conventional phytohormones, highlighting that DMHDA does not mimic jasmonic acid but induces an alternative pathway. This is a novel aspect in the complex interactions between biotic and abiotic stresses.

Keywords: bacterial organic volatile compound; salicylic acid; iron deprivation; cross-talk in stress-response pathways

Introduction

Plants are sessile organisms that interact with biotic and abiotic factors. Biotic factors include both beneficial microorganisms, such as plant growth promoting rhizobacteria (PGPR) and phytopathogens, and herbivorous insects. Therefore, plants are vulnerable to various biotic attacks [1]. To cope with biotic stress, plants have a well-developed immune system. Through the presence of membrane proteins known as pattern recognition receptors, plants can recognize diverse elicitors, such as molecular patterns associated with microbes. These elicitors include flagellin, lipopolysaccharides, peptidoglycan, elongation factors, and siderophores, which are present in beneficial microorganisms and pathogens [2]; molecular patterns associated with herbivores, which include saliva and regurgitants of herbivorous insects [3]; and molecular patterns associated with damage, which include DNA, extracellular ATP, systemin, and oligogalacturonides produced in response to damage in plant cells [4].

Once elicitors have been recognized, plants activate pattern-triggered immunity (PTI) as a first basal defense mechanism [5]. In addition to PTI, plants have systemic defense mechanisms that protect

organs not exposed to microorganisms [6]. These are known as systemic acquired resistance (SAR) and induced systemic resistance (ISR) [7,8]. SAR is activated in response to pathogenic microorganisms with a biotrophic lifestyle and depends mainly on the production of salicylic acid (SA) [9,10]. ISR is activated in response to necrotrophic pathogens, herbivores, and even PGPR by increased synthesis of jasmonic acid (JA) [10,11].

Plants also interact with abiotic factors, mainly through nutrient provision, which includes iron, an essential metal for vital metabolic processes in plants [12]. Although iron is abundant, its bioavailability is restricted in alkaline or calcareous soils because it forms insoluble Fe^{3+} oxyhydroxide complexes, which are not available for plant uptake [13,14]. Therefore, plants have developed two strategies to increase iron uptake. Strategy I is used by dicotyledonous and monocotyledonous nongraminaceous plants and is based on rhizosphere acidification through proton release by an ATPase [15], followed by the reduction of Fe^{3+} to Fe^{2+} by the membrane protein ferric-chelate reductase. This is encoded by the ferric reduction oxidase 2 gene (*FRO2*) [16] and the subsequent internalization of Fe^{2+} to plant root epidermal cells by the protein iron-regulated transporter 1 (IRT1) [17]. The expression of *FRO2* and *IRT1* genes is regulated by the basic helix-loop-helix (bHLH) transcription factor FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT1) [18]. Additionally, FIT forms heterodimers with bHLH38 and bHLH39 [19], and bHLH100 and bHLH101 [20]. Strategy II is used by monocotyledonous grass plants and consists of the production and release of phytosiderophores that chelate iron to internalize the Fe^{3+} phytosiderophore complex through the protein yellow stripe 1 (YS1) [14].

Previous studies have related SAR and ISR with iron-deficiency response. In strategy I, Fe deficiency promotes the release of phenolic compounds through the protein pleiotropic drug resistance 9 (PDR9) to chelate and solubilize Fe^{3+} [21]. Additionally, these compounds exhibit both antimicrobial and antifungal activities [22]. At the genetic level, *Arabidopsis thaliana* plants grown under iron-deficient conditions show an increase in the expression of the *pathogenesis related 1* (*PR1*) and *plant defensin 1.2* (*PDF1.2*) genes, which are markers of the SAR and ISR pathways, respectively. Furthermore, when these plants were inoculated with *Botrytis cinerea*, there was a synergistic effect [23]. Similarly, there are pathogens capable of activating the response to iron deficiency, such as *Dickeya dadantii*, which, when inoculated in *A. thaliana*, increase the expression of the genes "*Natural Resistance-Associated Macrophage Protein 3*" (*NRAMP3*), *IRT1,* and *FRO2* [24].

PGPR also increase resistance to iron-deficiency stress, such as in the case of *Paenibacillus polymyxa* BFKC01, which, when inoculated in *A. thaliana,* induces the expression of *FIT1*, *FRO2,* and *IRT1* genes [25]. Similarly, these PGPR increase the expression of the *PR1*, *PR2,* and *PDF1.2* defense genes. Another beneficial microorganism that can activate both iron-deficiency response and defense pathways is *Arthrobacter agilis* UMCV2, which, in addition to promoting the growth of *Medicago sativa* [26], increases the expression of *MtFRO1*, *MtFRO2*, *MtFRO3*, *MtFRO4*, *MtFRO5,* and *MtDef*2.1 genes in *M. truncatula* plants, and the effect was synergistic when plants were grown under iron-deficient conditions [27].

Some microorganisms may activate iron-deficiency response and defense pathways through the emission of volatile organic compounds (VOCs) [28]. Treatment of *A. thaliana* plants with VOCs from the fungi *Trichoderma asperellum* and *T. harzianum* increases the expression of *bHLH38*, *bHLH39*, *FRO2,* and *IRT1* genes, which are involved in iron uptake, in addition to increasing the expression of the *PDF1.2* gene of the ISR pathway, and therefore, *A. thaliana* resistance to the fungus *B. cinerea.* The effect is similar in *Solanum lycopersicum* plants.

PGPR, such as *A. agilis* UMCV2 [26], *Sinorhizobium meliloti* 1021 [29], or *Pseudomonas fluorescens* UM270 [30], produce VOCs that induce plant growth. Of the VOCs profiled for these bacteria, *N*,*N*-dimethylhexadecylamine (DMHDA) has been highlighted. DMHDA promotes plant growth [26,31,32] and activates responses to iron deficiency through, e.g., the acidification of the rhizosphere in *M. truncatula* [31] and the induction of *SbFRO1* genes in *Sorghum bicolor* [32]. Additionally,

DMHDA activates the expression of the *LOX2* gene that participates in the synthesis of JA in *A. thaliana* [33].

Recently, our research group has shown that VOCs produced by *A. agilis* UMCV2 increase the expression of *SbIRT1* and *SbIRT2* genes involved in iron transport, of *SbCOI1* involved in the ISR pathways, and *SbPR1* involved in SAR pathways [34]. However, whether DMHDA is responsible for inducing these genes remains unclear. Thus, we hypothesized that DMHDA triggers the JA pathway in plants, and through this mechanism, iron-deficiency and defense responses are induced. In the present study, we identified key genes in the defense and iron-deprivation response of the legume model *M. truncatula* and evaluated the effect of DMHDA on the expression of genes involved in the iron-deficiency and defense pathways of *M. truncatula* plants exploring the interaction between biotic and abiotic stresses.

Results

. Effect of Iron Deficiency on M. truncatula Growth

We employed iron-deficient growth conditions to induce iron-deficiency stress in *M. truncatula* plants. To verify the system utility, first, we analyzed the phenotype caused by our plant growth conditions. It was observed that the iron-deficient conditions decreased the length and weight of plant roots and shoots, the number of lateral roots and trifoliate leaves, and chlorophyll content compared with those of the control (Figure 1). Based on these results, we concluded that the system had effectively induced iron-deprivation stress in the plants [35].

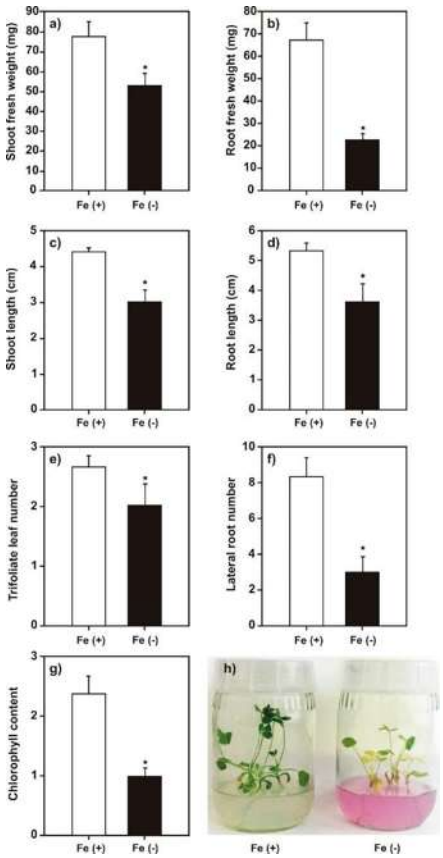

Figure 1. Effect of iron deprivation on *Medicago truncatula* growth. The *M. truncatula* plants cultured under iron sufficiency (control) or iron deficiency for 14 days. (**a**) Shoot fresh weight; (**b**) root fresh weight; (**c**) shoot length; (**d**) root length; (**e**) trifoliate leaf number; (**f**) lateral root number; and (**g**) chlorophyll

content. Panel (**h**) show the phenotypes of plants in the treatments. The asterisk above the standard error bars indicates a significant difference between treatments calculated using Student's *t*-test ($p < 0.05$; $n = 9$).

Effect of SA, JA, and DMHDA on M. truncatula Growth

We analyzed the effect of SA (100 µM), JA (20 µM), and DMHDA (8 µM) on plant growth under both iron-sufficient and iron-deficient conditions. Plants treated with 100 µM of SA (Figure 2) and 20 µM of JA (Figure 3) showed a decrease in the length and weight of shoots and roots, the number of lateral roots, and chlorophyll content. The effect on growth was greater in plants treated with JA. The combination of SA or JA with iron deficiency had a synergistic effect on the decrease in shoot and root length, number of lateral roots, and chlorophyll content. A less clear effect was observed on the number of trifoliate leaves (Figures S1 and S2).

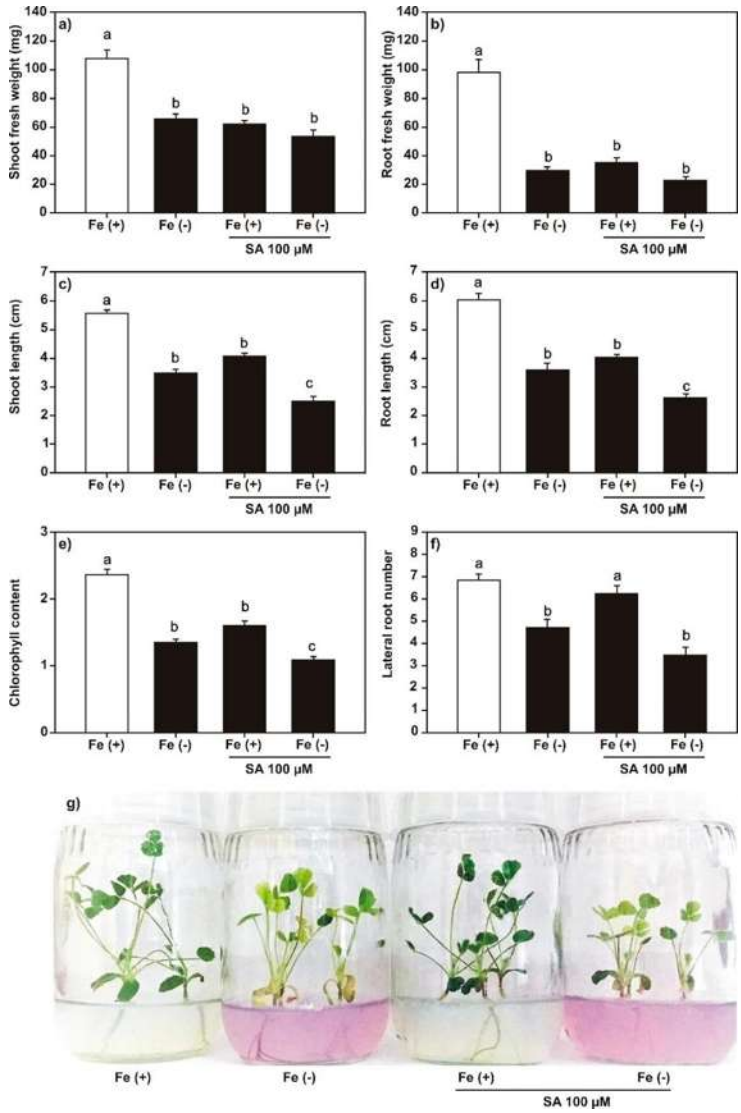

Figure 2. Effect of salicylic acid (SA) on *Medicago truncatula* growth. The *M. truncatula* plants were cultured in MS medium with SA (100 µM) under both iron sufficiency (control) and iron deficiency for

14 days. (**a**) Shoot fresh weight; (**b**) root fresh weight; (**c**) shoot length; (**d**) root length; (**e**) chlorophyll content; and (**f**) lateral root number. Panel (**g**) shows the phenotypes of plants in the treatments. Different lowercase letters above the standard error bars from all graphics indicate significant differences between treatments calculated with two-way ANOVA and Tukey's test ($p < 0.5$; $n = 9$).

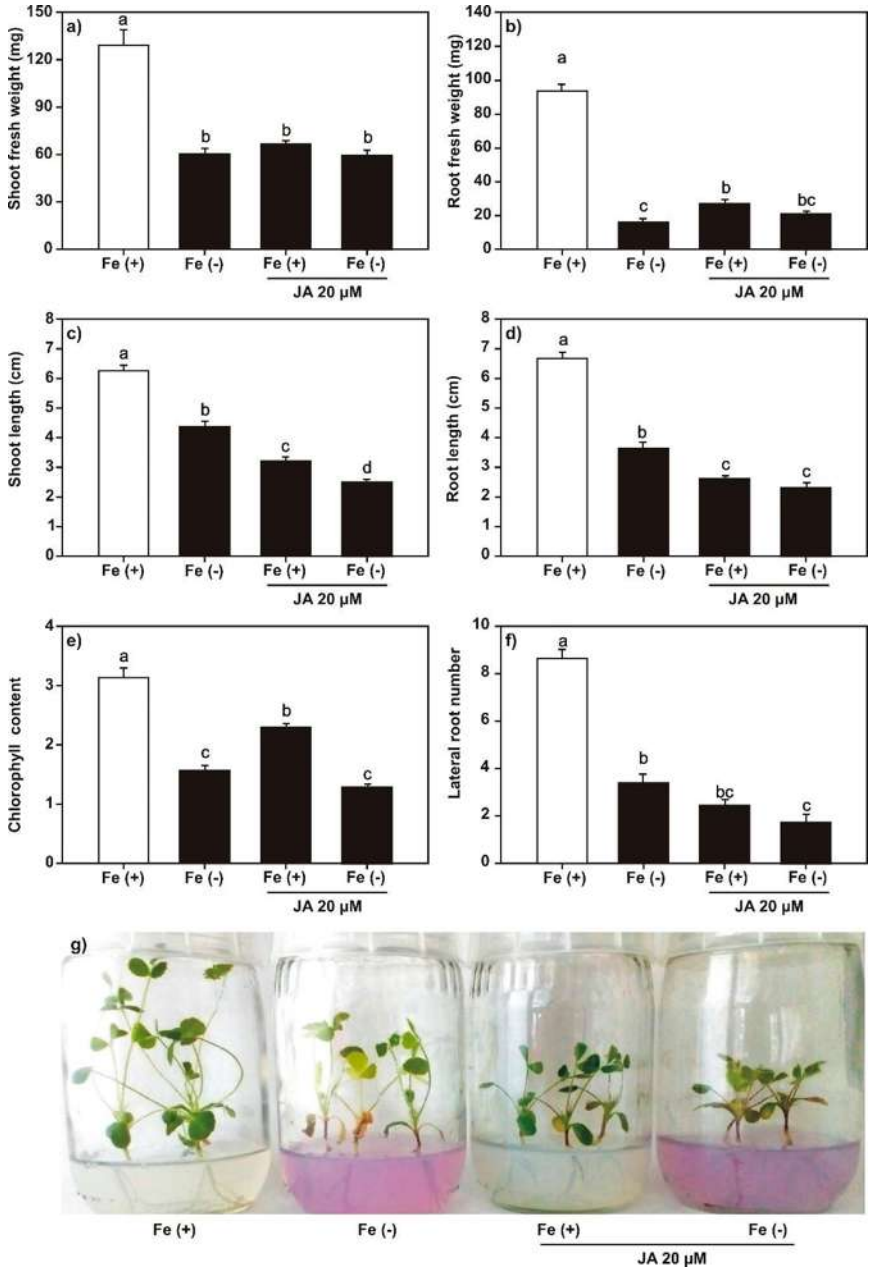

Figure 3. Effect of jasmonic acid (JA) on *Medicago truncatula* growth. The *M. truncatula* plants were cultured in MS medium with JA (20 µM) under both iron sufficiency (control) and iron deficiency or 14 days. (**a**) Shoot fresh weight; (**b**) root fresh weight; (**c**) shoot length; (**d**) root length; (**e**) chlorophyll content; and (**f**) lateral root number. Panel (**g**) shows the phenotypes of plants in the treatments. Different lowercase letters above the standard error bars indicate significant differences between treatments calculated with two-way ANOVA and Tukey's test ($p < 0.5$; $n = 9$).

In contrast, the application of DMHDA (8 µM) increased the length and weight of shoots and roots, the number of trifoliate leaves and lateral roots, and chlorophyll content (Figure 4 and Figure S3). Finally, the DMHDA combined with iron deficiency protected the plants against this stress because we observed that the weight and length of shoots, number of lateral roots, and chlorophyll content were significant higher in these plants than plants under iron-deficient conditions without DMHDA.

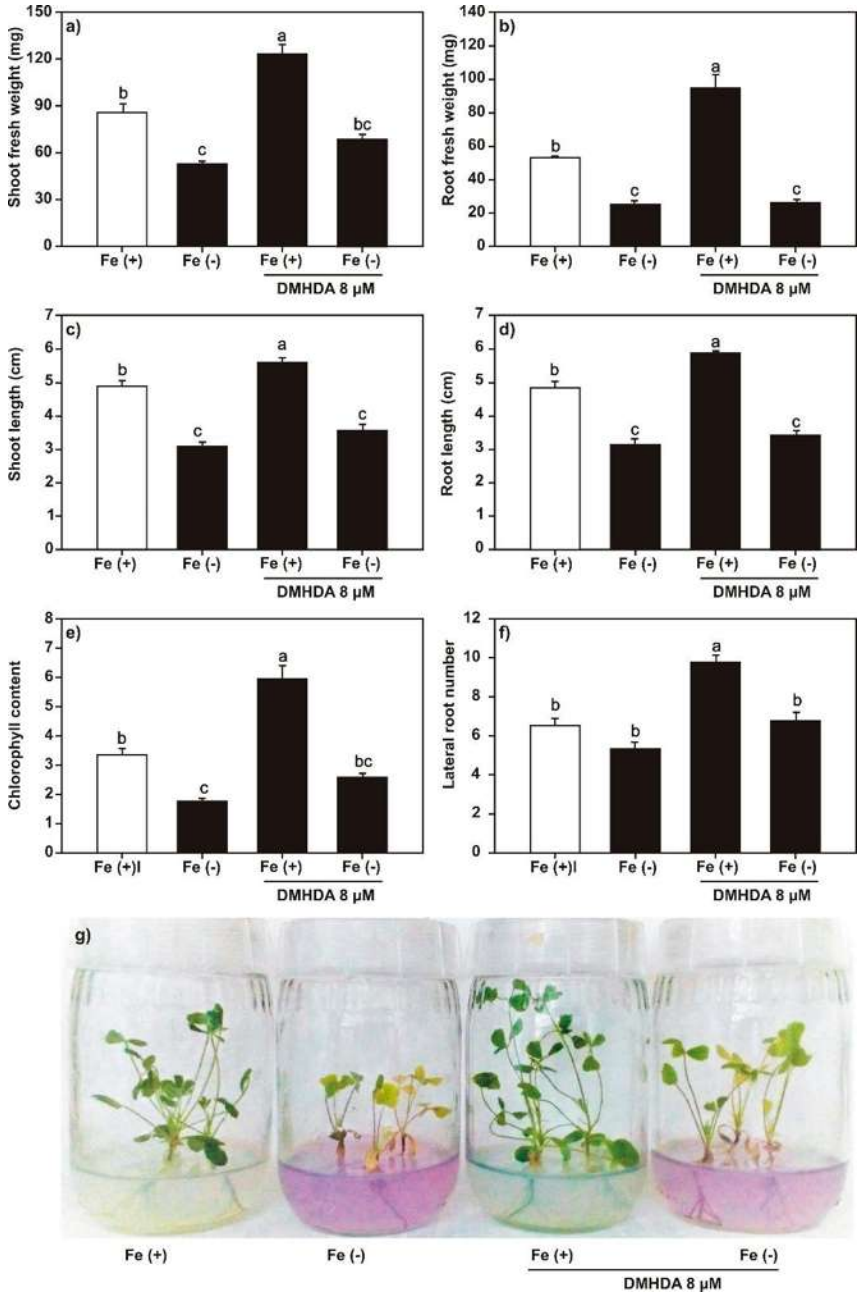

Figure 4. Effect of *N,N*-dimethylhexadecylamine (DMHDA) on *Medicago truncatula* growth. The *M. truncatula* plants were cultured in MS medium with DMHDA (8 µM) under both iron sufficiency (control) and iron deficiency for 14 days. (**a**) Shoot fresh weight; (**b**) root fresh weight; (**c**) shoot length; (**d**) root length; (**e**) chlorophyll content; and (**f**) lateral root number. Panel (**g**) shows the phenotypes of plants in the treatments. Different lowercase letters above the standard error bars indicate significant differences between treatment calculated with two-way ANOVA and Tukey's test ($p < 0.5$; $n = 9$).

Effect of Iron Deficiency on the Expression of Iron-Deficiency and Biotic Stress-Response Genes

In the *M. truncatula* genome, we identified the genes *MtbHLH38*, *MtbHLH39*, and *MtFIT* as key genes in iron-deprivation response, *MtNPR4* and *MtWRKY70* as key genes in SAR response, and *MtMYC2* as a key gene in ISR response. Genes were identified by homology with *A. thaliana* genes. All the identified genes showed at least 60% identity with their *A. thaliana* orthologues and the sequences of the characteristic domains of each protein (Table S1). The key genes *MtFRO3* [27,36] (iron-deficiency response), and *MtDef2.1* [37] (ISR response) have been previously described.

We proceeded to evaluate the expression of the key genes in the defense pathways and the iron uptake in plants grown under iron deficiency. Previous studies have reported that the expression of iron-deprivation response genes is time dependent [38]; therefore, a time kinetic of *MtFIT* expression was performed (Figure S4). We observed that gene expression peaked at 48 h (although the differences between *MtFIT* expression at different times was not significant); thus, plants were maintained under treatment conditions for 48 h before we measured gene expression. First, we analyzed the expression of key genes under iron-deficient conditions. The expression of *MtbHLH38*, *MtbHLH39*, *MtFIT*, and *MtFRO3* ranged from 2.5- to 18.1-fold higher than that in the respective controls in plants cultured under iron-sufficient conditions (Figure 5). These results showed that the selected genes clearly responded to iron deprivation as expected.

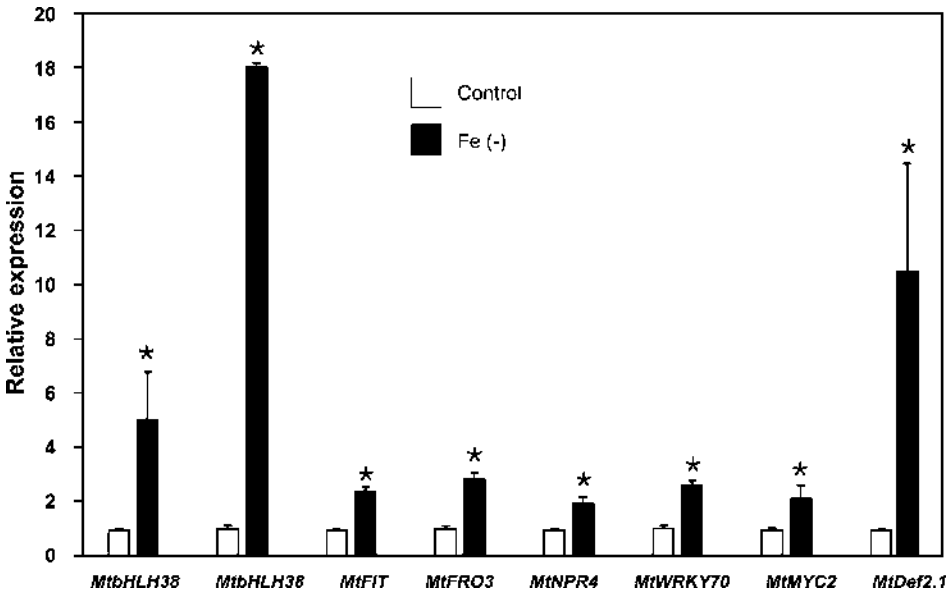

Figure 5. Relative expression of iron uptake and defense genes in *Medicago truncatula* plants grown under both iron-sufficient (control) and iron-deficient conditions for 48 h. Values represent mean ± standard errors of relative expression in reference to controls. Asterisks indicate significant differences between treatments calculated using Student's t test ($p < 0.5$; $n = 3$).

Several previous studies have related iron deficiency to defense responses in plants [23,39,40]. Therefore, the expression of *MtNPR4* and *MtWRKY70* in the SAR pathway, and *MtMYC2* and *MtDef2.1* in the ISR pathway was evaluated in plants subjected to iron-deficient conditions. Genes involved in the SAR response (*MtNPR4* and *MtWRKY70*) showed expressions ranging from 2.2- to 2.7-fold higher than those of the respective controls in plants cultured under iron-sufficient conditions (Figure 5).

Genes involved in the ISR response (*MtMYC2* and *MtDef2.1*) showed a 2.2- and 10.5-fold increase, respectively (Figure 5). These results indicated that iron deprivation, as well as induction of the expression of genes involved in iron uptake, activates the biotic stress-response genes involved in the signaling pathways of SAR and ISR.

Effect of SA and JA on the Expression of Biotic Stress and Iron-Deficiency Response Genes

As we observed that iron deficiency activates defense pathways at the transcriptional level, we decided to evaluate the positive feedback of SA and JA on iron-deficiency response genes. The expression of the *MtbHLH38*, *MtbHLH39*, *MtFIT*, and *MtFRO3* genes was repressed in plants treated with SA (Figure 6) or JA (Figure 7). However, when iron deprivation was combined with SA or JA treatment, the gene repression reverted to similar levels as the control (Figures 6 and 7).

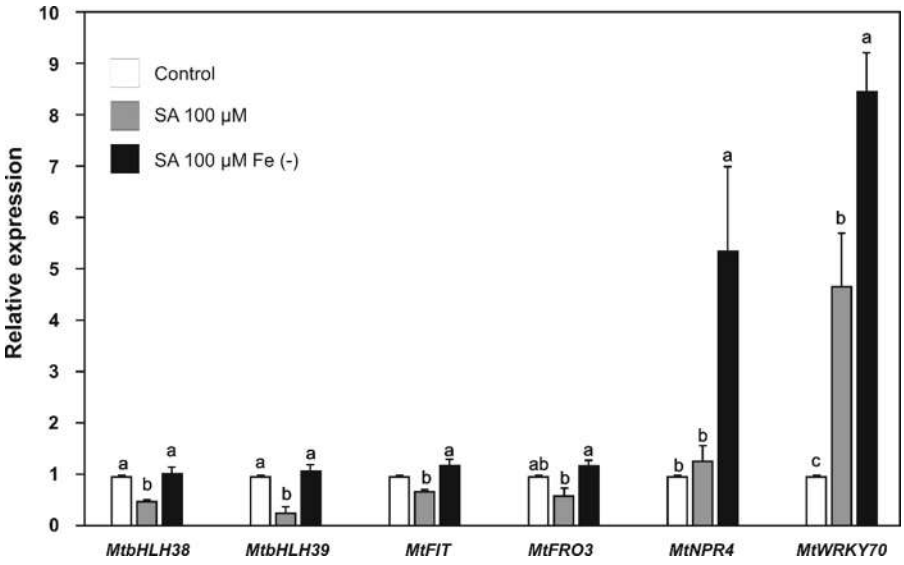

Figure 6. Relative expression of iron uptake and defense genes in *Medicago truncatula* plants grown with salicylic acid (100 µM) under both iron-sufficient and iron-deficient conditions for 48 h. Values represent mean ± standard errors of relative expression in reference to controls. Different lowercase letters indicate significant differences as determined by one-way ANOVA and Tukey's test ($p < 0.5$; $n = 3$).

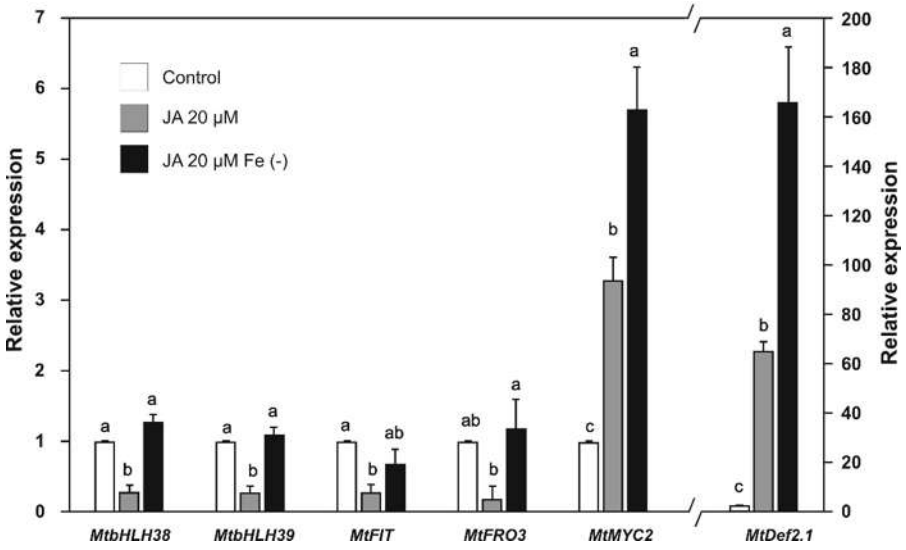

Figure 7. Relative expression of iron uptake and defense genes in *Medicago truncatula* plants grown with jasmonic acid (20 µM) under both iron-sufficient and iron-deficient conditions for 48 h. Values represent mean ± standard errors of relative expression in reference to controls. Values of *MtDef2.1* expression are shown on the axis on the right. Different lowercase letters indicate significant differences as determined by one-way ANOVA and Tukey's test ($p < 0.5$; $n = 3$).

We also analyzed the response of defense pathway genes to their corresponding phytohormone triggers. The *MtNPR4* gene was not induced significantly under iron sufficiency and SA treatment, but the combination of SA and iron deprivation resulted in a 5.4-fold increase in expression (Figure 6). This result suggests an additive effect, which is probably due to the increase in the endogenous levels of this phytohormone under iron deficiency. The expression of the *MtWRKY70* gene showed a 4.7-fold increase in the presence of SA and an 8.5-fold increase under iron deficiency combined with SA (Figure 6).

The expression of the *MtMYC2* gene showed a 3.3-fold increase after application of JA alone, and a 5.7-fold increase after application of JA combined with iron deprivation (Figure 7). The expression of the *MtDef2.1* gene showed a 65.3-fold increase after application of JA alone, and a 166.0-fold increase after application of JA combined with iron deprivation (Figure 7). These results confirm that the genes identified in the present study responded to the phytohormones involved in the defense pathways.

Effect of DMHDA on the Expression of Biotic Stress and Iron-Deficiency Response Genes

Aiming to establish a relationship between iron deficiency, SA, JA, and DMHDA, we evaluated the expression of genes involved in iron uptake and biotic stress response in plants treated with DMHDA. The expression of the *MtbHLH38*, *MtbHLH39*, *MtFIT,* and *MtFRO3* genes in DMHDA-treated plants was from 2.4- to 4.4-fold higher than that of the controls, and in plants treated with DMHDA combined with iron deprivation was from 4.7- to 52.2-fold higher than that of the controls (Figure 8). These results showed that DMHDA and iron deprivation have a synergistic effect on iron-deficiency response genes.

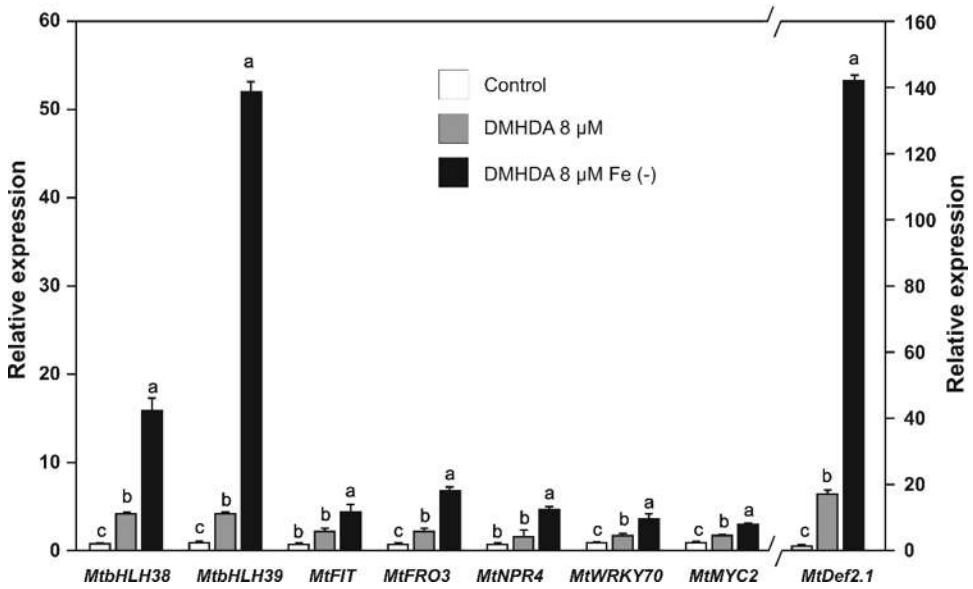

Figure 8. Relative expression of iron uptake and defense genes in *Medicago truncatula* plants grown with DMHDA (8 µM) under both iron-sufficient and iron-deficient conditions for 48 h. Values represent mean ± standard errors of relative expression in reference to controls. Values of *MtDef2.1* expression are referred to secondary axis. Different lowercase letters indicate significant differences as determined by one-way ANOVA and Tukey's test ($p < 0.5$; $n = 3$).

We observed that the plants treated with DMHDA also exhibited transcriptional activation of the biotic stress-response pathways. The *MtNPR4* and *MtWRKY70* genes showed a higher expression compared with the controls (1.9- to 2.1-fold), but plants treated with DMHDA under iron deprivation increased gene expression from 3.8 to 7.5-fold (Figure 8).

The *MtMYC2* and *MtDef2.1* genes were also induced by DMHDA, but to different magnitudes. The addition of DMHDA under iron deprivation induced a 142-fold increase in the expression of *MtDef2.1*,

which was similar to the results from plants treated with JA under iron deprivation (Figures 7 and 8). This result suggests that JA and DMHDA may share a common induction mechanism in ISR responses.

Effect of P. syringae and B. cinerea the Expression of Biotic Stress and Iron-Deficiency Response Genes

We evaluated the effect of *P. syringae* and *B. cinerea* inoculation on the expression of biotic stress and iron-deficiency response genes because these pathogens activate the SAR and ISR pathways under natural conditions. The expression of the genes *MtbHLH38*, *MtbHLH39*, *MtFIT*, and *MtFRO3* was strongly repressed when plants were inoculated with *P. syringae* (9- to 20-fold) and *B. cinerea* (3.2- to 10-fold) (Figure 9). These results indicate that, as JA and SA did, pathogen inoculation suppressed the iron-deficiency response pathway.

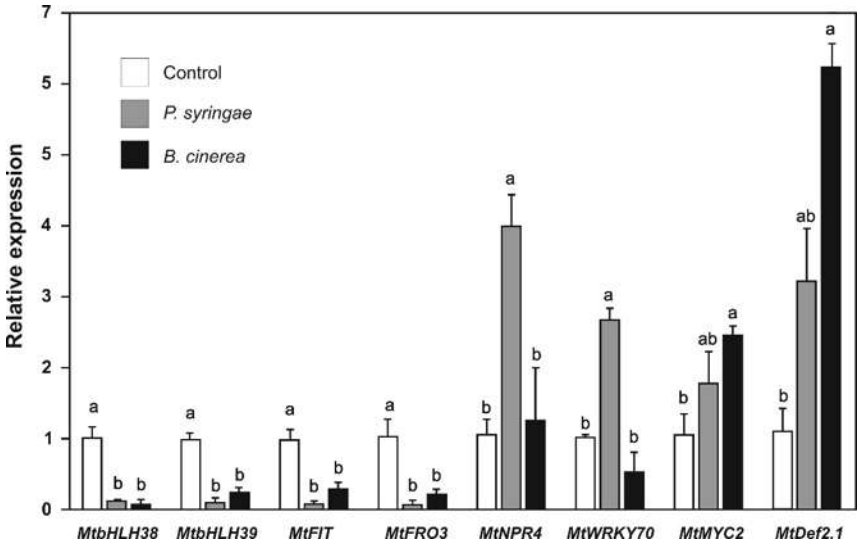

Figure 9. Relative expression of iron uptake and defense genes in *Medicago truncatula* plants inoculated with *Pseudomonas syringae* or *Botrytis cinerea*. Values represent mean ± standard errors of relative expression in reference to controls. Different lowercase letters indicate significant differences as determined by one-way ANOVA and Tukey's test ($p < 0.5$; $n = 3$).

The expression of the SAR and ISR pathway genes was also analyzed. We observed that the expression of the *MtNPR4* and *MtWRKY70* genes were strongly induced after *P. syringae* inoculation, whereas inoculation with *B. cinerea* did not affect the expression of these genes (Figure 9). These results indicate that the genes of the SAR pathway respond to the hemibiotrophic *P. syringae* pathogen. Regarding the genes of the ISR pathway, the expression of *MtMYC2* and *MtDef2.1* was strongly induced after *B. cinerea* inoculation; expression of *MtMYC2* and *MtDef2.1* also increased after *P. syringae* but to a lesser extent (Figure 9).

Effect of DMHDA on Growth of M. truncatula Plants Infected with P. syringae or B. cinerea

Finally, with the aim to determine if the gene induction produced by DMHDA conferred protection against biotrophic and necrotrophic pathogens, we evaluated the effect of *P. syringae* and *B. cinerea* inoculation on the growth parameters of *M. truncatula* plants cultured with DMHDA. As previously described, uninoculated plants treated with DMHDA (8 µM) increased the length and weight of shoots and roots, and chlorophyll content compared with controls (Figure 10), but there was no significant differences in the number of trifoliate leaves and lateral roots (Figure 10 and Figure S5). Plants inoculated with *P. syringae* or *B. cinerea* had lower shoot and root weights, shoot and root lengths, and number of lateral roots and trifoliate leaves compared with uninoculated controls. Chlorophyll content was strongly affected by *B. cinerea* inoculation but not by *P. syringae* inoculation (Figure 10 and

Figure S5). Plants cultured with DMHDA and inoculated with *P. syringae* or *B. cinerea* grew better than plants that were inoculated with the pathogens but cultured without DMHDA, as shown by the growth parameters (shoot and root fresh weight, root length, chlorophyll content, and trifoliate leaf number) (Figure 10 and Figure S5). In particular, plants cultured with DMHDA showed a higher root fresh weight and chlorophyll content than plants cultured without DMHDA (Figure 10b,e). This correlates with a healthier phenotype, comparable with the not infected controls (Figure 10e,g). Plants inoculated with *P. syringae* and cultured with DMHDA also showed a higher shoot length and lateral root number, but plants inoculated with *B. cinerea* with and without DMHDA did not differ in these parameters. These results showed that DMHDA may confer protection to *M. truncatula* plants against pathogens, although this protection was not complete in our experimental system.

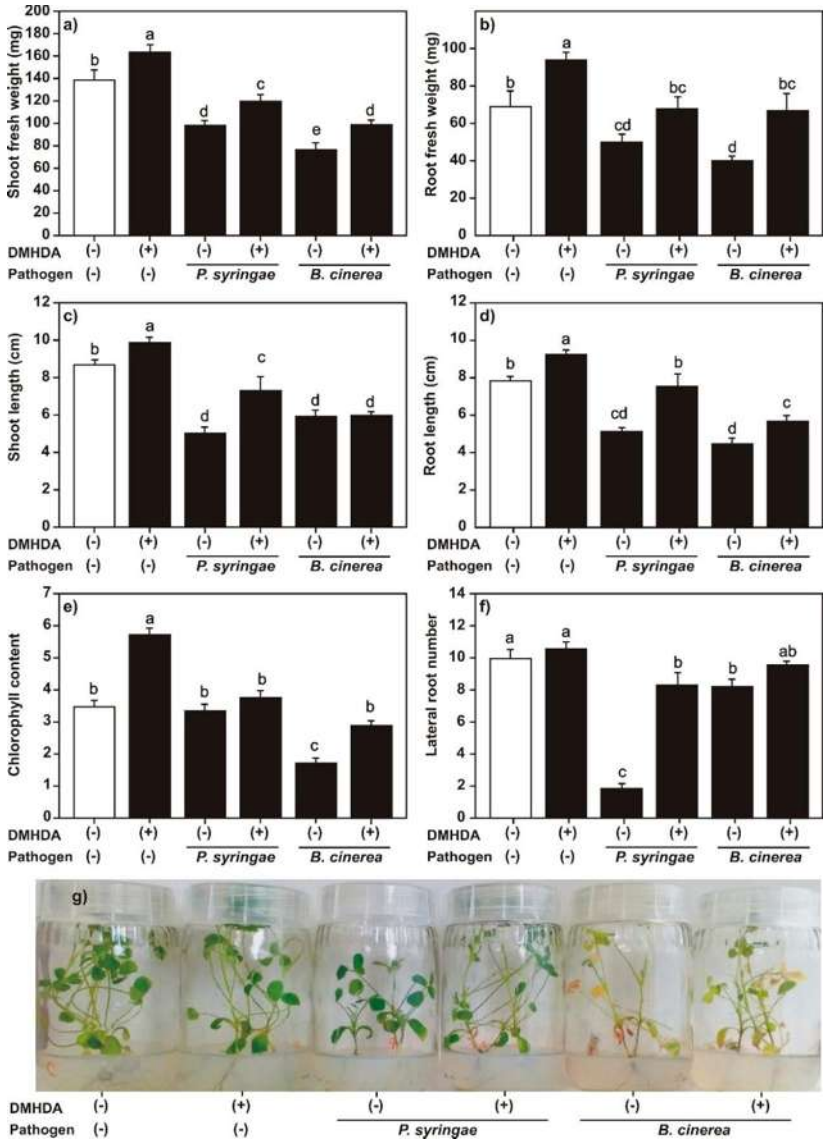

Figure 10. Effect of *N,N*-dimethylhexadecylamine (DMHDA) on growth of plants of *Medicago* infected with *Pseudomonas syringae* or *Botrytis cinerea*. The *M. truncatula* plants were cultured in MS medium with DMHDA (8 µM) and the cultured for 15 days. (**a**) Shoot fresh weight; (**b**) root fresh weight; (**c**) shoot length; (**d**) root length; (**e**) chlorophyll content; and (**f**) lateral root number. Panel (**g**) shows the phenotypes of plants in the treatments. Different lowercase letters above the standard error bars indicate significant differences calculated with two-way ANOVA and Tukey's test ($p < 0.5$; $n = 9$).

Discussion

In recent years, response to iron-deficiency stress has been linked to the defensive activation of both SAR and ISR pathways [23]. It has also been shown that the absence of iron decreases the development of symptoms caused by pathogens such as *D. dadantii* [39]. The mechanism underlying this phenomenon remains unknown; however, in *A. thaliana* it is suggested that the subunit of the mediator complex MED16 mediates these responses by interacting with MED25, which interacts with EIN3 and EIL1 (ethylene signaling transcription factors). EIN3 and EIL1 are involved in the JA signaling pathway and act to maintain a normal amount of FIT, which in turn regulates iron homeostasis [41]. Additionally, MED25 interacts with the transcriptional factor MYC2 through the trans-activation domain TAD [42]. MED16 is also involved in the activation of defense pathways through the regulation of SA and JA signaling [43].

In the present study, the expression of genes involved in both iron uptake and defense pathways in the legume model *M. truncatula* plants grown under iron deficiency was evaluated and a relationship was established between iron deficiency and DMHDA. In addition, we compared the effects produced by the phytohormones responsible for activating plant defense (SA and JA) and DMHDA. Before analyzing the expression of the genes, the phenotypes caused by the different conditions were characterized. It was observed that iron deficiency (Figure 1) generated a chlorotic phenotype with lower shoot and root weight and length compared to the control, as well as a decrease in the number of lateral roots. As iron is involved in metabolic reactions in organelles, such as respiration and photosynthesis, as well as in chlorophyll biosynthesis, iron deficiency affects all cellular metabolic processes [44].

In plants treated with SA and JA (Figures 2 and 3), a decrease was observed in the weight and length of shoots and roots and the number of lateral roots and chlorophyll content. This reduction effect was enhanced when phytohormones were combined with iron deprivation. The exogenous application of both SA and JA decreases the length of shoots and roots, and SA affects the photosynthetic capacity of plants [45,46].

Contrary to what was observed with application of SA and JA, the application of DMHDA increased the weight and length of shoots and roots, the number of trifoliate leaves and lateral roots, and chlorophyll content (Figure 4); similar results have been reported in previous studies [26,31]. The combination of DMHDA and iron deficiency resulted in higher values of the analyzed parameters compared with those observed under iron deficiency alone, but in lower values than in the control. DMHDA activates mechanisms involved in iron uptake, such as acidification [31] and the induction of *FRO* genes [32]. Therefore, the protective effect is probably due to this phenomenon.

Currently, there is increasing evidence indicating a sophisticated transcriptional regulatory network that maintain iron homeostasis in plants. In *A. thaliana*, 16 of the transcriptional factors integrating this network belong to the bHLH family. The bHLH transcriptional factors acts as homo- or heterodimers to regulate the expression of their target genes that frequently are other *bHLH* genes [47]. The bHLH transcription factors are also involved in the JA signaling pathway. In *A. thaliana* plants MYC2 (bHLH6) is often considered as a central transcriptional factor of the JA signaling pathway [42] and acts as a JA-dependent repressor of FIT [47]. The SA and JA signaling pathways pathway also cross-talk, and it has been pointed out that the transcriptional factor WRKY70 is involved in this interaction since it represses the JA-responsive genes, and activates the SA-responsive genes [48].

In the present study, we identified in the *M. truncatula* genome the orthologues of the key transcription factor genes *bHLH38*, *bHLH39*, and *FIT* (iron-deprivation response) [35,49]; *WRKY70* (SAR response) [1,48]; and *MYC2* (ISR response) [1,42], and employed them to analyze their signaling pathways together with *MtFRO3* [27], *MtNPR4* (this work), and *MtDef2.1* [37] placed downstream on those respective signaling cascades. We observed that iron deprivation up-regulated the expression of *MtbHLH38*, *MtbHLH39*, *MtFIT,* and *MtFRO3* genes (Figure 5), and had a greater effect on *MtbHLH38* and *MtbHLH39* with 5-fold and 18-fold increases, respectively. In previous reports, higher induction

results were observed in the orthologues of these genes over others involved in iron deficiency [35], which indicates that these genes play a key role in activating iron uptake response.

The genes of SAR and ISR were also induced by iron deprivation (Figure 5). We observed induced expression for all defense genes, which indicates that iron deficiency regulates this process at the transcriptional level. Iron deficiency increases the endogenous levels of JA [50] and SA [51]; thus, it can be speculated that the increased expression of defense genes is due to induced synthesis of JA and SA triggered by iron deficiency. Additionally, the expression of MYB72 a transcriptional factor essential to mounting ISR responses against phytopathogen microorganisms [52] is driven by FIT1 in *A. thaliana* [53].

The application of SA and JA prevented the up-regulation of the *MtbHLH38*, *MtbHLH39*, *MtFIT,* and *MtFRO3* genes when the plants were grown under iron deficiency combined with these phytohormones. In *A. thaliana,* SA decreased the expression of *FRO2* [54], whereas JA decreased *FIT (bHLH29), bHLH38, bHLH39*, *FRO2,* and *IRT* gene expression [55,56]. However, other studies have reported that SA increased the expression of the *bHLH38* and *bHLH39* genes [57], while in *Oryza sativa*, JA is involved in the positive regulation of the *IDEF1* and *IBP1.1* genes, which are involved in the activation of the iron uptake response [50]. Our results from the *M. truncatula* model support the negative regulation of iron uptake by SA and JA.

The effect of SA and JA on defense genes was analyzed. The *MtMYC2* and *MtDef2.1* genes were clearly induced by JA, and this induction was potentiated by iron deprivation as expected by ISR genes [23,53]. In the same way, SA induced *MtWRKY70* gene expression and iron deprivation enhanced this induction as expected [39,58]; however, *MtNPR4* was significantly induced when SA was combined with iron deprivation, and this was probably due to the increase in endogenous levels of SA triggered by iron deficiency. Previous research has shown that the expression of some *PR*s genes of different plants is activated by a combination of phytohormones, such as SA/methyl ester JA (MeJA) [59] or MeJA alone [60].

DMHDA induced *MtbHLH38*, *MtbHLH39*, *MtFIT,* and *MtFRO3* gene expression (Figure 8), and this induction was potentiated when DMHDA was combined with iron deprivation. Similar behavior was previously observed in the *M. truncatula* system in experiments on rhizosphere acidification (iron-deficiency response) [31]. Thus, our results showed that DMHDA also regulates the response to iron deficiency at the transcriptional level as expected. Additionally, DMHDA activates the two defense pathways, since the plants treated with this compound showed a marginal increase in *MtNPR4*, *MtWRKY70*, and *MtMYC2*, gene expression but a clear increase in *MtDef2.1.* The gene expression of all these genes was potentiated with the combination of iron deprivation and DMHDA. In particular, *MtDef2.1* showed considerable induction of expression after DMHDA treatment under iron deprivation, which was comparable to that of JA application under iron deprivation, suggesting that DMHDA may act through the JA pathway.

To produce not an exogenous but a physiological induction of SAR and ISR pathways, we infected the plants with *P. syringae* or *B. cinerea* and evaluated the iron-deprivation response, SAR pathway, and ISR pathway gene expression, and we obtained similar results to SA and JA addition to plants (Figure 9). The presence of these pathogens had a negative effect on the expression of genes involved in iron uptake, but *B. cinerea* triggered ISR pathway gene expression, whereas *P. syringae* induced SAR gene expression. These results demonstrated that, under experimental conditions that emulate natural conditions, the gene expression pattern produced by the addition of SA and JA was conserved. Furthermore, *M. truncatula* plants treated with DMHDA and infected with *P. syringae* and *B. cinerea* were healthier and grew better than plants not treated with DMHDA but infected with pathogens. This supports the idea that under real infection, defense pathways induced by DMHDA conferred protection against biotrophic and necrotrophic pathogens.

It is proposed that plants maintain an energetic balance between growth and defense [61]. In this way, the stimulation of defense pathways has a detrimental effect on growth [62,63]. Other bacterial VOCs, such as 2,3-butanediol, have the ability to activate both SAR and ISR defense pathways

simultaneously with plant growth promotion [64], as DMHDA did. DMHDA increases the expression of *AtLOX2*, a gene that responds to JA and is involved in JA biosynthesis [33], and both DMHDA and JA are antagonized by kinetin and correlated with an inhibition of cytokinin-related *ARR5*::*GUS* and *TCS*::*GFP* expression in *A. thaliana* [65]. Thus, we hypothesized that the effects of DMHDA in plants trigger the JA pathway. However, the results of the present study revealed a different process. The addition of SA or JA inhibited the growth of all the parameters that we recorded, and growth was more strongly inhibited when the addition of SA or JA was under iron-deficient conditions. However, in plants treated with DMHDA, growth was promoted, and when iron deprivation or infection with pathogens were combined with DMHDA treatment, the iron or biotic stresses effects were mitigated. Iron deprivation and DMHDA induced SAR, ISR, and iron-deficiency gene expression, especially when combined; however, SA and JA inhibited iron response genes (Figure 11).

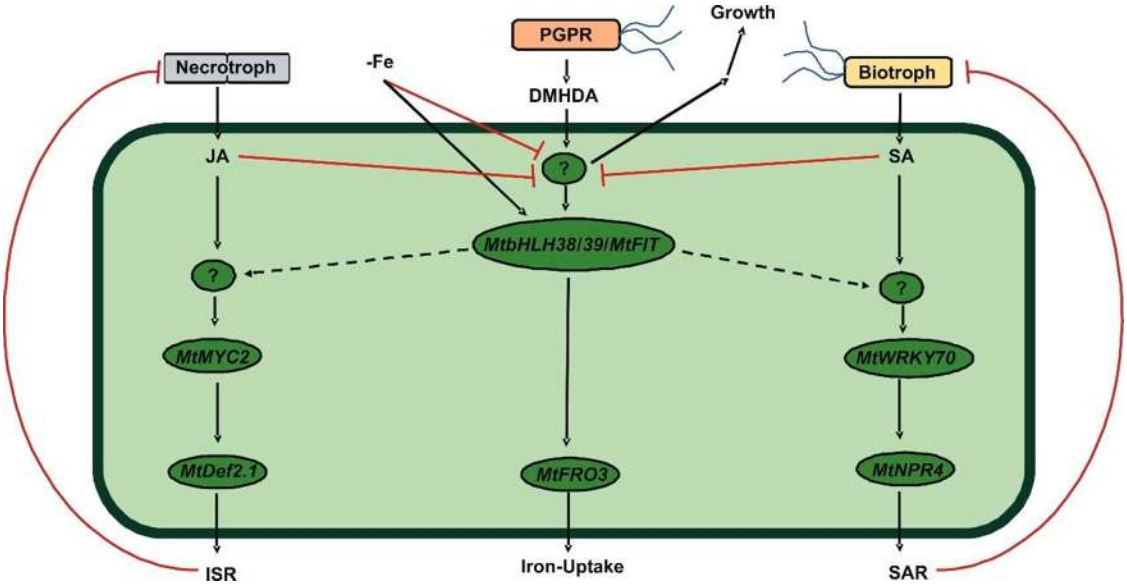

Figure 11. Proposed model of cross-talk between systemic acquired resistance (SAR), induced systemic resistance (ISR), and iron-deprivation response pathways mediated by *N,N*-dimethylhexadecylamine (DMHDA) in *Medicago truncatula*. Necrotrophic pathogens trigger jasmonic acid (JA) synthesis in plant cells, which downstream induces the expression of *MtMYC2* and *MtDef2.1* as ISR responses, which in turn inhibit the necrotrophic pathogen attack. Biotrophic pathogens trigger salicylic acid (SA) synthesis in plant cells, which downstream induces the expression of *MtWRKY70* and *MtPR4* as SAR responses, which in turn inhibit the biotrophic pathogen attack. Some plant growth promoting rhizobacteria (PGPR) produce DMHDA that activates a regulatory element upstream of *MtbHLH38/39/MtFIT* that induces *MtFRO3* as an iron-deprivation response. This regulatory element also promotes plant growth. Iron deprivation induces *MtbHlH38/39/MtFIT* but inhibits plant growth, probably through a regulatory element located upstream of *MtbHlH38/39/MtFIT*. JA and SA inhibit plant growth and iron-deprivation responses upstream of *MtbHlH38/39/MtFIT*. *MtbHlH38/39/MtFIT* induces ISR upstream of *MtMYC2* and SAR upstream of *MtPR4*.

In conclusion, we identified key genes in the defense and iron-deprivation responses of the legume model *M. truncatula* and reaffirmed the interactions between them. Taken together, our results showed that the VOC DMHDA produced by PGPR activates defense and iron-deprivation pathways, and exhibits a growth promoting effect unlike conventional phytohormones, highlighting that DMHDA does not mimic JA but induces an alternative pathway, revealing a novel aspect in the complex interactions between biotic and abiotic stresses.

Materials and Methods

M. truncatula Seed Germination

The *M. truncatula* ecotype Jemalong A17 was used in the present study. Seeds were submerged in a tube with 1 mL of sulfuric acid and were constantly shaken for 8 min. The excess of acid was removed, and the seeds were rinsed seven times with sterile deionized water. Sterilization was carried out with a 12% sodium hypochlorite solution for 2 min. Subsequently, the seeds were rinsed five times with sterile deionized water. The seeds were transferred to Petri dishes with Murashige and Skoog (MS) 0.2× medium at 6.5 pH with 6 g of agar L^{-1} and vernalized for 1 day at 4 °C. Finally, 2 days after germination, the seedlings were transplanted into glass jars with 30 mL of MS to apply the corresponding treatments.

MS Medium Preparation

The MS medium used for germination and growth was prepared as follows. In 1 L of water, we added 200 µl of solution 1 (25 g L^{-1} of $CaCl_2$), 2 mL of solution 2 (9.25 g L^{-1} of $MgSO_2$ and 4.25 g L^{-1} of KH_2PO_4), 1 mL of solution 3 (0.5 g L^{-1} of $FeSO_4$ and 0.7 g L^{-1} of NaEDTA), 200 µL of solution 4 (1.69 g L^{-1} of $MnSO_4$, 1.05 g L^{-1} of $ZnSO_4$, 0.62 g L^{-1} of H_3BO_3, 0.83 g L^{-1} of KI, 0.025 g L^{-1} of Na_2MoO_4, 0.0025 g L^{-1} of $CuSO_4$, and 0.0025 g L^{-1} $CoCl_2$), 2 mL of solution 5 (0.5 g L^{-1} of glycine, 0.0125 g L^{-1} of pyridoxine, 0.025 g L^{-1} of nicotinic acid, 0.0025 g L^{-1} of thiamine, and 1.5 g L^{-1} of myo-inositol), and 2 mL of solution 6 (41.25 g L^{-1} of NH_2NO_3 and 47.5 g L^{-1} KNO_3). To induce iron deficiency, solution 3 was omitted, and ferrozine was added to have a final concentration of 100 µM. Finally, the pH of the medium was adjusted to 6.5 and sterilized for 20 min in a pressure vessel.

Chemicals

SA, JA, and DMHDA were purchased from Sigma-Aldrich. SA and JA and were dissolved in water, and DMHDA in ethanol. Equal volumes of solvent used in treatments were added to controls.

Growth and Gene Expression Evaluation

Two days after germination, the seedlings were transplanted into glass jars containing 30 mL of MS. Three flasks were used per treatment, with three plants in each. Control (+Fe) and iron-deficiency (−Fe) conditions were used. Additionally, SA, JA, or DMHDA were added to the MS medium to a final concentration of 100 µM [57], 20 µM, and 8 µM [26], respectively, under both iron-sufficient and iron-deficient conditions. To evaluate the growth parameters, the plants were kept in an AR-66L2 growth chamber (Percival Scientific, Inc. Perry, IA, USA) for 14 days with a photoperiod of 16-h light/8-h dark and a light intensity of 200 µmol m^{-2} s^{-1} at 22 °C. After 14 days, the roots and shoots were measured and weighed, and then lateral roots, leaves, and chlorophyll content were quantified. To analyze gene expression, the germinated plants were grown for three days in MS and then transferred for 48 h to their respective treatment or to MS without phytohomones (controls). After 48 h, total RNA was extracted from the whole plant.

P. syringae and B. cinerea Inoculation

M. truncatula plants were inoculated according to a previously described protocol [30]. Plants were inoculated with 10 µM of 1 × 10^5 spores of *B. cinerea* and 10 µM of 1 × 10^7 CFU of *P. syringae* 5 days after being transferred to MS. Two days post inoculation, gene expression was evaluated. For *M. truncatula* growth assays, plants were transferred to MS without (controls) or with DMHDA and cultured for 5 days. Then plants were inoculated with *B. cinerea* or *P. syringae* and cultured for 15 days.

Growth Analysis and Quantification of Chlorophyll Content in M. truncatula

Roots and aerial parts were weighed using an analytical balance, and then their length was measured. The lateral roots and leaves were quantified manually. Chlorophyll content was quantified as previously described [32] using a CCM-200 chlorophyll meter (Opti-Sciences, Inc., Hudson, NH, USA) based on the rate of transmitted radiation (940 and 660 nm) through a leaf in arbitrary units.

Identification of Defense and Iron-Deficiency Genes

The genes evaluated in the present study were identified in the genome of *M. truncatula* (http://blast.jcvi.org/er-blast/index.cgi?project=mtbe) by performing a BLAST search against genes previously reported in *A. thaliana*. Additionally, the domains of the selected sequences were identified using the NCBI tool "Conserved domain search" (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Table S1).

RNA Extraction and cDNA Synthesis

The whole plant was macerated with liquid nitrogen. Total RNA was extracted with the TRI reagent (Catalog T9424, Sigma-Aldrich). Prior to its use, the RNA was treated with DNase to remove DNA residues. The samples were run on a 1% agarose gel at 90 V to determine the integrity of the molecule and absorbance. The quality and quantity of RNA was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA) to calculate the ratio of absorbance at 260 nm to absorbance at 280 nm (Table S2) and by examination on agarose gel electrophoresis (1% agarose gel at 90 V) (Figure S6). Finally, cDNA was synthesized according to the specifications of the "SuperScript™ First-Strand Synthesis System for RT-PCR" kit (Life Technologies/Gibco-BRL., Carlsbad, CA, USA).

RT-qPCR

RT-qPCR was performed in triplicate for each treatment and gene using the ABI StepOne™ System thermocycler (Applied Biosystems, Foster City, CA, USA). The oligonucleotides for the genes were designed using the NCBI tool "First Designing Tool" (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and are listed in Table S3. The RT-qPCR analysis was carried out with SYBR-Green PCR Master Mix (Applied Biosystems) in a volume containing 5 µL SYBR-Green PCR Master Mix, 1 µL of the oligonucleotide mixture (forward and reverse), 2 µL of cDNA, and 3 µL of deionized sterile water. The thermal cycling protocol was as follows: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 30 s. To verify the amplification of single, specific target cDNA, a dissociation curve analysis was included according to the thermal profile, as suggested by the manufacturer (Applied Biosystems). To prepare the dissociation curve, the reaction was terminated at 95 °C for 15 s followed immediately by annealing and extension at 60 °C for 1 min; finally, the temperature was increased to 95 °C at a rate of 0.3 °C s^{-1}. A specific target cDNA corresponding to a single dissociative peak was obtained in all the cases (Figure S7).

The amount of RNA in each sample was normalized using actin as the reference gene. Finally, gene expression was evaluated using the comparative $2^{-\Delta\Delta Ct}$ method [66].

Statistical Analysis

The results were analyzed using Student's t test or with one-way or two-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons ($p < 0.05$). Growth parameter experiments were performed with nine biological replicates. Gene expression was analyzed with three composite biological samples. Each composite sampled consisted of three plants. Each experiment was carried out at least twice with similar results.

Supplementary Materials: The following are available online at http://www.mdpi.com/2223-7747/9/5/624/s1, Figure S1: Effect of salicylic acid (SA) on *Medicago truncatula* trifoliate leaf number, Figure S2: Effect of jasmonic acid (JA) on *Medicago truncatula* trifoliate leaf number, Figure S3: Effect of *N*,*N*-dimethylhexadecylamine (DMHDA)

on *Medicago truncatula* trifoliate leaf number, Figure S4: Time kinetics of MtFIT gene expression, Figure S5: Effect of *N*,*N*-dimethylhexadecylamine (DMHDA) and *Pseudomonas syringae* or *Botrytis cinerea* infection on *Medicago truncatula* trifoliate leaf number, Figure S6: Representative images of RNA samples run on 1% agarose gel, Figure S7: Dissociation curves produced by RT-qPCR amplicons of genes listed in Table S1, Table S1: Genes identified in the present study, Table S2: Ratio of absorbance at 260 nm to absorbance at 280 nm of RNA samples used in the RT-qPCR measurements, Table S3: List of oligonucleotides employed in RT-qPCR.

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7. Discusión

Desde hace años se ha establecido la participación de elementos relacionados a la defensa en la regulación de la respuesta a deficiencia de Fe y viceversa en plantas. El ET, una de las fitohormonas que activan la vía de defensa, modula diversas respuestas de la deficiencia de Fe que van desde morfológicas y fisiológicas hasta genéticas (García et al., 2010; Lucena et al., 2015; Li y Lan, 2017; Romera et al., 2017). El ABA es otra de las fitohormonas que está involucrada en el control de la maquinaria para el transporte de hierro debido a que regula la expresión de los genes *NRAMP3*, *FRD3*, *YSL2* y *NAS1* (Lei et al., 2014). En el caso del AJ y AS, reguladores centrales del establecimiento de la respuesta de defensa (ISR y SAR, respectivamente), se tiene resultados que van desde efectos negativos sobre las plantas crecidas en deficiencia de Fe, por ejemplo la inhibición de la expresión de genes de involucrados en la reducción y transporte de Fe (Maurer et al, 2011; Maurer et al., 2014); hasta aquellas en las cuales, dichas fitohormonas aumentan el contenido de clorofila y la expresión de factores de transcripción que activan la expresión de genes de respuesta a deficiencia de Fe (Kang et al., 2003).

Por otro lado, la deficiencia de Fe induce la expresión de los genes involucrados en las vías de defensa (e.g, *PR1*, *PAD4*, *PDF1.2*) (Dellagi et al., 2009; Koen et al., 2014) además de disminuir los síntomas de infección causados por patógenos necrótrofos (Kieu et al., 2012).

El mecanismo por el cual se establece la relación entre defensa y deficiencia de hierro es desconocido, sin embargo se ha propuesto al complejo mediador como elemento clave para explicar dicho fenómeno, principalmente a las subunidades MED16 y MED25, que en conjunto interactúan con EIN2 y EIL1, involucrados en la señalización de ET y que además, regulan la degradación de FIT (Yang et al, 2014). Adicionalmente, MED25 interactúa con MYC2 a través del dominio TAD (Goossens et al., 2017) y MED16, regula la señalización de AJ y AS (Zhang et al, 2012).

En la presente investigación, se evaluó el efecto del COV DMHDA sobre la expresión de genes clave tanto de las vías de defensa, es decir ISR y SAR, así como aquellos de la vía de respuesta a deficiencia de Fe, utilizando la leguminosa modelo *M*. *truncatula*.

Con la finalidad de realizar una comparación entre el efecto de las fitohormonas que disparan la respuesta de defensa y la DMHDA, las plantas también fueron tratadas con AS y AJ.

El fenotipo de deficiencia de Fe se encuentra bien identificado en plantas estrategia I y estrategia II y se caracteriza principalmente por la presencia de clorosis intervenal así como una disminución en la masa del brote y raíz (Zhang et al., 2015; Kobayashi et al., 2019; Kaya y Ashraf, 2019). En el presente estudio, para establecer el sistema de deficiencia de Fe, las plantas fueron crecidas en medio MS en ausencia de dicho metal (0 µM) y adicionado con ferrozina. Se observó que la deficiencia de Fe generó plantas cloróticas, con un brote y raíz de menor tamaño y peso en comparación con los controles, además de una disminución en el número de raíces laterales. Lo anterior debido a que el Fe participa en diversas reacciones metabólicas de diferentes organelos (e.g. cloroplastos, vacuola, mitocondria), como son la respiración, fotosíntesis, biosíntesis de clorofila y transporte de oxígeno, por lo tanto la deficiencia de Fe afecta todo el metabolismo celular (Vigani et al., 2013).

Por otra parte, se sabe que la aplicación exógena tanto de AS como AJ, disminuye la longitud del brote y raíz, y que específicamente, el AS afecta la capacidad fotosintética de las plantas (Pancheva et al., 1996; Jang et al., 2017). En nuestra investigación, cuando las plantas fueron tratadas con las fitohormona AS o AJ, se observó un decremento en el peso y longitud de brote y raíz, así como una disminución en el número de raíces laterales, es decir, se obtuvo un fenotipo similar al causado por la deficiencia de Fe, por lo cual, cuando se combinaron ambos estímulos (AS o AJ y deficiencia de Fe), la reducción en los parámetros mencionados fue la mayor. Wang et al, (2007) señalan que ls aplicación de AS en plantas de *A*. *thaliana* inhibe la expresión del gen reportero de auxinas, *DR5*. Adicionalmente, plantas mutantes con una sobreacumulación de AS (*cpr5*, *cpr6* y *snc1*), exhiben fenotipos con una morfología apical reducida y un retraso en el crecimiento, característicos de la deficiencia de auxinas (Bowling et al., 1997; Li et al., 2001). En estas mutantes, los niveles de auxinas libres fueron menores que en plantas control. La aplicación exógena de AS también redujo el contenido de auxinas (Wang et al., 2007). Lo anterior indica que el fenotipo de reducción de

crecimiento observado en las plantas tratadas con AS, se debe a una inhibición de la vía de señalización de auxinas, como parte del establecimiento del mecanismo de defensa en las plantas. En el caso del AJ, se ha reportado un antagonismo con algunas fitohormonas involucradas en el crecimiento y desarrollo vegetal como son el ácido giberélico que participa en la elongación del tallo, la expansión de las hojas, en la floración, desarrollo y germinación de la semilla; y las citocininas involucradas en la división celular, iniciación y crecimiento del brote, senescencia de las hojas, dominancia apical, filotaxis, entre otras (Yang et al., 2012; Chen et al., 2011), por lo cual las plantas tratadas con AJ, presentan un disminución generalizada en el crecimiento.

Una vez que se caracterizó el fenotipo de las plantas de *M*. *truncatula* tratadas con las fitohormonas de defensa AS y AJ, se procedió a la aplicación de DMHDA. Contrario a los resultados obtenidos con AS y AJ, la DMHDA aumentó el peso y la longitud de brote y raíz, así como el número de hojas trifoliadas, raíces laterales y contenido de clorofila. En nuestro grupo de trabajo, el efecto promotor del crecimiento debido a la DMHDA así como su relación con el contenido de clorofila (que indica el estatus nutrimental por Fe) se ha conservado en diferentes especies de plantas, como *M*. *sativa*, *S*. *bicolor* y *P*. *devoniana* (Velázquez-Becerra et al., 2011; Castulo-Rubio et al., 2015; Valencia-Cantero et al., 2015). Se desconoce el mecanismo por el cual la DMHDA promueve el crecimiento vegetal, sin embargo se sabe que esta molécula funciona de manera independiente a la vía de señalización de las auxinas (Raya-González et al., 2017).

A. *thaliana* cuenta con 133 genes *bHLH*, de los cuales 16 son factores de transcripción en la vía de respuesta a deficiencia de Fe, esto representa el 12% de los miembros de dicha familia (Heim et al., 2003). Estos factores de transcripción interactúan *in vivo* formando homodímeros o heterodímeros, para regular de manera positiva la expresión de los genes *FRO2* e *IRT1* en la epidermis de la raíz (Colangelo et al., 2004). El factor transcripción bHLH6 también conocido como MYC2 es un regulador maestro de la vía de señalización del ácido jasmónico, cuya actividad afecta la expresión de los genes *bHLH18*, *bHLH19*, *bHLH20* y bHLH25 que interactúan con FIT y modulan su degradación proteosomal (Cui et al., 2018).

Adicionalmente, existe una comunicación cruzada entre el AS y el AJ mediante el factor de transcripción WRK70, cuya expresión es activada AS y reprimida por AJ. Este factor activa la expresión de los genes de respuesta a AS reprime a los genes de respuesta a AJ (Li et al., 2004). Tomando en cuenta que la mayoría de genes presentes en *Arabidopsis* tienen ortólogos en *M*. *truncatula*, incluyendo aquellos que participan en la toma de Fe (e.g. *FRO2*, *FIT1*, *BTS*, *bHLH38*), se decidió identificar en el genoma de la planta modelo de este estudio mediante alineamientos de secuencias, a los genes ortólogos para los factores de transcripción *bHLH38*, *bHLH39 y FIT* de la vía de respuesta a deficiencia de Fe; los genes WRKY70 y MYC2 de respuesta a AS y AJ, respectivamente.

Posteriormente se analizó la expresión de estos factores de transcripción y de los genes regulados por ellos como son *MtFRO3* (Montejano-Ramírez et al., 2018), *MtNPR4* (identificado en este trabajo), y *MtDef2.1* (Hanks et al., 2005). La ausencia de Fe indujo la expresión de los genes *MtbHLH38*, *MtbHLH39*, *MtFIT* y *MtFRO3*, observándose un efecto mayor sobre los factores de transcripción *MtbHLH38* y *MtbHLH39*, con un incremento de 5 y 8 veces respectivamente. En reportes previos, la expresión de estos genes llega a 107 y 46 en condiciones de deficiencia de hierro, mientras que *FIT* alcanza valores de 2 (Zhang et al., 2015). En conjunto con estos antecedentes, la expresión observada para *MtbHLH38* y *MtbHLH39*, resalta una función clave de estos factores de transcripción en la activación de la maquinara involucrada en la toma de Fe. Asimismo, existe evidencia de que la deficiencia de Fe en plantas, aumenta los niveles endógenos de las fitohormonas involucradas en el establecimiento de ISR, es decir ET y AJ así como del AS que activa SAR (Romera et al., 1999; Kobayashi et al., 2016; Shen et al., 2016). En el presente trabajo se observó que en las plantas crecidas en deficiencia de Fe, aumentó la expresión de los genes de defensa *MtWRKY70*, *MtNPR4*, *MtMYC2* y *MtDef2.1* por lo cual se podría especular que este efecto se debe a un incremento en la síntesis de las fitohormonas que regulan la expresión de dichos genes.

Después de observar un efecto positivo de la deficiencia de Fe sobre los genes de defensa, se decidió evaluar el efecto de las fitohormonas AS y AJ sobre los genes de respuesta a este metal. Las plantas tratadas con AS o AJ, presentaron una

inhibición en la expresión de los genes *MtbHLH38*, *MtbHLH39*, *MtFIT* y *MtFRO3* e impidieron la activación de estos en condiciones de deficiencia de Fe. A pesar de que la deficiencia de Fe aumenta los niveles endógenos de las fitohormonas de defensa (Romera et al., 1999; Kobayashi et al., 2016; Shen et al., 2016), los resultados difieren cuando se aplican estas fitohormonas de manera exógena.

En plantas de *A*. *thaliana* tratadas con AS, se ha observado una disminución en la expresión del gen *AtFRO2*, sin embargo, no existe ningún efecto en la expresión de los genes *AtbHLH38* y *AtFIT1* (Maurer et al., 2014). En el caso del AJ, la aplicación exógena de este, muestra efectos claros de inhibición sobre la expresión de los genes *AtFIT*, *AtFRO2* y *AtIRT1* tanto en condiciones de suficiencia como deficiencia de Fe (Maurer et al., 2011). En un estudio más reciente, se observó que el AJ, inhibe la expresión de los factores de transcripción *FIT*, *bHLH38*, *bHLH39*, *bHLH100* y *bHLH101* y que adicionalmente, el AJ promueve la degradación de *FIT* mediante la interacción con los factores *bHLH18*, *bHLH19*, *bHLH20* y *bHLH25*, lo cual da como resultado una inhibición en la activación de la respuesta a deficiencia de Fe (Cui et al, 2018). Por el contrario, existen estudios donde la aplicación de AS aumenta la expresión de los factores *AtbHLH38* y *AtbHLH39* (Kang et al., 2003) y el AJ tiene un efecto positivo en la respuesta temprana a deficiencia de Fe en *Oryza sativa* (Kobayashi et al., 2016) sin embargo los resultados del presente estudio apoyan el efecto de regulación negativa ejercida por la aplicación exógena de AS y AJ.

A fin de caracterizar la respuesta de los genes de defensa identificados en esta investigación, se evaluó el efecto de AS y AJ sobre los genes de las vías SAR e ISR. Como era de esperarse los genes *MtMYC2* y *MtDef2.1* tuvieron una inducción clara cuando las plantas fueron tratadas con AJ (Boter et al, 2004; Penninckx et al, 1996). Adicionalmente, el AS aumentó la expresión del gen *MtWRKY70*, en cambio la inducción de *MtNPR4* sólo fue clara cuando esta fitohormona se combinó con deficiencia de Fe, lo cual resalta la existencia de mecanismos adicionales disparados por Fe que participan en la vía ISR debido a que se ha demostrado que la deficiencia de este metal, aumenta los niveles endógenos de AS mediante la regulación de la expresión del gen "*Phytoalexin Deficient 4*" (*PAD4*) (Shen et al., 2016).

En comparación con el AS y el AJ, la aplicación de DMHDA indujo la expresión de los genes *MtbHLH38*, *MtbHLH39*, *MtFIT* y *MtFRO3*. El efecto de inducción fue mayor cuando se combinó la DMHDA con deficiencia de Fe. Recapitulando, en nuestro grupo de trabajo se ha reportado que la aplicación del COV DMHDA en plantas de *M*. *truncatula*, aumenta la acidificación de la rizosfera y la actividad férrico quelato reductasa, siendo estas respuestas mayores en combinación de la molécula con deficiencia de Fe (Orozco-Mosqueda et al., 2013). Por otra parte, también se ha demostrado que la DMHDA aumenta la expresión del gen *FRO1* de *S. bicolor* (Castulo-Rubio et al., 2015). En conjunto, nuestros resultados reafirman la participación de la DMHDA en la regulación transcripcional de la respuesta a deficiencia de hierro y que este proceso ocurre desde los principales factores de transcripción involucrados en la vía (*MtbHLH38*, *MtbHLH39* y *MtFIT).* Además, observamos que la DMHDA genera una ligera inducción en la expresión de los genes *MtNPR4*, *MtWRKY70* y *MtMYC2*, pero un aumento claro en la expresión de *MtDEF2.1* comparable con aquella observada en las plantas tratadas con AJ. La expresión de estos genes fue mayor en combinación con ausencia de hierro. Estos resultados resaltan la participación de la DMHDA las dos vías de defensa, es decir SAR e ISR, mediante la regulación de los factores de transcripción claves de éstas. Se ha propuesto que durante la activación de la defensa y con el fin de mantener un balance energético, las plantas presentan una inhibición en el crecimiento (Huot et al., 2014; Kobayashi et al., 2020), no obstante el COV 2,3-butanodiol activa tanto SAR como ISR además de promover el crecimiento vegetal (Kong et al., 2018) al igual que la DMHDA.

El hongo necrótrofo *Botrytis cinerea* y la bacteria hemibiótrofa *Pseudomonas syringae* son dos patógenos de gran importancia económica en la agricultura. *B*. *cinerea* infecta a más de 200 especies de plantas y es considerado el segundo fitopatógeno más importante, responsable del deterioro pre y postcosecha de los frutos (Dean et al., 2012). Este hongo infecta cultivos como fresa, tomate y flores ornamentales (Legard et al., 2000). Por su parte *P*. *syringae* con más de 50 patovares, infecta a casi todos las especies de cultivos de interés agrícola, por lo cual es uno de los patógenos más comunes en las plantas (Xin et al., 2018). Es bien

sabido que el AS presenta funciones importantes en el mecanismo de defensa contra patógenos biótrofos y hemibiótrofos como *P*. *syringae*, mientras que el AJ y el ET están activamente involucrados en la defensa contra insectos herbívoros y patógenos necrótrofos, incluyendo a *B*. *cinerea* (War et al., 2012; Glazebrook 2005). Con el propósito de activar las vías de SAR e ISR de manera fisiológica y a través de los patógenos correspondientes, se infectaron plantas de *M*. *truncatula* con *P*. *syringae* o *B*. *cinerea* y se evaluó la expresión de los genes de dichas vías, además de aquellos involucrados en la respuesta a deficiencia de Fe. Al igual que con la aplicación exógena de AS y AJ, la presencia de estos patógenos tuvo un efecto negativo en la expresión de los genes *MtbHLH38*, *MtbHLH39*, *MtFIT* y *MtFRO3.* Por otra parte, la inoculación con *B*. *cinerea* indujo la expresión de los genes *MtMYC2* y *MtDef2*.*1*, mientras que *P*. *syringae* tuvo este mismo efecto sobre los genes *MtNPR4* y *MtWRKY70.*

Finalmente, evaluamos el efecto protector de la DMHDA en *M*. *truncatula* ante la inoculación de los patógenos *P*. *syringae* y *B*. *cinerea*. Las plantas tratadas con DMHDA e infectadas con dichos patógenos mostraron un mejor crecimiento en comparación con las aquellas a las que no se les aplicó DMHDA. En nuestro grupo de trabajo ya se ha observado que la DMHDA protege a las plantas contra *B*. *cinerea*, además de *Phytophthora cinnamomi* e *Hipocrea* o *fusarium* (Velázquez-Becerra et al., 2013; Orozco-Mosqueda et al., 2015), sin embargo, no se conocía el mecanismo detrás de este fenómeno. Nuestros resultados demuestran que esta protección se debe a la activación de las vías ISR y SAR mediada por la DMHDA como se muestra en la inducción de la expresión de los genes *MtNPR4*, *MtWRKY70*, *MtPR1*, *MtMYC2* y *MtDef2*.*1.*

Los resultados previos a esta investigación indican que la DMHDA aumenta la expresión del gen *AtLOX2,* el cual responde a AJ y está involucrado en la síntesis de esta fitohormona (Raya-González et al., 2017), además de que está molécula y el AJ son antagonizados por la kinetina (Vázquez-Chimalhua et al., 2019) por lo que se hipotetizó que la aplicación de DMHDA en plantas dispara la vía de AJ. Nuestros datos revelan un comportamiento opuesto. Las plantas tratadas con DMHDA presentaron una promoción en el crecimiento vegetal, además de una inducción en

la expresión de los genes de respuesta a deficiencia de Fe; por otro lado, el tratamiento con AJ inhibió tanto el crecimiento como la expresión de los genes ya mencionados. Adicionalmente la DMHDA activó conjuntamente la expresión de los genes marcadores para ambas vías de defensa, es decir ISR y SAR, mientras que el AJ únicamente activó ISR.

8. Conclusión

La DMHDA activa las vías de defensa y deficiencia de hierro, promueve el crecimiento vegetal y la resistencia a patógenos, además, el efecto de la DMHDA no es a través de la activación de la vía del AJ, sino mediante la inducción de una vía alterna.

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10. Addenda

10.1. Volatile compounds from beneficial or pathogenic bacteria differentially regulate root exudation, transcription of iron transporters, and defense signaling pathways in *Sorghum bicolor.*

Artículo de investigación en el cual se participó como coautor y que fue publicado en la revista *Plant Molecular Biology*, la cual cuenta con un factor de impacto de 4.065.

10.2. Rhizobacterium Arthrobacter agilis UMCV2 increases organ-specific expression of FRO genes in conjunction with genes associated with the systemic resistance pathways of *Medicago truncatula*.

Artículo de investigación que fue publicado en la revista *Acta Phisiologiae Plantarum*, que tiene un factor de impacto de 2.078.

10.3. Fitohormonas en la defensa de las plantas: Una lucha estratégica.

Artículo de divulgación publicado en el número 42 de la revista “Saber Más” de la Coordinación de la Investigación Científica en la Universidad Michoacana de San Nicolás de Hidalgo. Con ISSN-2007-7041.

10.4. The volatile organic compound dimethylhexadecylamine affects bacterial growth and swarming motility of bacteria.

Artículo de investigación en el cual se participó como coautor y que fue publicado en la revista *Folia Microbiologica*, la cual cuenta con un factor de impacto de 1.639.

10.1 Addendum I

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Volatile compounds from beneficial or pathogenic bacteria differentially regulate root exudation, transcription of iron transporters, and defense signaling pathways in *Sorghum bicolor*

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Abstract

Key message **Our results show that *Sorghum bicolor* is able to recognize bacteria through its volatile compounds and differentially respond to beneficial or pathogens via eliciting nutritional or defense adaptive traits.**

Abstract Plants establish beneficial, harmful, or neutral relationships with bacteria. Plant growth promoting rhizobacteria (PGPR) emit volatile compounds (VCs), which may act as molecular cues influencing plant development, nutrition, and/or defense. In this study, we compared the effects of VCs produced by bacteria with different lifestyles, including *Arthrobacter agilis* UMCV2, *Bacillus methylotrophicus* M4-96, *Sinorhizobium meliloti* 1021, the plant pathogen *Pseudomonas aeruginosa* PAO1, and the commensal rhizobacterium *Bacillus* sp. L2-64, on *S. bicolor*. We show that VCs from all tested bacteria, except *Bacillus* sp. L2-64, increased biomass and chlorophyll content, and improved root architecture, but notheworthy *A. agilis* induced the release of attractant molecules, whereas *P. aeruginosa* activated the exudation of growth inhibitory compounds by roots. An analysis of the expression of iron-transporters *SbIRT1*, *SbIRT2*, *SbYS1*, and *SbYS2* and genes related to plant defense pathways *COI1* and *PR-1* indicated that beneficial, pathogenic, and commensal bacteria could up-regulate iron transporters, whereas only beneficial and pathogenic species could induce a defense response. These results show how *S. bicolor* could recognize bacteria through their volatiles profiles and highlight that PGPR or pathogens can elicit nutritional or defensive traits in plants.

Keywords Rhizosphere · Root exudates · Volatile compounds · Plant defense · Jasmonic acid

Introduction

Roots play important roles including anchorage, provision of nutrients and water, and release exudate molecules with regulatory, attractant or repellent properties for microbial populations (Bertin et al. 2003). Bacteria proliferate at the rhizosphere, the soil zone influenced by root exudation, and

are crucial components of the plant microbiome influencing

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rhizobia, or free-living bacteria (Badri and Vivanco 2009), or the induction of allelopathic responses in neighbor plants, thus providing adaptive or competitive advantages (Ohkama-Ohtsu and Wasaki 2010; Shi et al. 2011; Broeckling et al. 2008; ZhongQun et al. 2012).

Plants differentially recognize pathogenic or beneficial rhizobacteria by detecting diffusible substances, such as the quorum-sensing compounds *N*-acyl-*L*-homoserine lactones, which induce changes in the root secretion profile (Mathesius et al. 2003; Ortiz-Castro et al. 2011) and activate plant immunity (Schuhegger et al. 2006). On the other hand, volatile compounds (VCs) elicit growth and developmental traits via influencing phytohormone homeostasis (Velázquez-Becerra et al. 2011; Bailly and Weisskopf 2012; Castulo-Rubio et al. 2015; Zhang et al. 2007). Bacterial VCs reprogram the global gene expression network of *A. thaliana* (Zhang et al. 2007), reinforcing immunity (Ryu et al. 2004), and nutritional iron homeostasis (Zhang et al. 2009; Orozco-Mosqueda et al. 2013b; Zamioudis et al. 2015). Currently, the specificity and signaling mechanisms orchestrating these responses remain unknown.

Plants acquire iron (Fe) via two strategies. Non-graminaceous monocots and all dicots are strategy I plants, in which the expression of the iron-regulated transporter (IRT) is induced following low Fe exposure (Abadía et al. 2011). Graminaceous plants show strategy II, which involves secretion of phytosiderophores by roots to solubilize Fe^{3+} in soil and improve its uptake through specific yellow stripe (YS) (Enomoto and Goto 2013) and/or IRT transporters (Ishimaru et al. 2006). Diverse YS transporters are involved in the internal mobilization of the Fe-nicotianamine complex (Abadía et al. 2011). A relationship between iron homeostasis and disease resistance has been established as plants growing under iron deficient conditions develop induced systemic (ISR) and systemic acquired resistance (SAR) (Kieu et al. 2012; Koen et al. 2014; Zamioudis et al. 2015). In addition to the PGP effect of bacterial VCs, these molecules can be recognized as signatures from beneficial or pathogenic bacteria that influence iron nutrition and defense responses. Thus, roots may change their exudate profile to strengthen beneficial relationships or defend against possible attackers.

In this study, we compared the responses of *Sorghum bicolor* plants to a set of beneficial, pathogenic, or neutral bacteria to determine if bacterial VCs could induce differential root exudation, iron nutritional responses, or plant immunity. The results show that plants can sense bacteria through its VCs profile and differentially respond to PGPR or pathogens via attractant or defensive mechanisms.

Materials and methods

Plant growth conditions

Sorghum bicolor PIONEER MR 82W21 seeds were used in all experiments. Seeds were surface disinfected by vigorously shaking in 95% alcohol using a vortex for 5 min, they were then washed with sterilized deionized water, immersed in 20% sodium hypochlorite for 8 min, and finally, rinsed 5 times with sterilized deionized water. Seeds were germinated in Petri dishes containing agar-solidified Murashige and Skoog (MS) 0.2 × medium and incubated in a Percival plant growth chamber with a photoperiod of 16/8 h (light/darkness), and a light intensity of 200 µmol m^2 s^{-1} at 22 °C.

Embryonic axes were obtained from seedlings 120 h after germination, according to Peña-Uribe et al. (2012). The axes were carefully separated from endosperm using a scalpel, sterilized with 12% sodium hypochlorite for 1 min, rinsed four times with deionized sterilized water and then placed in a glass flask with MS 0.2 × medium and incubated in the growth chamber as previously described.

Bacterial strains and growth conditions

The bacterial strains *Arthrobacter agilis* UMCV2 (Valencia-Cantero et al. 2007), *Bacillus methylotrophicus* M4-96 (Pérez-Flores et al. 2017), *Sinorhizobium meliloti* 1021 (Orozco-Mosqueda et al. 2013a), *Pseudomonas aeruginosa* PAO1 (Ortiz-Castro et al. 2011), and *Bacillus* sp. L2-64 (Gutiérrez-Luna et al. 2010) were used in this study. All strains were grown on nutrient agar (NA; 3 g/L of meat extract, 5 g/L peptone, 1.5% agar) at 30 °C except *A. agilis* UMCV2, which was grown at 22 °C and *S. meliloti* 1021, which was grown on peptone yeast extract (PY; 5 g/L of peptone, 3 g/L of yeast extract, 0.7 g/L, 1.5% agar) at 30 °C.

Plant-bacteria co-cultivation experiments

To evaluate the effects of bacterial VCs on plant growth, embryonic axes were placed in 170 mL glass flasks containing 30 mL of MS 0.2 × medium after 6 days of germination, and a vial with 5 mL of NA or PY medium, depending on the type of bacterial strain, was introduced. The vial inside the flask was inoculated with 30 µL of bacterial suspensions of different strains corresponding to 1×10^6 CFU/mL. This system prevented physical contact between *S. bicolor* and the different bacteria and allowed plants to react to the bacterial volatile blends accumulating in the flask (Castulo-Rubio et al. 2015). Plants were maintained in the flask for 10 days, and at the end of the experiment, the fresh weight biomass was recorded using an analytical balance. The stem

length of plants was measured using a digital Vernier caliper (Mitutoyo Corporation, Tokyo Japan). The total chlorophyll content in arbitrary units was measured according to Castulo-Rubio et al. (2015), using a CCM-200 chlorophyll meter (Opti-Sciences, Inc.; Hudson, NH, USA).

Effect of bacterial VCs on root exudates

Three, 3 days-old embryonic axes were placed in glass flasks with 15 mL of sterile deionized water. The glass flask was protected from light with aluminum foil and the top of the flask was covered with sterile cotton wool allowing the leaf tissue to remain outside the bottle. A 5 mL vial with 3 mL of NA and PY medium was inoculated with 1×10^6 UFC of *A. agilis*, *Bacillus methylotrophicus* M4-96, *P. aeruginosa*, *S. meliloti*, and *Bacillus* sp. L2-264 as previously described (Orozco-Mosqueda et al. 2013b). Both the glass flask with *S. bicolor* and vials with inocula were placed into a 170 mL flask, closed, and transferred into the growth chamber for 8 days. The *S. bicolor* leaves were moistened with MS 0.2 × medium every 2 days with a brush and the root exudates were extracted.

Extraction and derivatization of organic acids

Root exudates were collected and adjusted to a pH of 3.0 with 1 N of HCl. The same volume of ethyl acetate was added and mixed using a vortex. The organic phase was transferred to a vial and evaporated to dryness under a stream of nitrogen. The organic acids were methyl esterified with 2 mL of acetyl chloride in dry methanol (500 µL/2 mL), sonicated for 15 min, and heated for 1 h at 75 °C. After cooling, the sample was dried under a stream of nitrogen and later, 1 mL of dichloromethane and 1.5 mL of acetic anhydride were added and the sample re-heated for 1 h at 75 °C. Finally, the sample was transferred to a 1.5 mL amber vial, dried under a stream of nitrogen, and re-suspended in 50 µL of ethyl acetate for Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

Analysis of root exudates via GC-MS

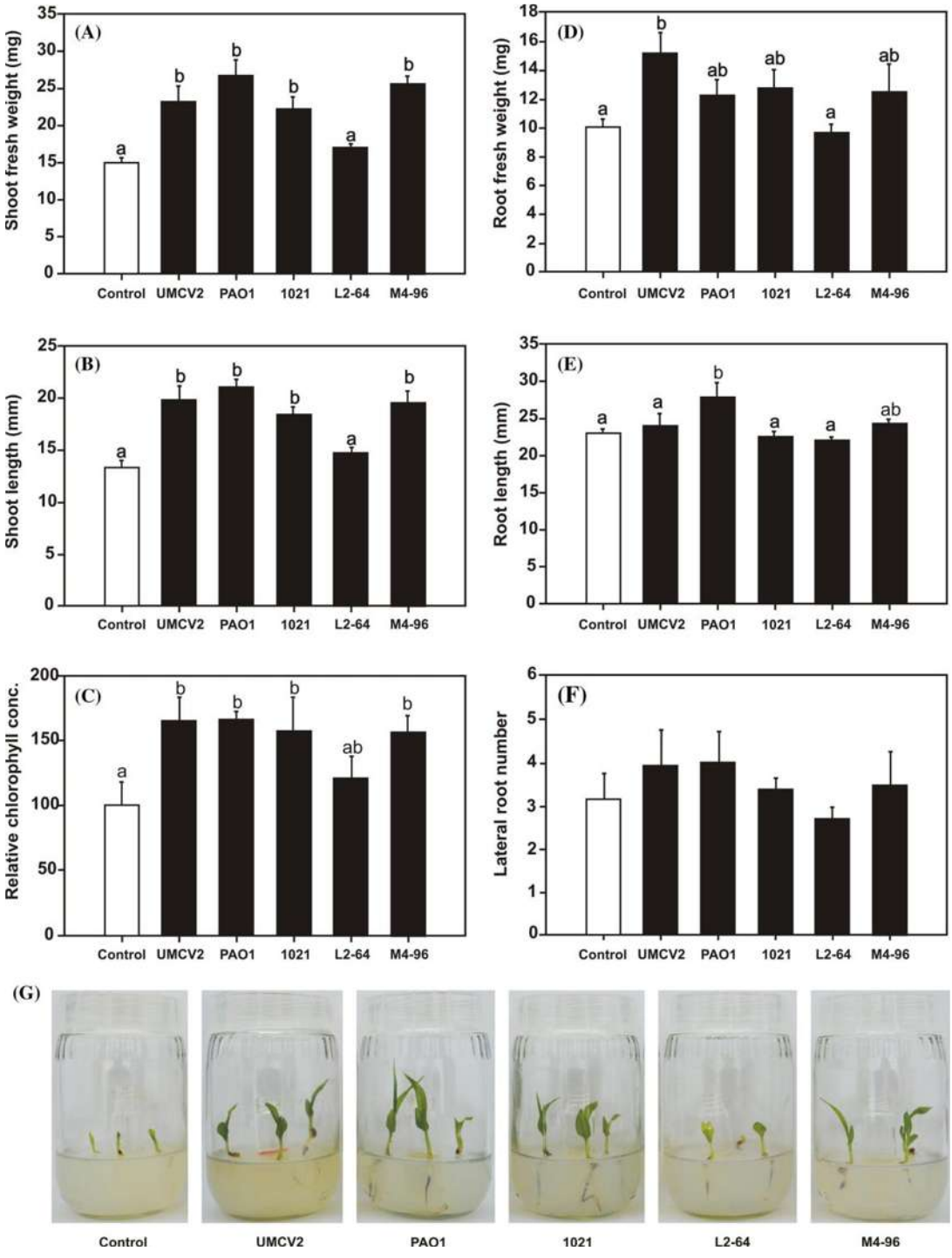
The organic acids were analyzed in an Agilent 6850 series II gas chromatograph (Agilent, Foster City, CA, USA) equipped with an Agilent MS detector model 5973, 30 m × 0.2 µm film thickness × 0.25 mm, and a 5% phenyl methyl silicone capillary column (HP5-MS, Agilent). Ultrapure helium ($1 \text{ mL} \times \text{min}^{-1}$) was used as a carrier gas and detector and injector temperatures were 300 and 250 °C, respectively. The column was held for 5 min at 150 °C and then programmed to increase 5 °C every 5 min until it reached a final temperature of 275 °C, which was maintained for 10 min. The compounds were identified by comparisons with mass spectra from the library (NIST/EPA/NIH Mass Spectral Database 11 and NIST Mass Spectral Search Program 2.0; Chem Station Agilent Technologies Rev. D.04.00.2002).

Identification and validation of expression of iron transporters and defense related genes

To search for the *S. bicolor* iron-regulated metal transporter 1 and 2 (*SbIRT1* and *SbIRT2*) sequences, we ran a homology Basic Local Alignment Search Tool (BLAST), employing the *OsIRT1* and *OsIRT2* gene sequences as target genes (Ishimaru et al. 2006), with accession numbers AB070226 and AB126086, respectively. To search for the *S. bicolor* YS 1 and 2 (*SbYS1* and *SbYS2*) sequences, we used the *S. bicolor* database (http://plantgdb.org/SbGDB/). We searched the open reading frames (ORFs) for recovery sequences using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and the ORFs were analyzed using the BLASTP suite. To search conserved domains in the putative sequences of iron-transporters, the nucleotide sequence from the Conserved Domains Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was employed. Predictions of transmembrane domains were performed using the HMMTOP 2.0 transmembrane topology prediction software at http://www.enzim.hu/hmmtop/ (Tusnady and Simon 2001). Finally, a phylogenetic analysis using the nucleotide and amino acid sequences was performed. The top putative homologues and orthologues of *IRT1* and *IRT2* in *S. bicolor* were identified using accessions XM_002464063.1 and XM_002464064.1 as a query against GenBank (BLASTN, threshold E-value $\leq 1 \times 10^{-50}$ and identity > 70). The same procedure was used to analyze the sequences of YS 1 and 2 using the accessions XM_002452787 and XM_002458662, respectively. The recovery sequences were aligned with CLUSTALW as part of the MEGA 6.06 software (Tamura et al. 2013). The phylogenetic tree was created in MEGA 6.06 using the maximum parsimony method, and bootstrap support values were obtained over 1000 replications.

We used the sequence of coronatine-insensitive protein 1 (*SbCOI1*) to test the activation of jasmonic acid signaling (Sen et al. 2016) and pathogenesis-related protein 1 (*PR-1*) as a salycilic acid inducible gene using the *S. bicolor* database (http://plantgdb.org/SbGDB/) and GenBank.

Gene specific primers were designed based on the characterized sequences of *S. bicolor SbIRT1* (XM_002464063.1), *SbIRT2* (XM_002464064.1), *SbYS1* (XM_002452787), *SbYS2* (XM_002464064.1), *SbCOI1* (XM_002456604.1), and *SbPR-1* (XM_002436360.2) (Supplementary Table S1) using the Primer Express Software Version 3.0 by Applied Biosystems (Foster City, CA, USA). The actin primer was used as in Castulo-Rubio et al. (2015). The efficiency of each primer set was verified


(A)
Shoot fresh weight (mg)
(D)
Root fresh weight (mg)
(B)
Shoot length (mm)
(E)
Root length (mm)
(C)
Relative chlorophyll conc.
(F)
Lateral root number
Control
UMCV2
PAO1
1021
L2-64
M4-96
(G)

◂**Fig. 1** Effects of VCs of *A. agilis* UMCV2, *P. aeruginosa* PAO1, *S. meliloti* 1021, *Bacillus* sp. L2-64, and *B. methylotrophicus* M4-96 on growth and development of *S. bicolor* seedlings. After 8 days of inoculation, shoot fresh weight (**a**), shoot length (**b**), relative concentration of chlorophyll (**c**), root fresh weight (**d**), root length (**e**), and lateral root number (**f**) were plotted. Bars represent the mean± standard error. Means with the same letter did not differ significantly after an ANOVA and Tukey post-hoc test

using a five-fold serial dilution of untreated samples cDNA. Linear regressions between the number of cDNA templates and the corresponding Ct values were calculated for each primer set to obtain the correlation coefficient ($R^2 > 0.98$) according to Schmittgen and Livak (2008). To verify the amplification of single, specific target cDNA, a melting-curve analysis was included according to the thermal profile, as suggested by the manufacturer (Applied Biosystems). The amount of *S. bicolor* RNA in each sample was normalized using actin as the reference gene. Finally, gene expression was calculated using the mathematical formula: $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

In order to validate the expression of *SbIRT1, SbIRT2, SbYS1*, and *SbIRT2* genes, we used embryonic axes, which 6 days after germination were placed in 170 mL glass flasks containing 30 mL of complete MS 0.2 × medium, MS 0.2 × medium without Fe (MS Fe-minus medium), or MS 0.2 × Fe-minus medium plus 100 μM *FerroZine* [3-(2-pyridyl)-5,6 diphenyl-1,2,4-triazine sulfonate; Sigma St Louis] (as an Fe scavenging agent). Plants were incubated for 48 h, then the plant roots were frozen using liquid nitrogen immediately after which RNA extraction was performed using Tri reagent (Sigma-Aldrich). Total RNA extracted was treated with DNase I. The integrity of the RNA was assessed by visualization in 1.2% agarose gel and the final concentration was estimated using NanoDrop (Thermo Scientific, Rockford, IL). cDNA synthesis was obtained using 500 ng of the RNA template with the Super Script First-Strand Synthesis System (Life Technologies/Gibco-BRL CA, USA). A quantitative PCR (qPCR) reaction (20 μL) was performed using the iQ SYBR Green kit (Applied Biosystems) with 10 μL of Master and 1 μL of gene specific primers (Supplementary Table S1).

To validate the gene expression of *COI1* and *PR-1*, 6 days after germination, seedlings were placed in 170 mL glass flasks containing 30 mL of MS 0.2 × medium. On day six, the plants were incubated for 24 h with an MS solution supplemented with methyl jasmonate (MeJA) or salicylic acid (SA) (Sigma, St Louis). Stocks of MeJA and SA were diluted using ethanol absolute and dimethyl sulfoxide, respectively. The final concentrations of MeJA and SA in the culture medium were 100 μM and 1 mM, respectively, according to Salzman et al. (2005). After 24 h induction, cDNA was obtained from roots and the qPCR reaction was performed as described above.

Gene expression analysis of iron transporters and defense genes

In 170 mL glass flasks containing 30 mL MS 0.2 × medium and a 5 mL vial with NA or PY, 6 days-old embryonic axes were placed. On day six, the vials were inoculated with *A. agilis* UMCV2, *P. aeruginosa* PAO1, *S. meliloti*, *Bacillus* sp. L2-64, and *B. methylotrophicus* M4-96. The plants were maintained in this interaction with bacteria for 8 days and then, total RNA was obtained from roots by grinding the tissue with liquid nitrogen. The RNA was extracted using TRI reagent (Sigma-Aldrich) and treated with DNase I to remove DNA contamination. The quality and purity of RNA samples was visualized on 1.2% agarose gel stained with GelRed (Biotium, USA) and quantified using NanoDrop (Thermo Scientific, Rockford, IL). Total RNA (500 ng) was synthesized into cDNA using the Super Script First-Strand Synthesis System (Life Technologies/Gibco-BRL CA USA) and diluted to a final concentration of 4 ng × μL^{-1}. The quantitative PCR (qPCR) reaction (20 μL) was performed using the iQ SYBR Green kit (Applied Biosystems; Foster City, CA, USA) with 10 μL of Master Mix.

Statistical analysis

Experiments were performed with n = 5 plants per treatment and replicated three times. The results were analyzed using a one way ANOVA and Tukey's means separation test for multiple comparisons ($p < 0.05$) using the Statistica 7 software (Statsoft Inc.). This was performed using PAST Software (Hammer et al. 2001). To calculate the similarities in the chemical compositions of root exudates stimulated by VCs, an analysis cluster based on the UPGMA algorithm using a matrix of similarity of Horn supported by 1000 bootstraps was performed in PAST. To analyze the transcription of *IRT1, IRT2, YS1*, and *YS2*, a generalized linear model with Poisson errors was used to test differences in mean values of gene expressions between bacterized and untreated (control) plants, while for defense genes, an ANOVA and Tukey's post hoc test were performed with JMP® V. 8.0 (SAS Institute Inc., Cary, NC, 1989–2007).

Results

Volatiles from either beneficial or pathogenic bacteria promote plant growth

To determine the effect of VCs from beneficial, commen- sal (neutral) or pathogenic bacteria on sorghum plants, we first measured plant variables related to growth, develop- ment and nutrimental status using a compartmentalized sys- tem previously described by Castulo-Rubio et al. (2015).

The VCs from *A. agilis* UMCV2, *P. aeruginosa* PAO1, *S. meliloti* 1021, and *B. methylotrophicus* M4-96, increased shoot fresh weight ($p < 0.001$) (Fig. 1a–g), shoot length ($p < 0.001$) (Fig. 1b), and chlorophyll content ($p < 0.05$) (Fig. 1c), whereas VCs from *Bacillus* sp. L2-64 did not significantly increase any of these traits. The effects of bacterial volatiles on roots were moderate (Fig. 1), and only *A. agilis* UMCV2 was able to significantly increase root fresh weight ($p < 0.05$) (Fig. 1d). The root length only increased when the plants were exposed to volatiles from *P. aeruginosa* PAO1 ($p < 0.05$) (Fig. 1e), while no significant effects were evident for root branching (Fig. 1f). Representative images of plants in the experimental system clearly show the beneficial effects of some of the bacteria on plant size (Fig. 1g), suggesting that perception of VCs orchestrates a growth response.

Bacterial VCs induce specific root exudation profiles

Since we observed that VCs from all tested bacteria, except *Bacillus* sp. L2-64, produce a plant growth promoting effect on sorghum, we next analyzed the root exudates as a possible response to the type and lifestyle of interacting bacteria. It was found that root exudates of plants exposed to VCs from different bacterial strains were distinct in terms of type, number, and concentration (Fig. 2, Supplementary Table S2). Exudates produced by plants exposed to VCs from *A. agilis* UMCV2 were more diverse and accumulated at higher concentrations than those of plants exposed to other bacterial strains (Fig. 2). We found 39 differential compounds produced as root exudates. In un-inoculated and *A. agilis* UMCV2 inoculated plants, 21 and 29 different compounds, respectively, were released (Table 1). Plants

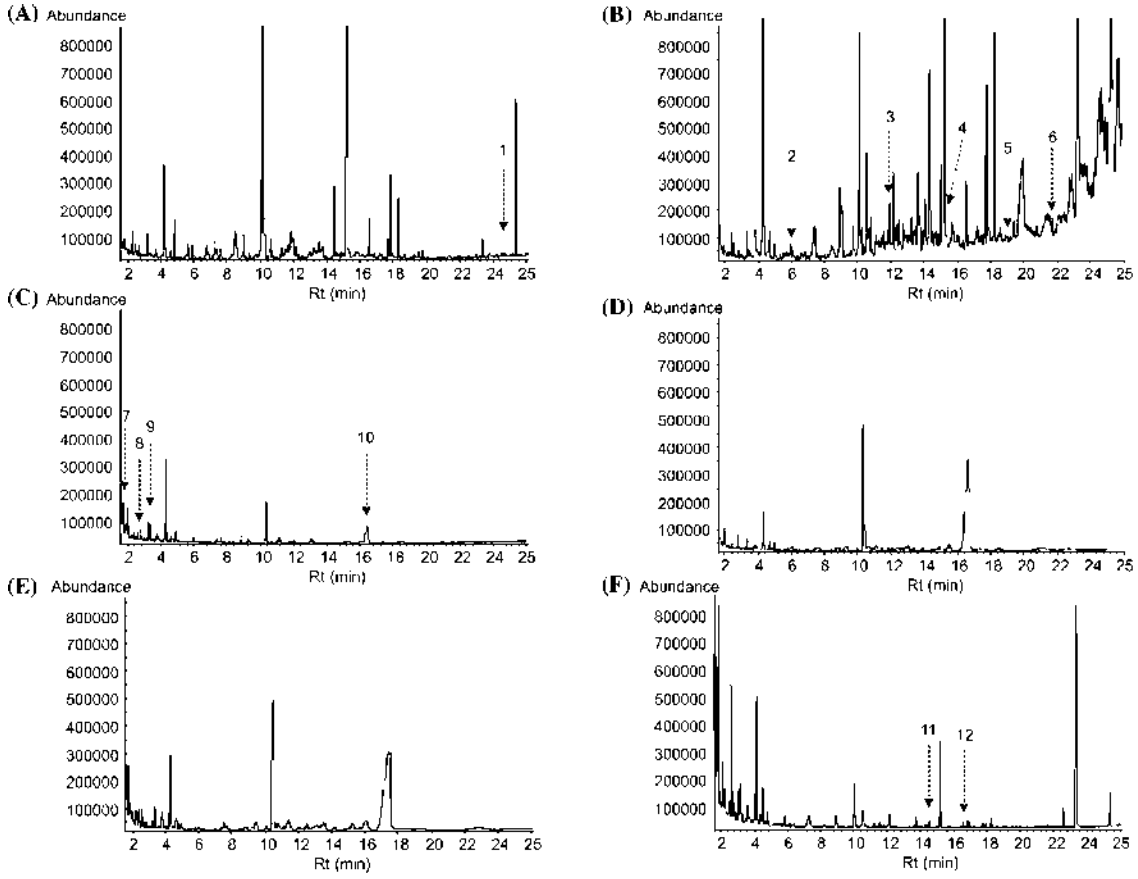


Table 1 Summary of root exudates from *S. bicolor* plants and those promoted by interactions between VCs of *A. agilis* UMCV2, *P. aeruginosa* PAO1, *S. meliloti* 1021, *Bacillus* sp. L2-64, and *Bacillus* M4-96

Treatment	Total number of exudates	Number of specific exudates	Most abundant compound in exudate	Compound only present in exudates of plants treated with the respective strains
Control	21.7 ± 0.577	1	1-Butyl-2-isobutyl phthalate	10-Nonadecenoic acid
UMCV2	29 ± 0.00	6	Tri-(butoxyethyl) phosphate	Citric acid Pentaerythritol Ferulic acid Nonadecanoic acid Eicosanoic acid Tetracosanoic acid
PAO1	11.7 ± 1.15	7	Diisobutyl phthalate	1,2-Ethanediol 2,6-Dimethylbenzaldehyde *n*-Propyl benzoate Benzoylformic acid Phthalic anhydride Phthalic acid Diisobutyl phthalate
1021	14 ± 0.00	0	1-Butyl-2-isobutyl phthalate	None
L2-64	12.7 ± 0.577	0	1-Butyl-2-isobutyl phthalate	None
M4-96	20 ± 1.00	2	Hexadecanoic acid	Heptadecanoic acid 1,2-Ethanediol diacetate Methyl 2-ethylhexyl phthalate

exposed to VCs from *B. methylotrophicus* M4-96, *S. meliloti* 1021, *Bacillus* sp. L2-64, and *P. aeruginosa* PAO1 produced fewer root exudates (Table 1; Fig. 2).

In general, we found a high similarity within groups of root exudates exposed to the same bacterial VCs and a low similarity among exudates released from plants exposed to VCs from different bacteria. The intragroup similarity of roots exudates promoted by VCs from *P. aeruginosa* PAO1 ranged from 0.903 to 0.92, but was ~ 0.3 for root exudates of plants treated with other microorganisms. The root exudates of *Sorghum* treated with VCs of *A. agilis* UMCV2 were similar (0.88–0.94), whereas their similarity with other groups ranged from 0.22 to 0.86 (Supplementary Table S3). The similarity of root exudate intra-treatments among controls was nearly 1 while that of root exudates between treatments varied from 0.193 to 0.95 (Supplementary Table S3). In general, the chemical composition of root exudates of *Sorghum* plants treated with *S. meliloti* 1021, *B. methylotrophicus* M4-96, and *Bacillus* sp. L2-64 were very similar to the exudates in axenically-grown plants (Supplementary Table S3).

Identification of iron-transporters IRT and YS in *S. bicolor*

We observed an increased chlorophyll concentration in sorghum plants exposed to VCs from beneficial and pathogenic bacteria, and it has been shown that some beneficial rhizobacteria trigger iron uptake mechanism in plants with strategy I via VCs emission (Zhang et al. 2009). With this in mind, we determined the effects of VCs from different bacteria on iron transporter gene expression in *S. bicolor* (strategy II plant). We first performed an *in silico* search to identify sequences of iron transporters homologous to *IRT* and *YS*. We found the putative *SbIRT1*, *SbIRT2*, *SbYS1*, and *SbYS2* sequences in the *S. bicolor* genome database (http://www.plantgdb.org/SbGDB/) and the NCBI (Table 2).

The ORFs corresponding to *SbIRT1* (XM_002464063) encoded a predicted protein of 392 amino acids and eight trans-membrane regions, whereas the *SbIRT2* sequence (XM_002464064) encoded a putative protein of 374 amino acids and nine trans-membrane regions (Table 2), both sequences had homologous and conserved domains of metal transporters (Table 2). Owing to its highly con- served zinc/iron transporter domain, the putative *SbIRT1* sequence XM_002464063 showed significant similarity to other Fe^{2+} transport proteins 1-like or zinc transport proteins, such as *Triticum urartu IRT1*, *Hordeum vulgare IRT1*, *Brachypodium distachyon IRT1*, and *Setaria italica IRT1*, and with *Aegilops tauschii* zinc transporter 10 and *Zea mays* zinc transporter 10 (Supplementary Fig. S4a). The *SblRT2* XM_002464064 sequence was similar to the Fe^{2+} transport protein 2-like of *Zea mays*, *Serratia italica*, *Oryza sativa*, and *Oryza branchiata* (Supplementary Fig. S4a). All of these sequences contained a metal transporter domain annotated as belonging to the zip zinc/iron super- family and Fe^{2+} transporter protein accessions TGR00820 and PLN02159, respectively (NCBI-conserved domain

Table 2 Features of open reading frames (ORFs) to *SbIRT1, SbIRT1, SbYS1, and SbYS2*

Source of sequences (Accession in the GenBank)	ORF size	Transmembrane regions	Conserved domains found	(E-value)	Accessions in conserved domain database
SbIRT1 XM_002464063	392aa − 2	8	Zip zinc/iron transport family	1.23e−143	TIGR00820
			Fe(2+) transport protein	2.21e−127	PLN02159
			Zip zinc transporter	5.51e−75	pfam02535
SbIRT2 XM_002464064	374aa + 1	9	Zip zinc/iron transport family	3.67e−143	TIGR00820
			Fe(2+) transport protein	6.71e−132	PLN02159
			Zip zinc transporter	1.26e−71	pfam02535
SbYS1 XM_002452787	678aa + 1	14	OPT oligopeptide transporter protein	1.66e−129	pfam03169
			OPT superfamily	7.03e−86	TIGR00728
			Uncharacterized membrane protein OPT	6.96e−23	COG1297
SbYS2 YS2_XM_002458662	680aa + 1	14	OPT oligopeptide transporter protein	4.38e−136	pfam03169
			OPT superfamily	1.89e−62	TIGR00728
			Uncharacterized membrane protein OPT	3.87e−27	COG1297

database) (Table 2). The putative homologous *SbIRT1* and *SbIRT2* were divergent sequences in the phylogenetic analysis. However, sequences of *IRT1* and *IRT2* in oth- ers species were orthologous in their relationship with *S. bicolor* iron regulated transporters (Supplementary Fig. S4a).

The *SbYS1* and *SbYS2* ORFs comprised 678 and 680 amino acids, respectively, and possessed 14 trans-membrane spanning regions (Table 2). Both nucleotide sequences were highly similar to oligopeptide transporter proteins. Phylogenetic analysis of the *YS1* gene of *S. bicolor* showed that the hypothetical sequence XM_002452787 formed a cluster close to the putative sequences of *YS1* of *Zea mays* and *Serratia italica* (Supplementary Fig. S4b). The *YS1* sequence was closely related to *YS15* of *Brachypodium distachyon*, *Oryza sativa*, and *Oryza brachyantha* (Supplementary Fig. S4b). The putative sequence *SbYS2* accession XM_002458662 was more related to variants of *YS-like* 18 (Supplementary Fig. S4b). However, the sequence of *SbYS2* was closely related to the *YS2* of different species. Both nucleotide sequences had domains related to the oligopeptide transporter family (accession pfam03169) and oligopeptide transporter superfamily (accession TIGR00728) (Table 2).

Plants transferred to the MS Fe-minus medium showed an induction ranging from 5.3 to ninefold of *SbIRT1, SbIRT1, SbYS1*, and *SbYS2* genes in the controls (Fig. 3a, b). The addition of *FerroZin*e to the MS Fe-minus medium produced a characteristically, slightly purple coloration, indicating that this medium contained traces of iron (Fig. 3c). Similarly, according to this observation, plants transferred to the MS Fe-minus medium plus *FerroZine* showed over two-fold more *SbIRT1, SbIRT1, SbYS1*, and *SbYS2* expression than plants transferred to the MS Fe-minus medium without *FerroZine* (Fig. 3a, b), indicating its overregulation by iron scarcity.

Bacterial VCs induce transcription of iron transporters

To investigate whether VCs of different bacteria could induce the expression of iron-uptake genes in roots, the levels of *SbIRT1, SbIRT2, SbYS1*, and *SbYS2* transcripts were quantified by Q-RT-PCR (Fig. 4). The transcript levels of *SbIRT1* in relation to its expression in axenically-grown plants were significantly up-regulated ($p < 0.0001$) in response to VCs of *A. agilis* UMCV2 (35-fold), *P. aeruginosa* PAO1 (56 fold), and *Bacillus* sp. L2-64 (35 fold). The VCs of *S. meliloti* 1021 exerted the greatest induction on levels of *SbIRT1* gene transcripts (140 fold) (Fig. 4a). The VCs of *B. methylotrophicus* M4-96 down-regulated significantly the transcription level of all genes tested (Fig. 4a–d). The transcription of *SbIRT1, SbYS1*, and *SbIRT2* was up-regulated ($p < 0.0001$) in response to VCs of *P. aeruginosa* PAO1 (14-fold), *Bacillus* L-254 (12-fold), and *S. meliloti* 1021 (16-fold) (Fig. 4a–d). We did not find any effects of VCs of *A. agilis* UMCV2 on the transcription of *SbIRT2, YS2*, and *SbYS1* (Fig. 4b–d), but statistical differences in the expression of *SbIRT2* and *YS2* ($p < 0.0001$) were evident in response to most bacterial VCs (Fig. 4c, d). The data show the differential regulation of iron transporters by the bacterial volatiles.

Effects of bacterial volatiles on *COI1* and *PR-1* expression in *S. bicolor*

Owing to the fact that that VCs from beneficial and pathogenic bacteria differentially regulate root exudation, we

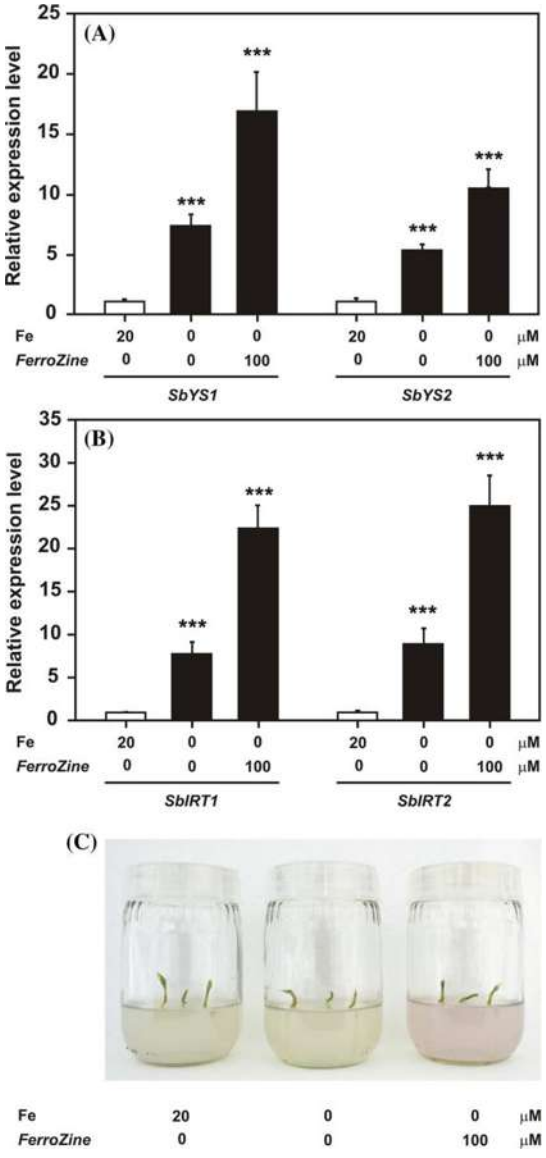


Fig. 3 Effect of iron deficiency on expression of iron transporter genes in *S. bicolor*. **a** Fold-change expression of *SbYS1* and *SbYS2* (**a**), or *SbIRT1* and *SbIRT2* (**b**) on *S. bicolor* embryonic axes trans- ferred to media with Fe, without Fe, or without Fe plus *FerroZine*. **c** The phenotypes of plants in the treatments. Asterisk above the standard error bars from all graphics indicate statistical significances calculated using a generalized linear model with Poisson errors.
**p* < 0.05; ***p* < 0.01; ****p* < 0.001

hypothesized that defense pathways could be influenced as part of the adaptive networks elicited in plants. There- fore, we analyzed the transcription of *SbCOI1* and *SbPR-1* genes in response to MeJA and SA using qPCR. Seedlings transferred to media with MeJA showed a *SbCOI1* induction 15.8 fold higher than in control seedlings, but also showed a *SbPR-1* repression (a transcription of 0.6 folds compared to solvent supplied seedlings; Fig. 5a). On the other hand, seedlings transferred to media with SA showed a 57-fold *SbPR-1* induction, although *SbCOI1* was 10-fold less induced (Fig. 5b). From these data, we conclude that *SbCOI1* and *SbPR-1* are regulated in opposite manners by two canonical phytohormones involved in plant defense. Next, an analysis of the transcription of *SbCOI1* and *SbPR-1* in response to bacterial VCs, revealed statistical differences in expressions of *SbPR-1* ($F = 41.55$, $df = 5$, $p < 0.0001$) and *SbCOI1* ($F = 14.1$, $df = 5$, $p < 0.0001$). Through a multiple comparison post hoc tests, differences in *PR-1*, induction were established for plants exposed to VCs from *A. agilis* UMCV2, *P. aeruginosa* PAO1, and S. *meliloti* 1021 when compared to the control (Fig. 6a). The relative expression of *PR-1* was similar in plants treated with *Bacillus* sp. L2-64, *B. methylotrophicus* M4-96 and the control (Fig. 6a). The induction of *COI1* was higher in plants treated with *A. agilis* UMCV2 and *S. meliloti*1021 than in the control (Fig. 6b), and no statistical differences were found between the relative expression of *COI1* in the control, *P. aeruginosa* PAO1, *Bacillus* sp. L2-64, and *B. methylotrophicus* M4-96 (Fig. 6b).

Discussion

In this study, we investigated the differential effects of VCs from beneficial or pathogenic rhizobacteria on growth as well as nutritional and defensive gene expression in *S. bicolor* plants. Beneficial rhizobacteria included *A. agilis* UMCV2, *S. meliloti* 1021, and *B. methylotrophicus* M4-96, which are characterized by their production of VCs with potent effects on the reconfiguration of the root system (Velázquez-Becerra et al. 2011; Orozco-Mosqueda et al. 2013a; Pérez-Flores et al. 2017). We also tested the VCs of *P. aeruginosa* PAO1, a bacterium employed as a model pathogen on plants including *A. thaliana*, *M. truncatula*, and *Hordeum vulgare* (Mathesius et al. 2003; Attila et al. 2008; Starkey and Rahme 2009; Ortiz-Castro et al. 2011), and *Bacillus* sp. L2-64, a commensal rhizobacterium, it means "neither harm nor benefit the plant directly" (Berendsen et al. 2012). Interestingly, VCs from all these bacteria, except *Bacillus* sp. L2-64, promoted *S. bicolor* growth and chlorophyll accumulation, suggesting some specificity in the phytostimulation mechanisms. One of the growth traits influenced by VCs was the chlorophyll content in leaves, which was used as an indirect indicator of the iron nutritional status of plants (Masalha et al. 2000; Radhamani et al. 2016). Orozco-Mosqueda et al., (2013a) found that *S. meliloti* promotes the growth of *M. truncatula* via VC emissions, rhizosphere acidification, and enhanced root ferric

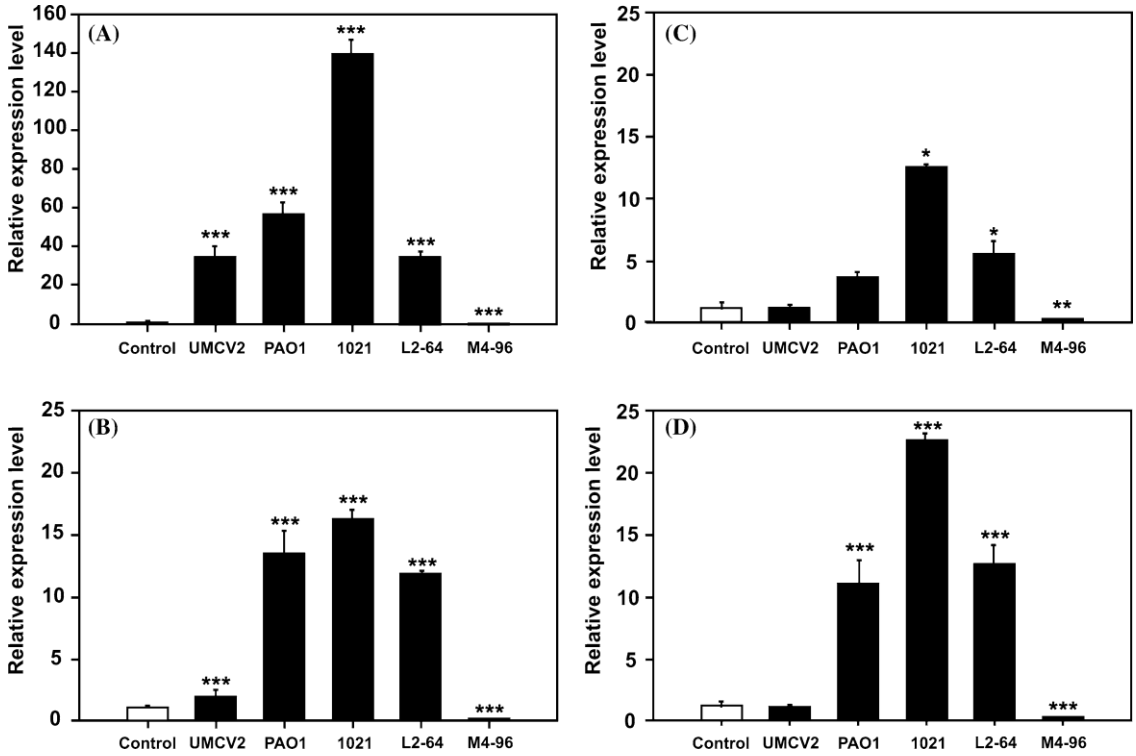


Fig. 4 Effect of bacterial volatiles on expression of iron transporter genes in *S. bicolor*. Fold-change expression of *SbIRT1* (**a**), *SbYS1* (**b**), *SbIRT2* (**c**), and *SbYS2* (**d**). The relative expression was calcu- lated based on the gene expression in the control (represented as 1). Data points are an average of at least three biological replicates and nine technical replicates. Asterisk above the standard error bars of all graphics indicate statistical significances calculated using a generalized linear model with Poisson errors. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

reductase activity even when plants were grown under iron sufficient conditions. Thus, the VCs of *S. meliloti* contain signaling molecules perceived by its natural-specific symbiont *M. truncatula* to improve iron acquisition, which may be essential for the nodulation process and symbiotic nitrogen fixation.

Sorghum bicolor is a Fe-uptake strategy II plant, and currently, no specific associative interactions with rhizobia are known. Our study showed that VCs of *S. meliloti* played a role in *Sorghum* growth promotion and that the induction of the Fe-uptake mechanism may be involved in these beneficial effects. Surprisingly, the highest fresh weight, stem length, and root length of plants were induced by VCs from *P. aeruginosa* PAO1, a gram-negative bacterium commonly isolated from soil and water environments. *Pseudomonas aeruginosa* produces phytoregulators, such as indole-3-acetic acid and cytokinins, as well as bioactive volatiles (Aka and Babalola 2016; Khara and Arora 2010; Blom et al. 2011b). Volatile compounds of *Bacillus methylotrophicus* M4-96 also increased shoot biomass and chlorophyll content. Our data were in agreement with those of Ryu et al. (2003), who demonstrated that the airborne volatile 2, 3-butanediol from related species *B. subtilis* and *B. amyloliquefaciens* could stimulate the growth of *A. thaliana*. A similar mechanism involving volatile sensing may occur in sorghum plants inoculated with *Bacillus methylotrophicus* M4-96.

The reported effects of bacterial VCs range from large biomass increases to plant death (Ryu et al. 2003; Blom et al. 2011a). The production of CO_2 by microbial respiration was common in all the tested bacteria and likely contributed to the growth promotion observed in Petri dish assays (Kai and Piechulla 2009). However, since *Bacillus* sp. L2-64 had no effect on the growth of *S. bicolor*, CO_2 emissions alone cannot explain the effects of all beneficial and pathogenic bacterial VCs on plant growth. Thus, we proposed that the plants were able to sense volatile blends and respond in a specific manner.

Plants are able to recruit beneficial microorganisms and suppress the growth of pathogens in the rhizosphere thereby

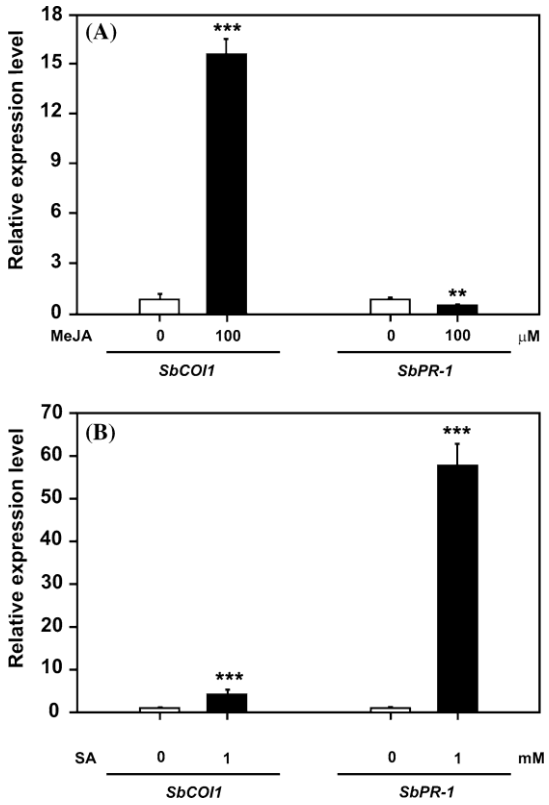


Fig. 5 Effect of MeJA and SA on expression of *COI1* and *PR-1* genes in *S. bicolor*. *Sorghum bicolor* seedlings were transferred to media with MeJA or SA and relative expression of *COI1* (**a**) and *PR-1* (**b**) determined by qPCR. Asterisk above the standard error bars of all graphics indicate statistical significances calculated using a generalized linear model with Poisson errors. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

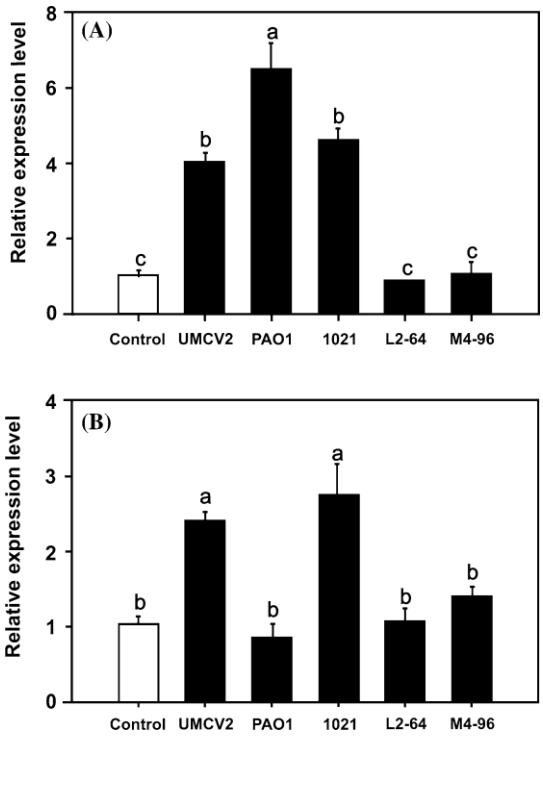


Fig. 6 Effect of bacterial volatiles on expression of *COI1* and *PR-1* genes in *S. bicolor*. Relative expression values of pathogenesis- related protein 1 (*PR-1*) (**a**) and coronatine-insensitive 1 (*COI1*) (**b**) genes. The relative values were calculated based on the gene expres- sion in the control. Bars represent means of three biological replicates and nine technical replicates ± standard errors. Letters above bars indicate means that differ significantly based on a Tukey's HSD post hoc test ($p < 0.05$)

modifying root exudation (Berendsen et al. 2012). With this in mind, we analyzed the exudate compounds of *S. bicolor* plants exposed to the VCs emitted by the different rhizobacteria tested. From the 28 different organic compounds present in the exudates from plants exposed to *A. agilis* UMCV2 VCs, we found that citric acid, an organic acid likely used as a carbon source from bacteria, ferulic acid a chemo-attractant signal compound for beneficial rhizospheric bacteria (Yuan et al. 2015), and three fatty acids (nonadecanoic, eicosanoic, and tetracosanoic acids), which may affect plant growth (Bertin et al. 2003), were released as specific exudates. Thus, exudates produced by plants treated with *A. agilis* UMCV2 VCs may act as attractants for bacteria. The root exudates induced by VCs of *P. aeruginosa* PAO1 largely differed. We found three phthalate derivatives that may exhibit allelopathic effects on the growth of other plants (ZhongQun et al. 2012). Another group of compounds produced were benzoic acid derivatives (2, 6-dimethylbenzaldehyde, *n*-propyl benzoate, and benzoylformic acid), reported as mediators of plant response to biotic and abiotic stress (Wildermuth 2006), and with antibacterial and antifungal properties (Park et al. 2001; López et al. 2002). Thus, the exudates produced by plants exposed to *P. aeruginosa* PAO1 VCs were compounds with an inhibitory effect on microorganisms, and is possible that these compounds play a role in defense responses.

The induction of iron uptake mechanisms by bacte- rial VCs has been previously demonstrated in *A. thaliana* (Zhang et al. 2009) and *M. truncatula* (Orozco-Mosqueda et al. 2013b) and an overlap between iron deficiency and defense responses is common. In *A. thaliana* roots, the transcription factor MYB72 is required for ISR activation via VCs and for the expression of a gene module involved in the synthesis and exudation of iron-mobilizing phe- nolic compounds (Zamioudis et al. 2015). In rice plants,

JA signaling was activated during the early stages of the iron deficiency response, and both were negatively regu- lated by the ubiquitin ligases OsHRZ1 and OsHRZ2, close homologs of the *Arabidopsis* BTS protein, which is the master regulator of low iron adaptive responses (Kob- ayashi et al. 2016).

To investigate the role of bacterial VCs on the regulation of iron transporter genes, we quantified the expression of *IRT1, IRT2, YS1,* and *YS2* in axenically-grown plants and in response to bacterization. The expression patterns indicated a clear induction, by bacterial VCs, of strategy I and II iron transporters. Accordingly, *A. agilis* UMCV2, *P. aeruginosa* PAO1, *S. meliloti* 1021, and *Bacillus* sp. L2-64 VCs induced the transcription of *SbIRT1 SbIRT2, SbYS1,* and *SbYS2*. IRT1 is a member of the zip family of broad range metal transport proteins identified from eukaryotes (Eng et al. 1998). *AtIRT1* transcription is predominant in roots and is induced upon iron deficiency (Eide et al. 1996). Loss of function of IRT1 leads to reduced viability in *Arabidopsis* unless iron is re-supplied (Vert et al. 2002). The co-regulation of the *IRT2* gene with the genes encoding the components of iron uptake machinery (*IRT1* and *FRO2*) suggests the role of IRT2 in the same iron absorption mechanism. The results of the expression of iron transporters showed that bacterial VCs played critical roles in improving Fe nutrition by increasing iron-uptake mechanisms through the up-regulation of IRT1, but also directly activating strategy II iron transporters YS1 and YS2. *Yellow Stripe*-1 controls the root acquisition of Fe(III)-phytosiderophore, while YS2 controls the uptake of Fe(II)-phytosiderophore in phloem (Curie et al. 2001; DiDonato et al. 2004; Koike et al. 2004). The function of *SbIRT1* may be to supplement Fe uptake if low amounts of phytosiderophores are excreted by sorghum plants or under increased iron demands caused by plant growth stimulation or effective microbial root colonization.

A link between iron homeostasis and enhanced disease resistance has been reported in *Arabidopsis* plants, since the *AtPR1 and AtPDF1* genes employed as genetic markers of SAR and ISR activation are inducible upon iron deficiency (Kieu et al. 2012; Koen et al. 2014). We previously found that the VC, dimethylhexadecylamine, produced by *A. agilis* UMCV2 and *S. meliloti* 1021, induced the jasmonic acid signaling pathway (Raya-González et al. 2017) and the bacteria activated iron deficiency responses in crop plants (Orozco-Mosqueda et al. 2013b; Castulo-Rubio et al. 2015). Interestingly, VCs from the beneficial bacteria *A. agilis* UMCV2 and *S. meliloti* 1021 but not from the commensal L2-64 strain up-regulated the transcription of *SbCOI1* and *PR-1* genes. Although plant pathogenic bacteria typically activate SAR immune responses (García-Gutierrez et al. 2013; Pieterse et al. 2014), beneficial bacteria may induce both ISR and SAR immune mechanisms. In accordance with this premise, VCs produced by the pathogen *P. aeruginosa* PAO1 strongly induced *PR-1*, but not *COI1*, whereas the PGPR tested could induce both of these genetic markers.

Altogether, our results suggested that *S. bicolor* was able to recognize bacteria through its VC profile and plants differentially responded to beneficial or pathogens bacteria by eliciting nutritional or defensive adaptive traits. Sensing of VCs may be part of an adaptive mechanism to boost plant nutrition and defense depending on the composition of the root microbiome.

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Author contributions EHC: data collection, data analysis and interpretation, drafting the article; MEAG: data collection; DYCR: data collection; LMR: data collection, data analysis and interpretation; VMR: data collection; GS: data analysis and interpretation; JLB: data analysis and interpretation, Drafting the article and EVC: conception or design of the work, data analysis and interpretation, final approval of the version to be published.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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10.2. Addendum II

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ORIGINAL ARTICLE

Rhizobacterium *Arthrobacter agilis* UMCV2 increases organ-specific expression of *FRO* genes in conjunction with genes associated with the systemic resistance pathways of *Medicago truncatula*

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Abstract
Ferric-chelate reductase (*FRO*) genes are essential for iron uptake in strategy I plants. In this study, *Medicago truncatula* plants were grown in a greenhouse under iron-sufficient and iron-deficient conditions with inoculation of the beneficial rhizobacterium *Arthrobacter agilis* UMCV2. The expression of five *MtFRO* genes and the marker genes *MtDef2.1* and *MtPR1*—involved in systemic resistance pathways—was quantified by RT-qPCR in plant organs. *MtFRO1* and *MtFRO3* were expressed in roots, and *MtFRO2* was expressed in leaves, flowers, and pods. *MtFRO4* was expressed in leaves and roots, and *MtFRO5* was expressed in roots, stems, and leaves. *A. agilis* UMCV2 and iron deficiency induced the expression of all *MtFRO* genes and systemic defense marker genes. The defense genes exhibited expression profiles similar to those of the *MtFRO* genes, and strong synergistic gene induction was observed in *A. agilis* UMCV2-inoculated plants grown under iron-deficient conditions. Our data supported the existence of a relationship between systemic defense responses and systemic iron deficiency responses.

Keywords *Medicago truncatula* · *Arthrobacter agilis* UMCV2 · *MtFRO* · ISR · SAR

Introduction

Iron plays a central role in several essential biochemical reactions that use oxygen, hydrogen, or water as substrates, including electron transport during respiration and photosynthesis and as a cofactor for metalloproteins (Johnson et al. 2005; Sánchez et al. 2017). In plants, iron functions in vital metabolic reactions, and its imbalance affects cellular metabolism (Vigani et al. 2013).

Although iron abundance is commonly high in the geosphere, its low solubility in the soil restricts its availability for plant roots (Mimmo et al. 2014). Approximately 30% of the world's soils are considered iron-limited (Wu et al. 2002). To address the low iron availability, plants use two mechanisms aimed at mobilizing this metal from the rhizosphere and transporting it through the plasma membrane of root cells. Strategy I, which is used by all dicotyledons and non-grass monocotyledons, depends on rhizosphere acidification to increase iron solubility via proton release through an ATPase enzyme (Curie and Briat 2003). Subsequently, there is a secretion of phenol compounds, carboxylates, and flavonoids from the pleiotropic drug resistance 9 (PDR9) protein that chelates Fe(III) (Ito and Gray 2006). Free or chelated Fe(III) is reduced to Fe(II) by the action of the enzyme ferric-chelate reductase that is encoded by the ferric reductase oxidase 2 gene (*FRO2*) (Robinson et al. 1999). Finally, reduced iron is internalized to root cells through the iron regulated transporter 1 (IRT1) protein (Eide et al. 1996).

In strategy II, used by grasses, there is a release of phytosiderophores [members of the mugineic acid (MA) family] through the transporter of MA (TOM) to solubilize iron in the rhizosphere (Nozoye et al. 2011) and the subsequent transport of the Fe(III)–phytosiderophore complex through the plasma membrane of root epidermal cells via the yellow stripe 1 (YS1) carrier protein (Curie et al. 2001).

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Iron is required in each type of plant tissue, and its transport inside the plant involves the formation of chelates with the non-proteinogenic amino acid nicotianamine, as well as with citrate (Morrissey and Guerinot 2009). Ferric-chelate reductase activity is found in aerial tissues (Feng et al. 2006; Solti et al. 2014), and it is essential for plant survival under Fe-limited conditions (Jeong et al. 2008).

In addition to the strategies used by plants for iron uptake, there are soil microorganisms involved with the mineralization and transformation of nutrients inside the rhizosphere. Among these microorganisms are the so-called plant growth-promoting rhizobacteria (PGPRs), which are able to modify the availability of nutrients through changes in the environment (Pii et al. 2015a; Terrazas et al. 2016). For instance, PGPRs alter the secretion of molecules with chelating and reducing capacities, and these capacities allow plants to uptake nutrients, including iron (De Nobili et al. 2001; Pii et al. 2015b). Therefore, beneficial soil microorganisms (fungus and rhizobacteria) play a central role in plant iron acquisition (de Santiago et al. 2013; Masalha et al. 2000) through different mechanisms, including the mobilization of soil iron via siderophore release (Pii et al. 2015b; Zhao et al. 2014), direct iron reduction in soil (Valencia-Cantero et al. 2007; Zhao et al. 2014), and induction of plant iron uptake mechanisms such as ferric-chelate reductase activity in plant roots (Orozco-Mosqueda et al. 2013a; Zhang et al. 2009) and leaves (Castulo-Rubio et al. 2015; Zhao et al. 2014).

PGPRs also eliminate or reduce the damage to plants caused by deleterious microorganisms antagonizing them (Berendsen et al. 2012) or triggering plant systemic defense pathways "induced systemic resistance" (ISR) mediated by jasmonic acid (JA), and "systemic acquired resistance" (SAR) mediated by salicylic acid (SA) (Farag et al. 2013; Pieterse et al. 2014).

Recent studies suggest that plant iron deficiency responses and systemic resistance responses are related. The JA and SA response genes, *PR1* and *PDF1.2*, are induced in *Arabidopsis* plants that are subjected to iron deficiency, and the effect is synergistic in the presence of pathogens (Koen et al. 2014). Volatile compounds released by *Trichoderma* spp. stimulate iron uptake responses in roots resulting in priming of JA-dependent defenses in shoots of *A. thaliana* and *Solanum lycopersicum*, in vitro (Martínez-Medina et al. 2017).

We observed that volatile compounds emitted from the beneficial rhizobacteria *Arthrobacter agilis* UMCV2 also induce the marker genes *SbCOI1* and *SbPR-1*, involved in systemic resistance pathways in *Sorghum bicolor* plants, simultaneously with the induction of the iron transporter genes *SbYS1* and *SbIRT1* (Hernández-Calderón et al. 2017). Volatile organic compounds (VOC) produced by *A. agilis* UMCV2, mainly dimethylhexadecylamine (DMHDA), increased the length and biomass of shoots and roots as well as the chlorophyll content in *Medicago truncatula* (Orozco-Mosqueda et al. 2013b) and *S. bicolor* (Castulo-Rubio et al. 2015). Both a VOCs cocktail and DMHDA increased the expression of five *M. truncatula FRO* genes in shoots and roots (data not published) and a *FRO* gene in *S. bicolor* (Castulo-Rubio et al. 2015) under in vitro conditions. Furthermore, plants grew equally well under iron-sufficient and iron-deficient conditions, but the effect of *A. agilis* UMCV2 inoculation on plants grown under greenhouse conditions is unknown.

In the present work, we evaluated the expression of *MtPR1* (Peleg-Grossman et al. 2012) and *MtDef2.1* (Hanks et al. 2005) genes, which are markers of SAR and ISR, respectively. At the same time, we evaluated the *MtFRO* gene expressions under greenhouse conditions. Moreover, these markers were used to explore the crosslinks between iron deficiency and defense pathways. We hypothesized that *A. agilis* UMCV2 increases the organ-specific expression of *M. truncatula FRO* under conditions of iron deficiency and sufficiency in the same way that it induces elements of the *M. truncatula* systemic resistance pathways.

Materials and methods

Plant materials and growth conditions

Plants of *M. truncatula* ecotype Jemalong A17 were used. Seeds were subjected to chemical scarification as described by Orozco-Mosqueda et al. (2013b). Briefly, seeds were immersed in a vial containing 1 mL of sulfuric acid and were constantly shaken until small black spots appeared in the tegument (approximately 8 min). The acid was removed, and the seeds were rinsed seven times with sterile deionized water. Sterilization was carried out with 12% sodium hypochlorite solution for 2 min, and the seeds were subsequently rinsed five times with sterile deionized water. Seeds were germinated in Petri dishes with 0.2 × MS medium (Murashige and Skoog Basal Salts Mixture, Sigma-Aldrich catalogue no. M5524) at 6.5 pH with 6 g of agar/L. Seeds were then transferred to greenhouse conditions in pots containing sterile peat moss (sterilized twice in a pressure vessel for 1 h) 5 days after germination.

Greenhouse assays

Three seedlings that had been germinated for 6 days were placed in pods containing 1 kg of peat moss under greenhouse conditions, and three pots per treatment were used. Seedlings were inoculated (with the exception of control specimens) with 3 mL of an *A. agilis* UMCV2 suspen- sion with an approximate density of 1×10^9 CFU mL^{-1} three times, at 15 days after transplantation, 30 days after

transplantation, and 145 days after transplantation. The plants were watered twice per week with deionized water and once per week with complete Hoagland nutrient solution (Hoagland and Arnon 1950), which had the following composition (all concentrations are in mmol L^{-1}): $NH_4H_2PO_4$ (1), KNO_3 (6), $Ca(NO_3)_2$ (4), $MgSO_4$ (2), H_3BO_3 (0.05), $MnCl_2$ (0.0015), $ZnSO_4$ (0.0015), $CuSO_4$ (0.0005), H_2MoO_4 (0.00015), and 100 µM Fe(III)-EDTA (treatments corresponded to iron sufficient conditions for controls) or with Hoagland solution minus iron (treatments corresponded to iron deprivation). Plants were harvested and samples were obtained 150 days after transplantation.

Chlorophyll quantification

Chlorophyll quantification was conducted as previously described (Castulo-Rubio et al. 2015) using a CCM-200 chlorophyll meter (Opti-Sciences, Inc.; Hudson, NH, USA) to measure the chlorophyll concentration based on the rates of transmitted radiation (940 and 660 nm) through a leaf in arbitrary units.

RNA extraction

RNA extraction was performed in three biological composite samples of roots, stems, leaves, flowers, and pods; each biological sample was composed of the mixture of the organ of interest from the three plants in each pot. Total RNA extraction was conducted with the TRI reagent (Catalogue T9424, Sigma-Aldrich, St. Louis, MO, USA), and RNA was treated with DNaseI to remove residual genomic DNA. Finally, the samples were run on a 1% agarose gel at 90 V to determine specimen integrity.

Bacterial DNA quantification

Bacterial DNA quantification in seeds was conducted as described by Aviles-Garcia et al. (2016) with minimum modifications. In brief, seeds were aseptically extracted from mature pods and ground in liquid nitrogen, DNA from 100 mg of powdered seeds was extracted employing the methodology reported by Mahuku (2004), and then specific primers 2CV2F and 2CV2R designed to amplify an amplicon in the *A. agilis* UMCV2 16S-23S ITS were used to perform absolute quantifications of bacterial DNA by qPCR.

Phylogenetic analysis

The phylogenetic tree and molecular evolutionary analyses of the *MtFRO* genes and related sequences were performed using MEGA v6 software (Tamura et al. 2013) employing the "maximum parsimony" algorithm with 1000 repetitions for bootstrapping. Sequences of previously reported *FRO* genes were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Sequences of previously reported *MtFRO* genes (Orozco-Mosqueda et al. 2012) were identified on the *M. truncatula* Genome Database (http://www.medicagogenome.org/), with the exception of *MtFRO5*, for which the predicted products were located on GenBank.

Gene expression analysis

Reverse transcription quantitative real-time PCR (RT-qPCR) were performed in triplicate for each organ and gene using an ABI StepOne ™ System thermocycler (Applied Biosystems, Foster City, CA, USA). Oligonucleotides designed by Orozco-Mosqueda et al. (2012) were used to amplify *MtFRO1*, *MtFRO3*, and *MtFRO4*, genes and *MtACT* as normalizer gene. The expression of *MtPR1* was evaluated using the oligonucleotides reported by Peleg-Grossman et al. (2012). In addition, oligonucleotides MtFRO2F (5′ACT ATG CAG GCT CTT GCA GC3′), MtFRO2R (5′TAA TCC AAT CCC GCC CGA AC3′), MtFRO5F (5′GATATA TTA GCT CAT AGT GGT TGG C3′), MtFRO5R (5′ATC TTC ACT TGA CGA AAT TGG C3′), MtDef2.1F (5′ACT TTA ATA CAC ACA CCC ATT TGC 3′), and MtDef2.1R (5′TCA GTT AAG ATC TAG AGT CCC ACA3′) were used to amplify *MtFRO2* and *MtFRO5* (Orozco-Mosqueda et al. 2012) and *MtDef2.1* (Hanks et al. 2005), and the primers were designed using NCBI's Primer Designing Tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast). *MtPR1* and *MtDef2.1* expression was only measured in leaves. The RT-qPCR analysis was performed using the SYBR-Green kit (Applied Biosystems) and the following protocol: 10 µL SYBR-Green, 1 µL each of forward and reverse oligo's, 1 µL cDNA, and 7 µL water. Samples were run using the following protocol: 95 °C for 4 min, 40 cycles at 95 °C for 15 s, and 60 °C for 30 s. To prepare the melting curve, samples were run at 95 °C for 15 s, 60 °C for 1 min, and the temperature was subsequently raised to 95 °C at a rate of 0.3 °C/s. Gene expression was evaluated using the comparative ΔΔCt method according with Livak and Schmittgen (2001).

Statistical analysis

The results were analyzed using the Student's *t* test or with analysis of variance and Duncan's means separation test for multiple comparisons ($p \leq 0.05$).

Results

Phylogenetic relationships of *MtFRO* genes

To date, no studies have evaluated the organ-specific expression of *M. truncatula FRO* genes. However, the expression

of these genes has been observed in other strategy I plants, and *FRO* genes were also reported in strategy II plants. Aiming to identify possible orthological relationships between *MtFRO* genes and previously reported *FRO* genes, a phylogenetic analysis was performed using the maximum parsimony algorithm (Fig. 1), and three clusters were identified. The first cluster included genes expressed in shoots: *HvFRO1* and *HvFRO2* (Mikami et al. 2011); *OsFRO1* and *OsFRO2* (Ishimaru et al. 2006); *SbFRO1* (Castulo- Rubio et al. 2015); *AtFRO7* and *AtFRO6* (Mukherjee et al. 2006); and *MtFRO2* and *MtFRO5* (Orozco-Mosqueda et al. 2012). *MtFRO2* and *MtFRO5* were most closely related to *AtFRO7*, which codes for a protein that is located in the chloroplast. The second cluster was composed of the fol- lowing genes with their expression localized in the roots: *AtFRO5, AtFRO4, AtFRO2, AtFRO1, AtFRO3* (Mukherjee et al. 2006); *LsFRO1* (Li et al. 2004); *CsFRO1* (Waters et al. 2007); and *MtFRO1, MtFRO3*, and *MtFRO4* (Orozco-Mosqueda et al. 2012). A sequence reported by Wang et al. (2017) as *MtFRO2* (GeneBank Accession number KX641482.1) was grouped together with *MtFRO1* (Andaluz et al. 2009). Both sequences are 99% homologues, they were reported to be expressed in roots, and their expression is induced by iron deficiency. With these bases, both were considered as the same gene. The third cluster contained *AtFRO8, gp91phox, FRE1*, and *MtFRO6* next to *AtRBOHA*. *AtRBOHA* codes for a respiratory burst-oxidase that differs structurally from most FRO reported proteins, although it contains the motifs needed to perform iron reduction (Baxter et al. 2014). On this basis, we concluded that the *MtFRO6* gene is more closely related to respiratory burst-oxidase genes than to ferric-chelate reductases and was not included in further experiments.

Arthrobacter agilis UMCV2 modulates the organ-specific expression of *M. truncatula FRO* genes

Plants were inoculated three times (with the exception of control specimens), at 15 days after transplantation, 30 days after transplantation, and 145 days after transplantation. Plants were harvested and samples were obtained 150 days after transplantation. Chlorophyll has been reported as an indicator of the iron nutritional status of plants (Masalha et al. 2000; Radhamani et al. 2016). Therefore, from iron-deficient (or iron-sufficient) *M. truncatula* plants that were inoculated (or not) with *A. agilis* UMCV2, the chlorophyll was quantified (Fig. 2). Plants inoculated with *A. agilis* UMCV2 and under iron-sufficient conditions had a chlorophyll content that was 9.7 arbitrary units (au) (approximately 25%) higher than that of control plants grown under iron-sufficient conditions. Plants grown under iron-deficient conditions had a chlorophyll content that was 11.8 au (approximately 40%) lower than that of iron-sufficient controls.

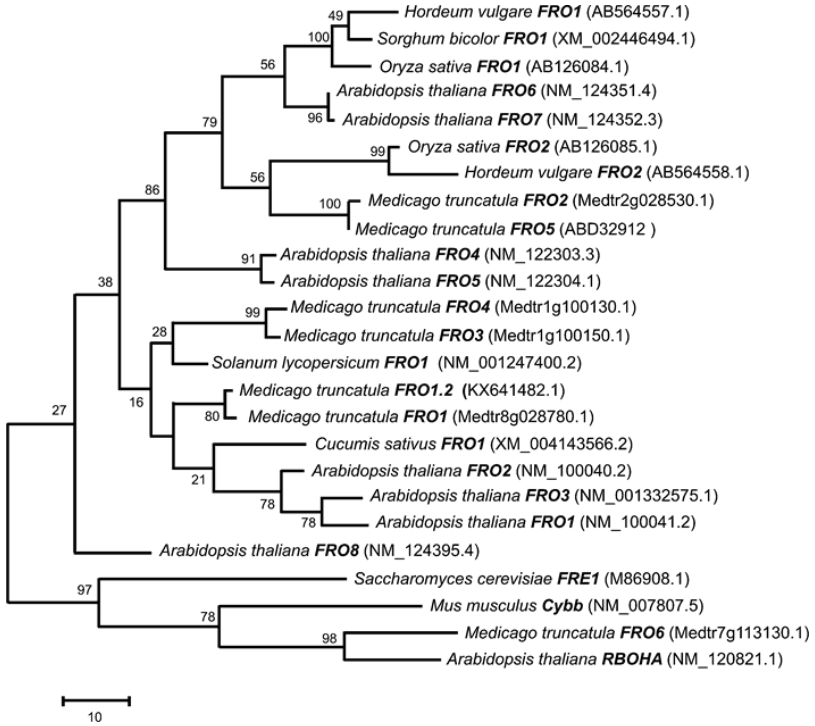

Fig. 1 Phylogenetic analysis of *MtFRO* genes. A phylogenetic tree of *MtFRO* genes and related genes from various species was constructed using maximum parsimony. Numbers on nodes represent bootstrap values that are based on 1000 replicates. GenBank Accession Numbers are indicated in parentheses. *MtFRO* genes are referred to in *Medicago truncatula* Genome Database, with exception of *MtFRO5*, which predicted product was located on GenBank

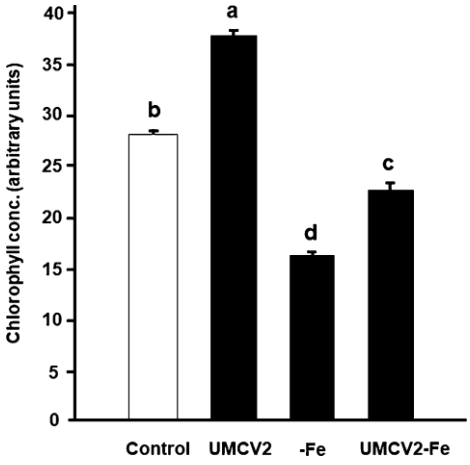

Fig. 2 Effects of A. *agilis* UMCV2 inoculation in plants grown under iron-sufficient and iron-deficient conditions on leaf chlorophyll content. Chlorophyll content was measured in leaflets, and values represent data from triplicate tests of nine 5-month-old *M. truncatula* plants from each treatment. Control represents iron-sufficient conditions without bacteria; UMCV2 indicates iron-sufficient conditions in presence of bacteria; -Fe refers to iron-deficient conditions with- out bacteria, and UMCV2-Fe indicates iron-deficient conditions with bacteria. Letters are used to indicate significant statistical differences determined by Duncan's multiple range test ($p \leq 0.5$; $n = 9$)

However, *A. agilis* UMCV2-inoculated plants that were grown under iron-deficient conditions had a chlorophyll content that was 6.4 au (approximately 25%) higher compared to that of plants that were not inoculated and grown under iron-deficient conditions.

Furthermore, the successful colonization of *A. agilis* UMCV2 in plants was confirmed through the quantification of DNA from *A. agilis* UMCV2 in seeds. Seeds of plants inoculated with *A. agilis* UMCV2 under iron-sufficient and iron-deficient conditions contained (medium ± standard error) 0.61 ± 0.10 and 0.12 ± 0.02 ng of *A. agilis* DNA/g seed, respectively. None of the *A. agilis* DNA was detected from the seeds harvested from non-inoculated control specimens.

Previous research has shown that *FRO* gene expression and ferric-chelate reductase activity are not constant but induced by a stimulus and followed by a down regulation (Andaluz et al. 2009; Connolly et al. 2003), with this in mind, plants were reinoculated 5 days prior to analyzing *MtFRO* expression.

The organ-specific expression patterns for *MtFRO* genes were as follows: *MtFRO1*, *MtFRO3*, *MtFRO4*, and *MtFRO5* were expressed in the roots; *MtFRO5* was expressed in the stems; *MtFRO2*, *MtFRO4*, and *MtFRO5* were expressed in the leaves; and *MtFRO2* was expressed in the flowers and pods (Fig. 3).

Inoculation with *A. agilis* UMCV2 induced the expression of all *MtFRO* genes in their corresponding tissues. In the roots, the expression of *MtFRO1*, *MtFRO3*, *MtFRO4*, and *MtFRO5*, ranged from 1.4- to 3.1-fold higher than controls, while in the stems, *MtFRO5* was expressed 3.7-fold higher than controls. In the leaves, the expression of *MtFRO2*, *MtFRO4*, and *MtFRO5* were 4.4-, 2.5-, and two-fold higher than controls, respectively. In flowers and pods, *MtFRO2* was induced between 6 and threefold higher than controls, respectively (Fig. 3).

Iron-deficient conditions produced a more conspicuous effect with regard to the induction of *MtFRO* genes in their corresponding tissues than that observed after bacterial inoculation. In the roots, the expression of *MtFRO1*, *MtFRO3*, *MtFRO4*, and *MtFRO5* ranged from 18- to 35-fold higher than in the controls. In the stems, *MtFRO5* was expressed 19-fold higher than in the controls. In the leaves, *MtFRO2*, *MtFRO4*, and *MtFRO5* expression was 24-, 12-, and 10-fold higher than in the controls, respectively. In the flowers and pods, the expression of *MtFRO2* was between 29 and nine-fold higher than in the controls, respectively (Fig. 3).

Interestingly, when plants were grown under iron-deficient conditions and inoculated with *A. agilis* UMCV2, the *MtFRO* induction effect was synergistic. In this way, the expression of *MtFRO1*, *MtFRO3*, *MtFRO4*, and *MtFRO5* ranged from 48- to 548-fold higher than in the controls. *MtFRO5* expression in the stems was 49-fold higher than in the controls. *MtFRO2*, *MtFRO4*, and *MtFRO5* in the leaves ranged between 36- and 89-fold higher than in the controls, and *MtFRO2* expression in the flowers and pods ranged from 72- to 126-fold higher than in the controls, respectively (Fig. 3).

Inoculation of *A. agilis* UMCV2 induces the expression of defense pathways genes in *M. truncatula*

Previously, we reported that volatile compounds from *A. agilis* UMCV2 induced SAR and ISR responses in *S. bicolor* (Hernández-Calderón et al. 2017). With this in mind, the following qPCR assays for defense-related genes were performed: *MtDef2.1* (a JA response gene involved in ISR) and *MtPR1* (an SA response gene involved in SAR). Following *A. agilis* UMCV2 inoculation, an increase of 12.0-fold was observed in the expression of *MtDef2.1* with respect to the control. However, iron deficiency also induced the expression of the gene, which increased fourfold without bacteria and 29.1-fold under iron-deficient conditions and in the presence of *A. agilis* UMCV2 (Fig. 4a). The expression of *MtPR1* also increased but was lower compared to that of *MtDef2.1*. A 12.7-, 3.2-, and 14.4-fold increases were

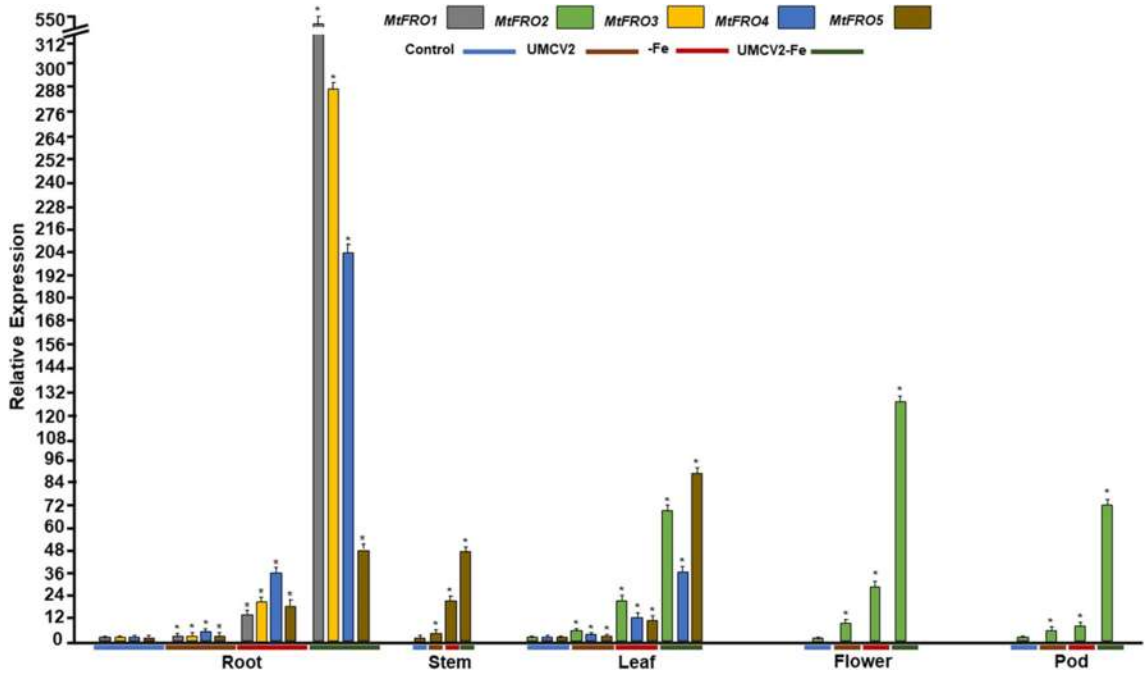

Fig. 3 RT-qPCR analysis of *MtFRO* gene expression in plants inoculated with *A. agilis* UMCV2 and grown under iron-sufficient and iron-deficient conditions. Using cDNA from different organs, RT-qPCRs were performed to evaluate effects of *A. agilis* UMCV2 inoculation on *MtFRO* gene expression. Control represents iron-sufficient conditions without bacteria; UMCV2 indicates iron-sufficient conditions in presence of bacteria; -Fe refers to iron-deficient conditions without bacteria, and UMCV2-Fe indicates iron-deficient conditions with bacteria. Asterisks represent significant statistical differences with respect to control, based on Student's *t* test ($p \leq 0.05$; $n = 3$)

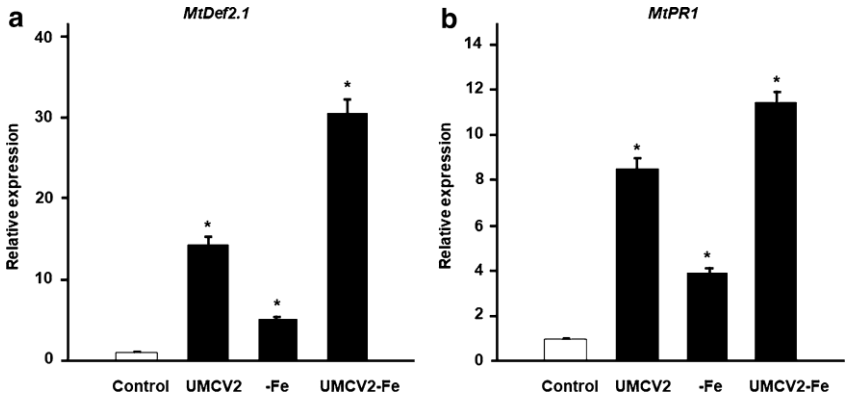

Fig. 4 RT-qPCR analysis of *MtDef 2.1* and *MtPR1* gene expression in *M. truncatula* plants inoculated with *A. agilis* UMCV2 and grown under iron-sufficient and iron-deficient conditions plants. Using cDNA from leaves, RT-qPCRs were performed to evaluate effects of *A. agilis* UMCV2 on *MtDef 2.1* and *MtPR1* gene expression. **a** *MtDef2.1* expression in different treatments. **b** *MtPR1* expression in different treatments. Control represents iron-sufficient conditions without bacteria; UMCV2 indicates the iron-sufficient conditions in presence of bacteria; -Fe refers to iron-deficient conditions without bacteria, and UMCV2-Fe indicates iron-deficient conditions with bacteria. Asterisks represent significant statistical differences with respect to control, based on Student's *t* test ($p \leq 0.05$; $n = 3$)

observed in the presence of bacteria under iron-deficient conditions and iron-deficient conditions combined with *A. agilis* UMCV2 inoculation, respectively (Fig. 4b).

Discussion

It has been established that some but not all bacteria are able to trigger the iron deficiency responses on plants, even in plants grown under iron-sufficient conditions (Scagiola et al. 2016; Orozco-Mosqueda et al. 2013b). The mechanisms that mediate this phenomenon are unknown, but recent observations have contributed to the clarification of the question.

Since *FRO* genes are key elements in the iron uptake process in strategy I plants, they have been studied in several plants, including *A. thaliana* (Robinson et al. 1999). In *M. truncatula*, six *FRO* genes have been identified (Orozco-Mosqueda et al. 2012), and the expression of these genes has been observed in both shoots and roots but not in an organ-specific form. In the present study, we evaluated the organ-specific expression of these genes and the effects of the beneficial rhizobacterium *A. agilis* UMCV2 on these expression profiles.

Prior to evaluating the organ-specific expression of *M. truncatula FRO* genes, a phylogenetic tree was constructed to compare the *MtFRO* genes with previously reported *FRO* genes and to detect possible orthological relationships. Three clusters were identified, including one with genes expressed in the roots, another with genes expressed in the shoots, and one with genes that expressed proteins that differed structurally from other *FRO* genes. *MtFRO6* was found in the third cluster, thus suggesting that the gene was related to *AtRBOHA* and was not considered in further experiments. In addition, the *FRO* genes of monocotyledonous plants are related to among them as well as to the *FRO* genes of dicotyledonous plants that are expressed in the shoots, indicating that these genes arose before the separation of the plant lineages.

Although no previous studies related to the organ-specific expression of the *FRO* genes in *M. truncatula* have been conducted, Wu et al. (2005) and Mukherjee et al. (2006) examined *FRO* gene expression profiles in the different organs of *A. thaliana*. While they did not obtain completely similar results, both works indicated that *AtFRO2* and *AtFRO3* were expressed in the roots; *AtFRO3, AtFRO6, AtFRO7*, and *AtFRO8* were expressed in the aerial tissues; and *AtFRO6, AtFRO7*, and *ATFRO8* were expressed in the flowers.

The following was discovered in the present study: *MtFRO1* and *MtFRO3* were expressed in the roots; *MtFRO2* was expressed in the leaves, flowers, and pods; *MtFRO4* was expressed in the roots and leaves; and *MtFRO5* was expressed in the roots, stems, and leaves. These data suggested that functional redundancy may exist between members of the *MtFRO* gene family. However, *A. thaliana* research has shown that different *FRO* genes are expressed in different tissues in the same organ. For instance, *AtFRO2* is expressed in the epidermal cells of roots, and *AtFRO3* is expressed in the vascular cylinder in roots (Mukherjee et al. 2006). Our data also show that at least one *MtFRO* gene is expressed in each studied organ, and that the genes are likely involved in iron reduction and homeostasis throughout the plant.

In all of the evaluated organs of the plants grown under greenhouse conditions, *MtFRO* expression increased under iron-deficient conditions, and the increased expression ranged from 9- to 35-fold higher than that observed in controls. *FRO* genes induced by iron deficiency have been detected in *A. thaliana*. Connolly et al. (2003) and Ye et al. (2015) observed that, when plants were grown under iron deficiency in vitro, *AtFRO2* was induced 2720- and 147-fold higher, respectively, compared to the expression levels in the controls. The induction levels observed in our iron deficiency treatments were clearly lower, and this was likely due to the use of a different species and differences between the iron-deficient conditions in our greenhouse and the in vitro conditions of Connolly et al. (2003) and Ye et al. (2015).

Under iron-sufficient conditions, inoculation with the rhizobacterium *A. agilis* UMCV2 also increased *MtFRO* gene expression by 1.3- to sixfold with respect to the control plants, but the induction of gene expression was lower than that observed under iron-deficient conditions. Moreover, the chlorophyll content in inoculated plants grown under iron-sufficient conditions was approximately 25% higher than that observed in control plants, thus suggesting a better iron nutrimental status than control plants (Radhamani et al. 2016). Inoculation with *A. agilis* UMCV2 induced *MtFRO* genes throughout all plant tissues, strongly suggesting that the bacteria not only enhanced iron uptake, as was previously shown *Phaseolus vulgaris* plants (Valencia-Cantero et al. 2007), but that it also promoted iron reduction across all plant tissues, thus modulating plant iron homeostasis.

In our laboratory, we have conducted several experiments with *A. agilis* UMCV2 and observed that the bacteria use VOCs to promote plant growth in *M. sativa* (Velázquez-Becerra et al. 2011), *M. truncatula* (Orozco-Mosqueda et al. 2013b), and *S. bicolor* (Castulo-Rubio et al. 2015) under in vitro conditions. The effect is mainly attributed to the aminolipid DMHDA. We observed an increase in the expression of the *S. bicolor SbFRO1* gene (Castulo-Rubio et al. 2015) in the presence of *A. agilis* UMCV2 VOCs or pure DMHDA when in a closed system, and the greatest effect occurred when plants grew with iron deficiencies.

In the present study, we also observed that the iron-deficient condition together with *A. agilis* UMCV2 inoculation produced a synergistic effect on *MtFRO* gene induction, and

the expression values ranged from 44- to 493-fold relative to the control plants. A greenhouse system was used in this study, so the increased expression of *MtFRO* genes cannot be explained by an accumulation of VOCs in a confined space, such as that used by Orozco-Mosqueda et al. (2013a, b) and Castulo-Rubio et al. (2015). Therefore, the expression of genes in the aerial tissues requires another explanation. One possibility that we explored is the systemic effect related to the induction of defense genes such as *MtDef 2.1* and *MtPR1*.

It is known that PGPR bacteria induce ISR defenses, so the expression of related genes (*PDF1.2* and *Hel*) (Pieterse et al. 2014; Van Loon et al. 1998) is also known to induce the parallel expression of SAR- and ISR-related genes (García-Gutiérrez et al. 2013). Conn et al. (2008) reported that endophytic actinobacteria isolated from wheat induce the SAR-related genes *PR1* and *PR5* and the ISR-related genes *PDF1.2* and *Hel* in *Arabidopsis*. These results are in agreement with those of the present study, since mark- ers of both ISR and SAR responses were induced by the endophytic actinobacteria *A. agilis* UMCV2. Interestingly, PGPRs also induce a phenomenon called induced systemic tolerance (IST), which triggers resistance to drought, salinity, and high or low temperatures (Yang et al. 2009). ISR, SAR, and IST are related responses. In a model proposed by Farag et al. (2013), the VOCs of PGPRs are thought to trigger SAR and ISR responses by increasing the synthesis of ethylene and salicylic acid, thus inducing the expression of *PR* and *DEF* genes and establishing such responses. On the other hand, VOCs induce the expression of *FIT1* that codes for a transcription factor throughout the plant, thus inducing the expression of *FRO2* and *IRT1*, improving the iron deficiency response, and triggering IST (Farag et al. 2013). Based on our results, the relationship between iron systemic responses and systemic resistance was observed because of an increase in the expression of *MtDef 2.1* and *MtPR1* under iron-deficient conditions and a synergistic effect associated with iron deficiency and *A. agilis* UMCV2 inoculation. Kobayashi et al. (2016) proposed a model in strategy II plants that related ISR to iron uptake (IST), and they observed that iron deficiency increased the expression of inducible JA genes. Furthermore, the transcription factor *IDEF1* that modulates the expression of iron responsive genes also increased the expression of JA response genes, and this, in turn, increased the expression of the aforementioned *IDEF1* factor, indicating that the signaling pathway response to iron deficiency and the jasmonate signaling pathway are connected. Because iron-deficient conditions resulted in an induction of *MtDef2.1* expression, which responds to jasmonate, it can be concluded that aregulation phenomenon occurs both at the defense level and in the iron uptake of the strategy I plant, *M. truncatula*. Furthermore, this phenomenon is similar to what happens in the strategy II plant, *Oryza* sativa. Koen et al. (2014) demonstrated that iron deficiency in *A. thaliana* induced the expression of *PR1* and *DEF1.2*. Moreover, the presence of the phytopathogenic fungus *Botrytis cinerea* produced an additive effect in the induction of *PR1* and *DEF1.2*. Our results demonstrate that the induction of the expression of both defense genes also occurs under iron-deficient conditions and that the effect is enhanced in the presence of the PGPR *A. agilis* UMCV2. Recently, Raya-González et al. (2017) found that DMHDA induced the expression of the jasmonate synthesis gene *LOX2* in *A. thaliana*. Furthermore, mutant plants affected in the conjugation (*Jar1*), perception (*coi1-1*), and expression of genes responding to JA (*myc2*), were resistant to DMHDA. Based on this observation, it is probable that DMHDA is involved in the induction of *MtDef2.1* via *A. agilis* UMCV2 inoculation, and that it is involved in the iron deficiency responses in *M. truncatula* (Orozco-Mosqueda et al. 2013a, b).

In conclusion, our data demonstrate that *MtFRO* genes are expressed virtually throughout the *M. truncatula* plant, and the PGPR *A. agilis* UMCV2 systemically induced the expression of *MtFRO* genes (likely through associated VOCs such as DMHDA). Furthermore, the bacteria induced marker genes of the ISR and SAR pathways, thus triggering resistance responses to biotic stresses, and these effects were considerably greater under iron-deficient conditions (Fig. 5). Therefore, our data support the existence of a relationship between systemic iron deficiency responses and systemic defense responses.

Author contribution statement VMR: Data collection, data analysis and interpretation, drafting the article. RMC: Data collection. EGP: Data analysis and interpretation. EVC: Conception or design of the work, data analysis and interpretation. Final approval of the version to be published.

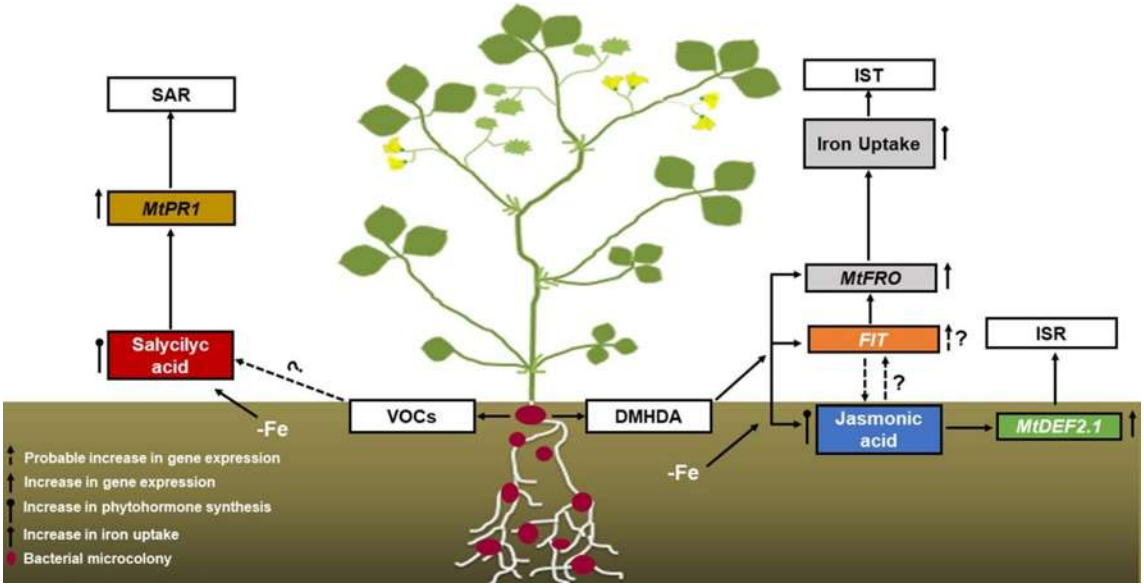

Fig. 5 General model of iron deficiency and *A. agilis* UMCV2 inoculation effects on iron uptake and resistance pathways in *M. truncatula*. Based on the results and background information, we propose a model in which elements of the ISR pathway are related to elements of the iron uptake pathway

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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10.3. Addendum III

ARTÍCULO

Fitohormonas en la defensa de las plantas: Una lucha estratégica

Vicente Montejano Ramírez

Foto: Miguel Gerardo Ochoa Tovar

Imagina que te encuentras inmóvil en un ambiente hostil, con microorganismos potencialmente patógenos para los humanos, ¿Cuál sería tu primera reacción?

Considera que tu única herramienta de defensa, eres tú mismo. Inicialmente, las barreras naturales presentes en tu organismo, como la piel y las mucosas, intentarán retener el ingreso de dichos patógenos a tu cuerpo, sin embargo, si este mecanismo de defensa es superado; se elevará la temperatura corporal para matar al huésped y se inflamarán las áreas de infección, para impedir su dispersión.

Estas respuestas iniciales, no siempre son exitosas, por lo cual, se desarrollan anticuerpos específicos contra el atacante. Las plantas también presentan mecanismos de defensa que se activan en respuesta a fitopatógenos. Pero...

¿Cómo se defienden las plantas?

De manera similar a la piel de los humanos, las plantas tienen estructuras que actúan como barreras contra atacantes, tales como tricomas y espinas. Ante la presencia de organismos biótrofos, que establecen relaciones a largo plazo con las células de las plantas debido a que obtienen sus nutrientes de éstas; la

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planta activa diversos mecanismos con la finalidad de evitar la dispersión del patógeno. Por otra parte, frente a organismos necrótrofos, éstos que destruyen las células vegetales a fin de alimentarse de su contenido, las plantas activan la producción de diversos grupos de compuestos, para detenerlos o incluso matarlos.

No todos los enemigos de las plantas son microscópicos, por lo tanto, ante la presencia de insectos, la planta produce metabolitos denominados secundarios que son tóxicos y afectan la digestión, crecimiento y supervivencia de estos herbívoros; adicionalmente, la planta emite compuestos orgánicos volátiles (compuestos que se evaporan a temperatura ambiente) para atraer depredadores naturales contra el insecto atacante (*Saber Más 36:22-26*).

La defensa en las plantas es un mecanismo bien estructurado

Para que la planta puede emitir las respuestas de defensa previamente mencionadas, existe una estrategia molecular bien estructurada que interacciona con sus atacantes. Todos estos organismos patógenos de las plantas (fitopatógenos), producen moléculas que alertan a la planta de su presencia. En la célula vegetal hay otras moléculas como las proteínas de detección que reconocen la señal de ataque y las plantas activan su primer plan de defensa: se producen especies reactivas de oxígeno (ERO), se activan proteínas y se libera la fitohormona ***etileno***, para combatir al patógeno.

Sin embargo, existen fitopatógenos con capacidad de burlar este primer mecanismo de defensa a través de la producción de moléculas más específicas del atacante que inhiben este primer plan de ataque.

Pero las plantas continúan defendiéndose y echan andar un segundo mecanismo de defensa en el que ocurre una muerte celular programada, con la que se induce la muerte de las células alrededor del ataque, para detener la proliferación del patógeno. Este mecanismo también es regulado por fitohormonas, que son moléculas pequeñas requeridas por las plantas en bajas concentraciones para regular crecimiento, desarrollo, reproducción y respuesta de defensa.

Fitohormonas que regulan los mecanismos de defensa en las plantas

Ante la presencia de insectos masticadores, en los tejidos dañados de las plantas se aumenta la síntesis de ácido ***abscísico*** y ácido jasmónico, que inducen

la producción de una proteína fosfatasa ácida, una enzima que libera grupos fosfato adheridos a otras moléculas, en un pH ácido, que daña los intestinos de los insectos atacantes.

Esquema de defensa y fitohormonas

La presencia de microorganismos benéficos, éstos que favorecen el crecimiento vegetal de la planta y ayudan en la defensa contra los fitopatógenos, incrementa también la síntesis de ácido jasmónico y del etileno, que inducen la producción de pequeñas proteínas (péptidos) como las defensinas, las que atraviesan la membrana celular de los fitopatógenos, formando poros en ésta lo que ocasiona la pérdida de iones y nutrientes, llevándolos a la muerte. Estos péptidos se denominan "péptidos antimicrobianos".

Por otra parte, cuando la planta detecta fitopatógenos que se alimentan de tejidos conductores como el floema, dispara la síntesis de ácido salicílico, fitohormona que induce la producción de diversas familias de enzimas como las quitinasas que atacan la pared celular de los hongos, las muramidasas que atacan la pared celular de bacterias, las proteasas que rompen enlaces peptídicos y degradan las proteínas de sus atacantes, entre otras.

Durante el proceso de defensa, el etileno además realiza una función de control durante el ataque de estos fitopatógenos, ya que esta fitohormona promueve el aumento de la síntesis de ácido salicílico, favoreciendo la respuesta contra ellos. En otro patógenos como los microorganismos necrótrofos, el etileno inhibe la síntesis del ácido salicílico, pero favorece la defensa mediada por defensinas.

Como ves, las plantas presentan diversas estrategias para defenderse de quienes las atacan, aquí te mostré la función de las fitohormonas como parte de su arsenal de defensa. Cuando camines por el parque, cuando riegues tus hortalizas o cuando admires las flores de un jardín, seguramente se está efectuando una batalla majestuosa entre plantas y fitopatógenos, cuyo único objetivo es persistir.

Foto: Miguel Gerardo Ochoa Tovar

SaberMás

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10.4. Addendum IV

Folia Microbiologica
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ORIGINAL ARTICLE

The volatile organic compound dimethylhexadecylamine affects bacterial growth and swarming motility of bacteria

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Abstract
Bacteria have developed different intra- and inter-specific communication mechanisms that involve the production, release, and detection of signaling molecules, because these molecules serve as the autoinducers involved in "quorum sensing" systems. Other communication mechanisms employ volatile signaling molecules that regulate different bacterial processes. The *Arthrobacter agilis* strain UMCV2 is a plant growth promoting actinobacterium, which induces plant growth and inhibits phytopathogenic fungi by emitting the dimethylhexadecylamine (DMHDA). However, little is known about the effect of this volatile compound on *A. agilis* UMCV2 itself, as well as on other bacteria. By exposing *A. agilis* UMCV2 and bacteria of the genus *Bacillus* and *Pseudomonas* to different concentrations of DMHDA, this study showed the dose-dependent effects of DMHDA on *A. agilis* UMCV2 growth, cellular viability, swarming motility, and expression of marker genes of the flagellar apparatus of bacteria. DMHDA was found to also modulate swarming motility of *Bacillus* sp. ZAP018 and *P. fluorescens* UM270, but not that of *P. aeruginosa* PA01. These data indicate that DMHDA is involved in both intra- and inter-specific bacterial interaction.

Introduction

In all ecosystems, species coexist and maintain different interactions with each other, and chemical signals are the oldest and most widespread mode of interaction. All living organisms emit, detect, and respond to chemical cues. Thus, a countless number of interactions exist (Crespi 2001; Meinwald and Eisner 2008; Brunetti et al. 2018). Quorum sensing (QS) is a cell-to-cell communication mechanism that involves the production, release, and detection of low molecular weight compounds, known as autoinducers. Autoinducers control the expression of genes in different bacterial processes, such as luminescence, the formation of biofilms, and swarming motility.

The most studied QS systems are those regulated by N-acyl homoserine lactones (AHLs) in gram-negative bacteria, and those regulated by autoinducer peptides (AIP) in gram-positive bacteria (Ryan and Dow 2008; Schuster et al. 2013; Monnet and Gardan 2015). Similar processes occur in both mechanisms, whereby a self-inducing molecule is produced and released to the extracellular medium, and as the bacterial population density increases, the quantity of the autoinducer also increases until it reaches a critical concentration that is recognized by a receptor which then initiates the expression of the genes regulated by the system (Galloway et al. 2011; Rutherford and Bassler 2012). However, other molecules have also been observed to act as signals, for example, volatile organic compounds (VOCs), which can regulate some of the processes in a similar way to the self-inducing molecules. The communication mechanisms mediated by VOCs, as well as the behaviors regulated by them, are largely unknown (Schulz-Bohm et al. 2017; Xie et al. 2018). Resistance to antibiotics in *Escherichia coli*, virulence in *Pseudomonas aeruginosa*, and motility in *Burkholderia glumae* are some examples of behaviors mediated by VOCs (Enkataraman et al. 2014; Kim et al. 2013; Mansurova et al. 2018).

Arthrobacter agilis UMCV2 is a rhizobacterium with a plant growth–promoting activity, that are linked to the production of their VOCs, mainly dimethylhexadecylamine (*N*,*N*-

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dimethyl hexadecylamine; DMHDA) (Velázquez-Becerra et al. 2011). In previous studies, the DMHDA emitted by *A. agilis* UMCV2 was observed to accumulate in the extracellular environment. In addition to being bioactive in plants and fungi, DMHDA can affect the growth of *A. agilis* UMCV2 itself in a dose-dependent manner (Velázquez-Becerra et al. 2011; Orozco-Mosqueda et al. 2013; Velázquez-Becerra et al. 2013; Raya-González et al. 2017). Therefore, the aim of the present study was to explore the function of DMHDA in intra- and inter-specific bacterial interactions by evaluating its effect on the motility behavior of *A. agilis* UMCV2, as well as bacteria belonging to the other two largest bacterial divisions, namely *Bacillus* sp. AZP018 (firmicutes) and *P. fluorescens* UM270 (proteobacteria).

Materials and methods

Biological material

The strains used in this work were *A. agilis* UMCV2 (Valencia-Cantero et al. 2007), *Bacillus* sp. ZAP018 (Prieto-Barajas et al. 2017), and *Pseudomonas fluorescens* UM270 (Hernández-León et al. 2015). These strains were deposited in the Microorganism Collection of the National Center of Genetic Resources (Boulevard de la Biodiversidad 400, Rancho las Cruces, 47600 Tepatitlán de Morelos, Jalisco, México). *P. aeruginosa* PA01 (Ortiz-Castro et al. 2011) is collection strain available from the American Type Culture Collection (Manassas, VA, USA) under the accession number ATCC BAA-47. The bacteria were cultured routinely in nutrient agar (NA) or nutrient broth (NB) at 23 °C (*A. agilis* UMCV2), 30 °C (*P. fluorescens* UM270), or 37 °C (*Bacillus* sp. ZAP018 and *P. aeruginosa* PA01).

Chemicals

The high purity, volatile chemical compound DMHDA and [3-(4,5-dimethylthiazol-2-yl)]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich. DMHDA was dissolved in ethanol. Equal volumes of solvent were used in all treatments.

Effect of DMHDA on the growth of *A. agilis* UMCV2, *Bacillus* sp. ZAP018, *P. fluorescens* UM270, and *P. aeruginosa* PA01

To measure the effect of DMHDA on bacterial growth, NA plates with concentrations of 0, 0.4, 0.8, 1.5, 3.0, 6.0, and 12.0 μM DMHDA were prepared. Each plate was inoculated with 100 μL of the bacterial suspension (OD_{595} = 1). *A. agilis* UMCV2 plates were cultured for 72 h, while those of *Bacillus* sp. ZAP018, *P. fluorescens* UM270, and *P. aeruginosa* PA01 were cultured for 12 h. After culturing, the cells were carefully collected and resuspended in 5 mL deionized water. The optical densities of the solutions were determined at 595 nm using a microplate spectrophotometer (iMarK Microplate Reader, BIO-RAD, Hercules, CA, USA) and compared with the control treatments.

Effect of DMHDA on the viability of *A. agilis* UMCV2

To analyze the effect of DMHDA on the viability of *A. agilis* UMCV2, NB tubes supplemented with different concentrations 0, 0.4, 0.8, 1.5, 3.0, 6.0, and 12.0 μM of DMHDA were prepared. Each tube was inoculated as previously described and then incubated for 72 h at 23 °C. The optical density was then adjusted as previously described (OD_{595} = 0.3) for all cultures; thereafter, the bacterial suspensions were seeded in 96-well plates. MTT solution (10 μL of 5 mg/mL) in a phosphate-buffered saline (PBS) was added to each well, and they were then incubated for 4 h at 23 °C. Finally, acid isopropanol (100 μL of 95% isopropanol and 5% of 1 N of HCL) was added to dissolve the formazan crystals. Absorbance was measured at 595 nm as previously described (Vázquez-Rivera et al. 2015). Un-inoculated controls with NB plus MTT and 12 μM of DMHDA solution were included to assess lack of abiotic reduction of MTT.

Bacterial motility assay

The effect of DMHDA on the motility of bacteria was tested on NB plates with DMHDA at concentrations of 0, 0.4, 0.8, 1.5, 3.0, 6.0, and 12.0 μM. For this assay, 5 μL of bacterial suspension (OD_{595} = 1) was dropped in each plate center. Three forms of bacterial motility, swarming, swimming, and twitching, were assessed following the protocols described by Combes-Meynet et al. (2011) with a few modifications, i.e., Petri dishes prepared with NB plus 0.2% (for swimming motility), 0.7% (swarming motility), and 1.6% (for twitching motility) of bacteriological agar. The plates were incubated for 120 h at 23 °C for *A. agilis* UMCV2, 24 h at 37 °C for *Bacillus* sp. ZAP018, 24 h at 30 °C for *P. fluorescens* UM270, and 72 h at 37 °C for *P. aeruginosa* PA01. The diameters of motility halos were then measured and the results reported in centimeters of bacterial extension on the media.

In silico search and identification of genes *motA*, *fliC*, *fliM*, and *flgL* of *A. agilis* UMCV2

motA, *fliC*, *fliM*, *flgL*, and *recA* genes were localized in the draft genome of *A. agilis* UMCV2 (GenBank Accession number: CP024915.1) by performing a Basic Local Alignment Search for protein-protein (BLASTP) homologs at the following web page https://blast.ncbi.nlm.nih.gov/Blast.cgi. The translations to proteins of previously identified *motA*, *fliC*,

fliM flgL, and *recA* gene sequences from *Salmonella enterica* LT2 (GenBank Accesion AE006468.2) (McClelland et al. 2001) were used as the probe sequences. *fliC*, *fliM flgL*, *motA*, and *recA A. agilis* UMCV2 genes were compared with their orthologues from 14 other bacteria and archaea species (Table 1). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al. 2013) and the "maximum parsimony" algorithm with 500 repetitions in the bootstrap calculations.

Gene expression analysis

RNA extraction was performed for the three biological replicates of the *A. agilis* UMCV2 from the swarming motility assay, as previously described (Hernández-Calderón et al. 2018). sRNA was extracted using TRI reagent (Sigma Aldrich) and then treated with DNaseI to remove the remaining genomic DNA. The integrity of the RNA was assessed by visualization in 1.2% agarose gel and the final concentration was estimated using NanoDrop (Thermo Scientific, Rockford, IL). cDNA synthesis was performed using 500 ng of the RNA template and the Super Script First-Strand Synthesis System (Life Technologies/Gibco-BRL CA, USA). The quantitative real-time PCRs (qPCR) were performed using an ABI StepOne™ System thermocycler (Applied Biosystems). Oligonucleotides were designed (using the NCBI's primer design tool) to amplify the *fliC*, *flgL*, *fliM*, and *motA* genes and *recA* was used as the normalizer gene (Table 2). The RT-qPCR analysis was performed using the SYBR-Green kit (Applied Biosystems) and the following protocol: 10 µL SYBR-Green, 1 µL of direct and inverse oligo, 1 µL of cDNA, and 7 µL of water. Samples were run using the following protocol: 95 °C for 4 min, 40 cycles at 95 °C for 15 s, and 60 °C for 30 s. To prepare the melting curve, samples were run at 95 °C for 15 s, 60 °C for 1 min, and the temperature was subsequently raised to 95 °C at a rate of 0.3 °C/s. Gene expression was evaluated using the comparative ΔΔCt method according with Livak and Schmittgen (2001).

Statistical analysis

The results were analyzed using a Student's *t* test or an analysis of variance and Duncan's Multiple Range Test for multiple comparisons ($p \leq 0.05$).

Results

Effect of dimethylhexadecylamine on *A. agilis* UMCV2 growth and viability

We analyzed the effect of DMHDA on the growth of *A. agilis* UMCV2 exposed to a range of concentrations from 0.4 to 12 µM. Although increased growth was observed at 1.5 µM DMHDA, inhibitory effects of 92% and 99% were observed on cultures with 6.0 and 12.0 µM, respectively, compared with the control (Fig. 1A). Growth inhibition at 6 and 12 µM led us to test whether bacterial viability was affected,

Table 1 Orthologous genes *fliC*, *flgL*, *fliM*, and *motA* located in the genomes of diverse prokaryotes

Bacterial strain	GenBank accesion	Locus tag			
		fliC	*flgL*	*motA*	*fliM*
Arthrobacter agilis UMCV2	CP024915	CVO76_02240	CVO76_02220	CVO76_10055	CVO76_10045
Arthrobacter agilis 4042	NZ_NFSD01000030.1	B8W74_RS06165	B8W74_RS06130	B8W74_RS06235	B8W74_RS06245
Azospirillus brasilense SP7	CP012915.1	AMK58_18285	AMK58_14830		
Azospirillus brasilense SP7	CP012917.1			AMK58_25375	AMK58_25290
Sinorhizobium meliloti 1021	AL591688.1	SMc03038	SMc03049	SMc03022	SMc03021
Rhodobacter sphaeroides 2.4.1	CP000143.2	RSP_0069	RSP_0073	RSP_0233	RSP_0060
Pseudomonas aeruginosa PAO1	AE004091.2	Pa1092	Pa1087	Pa4954	Pa1443
Burkholderia pseudomallei K96243	BX571965.1	BPSL3319	BPSL0281	BPSL3309	BPSL0027
Escherichia coli K-12	U00096.3	b1923	b1083	b1890	b1945
Salmonella enterica LT2	AE006468.2	STM1959	STM1184	STM1923	STM1976
Salmonella enterica UK-1	CP002614.1	STMUK_1938	STMUK_1151	STMUK_1903	STMUK_1975
Thermotoga maritima MSB8	CP007013.1	THEMA_00860	THEMA04395	THEMA 01285	THEMA_01270
Bacillus subtilis 168	AL009126.3	BSU_35360	BSU_35400	BSU_13690	BSU_16310
Borrelia burgdorferi	X16833	X16833			
Borrelia burgdorferi B31	AE000783.1		BB_0182	BB_0281	BB_0278
Bacillus thuringiensis 97-27	AE017355.1	BT9727_1546	BT9727_1527	BT9727_4248	BT9727_1550
Methanococcus maripaludis C6	CP000867.1	MmarC6_1007		MmarC6_0754	

Table 2 Summary of primers employed in RT-qPCR analysis

Primer	Sequence (5′ to 3′)	Target gene
FMTfliCF	GAAGGAGATGGCGAGCTTCAC	*fliC*
RMTfliCR	GAAGGACACCCTGGTTCATCTG	
FMTflgLF	CTCGAAGCCAAGGATACCGTC	*flgL*
RMTflgLR	GCAGTTTGAGGTCCAGGATGA	
FQTfliMF	GGTCCTCATGCAGACGTACAAC	*fliM*
RQTfliMR	GAACTGGATGACCGCCTTGC 3'	
FQTmotAF	CGTCGAAGTTCTTCATGAGCCT	*motA*
RQTmotAR	GATCATGTGGCCCAGTTCGTC	
FNDrecAF	GGAGAACGGACTGGTCAAGAAG	*recA*
RNDrecAR	GCTTGACCCTGATCTTCTGCTC	

and thus, resulted in the observed inhibition. Therefore, we employed the MTT assay (which determines bacterial activity by the degree of reduction of the MTT) to determine the effect of DMHDA on the viability of *A. agilis* UMCV2. The DMHDA concentrations 0.4, 0.8, and 1.5 μM did not significantly reduce bacterial viability, but 3.0 μM DMHDA decreased the viability to 60%. It is noteworthy that at 3.0 μM DMHDA, bacterial growth (measured as the optic density) was not significantly affected. DMHDA at 6 μM decreased the viability to 40%; however, the higher DMHDA concentration 12 μM did not decrease the viability of the cultures compared with 6 μM (Fig. 1B). These results indicated that although the DMHDA altered the growth in a dose-dependent manner, it was not lethal even at the highest concentration used in the present study.

Effect of DMHDA on *A. agilis* UMCV2 motility

Once the effect of DMHDA on *A. agilis* UMCV2 growth had been determined, we measured its effect on motility. After 120 h of incubation, the swarming motility of *A. agilis* UMCV2 had increased under the DMHDA except 0.4 μM concentration, which behaved in a similar manner to that of the control (Fig. 2A). Compared with the controls, DMHDA only induced a significant difference in the swimming motility at 1.5 μM, but did not cause any changes in the twitching motility at any of the concentrations used (Fig. 2B and C). These results indicated that DMHDA modified the motility

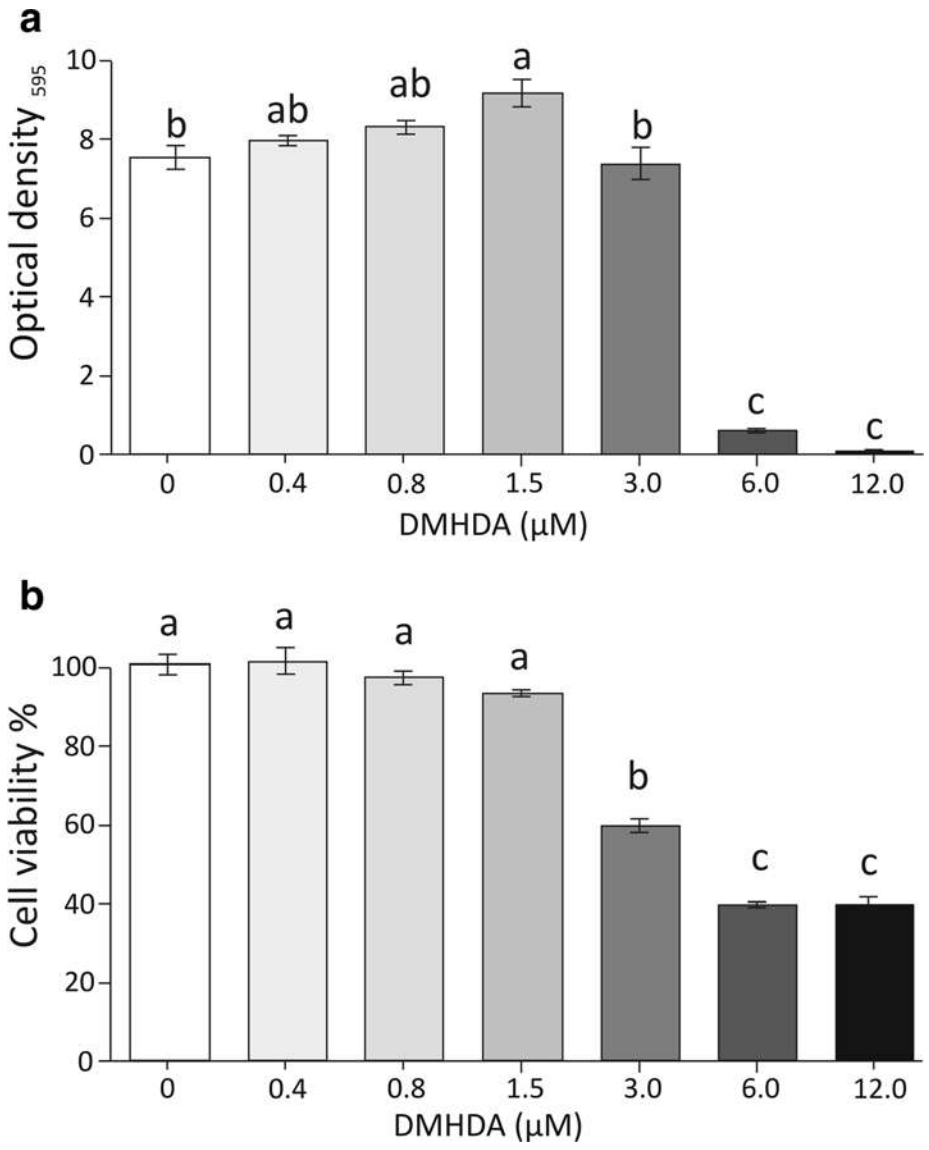

Fig. 1 Effect of dimethylhexadecylamine (DMHDA) on *Arthrobacter agilis* UMCV2 growth and cellular viability. A Nutrient agar plates were prepared with various concentrations of DMHDA and inoculated with the bacteria. After 72 h of culturing, bacteria were carefully harvested, diluted in 5 mL deionized water and the absorbance was determined at 595 nm. B Bacteria were cultured for 72 h in nutrient broth prepared with the same concentrations of DMHDA. The cells were then collected and the viability was determined using the MTT method. Bars and error bars represent the mean ± standard error values, respectively, for three biological replicates. Letters indicate means that differ significantly, following a Duncan's multiple range test ($p < 0.05$)

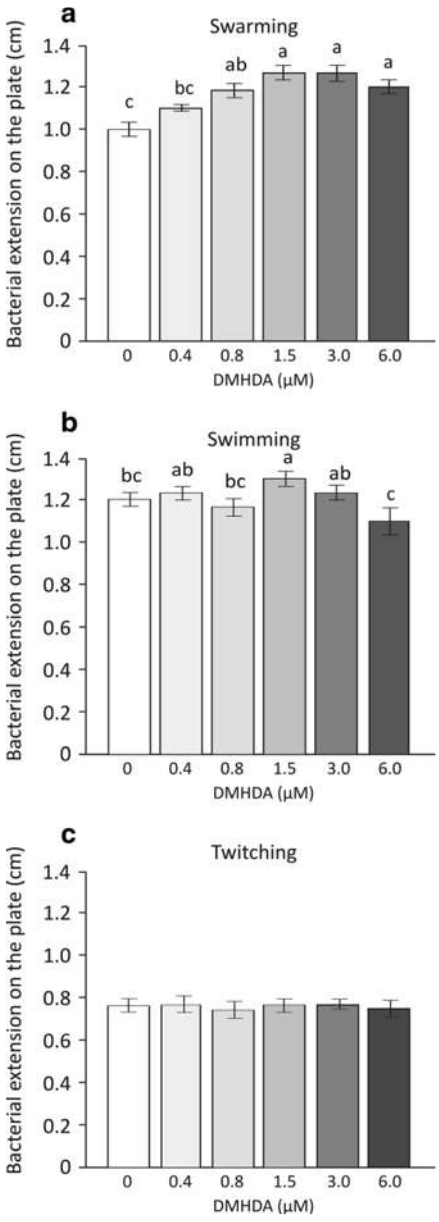

Fig. 2 *Arthrobacter agilis* UMCV2 motility in response to dimethylhexadecylamine (DMHDA). Nutrient agar plates were prepared with A 0.2% (for swimming motility), B 0.7% (for swarming motility), and C 1.6% (for twitching motility) of bacteriological agar, and were inoculated with cultures of *A. agilis* UMCV2. After 120 h, bacterial extensions on the plates were recorded. Bars and error bars represent the mean ± standard error values, respectively, for three biological replicates. Letters indicate means that differ significantly, following a Duncan's multiple range test ($p < 0.05$)

of *A. agilis* UMCV2 that involves the flagellar apparatus (Wang et al. 2004) but not necessarily the pili type IV apparatus involved in twitching (Mattick 2002).

Effect of DMHDA on expression of marker genes associated to flagellar apparatus

We first performed an in silico search to identify orthologue sequences of the genes *fliC*, *flgL*, *fliM*, and *motA* and in the genome of *A. agilis* UMCV2 (Table 1). The putative orthologoues of the genes *fliC*, *flgL*, *fliM*, and *motA* were consistently grouped in a cluster among the orthologous genes experimentally characterized from *S. enterica* Lt2, *E. coli* K-12 and *P. aeruginosa* PAO1 (Kuwajima et al. 1986; Dasgupta et al. 2003; Wang et al. 2004), and other orthologue genes from *Azospirillum brasilense* Sp7, *Sinorhizobium meliloti* 1021, *Rhodobacter sphaeroides* 2.4.1, and *Burkholderia pseudomallei* K96243. Orthologous genes from *Thermotoga maritima* MSB8, *B. subtilis* 168 (Barilla et al. 1994), *Borrelia burgdorferi* , *Bacillus thuringiensis* 97-27, and *Methanococcus maripaludis* C6 belonged to the second cluster. These observations confirmed the in silico identification of *A. agilis* UMCV2 genes *fliC*, *flgL*, *fliM*, and *motA*, and revealed that they are more closely related to the orthologous genes of the proteobacteria *S. enterica* and *P. aeruginosa* than the firmicutes *B. thuringiensis* and *B. subtilis* (Fig. 3A–D).

The gene expressions of *fliC*, *flgL*, *fliM*, and *motA* were quantified in the bacterial samples from the swarming motility assays prepared with the concentration of DMHDA that produced the highest swarming motility induction (i.e., 3 μM), and were compared with the expression in the controls (i.e., 0 μM DMHDA). Compared with the control, the expression of *fliC* showed a 5-fold increase (Fig. 4A), the expressions of *fliM* and *flgL* showed 4-fold increases (Fig. 4B and C), and the expression *motA* showed a 9-fold increase in cultures with 3 μM DMHDA (Fig. 4D). These data showed that DMHDA induced the expression of flagellar and swarming motility genes in *A. agilis* UMCV2.

Effect of DMHDA on growth and the swarming motility of *Bacillus* sp. ZAP018, *P. fluorescens*, and *P. aeruginosa*

With the aim of determining the effect DMHDA on the growth and motility of other bacteria, we cultured the strains *Bacillus* sp. ZAP018, *P. fluorescens* UM270, and *P. aeruginosa* PA01 under the various (previously stipulated) range of concentrations of DMHDA (from 0.4 to 12.0 μM). The *Bacillus* sp. ZAP018 population increased under the 0.4, 0.8, 1.5, and 3.0 μM DMHDA, compared with controls without DMHDA; however, growth inhibition (i.e., by 37%) was observed under the 12.0 μM DMHDA (Fig. 5A). Neither *P. fluorescens* UM270 nor *P. aeruginosa* PA01 shows a dose-dependent response, and DMHDA was not observed to affect the growth of this bacterial species (Fig. 5C). These data

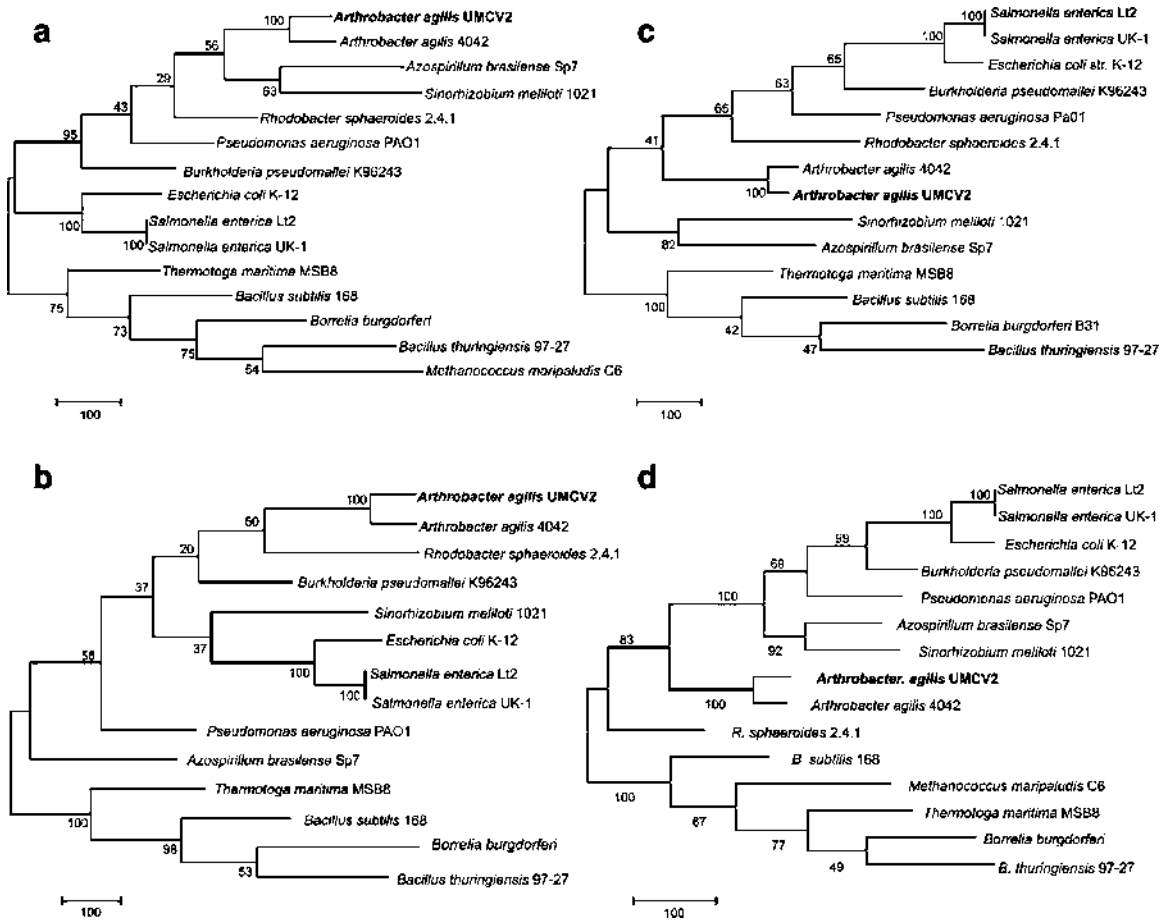

Fig. 3 Phylogenetic trees of the sequences of *fliC*, *flgL*, *fliM*, and *motA* of *Arthrobacter agilis* UMCV2. Sequences of *A. agilis* UMCV2 *fliC* (A), *flgL* (B), *fliM* (C), and *motA* (D) genes and their orthologues from diverse bacterial species were used to construct phylogenetic trees by employing the "maximum parsimony" algorithm. Numbers next to the branches represent bootstraps in bases from 500 repetitions

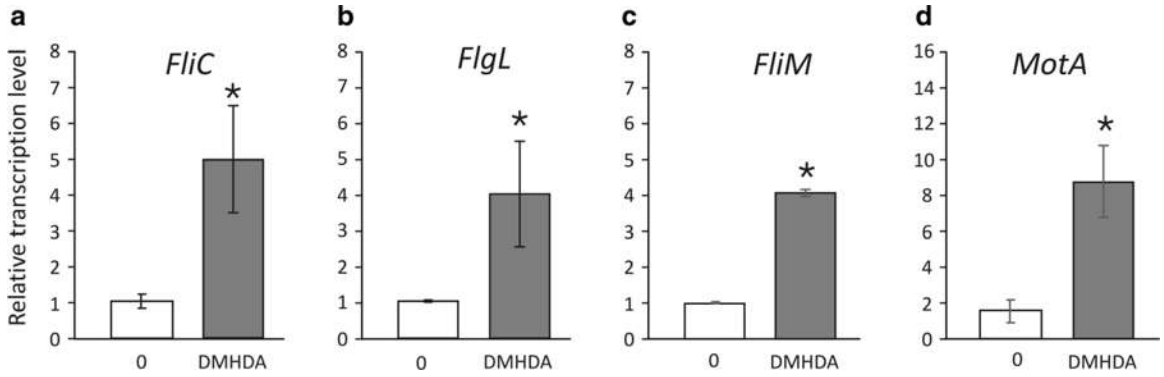

Fig. 4 Effect of dimethylhexadecylamine (DMHDA) on gene expression of *fliC*, *flgL*, *fliM*, and *motA* of *Arthrobacter agilis* UMCV2. A RT-qPCR analysis of the expression of *A. agilis* UMCV2 *fliC* (A), *flgL* (B), *fliM* (C), and *motA* (D) genes was performed with the total RNA isolated from *A. agilis* UMCV2 cells collected from the swarming motility assays performed on nutrient agar prepared with the 3 μM concentration of dimethylhexadecylamine (DMHDA). The relative expression was calculated based on the gene expression in the controls. Bars and error bars represent the mean ± standard error values, respectively, for three biological replicates. Asterisks above the standard error bars indicate significant differences, following a Student's *t* test ($p < 0.05$)

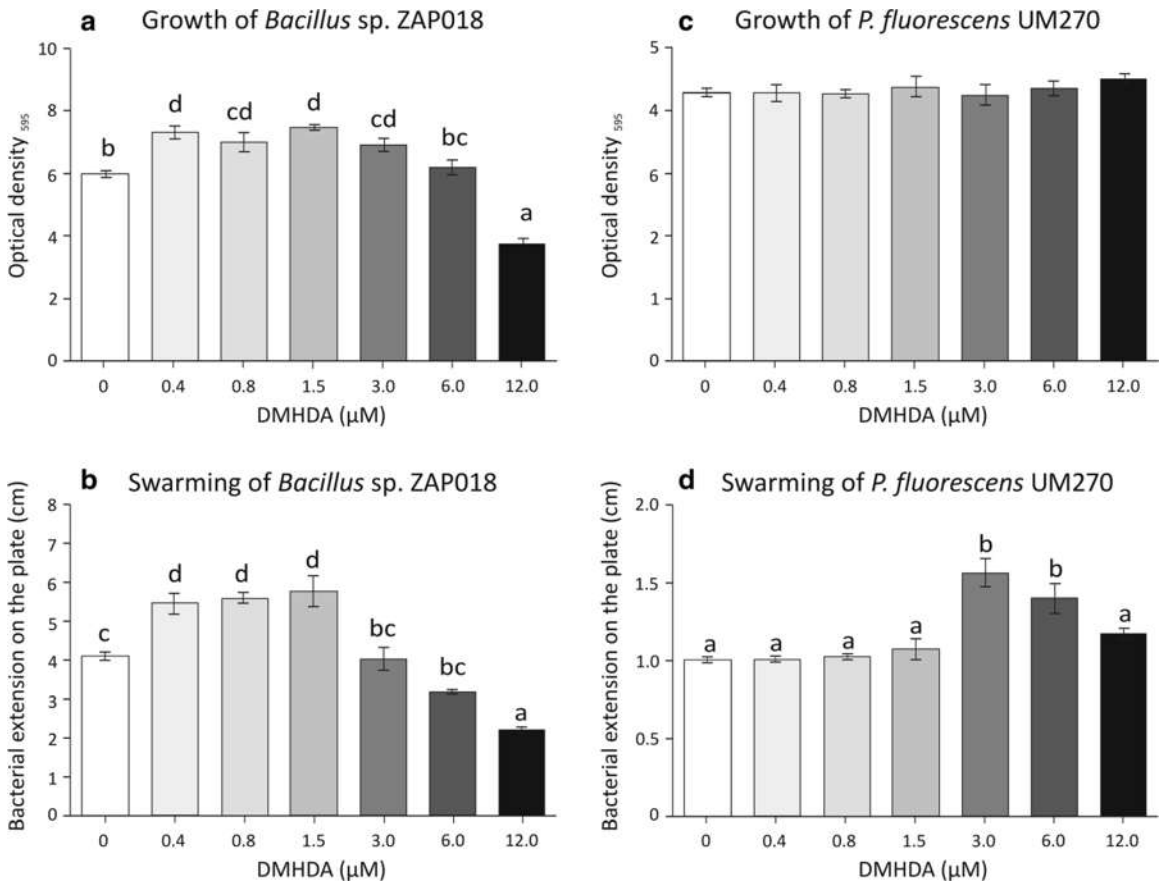

Fig. 5 Effect of DMHDA on *Bacillus* sp. ZAP018 and *Pseudomonas fluorescens* UM270 growth and motility. Nutrient agar (NA) plates were prepared with various concentrations of dimethylhexadecylamine (DMHDA) and then inoculated with *Bacillus* sp. ZAP018 (A) and *P. fluorescens* UM270 (C). After 12 h, the bacteria were carefully harvested, diluted in 5 mL deionized water, and the absorbance was measured at 595 nm. To observe swarming motility, NA with 0.7% of bacteriological agar was inoculated with the bacteria. After 24 h, bacterial extensions on the media were recorded for *Bacillus* sp. ZAP018 (B) and *P. fluorescens* UM270 (D). Bars and error bars represent the mean ± standard error values, respectively, for three biological replicates. Letters indicate means that differ significantly, following a Duncan's multiple range test ($p < 0.05$)

show that DMHDA did not have a general effect on bacterial growth.

In response to DMHDA, *Bacillus* sp. ZAP018 and *P. fluorescens* UM270 did exhibit different swarming behavior. Compared with the controls, the *Bacillus* sp. ZAP018 showed an increase in motility, from 58 to 63%, under the DMHDA concentrations of 0.4, 0.8, and 1.5 μM, but the swarming motility significantly decreased under the DMHDA concentration 12.0 μM (Fig. 5B). *Pseudomonas fluorescens* UM270 showed an increase in motility of 57% and 40% at concentrations of 3 and 6 μM compared with controls, respectively (Fig. 5D), in contrast, the swarming motility of *P. aeruginosa* PA01 only showed a slight decrease under the 3, 6, and 12 μM DMHDA concentrations (Fig. S1). These findings showed that the bacterial VOC DMHDA is recognized by a diverse array of bacteria, but its effect differs in each distinct strain.

Discussion

All bacteria are known to produce VOCs; however, the types of VOCs produced may vary depending on the type of bacteria as well as on the medium in which they develop (Brunetti et al. 2018). These volatile compounds assist in communication mechanisms and can thus great- ly affect nearby organisms; however, the mechanisms involved in such regulations are only just beginning to be understood. In this study, we evaluated the effect of the volatile organic compound DMHDA produced by *A. agilis* UMCV2 on itself, as well as the effect it can have on representatives of other bacterial divisions, such as the firmicutes (*Bacillus* sp. ZAP018) and gamma-proteobacteria (*P. fluorescens* UM270 and *P. aeruginosa* PA01).

The volatiles of some bacteria have been shown to promote the growth of some plants; for example, *B. subtilis* GB03 promotes the growth of *Arabidopsis thaliana* due to the effects of 2,3-butanediol and acetoin (Ryu et al. 2003). Bacterial VOCs are known to regulate processes in other bacteria. The VOCs of *Bacillus amyloliquefaciens* SQR-9 inhibited the growth and the motility traits of *Ralstonia solanacearum*, and a proteomic approach showed that the production of antioxidant enzymes and exopolysaccharides, biofilm formation, and tomato root colonization were depressed by *B. amyloliquefaciens* SQR-9 VOCs (Raza et al. 2016). Other bacterial VOCs, such as 1,2-benzisothiazol-3(2 H)-one and 3,5,5-trimethylhexanol, also affect growth but drastically affect the cellular morphology, which has an antibacterial effect and alters the expression of genes related to motility (Tahir et al. 2016; Xie et al. 2018). The VOCs 2,3-butanedione and glyoxylic acid emitted by *B. subtilis* 168 decreased the motility of *E. coli* K-12, *B. glumae* BGR1, *P. aeruginosa* PA14, and *Paenibacillus polymyxa* E681, and significantly inhibited the expression of motility-related genes (Kim et al. 2013) but did not affect *E. coli* growth.

DMHDA had a dosage-dependent effect on *A. agilis* UMCV2 growth, ranging from growth stimulation at low concentrations (1.5 μM) until almost total growth inhibition at high concentrations (6 and 12 μM), where in a significant proportion of the cells still remained viable (Fig. 1). A previous study showed that physiological concentrations of DMHDA fluctuate in an intermedia interval of concentrations. When *A. agilis* UMCV2 actively grew, the concentration of DMHDA was close to 3 μM in the culture media, and when the growth stopped, concentration reached 6 μM (Velázquez-Becerra et al. 2013). The results of this previous study are consistent with those reported in present study, where in culture media prepared with 3 μM DMHDA did decrease the viability but did not significantly inhibit the growth of *A. agilis* UMCV2.

Signaling molecules, such as the autoinducer AHLs, have shown to modulate bacterial growth. AHLs have been shown to increase the growth rate at low concentrations, but decrease the growth rate at high concentrations, compared with a control without AHLs (Wang et al. 2018). AHLs also control the cooperative motility in bacteria (Daniels et al. 2004; Atkinson et al. 2006). With the findings of these previous studies in mind, we investigated the effect of DMHDA on the swimming, swarming, and twitching motility of *A. aglilis* UMCV2. We found that swarming motility was modified by DMHDA, especially at the typical physiological range of concentrations. Because swarming motility employs the flagellar apparatus (Kearns 2010), we identified the genes *fliC*, *fliM*, *flgL*, and *motA*, and analyzed their expression in response to DMHDA. We found the expression of the four genes increased 5- to 9-fold compared with the controls; which clearly indicated that the effect that DMHDA had on the swarming motility included the transcriptional level. These suggest that DMHDA could act as a signaling molecule in the interspecies communication in *A. agilis* UMCV2, although further work is required to support this hypothesis.

Other bacterial compounds, AHLs, 2,3-butanedione, glyoxylic acid, or even 2, 4-diacetylphloroglucinol act as interspecies signaling molecules and can modulate motility in different bacterial species (Combes-Meynet et al. 2011; Kim et al. 2013; Martínez et al. 2015). We evaluated the effect of DMHDA on other bacterial strains from different phyla (firmicutes and proteobacteria). We found that *Bacillus* sp. ZAP018 modified its growth and swarming motility in response to DMHDA in a dose-dependent manner, similar to the responses seen by *A. agilis* UMCV2 (Fig. 5). It is noteworthy that the concentration intervals of DMHDA that induced growth and swarming motility were wider for *Bacillus* sp. ZAP018 than for *A. agilis* UMCV2, and that DMHDA inhibited the growth of *Bacillus* sp. ZAP018 to a lower degree than that observed in *A. agilis* UMCV2. These findings showed that the actinobacteria *A. agilis* UMCV2 and the firmicute *Bacillus* sp. ZAP018 had different sensitivities to DMHDA. Furthermore, DMHDA did not have a clear effect on the growth of *P. fluorescens* UM270 or *P. auruginosa* PAO1, but induce swarming motility *P. fluorescens* UM270, and not in *P. auruginosa* PAO1. This last observation suggests a possible ecological role of DMHDA in rhizopheric environments since *P. fluorescens* UM270 (as *A. agilis* UMCV2) is a rhizospheric isolate (Hernández-Salmerón et al. 2016), while *P. auruginosa* PAO1 is a pathogenic clinical isolate (Pires et al. 2011).

Differences in response thresholds to interspecies signaling molecules have been proposed as mechanisms to maintain slower growing species within the community (Juhász et al. 2017), although differences in the sensitivities to signaling molecules may also act as adaptations in complex bacterial communities, for example, in the soil and the rhizosphere, where bacterial populations not only cooperate, but also compete. Therefore, in a rhizospheric environment, DMHDA may act as a molecule that induces responses not only in plants but also in intra- and inter-specific bacterial interactions.

The present study showed that the bacterial VOC can modulate the growth and swarming motility in the plant growth–promoting rhizobacterium *A. agilis* UMCV2 by inducing the expression of genes related to the flagellar apparatus. Motile flagellated bacteria possess a competitive advantage in colonization of plant root surfaces, which is a prerequisite for the establishment of beneficial associations, and even to actively endophytic movement to developing seeds and the vertical transmission of such endophytes to successive plant generations (Scharf et al. 2016; Malinich and Bauer 2018). We also showed that DMHDA acts on different bacterial species and that different bacteria have different sensitivities (and thus responses) to DMHDA. Current efforts in our lab are aimed at characterizing

the effects of DMHDA on the rhizosphere environment wherein multiple bacterial species compete and cooperate and also interact with the plant root. Other potential plant growth promoting effects of DMHDA, i.e., not only those directly acting on the plants but also the indirect effects on the rhizospheric microbiome, should also be considered to more accurately evaluate the impact of DMHDA on plant growth.

Authors' contribution Ramiro Mártínez-Cámara Performed research, analyzed data, wrote the paper,
Vicente Montejano-Ramírez Performed research
Gabriel Moreno-Hagelsieb Analyzed data, contributed new methods or models
Gustavo Santoyo Analyzed data, contributed new methods or models
Eduardo Valencia Cantero Conceived of or designed study, analyzed data, wrote the paper

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any author.

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