UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

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MODULACIÓN DE LOS MECANISMOS DE RESPUESTA A LA ESCASEZ DE HIERRO EN *Medicago truncatula* MEDIADA POR RIZOBACTERIAS PROMOTORAS DEL CRECIMIENTO VEGETAL

TESIS

QUE PRESENTA

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ABREVIACIONES

AHA	Bomba de extrusión de protones
AN	Agar nutritivo
DMHDA	N-N-dimetilhexadecilamina
D.O.	Densidad óptica
Fe	Hierro
FBN	Fijación biológica de nitrógeno
FQR	Férrico quelato reductasa
FRO	Gen que codifica a una férrico quelato reductasa
FW	Peso fresco
GCMS	Cromatografía de gases acoplada a espectrometría de masas
IRT	Proteína transportadora de hierro
Mb	Mega bases
Mpb	Mega pares de bases
mm	milímetros
m.o.	microorganismos
pb	Pares de bases
PBM	Membrana peribacteroidal
PEPC	Fosfoenol piruvato carboxilasa
PGPR	Bacteria promotora del crecimiento vegetal
PS	Fitosideróforos
S	azufre
SPME	Microextracción en fase sólida
VOCs	Compuestos orgánicos volátiles

INDICE

1. RESUMEN

El hierro es un nutriente esencial para el desarrollo vegetal. Comúnmente es un elemento que se encuentra en forma oxidada muy poco disponible. Para contender con la deficiencia, las plantas han desarrollado estrategias para adquirir el hierro del suelo; de acuerdo con esto se agrupan como estrategas I y estrategas II. Las plantas estratega I toman y movilizan el hierro por acidificación, reducción e internalización celular; mientras que las estrategas II lo hacen por medio de la quelación (PS-Fe). En este trabajo se investigaron las respuestas a la deficiencia de hierro en plantas de *Medicago truncatula* expuestas a los compuestos orgánicos volátiles de *Arthrobacter agilis* UMCV2 y *Sinorhizobium meliloti* 1021. Los análisis de cromatografía de gases permitieron identificar el perfil de compuestos volátiles producidos por las bacterias. En particular se identificó el compuesto N-N-dimetilhexadecilamina durante la interacción con *M. truncatula*. El efecto de la inducción en *M. trunctula* con los VOCs de *A. agilis* y *S. meliloti* favoreció un incremento en la capacidad de acidificación rizosférica, incrementada actividad reductasa de la raíz, mayor contenido de biomasa así como un incremento en el contenido de clorofila y hierro endógeno. Los resultados también muestran que los VOCs producidos por *A. agilis* tuvieron mayor capacidad de inducir respuestas de escasez de hierro en *M. trunctula* que los producidos por *S. meliloti*; sin embargo, siempre fueron valores mayores respecto del control sin inducir. Adicionalmente, análisis *in silico* permitieron identificar 5 nuevos genes *MtFRO* en el genoma de *M. truncatula* adicionales a 1 ya descrito. Los 6 genes poseen elevadas identidades con otros genes *FRO* descritos en las bases de datos, además muestran los motivos FAD, NADPH y oxidorreductasa altamente conservados característicos de dichos genes. Datos de RT-PCR en 5 genes muestran modulación de expresión en deficiencia de hierro en distintos tejidos de *M. trunctula*. En conclusión, los VOCs de *A. agilis* y *S. meliloti* inducen respuestas de deficiencia de hierro en *Medicago truncatula* vía el compuesto dimetilhexadecilamina.

2. SUMMARY

Iron is an essential nutrient for plant growth. Is an element commonly found in an oxidized form, and therefore is very little available for plants. To contend with the deficiency, plants have developed mechanisms to acquire iron from soil. According to this, plants have been grouped as strategy I and II.Strategy I plants cope with this situation by mobilizing iron by acidification, reduction and cellular internalization, while strategy II plants do so by means of chelation (PS-Fe). In this work we investigated the responses to iron deficiency in *Medicagotruncatula* plants exposed to volatile organic compounds (VOC) produced by *Arthrobacteragilis* UMCV2 and *Sinorhizobiummeliloti* 1021.The gas chromatographic analysis allowed us to identify the profile of volatile compounds produced by bacteria. In particular, the compound N-N-dimethylhexadecylamine was identified during the interaction with *M. truncatula*.The VOCs emitted by *A. agilis* and *S. meliloti* increased the rhizosphere acidification capacity and reductase activity in *M truncatula*. Also, it was observed a higher biomass content, chlorophyll and iron content in treated plants compared to controls.The results also show that the VOCs produced by *A. agilis* had a greater ability to induce iron deficiency responses in *M. trunctula* than those produced by *S. meliloti*, howeverthe values were always higher with respect to controls without inducing.*In silico* analysis allowed us to identify five new *MtFRO* gene copies in the genome of*M. truncatula*, in addition to *FRO1*, which was already reported. The six *FRO* gene reiterations had high identity with other*FRO* genes from different plants, containing highly conserved FAD, NADPH and oxidoreductase motifs. RT-PCR dataof*FRO* genes in *M. truncatula*showeddifferential expression under iron deficiency in different plant tissues.In conclusion, the VOCs of *A. agilis* and *S. meliloti* induce iron deficiency responses in *Medicagotruncatula* via the compound dimethylhexadecylamine.

3. INTRODUCCIÓN GENERAL

3.1. PLANTAS LEGUMINOSAS

Las leguminosas son una importante fuente de proteínas para la nutrición ya que contienen del 20 al 50% de proteína en base a su peso seco. Son el segundo grupo de importancia agrícola solo después de las gramíneas. Poseen una amplia distribución geográfica además de que cuentan con una extensa diversidad morfológica, fisiológica y química y son parte integral de la civilización humana (Singh *et al*, 2007). Dichas plantas crecen en amplias condiciones agroecológicas y su morfología puede variar desde hierbas hasta gigantescos árboles. Su fruto es una vaina, característica que las hace únicas entre las especies vegetales. Son miembros de la familia Fabaceae o Leguminoseae los cuales incluyen 727 géneros y 19,325 especies representadas en 3 subfamilias: Papilionoideae, Caesalpinioideae y Mimosoideae (Fig. 1) (Singh *et al*, 2007). Son cultivadas en 180 millones de hectáreas lo que representa el 12% de campos arables en la tierra y aproximadamente el 27% de la producción primaria de cultivos en el mundo. Incluyen especies empleadas como forraje, ornamentales, con aplicaciones medicinales y agroforestales además de que también son reconocidas como una fuente valuable de metabolitos secundarios (Hong-Kyu *et al* 2004).

Figura 1.- Variedad de semillas de plantas leguminosas.

Las plantas leguminosas también son utilizadas como alimento para animales o pueden tener aplicaciones comerciales como ejemplo en la industria farmacéutica para la elaboración de jabones, pinturas, resinas, cosméticos, linóleo, lubricantes químicos y etanol. Además, se ha documentado que el consumo de estas plantas puede ayudar a prevenir enfermedades cardiovasculares, ataques de apoplejía, Parkinson, Alzheimer, Huntington, enfermedades del hígado y algunos tipos de cáncer (Singh *et al*, 2007). Por lo anterior varias leguminosas son consideradas como cultivos modelo para estudios genéticos y moleculares, entre dichas especies podemos citar a *Medicago truncatula*.

3.2. *Medicago truncatula*

En este trabajo se utilizó la planta *Medicago truncatula* como modelo de estudio (Fig. 2). Es una leguminosa cercanamente relacionada a una planta forrajera de gran importancia económica en nuestro país: la alfalfa (*M. sativa*). En Australia es usada como planta forrajera y como mejoradora de suelo. Es una fuente importante de proteína ya que puede contener del 20 al 50% en base a su peso seco.

Figura 2.- Aspecto general de *Medicago truncatula*

La clasificación de esta especie vegetal es como sigue:

Linaje: *Eucariota; Viridiplantae, Estreptofita; Embriofita; Traqueofita; Espermatofita; Magnoliofita, eudicotiledones; core eudicotiledones; rosides; eurosidesI; Fabales; Fabaceae; Papilionoideae; Trifolieae; Medicago; Medicago truncatula*.

Entre las leguminosas, *M. truncatula* es una planta que posee atributos genéticos favorables para estudios en laboratorio. Cuenta con 8 cromosomas, su genoma diploide es pequeño (500 Mpb), tiene un tiempo generacional corto, autofertilización y elevada eficiencia de transformación (Bell *et al*, 200; Callum *et al*, 2001). Es una especie anual, autógama y posee un rango geográfico amplio para su crecimiento (Bataillon y Ronfort, 2006).

3.2.1. MORFOLOGÍA Y DESARROLLO DE *M. truncatula*.

La morfología y arquitectura de esta planta varía entre genotipos y en base a las condiciones ambientales, sin embargo las características generales de la especie pueden ser la formación de un eje principal que puede organizarse en roseta o en un eje elongado o puede estar formada por ramas de distintos órdenes (primaria, secundaria, terciaria etc.). Cada uno de los ejes está compuesto de fitómeros (unidad funcional elementaria del eje). Los fitómeros a su vez se componen de Internodos, hojas y meristemo axilar. El internodo es la sección entre dos tallos adyacentes y para el caso de *M. truncatula* éstos son angulares; las hojas son trifoliadas, a excepción de la primera hoja verdadera que es unifoliada. Cada hoja se compone de un peciolo, el trifolio es dentado y con tricomas en la parte superior, algunas hojas con algunas manchas negras en el centro y dos estípulas basales cuya función es proteger a la yema axilar (Fig. 3). El meristemo axilar funciona para producir ramas u órganos reproductivos.

Figura 3.- Morfología de *M. truncatula*.

Durante el desarrollo vegetal, los fitómeros de un eje son iniciados sucesivamente a partir del meristemo apical (Fig. 4). Así, cada eje está construido de fitómeros apilados cuya edad es diferente, los fitómeros más viejos se encuentran en la base y los fitómeros mas jóvenes en la punta del eje (Moreau *et al*, 2006).

La germinación en las especies de *Medicago* es epigeal (cotiledones expandidos sobre el suelo) y una vez que los cotiledones emergen, las hojas que producen se organizan en una roseta la cual formará el eje principal de la planta.

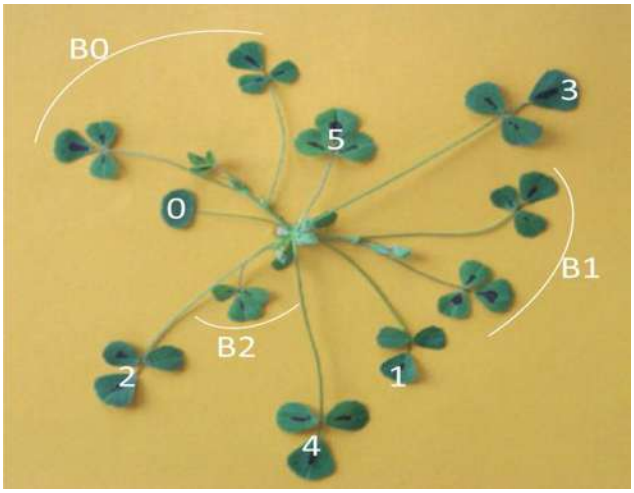


Figura 4.- Filotaxia del eje principal y arreglo de las ramas primarias en *M. truncatula*.

Las hojas que se producen son entonces organizadas en una filotaxia alternada (dos hojas sucesivas son acomodadas alternadamente a uno y otro lado del eje).

Durante el desarrollo vegetal, las ramas primarias se desarrollan en las uniones de las hojas del eje principal. La rama primaria se forma a partir de la unión de la primera hoja desarrollada (hoja unifoliada). Entonces aparecen sucesivamente las otras ramas primarias a partir de la unión de la cual se desarrollaron y su arreglo en la planta es parecido a la filotaxia de la raíz principal. Las ramas secundarias y terciarias generalmente se desarrollan de la unión de las hojas de las ramas primaria y secundaria respectivamente. Las ramas poseen también una filotaxia discontinua, sin embargo, debido a la torción de los ejes, pueden ser posicionadas dos hojas sucesivas en el mismo lado (Lesins y Lesins, 1979 en Moreau, 2006).

Las ramas son nombradas de acuerdo a su posición en la planta, así que las 2 ramas cotiledonarias son nombradas como CB1 y CB2. Las ramas primarias son nombradas como B1 de acuerdo a la unión de la cual se desarrollan (Fig. 4 y 5). Las ramas secundarias son conocidas comoB1-j ya que son nombradas por el identificador de la rama primaria que comparten (B1) y por el rango de la hoja de la rama primaria de la unión de la cual se desarrollaron (j iniciando en 1). Similarmente las ramas terciarias son nombradas como B1-j-k, es decir, se identifican a partir de la rama secundaria que comparten (B1-j) y por el rango de la hoja de la rama secundaria de la unión de la cual se desarrollaron (k iniciando en 1) (Lesins y Lesins, 1979).

La primera hoja verdadera que se desarrolla (hoja redonda) es nombrada como hoja 0 y las hojas trifoliadas son nombradas en orden de aparición a partir de la hoja 1.

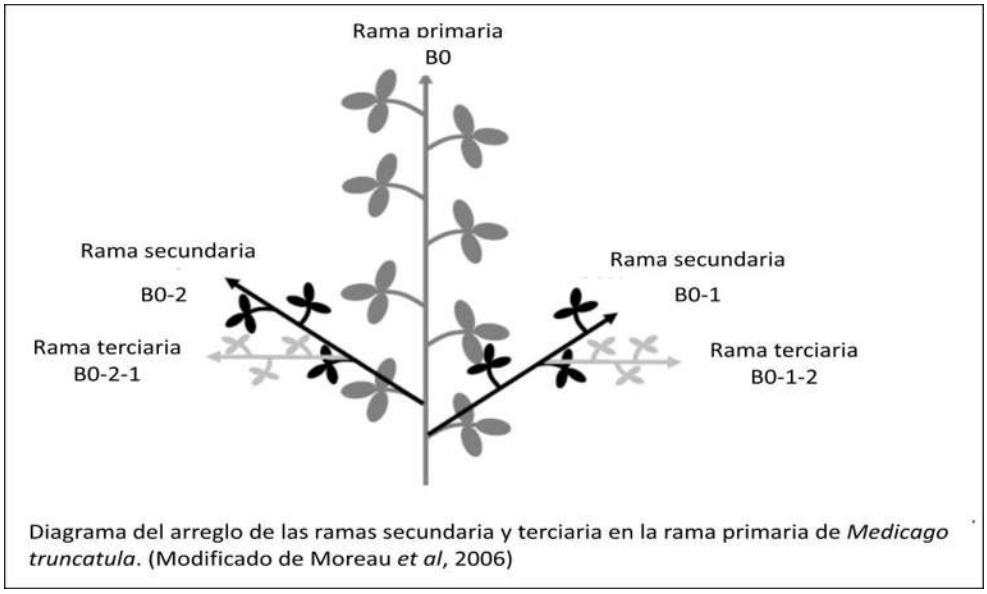


Diagrama del arreglo de las ramas secundaria y terciaria en la rama primaria de *Medicago truncatula*. (Modificado de Moreau *et al*, 2006)

Figura 5.- Nomenclatura de la arquitectura de la parte aérea de *M. truncatula* según.

El estado de desarrollo vegetativo se determina contando el número de hojas trifoliadas por eje. Mientras que el estado de desarrollo reproductivo se determina identificando para cada eje la posición del primer nodo reproductivo y contando el número de nodos reproductivos por eje. El nodo es considerado como reproductivo cuando al menos una flor está abierta (Moreau *et al*, 2006, *Medicago truncatula* handbook).

M. truncatula es una especie autógama. 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. El fruto es una pequeña vaina espinosa. La semilla es de color marrón claro.

Las flores poseen un “mecanismo explosivo”, que es una característica floral compartida en todo el género *Medicago* (Lesins y Lesins, 1979 en Moreau, 2006) el cual consiste en que los estambres y el pistilo de las flores están sometidos a una gran tensión sobre la quilla y son expulsados violentamente cuando el

polinizador ejerce presión sobre las alas y la quilla, por lo que solo es efectiva una visita (Rodríguez-Riaño *et al*, 1999).

3.3. BACTERIAS PROMOTORAS DE CRECIMIENTO VEGETAL

Las bacterias PGPR (**P**lant **G**rowth-**P**romoting **R**hizobacteria) son habitantes comunes de la rizósfera. Muchas bacterias han sido aisladas de una gran variedad de ambientes incluyendo especies vegetales cultivables (Persello-Cartieaux *et al*, 2003). Al estar en contacto con plantas, las PGPR´s producen efectos benéficos en el desarrollo vegetal, es por ello que muchas se han empleado como inoculantes para fabricar biofertilizantes, como fitoestimuladores o como biocontrol. El efecto benéfico en el desarrollo vegetal se da principalmente a través de la inducción del crecimiento y productividad de la planta mediante la producción de fitohormonas y antibióticos. Además pueden inducir programas de defensa en la planta para reducir el crecimiento de bacterias patógenas (Mantelin y Touraine, 2004; López-Bucio *et al*, 2007).

3.3.1. *Arthrobacter agilis* UMCV2

En este estudio una de las especies bacterianas empleadas fue *Arthrobacter agilis* UMCV2. *Arthrobacter* proviene del griego “arthros” que significa articulación (bacilos con forma rectangular articulada) (Fig. 6). Las especies del género Arthrobacter constituyen un grupo predominante de microorganismos en suelos del varias partes del mundo (Reddy *et al*, 2000). Son bacterias Gram positivas, con forma de esfera que pueden medir de 0.8 a 1.2μm de diámetro, pueden presentarse en pares y tétradas.

La clasificación de la especie es como sigue:

Linaje: Domain: *Bacteria;* Kingdom: *Bacteria*; Phylum: *Actinobacteria*; Class: *Actinobacteria*; Subclass: *Actinobacteridae;* Order: *Actinomycetales;* Suborder: *Micrococcineae*; Family: *Micrococcaceae*; Genus: *Arthrobacter;* Specific descriptor: *agilis;* Scientific name: *Arthrobacter agilis* (Koch *et al*, 1995)

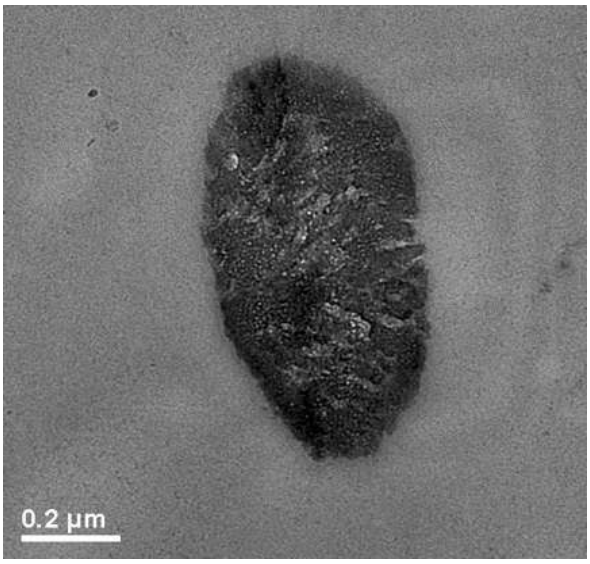


Figura 6.- Aspecto general de *Arthrobacter agilis* UMCV2 mostrado por microscopía electrónica de transmisión.

Las colonias crecidas en agar son circulares, enteras, ligeramente convexas, suaves y mate. Existe un buen crecimiento en obscuridad, con formación del pigmento en placas de agar inclinadas. El pigmento es insoluble en agua. La movilidad se lleva a cabo por uno o tres flagelos. Pueden existir cepas no móviles. No forma esporas. El polisacárido amino de la pared celular es glucosamina. Quimioorganotróficos, metabolismo respiratorio aerobio. Catalasa positivas, oxidasa positiva, P-galactosidasa positiva. No reducen nitrato. Hidrolizan gelatina, almidón y esculina. Rojo Metil negativas. El mejor crecimiento se lleva a cabo entre 20 y 30°C. No existe crecimiento a 37°C. Son suceptibles a penicilina, estreptomicina, cloramfenicol, tetraciclina, eritromicina, novobiocina, ampicilina, carbencilina y gentamicina. Son resistentes a lisozima. Son soprofiticas. Habitat: agua, suelo y piel humana (Koch *et al*, 1995).

Las especies de *Arthrobacter* son divididas en dos grupos de acuerdo a la pared celular de peptidoglicanos que poseen. Las especies del grupo I contienen la

variación A3α de peptidoglicano. En el grupo II se encuentran las especies de *Arthrobacter* con peptidoglucano tipo A4α (Stackebrandt *et al*,1983).

Arthrobacter agilis UMCV2 es una cepa aislada de la rizósfera de plantas de maíz, la cual favorece la adquisición de hierro en plantas bajo condiciones de estrés por deficiencia de hierro (Valencia-Cantero *et al*, 2007; Velázquez-Becerra *et al*, 2011), por lo cual es considerada como una bacteria promotora del crecimiento vegetal (PGPR).

3.4. BACTERIAS FIJADORAS DE NITRÓGENO

La principal vía de entrada de nitrógeno en sistemas agrícolas y naturales es llevada a cabo por plantas leguminosas en asociación con bacterias conocidas como rhizobias, en estructuras formadas en las raíces de las plantas conocidas como nódulos, por un proceso conocido como fijación biológica de nitrógeno (FBN) (Graham y Vance, 2003).

Para que ocurra este proceso debe haber un diálogo molecular entre la planta y la bacteria. La raíz de la leguminosa exuda compuestos como los flavoniodes que inducen la expresión de un grupo de genes rhizobianos, los genes *nod*, los cuales son esenciales para la nodulación y determinación del rango de hospedero (Hirsch *et al*, 2001). Los factores Nod secretados por la bacteria activan múltiples respuestas en la planta hospedera. Estos compuestos inducen la formación del canal de infección, los cuales son tubulos delgados llenos con bacterias que penetran dentro del tejido cortical vegetal y libera a las bacterias en células blanco que desarrollan estructuras denominadas nódulos (Fig. 7). Las células vegetales internalizan a la bacteria invasora en compartimentos unidos a la membrana conocidos como simbiosomas. Entonces la bacteria internalizada se desarrolla en un bacteroide, una forma diferenciada que es capaz de fijar nitrógeno (Kathryn *et al*, 2007).

Un ejemplo de interacción simbiótica es el que se lleva a cabo con la leguminosa *Medicago truncatula* que establece interacciones simbióticas con *Sinorhizobium meliloti* (Cook *et al*., 1997).

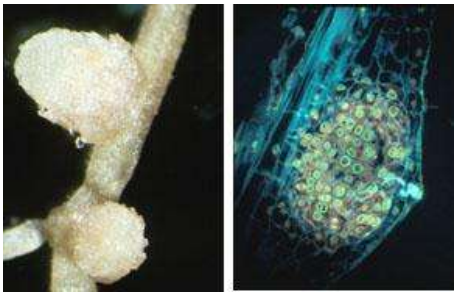


Figura 7.- Nódulos formados durante la interacción *M. truncatula-Sinorhizobium meliloti*. La imagen de la izquierda muestra un nódulo generado a partir del tejido cortical de la raíz, mientras que la imagen de la derecha muestra los simbiosomas dentro del nódulo.

3.4.1. *Sinorhizobium meliloti*

Sinorhizobium meliloti es otra de las especies bacterianas utilizadas en este trabajo. Es una bacteria aerobia, gran negativa, que puede encontrarse en forma de vida libre en el suelo o en los nódulos de la raíces de plantas leguminosas. Al igual que otras especies bacterianas, *S. meliloti* posee una importancia vital para la sobrevivencia de muchas especies vegetales ya que favorece la utilización del nitrógeno como elemento esencial para sus procesos biológicos. La atmósfera está constituída por aproximadamene 85% de nitrógeno, sin embargo, éste se encuentra en forma de dinitrógeno (N2) el cual es indisponible para las plantas. Por lo tanto, el papel de especies fijadoras de nitrógeno como *S. meliloti* es crucial ya que llevan a cabo la conversión de N2 en nitrógeno orgánico (Capela *et al*, 2001). A cambio, las células vegetales excretan compuestos carbonados que la bacteria utiliza como fuente de carbono, algunos de estos compuestos son ácidos dicarboxílicos como succinato, fumarato y malato (Portais *et al*, 1999).

La clasificación de esta especie es como sigue:

Linaje: Bacteria; Protobacteria; Alphaprotobacteria; Rhizobiales; Rhizobiaceae; Sinorhizobium; *Sinorhizobium meliloti*; *Sinorhizobium meliloti* 1021 (Barnet *et al*, 2004). El genoma de *S. meliloti* contiene 3 replicones: un cromosoma de 3.65Mb y 2 megaplásmidos pSyma (1.35Mb) y pSymb (1.68Mb) (Burkardt *et al*, 1987)

Figura 8.- Imagen de microscopía de barrido mostrando células de *Sinorhizobium meliloti*

S. meliloti (Fig. 8) forma nódulos fijadores de nitrógeno en las raíces de plantas leguminosas hospederas, incluyendo *Medicago*, *Melilotus* y *Trigonella* donde llevan a cabo la fijación biológica de nitrógeno. La formación del nódulo en la raíz vegetal, es el resultado de un elaborado programa de desarrollo dirigido por un cambio de señales entre los dos organismos. La planta excreta por la raízcompuestos como flavonoides que inducen la expresión de un grupo de genes bacterianos conocidos como genes nod, los cuales son esenciales son esenciales para la nodulación y para determinar el rango de hospederos (Macchiavelli y Brelles-Mariño, 2004). Posteriormente, las células bacterianas se dirigen hacia el pelo de la raíz e induce el encurvamiento de la punta del pelo. Enseguida, se forma un puente citoplasmático a partir de microtúbulos y citoplasma de la célula vegetal. El puente guía un canal de infección que se extiende de la raíz al córtex de la célula bacteriana. Finalmente, las células de *S. meliloti* entran al citoplasma de las células de la raíz a través de endocitosis (Geurts y Bisseling, 2002)

3.5. COMPUESTOS ORGÁNICOS VOLÁTILES

Los compuestos orgánicos volátiles son producidos por animales, humanos, hongos, plantas y bacterias (Schulz y Dickschat, 2007).

Las bacterias pueden producir una amplia variedad de compuestos volátiles. Estos pueden ser liberados por distintos factores como: ruptura celular, para inhibir el crecimiento de otros microorganismos, como atrayentes, repelentes humanos para inducir resistencia vegetal contra especies bacterianas patógenas o para promover el crecimiento vegetal. Las moléculas señal liberadas durante la interacción planta-bacteria sugieren que existe comunicación entre ambos durante varios estados del desarrollo vegetal y es justamente este diálogo el que determina el curso final de la relación, ya sea de patogenicidad o simbiosis (Ortiz-Castro *et al*, 2009).

El papel ecológico de estos compuestos aun no está claramente establecido, pero es razonable pensar que algunos compuestos en particular, funcionan como defensa o señalización (Schulz y Dickschat, 2007).

3.5.1. N-N- dimetilhexadecilamina

La dimetilhexadecilamina (Fig. 9) es un compuesto volátil producido por la bacteria *Arthrobacter agilis* UMCV2. Es un lípido aminado de cadena media de 16 carbonos.

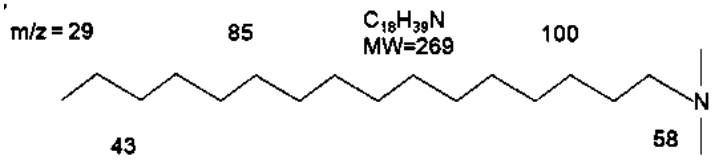


Figura 9.-Estructura de la N-N-dimetilhexadecilamina.

Este compuesto media efectos de crecimiento y desarrollo al inocular plantas leguminosas con UMCV2 (Velázquez-Becerra *et al*, 2011). La dimetilhexadecilamina es capaz de modular el crecimiento vegetal en concentraciones micromolares y de una manera dosis dependiente. De igual manera la hexadecilamina producida por la bacteria favorece el crecimiento de raíces laterales cuando interacciona con plantas. El compuesto también muestra actividad en la bacteria que la produce, ya que los intervalos de concentraciones de bioactividad es similar al de las plantas. Con todo esto, se propone que la dimetilhexadecilamina constituye una molécula de comunicación bacteria-planta que provee una ventaja en la planta para incrementar la capacidad exploratoria de la raíz, además de que, al ser percibida la planta puede modular procesos de crecimiento y desarrollo, mientras que a la bacteria le permite regular su crecimiento o actuar como una señal de competencia (Velázquez- Becerra et al, 2011).

3.6. HIERRO

El hierro es un elemento versátil en la naturaleza. Como metal de transición, posee la capacidad de ganar o perder electrones lo que le confiere un importante papel en reacciones tipo rédox (Vert *et al*, 2003). También puede formar complejos octahédricos con varios ligandos y variar su potencial rédox en respuesta a varios ligandos ambientales. Dichas características le permiten funcionar en reacciones esenciales de la vida como la respiración, biosíntesis de clorofila, transferencia fotosintética de electrones (Haruhiko *et al* 2003), síntesis de ADN y síntesis de hormonas (Stephan y Hell, 2003; Wu *et al*, 2005). Algunas de las proteínas vegetales que contienen hierro son por ejemplo la adenosín-5´-fosfosulfato reductasa que participa en la asimilación de sulfatos (4Fe-4S; Kopriva *et al*, 2002), la aminociclopropano-1-acido carboxílico oxidasa en la síntesis de etileno (6 Fe coordinados; Zhou *et al*, 2002) así como las oxidasas alternativas en la respiración (centros di-Fe; Albury *et al*, 2002).

El hierro es un elemento abundante en el suelo, representando aproximadamente el 5% de la corteza terrestre. La adquisición de Fe es esencial para todos los organismos ya que les permite mantener la homeóstasis celular. Sin embargo, la baja disponibilidad de este metal en suelos calcáreos es un problema para la nutrición vegetal (Ishimaru *et al*, 2007), ya que a pesar de su abundancia, éste no se encuentra fácilmente disponible en el suelo y un exceso puede ser tóxico (Mukherjee *et al*, 2006). Esto se debe a que el Fe (II) es relativamente soluble pero se oxida rápidamente con el oxígeno atmosférico (Fig. 10) y la solubilidad del Fe (III) disminuye dramáticamente en suelos alcalinos con pH elevado (Vasconcelos *et al*, 2006; Valencia-Cantero *et al*, 2007), lo que convierte al hierro en un elemento poco disponible para la planta, condición común en la mayoría de los suelos cultivables (Hell y Stephan, 2003).

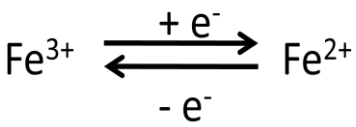


Figura 10.- Reacción reversible de ganancia y pérdida de electrones del Fe.

La deficiencia de Fe resulta en una disminución en el contenido de proteínas que contienen clorofila causando clorosis en hojas jóvenes (Wu *et al*, 2005) y como consecuencia pérdidas económicas en cultivos agrícolas. Además, las mismas características que le permiten a este metal actuar como un eficiente cofactor y catalizar reacciones controladas, también le permiten actuar como una potente toxina, catalizando la generación de radicales hidroxilo que dañan a los constituyentes celulares (Stephan y Hell, 2003). Esto nos indica que la problemática del hierro no es una cuestión de escases de hierro, sino más bien de adquisición y distribución celular. Po lo que es fundamental conocer los componentes genéticos involucrados en la adquisición de hierro en plantas, lo que conllevará a mejorar la nutrición férrica en humanos.

3.6.1. ESTRATEGIAS VEGETALES PARA LA ADQUISICIÓN DE HIERRO

Las plantas han desarrollado mecanismos efectivos para adquirir el hierro del suelo. Con base en el mecanismo de adquisición de Fe las plantas pueden ser agrupadas como plantas estratega I y estratega II (Römheld y Marschner, 1986). Las plantas estratega II incluyen a las gramíneas cuyas raíces secretan compuestos conocidos como fitosideróforos (PS) que quelan el Fe^3 de la rizósfera. Una vez formado el complejo Fe^3-PS, éste es introducido a la célula a través de una proteína transportadora en el plasmalema conocida como YS1 (Stephan y Hell, 2003). Por otra parte las plantas estratega I son todas las plantas superiores (excepto las poales y entre ellas las gramíneas) y la adquisición de hierro ocurre en tres reacciones principales: i) Excreción de protones a través de la membrana plasmática por medio de una ATPasa para acidificar la rizósfera y dirigir más hierro en solución. La ATPasa ha sido identificada en plantas de *Cucumis sativus* (CsHA1) (Santi et al, 2005) y en *Arabidopsis thaliana* AHA7 (Colangelo y Guerinot, 2004), ii) Reducción de Fe^{3+} a la forma ferrosa (Fe^{2+}) por la expresión de una proteína férrico quelato reductasa (FRO) en raíz (Robinson et al, 1999) y iii) Transporte del Fe^{2+} al interior de las células de la raíz por medio del transportador IRT a través de la membrana plasmática (Eide *et al*, 1996). (Tabla I)

COMPONENTES MOLECULARES EN ESTRATEGAS I		COMPONENTES MOLECULARES EN ESTRATEGAS II		COMPONENTES MOLECULARES COMPARTIDOS	
FRO	Enzima férrico quelato reductasa. Regulado por los factores transcripcionales FIT/FER	MA	(Ácido Mugineico). Familia de PS (fitosideróforos). Quelantes de hierro	IRT Y FeIII-PS	iTransportadores Inducidos en arroz y cebada en deficiencia de Fe
IRT	(Iron root transporter). Transportador de Fe en raíz. Regulado por FIT/FER	TOM1	(Transporter Of MA). Transportador de PS. Sobreregulado por deficiencia de Fe	AtYSL3	Transportador sobreregulado en raíces de *Arabidopsis* en deficiencia de Fe
POPEYE/BRUTUS	Factores de transcripción que regulan homeóstasis del hierro en *A. thaliana*	YS1	(Yellow Stripe). Funciona como transportador del tipo simporter del complejo FeIII-PS. Puede funcionar transportador del tipo protón-acomplado para otros metales como Zn, Cu y Ni	IREG1/FPN1	(Iron Regulated 1/Ferroportina1). Transportadores que funcionan en el cargado de Fe al xilema
AHA	(*Arabidopsis* H^+ ATPasa). Extrusión de protones	HvYS1	Transportador homólogo de YS1 en cebada	NA	(NicotiAnamina). Ácido que acompleja FeIII (FeIII-NA) y es cargado en xilema por un transportador como YSL
MATE/ ABC	(Multidrug And Toxic compound Extrusion) (ATP-Binding Cassette). Transportadores de compuestos orgánicos.	IDE1/IDE2	(Iron Deficiency-responsive Element). Elementos en cis, confieren inducción de expresión por deficiencia de hierro.	CITRATO	Quelante de FeIII en xilema
PEZ1	(Phenolics Efflux Zero). Miembro de subfamilia MATE inducido por hierro. Participa en el eflujo de ácido dihidroxibenzoico y ac. cafeico	IDEF1/IDEF2	Factores en trans que se unen a elementos como IDE1 e IDE2	FRD3	(Ferric Reductase Defective). Transportador de la familia MATE. Lleva a cabo el acarreo en el flujo de citrato hacia la vasculatura de raíz de A. thaliana, arroz y centeno
AtPDR9	(Pleiotropic Drug Resistance). Miembro de la subfamilia ABC. Exporte de compuestos fenólicos como ac. cafeico o clorogénico. Mejoran la utilización de hierro en apoplasto	OsIRO	(Iron-Related transcription factor 2). Factor de transcripción, regula el sistema de Fe mediado por PS	MAR1	(Multiple Antibiotic Resistance 1). Homólogo de IREG1. Se propone que transporta Fe-NA en cloroplasto
DMRL	(DiMetil RibitiLumazina sintasa). Enzima de síntesis de rivoflavinas. Sobreregulada en deficiencia de Fe			ZmFRD3	Transporte de Fe en tilacoides
ITP	(Iron Transport Protein). Proteína que une Fe en el floema de *Ricinus*			AtYSL6	Transportador que participa en el eflujo de Fe en cloroplastos

PIC1	(Permeasa en Cloroplasto). Transportador que media el transporte de Fe en el cloroplasto			MIT1	(Mitocondrial Iron Transporter). Transportador en mitocondria esencial para crecimiento y desarrollo
STA1	(STARIK1/AtATM3). Transportador ABC implicado en exporte de clusters Fe-S			VIT1	(Vacuolar Iron Transporter 1). Transportador de influjo de hierro en vacuola
				IREG2	Transportador para el Importe de hierro en vacuola
				NRAMP2 y NRAMP4	(Natural Resistance-Associated Macrophage Protein). Transportadores que exportan Fe de la vacuola al citoplasma

Tabla 1.- Componentes moleculares descritos en plantas estratega I y II

Los 3 principales genes en este tipo de estrategia activan su inducción bajo las siguientes condiciones: en deficiencia de hierro (Stephan y Hell, 2003), en presencia de ácido húmicos (Aguirre *et al*, 2009) o por la presencia de microorganismos (Zhang *et al*, 2009). La deficiencia de hierro también promueve la excreción de compuestos fenólicos, ácidos orgánicos y flavinas, los cuales facilitan la reducción y solubilidad de hierro externo (Welkie *et al*, 1988; Susin *et al*, 1994).

Adicionalmente, existen rhizobacterias promotoras del crecimiento vegetal que pueden incrementar la toma de hierro por las plantas, mediante la reducción de Fe (III) a Fe (II) en la superficie de la raíz, lo que sugiere un importante papel bacteriano para suplir a la planta de hierro en suelos alcalinos (Valencia-Cantero *et al*, 2007). Por lo anterior, es importante identificar los genes *fro*, conocer su expresión tejido-específica así como identificar la participación bacteriana. De este modo aportar conocimientos sobre la homeóstasis del hierro en plantas modelo como *M. truncatula*.

4. ANTECEDENTES

La reducción del hierro en la membrana plasmática de células de la raíz vegetal es un paso obligatorio en las plantas estratega I, debido a ello es necesario el estudio de los genes férrico quelato reductasa (*fro*) responsables de dicha actividad. En plantas de chícharo (*Pisum sativum*) se ha identificado el gen FRO1, el cual codifica para una férrico quelato reductasa de 712 aminoácidos con dominios FAD, NADH y oxido reductasa. La expresión de FRO1 en células de levadura mostró niveles incrementados de actividad férrico quelato reductasa en medio deficiente de hierro comparado con el control, mostrando que FRO1 codifica para una férrico quelato reductasa funcional. De igual manera se analizó la expresión de FRO1 en varios tejidos. Los niveles de RNAm de FRO1 en plantas crecidas en deficiencia de hierro fueron identificados en células epiteliales de la raíz, en células del mesófilo de las hojas así como en los nódulos, particularmente en la zona de infección y de fijación de nitrógeno. Los autores sugieren que el gen FRO1 participa en la toma de hierro por parte de la raíz, además de que se le atribuye un papel fundamental en la distribución de Fe a través de toda la planta (Waters *et al*, 2002).

Los genes *fro* han sido estudiados extensamente en *Arabidopsis thaliana* (Wu *et al*, 2005; Mukherjee *et al*, 2006). En el genoma de esta planta se identificó una familia de genes *fro* con 8 miembros, los cuales están localizados en el cromosoma I (3 genes) y en el cromosoma V (5 genes) y muestran un alto grado de similitud a nivel de aminoácidos. Dichos genes fueron clonados y expresados en células de levadura mostrando elevada actividad reductasa, lo que permitió concluir que dichos genes codifican para una quelato reductasa de hierro y funcionan en la homeóstasis de dicho metal. De igual manera, estudios de RT-PCR en tiempo real permitieron demostrar que los genes *fro* tienen una expresión tejido-específica: *AtFRO2* y *AtFRO3* se expresaron en raíces de Arabidopsis, *AtFRO5* y *AtFRO6* en brotes y flores, *AtFRO7* en cotiledones y tricomas y *AtFRO8* en venas de las hojas (Wu *et al*, 2005). Posteriormente, fue publicado un trabajo

similar por Mukherjee *et a.l* (2006) donde estudian la expresión de los genes *fro* en *A. thaliana*. Dichos autores también identificaron 8 genes *FRO* y analizaron su expresión por RT-PCR en tiempo real. Sin embargo, a diferencia de Wu *et al*, identificaron distintos patrones de expresión: *FRO2* y *FRO5* se expresan en raíz, *FRO3* se expresa en altos niveles en cilindro vascular de la raíz y brotes, *FRO6* y *FRO7* en partes verdes de la planta y *FRO8* en brotes. Esta diferencia de expresión tejido-específica de los genes *fro* indica que el estudio de dichos genes aún está en proceso, mientras que para la planta *Medicago truncatula* no existen investigaciones sobre la identificación y expresión de los genes FRO.

Con la finalidad de reducir el estrés por hierro en cultivos de importancia agrícola, Vasconcelos *et al.* (2006) llevaron a cabo la caracterización fenotípica de una planta transgénica de frijol-soya expresando el gen *FRO2* de *A. thaliana*. Las plantas de frijol-soya transgénico crecidas en deficiencia de Fe incrementaron significativamente la reducción de Fe(III) en raíces (10 veces más) y hojas (3 veces más). Esta incrementada actividad reductasa también redujo clorosis, incrementó los niveles de clorofila y el contenido de Fe en las semillas cuando se compararon con las plantas control. Sin embargo, los resultados mostraron que cuando no hay estrés por deficiencia de Fe la expresión constitutiva de *FRO2* condujo a un descenso en la productividad de la planta. Los autores concluyen sugiriendo que la expresión heteróloga de una férrico quelato reductasa provee una ruta para aliviar la clorosis por deficiencia de Fe.

Adicionalmente, se sabe que los microorganismos participan activamente en la adquisición de Fe para el desarrollo de las plantas (Masalha *et al*, 2000; Rroco *et al*, 2003; Valencia-Cantero *et al*, 2007) y tienen la capacidad de modular la expresión de los genes que participan en la toma y homeóstasis del Fe (Zhou y Yang, 2004; Elena *et al*, 2009).

Masalha *et al.* (2000) investigaron el papel de la actividad microbiana en la adquisición de Fe en plantas de maíz y girasol. Los autores crecieron plantas en suelo estéril y no estéril (es decir, en presencia de microorganismos). Observaron que aquellas plantas crecidas en presencia de microorganismos (m.o.), tuvieron

mayor biomasa en base al peso fresco y seco, no mostraron síntomas de deficiencia de Fe y el contenido de Fe en raíz, apoplasto de la raíz y hoja así como el contenido de clorofila fueron mucho mayores comparadas con las plantas crecidas en suelo estéril. A pesar de que la planta de maíz es una estratega II y tiene la capacidad de secretar al medio fitosideróforos para quelar al Fe(III), dichos compuestos de las plantas de maíz crecidas en esterilidad fueron menos efectivos en prevenir clorosis por deficiencia de Fe. Los autores asumen que la actividad microbiana en hábitats naturales del suelo posee gran importancia para la adquisición de Fe en plantas. Posteriormente, este mismo grupo de investigación (Rroco *et al*, 2003) analizó la actividad microbiana del suelo en el crecimiento de plantas de sorgo y *Brassica napus*. Nuevamente observaron que las plantas crecidas en suelo estéril mostraron muy poco crecimiento, aún cuando éstas fueron crecidas en alto contenido de Fe, mientras que aquellas crecidas en presencia de m.o. pero con poco Fe tuvieron mayor contenido de Fe en raíz, apoplasto de la raíz y hoja, además de mayor contenido de clorofila. Estos resultados permiten concluir que la actividad microbiana es esencial para la adquisición de Fe.

Un trabajo más reciente llevado a cabo por Valencia-Cantero *et al.* (2007) muestra que la actividad microbiana es esencial para la toma de Fe en plantas de frijol. Los autores identificaron cepas bacterianas con una elevada actividad de reducción de Fe(III). Aislaron 3 cepas (UMCV1, UMCV2, UMCV3) de la rizósfera de maíz y 1 cepa (UMCV4) de la rizósfera de frijol. Mediante estudios de amplificación de los ADNr 16S se clasificaron como: UMCV1 *Bacillus megaterium*, UMCV2 *Arthrobacter spp*, UMCV3 y UMCV4 *Stenotrophomonas maltophilia*. Las 4 cepas mostraron elevada capacidad para reducir Fe(III) a Fe(II), pero la UMCV1 mostró un incremento de 8 veces más y la UMCV2 9 veces más contenido de Fe(II) comparadas con el control. Al inocular plantas de frijol con las cepas bacterianas, éstas estimularon el crecimiento vegetal in vitro y en suelo, ya que las plantas fueron más grandes y mostraron mayores contenidos de Fe. Los autores

concluyen que estas cepas bacterianas contribuyen a la toma de Fe por las plantas a través de una incrementada reducción de Fe (III) en la rizósfera.

La actividad microbiana no solo favorece la adquisición de Fe para el desarrollo vegetal, se sabe que los m.o. pueden modificar la expresión de los genes relacionados con la toma y distribución de Fe. Los genes *Nramp* (1, 2 y 3) codifican para proteínas transportadoras de metales, entre ellos el Fe. Zhou y Yang (2004) analizaron la expresión de los genes *OsNramp* en células en suspensión de arroz en presencia de los patógenos *Burkholderia glumae* y *Magnaporthe grisea*. Observaron que la bacteria *B. glumae* reprime la expresión de Nramp1, mientras que induce la expresión de Nramp 2 y 3. Por otra parte, el hongo *Magnaporthe grisea* induce la expresión de los 3 genes *Nramp* después de 4 días de tratamiento. Interesantemente, cuando las células fueron crecidas en presencia de ión ferroso, disminuyó la expresión de *OsNramp* 1 y 2, mientras que *Nramp* 3 se induce después del tratamiento. Por lo anterior los autores sugieren que *OsNramp1* y posiblemente *OsNramp2* funcionan en la toma de Fe, mientras que *Nramp 3* pudiera participar en el proceso de eflujo de Fe. En conclusión demuestran que los genes *OsNramp* modulan su expresión en respuesta a infección por patógenos y pueden ser modulados también por la adición de iones en el medio.

La modulación en la expresión de los genes de toma y distribución de Fe no solo se lleva a cabo por m.o. Un estudio reciente muestra que los ácidos húmicos modulan la regulación transcripcional de los genes que participan en la asimilación del Fe en plantas de pepino (*Cucumis sativus*) crecidas en deficiencia de Fe (Elena *et al*, 2009). Específicamente analizaron la expresión de *Csfro1* (férrico quelato reductasa), *CsHa1* y *CsHa2* (codifican para una proteína H-ATPasa para acidificación del medio) y *CsIRT1* (codifica para el transportador de alta afinidad de Fe(II)). Los resultados del qRT-PCR mostraron que los ácidos húmicos causaron un incremento transitorio en *CsHa2*, mientras que *CsHa1* no se afectó o disminuyó su expresión. *CsFRO1* y *CsIRT1* sobre regularon su expresión a las 48 y 72 hrs después del tratamiento, efectos que estuvieron asociados con el

incremento en la actividad férrico quelato reductasa. Este estudio muestra que la aplicación de ácidos húmicos afecta respuestas fisiológicas de las plantas y que estos efectos difieren dependiendo del gen estudiado.

Estudios recientes han mostrado que la expresión de los genes *fro* también puede ser modulada por actividad microbiana. Zhang *et al.* (2009) observaron que la bacteria PGPR *Bacillus subtilis* (GB03) activa la maquinaria de adquisición de hierro para incrementar la asimilación del metal en plantas de *A. thaliana*. Determinaron que GB03 sobre regula transcripcionalmente al factor de transcripción FIT1, el cual es necesario para la actividad de FRO2 (reductasa de hierro) y del transportador de hierro IRT1. Observaron que cuando la bacteria creció sin que existiera contacto físico con la planta (compartimentos separados), la bacteria fue capaz de causar acidificación de la rizósfera (por un aumento en la liberación de protones en la raíz), un incremento en los niveles de hierro así como un incremento en la capacidad fotosintética de las plantas. Por lo que los autores concluyen que el coctel de compuestos orgánicos volátiles (VOCs) emitidos por la bacteria son los responsables de los efectos observados en la planta y proponen que en específico el ácido glioxílico, ácido dietil acético y el ácido 3-metil butanoico pueden ser los compuestos responsables de dicho efecto.

Velázquez-Becerra *et al.* (2011) analizaron los efectos de la inoculación de la bacteria PGPR *A. agilis* (UMCV2) en el crecimiento y desarrollo de plantas de *M. sativa*. El crecimiento de *A. agilis* en compartimentos donde solo hubo intercambio del espacio gaseoso con *M. sativa*, fue suficiente para modificar el desarrollo de la raíz de la planta, demostrando que los compuestos orgánicos volátiles (VOCs) producidos por *A. agilis* fueron los responsables de dicho efecto. El análisis de los VOCs producidos por *A. agilis* mostró que en particular la N-N-dimetil-hexadecilamina es el compuesto responsable de modular el crecimiento bacteriano así como el desarrollo de la planta. Los autores muestran que los VOCs bacterianos son percibidos por la planta para modular procesos morfogenéticos y de crecimiento mediante el reconocimiento a través de una molécula señal novedosa (Velázquez-Becerra *et al*, 2011).

Las señales bacterianas que desencadenan respuestas en plantas han sido ampliamente estudiadas, tal es el caso de la red de señalización que desencadenan bacterias simbióticas con plantas leguminosas como el frijol.

La fijación biológica de nitrógeno (FBN) es un proceso que requiere una coordinada regulación ya que la demanda de micronutrientes incrementa durante el establecimiento de la simbiosis, debido a que algunos metales son utilizados para la síntesis de varias proteínas que requieren, por ejemplo, el hierro tanto para la planta como para el bacteroide. Se han llevado a cabo estudios para identificar fuentes de hierro en bacteroides y simbiosomas (bateroides en el espacio peribacteroidal encerrados en la membrana peribacteroidal, PBM) en frijol soya (*Glycine max*). Los autores observaron que el hierro presente como FeIII fue transportado a tasas muy elevadas a través de la PBM que atraviesa la membrana del bacteroide sugiriendo la existencia de un pool de almacén de hierro en el espacio peribacteroidal. De igual manera los autores identificaron actividad ferrico reductasa asociada a la PBM con lo que sugieren que la reducción de Fe(III) a Fe(II) juega un papel en el movimiento de hierro dentro de los simbiosomas de frijol soya (LeVier *et al*, 1996).

También se ha estudiado el efecto de la deficiencia de hierro en la actividad de algunas de las proteínas que participan en la estrategia I como la férrico quelato reductasa (FQR), H+ATPasa, la fosfoenol piruvato carboxilasa (PEPC) y del transportador IRT1 en raíces y nódulos de dos cultivares (resistente y suceptible) de frijol común *Phaseolus vulgaris* (Tarek Slatni *et al*, 2011). Los autores identificaron que el cultivar tolerante mostró actividades incrementadas de FQR y H+ATPasa tanto en nódulos como en raíces lo que conllevó a un eficiente contenido de hierro en los nódulos. De igual manera se observó un incremento en la actividad de la PEPC y el contenido de ácidos orgánicos en los nódulos del cultivar tolerante. Los autores sugieren que los nódulos en deficiencia de hierro tienen la capacidad de modular su metabolismo para mantener las actividades necesarias para adquirir el hierro directamente de la solución del suelo (Tarek

Slatni *et al*, 2011). Por lo tanto el hierro es un componente importante en los nódulos de las leguminosas fijadoras de nitrógeno.

Por lo anterior se sabe que el estudio de los genes que participan en la toma y homeóstasis del hierro (*FRO*) son fundamentales y la disponibilidad de genomas de plantas como *M. truncatula* permite estudiar la presencia de genes ortólogos (*A. thaliana*) mediante el uso de herramientas bioinformáticas. Estudios *in silico* en el genoma de maíz (*Zea mays*) han permitido predecir la identificación de 33 genes que participan en el transporte de hierro y zinc; de éstos, 15 genes pertenecen a la familia *YS* (Yellow stripe, media el transporte de Fe-fitosideróforo en estrategas II), 9 genes a la familia *ZIP* (superfamilia de transportadores de iones), 6 genes a la familia *Nramp* (proteína de membrana transportadora de cationes multiespecíficos), 2 genes a la familia de las ferritinas (proteína de almacenaje de Fe) y 1 gene a la familia *FRO* (férrico quelato reductasa). Estas secuencias de genes candidatos pueden ser estudiadas para conocer su expresión, como marcadores genéticos o utilizados en ingeniería genética para incrementar el contenido de Fe en maíz (Chauhan, 2006).

5. JUSTIFICACIÓN

Las plantas leguminosas son ampliamente utilizadas para la nutrición humana y animal además de que enriquecen el suelo a través de la fijación de nitrógeno o cuando son usadas como abono verde (Singh *et al*, 2007). En Australia la leguminosa *M. truncatula* es usada como forraje y como mejoradora del suelo, lo que ha motivado numerosos programas de investigación dirigidos a caracterizar las poblaciones naturales, en un principio con un enfoque netamente agronómico. Sin embargo, desde hace 10 ó 15 años *M. truncatula* ha sido reconocida como una especie modelo interesante y actualmente los estudios se han enfocado a su diversidad genética (Bataillon y Ronfort, 2006). Uno de los aspectos genéticos a estudiar en esta planta es el status del hierro, es decir su capacidad para tomar el hierro del suelo, ya que este elemento es uno de los nutrientes más limitantes en el crecimiento y desarrollo vegetal debido a su extremada baja solubilidad en ambientes aeróbicos a pH neutrales o alcalinos. Se sabe que las plantas deficientes de hierro muestran un decremento en las proteínas, en los pigmentos que contienen hierro y en el contenido de clorofila, causando clorosis en hojas jóvenes. La toma de hierro por la raíz de las planas con estrategia I es llevada a cabo por la enzima férrico quelato reductasa y en la planta *Arabidopsis thaliana* se han identificado 8 genes *FRO* que codifican para una férrico quelato reductasa activa, las cuales exhiben una expresión tejido-específica, por lo que se les atribuye una función en la homeóstasis del hierro en la planta (Wu *et al*, 2005).

M. truncatula tiene la capacidad de establecer relaciones simbióticas con bacterias fijadoras de nitrógeno y actualmente solo cuanta con un gene *FRO* caracterizado, de aquí el interés por conocer si la bacteria simbionte *Sinorhizobium meliloti* y las bacterias PGPRs favorecen la adquisición de hierro mediante la toma y distribución de dicho metal vía la inducción de las respuestas a la escasez de hierro de las plantas estrategas I o solamente como ya se ha descrito para cepas de *B. megaterium*, *A. agilis* y *S. maltophilia* por la reducción directa del Fe(III), especialmente en suelos alcalinos, cuando la férrico quelato reductasa vegetal es menos activa (Valencia-Cantero *et al*, 2007).

6. HIPÓTESIS

LAS BACTERIAS *Artrhobacter agilis* UMCV2 Y *Sinorhizobium meliloti 1021*, MODULAN LOS MECANISMOS DE RESPUESTA DE *Medicago truncatula* A LA ESCASEZ DE HIERRO

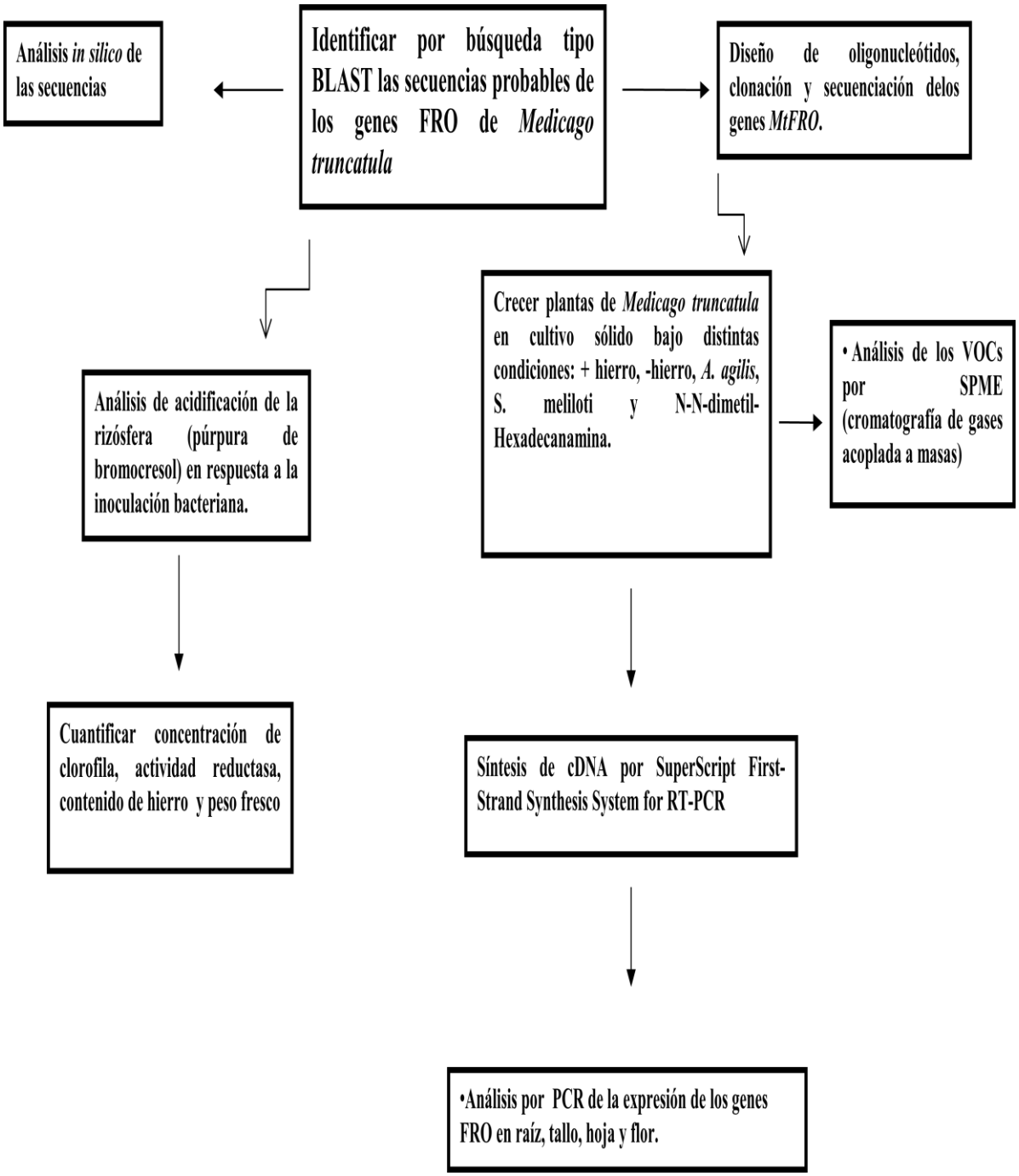
7. OBJETIVO GENERAL

Analizar la modulación de los mecanismos de respuesta a la escasez de hierro de *Medicago truncatula* mediada por rizobacterias promotoras del crecimiento vegetal

8. OBJETIVOS ESPECÍFICOS:

- Identificar los genes *fro* de *Medicago truncatula.*
- Determinar la capacidad de las PGPRs *Arthrobacter agilis* UMCV2 y *Sinorhizobium meliloti* para inducir los mecanismo de toma de hierro en *Medicago truncatula*.
- Identificar los metabolitos bacterianos causantes de la inducción de los mecanismos de toma de hierro de *Medicago truncatula*.
- Aislar y clonar las secuencias parciales de los genes FRO de *Medicago truncatula*.
- Analizar la modulación de la expresión de los genes *FRO* en distintos tejidos de la planta *Medicago truncatula* por efecto de la escasez de hierro.

9. METODOLOGÍA



10. RESULTADOS

CAPÍTULO I

Arthrobacter agilis UMCV2 induces iron acquisition in *Medicago truncatula* (strategy I plant) *in vitro* via dimethylhexadecylamine emission

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REGULAR ARTICLE

Arthrobacter agilis UMCV2 induces iron acquisition in *Medicago truncatula* (strategy I plant) in vitro via dimethylhexadecylamine emission

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Abstract
Background and aims Iron is an essential nutrient for plant growth. Although abundant in soil, iron is poorly available. Therefore, plants have evolved mechanisms for iron mobilization and uptake from the rhizospheric environment. In this study, we examined the physiological responses to iron deficiency in *Medicago truncatula* plants exposed to volatile organic compounds (VOCs) produced by *Arthrobacter agilis* UMCV2.
Methods The VOC profiles of the plant and bacterium were determined separately and during interaction assays using gas chromatography. *M. truncatula* plants exposed to *A. agilis* VOCs and pure dimethylhexadecylamine were transferred to conditions of iron deficiency, and parameters associated with iron nutritional status were measured.
Results The relative abundance of the bacterial VOC dimethylhexadecylamine increased 12-fold when in co-cultures of *A. agilis* and *M. truncatula*, compared to axenic cultures. Plants exposed to bacterial VOCs or dimethylhexadecylamine exhibited a higher rhizosphere acidification capacity, enhanced ferric reductase activity, higher biomass generation, and elevated chlorophyll and iron content relative to controls.
Conclusions The VOCs emitted by *A. agilis* UMCV2 induce iron acquisition mechanisms in vitro in the Strategy I plant *M. truncatula*. Dimethylhexadecylamine is the signal molecule responsible for producing the beneficial effects.

Keywords Iron · *Medicago truncatula* · Ferric chelate reductase activity · Dimethylhexadecylamine

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Introduction

Iron (Fe) is an essential nutrient for plants as it allows them to maintain cellular homeostasis (Hell and Stephan 2003). Although iron is abundant in the soil, its low solubility restricts its availability in aerobic soils to levels low enough to limit plant growth. This problem of solubility is due to the chemical nature of iron. As a transition metal, iron possesses the ability to gain or lose electrons, which gives it important properties in redox reactions (Vert et al. 2002). Fe^{2+} is the

form available to plants because it is relatively soluble, but is rapidly oxidized to Fe^{3+} by atmospheric oxygen. The solubility of Fe(III) dramatically decreases with increasing pH due to hydrosilation, polymerization, and precipitation with inorganic anions (Hell and Stephan 2003). The scarcity of iron reduces agricultural production and causes a decline in the nutritional value of crops (Schmidt 1999).

Plants require effective mechanisms to acquire iron from the soil to fulfill growth and developmental demands, while avoiding toxicity due to excess. Efficient acquisition of iron in plants occurs via 2 mechanisms: Strategy I and Strategy II (Römheld and Marschner 1986). Strategy II plants include grasses whose roots secrete compounds known as phytosiderophores (PS) that chelate Fe^{3+} in the rhizosphere. Subsequently, the Fe^{3+}-PS complex is introduced into the cell via a carrier protein in the plasmalemma known as YS1 (Hell and Stephan 2003). Strategy I plants are all higher plants (except grasses), and iron acquisition occurs via 3 mechanisms: i) excretion of protons across the plasma membrane through an ATPase to acidify the rhizosphere and enhance Fe^{3+} solubilization; ii) reduction of Fe^{3+} to the ferrous form (Fe^{2+}) by the expression of the ferric chelate reductase protein, encoded in *Arabidopsis* by the *FRO2* gene (Robinson et al. 1999), in roots; and iii) transfer of Fe^{2+} into the root cells through the plasma membrane by the transporter IRT (Eide et al. 1996). Besides the 3 main mechanisms described above, iron deficiency also promotes the excretion of phenolic compounds, organic acids, and flavins, which facilitate the reduction and solubility of external iron (Susín et al. 1994; Welkie and Miller 1988). In Strategy I plants, the 3 main inducing conditions are: iron deficiency (Hell and Stephan 2003), presence of humic acids (Aguirre et al. 2009), and presence of growth-promoting microorganisms (Zhang et al. 2009). Regarding the latter, the molecules responsible for the induction of iron acquisition in Strategy I plants are unknown.

Plant **g**rowth-**p**romoting **r**hizobacteria (PGPR) stimulate plant growth via different mechanisms, including the synthesis of plant growth regulators, such as auxins, cytokinins, and even cyclopeptides (Ortíz-Castro et al. 2008; 2011; Spaepen et al. 2009). Recently, we have shown that volatile organic compounds (VOCs) produced by PGPR alter plant development, particularly the root system (Gutiérrez-Luna et al. 2010). Further, a previous study by our group showed that *Arthrobacter agilis* UMCV2 can improve the nutritional status of leguminous plants by promoting iron acquisition mechanisms involving the reduction and dissolution of Fe^{3+} present in the soil (Valencia-Cantero et al. 2007), and that emission of the VOC N, N-dimethylhexadecylamine (dimethylhexadecylamine or DMHDA) acts as a signal to promote the growth of the legume *Medicago sativa*, besides drastically modifying the roots (Velázquez-Becerra et al. 2011).

To dissect the mechanisms and determine the compounds involved in the modulation of iron acquisition responses in Strategy I plants, we examined the overall effect of the VOCs emitted by the PGPR *A. agilis* UMCV2 on the development of the model legume *M. truncatula*. In this study we used the legume model *M. truncatula* instead of *M. sativa* for 2 reasons: 1) it is easier to compare our results with those of other studies employing *M. truncatula*, particularly those related to plant-bacteria interactions and plant iron acquisition in vitro; 2) the genome of *M. truncatula* has been recently sequenced and released, which will facilitate future studies on the components of the strategy I iron uptake at genetic or genomic level.

The results confirm that the growth-promoting effect of the VOCs produced by *A. agilis* UMCV2 can be extended to other legumes, such as *M. truncatula*, and also show that, regardless of its effect on root development, the VOCs produced by the strain UMCV2, and dimethylhexadecylamine in particular, induce at least 2 Strategy I components for iron acquisition in plants.

Materials and methods

Biological material and growth conditions

M. truncatula seeds (ecotype Jemalong A17) were subjected to chemical scarification (Boisson-Dernier et al. 2005) and immersed in a vial containing 1 to 2 mL of concentrated anhydrous sulfuric acid with intermittent agitation until the appearance of small black spots on the integument (5–15 min). Excess acid was removed and the seeds were rinsed with 5 washes of sterile deionized water. For sterilization, seeds were soaked in a solution of sodium hypochlorite (12 %) for 3 min and rinsed with 6 washes of sterile deionized water. Seeds were germinated in Petri dishes with

Murashiege and Skoog MS medium and transferred to a Percival growth chamber with a photoperiod of 16 h light/8 h dark at a light intensity of 200 mol m^2 s^{-1} at 22°C.

The PGPR strain *A. agilis* UMCV2 was isolated from lightly acid soil, as previously described (Valencia-Cantero et al. 2007). The bacterium was grown on nutrient agar (NA) at 26°C.

Effect of UMCV2 on the growth of *M. truncatula*

Newly scarified seeds were placed in plastic Petri dishes containing MS medium at 4°C for 48 h and then placed in a Percival growth chamber with a photoperiod of 16 h light/8 h dark at a light intensity of 200 mol m^2 s^{-1} at 22°C. The germinated sprouts were placed in glass flasks of 170 mL containing 25 mL of MS nutrient medium, 6 g of agar (Phytotechnology, Shawnee Mission KS, US) per liter (L), and a vial with 5 mL of NA (Fig. 1d). After 5 days, half the flasks were incubated with small vials containing an *A. agilis* UMCV2 inoculum, and the other half were incubated with vials devoid of bacteria (axenic) and were used as controls. The experiment was maintained until the plants were 10 days old. After this time, plants were measured and weighed. Chlorophyll concentration in the plant shoots was determined using a spectrophotometric method described below.

Analysis of volatile compounds

For chromatographic analysis, the following treatments were carried out: I) flasks were incubated with vials containing UMCV2 for 5 days; II) 3 plants were axenically grown in flasks for 10 days; III) plants were grown for 5 days in flasks, followed by addition of a vial containing the bacterial inoculum and co-incubation of plant and bacteria for 5 days; and IV) controls. At the end of the incubation, gas chromatographic analysis was performed to detect bacterial and plant VOCs (treatments I and II), VOCs emitted during the plant-bacteria interaction (treatment III), and VOCs from controls. VOCs from axenic media were also analyzed to be discarded in the final table of results.

The gas chromatography was performed as previously described (Velázquez-Becerra et al. 2011). Briefly, the analysis (Gas Chromatographer Agilent 6850 Series II; Agilent, Foster City, CA, U.S.A.) was performed using the solid-phase microextraction technique (SPME), exposing the PDMS/DVB fiber (Supelco, Inc., Bellafonte, PA, USA.) to the sample headspace for 30 min at 30°C, and desorbing at 180°C for 30 s in the injection port of the gas chromatographer coupled to a mass spectrometer (Agilent 5973). A 25 mm×0.52 mm capillary column with 0.32 μm film thickness (HP-FFAP; Agilent) was used; ultra pure helium (1 mL·min^{-1}) was used as the carrier gas and the detector temperature was 250°C. The column was held for 13 min at 40°C, and then programmed to rise at a rate of 3°C per min to a final temperature of 180°C, which was maintained for 25 min. The source pressure and filament voltage were 7 Pa and 70 eV, respectively, and the scan rate was 1.9 scan·s^{-1}. The compounds were identified by comparing with mass spectra from the library (NIST/EPA/NIH, "Chem Station'Agilent Technologies Rev. D.04.00 2002). The identity of dimethylhexadecylamine was further confirmed by comparing the retention time in the VOC profiles to a sample of the pure standard (Sigma-Aldrich catalog 40460; CAS: 112-69-6). Dimethylhexadecylamine was quantified using an external standard. Five microliters of dimethylhexadecylamine (100 μ*M*) was spotted on a vial with 5 mL of AN inside the flask, as described above. The peak area of the pure standard compound was recorded and compared with the axenic and bacterial compound peak areas, as well as with that from the interaction experiment.

Analysis of rhizosphere acidification

The pH change in the root environment or in the plant rhizosphere was measured by a change in the color of the pH indicator bromocresol purple. Plants were grown in culture flasks containing MS medium with iron sufficiency (100 μ*M*) in a growth chamber for 5 days. The induction was conducted as follows: an *A. agilis* UMCV2 inoculum (1×10^6 CFU) was placed in a vial, and dimethylhexadecylamine (5 μL to 100 μ*M*) and water (control) were placed separately in glass vials with 5 mL of NA and then placed into culture flasks containing the plants, so that there was no physical contact between *M. truncatula* and the bacterium. Plants were induced for 2 days, after which a group of plants was transferred to Petri dishes

containing MS medium (0.6 % sucrose and 0.8 % agar) with iron sufficiency (100 μ*M*). Another group was transferred to MS medium with iron deficiency (1 μ*M*); the colorant bromocresol purple (0.006 %) (modified from Zhang et al. 2009) was included in both cases. Photographs were taken at 24 and 48 h after initiation of iron stress. The color of bromocresol purple was compared with a colorimetric scale prepared with culture media plates containing bromocresol purple at a pH range of 4 to 7.

Quantification of ferric chelate reductase activity

Ferric chelate reductase activity was analyzed using a spectrophotometric quantification method for the formation of Fe^{2+}-ferrozine complex (Yi and Guerinot 1996). Five-day-old seedlings grown in MS medium were exposed to the volatile compounds of *A. agilis* or dimethylhexadecylamine for 48 h in the system described above. After the induction, a group of plants was transferred to plates containing MS medium with iron sufficiency or deficiency at different times according to the experiment. The root system was immersed in a solution containing 0.5 m*M* Fe(III)-EDTA and 4.4 mM ferrozine at pH 6.5. The absorbance was read at 562 nm after 1 h of incubation at room temperature in darkness. The Fe(II)-ferrozine concentration was calculated from a previously formulated standard curve equation. An identical solution but without the root system was used as a blank. The pH of the solution was measured using a potentiometer.

Determination of chlorophyll content

Quantification of chlorophyll was carried out according to the method of Lichtenthaler and Wellburn (1983). The plant tissue was ground in a mortar with a solution of 80 % acetone. The solution containing the pigment was filtered with Whatman No. 1 paper. The samples were gauged to 5 mL with 80 % acetone and read in a spectrophotometer at 663 and 646 nm. The concentration of chlorophyll was calculated using the following formulas: Chlorophyll *a*=(12.21)·(E663) - (2.81)·(E646); chlorophyll *b*=(20.13) (E646) - (5.03) (E663); Total chlorophyll = ([mL acetone]·[pigment content])/sample weight

Determination of endogenous Fe content

Endogenous iron in plants of *M. truncatula* was quantified using the AY-5 analysis of plant tissue wet digestion method (Perkin-Elmer Corp. 1996). For every 1 g of plant tissue powder, 10 mL of HNO_3 was added and allowed to stand overnight. The solution was carefully heated in a water bath until the production of nitrous oxide fumes ceased. Liquid solutions were allowed to cool at room temperature and 4 mL of hydrogen peroxide (trace metal grade) was added. The mixture was reheated to evaporate into a small volume of approximately 3 μL and transferred to 50 mL of sterile deionized water. We generated a standard curve for Fe according to the recommended conditions for the equipment (Atomic Absorption Spectrometer AAnalyst 200; Perkin-Elmer Corporation, USA).

Statistical analysis

All experiments were performed 2–3 times. The results were analyzed using the Statistica 6.0 software (Statsoft Inc. 2001). The Student's *t* test was used to compare the means of 2 groups, and the ANOVA test and Duncan's means separation test were used for multiple comparisons ($p<0.05$).

Results

A. agilis promotes the growth of *M. truncatula* seedlings via VOC emission

To determine the effects of VOCs produced by *A. agilis* UMCV2 on *M. truncatula* growth we employed a separate compartment system (Fig. 1d), where both organisms were grown in the same gas phase but without physical contact. Under these conditions, bacterial VOCs had a stimulatory effect on the growth of shoots and roots (Fig. 1a, e, and f) and particularly on the plant biomass. In general, plants grown in the presence of bacterial VOCs exhibited 40 % and 35 % increases in shoot and root fresh weights compared to control plants (Fig. 1b).

Plants grown in the presence of UMCV2 also exhibited a 35 % increase in chlorophyll concentration, compared to control plants (Fig. 1c). This is particularly interesting, given that chlorophyll

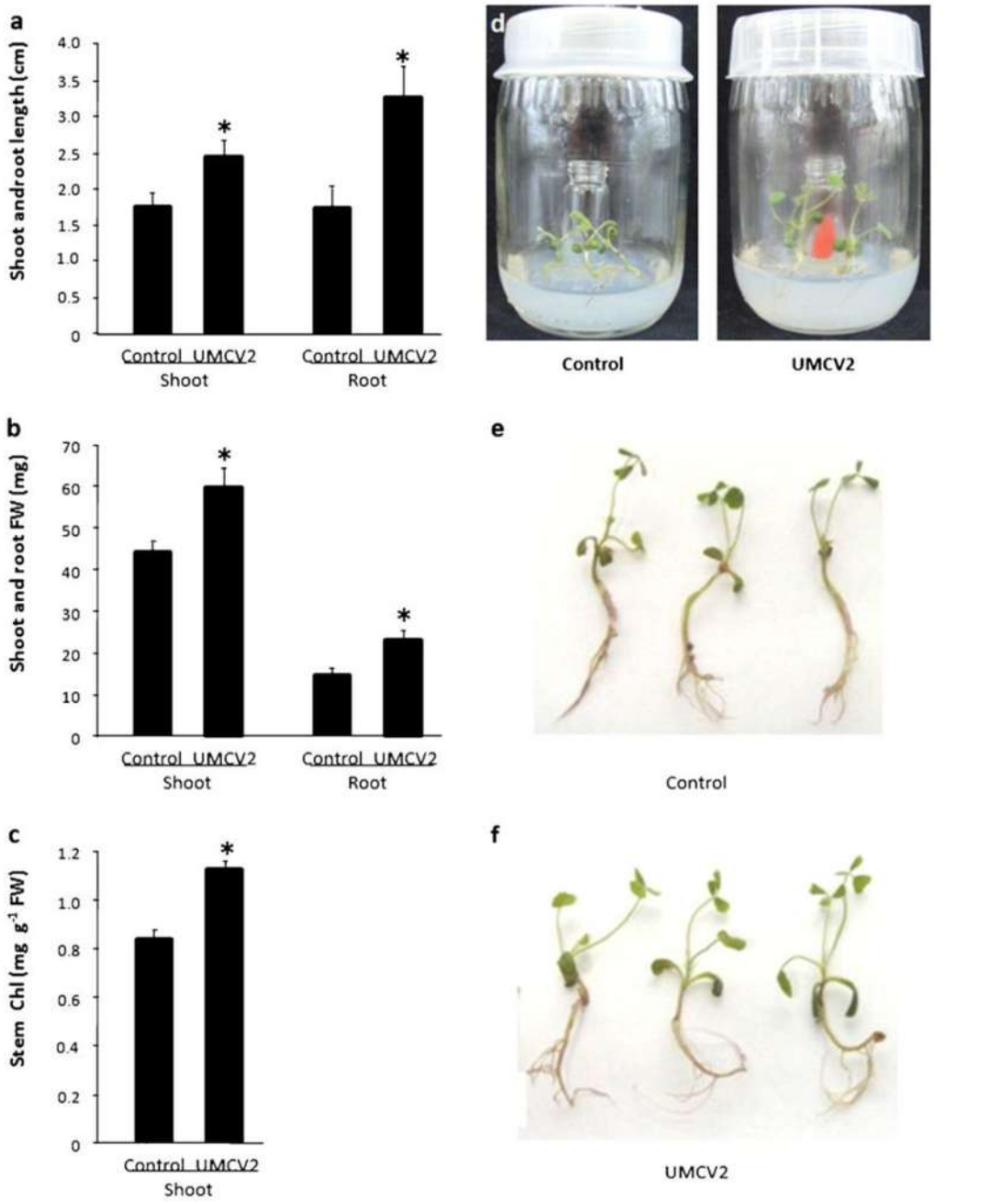

Fig. 1 *Arthrobacter agilis* UMCV2 VOCs promote the growth of *Medicago truncatula*. Newly germinated *M. truncatula* seedlings were placed in glass flasks containing MS medium. After 5 days, *A. agilis* was inoculated in vials containing nutrient agar (NA) medium, and the systems were incubated for 5 days. Values represent mean (standard error [SE]) (n 9). **a** Shoot and root length, **b** plant biomass fresh weight (FW), **c** chlorophyll content, **d** general view of the plant-bacteria interaction system, **e** 10-day-old uninduced plants, and **f** *A. agilis* UMCV2 VOC-induced plants. Asterisks indicate statistically significant differences ($p<0.05$; Student t test)

concentration is considered an indicator of iron nutritional status in plants (Masalha et al. 2000; Terry and Abadia 1986). Thus, *A. agilis* is able to increase the chlorophyll concentration and promote

the overall growth of *M. truncatula* via mechanisms that involve volatile compounds.

A. agilis UMCV2 and *M. truncatula* modify their volatile cocktail production during interaction with each other

To determine whether *A. agilis* UMCV2 and *M. truncatula* are able to interact with each other through their VOCs and examine the potential modulation of iron metabolism by these compounds, we used the separate compartment system described above. Four-day old plants were incubated with a small vial containing nutrient agar inoculated (or not inoculated in the case of controls) with *A. agilis* UMCV2 for 3 days. This technique prevented direct contact between the plant and bacterium while allowing the plants to perceive the bacterial VOCs, and vice versa. VOCs in axenic cultures of the bacterium and the plant were independently identified by GC-MS. The mixture of compounds emitted by the bacterium *A. agilis*UMCV2 comprised different types of VOCs: the ketones 2-butanone, 2-pentanone, 2-octanone, and 5-methyl 2-hexanone (Claeson and Sunesson 2005; Müller et al. 2008; Zou et al. 2007); the alcohols ethanol alcohol, benzyl alcohol, and phenylethyl alcohol (Kai et al. 2007; Thorn et al. 2011); 2.5-dimethylpirazyne pyrazyne, previously reported by Xu et al. (2004); the terpenes terpinolene and camphor (Dickschat et al. 2005; Wilkins and Schöller 2009);and the amine dimethylhexadecylamine (Velázquez-Becerra et al. 20011). Among acid VOCs, we only detected benzeneacetic acid ethyl ester (Table 1). In axenic cultures, we also detected 11 different VOCs produced by *M. truncatula* (Table 1) among them: 2-ethyl 1-hexanol, 1-dodecanol, eucalyptol (1,8-cineole), and 1-octen-3-ol (Table 1).

Furthermore, a series of 12 compounds, including nonanal and 3-octanone, were detected during plant-bacteria interactions, but not in axenic treatments. The VOC 3-octanone is commonly produced by fungi, and also by some leguminous plants (Boué et al. 2005). Additionally, dimethyl disulfide and dimethyl trisulfide compounds with antifungal (Kai et al. 2008; Zou et al. 2007) and anti-microbial effects (Bendimerad et al. 2005; Wang et al. 2009) have been reported among bacteria and plant VOCs. Unfortunately, we were unable to determine which of the 2 organisms produced

Table 1 Volatile compounds produced by *A. agilis* UMCV2 and *M. truncatula* in axenic and interacting cultures

Compound[a]	*A. agilis*	*M. truncatula*	*A. agilis* × *M. truncatula*
Phenylethyl alcohol	+	−	−
Ethanol	+	−	−
2-Butanone	+	−	−
Benzeneacetic acid	+	−	−
2,5-Dimethyl pyrazine	+	−	−
Benzyl alcohol	+	−	−
5-methyl 2-hexanone	+	−	−
2-Octanone	+	−	−
2-Pentanone	+	−	−
Terpinolene	+	−	−
Camphor	+	−	−
3-Methyl quinoline	+	−	−
Dimethyl-N-hexadecylamine	+	−	−
3-Methyl-butanal,	+	−	−
2-Ethyl 1-hexanol	−	+	+
1-Dodecanol	−	+	+
2-Decenal	−	+	+
p-Menth-1-en-8-ol	−	+	−
1-Octanol	−	+	+
1-Octen-3-ol	−	+	+
Acetic acid	−	+	+
3-Cyclohepten-1-one	−	+	+
1-Hexadecanol	−	+	+
p-Mentha-6,8-dien-2-one	−	+	+
Eucalyptol	−	+	+
Dimethyl disulfide	−	−	+
3-Heptanone	−	−	+
2-Ethyl hexanal	−	−	+
3-Octanone	−	−	+
Dimethyl trisulfide	−	−	+
Nonanal	−	−	+
1,4-Dichloro benzene	−	−	+
2,6-Di-tert-butyl-4-sec-butylphenol	−	−	+
4-Octadecyl morpholine	−	−	+
p-Amino acetophenone	−	−	+
2-Morpholinomethyl-1,3-diphenyl-2-propanol	−	−	+

[a] Most abundant VOCs present in 3 replicated cultures. Compounds with less than 0.5 % area under the curve in at least 1 column were omitted

the specific VOCs. The above data suggest that *M. truncatula* is able to perceive the *A. agilis* VOCs and respond by modifying its own VOC cocktail.

Dimethylhexadecylamine was previously reported as an *A. agilis* UMCV2-derived volatile compound capable of promoting plant growth (Velázquez-Becerra et al. 2011). Therefore, its detection in axenic cultures of *A. agilis* was not surprising. However, the concentration of dimethylhexadecylamine in the *M. truncatula-A. agilis* interaction system increased 12-fold, compared to that of axenic *A. Agilis* cultures (Fig. 2). This suggests that *A. agilis* may perceive *M. truncatula* through VOCs and respond with an increase in dimethylhexadecylamine emission.

VOCs produced by *A. agilis* induce acidification of the *M. truncatula* rhizosphere

Considering that the VOCs produced by *A. agilis* UMCV2 elicited an increase in chlorophyll concentration of *M. truncatula*, and given that this measurement is considered as an indicator of the nutritional status of iron in plants, we analyzed rhizosphere acidification. In Strategy I plants, the first response to iron deficiency is an increased extrusion of protons from the roots into the surrounding environment (rhizosphere), thereby facilitating iron transport and mobility.

We tested the ability of *A. agilis* VOCs and pure dimethylhexadecylamine to induce acidification of the root environment using a colorimetric method (see Materials and methods). Seven-day-old *M. truncatula* plants were exposed to *A. agilis* VOCs or dimethylhexadecylamine for 48 h. After this time, a group of plants from each treatment was transferred to medium with iron sufficiency (100 μM) or iron deficiency (1 μM). Plants exposed to *A. agilis* VOCs and transferred to iron sufficiency exhibited a small change in rhizosphere acidification after 24 h (Fig. 3), whereas plants exposed to bacterial VOCs and transferred to iron deficiency showed a strong acidification as early as 24 h and more so at 48 h after iron stress. Plants exposed to dimethylhexadecylamine exhibited a similar pattern, acidifying the rhizosphere after 24 h of growth in iron-sufficient medium and producing a higher acidification area at 48 h after transfer to iron deficiency conditions. These data were compared with those of control experiments where the plants were not induced. Acidification of the medium was not observed when control plants were transferred to 100 μM iron, and only a slight acidification zone was observed at 48 h of growth in iron-deficient conditions (Fig. 3).

Acidification was evidently produced by the *M. truncatula* roots induced by bacterial VOCs and volatile dimethylhexadecylamine during plant-bacteria interactions, because this acidification corresponded to the area immediately around the roots and not to a general acidification that would be expected if acidification was directly caused by the bacterial VOCs or dimethylhexadecylamine. In a further experiment, we could not observe a direct change in the pH of the MS medium when the bacterium or dimethylhexadecylamine was grown or added in a separate compartment, respectively (Online Resource 1).

The results show that both the volatiles of *A. agilis* and dimethylhexadecylamine promote proton extrusion into the surrounding root area during interaction with *M. truncatula* plants, because both treatments elicited a change in pH from 6.5 to approximately 4 under conditions of iron deficiency; a change in pH was also observed in iron sufficiency but was less intense.

VOCs produced by *A. agilis* UMCV2 stimulate ferric chelate reductase activity in the roots of *M. truncatula*

After determining that the VOCs produced by *A. agilis* and dimethylhexadecylamine have the ability to induce the plant to acidify the root environment, we tested the effect of these same compounds on the activity of ferric chelate reductase, a second component of the iron uptake system in Strategy I plants.

Clearly, plants that were induced by *A. agilis* and dimethylhexadecylamine exhibited an increase in ferric reductase activity from 0 h (time of transfer) to 72 h after induction, compared to uninduced control plants in both conditions of iron (Fig. 4a and b). This increment in the ferric reductase activity ranged between 125 and 20 % depending on whether plants were induced with VOCs of *A. agilis* or dimethylhexadecylamine, and whether the plants were transferred to an iron deficiency or sufficiency condition after induction. Interestingly, a maximum reduction in activity was observed at 24 h after transfer, particularly in plants that were placed in

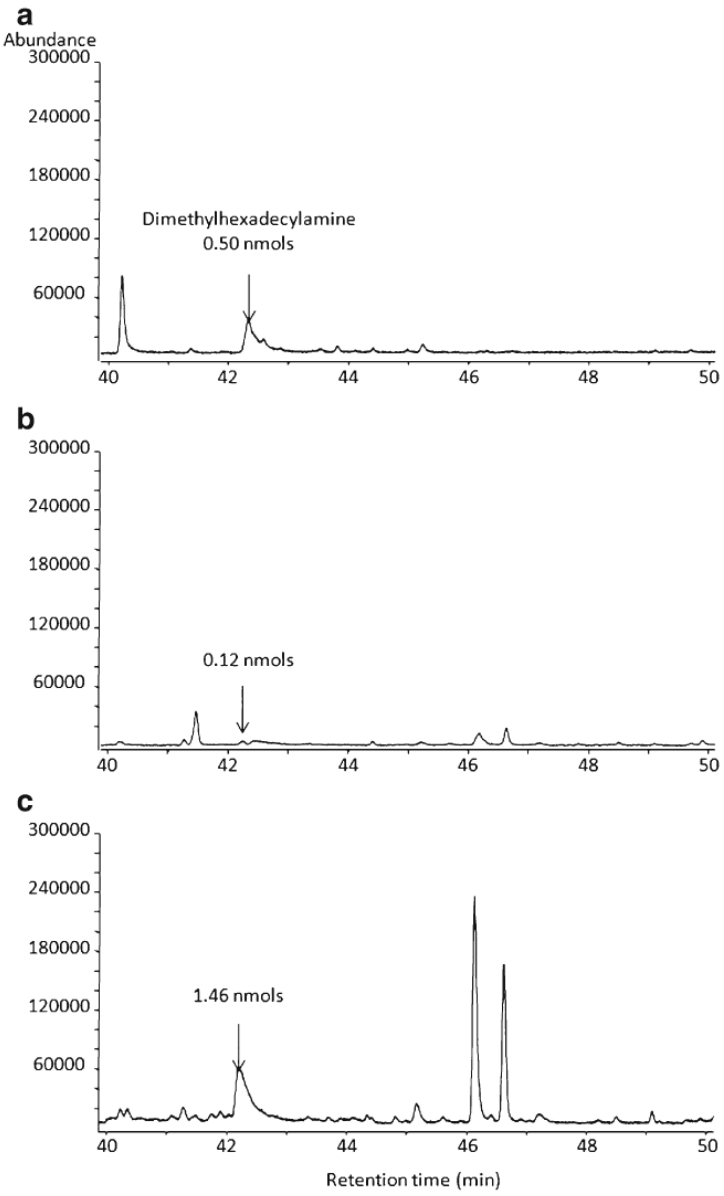

Fig. 2 Determination of dimethylhexadecylamine content in control, *A. agilis* UMCV2, and *M. truncatula*-*A. agilis* UMCV2 interaction systems by SPME-GC-MS. Total ion chromatogram of the pure standard at 0.50 nmol (Rt 42.3 min) obtained from control flasks (**a**), with bacteria (**b**), and from the plant-bacteria interaction system (**c**)

iron deficiency. Under this condition, plants induced by *A. agilis* VOCs exhibited a 120 % increase in ferric reductase activity compared to controls; however, this peak of elevated activity was also present in plants transferred to conditions of iron sufficiency.

Plant growth, measured as total fresh weight, was also greater in the induced plants, even at time points as short as 3 days of iron stress. Thus, *A. agilis* VOCs promoted plant growth independently of the iron status (Fig. 4c and d). Similarly, chlorophyll content was quantified as an indirect measure of the nutritional status of iron in plants. As shown in Fig. 4e, plants grown in iron sufficiency exhibited a gradual increase in chlorophyll content; however, the amount of pigment was higher in plants that were induced with *A. agilis*. Under iron deficiency conditions, control (Fig. 4f) plants began to show slight signs of chlorosis after 72 h of iron stress, whereas the chlorophyll

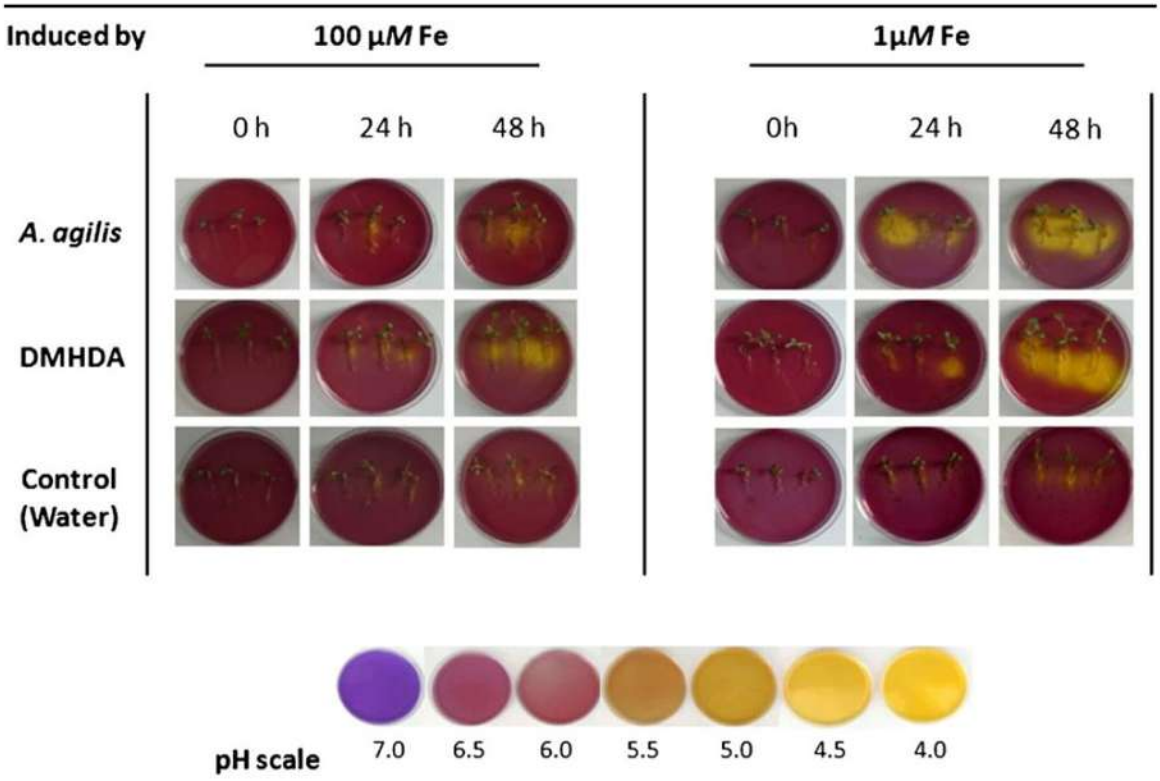

Fig. 3 Acidification of the *M. truncatula* rhizosphere in response to *A. agilis* VOCs and dimethylhexadecylamine (DMHDA). Five-day-old plants were induced with *A. agilis* or DMHDA for 48 h and transferred to MS medium containing bromocresol purple with iron sufficiency (100 µ*M*) or deficiency (1 µ*M*). Photographs were taken at 24 and 48 h after transfer to these media, and are representative of 6 replicates

content of plants induced with *A. agilis* VOCs or dimethylhexadecylamine was similar to that of plants maintained in medium with iron sufficiency.

To determine whether this effect of *A. agilis* VOCs and dimethylhexadecylamine on *M. truncatula* plants was sustained over the long term, seedlings were grown under the same conditions but under iron-deficiency stress for 7 days. *A. agilis* VOCs induced ferric chelate reductase activity in *M. truncatula* seedlings in iron sufficiency and deficiency, with the activity being higher in iron deficiency. Similarly, dimethylhexadecylamine enhanced ferric reductase activity in iron-deficient plants compared to uninduced controls (Fig. 5a). Growth was also promoted in induced plants. The weights of plants induced by *A. agilis* VOCs and grown in iron sufficiency were higher; however, it is noteworthy that in iron deficiency, the weights of the plants induced with *A. agilis* and dimethylhexadecylamine were always higher than those grown in uninduced iron sufficiency conditions even when the induction occurred 7 days prior (Fig. 5b).

Once the increase in iron reductase activity was identified, quantification of pH and chlorophyll content of plants were performed at 7 days post-induction. Figure 5c shows that plants exposed to *A. agilis* VOCs in iron deficiency produced a more intense acidification, reducing the pH from its initial value of 6.5 to 5.2, than plants from other treatments. Induced plants grown in iron sufficiency were also able to decrease the pH to 5.7, indicating an increased extrusion of protons into the medium by the induced plants.

Furthermore, plants exposed to dimethylhexadecylamine did not exhibit significant differences in acidification compared to control plants induced in both conditions (iron sufficiency and deficiency) (Fig. 5c). The differential effect of the bacterial VOCs mixture and dimethylhexadecylamine may be due to 2 reasons: a higher concentration of dimethylhexadecylamine in

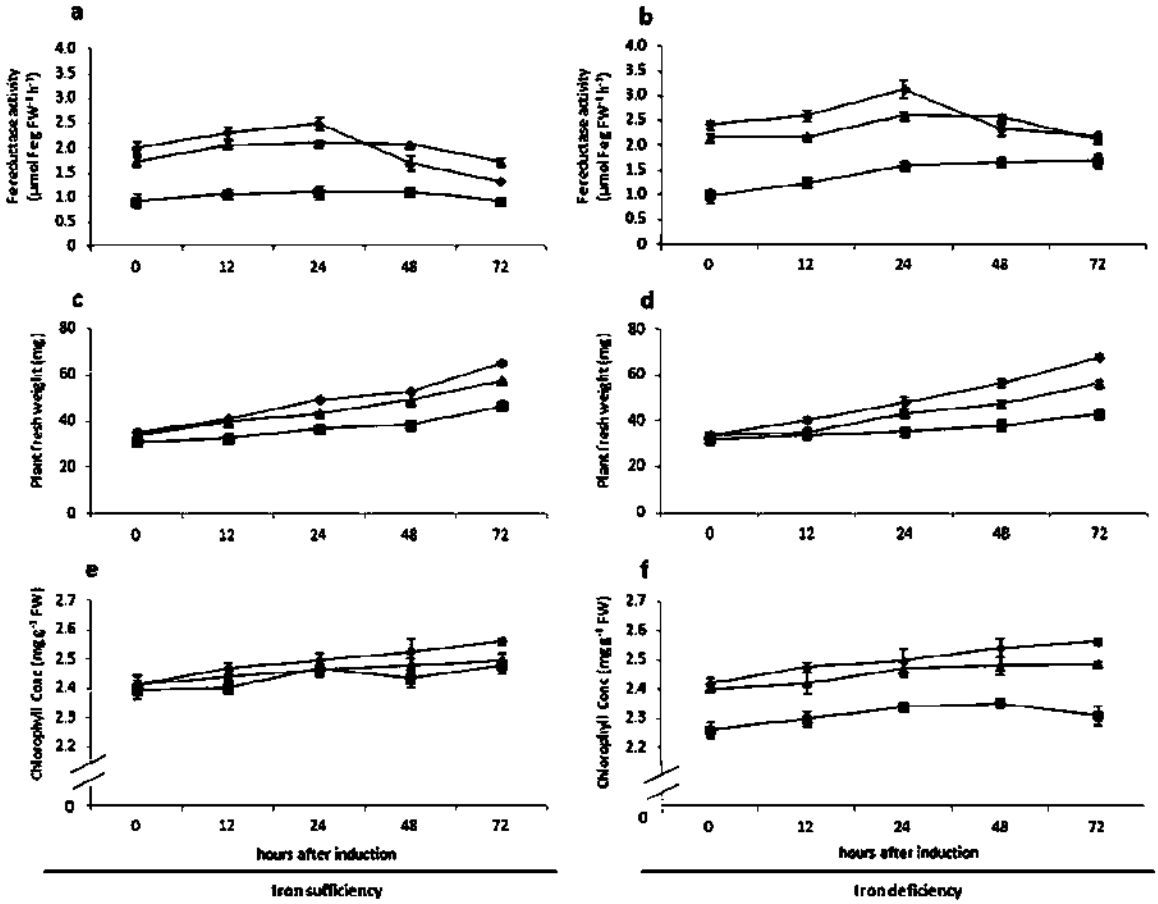

Fig. 4 Inductive effect of *A. agilis* VOCs and DMHDA on ferric chelate reductase activity (**a** and **b**), biomass (**c** and **d**), and chlorophyll content (**e** and **f**) in iron-sufficient (**a**, **c**, and **e**) or -deficient (**b**, **d**, and **f**) media as indicated. *M. truncatula* seedlings were induced for 2 days with UMCV2 or DMHDA and were transferred to medium with or without iron. (♦) UMCV2, (▲) DMHDA, (■) Control

the VOC mixture or synergistic action of other VOCs with dimethylhexadecylamine.

Finally, the induced plants showed no symptoms of chlorosis at 7 days post-induction; the chlorophyll concentration of plants exposed to *A. agilis* VOCs was higher, irrespective of the iron treatment. Similarly, plants exposed to dimethylhexadecylamine exhibited a higher chlorophyll concentration even in iron deficiency, compared to uninduced plants grown in iron sufficiency (Fig. 5d).

These results demonstrate that VOCs produced by *A. agilis* UMCV2, and dimethylhexadecylamine in particular, have the ability to induce ferric chelate reductase activity and acidification of the rhizospheric environment, and that this induction persists even 7 days after removal of the stimulus. The increase in both activities resulted in a greater generation of biomass and higher chlorophyll content with time.

VOCs produced by *A. agilis* UMCV2 promote iron accumulation in *M. truncatula*

Because the plants induced with *A. agilis* and dimethylhexadecylamine showed an increase in chlorophyll content at 24 h after induction and up to 7 days, we analyzed the Fe content in plants. *M. truncatula* plants were grown under the same conditions as in the experiments for reductase activity. After 48 h of induction with *A. agilis* or dimethylhexadecylamine, the plants were transferred to medium with iron sufficiency or

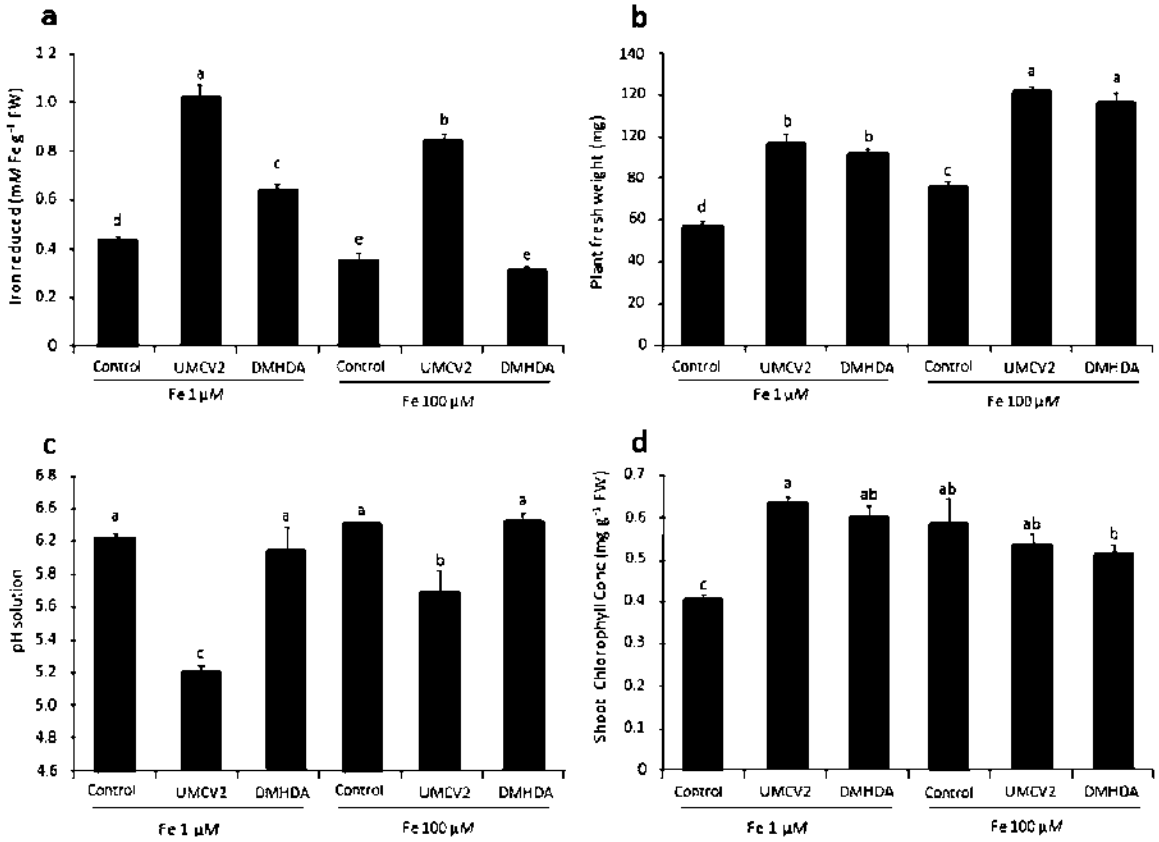

Fig. 5 *A. agilis* and DMHDA increase iron deficiency responses in *M. truncatula* plants up to 7 days after induction. Plants were transferred to iron-sufficient medium (100 µ*M*) or iron-deficient medium (1 µ*M*). Bars represent mean (SE) values (n 16) of iron reductase activity (**a**), biomass (**b**), acidification capacity (**c**), and chlorophyll content (**d**). Iron reductase activity was quantified using the ferrozine method. pH was directly measured in the solution of the root with a potentiometer. Chlorophyll content was measured using a spectrophotometric method, as described in Materials and methods. Lower-case letters indicate significant differences ($p<0.05$; Duncan's multiple range test)

deficiency. After 7 days, plants were treated according to the AY-5 wet digestion method and the samples were analyzed by atomic absorption spectroscopy (Fig. 5).

Plants grown in iron-deficient medium and induced with *A. agilis* showed increased Fe levels in roots, compared to uninduced controls. Also, plants induced with dimethylhexadecylamine in iron-sufficient medium exhibited a 2.3-fold increase (per root basis; Fig. 6a) or a 1.5-fold increase (concentration basis; Fig. 6c) in Fe content, whereas plant roots induced by *A. agilis* VOCs showed a 1.6-fold increase (per root basis) or 1.3-fold increase (concentration basis) in Fe content compared to their respective controls.

The shoots of control and *A. agilis* UMCV2-treated plants grown in iron-deficient medium exhibited significantly different Fe content. In iron sufficiency conditions, the Fe content of dimethylhexadecylamine-treated shoots was between 3.7- and 2.5-fold that of controls (Fig. 6b and c). Similarly, *A. agilis* UMCV2 elicited a 3-fold increase in iron accumulation (Fig. 6b).

These data confirm that *A. agilis* VOCs and dimethylhexadecylamine induce iron acquisition responses, such as acidification of the rhizospheric area, reductase activity, and increased Fe content. These findings are consistent with the observed increase in chlorophyll content and biomass in *M. truncatula*.

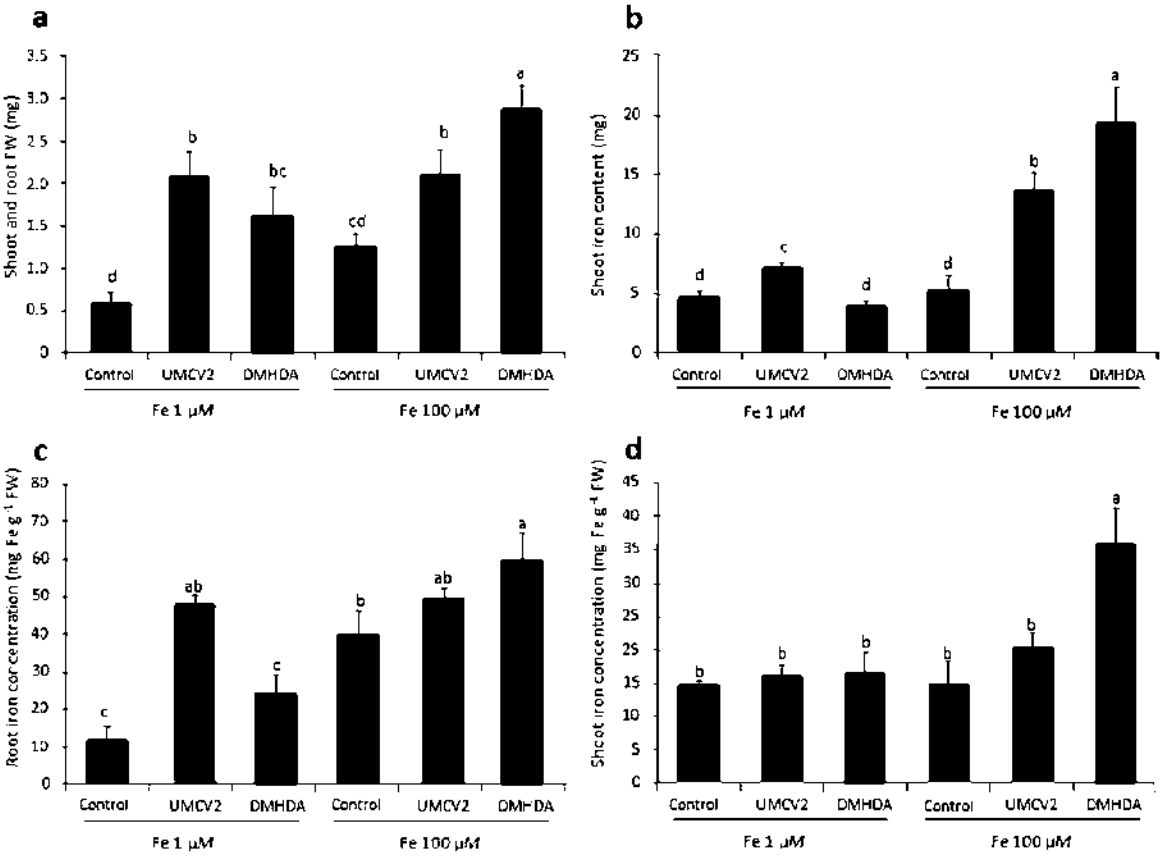

Fig. 6 Effect of *A. agilis* VOCs and DMHDA on total Fe content in *M. truncatula* plants. Bars represent mean (SE) values (n 16) of Fe content in roots (**a**) and shoots (**b**), and Fe concentration in roots (**c**) and shoots (**d**) of 7-day-old plants after transfer to medium with iron sufficiency (100 μ*M*) or deficiency (1 μ*M*). Iron was quantified using an atomic absorption spectrometer, as described in Materials and methods. Lower-case letters indicate significant differences ($p<0.05$; Duncan's multiple range test)

Discussion

Growth promotion of *M. sativa* due to emission of *A. agilis* UMCV2 VOCs has been previously established (Velázquez-Becerra et al. 2011). Therefore, the promotion of *M. truncatula* growth was an expected result (Fig. 1). In this sense, it is noteworthy that *M. truncatula* plants grown in the presence of *A. agilis* UMCV2 had a chlorophyll concentration 35 % higher than that of plants grown in axenic cultures, because chlorophyll concentration is considered as an indicator of the iron nutritional status of plants. This result led us to test the hypothesis that the VOCs of *A. agilis* UMCV2 influence some of the Strategy I components of iron acquisition (i.e., rhizosphere acidification and induction of ferric chelate reductase) via the emission of VOCs that act as signal molecules.

In a pioneering work, Mathesius et al. (2003) showed that *M. truncatula* perceives its symbiont *Sinorhizobium meliloti* through bacterial N-acyl homoserine lactones and responds via changes in the accumulation of more than 150 proteins in the presence of each bacterial species. In this work, we presented evidence suggesting that *M. truncatula* can perceive the rhizobacterium *A. agilis* UMCV2 through VOCs and respond by modifying its own VOC profile. We detected 14 VOCs produced by *A. agilis* UMCV2 axenic cultures, and 11 VOCs produced by axenic *M. truncatula* plants; however, a group of 11 compounds was produced only when the bacterium and the plant were allowed to interact with each other through their respective VOC emissions. The latter indicates that at least one of the 2 organisms, if not both, can sense the other via VOC emission.

In parallel, we found that the axenic cultures of *A. agilis* UMCV2 produced the VOC dimethylhexadecylamine, as previously reported (Velázquez-Becerra et al. 2011). Interestingly, we also detected a 12-fold increase in dimethylhexadecylamine in the interacting atmosphere. This also suggests that the bacterium perceives the plant through volatile compounds and responds by increasing the emission of a compound that acts as a signal molecule between the 2 organisms.

The mechanism by which VOCs from *A. agilis* stimulate plant growth is unknown. In this study, we demonstrated the involvement of VOCs produced by the PGPR *A. agilis* UMCV2 and the specific compound dimethylhexadecylamine in the activation of iron deficiency stress response mechanisms. First, we found that acidification of the *M. truncatula* rhizospheric area was increased in plants induced with bacterial VOCs or dimethylhexadecylamine, compared to control uninduced plants (Fig. 3). However, neither *A. agilis* UMCV2 nor dimethylhexadecylamine alone acidified the culture medium, which indicates that rather than directly acidifying the medium, the VOCs of *A. agilis* and dimethylhexadecylamine act as a signal molecule from the bacterium to the plant, which responds by acidifying the rhizosphere in both iron-deficient and -sufficient conditions, with a clearly higher acidification in iron-deficient conditions.

The ability to acidify the rhizosphere has been linked primarily to the activation of one or more members of the ATPase AHA family, which have already been extensively described (Colangelo and Guerinot 2004; Santi et al. 2005). Plants induced with VOCs from *A. agilis* UMCV2 showed an increased capacity for medium acidification, generating a pH close to 4 at 48 h after induction and a pH of 5.2 after 7 days of induction. This suggests that in our experiments, *M. truncatula* may activate the ATPase AHA enzymes during rhizosphere acidification.

A similar acidification effect was reported by Zhang et al. (2009) using a separate compartment system, but employing the plant *Arabidopsis thaliana* and the commercial bacterium *Bacillus subtilis* GB03. The authors showed that the increase in chlorophyll concentration was due to an increase in the expression of the *IRT1* and *FRO2* genes (required for iron reduction and uptake by plants), which was related to the acidification of the medium by bacterial VOCs, including glyoxylic acid, methyl butanoic acid, and diethyl acetic acid. In addition, the authors showed that GB03 can acidify the medium through VOCs from a separate compartment. In our study employing a similar system of separate compartments, this effect was not observed with *A. agilis* UMCV2 or dimethylhexadecylamine in the other compartment (Online Resource 1). Therefore, *A. agilis* may induce the acidification of *M. truncatula* rhizosphere via emission of different VOCs. Additionally, benzeneacetic acid ethyl ester, the only organic acid found in the VOC profile of axenic *A. agilis* UMCV2 cultures, was not found during interaction with *M. truncatula*. However, it cannot be ruled out that the increase in the chlorophyll concentration of *M. truncatula* plants was probably due to variations in the expression of iron acquisition genes induced by VOCs.

Rhizosphere acidification promotes iron solubility (Römheld 1987); however, an acidic environment is not sufficient for iron uptake. Iron is a transition metal and its reduction is necessary before its transport into root hairs through the plasma membrane. Therefore, given the idea that the VOCs of *A. agilis* and particularly dimethylhexadecylamine promote the nutritional status of *M. truncatula*, we measured the activity of the ferric chelate reductase enzyme after inducing *M. truncatula* plants with bacterial VOCs and pure dimethylhexadecylamine.

Interestingly, induction with *A. agilis* VOCs and dimethylhexadecylamine increased the activity of ferric chelate reductase immediately after induction, in both iron-deficient and -sufficient conditions (Fig. 4). This was particularly evident at 24 h after transferring plants to iron sufficiency; the enzyme activity was between 2.25- and 1.8-fold higher than that in the uninduced controls. Enzyme activity in induced plants transferred to iron-deficient medium was between 2- and 1.6-fold higher than that of controls. The inductive effect diminished over time, with the ferric reductase activity in induced plants being between 1.7- and 1.3-fold higher than that of uninduced plants at 72 h after induction. Although the induction with VOCs or dimethylhexadecylamine increased the ferric reductase activity in conditions of iron deficiency and sufficiency, it was clear that the ferric reductase activity was higher in iron-deficient conditions, as seen with the acidification.

However, during short periods of iron deficiency stress, we did not observe clear signs of chlorosis, even in the uninduced controls. Andaluz et al. (2009) showed that between 3 and 5 days of iron stress, *M. truncatula* seedlings begin to exhibit yellow patches as symptoms of iron chlorosis. They noted that the ferric reductase activity decreased dramatically after the seventh day of iron stress. Consistent with their results, control plants

exhibited clear signs of iron deficiency, including decreased reductase activity, up to 7 days post-induction (Fig. 5a). However, the plants induced with VOCs from *A. agilis* showed a 2.4-fold higher activity compared to the uninduced controls, in either condition of iron sufficiency or deficiency. A similar effect was reported for the *A. thaliana* ferric reductase activity in, which remained high after 7 days of treatment with a mixture of VOCs from *B. subtilis* GB03 (Zhang et al. 2009). Thus, we show an inductive effect of *A. agilis* VOCs on *M. truncatula* ferric reductase activity, which was sustained even after a week of growth in iron-deficient medium with a smaller effect observed in iron-sufficient conditions.

Additionally, in all cases the induced plants exhibited higher chlorophyll concentrations in the presence of bacterial VOCs compared with untreated controls (Fig. 1c), or when the plants were induced 72 h before the chlorophyll quantification (Fig. 4e–f). The effect was observed as late as 7 days post-induction in plants maintained on iron deficiency. Chlorophyll synthesis requires many iron-dependent enzymatic reactions (Hansen et al. 2003; Lin et al. 2000), including thylakoid synthesis and chloroplast development (Buchanan et al. 2000). The development and growth of *M. truncatula* was promoted by *A. agilis*-derived VOCs and dimethylhexadecylamine, whereas uninduced plants showed a photosynthetic capacity that was vulnerable to iron deficiency (Varsano et al. 2006). This situation may at least partially explain the elevated chlorophyll concentration and increased biomass generation observed in induced plants.

A general observation in our experiments was the greater effect of the *A. agilis* VOCs cocktail compared to pure dimethylhexadecylamine. This may be explained by the concentration of dimethylhexadecylamine in the VOC cocktail. The concentration of dimethylhexadecylamine in the plant-bacteria interaction system was 3-fold higher than the concentration of the pure standard used in the induction experiments (Fig. 2a vs. c). A previous logarithmic scan with different concentrations showed that a higher concentration of dimethylhexadecylamine provoked a deleterious effect on plant growth (Online resource 2). Therefore, an optimal concentration of dimethylhexadecylamine is important for its plant growth-promoting effects. An alternative explanation is that *A. agilis* UMCV2 produces VOCs other than dimethylhexadecylamine, and one or more of these VOCs may contribute to the inductive effect. Furthermore, it has been demonstrated that CO_2 produced by bacteria significantly promote plant growth in vitro (Kai and Piechulla 2009). Therefore, the possible participation of *A. agilis*-emitted CO_2 in plant growth induction cannot be ruled out.

A second general observation in our experiments was the higher inductive effect observed in plants grown in iron deficiency, compared to those grown in iron-sufficient conditions. This observation suggest that the plants grown in iron deficiency switch on strategy I mechanisms for iron uptake, with an additive effect observed in the presence of the *A. agilis* UMCV2 VOCs or pure dimethylhexadecylamine. However, iron sufficiency does not prevent the inductive effect of the bacterial VOCs or dimethylhexadecylamine.

The inductive effect of VOCs on iron-acquisition mechanisms of Strategy I plants were initially described in *Arabidopsis*. In that system, bacterial VOCs modulate genes encoding both the ATPase *AHA7* responsible for acidification of the rhizosphere, and ferric chelate reductase (*FRO*), which is activated by the transcriptional regulator *FIT1* (Colangelo and Guerinot 2004; Zhang et al. 2009). Thus, it is possible that the volatile compound dimethylhexadecylamine and is a potential homolog of *FIT1* in *M. truncatula*, and acts as a signal initiator to induce the expression and activity of *FRO* and *AHA* genes (Robinson et al. 1999). Experiments are currently underway to show that dimethylhexadecylamine induces the expression of *FRO* genes in the roots of *M. truncatula*.

Previously, our group showed that *A. agilis* UMCV2 increases iron levels in *Phaseolus vulgaris* plants (Valencia-Cantero et al. 2007). *Phaseolus* plants grown in alkaline soil and inoculated with *A. agilis* UMCV2 exhibited a 1.76-fold increase in Fe content in whole plants compared to control plants. Consistent with these data, *A. agilis* increased the Fe content in *M. truncatula* shoots. Interestingly, the Fe content in the roots of *M. truncatula* grown in iron-deficient medium was higher than that of control plants. These results together with the observed increase in chlorophyll content suggest that *A. agilis* UMCV2 and dimethylhexadecylamine facilitate the uptake and storage of iron, mainly in roots. The iron is then redistributed to the aerial parts where it can be used in processes such as photosynthesis. To our knowledge, this is the first study to elucidate the modulation of rhizosphere acidification capacity and ferric reductase activity by a bacterial volatile compound.

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11. RESULTADOS

CAPÍTULO II

Medicago truncatula increases its iron uptake mechanisms in response to volatile organic compounds produced by *Sinorhizobium meliloti*

Medicago truncatula increases its iron uptake mechanisms in response to volatile organic compounds produced by *Sinorhizobium meliloti*

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ABSTRACT

Medicago truncatula represents a model plant species for understanding legume-bacteria interactions. *M. truncatula* roots form a specific root-nodule symbiosis with the nitrogen-fixing bacterium *Sinorhizobium meliloti*. Symbiotic nitrogen fixation generates high iron (Fe) demands for bacterial nitrogenase holozyme and plant leghemoglobin proteins. Leguminous plants acquire Fe via "Strategy I," which includes mechanisms such as rhizosphere acidification and enhanced ferric reductase activity. In the present work, we analyzed the effect of *S. meliloti* volatile organic compounds (VOCs) on the Fe uptake mechanisms of *M. truncatula* seedlings under Fe-deficient and Fe-rich conditions. Axenic cultures showed that both plant and bacterium modified VOC synthesis in the presence of the respective symbiotic partner. Importantly, in both Fe-rich and -deficient experiments, bacterial VOCs increased the generation of plant biomass, rhizosphere acidification, ferric reductase activity, and chlorophyll content in plants. On the basis of our results, we propose that *M. truncatula* perceives its symbiont through VOC emissions and, in response, increases Fe uptake mechanisms to facilitate symbiosis.

Key words: legume-rhizobia symbiosis, iron-uptake, volatile organic compounds

INTRODUCTION

Medicago truncatula represents an ideal legume model plant for understanding plant-bacteria interactions (Benedito et al. 2008). In the rizhosphere, the roots of *M. truncatula* exude flavonoid compounds, which induce *nod* genes in the rhizobacterium *Sinorhizobium meliloti* to synthesize Nod-factors. Nod-Factors (lipo-oligosaccharides) communicate the presence of the symbiont to the plant, causing the root nodulation process to be initiated (Gage 2004; Bensmihen et al. 2011). Lipo-oligosaccharides are molecules that have specific modifications at their reducing and non-reducing ends, facilitating the specific communication in the *M. truncatula-S. meliloti* symbiotic interaction (Bensmihen et al. 2011; Dénarié et al. 1996). Plant responses include the induction of cortical cell division in the roots, which results in the initiation of nodule formation by the curling of root hairs. Bacterial cells trapped in the root hair tip subsequently induce the formation of an infection thread, which progressively extends and penetrates into the underlying cell layers. The bacterial cells are then housed inside host cell compartments where they differentiate into bacteroides, which carry out the symbiotic nitrogen fixation (for review, see Jones et al. 2007).

However, different environmental factors have a major influence on bacterial infection and the development of root nodules, including the type of soil where plants grow, high pH levels, and low Fe concentrations (Tang et al. 1990; LeVier et al. 2006; Valencia-Cantero et al. 2006). Iron availability in soil determines the ability of the symbionts to form functional nodules, because the bacterial enzymes responsible for nitrogen fixation (nitrogenases) contain large amounts of iron, and are irreversibly inactivated by the presence of oxygen. To avoid the inactivation of nitrogenase, legumes contain leghemoglobin proteins that have an iron core, which regulates oxygen tension in the nodule (Ott et al. 2005; Hoy and Hargrove 2008). In addition, iron is required in various enzymes that are involved in the synthesis of chlorophyll, with high quantities also being present in chloroplasts. Thus, iron deficiency is directly related to low concentrations of chlorophyll in plants, causing the yellowing of young leaves, a condition termed iron or ferric chlorosis (Terry and Abadía 1986; Masalha et al. 2000). Although iron is the fourth most abundant element in the earth's crust, its bioavailability to plants is very low because it has low solubility in basic or alkaline soils (Lindsa and Schwabl. 1982). To address this deficiency, plants have

developed two known strategies to acquire iron. Plants using Strategy I (including all higher plants, except grasses) elevate the activity of a root ferric reductase protein to reduce Fe (III) to Fe (II), while an ATPase acidifies the root environment to improve Fe solubilization and the functioning of the ferric reductase protein. Plants using Strategy II (including grasses) secrete compounds known as phytosiderophores (PS) from the roots, which chelate $Fe3^{+}$ in the rhizosphere (Abadía et al. 2004; Jeong and Connolly 2009).

Recent studies have shown that volatile organic compounds (VOCs) produced by plant growth-promoting rhizobacteria (PGPR) are able to increase the activity of iron-uptake mechanisms in *Arabidopsis thaliana* plants (Zhang et al. 2009). In parallel, our research group has shown that VOCs produced by the PGPR *Arthrobacter agilis* UMCV2 increase the growth of *Medicago sativa* and *M. truncatula*, in addition to improving iron nutritional status (Velázquez-Becerra et al. 2011; Orozco-Mosqueda et al. 2012).

Beneficial bacteria, such as *S. meliloti*, communicate with *M. truncatula* through well-studied Nod-factors, initiating a dialogue to form nitrogen-fixing nodules. However, the bacterium and plant may also communicate in other ways, such as through volatile compounds. This form of communication might have an additional beneficial effect on the plant. Therefore, in the present study, we tested the hypothesis that *S. meliloti*, the natural rhizobacterial symbiont of *M. truncatula*, is able to enhance the iron-uptake mechanisms of *M. truncatula* through the synthesis and emission of VOC compounds, which act as signal molecules.

MATERIALS AND METHODS

For biological material, we used the wild-type strain *S. meliloti* 1021 and seeds of *M. truncatula* cultivar Jemalong A17. *S. meliloti* was grown in peptone-yeast extract medium (PY medium) at 28°C for 48 h before use. *M. truncatula* seeds were subjected to chemical scarification (Boisson-Dernier et al. 2005), and immersed in a vial containing 1 to 2 mL of concentrated anhydrous sulfuric acid, with intermittent agitation until small black spots appeared on the integument (after approx. 5–15 min). Excess acid was removed, and the seeds were rinsed with 5 washes of sterile deionized water. For sterilization, seeds were soaked in a solution of sodium hypochlorite (12%) for 3 min, and rinsed with 6 washes of sterile deionized water. The

seeds were germinated in petri dishes containing Murashige and Skoog (MS) medium plus 6 g of agar (Phytotechnology,

Shawnee Mission KS, US) and 6 g sucrose per liter (L), and transferred to a Percival growth chamber, with a 16-h light/8-h dark photoperiod at a light intensity of 200 mol m^{-2} s^{-1} at 22°C.

Analysis of volatile compounds

Several treatments were carried out for chromatographic analysis. For bacterial VOCs, the *S. meliloti* strain was grown in petri dishes containing PY for 4 days (I). For plant VOCs, *M. truncatula* plants were placed into compartments of divided petri dishes containing either iron-rich MS medium (100 µM) (II) or iron-deficient MS medium (1 µM) (III). For a mixture of VOCs with the plant-bacteria interaction, *M. truncatula* plants were placed in petri dish compartments containing iron-rich MS medium (100 µM) (IV) or iron-deficient medium (1 µM) (V), while *S. meliloti* was streaked out on PY medium in the other compartments. VOCs from controls (VI). VOCs from axenic media were also analyzed to be discarded in the final table of results.

The analysis was performed using the solid-phase micro-extraction technique (SPME), exposing the PDMS/DVB fiber (Supelco, Inc., Bellafonte, PA, USA.) to the sample headspace for 30 min at 30°C, and desorbing it at 180°C for 30 s in the injection port of the gas chromatographer coupled to a mass spectrometer (Agilent 6850 Series II; Agilent, Foster City, CA, U.S.A.). A 25 mm × 0.52-mm capillary column of 0.32-µm film thickness (HP-FFAP; Agilent) was used; pure helium (1 mL·min^{-1}) was used as the carrier gas. The column was maintained at 40°C for 13 min, and then programmed for a temperature increase of 3°C per min to a final temperature of 180°C, which was maintained for 25 min. The treatments were sampled 2 days later.

The SPME technique does not allow the quantification of VOCs, and thus should be considered semi-quantitative. VOC abundance was accordingly expressed on a normalized percentage basis. For the normalization, we used the following formula: Normalized volatile compound amount = (peak area of volatile compound)/(total peak area of all volatiles).

Analysis of rhizosphere acidification

Recently germinated seeds were grown in 185-mL culture flasks containing 15 mL of iron-rich MS medium (100 µM) in a growth chamber for 5 days. The flasks also contained a 5 mL glass vial with 2 mL of PY, which was kept uncovered throughout the experiment. On the 5^{th} day of plant growth, a cell suspension of *S. meliloti* [1×10^6 colony forming units (CFU)] was dripped into the glass vial. For the control, 5 µL of water was dripped into the glass vial. The suspension was dripped to prevent any physical contact between *M. truncatula* and bacteria, while allowing the organisms to interact through the gas space.

The volatile interaction system was maintained in a growth chamber for 2 days, after which, a set of plants was transferred to petri dishes containing iron-rich (100 µM) MS , while the other set was transferred to petri dishes containing iron-deficient medium (1 µM). Both sets of dishes contained the dye bromocresol purple (0.006%) (modified from Zhang et al. 2009). Photographs were taken at 24 and 48 h after transferring the plants to these conditions. The color of bromocresol purple was compared using a colorimetric scale prepared with culture media plates containing bromocresol purple at a pH range of 4 to 7.

Analysis of ferric reductase activity

Ferric chelate reductase activity was analyzed for the formation of Fe^{2+}-ferrozine complex using spectrophotometric quantification (Yi and Guerinot 1996). Seedlings grown for 5 days in MS medium were exposed to the volatile compounds of *S. meliloti* for 48 h, as described in the previous section. After induction, a set of plants was transferred to iron-rich medium and iron-deficient medium for 48 h. The root systems were immersed in a solution containing 0.5 mM Fe(III)-EDTA and 4.4 mM ferrozine at pH 6.5. The absorbance was read at 562 nm after a 24-h incubation at room temperature in darkness. The Fe(II)-ferrozine concentration was calculated from a previously formulated standard curve equation. An identical solution, but without the root system, was used as the control. The pH of the solution was measured using a potentiometer.

Chlorophyll content

Chlorophyll content was quantified using a CCM-200 chlorophyll meter (Opti-Sciences, Inc., Hudson, NH, USA), which measured chlorophyll concentrations (in arbitrary units) based on the rates of transmitted radiation (940 and 660 nm) through a leaf.

Statistical analyses

All experiments were independent and repeated in triplicate. Fresh weight biomass was measured using an analytical balance, after carefully removing the plants from the culture medium. Plant height was determined on a scale in mm. The results for acidification of the rhizosphere, ferric reductase activity, pH, fresh weight, and plant length were analyzed using a factorial design, comprising 2 factors and 2 levels (iron-rich and iron-deficient, and the presence and absence of *S. meliloti*), followed by Duncan's multiple range test ($p \leq 0.05$).

RESULTS

M. truncatula and *S. meliloti* interaction through VOC emissions

To determine whether *M. truncatula* and *S. meliloti* are able to interact through their respective VOCs, both organisms were grown at opposite sides of divided petri dishes. We found that a mixture of VOCs composed of 2-butanone, 2-nonanone, and ethanol were present in all treatments (axenic *S. meliloti* and *M. truncatula*, and interaction of the two) (Fig. 1). A second mixture of 14 VOCs was attributed to *S. meliloti*, because they were present in the interacting system, but not in the axenic cultures of *M. truncatula*. Among these compounds, a number of methylated esters were present, such as 2- and 3-methyl butanoate, methyl heptanoate, methyl decanoate, and 3-methyl-1-butanol (Fig. 1). These compounds have previously been reported as VOCs in several rhizobacterial species (Schulz and Dickschat 2007; Wilkins and Schöller 2009). A third mixture of 14 compounds was attributed to *M. truncatula*, which were also present in axenic cultures of the plant and in the interaction with *S. meliloti*; however, these compounds were not present in the axenic cultures of the bacterium. These volatile mixtures were characterized by an abundance of tridecane and tetradecane (Fig. 1), both of which have been reported as VOCs in many other plants (Knudsen et al.

2006). A fourth mixture of 24 compounds was attributed to the plant-bacteria interaction, because they were only detected in these treatments. These compounds included 1,4-cineole, 1-methylnaphthalene and *N*, *N*-dimethylhexadecylamine (hexadecylamine). (Fig. 1). The presence of these compounds indicates that either *M. truncatula* or *S. meliloti*, or possibly both species, are able to detect and respond to one another through their respective VOCs.

Effect of *S. meliloti* VOCs on *M. truncatula* development

Having determined that there is an interaction between *M. truncatula* and *S. meliloti* via VOC emissions, the plants were exposed to bacterial VOCs (induced plants) and compared against non-induced controls. Induced plants had an increased ability to acidify the rhizospheric environment. For instance, non-induced plants that were grown on iron-rich medium exhibited minor color changes in the rhizosphere zone after 48 h, with pH values changing to 5.0 and 4.5. In comparison, induced plants that were grown on iron-rich medium produced larger zones of acidification after 48 h (Fig. 2).

In parallel, control plants that were transferred to an iron-deficient medium produced large areas of acidification around the roots, with pH values of 5.0 and 5.5, after 48 h. In comparison, plants induced with bacterial VOCs that were also transferred to an iron-deficient medium produced areas of acidification that were up to 3 times higher compared to the controls, producing pH values of 4.0 and 4.5 (Fig. 2). These results clearly show that bacterial VOCs increase acidification activity in the *M. truncatula* root system, particularly in iron poor environments, and that this effect is conserved (but attenuated) in a medium where iron is abundant.

The induction of *M. truncatula* plants with bacterial VOCs also had a clear effect on iron reduction activity. Factor analysis indicated that induction with bacterial VOCs had a highly significant effect (Table 1), because induced plants that were transferred to either iron-rich or iron-poor media exhibited higher iron reduction activity compared to the non-induced controls. The iron status also had a significant effect on the medium (Table 1), because plants in iron-deficient medium showed a 20% increase in ferric reductase activity compared to those in iron-rich medium (Fig. 3b and e).

Chlorophyll production in plants also increased as a result of *S. meliloti* VOC emission (Fig. 3c). Factor analysis showed a significant increase in the biomass production of plants exposed to bacterial VOCs compared to the non-induced controls (Table 1); however, in the short time before the plants were harvested, the concentration of iron in the medium did not produce a significant effect on chlorophyll concentrations.

A similar result was found for biomass production (Fig. 3a and f), with factor analysis showing that plants induced with *S. meliloti* VOCs exhibited significant growth (Table 1) compared to non-induced plants, an effect that was not observed for iron abundance. Finally, there was no significant difference in *M. truncatula* root size among treatments (Fig. 1d).

DISCUSSION

PGPR stimulate plant growth and health via several mechanisms, including the fixation of atmospheric nitrogen, nutrient solubilization, and the production of plant growth-regulating compounds (such as auxins, cytokinins, or even cyclodipeptides) (Ortiz-Castro et al. 2008, 2011; Spaepen et al. 2009; Santoyo et al. 2012). Recently, VOCs produced by PGPRs have been shown to modify plant development, particularly the root system (Gutierrez-Luna et al. 2010). Previous work has shown that the bacterium *A. agilis* UMCV2 produces VOCs that promote *M. sativa* growth, in addition to significantly modifying the root structure (Velazquez-Becerra et al. 2011). In a recent study, we also showed that the strain UMCV2 may improve the iron nutritional status of *M. truncatula* plants via the emission of volatile compounds (Orozco-Mosqueda et al. 2012).

In the current study, we accordingly tested the ability of *S. meliloti* to promote *M. truncatula* plant growth via the emission of VOCs. *S. meliloti* is a PGPR known for its ability to fix atmospheric nitrogen in symbiosis with *M. truncatula* roots. Here, we measured the effect of VOCs on 2 of the components used for iron-uptake in this plant, in addition to analyzing the nutritional requirement for this metal. Initially, we tested the ability of the plant and the symbiont to interact separately via VOCs (i.e., no physical contact) by using a system of divided petri dishes (Orozco-Mosqueda et al. 2012). Our data showed that the profile of *S. meliloti*

VOCs consisted of a mixture of 14 compounds found in axenic cultures, 7 of which were also present in the plant-bacterium interaction system. Analogously, the plants produced a mixture of 14 VOCs that were present in axenic cultures (i.e., without the bacterium), with all 14 VOCs also being present in the interacting system. However, as these latter compounds did not all occur in bacterial cultures, a plant origin was indicated. Furthermore, a mixture of 24 VOCs was also detected only in the plant-bacterial interaction systems. This shows that the plant, bacterium, or both organisms, may detect its symbiotic partner, and modify its metabolism to produce new volatile compounds. Given that the only form of contact between the plant and bacterium was through their VOCs, we concluded that at least one of the 2 organisms, but more likely both, is able to detect and respond to the other organism via a biochemical reaction.

The presence of the hexadecylamine in the VOC mixture detected from the *M. truncatula-S. meliloti* interaction is very interesting, since this compound has previously been reported as a volatile that is produced by the PGPR *A. agilis*. This volatile compound is able to induce rhizosphere acidification and iron reduction activity in *M. truncatula* (Orozco-Mosqueda et al. 2012). Once we confirmed that the plant and the bacterium could interact via the VOC mixtures, we subsequently investigated the effect of *S. meliloti* VOCs on the two components of the *M. truncatula* iron-uptake system. Thus, the rhizosphere acidification capacity of plants induced with bacterial VOCs, under both iron-rich and iron-deficient conditions, was investigated in comparison with non-induced controls. Induced plants consistently exhibited a better ability to acidify their rhizospheric environment compared to non-induced plants. Zhang et al. (2009) showed that a mixture of VOCs produced by *Bacillus subtilis* GB03 enhances the extrusion of protons (and iron reduction activity) in the roots of *A. thaliana* in iron-deficient media; however, we found that the VOC mixture of *S. meliloti* induced acidification of the *M. truncatula* rhizosphere, even when iron was abundant. The acidification capacity in plants (induced and non-induced) was always higher in iron-deficient media, supporting reports that iron deficiency induces acidification of the rhizosphere in Strategy I plants, such as *M. truncatula* (Andaluz et al. 2009).

Consistent with this observation, non-induced plants placed in iron-rich medium produced very limited acidification of the rhizosphere; however, induced plants in an iron-rich media had a significantly larger

rhizosphere acidification zone (Fig. 2). This outcome indicates that, while the abundance of iron is a factor in the acidification of the medium by the plant roots, induction with bacterial VOCs may serve as a second factor with an additive effect that increases acidification in both iron-rich and iron-deficient conditions. The fact that plants induced with *S. meliloti* VOCs placed in iron-rich and iron-deficient media exhibited a similar capacity for rhizosphere acidification clearly shows that iron did not negatively impact the effect of VOCs. In a previous study, we reported that dimethylhexadecilamine produced by *A. agilis* UMCV2 had a similar effect on *M. truncatula* plants (Orozco-Mosqueda et al. 2012).

The mixture of VOCs produced by *S. meliloti* also promoted a significant increase in *M. truncatula* chlorophyll concentrations, which is an indicator of nutritional iron status in plants (Terry and Abadia 1986). In addition, the VOCs also induced an increase in plant biomass. It has been reported that when the nutritional status of a plant is improved by iron, better photosynthetic capacity and higher growth rates are obtained (Pestana et al. 2011); however, in the current study, the iron concentration in the medium did not have a significant effect on chlorophyll concentrations or on plant biomass (Table 1). This outcome may be due to the short incubation time of the experiment, which did not allow sufficient time for the deficiency of iron in the medium to significantly impact the production of chlorophyll and plant biomass. An alternative explanation is that VOCs from *S. meliloti* trigger additional growth-promoting mechanisms for iron uptake. Hence, the volatiles acetoin (Ryu et al. 2003) and dimethylhexadecilamine (Velazquez-Becerra et al. 2011) produced by rhizobacteria may serve as signal molecules that induce plant growth; although, the mechanisms have yet to be fully clarified. However, it is known that CO_2 produced by bacteria may promote plant growth *in vitro* (Kai et al. 2007).

Overall, the data assimilated in this study indicate that *S. meliloti* VOCs contain one or more signal molecules, which are perceived by *M. truncatula*, consequently altering its metabolism to produce a different VOC mixture. This modification induces mechanisms for iron uptake, mainly by the acidification of the rhizosphere and increasing iron-reduction activity, which improves the iron nutritional status of the plant. This improved status may indicate that the plant is preparing to support the establishment of the nodule symbiosis with *S. meliloti*, which is a process with iron requirements. Of course, this hypothesis

requires validation; however, it was recently reported (Slatni et al. 2011) that flamingo bean plants may modify their metabolism to improve iron uptake when nodules are iron deficient. Iron is essential for the synthesis of several iron-containing proteins (i.e., nodule nitrogenase or leghemoglobins); hence, it would be interesting to investigate whether *S. meliloti* increases iron absorption of *M. truncatula* through VOCs during nodule formation. In summary, our study shows that the legume *M. truncatula* is able to perceive its symbiont *S. meliloti* via the emission of a mixture of VOCs, and in response increases its mechanisms for iron uptake, indicating preparation for the establishment of symbiosis with the bacterium.

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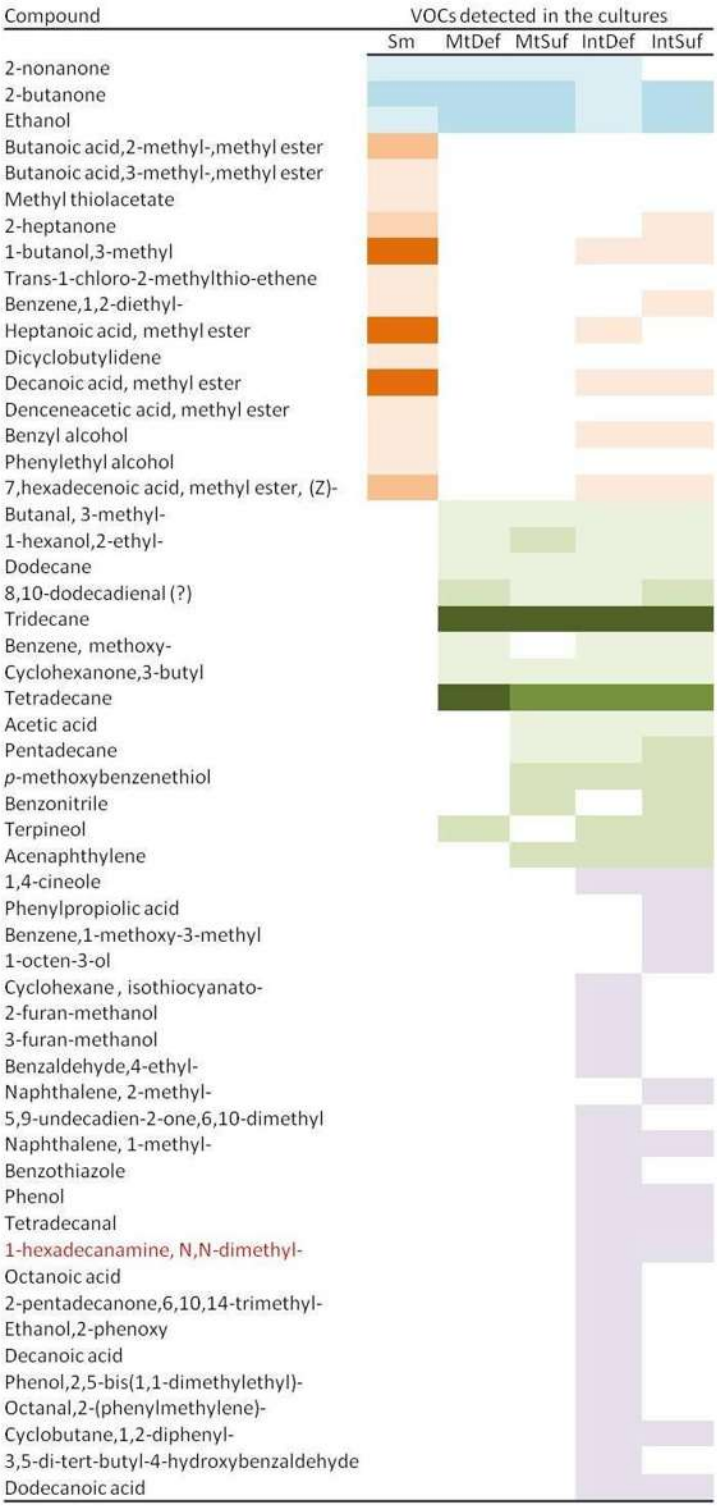
Table 1. Statistical significance of how Fe abundance factors and induction with VOCs affect different variables in *M. truncatula* plants

Factor	Statistical significance (*p*)
pH of the media	
Fe	0.001
VOCs	0.049
Fe × VOCs	0.765
Ferric reduction activity	
Fe	0.001
VOCs	0.000
Fe × VOCs	0.956
Chlorophyll concentration	
Fe	0.157
VOCs	0.001
Fe × VOCs	0.314
Fresh weight	
Fe	0.280
VOCs	0.017
Fe × VOCs	0.984
Root length	
Fe	0.298
VOCs	0.101
Fe × VOCs	0.480



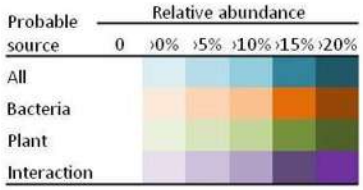


Figure 1. Volatile compounds produced by *S. meliloti* and *M. truncatula* in axenic and interacting cultures. The columns indicate *S. meliloti* (Sm); *M. truncatula* grown in Fe-deficient medium (MtDef); *M. truncatula* grown in Fe-rich medium (MtSuf); interacting cultures with *M. truncatula* grown in Fe-deficient medium (IntDef); and interacting cultures with *M. truncatula* grown in Fe-rich medium (IntSuf). The most abundant VOCs were present in 3 replicated cultures. Compounds with less than 0.5% area under the curve in at least 1 column were omitted.

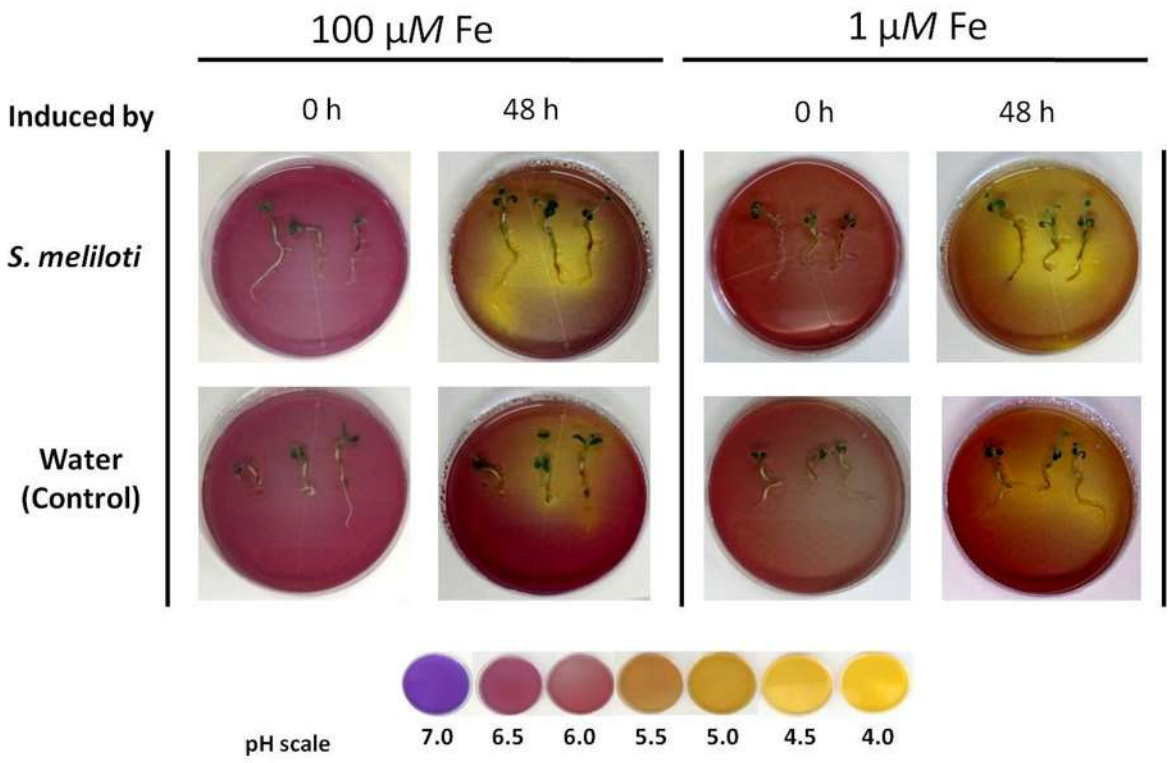


Figure 2. Acidification of the *M. truncatula* rhizosphere in response to *S. meliloti* VOCs. Five-day-old plants were induced with *S. meliloti* VOCs for 48 h, and transferred to MS medium containing bromocresol purple that was rich (100 µM) or deficient (1 µM) in iron. Photographs were taken at 0 and 48 h after transfer to these media, and are representative of 3 replicate plates.

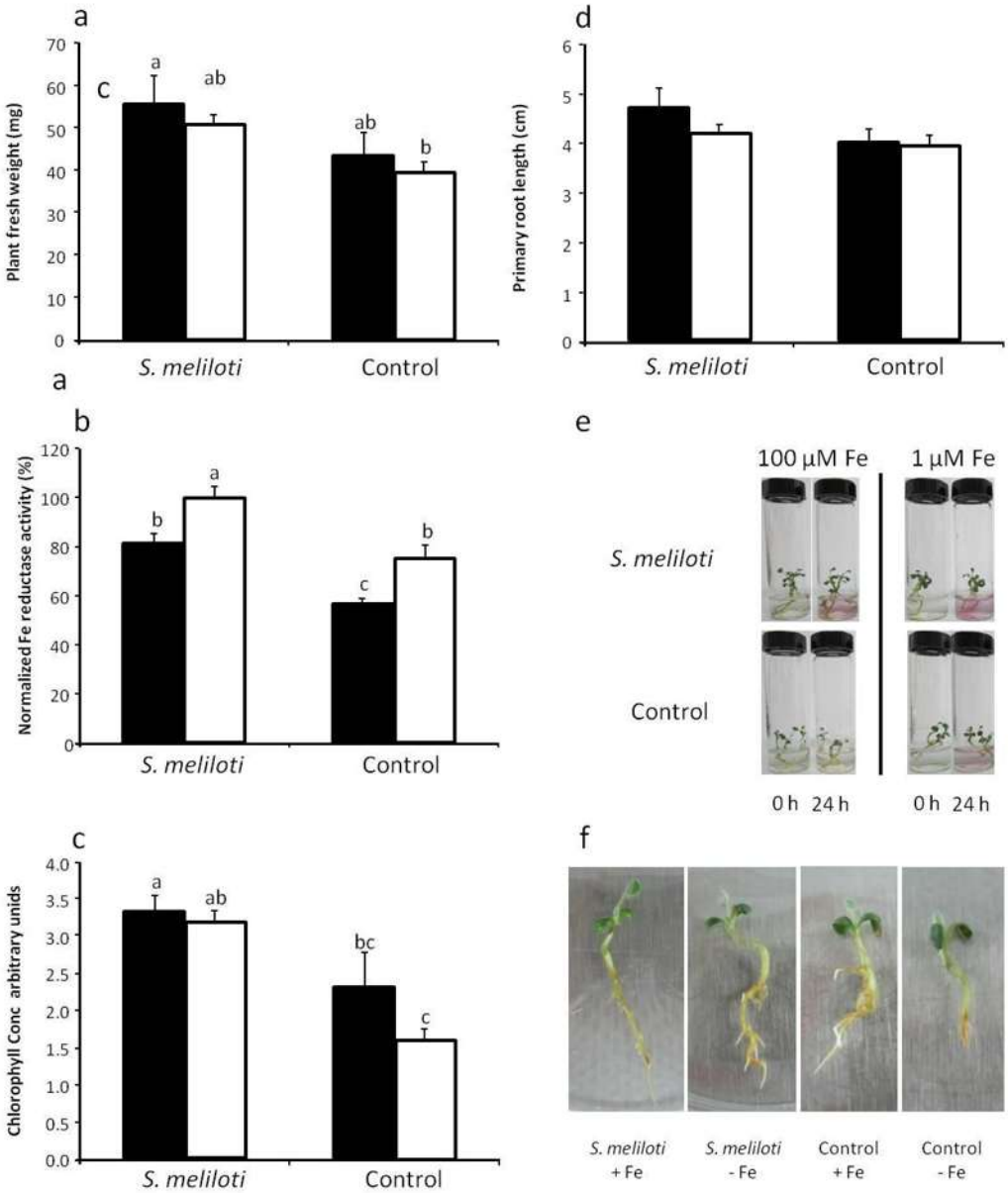
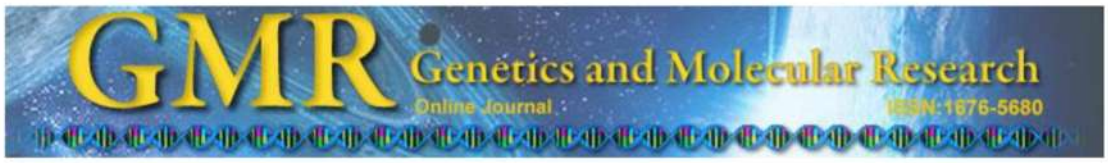


Figure 3. *S meliloti* VOCs (shown in Fig. 1) promote the growth and Fe reductase activity of *M. truncatula*. Newly germinated *M. truncatula* seedlings were placed in glass flasks containing MS medium. After 5 days, *S. meliloti* was inoculated in vials containing PY medium, and the systems were incubated for a further 5 days. The values represent mean ± standard error (n = 9). **a** plant biomass fresh weight (FW), **b** ferric chelate reductase activity normalized to iron-deficient controls, **c** chlorophyll concentration (arbitrary units) determined using a CCM-200 chlorophyll meter, **d** primary root length, **e** general view of the system employed to determine ferric reductase activity by ferrozine-plants were incubated for 24 h in the dark and then Fe(II)-ferrozine complex was measured spectrophotometrically, **f** 10-day-old non-induced plants and *S. meliloti* VOC-induced plants grown in iron-rich and iron-deficient medium. Asterisks indicate statistically significant differences ($p < 0.05$; Student t test).

12. RESULTADOS

CAPÍTULO III

Identification and expression analysis of multiple *FRO* gene copies in *Medicago truncatula*



Ribeirão Preto, May 21, 2012

Dear Authors,

We are informing you that your paper titled **"Identification and expression analysis of multiple FRO gene copies in Medicago truncatula", GMR 2029,** authored by **Ma. del C. Orozco-Mosqueda, G. Santoyo, R. Farías-Rodríguez, L. Macías-Rodríguez, and E. Valencia-Cantero,** was accepted for publication in *Genetics and Molecular Research (GMR)*.

We take the opportunity here to further inform you that GMR is indexed in 74 databases, including Index Medicus, PubMed, Medline and ISI. And it has an impact factor 1.083 according to JCR, June - 2011.

Sincerely,

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Identification and expression analysis of multiple *FRO* gene copies in *Medicago truncatula*

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Running title: "Identification and expression of multiple *MtFRO* gene copies".

ABSTRACT.

Iron (Fe) is an essential element for plant growth. Commonly, this element is found in an oxidized form in soil, which is poorly available for plants. Therefore, plants have evolved ferric-chelate reductase enzymes (FRO) to reduce iron into a more soluble ferrous form. Fe scarcity in plants induce the FRO enzymes activity. Although the legume *Medicago truncatula* has been employed as a model for FRO activity studies, only one copy of the *M. truncatula MtFRO1* gene has been characterized so far. In this work, we identified multiple gene copies of the *MtFRO* gene in the genome of *M. truncatula* by an *in silico* search, using blast analysis in the database of the *M. truncatula* Genome Sequencing Project and the National Center for Biotechnology Information, and also determined whether they are functional. We identified five genes apart from *MtFRO1*, which had been already characterized. All of the *MtFRO* genes exhibited high identity with homologous *FRO* genes from *Lycopersicon esculentum*, *Citrus junos* and *Arabidopsis thaliana*. The gene copies also presented characteristic conserved FAD and NADPH motifs, transmembrane regions and oxidoreductase signature motifs. We also detected expression in five of the putative *MtFRO* sequences by semiquantitative RT-PCR analysis, performed with mRNA from root and shoot tissues. Iron scarcity might be a condition for an elevated expression of the *MtFRO* genes observed in different *M. truncatula* tissues.

Key words: *Medicago truncatula*; *FRO* genes; Iron

INTRODUCTION

Iron (Fe) is an essential element for plant growth; however, due to the low solubility of Fe in the soil, plants require effective mechanisms to obtain it. Based on the mechanism for Fe acquisition, plants can be grouped as either strategy I or strategy II plants (Römheld and Marschner, 1986). Strategy II plants include grasses whose roots secrete compounds known as phytosiderophores (PS) that chelate Fe^{3+} from the rhizosphere. Thereafter, the Fe^{3+}-PS complex is introduced into the cell via the Yellow Stripe 1 (YS1) carrier protein in the plasmalemma (Römheld and Marschner, 1986). In contrast, strategy I plants are all higher plants (except grasses) and the acquisition of Fe occurs in 3 main reactions; (I) proton excretion via a self-phosphorilated-type adenosine triphosphate (ATP)ase, thus acidifying the surrounding soil to increase Fe solubility; (II) reduction of Fe^{3+} to Fe^{2+} by a ferric chelate reductase (FRO) (Robinson et al., 1999; Schmidt, 1999); and (III) transport of Fe^{2+} by the Iron Regulated Transporter (IRT) through the plasmalemma membrane (Römheld, 1987; Eide et al., 1996).

It is known that iron deficiency promotes the excretion of phenolic compounds, organic acids, and flavins, which also contribute to Fe reduction and solubility (Welkie et al., 1988; Susin et al., 1994). The *FRO* gene, is a key enzyme in Fe acquisition, and the first *FRO2* gene was identified in the model plant *Arabidopsis thaliana* (Robinson et al., 1999). Eight other copies of the *A. thaliana (At)FRO* gene family have also been identified and characterized; these genes exhibit tissue-specific expression (Wu et al., 2005), although there is some discrepancy in the expression patterns of different *AtFRO* copies in various tissues (Mukherjee et al., 2006). Other *FRO* genes have been reported in plants like *Pisum sativum* (Waters et al., 2002), *Solanum lycopersicum* (Holden et al., 1991), *Citrus junos* (Li et al., 2002), *Cucumis sativus* (Waters et al., 2007), *Arachis hypogaea* (Ding et al., 2009), and *Oryza sativa* (Gross et al., 2003).

Interestingly, it has recently reported the presence and expression of *FRO* genes in grasses such as barley and sorghum (Mikami et al., 2011), which raises the question of whether some other species of agricultural interest also present functional *FRO* genes, thus opening the possibility of improving their capabilities for iron acquisition. To date, no FRO activity has been observed in roots; this activity has only been detected in shoots and specific cellular organelles, thus likely contributing to Fe homeostasis and photosynthetic capacity (Mikami et al., 2011).

Medicago truncatula is a model legume that acquires Fe via strategy I. This plant has a small diploid genome, short generation time, is self-fertilizable, highly transformable, and its complete genome was recently reported (Cook, 1999; Trieu et al., 2000; Young et al., 2011). Additionally, it has the ability to establish symbiotic interactions with nitrogen-fixing bacteria of the genus *Shizorhizobium*, in addition to colonization by arbuscular mycorrhizal fungi (Harrison and Dixon, 1993). Recently it has been demonstrated that dimethylhexadecilamine an organic volatile compound produced by the rhizobacteria *Arthrobacter agilis* induce the FRO activity in *M. truncatula* plants (Orozco-Mosqueda et al., 2012). So far only 1 copy of the *M. truncatula (Mt)FRO1* gene has been characterized in this model plant (Andaluz et al., 2009). To explore the possibility that more copies of *FRO* genes are present and functional in the genome of *M. truncatula*, we carried out an *in silico* search for genes encoding FRO functions. Our results show that, apart from *MtFRO1*, there are at least 5 additional copies, which show highly conserved flavin adenine

dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate-oxidase NADPH motifs, transmembrane regions, and oxidoreductase signature motifs, a feature of characterized *FRO* genes (Andaluz et al., 2009; Mukherjee et al., 2006). Additionally, *MtFRO* gene expression was confirmed by semiquantitative real-time polymerase chain reaction RT-PCR in conditions of Fe sufficiency and deficiency, showing a differential expression pattern. The identification of the *MtFRO* gene family opens the possibility to better understand the Fe uptake mechanisms in strategy I plants.

MATERIAL and METHODS

In silico search and identification of *FRO* gene copies

To search for unidentified *FRO* gene copies, we performed a homology blast search by employing the previously identified *FRO1* gene sequence as a probe, obtained from GenBank with accession number AY439088. The blasts were carried out in the database of the *M. truncatula* Genome Sequencing Resources at the following webpages: http://www.medicagohapmap.org, the latest version of the genome of *M. truncatula* (Mt3.5) and http://blast.ncbi.nlm.nih.gov. Other *FRO* sequences from *P. sativum, S. lycopersicum, C. junos, C. sativus, A. hypogaea,* and *O. sativa* were also employed in blast searches, although better results were obtained with the *MtFRO1* sequence. We initially used Mt3.5 employing default parameters with an e-value of 0.01, which was modified to become either more or less restricted in various searches to open the possibility of detecting homologous genes with low or high identity. Five *FRO* putative sequences (open reading frames; ORFs) were obtained with different identities, which were depurated of introns with help of the GENSCAN program, available at http://genes.mit.edu/GENSCAN.html (The Board of Regents of the University of Wisconsin System, 2003). Prediction of transmembrane domains was performed using the Hidden Markov Model for TOpology Prediction (HMMTOP) 2.0 transmembrane topology prediction program at http://www.enzim.hu/hmmtop. The obtained putative gene sequences were also analyzed to search for FAD and NADPH domains, transmembrane regions, and the oxidoreductase signature motifs through the Basic Local Alignment Search Tool (BLAST); Conserved Domain Architecture (cdart; National Center for Biotechnology Information).

Growth conditions of *M. truncatula* plants

We used plants of *M. truncatula* ecotype Jemalong (A17-1). The seeds were scarified with concentrated anhydrous sulfuric acid. The excess of acid was removed by 6 washes with sterile deionized water and sterilization was carried out with a 12% sodium hypochlorite solution for 2 min, rinsed with sterile distilled water, left for 3 to 4 days at 4°C, then placed in complete Murashige-Skoog (MS) medium (with 0.6% of fitagar) in Petri dishes. The Petri dishes were transferred to a Percival growth chamber (16-h light/ 8-h dark cycle), with a light intensity of 200 mol m^2 s^{-1} at 22°C for 7 days. Subsequently, plants were transferred to Petri dishes containing MS with Fe sufficiency (100 μM) and deficiency (1 μM) and were kept in these conditions for 24 h in a growth chamber.

RNA extraction

After 24 h of growth in Fe-sufficient or deficient media, total RNA extraction was carried out with TRI reagent (Catalogue T9424, Sigma-Aldritch, St. Louis, MO. USA). The RNA was treated with RNase-free DNase I to remove residual genomic DNA (Mukherjee et al., 2006). The RNA samples were run on a 1.2% agarose gel, then stained with ethidium bromide to confirm the quality of the RNA. Finally, we carried out the cDNA synthesis with the SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies/Gibco-BRL Grand Island, NY, USA).

Semiquantitative RT-PCR conditions

The oligonucleotides used in this study were designed with the Beacon Designer 4.02 program (Biosoft International Premier, Palo Alto, CA). The cDNA had 1:2 dilution factors for the standard curves for each gene. The PCR conditions were used as follows: initial denaturation at 95°C for 3 min; 1 min at 95°C for denaturation, 1 min at 60°C for annealing, and 2 min at 72°C for extension for 30 cycles; and a final extension step at 72°C for 10 min. PCR amplifications were performed with a TC-412 Techne Thermal Cycler (Keison Products Chelmsford, Essex, CM1 3UP, England). GoTaq® Master Mixes tubes (Promega Corporation Fitchburg, Madison Wisconsin USA) were used (tubes are supplied with enzyme, magnesium, dNTPs, and buffer). Only 0.1 μg of template cDNA and 50 pM of each primer were added to each tube.

Sequencing of PCR products

The RT-PCR products were additionally purified by using the Wizard® SV Gel and PCR Clean-Up System (Promega), following the manufacturer's instructions and were sequenced at the Laboratorio Nacional para la Diversidad Genomica, Centro de Investigación y de Estudios Avanzados -Irapuato, Mexico. The sequencing results were employed to corroborate the homology of the *MtFRO* genes by using the BLAST program as previously described.

RESULTS

Identification of *MtFRO* gene copies

In this study, we conducted an *in silico* search for multiple copies of *FRO* genes in the genome of *M. truncatula*. To achieve this goal, we employed the previously identified *MtFRO1* sequence as a probe in blast searches at the Genome Sequencing *M. truncatula* Resources database. The results revealed 5 additional sequences with high identity to *FRO* genes (ranging from 67% to 76%), and contained the highly conserved motifs for FAD (HPFT), NADPH (GPYG), transmembrane regions, and the oxidoreductase signature motif (LVMVCGGSGITPFIS), which are known characteristics of *FRO* genes from diverse plants (Table 1). The number of transmembrane helices that were identified varied from 4 to 13, depending on the gene that was analyzed. The *FRO1* gene encodes a protein of 703 amino acids (aa), while *FRO2* and *FRO3* sequences showed similar lengths of 792 aa and 740 aa, respectively. Notably, we found other sequences with shorter lengths, which we

also named *FRO4* (476 aa), *FRO5* (417 aa), and *FRO6* (489 aa). *FRO* gene numbers were arbitrarily named according to the order in which they were found in our blast search. It is worth pointing out that we consider these smaller sequences as potential genes since they contain all of the motifs outlined above, as well as high identity with *FRO* genes.
The above results suggest the possibility that the genome of *M. truncatula* contains multiple copies of the *FRO* gene, thus we decided to design primers to amplify them. The primer sequences that were used are found in Table 2. Once we amplified the putative *FRO* sequences, the RT-PCR products were sequenced; of note, 5 of the 6 possible genes gave a positive result, except the gene sequence identified as *FRO2*. The sequencing results demonstrated that all of the amplified and partially sequenced genes showed 100% identity with the *M. truncatula* genome, as well as high identity with *FRO* genes from other plant species, such as *A. thaliana*, *L. esculentum*, and *Citrus junos* (Table 3).

The multiple *MtFRO* copies are functional

Once we identified the different copies of putative *MtFRO* genes and confirmed them by sequencing and Blast identity, we explored whether these gene sequences are functional. We performed semiquantitative RT-PCR to determine their expression in roots and shoots of plants grown under conditions of Fe sufficiency and deficiency. The results suggest that all of the sequences identified in this study, except *FRO2*, are functional at the level of expression. After designing 3 different sets of oligonucleotides to detect *FRO2* expression, we were unable to detect a band by semiquantitative RT-PCR (data not shown). In addition, we also observed that expression of the different *MtFRO* copies is inducible in Fe-deficient growth conditions and might be tissue-specific (Figure 1). In particular, *MtFRO1* was basally expressed in Fe-sufficient conditions, but when the plants are transferred to Fe-deficient media we noted an elevated expression level, especially in shoots. It was interesting to note that in conditions of Fe sufficiency, expression of the *MtFRO3*, *MtFRO4*, *MtFRO5*, and *MtFRO6* genes was detected in shoots but not in roots. However, under Fe deficiency conditions the expression of these genes was increased in both root and shoot parts. This suggests that the genome of *M. truncatula* contains multiple functional *FRO* gene copies, and that their expression pattern is likely tissue-specific and dependent on the concentration of Fe in the medium.

DISCUSSION

Plant genomics allows us to identify and further characterize genes that are involved in diverse cellular processes, such as Fe deficiency stress responses. Fe is one of the most abundant elements on earth, although its availability may be limited in plants; therefore, plants have developed efficient mechanisms for Fe acquisition (Römheld and Marschner, 1986). It is generally accepted that there are 2 strategies for Fe acquisition in plants. *M. truncatula* is a strategy I plant, which can acidify the rhizosphere and increase the bioavailability of Fe (Orozco-Mosqueda et al., 2012). In the present *in silico* study, we identified 5 putative sequences with high identity to *FRO* genes in the genome of *M. trucatula* (apart from *FRO1*, which was previously described; Andaluz et al., 2009). All

sequences described herein showed highly conserved motifs characteristic of FRO proteins, such as FAD, NADPH, transmembrane regions, and the oxidoreductase signature motifs (Table 1). *FRO* genes are present in various plant species and are responsible for reducing Fe^3 to Fe^2, a more soluble form that can taken up by the roots (Hell and Stephan, 2003). Apart from FRO, other proteins such as IRT1, which is a high affinity transporter for Fe^{2+}, play an essential role in Fe acquisition in plants such as *A. thaliana* (Varotto et al., 2002). The expression of *FRO2* and *IRT1* is regulated by Fe deficiency conditions in Strategy I plants (Connolly et al., 2003).

The *FRO1* gene was the first to be characterized in *A. thaliana* (Robinson et al., 1999), 7 other functional copies of the same family have been characterized (Wu et al., 2005; Mukherjee et al., 2006). In this sense the question arises: why do plants such as *A. thaliana* or *M. truncatula* contain multiple *FRO* copies in their genomes? One answer has been widely discussed in various studies, suggesting that the existence of multiple copies of the *FRO* gene are required for Fe reduction, uptake, and homeostasis in different plant tissues or organs, which are differentially regulated by the availability of Fe (or other factors) (Wu et al., 2005, Mukherjee et al., 2006). Likewise, it has been postulated that the expression of *FRO* genes in plant roots may play other roles, apart from reduction of Fe (Muherjee et al., 2006), since other elements can regulate their gene expression.

The presence of *FRO* genes is not unique to strategy I plants, as other grasses such as rice contain 2 *FRO* genes (Ishimaru et al., 2006). It has been argued that *Oriza sativa (Os)FRO2* and *OsFRO1* may not code for ferric chelate reductase functions, even when these activities have been reported only in mesophyll cells in other plant grasses such as barley and sorghum (Mikami et al., 2011). Interestingly, the expression of *FRO1* and *FRO2* genes in barley and sorghum was detected in leaves but not in roots. The above results suggest the possibility of detecting more gene copies in other grasses, and that the *FRO* genes may encode for FRO proteins with functions not only in leaves or shoots but in roots, which would break the exclusivity of Fe acquisition strategy I for non-grass plants.

In this study, we report evidence that the multiple copies of *FRO* genes in *M. truncatula* are expressed in low Fe concentrations and are present in root and/or shoots. This agrees with other studies in *A. thaliana*, which demonstrated that genes such as *AtFRO2* and *AtFRO3* are expressed under low Fe conditions mainly in roots, while *AtFRO5*, *AtFRO6*, *AtFRO8*, and *AtFRO7* are exclusively found in shoots and not regulated by this condition (Wu et al., 2005). Besides, the expression of each gene is tissue specific; for example, *AtFRO2* and *AtFRO3* are mainly expressed in roots, while *AtFRO5* and *AtFRO6* were detected in shoots and flowers, *AtFRO6* and *AtFRO7* in cotyledons and trichomes, and *AtFRO8* is specifically expressed in veins of leaves (Wu et al., 2005), although slight differential expression has been proposed by other authors (Mukherjee et al., 2006). In *M. truncatula*, we also observed a differential expression pattern for each *MtFRO* gene with respect to the Fe condition. In conditions of Fe sufficiency, *MtFRO1*, *MtFRO4*, and *MtFRO5* showed expression that can be considered basal, but *MtFRO3* and *MtFRO6* showed a strong expression level at the shoots. In Fe-deficient conditions, all of the *MtFRO* genes showed increased expression at both the roots and the shoots. It was very interesting to note the strong expression level of the *MtFRO3* and *MtFRO6* in the shoots, since experiments suggest that the ferric-chelate reduction is performed in aerial tissues. The role of the chelate Fe^{3+}-nicotianamine on the plant Fe transport via the phloem has been demonstrated (Takahashi et al., 2003), and it has been suggested that Fe could be internalized in the cells

via the Fe^{3+}-nicotianamine transporter in both strategy I and strategy II plants (Koike et al., 2003). Our results provided molecular evidence that the FRO is strongly expressed in aerial tissues and indicates that Fe is reduced before it can be internalized in the plant cells as the results of Wu et al., (2005) and Mukherjee et al., (2006) suggest. Our work also showed that different members of the *MtFRO* genes family are expressed in the same organs (roots, leaves, and stems), thus their physiological functions could be redundant. Further studies regarding the role of FRO for plant iron homeostasis utilizing loss-of-function mutants must now consider these redundant genes. We are currently conducting experiments to determine the iron-reducing functions of the multiple *MtFRO* copies in different tissues, as well as their regulation by different factors, including the presence of plant growth-promoting rhizobacteria.

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Table 1 Summarized features of the *MtFRO* family open reading frames (ORFs) and other ORFs from *A. thaliana* and *P. sativum* used as a reference.

Gene	Size (aa)	Transmem-brane regions	FAD Motif	NADPH Motif	Oxidoreductase Motif	Reference
MtFRO1	703aa	10	W**HPFT**I	E**GPYG**P	**LVMVSGGSGITPFIS**	Andaluz et al, 2009
MtFRO2	740aa	13	W**HPFS**V	E**GPYG**H	**LILVAGGIGLSPFLA**	This work
MtFRO3	792aa	13	W**HPFT**V	E**GPYG**P	**IAMVSGGSGITPFIS**	This work
MtFRO4	476aa	6	W**HPFT**V	E**GPYG**P	**IVMVSGGSGVTPFIS**	This work
MtFRO5	417aa	4	W**HPFS**V	E**GPYG**H	**LILVAGGIGLSPFLA**	This work
MtFRO6	489aa	6	W**HPFS**I	D**GPYG**A	**ILLVGLGIGATPLIS**	This work
AtFRO1	704aa	10	W**HPFT**I	E**GPYG**P	**LVMVSGGSGITPFIS**	Wu et al, 2005
AtFRO2	725aa	10	W**HPFT**I	E**GPYG**P	**LVMVCGGSGITPFIS**	Robinson et al, 1999
PsFRO1	712aa	10	W**HPFT**I	E**GPYG**P	**LVMISGGSGITPFIS**	Waters et al, 2002

The access numbers (GenBank) for the FRO putative sequences are in parenthesis. MtFRO1 (AY439088.1), MtFRO2 (XM_003594382.1), MtFRO3 (AC121237.19), MtFRO4 (AC121237.19), MtFRO5 (AC151000.3), MtFRO6 (AC154391.1), AtFRO1 (NM_100041), AtFRO2 (NM_100040.2) and PsFRO1 (AF405422.2).

Table 2. List of primers designed and employed in this study.

Primer name	Nucleotide sequence (5´-3´)	Size of the PCR expected product
FRO1UPRT	TGTGTCGGTAGTTGTTGTTG	
FRO1LORT	TCAGGTCTTGTTCCATAATGC	207 bp
FRO2UPRT	GGTGGTATAGTGGTTGG	
FRO2LORT	CATTTCTCTGACATTGATTC	165 bp
FRO3UPRT	AGGCGTTAGAGTGGAGCAAGAC	
FRO3LORT	GAGAATGTAGAGATGGTGAGTGTAGAAG	145 bp
FRO4UPRT	ATAACAAGCATTCCACAAATAAGG	
FRO4LORT	AAGGCGAGCAGATAGTAACC	210 bp
FRO5UPRT	TTATATTAGTGGCAGGTGGTATTGG	
FRO5LORT	ACGGTTGAAAGAAGTGGAAGC	157 bp
FRO6UPRT	AAGACATAGAACAAGGAGTGGTAG	
FRO6LORT	TGATGAAGTGATTGAAGCATAGTG	126 bp
MtACTINUPRT	CCAATAGGGACAACAACACTTTC	
MtACTINLORT	ACCAAACAGCGGATAGTAAGC	209 bp

Table 3. Sequence identity of the fragments of *MtFRO* genes amplified by RT-PCR compared with other identified *FRO* genes.[a]

Gene name	Closest Match	Access Number (GenBank)	Identity
MtFRO1	*Medicago truncatula* ferrico reductasa (FRO1) RNAm	AY439088.1	99%
MtFRO3	*Lycopersicon esculentum* ferrico quelato reductasa (FRO1) RNAm	AY224079.1	81%
MtFRO4	*Citrus junos* Fe(III)-chelato reductasa RNAm	DQ985810.1	82%
MtFRO5	*Arabidopsis thaliana* ATFRO6 férrico reductasa RNAm	NM_124351.3	71%
MtFRO6	*Arabidopsis thaliana* ferrico reductasa RNAm	NM_114450.2	74%
MtACTINA (2)	*Medicago truncatula* gen actina2 promotor y exon 1	AJ809891.1	100%

[a] All the sequences amplified by RT-PCR showed ≥ 99% of identity with their own sequences found *in silico* (see table 1).

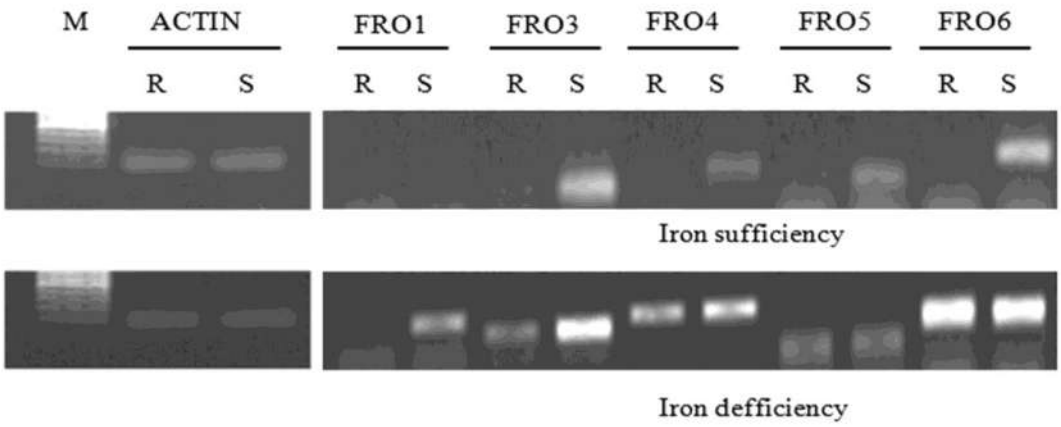


Figure 1. Semiquantitative RT-PCR detection of *MtFRO* genes in *Medicago truncatula* roots and shoots. *M. truncatula* plants were grown under iron sufficiency (100 μM) and deficiency (1 μM). Actin gene was employed as a control. R= roots and S= Shoots.

13. DISCUSIÓN GENERAL

La emisión de compuestos volátiles producidos por *A. agilis* actúan como moléculas señal e influyen en algunos componentes del sistema de toma de hierro en plantas estratega I. Previamente se mostró que los compuestos volátiles producidos por *A.agilis* promueven el crecimiento de *M. sativa*, en particular por efecto de la N-N-dimetilhexadecilamina (DMHDA) (Velázquez-Becerra *et al*, 2011). Esto sugiere que la bacteria percibe a la planta a través de sus compuestos volátiles y responde incrementando la emisión de compuestos que actúan como molécula señal entre ambos organismos. Otros trabajos han mostrado la participación de los VOCs en la señalización planta-bacteria. Por ejemplo, Mathesius *et al* (2003) mostraron que la planta *Medicago truncatula* tiene la capacidad de percibir a su simbionte *Sinorhizobium meliloti* a través del compuesto N-acil homoserina lactona y aun incluso de responder con cambios en la acumulación de más de 150 proteínas. De igual manera, Zhang *et al* (2009) mostraron una inducción en el crecimiento de plantas de *A. thaliana* a partir de los VOCs de *B. subtilis* GB03, así como el incremento en la expresión de IRT1 y FRO2, genes indispensables en la adquisición de hierro. Sin embargo, los autores suponen que el incremento en la actividad de estos genes se debe a la presencia de los VOCs como ácido glioxílico, ácido metilbutanoico y ácido dietil acético. En este trabajo mostramos que el efecto en la estimulación del crecimiento vegetal que observamos es debido a la DMHDA, sin embargo, no descartamos la posibilidad de que *A. agilis* pueda inducir respuestas como la acidificación de la rizósfera por medio de la emisión de otros VOCs. Lo anterior debido a que se observó un mayor efecto en los parámetros fisiológicos ligados al metabolismo del hierro en aquellos experimentos con los VOCs de *A. agilis* comparados con los experimentos donde solo se adicionó la DMHDA.

La acidificación de la rizósfera es la primer respuesta a la deficiencia de hierro en plantas estratega I debido a que promueve la solubilización del hierro (Römheld 1987). Nosotros pudimos observar que las plantas que estuvieron expuestas a los

VOCs de *A. agilis* así como a la DMHDA y fueron transferidas a deficiencia de hierro, mostraron un incremento en la capacidad de acidificación del área rizosférica, mostrando valores de pH cercanos a 4 después de 48 hr de haber sido transferidas al estrés por deficiencia de hierro y de 5.2 después de 7 días de estrés. Esta capacidad de acidificación del área rizosférica está relacionada a uno o más miembros de la familia de ATPasas AHA (Colangelo y Guerinot, 2004; Santi *et al*, 2005) y nuestros datos sugieren que *M. truncatula* puede activar enzimas de la familia ATPasa durante la acidificación de la rizósfera.

Una vez que habíamos observado la primera respuesta a la deficiencia de hierro (acidificación) llevamos a cabo un estudio para medir la actividad de la enzima férrico quelato reductasa. La inducción con los VOCs de *A. agilis* y la DMHDA incrementaron la actividad de la enzima tanto en suficiencia como en deficiencia de hierro. La respuesta en el incremento de la actividad enzimática fue evidente desde las 24hrs después de transferir las plantas al estrés por hierro. Interesantemente, notamos que la inducción con los VOCs o la DMHDA incrementaron la actividad de la férrico quelato reductasa en plantas transferidas a suficiencia como a deficiencia de hierro. Sin embargo, la mayor actividad de la enzima se observó en deficiencia de hierro, lo que concuerda con los datos observados en la capacidad de acidificación rizosférica. Estos resultados están de acuerdo con los reportados por Zhang *et al*. (2009) donde observan incremento en la actividad de la enzima férrico quelato reductasa en plantas de *A. thaliana* expuestas a los compuestos volátiles de *B. subtilis* GB03.

De igual manera se llevó a cabo la cuantificación de clorofila para conocer si la actividad incrementada de reducción de hierro influye sobre la capacidad fotosintética, ya que se sabe que la síntesis de clorofila requiere de enzimas dependientes de hierro (Lin *et al*, 2000; Hansen *et al*, 2003.) como las empleadas en la síntesis de tilacoides y desarrollo de cloroplastos (Buchanan et al, 2000). Interesantemente, el contenido de clorofila incrementó en las plantas inducidas con *A. agilis* y la DMHDA. De acuerdo con esto, pudimos observar en todos los casos que las plantas inducidas con los VOCs mostraron mayores

concentraciones de clorofila, mientras que las plantas control mostraron capacidad fotosintética vulnerable (Varsano *et al*, 2006) a la deficiencia de hierro. En síntesis, las plantas expuestas a *A. agilis* y a la DMHDA mostraron un incremento en la capacidad de acidificación, el crecimiento, desarrollo y contenido de clorofila respecto a las plantas control no inducidas.

Una vez que se obtuvieron estos datos, la siguiente pregunta era probar la habilidad de *S. meliloti* (el simbionte natural de *M. truncatula*) para promover el crecimiento de plantas hospederas vía emisión de compuestos volátiles. En el perfil de volátiles se detectaron 24 compuestos específicos de la interacción *M. truncatula-S. meliloti*, lo que nos sugiere que ambos organismos tienen la capacidad de detectar a su contraparte, modificar su metabolismo y producir nuevos compuestos volátiles. Interesantemente, también pudimos detectar entre estos compuestos a la DMHDA. Posteriormente, se probó si la inducción por los VOCs favorecía la acidificación del ambiente rizosférico. Las plantas inducidas con *S. meliloti* mostraron mejor capacidad de acidificación tanto en medios con suficiencia como en deficiencia de hierro comparadas con las plantas no inducidas. Estos resultados están de acuerdo con los que habíamos observado previamente con los VOCs de *A. agilis* y con los datos de Zhang (2009) con *B. subtilis* GB03. Respecto a los demás parámetros ligados al metabolismo del hierro, al igual que con las plantas inducidas con UMCV2 o DMHDA, aquellas que fueron inducidas con *S. meliloti* mostraron mayor capacidad de reducción de hierro, un incremento en el contenido de clorofila así como mayor peso seco respecto del control. El incremento en estos parámetros, puede sugerir que la planta inicia un proceso de preparación para el establecimiento de la simbiosis. De acuerdo con esta hipótesis, Slanti *et al* (2011) reportaron que nódulos de frijol crecidos en deficiencia de hierro tienen la capacidad de mejorar su metabolismo para consecuentemente mejorar la toma de hierro, ya que se sabe que las plantas requieren altas cantidades de este metal que es esencial para la síntesis de 2 enzimas fundamentales en la fijación simbiótica de nitrógeno: la nitrogenasa y la leghemoglobina. En este sentido sería muy interesante saber si el incremento en

los mecanismos de absorción de hierro inducidos por los VOCs de *S. meliloti* influyen directamente en el desarrollo del nódulo y en la fijación de nitrógeno (Bensmihen *et al*, 2011).

Todos estos datos sugieren que tanto *A. agilis* UMCV2 como su compuesto DMHDA así como el simbionte *S. meliloti* facilitan la adquisición de hierro por las raíces de las plantas, para posteriormente ser redistribuido a las partes aéreas para ser utilizado en procesos tales como la fotosíntesis. Para llevar a cabo la distribución de hierro hacia otros órganos vegetales las plantas requieren de la actividad de genes férrico reductasa, para reducir el FeIII, que es la forma en la que se transporta el hierro en plantas (Conolly y Guerinot, 2002). Por lo anterior consideramos indispensable saber si *M. truncatula* cuenta con genes FRO para llevar a cabo la reducción de hierro en otros organelos vegetales (Wu *et al*, 2005). Por lo anterior se llevó a cabo la búsqueda de genes *FRO* homólogos en el genoma de *M. truncatula*.

Se empleó el gen *MtFRO1* ya descrito (Andaluz *et al*, 2009) como gen de referencia. El análisis *in silico* permitió la identificación de 5 secuencias con elevada identidad con otros genes *FRO* descritos en las bases de datos. Interesantemente, todas las secuencias mostraron los motivos FAD, NADPH y Oxidorreductasa conservados característicos de las proteínas FRO. Previamente se reportó el gen *FRO1* de *Arabidopsis thaliana* (Robinson *et al*, 1999) y posteriormente se identificaron y caracterizaron 7 genes más de la misma familia (Wu *et al*, 2005; Mukherjee *et al*, 2006). La identificación de esta familia de genes diferencialmente regulada por deficiencia de hierro (Wu *et al*, 2005, Mukherjee *et al*., 2006) esta de acuerdo con nuestra hipótesis de que las pantas requieren múltiples copias *FRO* para reducción, toma y homeóstasis de hierro en distintos órganos vegetales.

Estudios de expresión en *A. thaliana* muestran que los genes *FRO* tienen patrones de expresión tejido específico. *AtFRO2* y *AtFRO3* son expresados en condiciones

de bajo hierro principalmente en raíz, mientras que *AtFRO5*, *AtFRO6* y *AtFRO7* son expresados exclusivamente en brotes (Wu *et al*, 2005), aunque se han reportado distintos patrones de expresión (Mukherjee *et al*, 2006). De acuerdo con estos datos, en este trabajo reportamos que miembros de la familia de genes *MtFRO* son expresados en bajas concentraciones de hierro tanto en raíz como en brotes. Específicamente, en suficiencia de hierro *MtFRO3* y *MtFRO6* muestran elevada expresión en los brotes mientras que *MtFRO1*, *MtFRO4* y *MtFRO5* muestran solo expresión basal. En condiciones de bajo hierro el patrón de expresión de todos los genes se ve incrementado tanto en raíz como en brotes. Los niveles de expresión incrementados en MtFRO3 y MtFRO6 en brotes sugieren que el hierro es reducido en partes aéreas donde puede ser requerido para procesos metabólicos como la fotosíntesis (Wu *et al*, 2005). Sin embrago, distintos miembros de la familia de genes *MtFRO* son expresados en los mismos órganos, lo que nos puede indicar que poseen funciones fisiológicas redundantes.

En conclusión, nuestros datos claramente muestran que los VOCs de *A. agilis* y *S. meliloti* tienen la capacidad de modular respuestas a la escasez de hierro en plantas de *M. truncatula* y que el menos en el caso de *A. agilis* el VOC dimetilhexadecilamina actúa como molécula señal en esta modulación y que *M. truncatula* posee una familia de genes FRO con 6 miembros inducibles por deficiencia de hierro.

14. PERSPECTIVAS

- Determinar la expresión tejido-específico de los genes *MtFRO* en distintos tejidos de la planta como raíz, tallo, hoja y flor bajo la inducción de *A. agilis* y la DMHDA.

- Determinar la presencia de genes *FRO* en nódulos de *M. truncatula*.

- Identificar la capacidad férrico reductasa en nódulos de *M. truncatula* durante la simbiosis con *S. meliloti*.

- Analizar la capacidad de los VOCs de *A. agilis* y la DMHDA en la actividad reductasa del nódulo.

- Identificar los principales componentes de adquisición de hierro en plantas estrategas II.

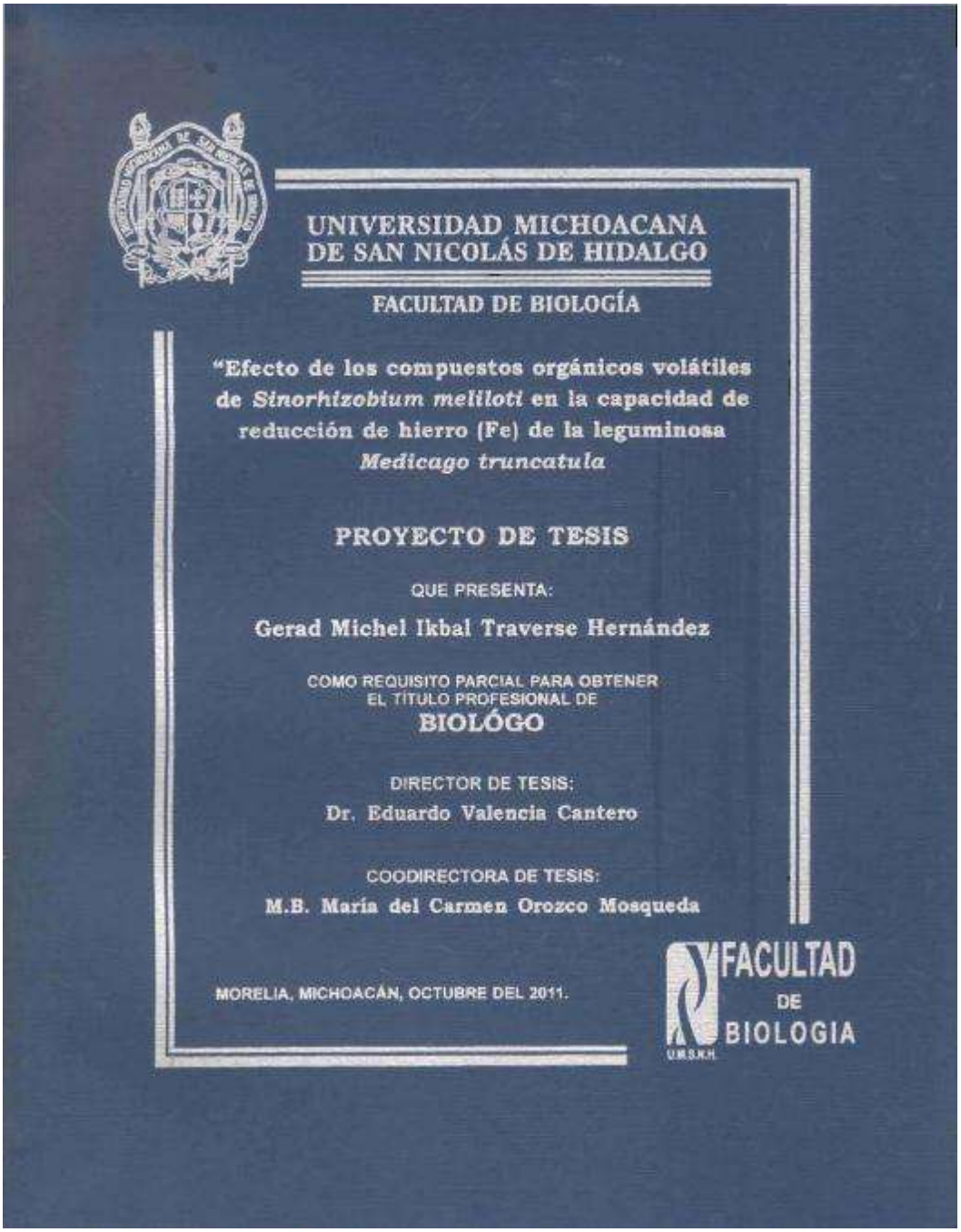
-Analizar la expresión de los componentes de adquisición de hierro en estrategas II por la inducción de los VOCs de *A. agilis* y la DMHDA.

15. OTRAS ACTIVIDADES ACADÉMICAS

Coautorías en otros artículos científicos durante el doctorado:

1. Martinez-Absalon, S.C., **Ma. del C. Orozco-Mosqueda**, M.M. Martinez-Pacheco, R. Farias-Rodriguez, M. Govindappa and G. Santoyo. 2012. Isolation and molecular characterization of a novel strain of *Bacillus* with antifungal activity from the sorghum rhizosphere. *Genetics and Molecular Research*. En prensa.
2. Velázquez-Sepúlveda, I., **Ma. del C. Orozco-Mosqueda**, C. M. Prieto-Barajas, M. Govindappa and G. Santoyo. 2012. Bacterial diversity associated with the rhizosphere of wheat plants (*Triticum aestivum*): Toward a metagenomic analysis.*Phyton, International Journal of Experimental Botany.* En prensa.
3. H.A. Marquez-Santacruz, R. Hernandez-Leon, **M.C. Orozco-Mosqueda**, I. Velazquez-Sepulvedaand G. Santoyo. 2010. Diversity of bacterial endophytes in roots of Mexican husk tomato plants (*Physalis ixocarpa*) and their detection in the rhizosphere. *Genetics and Molecular Research*. 9:2372-2380.
4. Suárez R, Wong A, Ramírez M, Barraza A, **Orozco M del C**, Cevallos MA, Lara M, Hernández G, Iturriaga G. 2008. Improvement of drought tolerance and grain yield in common bean by overexpressing trehalose-6-phosphate synthase in rhizobia. *Molecular Plant-Microbe Interaction*. 21:958-66.**(Artículo portada)**

16.- COASESORÍA DE UNA TESIS DE LICENCIATURA



UNIVERSIDAD MICHOACANA
DE SAN NICOLÁS DE HIDALGO

FACULTAD DE BIOLOGÍA

"Efecto de los compuestos orgánicos volátiles de *Sinorhizobium meliloti* en la capacidad de reducción de hierro (Fe) de la leguminosa *Medicago truncatula*

PROYECTO DE TESIS

QUE PRESENTA:

Gerad Michel Ikbal Traverse Hernández

COMO REQUISITO PARCIAL PARA OBTENER
EL TITULO PROFESIONAL DE
BIOLÓGO

DIRECTOR DE TESIS:
Dr. Eduardo Valencia Cantero

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17. BIBLIOGRAFÍA COMPLEMENTARIA

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