universidad de león

Departamento de Ingeniería y Ciencias Agrarias
Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad

Programa de Doctorado en Ingeniería de Biosistemas

Tesis doctoral

Evaluación de *Trichoderma* spp. en el control biológico de *Rhizoctonia solani* y la respuesta defensiva en plantas de alubia pertenecientes a la IGP "Alubia La Bañeza-León"

Evaluation of *Trichoderma* spp. in the biological control of *Rhizoctonia solani* and the defensive response in bean plants from PGI "Alubia La Bañeza-León"

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Memoria presentada para optar al título de

Doctor en Ingeniería de Biosistemas

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León, 2017

Esta memoria ha sido realizada gracias a la beca concedida (FPU12/00677) por el Ministerio de Educación, Cultura y Deporte (España) de acuerdo a la Resolución del 25 de abril de 2012 (BOE de 22 de mayo) y la Resolución del 20 de diciembre de 2012 y por la beca de movilidad para la realización de estancias breves con Resolución del 9 de diciembre de 2014 (BOE de 22 de diciembre). También al Ministerio de Economía y Competitividad por el proyecto nacional de "Farnesol as self-regulatory molecule in *Trichoderma*. Tyrosol and farnesol Signaling in *Trichoderma*-Bean interaction" (AGL2012-40041-C02-02). Y a la Junta de Castilla y León, Consejería de Educación por el proyecto "Effect of terpenes and physiologically related compounds produced by *Trichoderma parareesei* in the development of common bean (*Phaseolus vulgaris* L.) and in defensive responses of this plant". Además, a la Universidad de León por la beca proporcionada para la realización de una estancia breve de acuerdo al programa de "Ayuda a bolsas y estancias cortas 2014".


"Un científico en su laboratorio no es sólo un técnico: es también un niño colocado ante fenómenos naturales que le impresionan como un cuento de hadas."
Marie Curie
(1867-1934)

A mi familia, en especial a mis padres
porque sin ellos no sería lo que soy hoy

AGRADECIMIENTOS

Quisiera empezar agradeciendo a mis tutores Pedro Casquero y Santiago Gutiérrez por haberme dado la oportunidad de hacer un doctorado, por vuestra infinita paciencia, por hacerme ver la luz al final del túnel en momentos que ni siquiera sabía que estaba en uno y por enseñarme muchas más cosas de lo que está escrito en esta tesis.

A Piedad Campelo y Alicia Lorenzana, porque sin vuestro trabajo previo no habría ni siquiera podido iniciarlo y por mostrarme el apoyo y la ayuda incondicional en todo este tiempo. Al Laboratorio de Diagnóstico de Plagas y Enfermedades de la Universidad de León, en especial a Mari Fe Marcos, por tu ayuda indispensable en la determinación de aislamientos y sobre todo por haberme enseñado tantas cosas del trabajo con fitopatógenos.

A mis compañeros y amigos Óscar González y Álvaro Rodríguez, porque con vosotros empecé esta aventura y sin vosotros, esto no hubiera sido igual (no lo cambiaría por nada), porque he vivido experiencias que siempre llevaré grabadas. Desireé Rodríguez y Julia García, por ayudarme a desconectar y estar siempre dispuestas a echar un cable. También quiero agradecer a Paulo H. da Silva y Guzmán Carro, que, aunque llegasteis un poco después, me habéis ayudado mucho y habéis hecho que los días de trabajo duro no lo fueran tanto. Al igual que a Víctor Suárez y Samuel Álvarez que trajisteis aire fresco al laboratorio, a veces tan necesario.

A Mónica G. Malmierca por ensañarme todo lo que sabes a cerca de la extracción de RNA y DNA y hacer que fuera mucho más sencillo de lo que parecía.

Vorrei anche ringraziare i miei colleghi di Milano. Grazie a Francesca Sparvoli per avermi accolta presso il tuo laboratorio e avermi trattata al pari di tutti gli altri. A Eleonora Cominelli, per avermi insegnato tutto di qPCR e per la tua infinita pazienza con il mio povero italiano. Ai miei colleghi di studio, Alessia Losa, Roberto Russo e Remo Chiozzotto, e anche a Roberto Bollini e Gloria Daminati, per avermi capita nel mio "itagnolo" facendomi dimenticare che ero in Italia e facendomi sentire come a casa mia in Spagna. Ai miei colleghi di pranzo, Anna Paola Casazza, Elena Ponzzoni, Floriana Gabazzi, Luca Braglia, Aldo Grasso per avermi fatta sentire come una di voi e avermi aiutata a imparare molte cose in più dell'italiano. A Antonella Manca, per essere stata a mio fianco quando avevo bisogno di parlare in spagnolo con qualcuno.

Vorrei anche ringraziare i miei colleghi di Napoli. Grazie a Matteo Lorito e Sheridan L. Woo, per avermi accolta nel loro laboratorio. A Roberta Marra e Francesco Vinale per avermi insegnato tutto o quasi tutto sulla metabolomica e tante altre cose sulla cultura napoletana. A Adriana Saco

per avermi aiutata con il DNA e non solo. A Emanuela Monetti per tutti i momenti divertenti a tuo fianco ed a Mauro Senatore, Giada D´Errico, Stefania Lanzuise, Luigi de Vitto, Federica Lacatena, Filomena Grasso, Roberta Quadro, Roberta Panza, Susy Bottiglieri e Judyta Strakowska per avermi aiutata a fare più facile il mio soggiorno lì e, soprattutto, per i momenti indimenticabili a vostro fianco.

A todos los becarios que habéis trabajado conmigo y aportado vuestro grano de arena en los ensayos y todos me habéis enseñado algo: Julie Zamorano, Carla Escapa, Mónica Clemente, Juanjo Garrido, Cristian Domínguez, Daniel Martínez, Jessica Cavalheiro, Erica Losada, Laura Rodríguez, Raquel Domínguez, Héctor Izquierdo y Alejandra Porteous.

A los técnicos de laboratorio, Javier Quiroga, Elisa Baelo y Ruth Llorente, por vuestra predisposición a ayudarme en tareas tan tediosas pero necesarias de muchos ensayos.

A los agricultores por dejarnos entrar en vuestras fincas para tomar muestras, por vuestro tiempo y disposición para todo lo que se necesitara.

A mis amigas, Verónica Marbán, Mónica Ferrero, Silvia del Cojo, Marcia Barquero e Iris Marcano por estar tan pendientes de mí y entenderme incluso aquellas veces en las que ni yo misma me comprendía.

Por encima de todo, quiero dar las gracias a aquellos miembros de mi familia que día a día estáis a mi lado y siendo mi pilar en los buenos, pero, sobre todo, en los malos momentos.

RESUMEN

La alubia o judía grano (*Phaseolus vulgaris* L.) es la leguminosa de grano, dedicada al consumo humano, más importante a nivel mundial. Se ve afectada por numerosos fitopatógenos siendo *Rhizoctonia solani* el principal causante de pudrición de raíz en el 91,8 % de las plantas de alubia afectadas por el mal del pie de la judía en la provincia de León. Es importe encontrar una solución que proporcione un control de la misma, garantizando la seguridad alimentaria y ambiental sin mermar la calidad del producto. Conociendo la variabilidad de las enfermedades en los lugares originales y correlacionándola con las variedades locales de alubia se puede proporcionar información útil para controlar una fitopatología usando los propios recursos que el medio proporciona. Es por ello que la selección de un agente de biocontrol como *Trichoderma* es una opción para el control de dichas enfermedades, que aparece con frecuencia en el suelo, con alto potencial oportunista, simbionte no virulento, con una gran adaptabilidad a diversas condiciones ecológicas y capaz de reducir los efectos negativos de la enfermedad ya que está involucrado en la respuesta defensiva de la planta.

Se ha obtenido 58 aislamientos de *Trichoderma* spp. procedentes de las zonas de cultivo pertenecientes al sello de calidad IGP "Alubia La Bañeza-León", de los cuales 23 han sido extraídos de semilla y 35 de muestras de suelo. También se ha trabajado con otros 2 aislamientos que procedían de otros estudios.

Se ha evaluado cada aislamiento en condiciones *in vitro* para conocer la capacidad de inhibición de cada aislamiento de *Trichoderma* frente a *R. solani* mediante estudios de confrontación directa y de actividad antifúngica en membranas. Los porcentajes de inhibición de la mayoría de los aislamientos han variado entre 86 % y 50 %, sin que haya habido diferencias significativas entre los extraídos de semilla y los de suelo.

Posteriormente se ha evaluado una selección de aislamientos de *Trichoderma* en condiciones *in vivo*, siendo 15 de semilla y 30 de suelo. Se ha observado que existen diferencias significativas en el desarrollo de las plantas de alubia, siendo aquellas que han sido inoculadas con aislamientos extraídos de suelo las que han mostrado mayores dimensiones en todos los parámetros evaluados. Se han seleccionado dos aislamientos, *T. harzianum* T019 y *T. velutinum* T028, uno extraído de semilla y otro de suelo respectivamente, para el estudio de la respuesta defensiva en alubia.

Con el aislamiento *T. harzianum* T019, se ha visto que en la interacción con *R. solani* se ha inducido la expresión de 7 genes de defensa de la alubia y los niveles de ergosterol y escualeno han sido también elevados lo que puede explicar los posibles efectos en el desarrollo de la planta, así como su respuesta defensiva en presencia del patógeno.

En el caso de *T. velutinum* T028, se ha observado también una modificación en el nivel de expresión de los genes de defensa estudiados, dando una idea de la forma en la que la planta ha respondido frente a la presencia de *R. solani* y/o *T. velutinum*. Además, se ha estudiado la producción de metabolitos por parte de la planta de alubia como respuesta al agente de biocontrol y al patógeno, observándose 36 compuestos que han presentado diferencias significativas respecto al control en presencia de *R. solani* y/o *T. velutinum*.

ABSTRACT

The common bean (*Phaseolus vulgaris* L.) is the most important food legume crop worldwide. It is affected by numerous phytopathogens being *Rhizoctonia solani* the main cause of root rot in 91.8 % of bean plants affected by a disease in the Province of León. It is important to find a strategy to control this disease, ensuring also the food and environmental safety without compromising the quality of the product. By studying the variability of the disease at a particular place and connecting it with the local varieties of bean, it can provide useful information to control a phytopathology using resources that already exist in the same medium. Then, to select a biocontrol agent (BCA) as *Trichoderma* would be an option to control these diseases. This BCA is present in the soil, with high opportunistic potential, non-virulent symbiont, with great adaptability to various ecological conditions and can reduce the negative effects of the disease, due to its availity to elicit the plant's defensive response.

There have been extracted 58 isolates of *Trichoderma* spp. from the cultivated areas of the IGP "Alubia La Bañeza-León". Some 23 isolates were extracted from plant material and 35 from soil samples. Other two isolates from other studies have been also included in this work.

Each *Trichoderma* isolate was *in vitro* evaluated to know its inhibition capacity against *R. solani* using direct confrontation and evaluating the antifungal activity in membranes assays. The percentages of inhibition of most isolates varied among 86 % and 50 %, without significant differences between isolates obtained from plant material or from soil.

Afterwards, some *Trichoderma* isolates were evaluated *in vivo,* 15 coming from plant material and 30 from soil. There were significant differences in the development of the bean plants, being the plants inoculated with soil isolates those with the largest dimensions in all evaluated parameters. Two isolates, *T. harzianum* T019 and *T. velutinum* T028, from plant material and from soil, respectively, were selected for the study of their effect on the bean defensive response.

With the isolate *T. harzianum* T019, it was shown that, in interaction of *R. solani*, the expression of 7 defense genes of bean plants was induced. The level of ergosterol and squalene was also higher than in the control strain, which may explain the possible effects in the development of the plant, as well as its defensive response in the presence of the pathogen.

In the case of *T. velutinum* T028, the expression of the defense genes analyzed was also modified, giving an explanation of the way by which a bean plant responds to the presence of *R. solani* and/or *T. velutinum*. As well as the production of some metabolites by bean plants has been studied in response to the biocontrol agent and the pathogen, with 36 compounds showing significant differences respect to the control.

ÍNDICE GENERAL

JUSTIFICACIÓN Y OBJETIVOS

JUSTIFICACIÓN Y OBJETIVOS

Cada vez más el consumo en alimentación está dirigido por aspectos relacionados con la seguridad del producto, salubridad y las características nutricionales del mismo y además que las prácticas productivas permitan conservar los recursos naturales del planeta a largo plazo. La influencia negativa de los productos químicos de síntesis en la salud del trabajador agrícola, del consumidor y en el medio ambiente ha dado lugar al desarrollo de una normativa que antepone la defensa de la salud humana y la protección ambiental a la producción agrícola. Todo ello ha llevado a la eliminación, en los últimos años, de las listas de productos fitosanitarios un buen número de fungicidas que eran empleados por los agricultores en el control de hongos fitopatógenos que provocan graves daños en el cultivo de la alubia. Para ello es fundamental proporcionar un producto ecocompatible para el control de fitopatógenos en el cultivo de la alubia y a medio plazo poder desarrollar fungicidas biológicos eficientes.

El cultivo de la alubia representa en León una importante fuente de ingresos para muchos agricultores, haciendo de esta provincia la principal productora en España. Gracias a la tradición del cultivo cuenta con un sello de calidad, la Indicación Geográfica Protegida (IGP) "Alubia La Bañeza-León". Con el paso de tiempo se quiere incrementar de modo sostenible el rendimiento y dar un valor añadido a las variedades locales de alubia protegidas bajo este sello, mediante prácticas productivas basadas en el control biológico de hongos con cepas autóctonas de *Trichoderma,* reduciendo así el empleo de productos fitosanitarios. Este agente de biocontrol es un hongo que está presente en la mayoría de suelos, de rápido crecimiento, simbionte no virulento, lo que hace que sea idóneo para el control de fitopatógenos, como es el caso de *Rhizoctonia solani*. Este patógeno tiene un amplio abanico de huéspedes entre los que se encuentra la alubia.

Para el desarrollo de esta tesis se propusieron los siguientes objetivos:

1. Recolectar, identificar y caracterizar aislamientos de *Trichoderma* autóctonos procedentes de campos de cultivo de alubia pertenecientes a la IGP de "La Bañeza-León" procedentes tanto de semilla como de muestras de suelo.
2. Evaluar la capacidad de inhibición de cada aislamiento de *Trichoderma* spp. en el desarrollo de *R. solani* en condiciones *in vitro*, mediante la confrontación directa y la producción de metabolitos secundarios.
3. Evaluar en condiciones *in vivo* los aislamientos seleccionados de *Trichoderma* spp. entre los que mejor control ejerzan sobre *R. solani*. Así como determinar los aislamientos que, además de proteger a la alubia frente al fitopatógeno, favorezcan su desarrollo.
4. Estudiar la respuesta defensiva de la alubia en presencia o ausencia de *Trichoderma* y/o *R. solani* mediante el estudio de los genes de defensa, así como de la producción de metabolitos empleando para ello técnicas de qRT-PCR y HPLC-DAD-TOF-MS respectivamente.

INTRODUCCIÓN

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INTRODUCCIÓN

1. ALUBIA, *PHASEOLUS VULGARIS* L.

1.1. Origen, distribución y diversidad

La alubia es un cultivo que fue domesticado hace unos 7.000 años en las regiones de los Andes y de Mesoamérica (Méjico y América Central). Existen varias evidencias que demuestran que el ancestro de la alubia común se ha organizado en dos grupos geográficamente aislados y genéticamente diferenciados (mesoamericanos y andinos) que divergieron de una población silvestre ancestral común hace más de 100.000 años (Singh et al., 1991). De esas evidencias se extrae que la domesticación se llevó a cabo en las zonas centroamericana y sudamericana (Bitocchi et al., 2012, 2013; Gepts et al., 1986; Mamidi et al., 2011). En judías silvestres evaluadas, procedentes del Norte de Perú, han mostrado características comunes a las del germoplasma andino y centroamericano, por lo que se piensa que pudiera tratarse del antepasado común de la especie (Debouck et al., 1993; Kami et al., 1995). En lo que hoy es Méjico, la alubia común fue probablemente domesticada con maíz como parte del sistema de cultivo de la "milpa" (alubia común junto maíz y calabaza) (Zizumbo-Villarreal y Colunga-GarcíaMarín, 2010). El proceso de domesticación provocó cambios morfológicos en las judías como son el tamaño de semillas y hojas, cambios en el hábito de crecimiento, las respuestas del fotoperiodo, variación del color (McClean et al., 2002; Singh et al., 1991).

La llegada de la judía a Europa se sabe que fue tras el descubrimiento de América (a partir de 1492). Las que primero fueron introducidas eran las que procedían de la zona de Mesoamérica (tamaño de semilla pequeño-medio >25 g el peso de 100 granos). Posteriormente llegarían los cultivares de las regiones andinas (tamaño de semilla grande >40 g el peso de 100 granos) coincidiendo con la exploración de Perú en 1528. Una vez en el continente europeo, la alubia no tuvo una especial aceptación ya que existía otra especie muy similar, *Vigna unguiculata* (L.) Walp., comúnmente conocida como alubia de carilla o caupí (Lioi y Piergiovanni, 2013).

Pero no fue hasta 1753 cuando Linnaeus escoge el nombre de *Phaseolus* para determinar su especie y nombrándola *Phaseolus vulgaris* L. (Lioi y Piergiovanni, 2013). Se define su taxonomía como:

- Reino: Plantae
  - División: Magnoliophyta
    - Clase: Magnoliopsida
    - Subclase: Rosidae
      - Orden: Fabales
        - Familia: Fabaceae
        - Subfamilia: Faboideae
          - Tribu: Phaseoleae
          - Subtribu: Phaseolinae
            - Género: *Phaseolus*
              - Especie: *P. vulgaris* L.

Durante los siglos XV y XVI, este cultivo fue introducido en España para ser distribuido hacia el resto de Europa como consecuencia del floreciente comercio de este país (Rodiño et al., 2001). En la Península Ibérica se conservan un gran número de variedades locales adaptadas a las características ambientales, variando sus caracteres agronómicos, así como el aprovechamiento

(Casquero et al., 2005), la forma, tamaño o color del fruto y semilla según la región, provincia o incluso pueblo (Casquero et al., 2006). La Península Ibérica se considera un centro secundario de diversificación del cultivo de la judía (Santalla et al., 2002).

1.2. Situación actual del cultivo

La alubia (*P. vulgaris* L.) es la leguminosa grano, dedicada al consumo humano, más importante, ya que su cultivo se extiende por todos los continentes y es fuente de proteínas, vitaminas, minerales, fibra y otros compuestos como pueden ser polifenoles que podrían prevenir enfermedades cardiovasculares u obesidad. Estos beneficios para la salud y el medio ambiente, gracias a su asociación con otros microorganismos beneficiosos, han llevado a un renovado interés por su cultivo en países desarrollados, donde consumidores exigentes obligan a los cultivadores a considerar los rasgos culinarios y el valor sensorial, así como buenas prácticas ambientales en el cultivo de las plantas (Rivera et al., 2016).

A nivel mundial se produjeron 26.529.581 t en el año 2014 con una superficie de 30.612.841 ha. Los países con mayor superficie (Figura 1) dedicada a este cultivo en ese mismo año fueron India (10.000.000 ha lo que supone el 32,67 % de la superficie mundial de este cultivo), Brasil (3.185.745 ha, 10,41 %), Myanmar (3.017.250 ha, 9,86 %), Méjico (1.680.897 ha, 5,49 %) y Republica Unida de Tanzania (1.134.394 ha, 3,71 %). En cuanto a la producción de la alubia en 2014 (Figura 1), los países que tuvieron una mayor cantidad fueron Myanmar (4.651.094 t siendo el 17,53 % de la producción mundial), India (4.110.000 t, 15,49 %), Brasil (3.294.586 t, 12,42 %), Estados Unidos de América (1.311.340 t, 4,94 %) y Méjico (1.273.957 t, 4,80 %). El rendimiento de la alubia a nivel mundial en 2014 obtuvo como valor medio 1.500 kg/ha, aunque varía entre valores de 6.069 kg/ha en el caso de Irlanda y los 43,6 kg/ha en Eritrea (FAO, 2016).

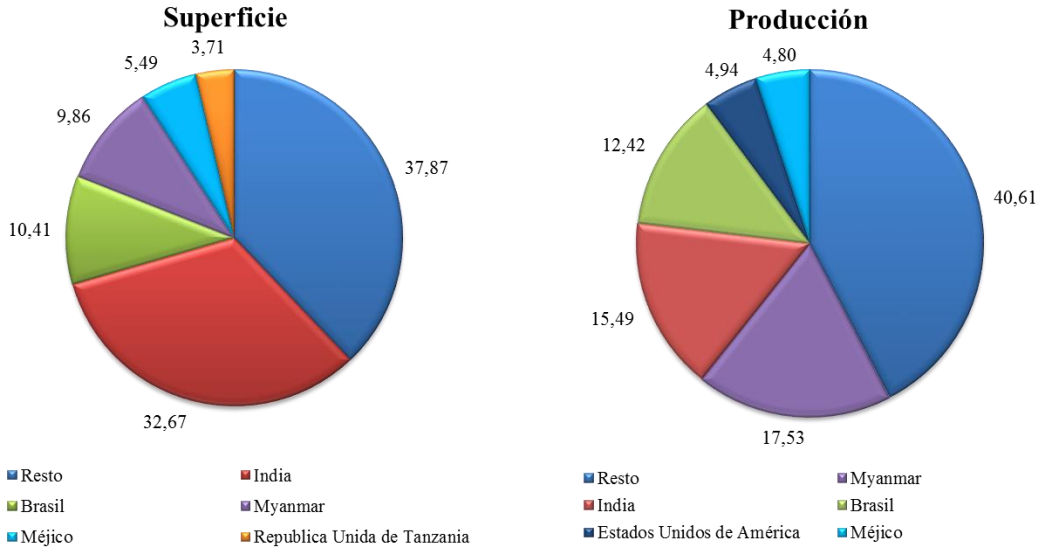

Figura 1: Porcentaje de la superficie y de la producción a nivel mundial en el año 2014 (FAO, 2016).

Si se observan los datos relativos al continente europeo en el 2014, se sembraron 311.014 ha que produjeron unas 701.576 t, siendo Bielorrusia, Ucrania, Rumanía, Lituania y Polonia los países con mayor superficie dedicada a la alubia y Bielorrusia, Lituania, Ucrania, Polonia y Albania los que

mayor producción obtuvieron (FAO, 2016). Analizando los datos de la Unión Europea, en 2014, la producción media fue de 228.571 t, de los cuales el 27,34 % es producido por Lituania, seguida de Polonia y Letonia (FAO, 2016). España, en 2014, obtuvo una producción de 12.629 t con una superficie de 7.737 ha situándola en el sexto puesto entre los países productores de alubia en la Unión Europea (Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente, 2016).

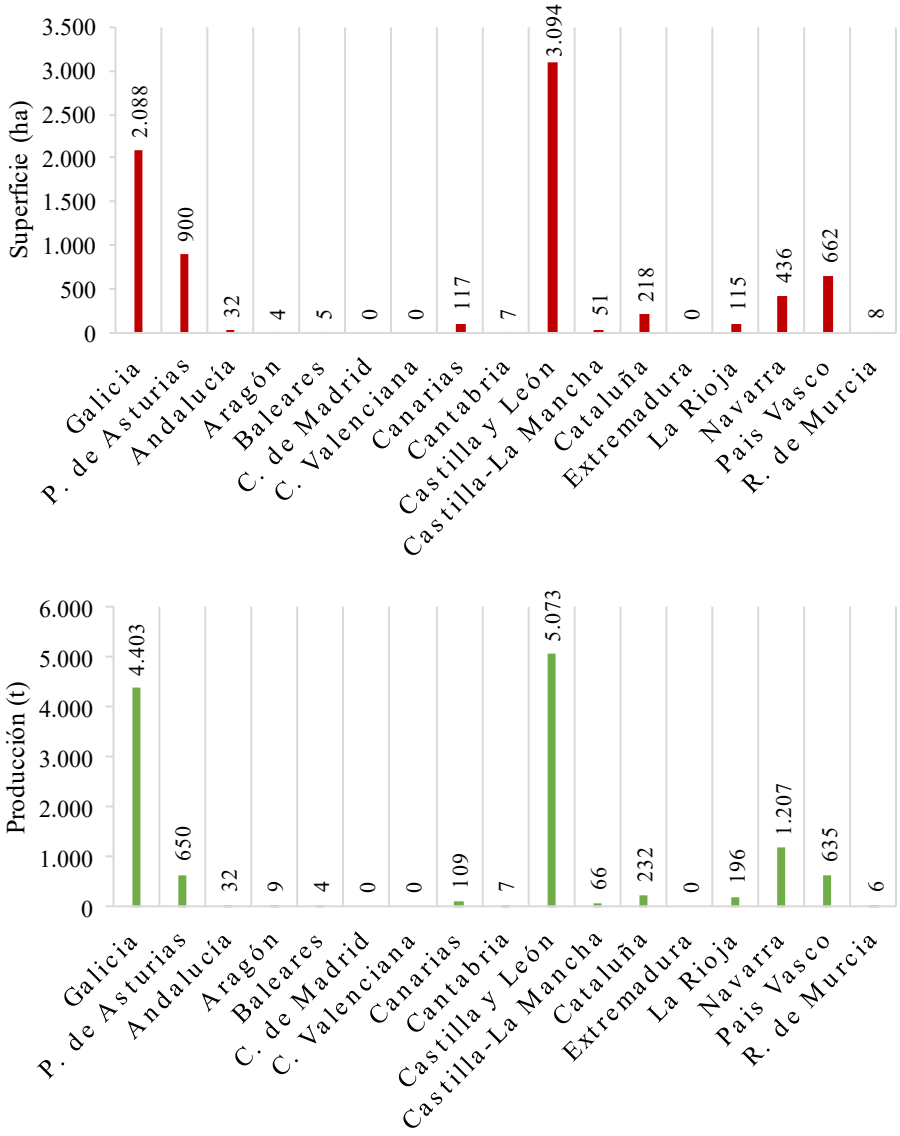

Figura 2: Superficie y producción de España distribuidas por Comunidades Autónomas (Ministerio de Agricultura Pesca Alimentación y Medio Ambiente, 2016).

En España (Figura 2), se observa que Castilla y León, en 2014, fue la comunidad con la mayor superficie dedicada al cultivo de alubia con 3.094 ha y una producción de 5.073 t, suponiendo el 40% de la producción total de España. A esta comunidad le siguió Galicia con el 34,86 % y Navarra con el 9,56% de la producción nacional (Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente, 2016).

Dentro de la Comunidad de Castilla y León, León fue la provincia que mayor superficie y producción tuvo en 2014, con 2.440 ha y 3.855 t, suponiendo el 75,97 % de la producción de este cultivo en la comunidad y 30,53 % de España. Le siguió Ávila con un 10,44 % de la producción de

Castilla y León. El resto de provincias presentaron una producción muy por debajo de las anteriormente mencionadas (Junta de Castilla y León, 2016).

En la provincia de León, la primera noticia que se halla sobre la existencia de la judía común data del Catastro en sus Comprobaciones de 1761 en los pueblos de Priaranza y Tabuyo del Monte. Existe constancia de que en los años '20 se exportaban desde La Bañeza las denominadas "alubias leonesas de riñón" y a lo largo del siglo XX destaca la expansión y establecimiento del cultivo en la comarca de El Páramo (Reinoso, 2001).

Por Orden Ministerial de 6 marzo de 1984 fue reconocida con carácter provisional la denominación específica "Judías de la Bañeza" y en la actualidad existe la Promotora y el Consejo Regulador de la Indicación Geográfica Protegida (I.G.P.) "Alubia de La Bañeza-León" (Orden A.P.A/289/2006; Orden A.Y.G./1254/2005), destinada a promocionar este producto tradicional y a velar por su calidad. La mayor superficie dedicada a este cultivo en 2015 (Tabla 1) en la provincia se encuentra en El Páramo, siguiéndole en orden decreciente de importancia la Bañeza, Esla-Campos, La Maragatería - La Cepeda, Sahagún, Tierras de León y La Cabrera (Junta de Castilla y León, 2015).

Tabla 1: Superficie y porcentaje dedicados al cultivo de la alubia en la provincia de León durante el año 2015 (Junta de Castilla y León, 2015).

Región o comarca	Superficie (ha)	%
León provincia	3.466*	--
Bierzo	0	0,00
La Montaña de Luna	0	0,00
La Montaña de Riaño	0	0,00
La Cabrera	5	0,14
La Maragatería - La Cepeda	172	4,96
Tierras de León	37	1,07
La Bañeza	195	5,63
El Páramo	2.841	81,97
Esla-Campos	177	5,11
Sahagún	39	1,13
*Superficie total de alubia en la provincia de León en 2015		

1.3. Características botánicas de la alubia

P. vulgaris L. es una planta herbácea anual perteneciente a la familia Fabaceae o Leguminosae. La germinación es epigea, y requiere 5-7 días para que se lleve a cabo. Presenta una raíz principal con numerosas raíces secundarias, cuya longitud puede variar entre 10 y 30 cm (Graham y Ranalli, 1997) (Figura 3a). Presenta dos cotiledones tras la germinación, los cuales son sustituidos por unas hojas verdaderas a los pocos días (Figura 3e). Las hojas son trifolioladas, con folíolos enteros o lobulados y con estípulas persistentes (Figura 3b).

Pueden tener diferentes hábitos de crecimiento (van Schoonhoven y Pastor-Corrales, 1987):

- Hábito de crecimiento I: porte erguido, tallo y ramas erectas y sin guía.
- Hábito de crecimiento II: porte erguido, con un tallo y las ramas erectas, y muchas veces sin una guía.
- Hábito de crecimiento III: hábito de arbusto con tallo débil y numerosas ramas, con una guía corta o larga y con capacidad para subir.

- Hábito de crecimiento IV: tallo largo y débil y con poca ramificación, posee guías con la capacidad de trepar por un tutor.

Los hábitos I y II tienen un crecimiento determinado, mientras que III y IV presentan un crecimiento indeterminado.

Las flores (Figura 3d) son zigomorfas, es decir, presentan un plano de simetría bilateral, y están dispuestas en racimos axilares insertados en los nudos hinchados, con brácteas persistentes o caducas. La flor, puede presentar una gran variedad cromática y contiene diez estambres y un solo ovario multiovulado, con una fecundación predominantemente autógama, obteniéndose un fruto o vaina recta o ligeramente curvada. La floración se inicia, normalmente entre los 28 y 42 días tras la siembra, aunque puede variar según las características climáticas de la zona. La duración de la floración varía en función de los hábitos de crecimiento: las de hábito determinado, tienen una floración concentrada en un periodo corto de 5-6 días. Sin embargo, las de hábito indeterminado presentan una floración escalonada que duraría entre 15 y 30 días. El fruto es una legumbre o vaina en cuyo interior se localizan de 4 a 8 semillas (Figura 3c) (Graham y Ranalli, 1997).

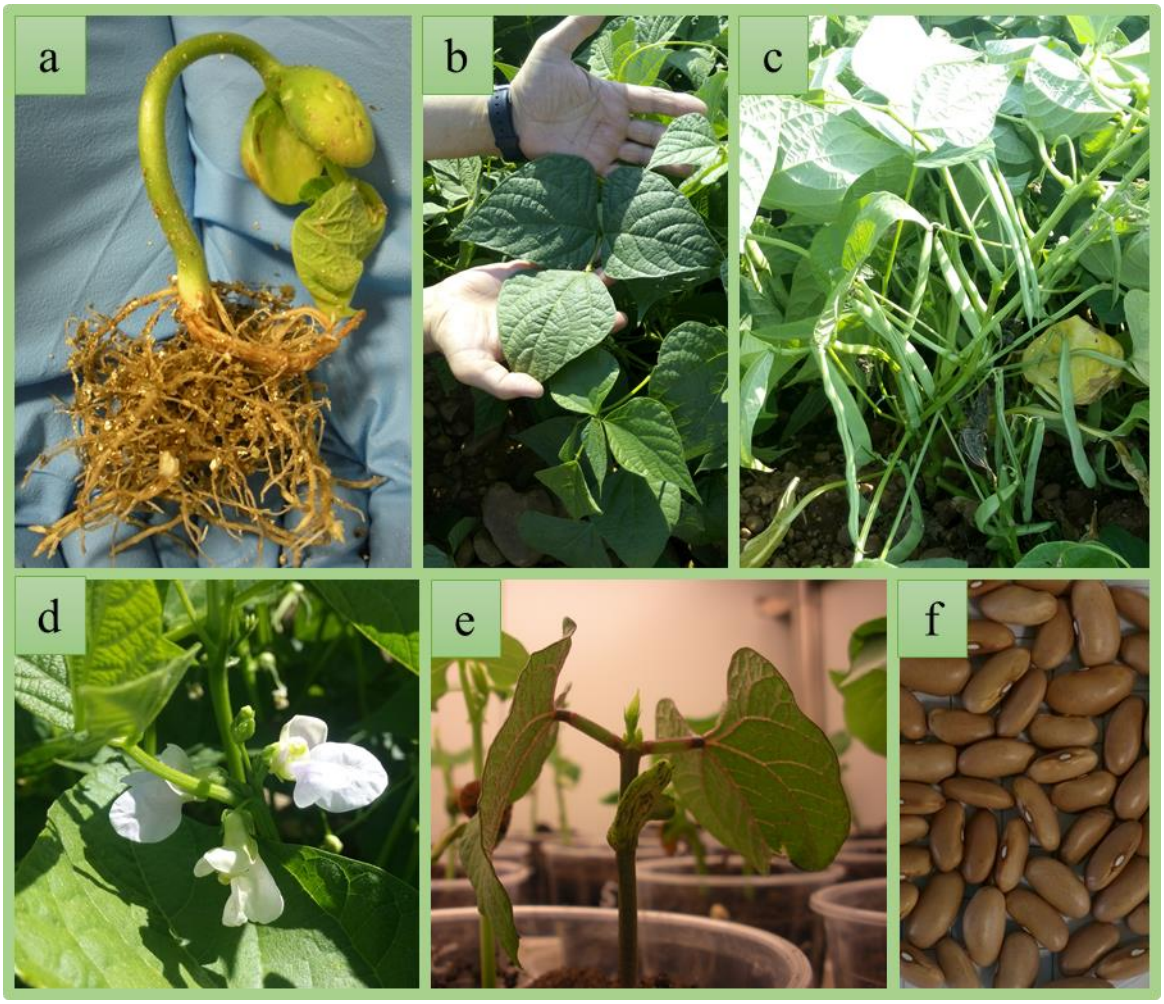

Figura 3: Detalle de la alubia, *P. vulgaris* L. a) Semilla germinada y detalle de las raíces; b) Hoja trifoliada; c) Fruto en forma de vaina o legumbre; d) Inflorescencia y flor; e) Cotiledones; f) Semillas de la variedad canela.

La formación de la semilla puede durar desde los 23 días en las cultivares de crecimiento determinado hasta casi 50 días en las de hábito indeterminado. La madurez fisiológica puede extenderse hasta los 200 días tras la siembra. Las semillas pueden ser redondas, elípticas, algo aplanadas o redondeadas de forma alargada, con una amplia variación de colores (Figura 3f).

1.4. Incidencia de los hongos en el cultivo de la alubia

El cultivo de la alubia se ve afectado por numerosas micosis (American Phytopathological Society, 2005). Se describen varias micosis, entre las que se podrían destacar las siguientes: alternariosis (*Alternaria alternata* (Fr.) Keissl.), antracnosis (*Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara), ascochyta (*Ascochyta boltshauseri* Sacc.), macrophomina (*Macrophomina phaseolina* (Tassi) Goid), mal del esclerocio (*Sclerotium rolfsii* Sacc.), mancha angular (*Phaeosiriopsis griseola* (Sacc.) Ferraris), mildiu velloso (*Phytophthora* sp Bary), oídio (*Oidium balsamii* Mont.), phoma (*Phoma exigua* Sacc.), podredumbre rosa de la vaina (*Trichothecium roseum* (Pers.) Link.) y roya (*Uromyces appendiculatus* F. Strauss).

Sin embargo, las que están más generalizadas en los campos leoneses son la fusariosis, causada por *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen y *Fusarium solani* (Mart.) Sacc., la podredumbre blanca (*Sclerotinia sclerotiorum* (Lib.) de Bary,), la podredumbre gris (*Botrytis cinerea* Pers. Fr.) y la rhizoctoniosis (*Rhizoctonia solani* Kühn). Las especies patógenas de *Fusarium* spp., junto con *R. solani* y *Pythium* spp., forman el denominado "complejo parasitario del pie de la judía" (Asensio, 1996; Berra y Arteaga, 1989; Tello et al., 1985), causante de la enfermedad conocida como "mal del pie de la judía" que origina daños importantes en la zona de producción de la provincia leonesa. De todos los fitopatógenos citados previamente, *R. solani* es el principal causante de pudrición de raíz en el 91,8 % de las plantas de alubia afectadas por el "mal del pie de la judía" en la provincia de León (Valenciano et al., 2006b).

2. *RHIZOCTONIA SOLANI* KÜHN

R. solani es un hongo fitopatógeno que provoca la muerte de las plántulas de numerosas especies como algodón, alfalfa, altramuz, arroz, berenjena, cacahuete, cebolla, cítricos, coliflor, fresa, guisante, alubia, maíz, melón, patata, pepino, pimiento, remolacha, soja, tabaco, tomate, trigo, zanahoria y otros, teniendo una distribución mundial ya que se encuentra en todos los suelos cultivables (Ogoshi, 1996).

2.1. Taxonomía y morfología del fitopatógeno

R. solani [teleomorfo: *Thanatephorus cucumeris* (Frank) Donk] es la especie más importante del género *Rhizoctonia*. Se caracteriza por la formación de micelio (Figura 4a) que no produce esporas, incoloro cuando pasa por su etapa juvenil pero que se torna amarillo o de color café claro conforme madura, y constituido por hifas que tienden a ramificarse en ángulo recto cerca de un tabique estrechándose ligeramente a nivel de la bifurcación y con un septo cerca de ella (Agrios, 2002). Las hifas son anchas (5 a 12 μm) con células multinucleadas (2 a 25 núcleos, siendo más común entre 4 y 8), carecen de fíbulas o conexiones "clamp" y pueden diferenciarse en cadenas de células hinchadas (hifas moniliáceas) (Figura 4c). A partir de éstas se pueden formar los esclerocios, con

tejido esclerotial sin diferenciar en corteza y médula, de color marrón a negro aplanados en la parte superior y de 1 mm de diámetro (Monte y Suárez, 2010).

El primero en establecer el género *Rhizoctonia* fue De Candolle (1815), pero no fue hasta 1970 cuando Parmeter y Whitney sentaron las bases de dicho género, las cuales fueron resumidas como la producción de esclerocios en una textura uniforme que está en una asociación con las raíces de las plantas (González García et al., 2006).

La taxonomía de este fitopatógeno es (Index Fungorum, 2017; International Mycological Association, 2016):

- Reino Fungi
  - División: Basidiomycota
    - Subdivisión: Agaricomycotina
      - Clase: Agaricomycetes
        - Orden: Cantharellales
          - Familia: Ceratobasidiaceae
            - Género: *Rhizoctonia.* Su etapa sexual o perfecta corresponde a *Thanatephorus*
              - Especie: *R. solani* Kühn

La temperatura óptima de crecimiento saprófito de *R. solani* es de 24 a 28 ºC, pero para que se produzca la infección se encuentra cerca de 15 a 18°C (Hagedorn, 1991), aunque algunas cepas muestran una mayor actividad a temperaturas superiores a 35°C. El pH óptimo de crecimiento es de 5 a 7, aunque puede crecer desde pH 3 hasta pH 8. La luz retrasa el crecimiento de las colonias del hongo que podría deberse más a la desecación debida a la fuente luminosa que al estímulo luminoso (Monte y Suárez, 2010).

R. solani tiene capacidad para parasitar a otros hongos. Sin embargo, al poseer un alto poder fitopatógeno y una elevada variabilidad genética se descarta como agente de control biológico. La capacidad de este fitopatógeno de sobrevivir por un tiempo indefinido en forma de organismo saprófito hace que pueda parasitar a una amplia gama de especies (Agrios, 2002).

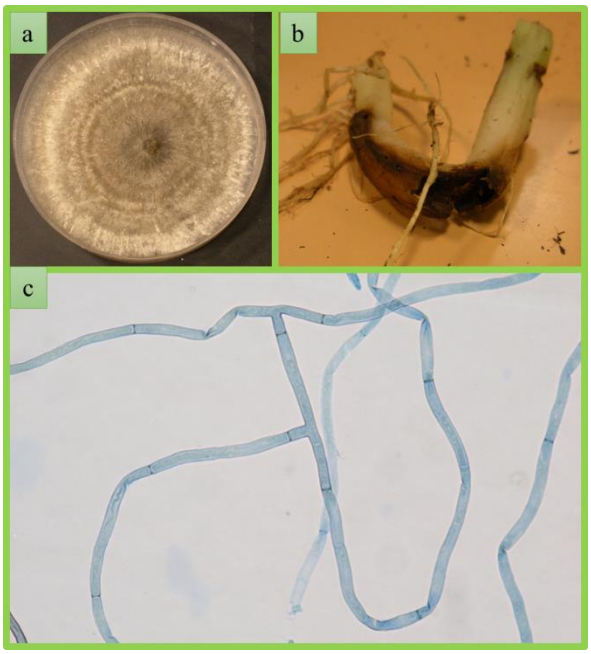

Figura 4: *R. solani* a) Aislamiento en medio patata-dextrosa-agar; b) Síntomas en una raíz de alubia; c) Hifas vistas al microscopio.

2.2. Diversidad genética de *R. solani*

Las primeras clasificaciones de este fitopatógeno, al no producir conidias, se realizaron en función del cultivo afectado, comportamiento patogénico y criterios ecológicos. Fue a partir de 1969 cuando se introdujo el criterio de "grupo de anastomosis" (AG) para su clasificación. Cada uno de ellos

estaría constituido por aislados de hongos que pueden establecer una fusión entre sus hifas al crecer entrelazados en un medio de cultivo, asumiendo que la compatibilidad para esta fusión implicaba una relación genética, mientras que aquellos que se mantuvieran aislados pertenecerían a otro AG (Monte y Suárez, 2010).

En la actualidad se han descrito 14 AG de *R. solani* (Tabla 2) y han sido clasificados en función de su patogenicidad y su morfología (González García et al., 2006). Se sabe que los AG que afectan al cultivo de la alubia son AG 1 IA y IB, AG 4 y AG 5, pudiendo causar pudriciones tanto en las plántulas, como en hojas, vainas y raíces.

Tabla 2: Resumen de los distintos grupos y subgrupos de anastomosis reconocidos por aislados de *R. solani* (González García et al., 2006).

Grupo/Subgrupo	Cultivos a los que afecta
AG 1 IA	Arroz, maíz, sorgo, **alubia**, soja, césped, plántulas de alcanforero
AG 1 IB	**Alubia**, arroz, soja, leguminosas arbustivas, lechuga, hortensia, repollo o berza
AG 1 IC	Trigo sarraceno, zanahoria, soja, lino, pino
AG 2-1	Crucíferas, fresa, tulipán
AG 2-2IIIB	Arroz, jengibre, césped, maíz, remolacha azucarera
AG 2-2IV	Remolacha azucarera, césped
AG 3	Patata, tabaco, tomate, berenjena
AG 4 (HGI, HGII y HGIII)	Tomate, guisante, patata, soja, cebolla, algodón, **alubia**, plántulas de *Pinus taeda*
AG 5	Patata, césped, **alubia**, soja
AG 6 (HG-I y GV)	No patogénico
AG 7	Soja
AG 8	*Poaceae* spp.
AG 9 (TP y TX)	Crucíferas, patata
AG 10	No patogénico
AG 11	Trigo
AG 12	Coliflor, rábano
AG 13	No patogénico
AGBI	No patogénico

2.3. Sintomatología

R. solani ataca en primer lugar a las partes subterráneas de la planta como son las semillas, hipocotilo y sistema radicular, aunque podría afectar a las partes aéreas de la planta como tallos, hojas y frutos.

El síntoma más destacable es la caída de plántulas o "damping-off" (Figura 5) que se caracteriza por la muerte de las semillas sin germinar o por la muerte de las plántulas en pre- o post-emergencia (Figura 4b) (Hagedorn, 1991). En el caso de las semillas, el hongo ataca y origina la muerte del ápice caulinar de crecimiento, mientras que en las plántulas, el ataque se produce en el hipocotilo, volviéndose acuoso, blando e incapaz de sostener a la plántula, la cual se desploma y muere (Monte y Suárez, 2010). Las plántulas maduras también son atacadas por el hongo, limitándose a invadir sus tejidos corticales externos en los que produce lesiones grandes y de color que va de canela a café rojizo. La longitud y anchura de dichas lesiones aumenta hasta que finalmente rodean al tallo y la planta puede morir (Agrios, 2002).

También puede producir pudriciones en las vainas de la alubia y en otros órganos que se encuentren próximos al suelo, siendo más frecuentes en climas húmedos y fríos. Estas lesiones en un principio toman el aspecto de áreas firmes y húmedas, en las que los tejidos en poco tiempo se colapsan y forman un área ligeramente hendida. Cuando el tiempo es húmedo, el micelio aparece sobre las manchas que inicialmente son de color blanco pero que después se tornan a colores más oscuros conforme pasa el tiempo. Los frutos y vainas afectados se tornan a colores marrones y se secan o pueden ser invadidos por otros microrganismos.

La infección de las plantas jóvenes es más severa cuando el crecimiento de la planta es lento, debido a las condiciones ambientales adversas para su desarrollo. Las plantas de crecimiento rápido tienen la posibilidad de escapar a la infección por *Rhizoctonia,* incluso cuando la humedad y la temperatura sean favorables para el hongo (Agrios, 2002).

2.4. Ciclo vital de *R. solani*

R. solani puede sobrevivir en el suelo en ausencia de plantas huésped durante periodos de tiempo largos ya que inverna casi siempre en forma de micelio o esclerocios en el suelo, en plantas perennes infectadas o en órganos de propagación (Figura 5). El hongo invade también a otros hospedantes y puede ir en la semilla. Se encuentra presente en la mayoría de los suelos y una vez que se ha establecido en un campo, permanece por tiempo indefinido (Monte y Suárez, 2010).

Cuando las condiciones son favorables, y la planta susceptible de ser infectada está presente, el micelio y/o los esclerocios germinan para colonizar la superficie de su huésped. Después del contacto, el hongo se desarrolla sobre la superficie de la raíz, hipocotilo u otra parte de la planta, ramificándose las hifas para formar estructuras como apresores y cojines de infección que, junto con la producción de enzimas como cutinasas, pectinasas y celulasas, propician la penetración en las plantas y la liberación de sustancias nutritivas para el fitopatógeno favoreciendo así su desarrollo (Figura 5).

Una vez que el hongo ha penetrado, mata las células y coloniza ese tejido muerto, formándose posteriormente los esclerocios. Sobre esta superficie necrosada se formaría un nuevo inóculo de *R. solani* lo que facilitaría el ataque de nuevas plantas o la supervivencia del patógeno. Se puede trasmitir en el suelo adherido en el calzado, maquinaria agrícola, aperos, vehículo, o bien en el agua de lluvia o de riego (Agrios, 2002).

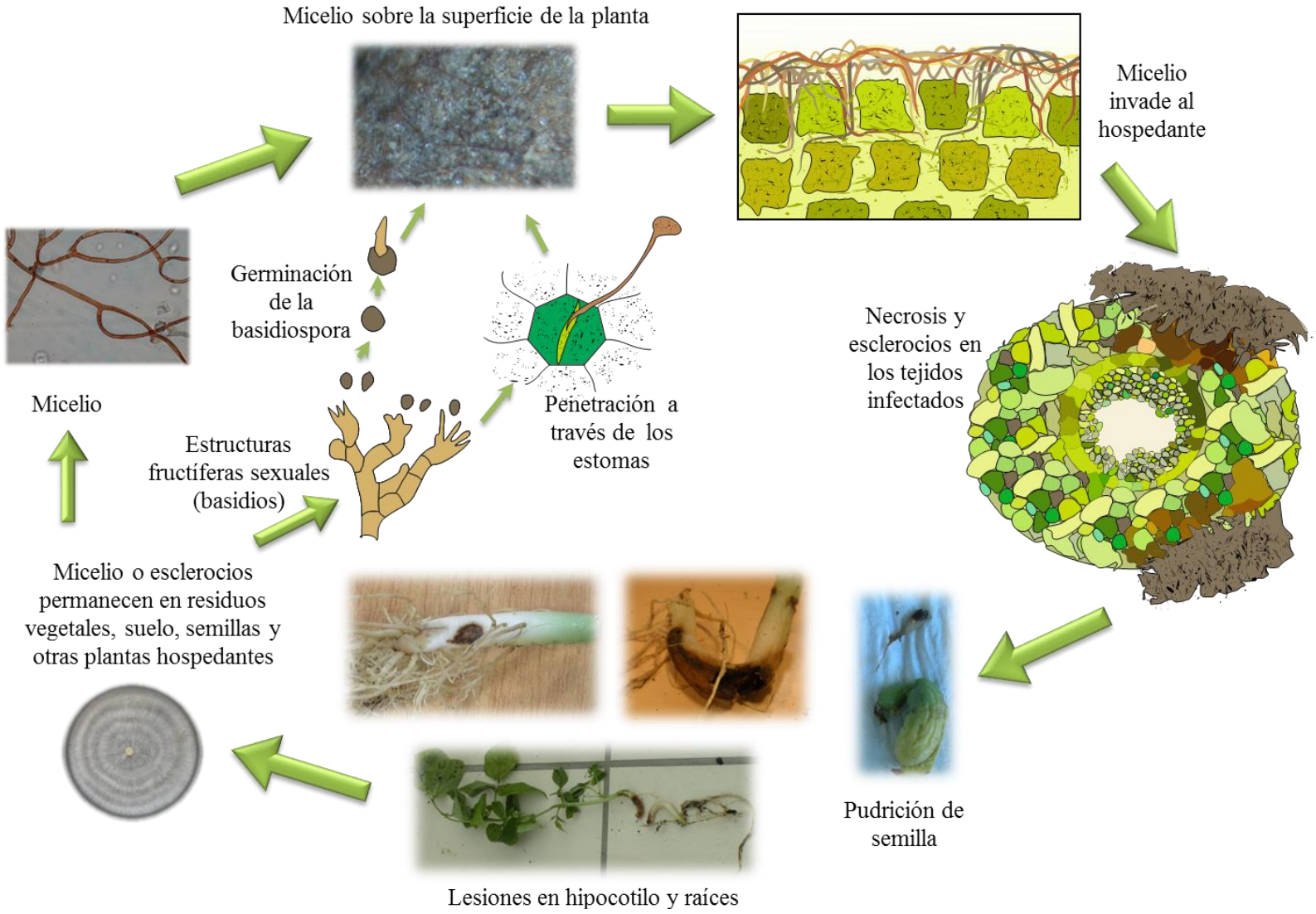

Figura 5: Ciclo vital de *R. solani* en el cultivo de la alubia.

3. CONTROL DE FITOPATÓGENOS EN EL CULTIVO DE LA ALUBIA

Existen distintas formas de prevenir o curar a una planta cuando ésta ha sido atacada por un fitopatógeno. Se pueden emplear productos de síntesis química, organismos con actividad antagónica, métodos culturales y otros. Debido a la Reforma de la Política Agraria Comunitaria se ha reducido el número de productos fitosanitarios de síntesis química autorizados con el fin de que prevalezca la seguridad alimentaria, así como que sea sostenible a largo plazo. Por ello se propone dar prioridad a los métodos no químicos descritos en producción integrada, cultivos ecológicos, y otros.

Una forma de controlar a los fitopatógenos, como es el caso de *R. solani*, en la alubia sería aplicando los fungicidas sobre la semilla o directamente al suelo pudiendo ser efectivos sobre los hongos que afectan al cultivo durante la germinación o en un periodo corto después de la misma (Beebe et al., 1991) pues reducen su incidencia (Gupta et al., 1999) y mejoran la emergencia de las plantas (Bordoloi et al., 1998; Valenciano et al., 2004). Sin embargo, las aplicaciones con fungicidas dirigidas a evitar los daños producidos por hongos que causan podredumbres radiculares o amarilleamientos y marchitez suelen ser ineficaces y usualmente impracticables debido al gran volumen de suelo en el que deberían aplicarse.

Otra forma de control de hongos en judía sería la mejora de las condiciones de establecimiento en campo de la alubia, desarrollando y evaluando nuevas técnicas de siembra combinadas con la utilización de pesticidas con el fin de optimizar las condiciones para la emergencia de las plántulas (Valenciano et al., 2006a) y así reducir la dependencia de los fitosanitarios. También hay estudios sobre la eficacia de los fungicidas comerciales para el control del "mal del pie de la judía" en variedades locales de alubia protegidas por la "I.G.P. Alubia de La Bañeza-León" (Campelo et al., 2007).

En España en el año 2014 (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, 2015), de las 38.393 t de fungicidas y bactericidas que se emplearon para el control de enfermedades, el 84,96 % fueron de fungicidas inorgánicos mientras que sólo un 0,04 % fueron biológicos o agentes de control biológico.

Una forma que podría ser sostenible a largo plazo para el control de fitopatógenos en el cultivo de la alubia sería el empleo de agentes de biocontrol u organismos distintos al hombre con capacidad para reducir la población del agente causante de la enfermedad o evitar sus efectos (Hjeljord y Tronsmo, 1998). Dichos agentes se pueden emplear solos o bien junto con dosis reducidas de fungicidas químicos para poder controlar a los patógenos que afecten a la planta y así reducir al mínimo el impacto sobre el medio (Chet y Inbar, 1994; Harman y Kubicek, 1998). Existen formulaciones de agentes biológicos formados por bacterias, como *Agrobacterium, Pseudomonas, Streptomyces* y *Bacillus*, y por hongos, como *Gliocladium, Trichoderma, Ampelomyces, Candida* y *Coniothyrium*. A partir de ellos se han patentado y comercializado diferentes productos comerciales para el control de distintas enfermedades vegetales en todo el mundo (Tabla 3).

Tabla 3: Ejemplo de productos comerciales a base de agentes de biocontrol.

Materia activa	Nombre comercial	Control / efectos	Casa comercial
Ampelomices quisqualis M10	AQ 10	Oídio	Agrichem (España)
Aureobasidium pullulans	BOTECTOR	*Botrytis* spp.	Manica Cobre (España)
Bacillus. amyloliquefaciens + B. licheniformis + B. pumilus	TRICHOBOT	*Botrytis* spp., *Monilia* spp. y hongos patógenos de suelo	AMC Chemical (España)
B. megaterium + B. laterosporus	FUSBACT	*Fusarium* spp.	AMC Chemical (España)
B. subtilis QST 713	SERENADE ASO	*Botrytis* spp.,	Bayer (EE.UU)
B. subtilis	SERENADE Max	*Botrytis* spp., *Pseudomonas* spp., *Sclerotinia* spp., *Venturia* spp, *Erwinia amylovora*, *Monilinia* spp., *Xanthomonas* spp.	Bayer (EE.UU)
B. subtilis GB03	KODIAK	*Fusarium* spp., *Rhizoctonia* spp., *Alternaria* spp. *Aspergillus* spp.	Bayer (EE.UU)
Coniothyrium minitans (CON/M/91-08)	CONTANS	*Sclerotinia* spp.	Bayer (EE.UU.)
Gliocladium catenulatum J1446	PRESTOP	Preventivo	C.Q. Massó (España)
G. virens	SOILGARD 12G	*Pythium* spp., *Rhizoctonia* spp.	Thermo Trilogy (EE.UU)
Streptomyces griseoviridis	MYCOSTOP	*Fusarium* spp., *Phytophthora* spp., *Alternaria* spp., *Pythium* spp., *Rhizoctonia* spp., *Botrytis* spp.	AgBio, Inc. (EE.UU.)
Trichoderma. asperellum + T. gamsii	BIOTEN	*Phytophthora* spp., *Verticillum* spp.	Isagro (España)
T. asperellum T18	PRODIGY	*Phytophthora* spp., *Sclerotinia* spp., *Rhizoctonia* spp.	AMC Chemical (España)
T. asperellum T34	T34 BIOCONTROL	Hongos patógenos de suelo	Biocontrol Technologies S.L. (España)

Tabla 3: Ejemplo de productos comerciales a base de agentes de biocontrol (continuación).

Materia activa	Nombre comercial	Control / efectos	Casa comercial
T. harzianum	ROOTSHIELD	*R. solani*, *Pythium* spp., *Fusarium* spp.,	BioWorks (EE.UU.)
T. harzianum	SUPRESIVIT	Estimulación del crecimiento	Borregaard Bioplant (Dinamarca)
T. harzianum (KRL-AG2)	TRI 002	Estimulación del crecimiento, fortalecimiento de las plantas frente a patógenos	Biowork (EE.UU.) Pant Support (Países Bajos)
T. harzianum (PV 5736-89)	SUPRESIVIT	*Pythium* spp., *Rhizoctonia* spp.	Fytovita (República Checa)
T. harzianum + *T. viride*	TUSAL	*Phoma* spp., *Pythium* spp., *Rhizoctonia* spp., *Phytophthora* spp.	Certis Europe (Países Bajos)
T. harzianum T22	PHC T-22	*Pythium* spp., *Rhizoctonia* spp., *Fusarium* spp., *Cylindroladium* spp., *Thielaviopsis* spp., *Sclerotinia* spp.	BioWork Inc (EE.UU.)
T. virens	ROOT MATE	*Phytophora* spp.	BioWork Inc (EE.UU.)
T. viride	ECOFIT	*Fusarium* spp., *Pythium* spp., *Rhizoctonia* spp.	Hoechst Schering AgrEvo (India)
T. viride	TRIECO	*Rhizoctonia* spp., *Pythium* spp., *Fusarium* spp.	Ecosense Labs (India)
Trichoderma spp.	TRICHOJET, TRICHOPEL, TRICHODOWELS, TRICHOSEAL	*Armillaria* spp., *Botryosphaeria* spp., *Chondrosternum* spp., *Fusarium* spp., *Nectria* spp., *Phytophathora* spp., *Pythium* spp., *Rhizoctonia* spp.	Agrimm Technologies (Nueva Zelanda)
Talamyces flavus	PROTUS	Estimulación del crecimiento, fortalecimiento de las plantas frente a patógenos como *Verticillum* spp.	Prophyta GmbH (Alemania)

4. *TRICHODERMA* SPP. PERSOON, FRIES

Trichoderma es un género de ascomicetes filamentosos que se encuentra entre los hongos saprofitos más aislados. Aparecen con frecuencia en el suelo y crecen sobre madera, corteza, otros hongos y muchos otros sustratos, teniendo un alto potencial oportunista y una gran adaptabilidad a diversas condiciones ecológicas (Harman y Kubicek, 2002).

4.1. Taxonomía y morfología de *Trichoderma*

El género *Trichoderma* fue definido en 1794 por Persoon. pero no fue hasta el siglo XX cuando se empezó a estudiar al llamar la atención de los agricultores y comprobar que era capaz de controlar hongos que causaban enfermedades en las plantas (Mukherjee et al., 2013; Weindling, 1932, 1934). Se ha definido su taxonomía como (Samuels, 1996):

- Reino: Fungi
- Subreino: Dikarya
  - División: Ascomycota
  - Subdivisión: Pezizomycotina
    - Clase: Sordariomycetes
    - Subclase: Hypocreomycetidae
      - Orden: Hypocreales
        - Familia: Hypocreaceae
          - Género: *Trichoderma* Persoon, Fries

En la actualidad se han definido 256 especies de este género (Bissett et al., 2015).

Los hongos de este género crecen rápidamente a una temperatura entre 25 y 30 ºC, y reducen su velocidad conforme se aumenta la temperatura, deteniendo su desarrollo a partir de 35 ºC. Las colonias cuando están en medio maíz-dextrosa-agar (CMD) presenten un aspecto transparente mientras que en medio patata-dextrosa-agar (PDA) el micelio es de una tonalidad blanca (Figura 6a). Pasados unos días, entre 3 y 10 según la especie, se forman los conidios tomando un aspecto que puede variar de amarillo a verde en sus distintas tonalidades o seguir con tonalidades blanquecinas (Figura 6a), aunque este último caso es menos frecuente. También puede segregar sustancias al medio, haciendo que éste torne a un color amarillento (Figura 6b), sobre todo si se emplea medio PDA (Samuels, 1996).

Los conidióforos son muy ramificados. Las hifas principales producen las laterales que pueden estar emparejadas o no, en función de la especie (Barnett y Hunter, 1998). Tanto las hifas principales como las secundarias surgen con un ángulo de 90º respecto al eje principal (Figura 6d). El típico conidióforo de *Trichoderma*, con hifas emparejadas, asume un aspecto piramidal.

Las fiálides pueden ser cilíndricas o casi globosas, aunque cuando crecen en medio se pueden agrandar. Las fiálides pueden mantenerse en espirales, con un ángulo de 90° respecto a otros miembros de la espiral, o pueden ser peniciladas (similares a *Gliocladium*), es decir, que en sus extremos presentan una especie de pincel. Las fiálides pueden estar densamente agrupadas en el eje principal ancho como *T. polysporum* y *T. hamatum* o pueden ser solitarias como *T. longibrachiatum* (Samuels, 1996).

Los conidios normalmente no presentan ningún líquido sobre ellos, pero en algunas especies pueden aparecer algunas gotas de líquido verde claro o amarillo como *T. virens* y *T. flavofuscum*. Los

conidios de la mayoría de las especies son elipsoidales (Figura 6e), con un tamaño 3-5 x 2-4 µm (relación de longitud/anchura ≥ 1,3) (Samuels, 1996). Los conidios son lisos (Barnett y Hunter, 1998).

El teleomorfo de *Trichoderma* pertenece al género *Hypocrea* Fr (Druzhinina et al., 2011). Se caracteriza por el estroma discoidal. Los peritecios están completamente sumergidos. Las ascosporas son bicelulares pero desarticuladas en el septo temprano en su desarrollo en 16 ascosporas parciales, de manera que puede contener 16 ascosporas. Éstas son hialinas o verdes y típicamente espinulosas (Samuels, 1996).

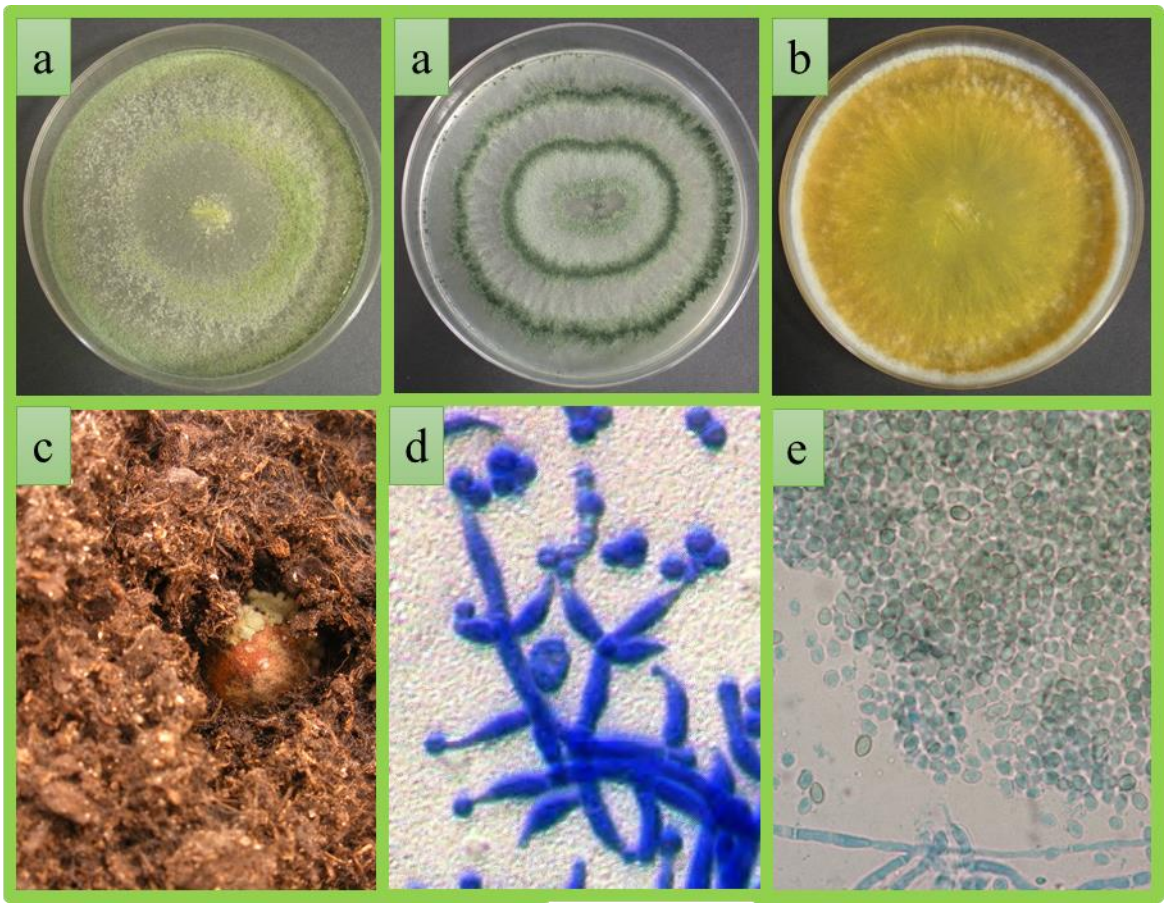

Figura 6: *Trichoderma* spp. a) Aislamientos creciendo en medio PDA en el que se aprecian distintas formas de desarrollo; b) Aislamiento que produce una tinción del medio PDA; c) Desarrollo de *Trichoderma* spp. sobre una semilla de alubia sembrada en sustrato; d) Detalle de los conidióforos vistos al microscopio de un aislamiento de *Trichoderma* (Imagen tomada de https://www.ars.usda.gov); e) Detalle de las esporas vistas al microscopio de *Trichoderma*.

Trichoderma se puede aislar de los suelos forestales o agrícolas en todas las latitudes. Aunque las especies de *Hypocrea* se encuentran más frecuentemente en la corteza o en la madera decorticada, algunas pueden crecer en hongos de soporte, como *H. pulvinata*, *H. sulphurea*, *H. latizonata, H. avellanea* (Samuels, 1996).

En la actualidad, el uso del teleomorfo (*Hypocrea*) ha quedado relegado en favor del anamorfo (*Trichoderma*) siendo válido desde del 1 de enero de 2013 (según la decisión tomada en el Congreso Internacional de Botánica en julio de 2011) (Druzhinina et al., 2011).

5. COEVOLUCIÓN ENTRE EL CULTIVO DE LA ALUBIA Y LOS FACTORES BIÓTICOS Y ABIÓTICOS

Las variedades de alubia común cultivadas en la actualidad han sido el resultado de un proceso de evolución y selección, en el que se han producido cambios morfológicos, fisiológicos y genéticos, como respuesta a las exigencias humanas o del medio ambiente (Casquero, 1997).

Como se ha expuesto anteriormente, la alubia ha sufrido una domesticación múltiple en parcelas que se mantuvieron aisladas entre sí durante miles de años, con lo que existirán importantes casos de coevolución entre el cultivo de la judía y los agentes bióticos y abióticos presentes en su ambiente (Gepts y Bliss, 1985), es decir las transformaciones que se producen durante la evolución de las especies y las influencias que interactúan en ellas (Moënne-Loccoz et al., 2015). Así Stavely (1984) observó una susceptibilidad diferencial de las variedades de alubia a las razas de roya (*Uromyces phaseoli*) y Rennie y Kemp (1983) detectaron, al ensayar con varias poblaciones de alubia la eficiencia en la fijación de nitrógeno usando cepas individuales de *Rhizobium*, que había diferencias significativas entre poblaciones e interacciones cepas-población.

Para muchos fitopatógenos, la falta de una resistencia efectiva junto con las limitaciones de los fitosanitarios hace que sea difícil su control con lo que se ven afectadas las producciones. Sin embargo, se han identificado suelos en los que hay una baja incidencia de enfermedades en condiciones aparentemente favorables para el desarrollo de la enfermedad (Mazzola, 2002). Esta supresividad natural y/o inducida se ha identificado, y los microorganismos de los suelos son críticos para el control de enfermedades de las plantas. La supresión de la enfermedad natural no se ve afectada por el huésped, sino que puede estar determinada por las características físico-químicas del suelo y sus efectos en las comunidades microbianas del suelo. Por el contrario, la supresión inducida depende no sólo de las características del suelo, sino también de las plantas cultivadas en el suelo, de la rotación de cultivos y de las estrategias de manejo (Bailey y Lazarovits, 2003). Así los suelos supresores de enfermedades se caracterizan frecuentemente por altas densidades y/o diversidad de microorganismos antagonistas (Adesina et al., 2007; Bonanomi et al., 2010; Mazzola, 2004). Existen diversos ejemplos en los que la disminución de una fitopatología se ve influenciada por factores bióticos y/o abióticos. Se ha visto que la disminución de la gravedad de la enfermedad causada por *R. solani* se debe a las sucesivas siembras de un huésped óptimo para este fitopatógeno y que se atribuye al aumento de la presencia de *Trichoderma* spp. en ese suelo (Henis, 1978; Liu y Baker, 1980). En otros estudios se ha demostrado que el pH del suelo tenía una influencia indirecta en *T. harzianum*, el crecimiento y la germinación de esporas se estimuló a pH bajo y la supresión a *R. solani* se indujo por monocultivo sólo en suelos acidificados o naturalmente ácidos (Chet y Baker, 1980).

Es útil conocer la variabilidad de las enfermedades y plagas en los lugares originales y correlacionar esta información con las variedades locales de alubia que han tenido una evolución conjunta en una región concreta. A tenor de las evidencias de coevolución de la alubia con factores bióticos y abióticos y de la concentración local de caracteres interesantes, los agricultores deberán trabajar con variedades locales para afrontar de modo sostenible las limitaciones bióticas y abióticas.

6. INTERACCIONES ENTRE ALUBIA, *R. SOLANI* Y *TRICHODERMA*

Cuando un organismo es sometido a un estrés, bien sea biótico o abiótico, éste va a producir una serie de cambios tanto estructurales como en producción de metabolitos, expresión de genes, y otros con el fin de responder a ese estímulo.

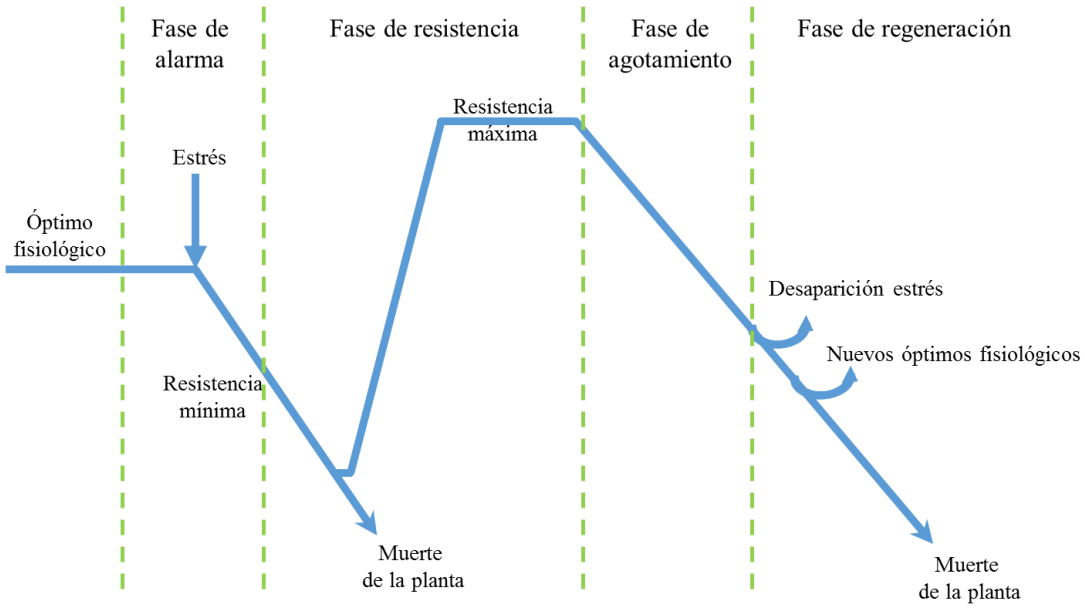

Figura 7: Respuesta de las plantas al estrés (Tadeo y Gómez-Cadenas, 2008).

Las plantas se desarrollan en un estado fisiológico óptimo (Figura 7) ya que se han adaptado a las condiciones en las que están creciendo. En cuanto se presenta el estrés las plantas ofrecen una resistencia mínima a éste, con lo que van a ralentizar sus funciones vitales reduciendo así su desarrollo; esta fase de alarma (Figura 7) es la que desencadenará todos los mecanismos que tiene para superarlo. Si no posee los adecuados se producirá la muerte de la planta, pero si tiene los mecanismos adecuados entrará en una fase de resistencia (Figura 7) alcanzando un nivel máximo en un momento dado. Si continúa la presencia del estrés, la planta entrará en una fase de agotamiento (Figura 7) pudiendo ocasionar la muerte de la planta si no desaparece. Si por el contrario finaliza, la planta restablece sus funciones fisiológicas, pudiéndose regenerar y alcanzar un nuevo estado fisiológico óptimo para las condiciones presentes, llegando a la fase de regeneración (Figura 7). Este proceso sería habitual a lo largo de la vida de la planta (Tadeo y Gómez-Cadenas, 2008).

6.1. Interacciones entre *R. solani* y alubia

Cuando un patógeno, en este caso *R. solani*, pretende invadir una planta pueden producirse distintas interacciones. Si hay una interacción incompatible, el patógeno no tendrá éxito en el ataque bien porque la planta no sea el huésped adecuado, bien porque el patógeno no pueda superar las barreras estructurales o químicas de la planta o bien porque reconozca en la planta mecanismos de defensa adecuados. Sin embargo si se produce una interacción compatible, el patógeno será virulento e invadirá la planta (Tadeo y Gómez-Cadenas, 2008).

Si *R. solani* detecta la planta huésped que va a colonizar, como es el caso de la alubia, empieza a liberar sustancias que degradan la pared celular que la planta, como pueden ser enzimas, o bien toxinas que afecten al desarrollo o al metabolismo de la misma.

Las enzimas segregadas por *R. solani* degradan las sustancias pécticas que unen las paredes celulares del parénquima de la alubia causando ensanchamiento y degradación de la pared celular y muerte de las células con antelación al contacto de las hifas invasoras. Las toxinas que se producen causan cambios en la permeabilidad de la membrana celular afectando al desarrollo óptimo de la planta (Jiménez-Díaz, 1996).

Una vez que la planta ha sido infectada o es susceptible de serlo, se desencadenan una serie de procesos para reprimir ese ataque y evitar así la invasión del fitopatógeno. La planta puede desarrollar mecanismos constructivos como la modificación de la composición de la cutícula, producción de cera epicuticular, y otros (Boller y Felix, 2009; Grotewold, 2005; Veitch, 2009). Otro mecanismo de actuación para evitar la acción del fitopatógeno es la participación de cationes, sobre todo del Ca^{2+} ya que éste se acumula alrededor de las lesiones dando lugar a la liberación de pectín-metil-esterasas de la pared celular del huésped, formándose pectatos de calcio u otros cationes multivalentes que son resistentes a la degradación por parte de la poligalacturonasa del patógeno (Jiménez-Díaz, 1996).

Cuando la planta detecta la presencia de un patógeno activa su sistema de defensa lo que implica la producción de ácido salicílico, molécula señal que conduce a la acumulación de proteínas relacionadas con la patogénesis que se cree que contribuyen a la resistencia. Este tipo de respuesta se conoce como respuesta sistémica adquirida (*Systemic Acquired Resistance*, SAR) (Druzhinina et al., 2011).

Otra respuesta que tiene la planta es la defensa necrótica o hipersensible, es decir, induce la muerte selectiva de algunas células para impedir el avance de *R. solani (Tadeo y Gómez-Cadenas, 2008)*. Los cambios en las reacciones hipersensibles comprenden la pérdida de la permeabilidad de las membranas celulares, aumento de la respiración y la producción de fitoalexinas. Como resultado final se produce la muerte y colapso de las células infectadas y las que las rodean. Los tejidos necróticos aíslan al fitopatógeno causándole la muerte ya que depende por completo de la planta para nutrirse, crecer y propagarse. Es probable que cuanto más rápido mueran las células del hospedador después de haber sido infectadas, éste se haga más resistente a la infección (Agrios, 2002).

La planta responde a la invasión de un fitopatógeno o bien de un agente de biocontrol activando la expresión de los genes de defensa y produciendo metabolitos secundarios como las fitoalexinas (fenoles, isoflavonas, terpenos, y otros) y varias sustancias que inhiben la expansión del patógeno como son ligninas y callosas (Chen et al., 2015). Las fitoalexinas son sustancias tóxicas que se producen en cantidades apreciables sólo después de haber sido estimuladas por los diferentes tipos de microorganismos o bien después de que la planta haya sufrido daños causados por agentes químicos o mecánicos. Se producen por las células sanas adyacentes a las células necróticas y dañadas, en respuesta a señales que difunden a partir de las células dañadas. La resistencia ocurre cuando las fitoalexinas alcanzan una concentración suficiente para inhibir el desarrollo del patógeno. La mayoría de las fitoalexinas conocidas son tóxicas para los hongos fitopatógenos ya que inhiben su desarrollo (Agrios, 2002).

6.2. Interacciones entre *Trichoderma* y alubia

Trichoderma al ser un organismo simbionte no virulento será beneficioso para la planta por muchas causas. Se encuentra presente en la mayoría de los suelos, demostrando su alto potencial oportunista y su adaptación a varias condiciones ecológicas.

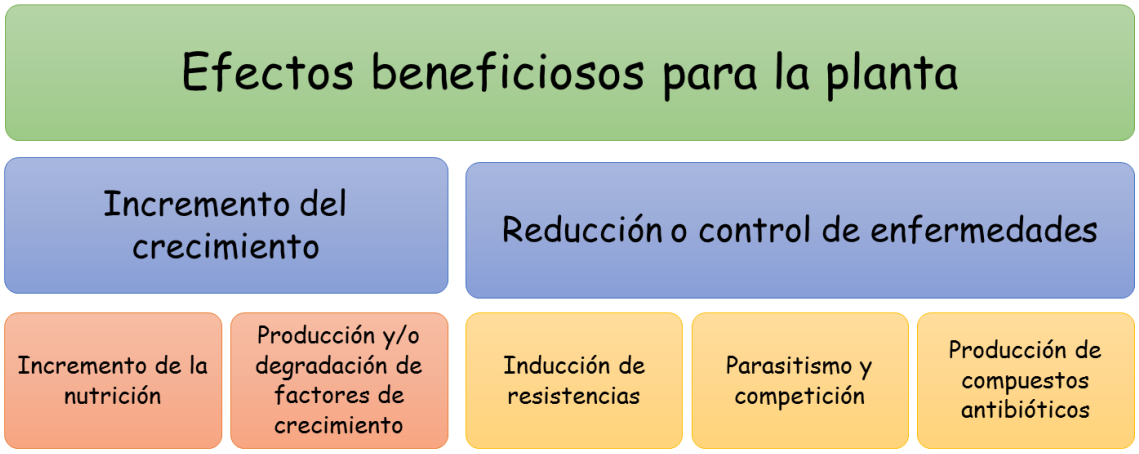

Figura 8: Efectos de *Trichoderma* sobre la planta.

Una de las múltiples formas que tiene *Trichoderma* de beneficiar a la planta de alubia es un incremento de su desarrollo (Figura 8 y Figura 9), aumentando los nutrientes disponibles para ella. *Trichoderma* produce ácidos orgánicos, como ácido cítrico o fumárico que reducen el pH del suelo y permiten la solubilización de fosfatos, micronutrientes y cationes como hierro, manganeso y magnesio (Benítez et al., 2004; Harman, et al., 2004). Se ha observado que *T. harzianum* generalmente reduce la presencia de Fe, Mn, Zn y Cu en la biomasa foliar de la alubia pero que se incrementa el contenido de Cu en las semillas (Öğüt y Er, 2006). Los nutrientes, y en particular el P, podrían incrementar su disponibilidad, favoreciendo así el crecimiento de la planta. En el caso de solubilización de compuestos insolubles o con baja solubilidad, se ha descrito que *Trichoderma* podría solubilizar Fe_2O_3, MnO_2, Zn y fosfatos, en condiciones *in vitro,* gracias a procesos de quelación y la actividad de oxidación-reducción (Altomare et al., 1999). *Trichoderma* también puede incrementar la capacidad fotosintética de las plantas ya que puede sobrerregular la producción de proteínas relativas a la fotosíntesis (Harman, 2000).

Otra forma de incrementar el desarrollo de las plantas es producir o degradar factores de crecimiento (Figura 8). *Trichoderma* puede mediar en la producción de etileno, con lo que al reducir su producción podría aumentar el desarrollo de la planta. *T. asperelloides* posee un gen α-1-aminociclopropano-1-carboxilato (ACC) que codifica una enzima que escinde ACC, un intermediario clave en la biosíntesis de etileno (Wang et al., 2002). Una asociación entre el incremento de desarrollo de la planta y la reducción de la síntesis de etileno podría deberse a un descenso de un precursor de ACC debido a la producción del ácido indol-3-acético (IAA) (auxina que promueve el desarrollo de las raíces) en la rizosfera y/o a la actividad de la ACC desaminasa (ACCD) (Hermosa et al., 2013; Shoresh et al., 2010). *Trichoderma* también produciría IAA y ACCD con lo que manipularía el patrón de regulación de estas fitohormonas (Contreras-Cornejo et al., 2009; Viterbo et al., 2010). Las nitrilasas serían otras enzimas que hidrolizarían β-ciano-l-alanina, que es un metabolito que se forma a partir de cianuro liberado durante la etapa final de biosíntesis de etileno (Piotrowski y Volmer, 2006).

Otro compuesto es una pirona volátil que produce *Trichoderma*, 6-pentyl-2*H*-pyran-1-one (6PP), la cual es responsable de la pigmentación amarilla y el olor a coco de algunos asilamientos (Hermosa et al., 2013). 6PP inhibe el desarrollo de fitopatógenos como *Fusarium oxysporum*, pero en bajas dosis regula el desarrollo de la planta, aumentando el crecimiento de las raíces, el peso y tamaño de la parte aérea e incrementando la germinación y el peso de las semillas (Rubio et al., 2009). El ácido harziánico, junto con 6PP y harzianolida , son otros compuestos que en función de la concentración en la que se encuentren afectarán al desarrollo de la planta además de tener una actividad antifúngica (Lorito, et al., 2010; Vinale et al., 2009). Otro de los compuestos que produce *Trichoderma* es farnesol, que se ha comprobado que en pequeñas dosis (10 µM y 100 µM) puede ser perjudicial para el desarrollo de la planta al alterar la síntesis del ácido abscísico, si bien cuando se incrementa la cantidad aportada a 2 mM, provoca un aumento en el desarrollo de las raíces de alubia (Mayo et al., 2016).

Trichoderma también es beneficioso para las plantas de alubia protegiéndolas frente a fitopatógenos empleando distintos métodos. Así, puede inducir los sistemas defensivos de la propia planta, competir con el fitopatógeno o bien producir compuestos antibióticos que inhiban el desarrollo de éste (Figura 8 y Figura 9).

Una vez que la planta ha detectado a *Trichoderma*, gracias a la producción de patrones moleculares asociados a microorganismos (*Microorganism-Associated Molecular Patterns*, MAMP) por parte del agente de biocontrol, se desencadena la producción de metabolitos que actuarían como respuesta para defenderse del ataque. Las moléculas que se pueden asociar como MAMP serían xilanasas, peptaiboles, swollenina, ceratoplatanina (Druzhinina et al., 2011) hidrofobinas, esteroles y otros metabolitos secundarios y enzimas (Lorito et al., 2010), los cuales desencadenan una respuesta defensiva en la planta. De esta forma se induce un sistema de resistencia inducida (*Induced Systemic Resistance*, ISR) que se trata de un proceso por el que la planta responde a un organismo no patógeno con una cascada de señales dependientes del jasmonato y del etileno como pueden ser la represión o sobreexpresión de genes o la producción de metabolitos involucrados en la respuesta defensiva de la planta (Shoresh et al., 2010). Ésta sería la primera fase por la que la planta responde a la invasión del hongo, ya que al tratarse de *Trichoderma,* en principio, sólo se genera una respuesta ISR, ya que en el caso de un fitopatógeno que ataca a la planta se desencadena una respuesta secundaria SAR.

La interacción de la raíz de la planta y *Trichoderma* conlleva una fase de reconocimiento, ataque, penetración, colonización y transferencia de nutrientes. Al tener una relación simbiótica, la planta también aporta una serie de beneficios al agente de biocontrol como es el transporte de sacarosa por parte de la planta y su posterior hidrólisis por *Trichoderma,* influyendo en su expansión en la rizosfera y su penetración en la raíz de la misma (Vargas et al., 2011).

6.3. Interacciones entre *R. solani* y *Trichoderma*

Una de las formas que tiene *Trichoderma* para beneficiar a la planta es protegiéndola frente a hongos fitopatógenos. Así puede realizar una acción micoparasitaria, pero también puede competir con éste por nutrientes, espacio, luz, y otros (Figura 9).

El micoparasitismo consiste en el reconocimiento del hongo, su ataque y penetración en él hasta causar la muerte del mismo. Este proceso conlleva distintas fases. *Trichoderma* detecta al patógeno, y sin llegar a establecer contacto físico con él, comienza a crecer por tropismo hacia su dirección (Chet et al., 1981; Lu et al., 2004). En este proceso *Trichoderma* secreta enzimas que hidrolizan la

pared celular del patógeno (Howell, 2003; Woo et al., 2006) tales como exoquitinasas que provocan la liberación de oligómeros del fitopatógeno, los que inducen la expresión por parte de *Trichoderma* de las endochitinasas que atacan a dicho patógeno incluso sin llegar a estar en contacto el agente de biocontrol con el mismo (Brunner et al., 2003; Lorito et al., 1993; Lorito, et al., 1994). Otras enzimas que segrega *Trichoderma* para degradar la pared celular del fitopatógeno son celulasas, chitinasas y glucanasas (Vinale et al., 2008). La mayor parte del sistema antifúngico de *Trichoderma* consiste en una producción de una gran variedad de enzimas líticas incluyendo endochitinasas, N-acetyl-β-glucosaminidasas, chitin 1,4-β-chitobiosidasas, proteasas, glucan β-1,3-glucosidasas, glucan β-1,6-glucosidasas, glucan α-1,3-glucosidasas, lipasas, xylanasas, mannanasas, pectinasas, pectin-lyasas, amylasas, fosfolipasas, RNAsas, DNAsas, etc (Sanz et al., 2004).

Trichoderma también excreta compuestos con actividad antimicrobiana al producir una mezcla de metabolitos secundarios que controlarían el desarrollo de fitopatógenos (Cardoza et al., 2005; Reino et al., 2007) como los derivados de la pirona [6-pentyl-2H-pyran-2-one (6-PP)] (Keszler et al., 2000) y otros peptaiboles (Vizcaino et al., 2005). Otros metabolitos secundarios son compuestos solubles en agua, como ácido heptelídico o ácido koníngico (Vinale et al., 2008).

La competencia por nutrientes con el fitopatógeno es otra interacción habitual. *Trichoderma* es un excelente competidor por espacio y recursos naturales ya que, al encontrarse en una gran cantidad de hábitats posee mejores mecanismos de adaptación al medio siendo así un excelente agente de biocontrol (Howell, 2003).

R. solani como respuesta a la acción de *Trichoderma*, produce compuestos que tratan de evitar el avance de este último, como moléculas con respuesta al estrés oxidativo [*Reactive Oxygen Species* (ROS)] que se producen durante la formación de esclerocios, y que junto con otros metabolitos antifúngicos (Aliferis y Jabaji, 2010) provocan una respuesta de estrés en *Trichoderma* y un proceso de desintoxicación (Druzhinina et al., 2011). Se ha comprobado que un aislamiento de *T. atroviridis,* al cual se le bloqueó un gen que codificaba transporte de metabolitos a través de la membrana, reducía su capacidad de biocontrol frente a *R. solani* ya que promovía la desintoxicación del mismo cuando era atacado por el patógeno (Ruocco et al., 2009).

6.4. Interacciones entre *R. solani,* alubia y *Trichoderma*

Anteriormente se han explicado las interacciones que existen entre patógeno y planta, agente de biocontrol y planta, y entre patógeno y agente de biocontrol (Figura 9). En la actualidad existen estudios en los que se plantea que puede existir una triple interacción entre los tres agentes implicados. Así se ha visto que la respuesta defensiva de la planta es diferente cuando están presentes ambos hongos, afectando tanto a la expresión génica, como a la metabolómica y a la proteómica.

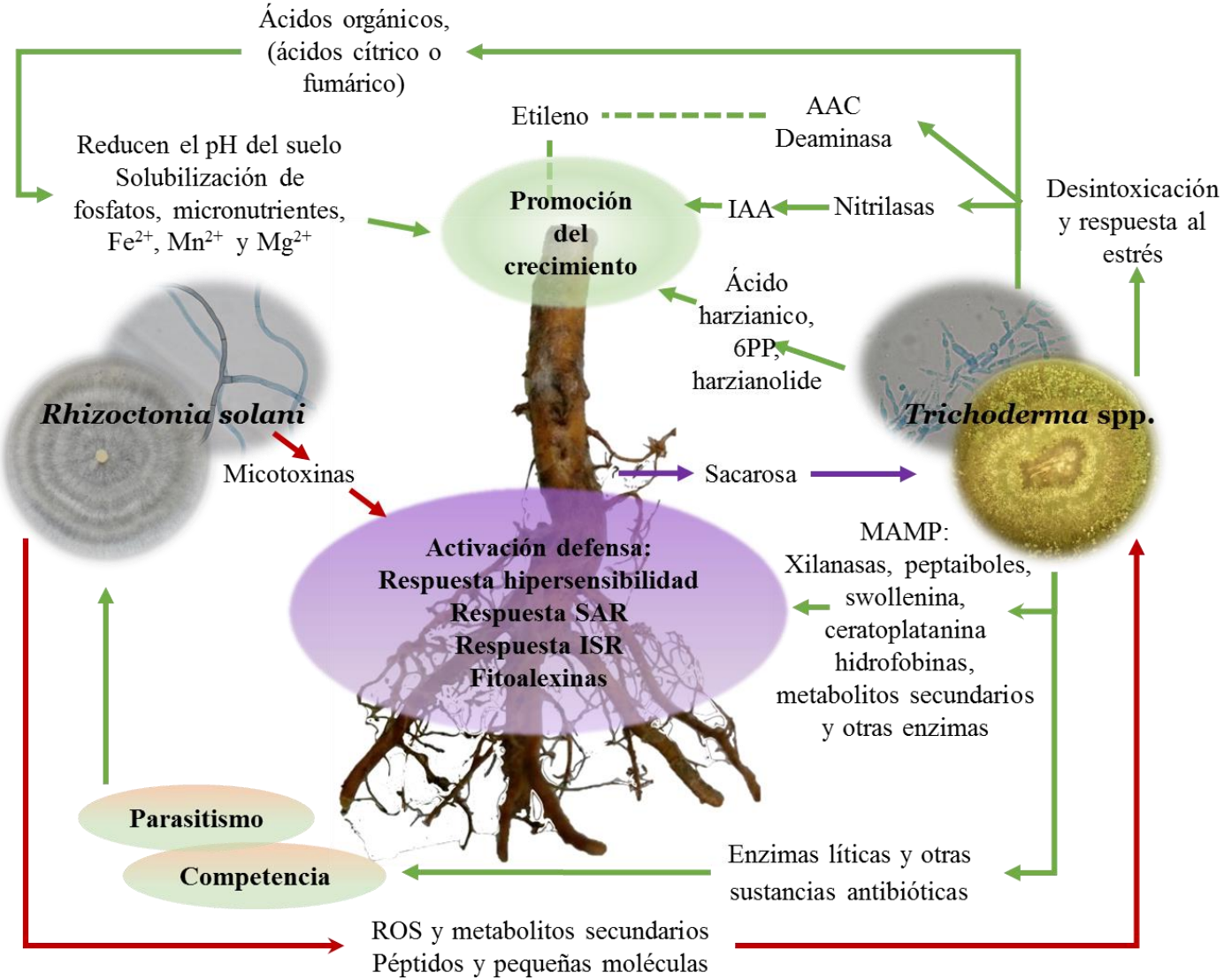

Figura 9: Interacciones entre el patógeno *R. solani*, la planta de alubia, *P. vulgaris*, y el agente de biocontrol *Trichoderma*. Las líneas y círculos de color verde son los compuestos, y las acciones que provoca *Trichoderma*. Las líneas de color rojo son los compuestos y respuestas que causa *R. solani*. Las líneas y círculos de color morado son los compuestos y las respuestas de la alubia a las actuaciones de ambos hongos (Altomare et al., 1999; Howell, 2003; Rubio et al., 2009; Vargas et al., 2011; Vinale et al., 2008).

7. BIBLIOGRAFÍA

Adesina, M. F., Lembke, A., Costa, R., Speksnijder, A., y Smalla, K. (2007). Screening of bacterial isolates from various European soils for *in vitro* antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum*: Site-dependent composition and diversity revealed. *Soil Biol. Biochem.* 39, 2818-2828. doi:10.1016/j.soilbio.2007.06.004.

Agrios, G. N. (2002). *Fitopatología*. 2ª ed. Limosa.

Aliferis, K. A., y Jabaji, S. (2010). Metabolite composition and bioactivity of *Rhizoctonia solani* sclerotial exudates. *J. Agric. Food Chem.* 58, 7604-7615. doi:10.1021/jf101029a.

Altomare, C., Norvell, W. A., Björkman, T., y Harman, G. E. (1999). Solubilization of phosphates and micronutrients by the plant-growth- promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Appl. Environ. Microbiol.* 65, 2926-2933.

American Phytopathological Society (2005). *Compendium of bean diseases*. 2nd ed. , eds. Schwartz HF, Steadman J R, Hall R, y Forster R L APS Press, American Phytopathological Society.

Asensio, C. (1996). «La judía I», en *El cultivo de las leguminosas grano en Castilla y León*, eds. F. Franco y A. Ramos (Valladolid: Junta de Castilla y León. Consejería de Agricultura), 295-318.

Bailey, K. ., y Lazarovits, G. (2003). Suppressing soil-borne diseases with residue management and organic amendments. *Soil Tillage Res.* 72, 169-180. doi:10.1016/S0167-1987(03)00086-2.

Barnett, H. L., y Hunter, B. B. (1998). *Illustrated genera of imperfect fungi*. 4.ª ed. St. Paul, Minnesota, USA: APS Press.

Beebe, S. E., Corrales, M. P., Schoonhoven, A. van, y Voysest, O. (1991). «Breeding for disease resistance», en *Common beans: research for crop improvement.*, ed. O. Schoonhoven, A. van;Voysest (Wallingford: CAB International), 561-617.

Benítez, T., Rincón, A. M., Limón, M. C., y Codón, A. C. (2004). Biocontrol mechanisms of *Trichoderma* strains. *Int. Microbiol.* 7, 249-260. doi:10.2436/IM.V7I4.9480.

Berra, D., y Arteaga, G. (1989). El complejo parasitario del pie de la judía en el País Vasco. *Cuad. Fitopatol.* 2, 53-57.

Bissett, J., Gams, W., Jaklitsch, W., y Samuels, G. J. (2015). Accepted *Trichoderma* names in the year 2015. *IMA Fungus* 6, 263-295. doi:10.5598/imafungus.2015.06.02.02.

Bitocchi, E., Bellucci, E., Giardini, A., Rau, D., Rodriguez, M., Biagetti, E., et al. (2013). Molecular analysis of the parallel domestication of the common bean (*Phaseolus vulgaris*) in Mesoamerica and the Andes. *New Phytol.* 197, 300-313. doi:10.1111/j.1469-8137.2012.04377.x.

Bitocchi, E., Nanni, L., Bellucci, E., Rossi, M., Giardini, A., Zeuli, P. S., et al. (2012). Mesoamerican origin of the common bean (*Phaseolus vulgaris* L.) is revealed by sequence data. *Proc. Natl. Acad. Sci.* 109, E788-E796. doi:10.1073/pnas.1108973109.

Boller, T., y Felix, G. (2009). A Renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379-406. doi:10.1146/annurev.arplant.57.032905.105346.

Bonanomi, G., Antignani, V., Capodilupo, M., y Scala, F. (2010). Identifying the characteristics of organic soil amendments that suppress soilborne plant diseases. *Soil Biol. Biochem.* 42, 136-144. doi:10.1016/j.soilbio.2009.10.012.

Bordoloi, D., Paul, S., Das, R., y Roy, A. (1998). Effect of pre-sowing seed treatment on emergence and seedling mortality of French bean. *Ann. Agric. Res.* 19, 348-349.

Brunner, K., Peterbauer, C. K., Mach, R. L., Lorito, M., Zeilinger, S., y Kubicek, C. P. (2003). The

Nag1 N-acetylglucosaminidase of *Trichoderma atroviride* is essential for chitinase induction by chitin and of major relevance to biocontrol. *Curr. Genet.* 43, 289-295.

Campelo, M. P., Lorenzana, A., Marcos, M. F., Ámez, M., Boto, J. A., y Casquero, P. A. (2007). Eficacia en campo de dos fungicidas para el control del «mal del pie» de la judía (*Phaseolus vulgaris*) en la La Bañeza (León). *Boletín Sanid. Veg.* 33, 137-142.

Cardoza, R. E., Vizcaíno, J. A., Hermosa, M. R., Sousa, S., González, F. J., Llobell, A., et al. (2005). Cloning and characterization of the *erg1* gene of *Trichoderma harzianum*: Effect of the *erg1* silencing on ergosterol biosynthesis and resistance to terbinafine. *Fungal Genet. Biol.* 43, 164-178. doi:10.1016/j.fgb.2005.11.002.

Casquero, P. A. (1997). Comportamiento agronómico, variabilidad genética y relaciones taxonómicas de las variedades de alubia (*Phaseolus vulgaris* L.) de la Península Ibérica. Tesis doctoral, Universidad de Santiago de Compostela, España.

Casquero, P. A., González, A. M., Santalla, M., y De Ron, A. M. (2005). Assessment of dual culinary use potential of common bean landraces in sustainable agriculture. *Biol. Agric. Hortic.* 22, 335-347. doi:10.1080/01448765.2005.9755296.

Casquero, P. A., Lema, M., Santalla, M., y De Ron, A. M. (2006). Performance of common bean (*Phaseolus vulgaris* L.) landraces from Spain in the Atlantic and Mediterranean environments. *Genet. Resour. Crop Evol.* 53, 1021-1032. doi:10.1007/s10722-004-7794-1.

Chen, X., Qi, X., y Duan, L.-X. (2015). «Overview», en *Plant Metabolomics*, eds. X. Qi, X. Chen, y Y. Wang (Dordrecht: Springer Netherlands), 1-24. doi:10.1007/978-94-017-9291-2_1.

Chet, I., y Baker, R. (1980). Induction of suppressiveness to *Rhizoctonia solani* in soil. *Phytopathology* 70, 994-998.

Chet, I., Harman, G. E., y Baker, R. (1981). *Trichoderma hamatum*: Its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microb. Ecol.* 7, 29-38. doi:10.1007/BF02010476.

Chet, I., y Inbar, J. (1994). Biological control of fungal pathogens. *Appl. Biochem. Biotechnol.* 48, 37-43.

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Cortes-Penagos, C., y López-Bucio, J. (2009). *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. *Plant Physiol.* 149, 1579-1592. doi:10.1104/pp.108.130369.

Debouck, D. G., Toro, O., Paredes, O. M., Johnson, W. C., y Gepts, P. (1993). Genetic diversity and ecological distribution of *Phaseolus vulgaris* (Fabaceae) in northwestern South America. *Econ. Bot.* 47, 408-423. doi:10.1007/BF02907356.

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., et al. (2011). *Trichoderma*: The genomics of opportunistic success. *Nat. Rev. Microbiol.* 9, 749-759.

FAO (2016). FAOSTAT. *Food Agric. Organ. United Nations*. Available at: http://www.fao.org/faostat/en/#data/QC [Accedido diciembre 29, 2016].

Gepts, P., y Bliss, F. A. (1985). F1 hybrid weakness in the common bean: Differential geographic origin suggets two gene pools in cultivated bean germplasm. *J. Hered.* 76, 447-450. doi:10.1093/oxfordjournals.jhered.a110142.

Gepts, P., Osborn, T. C., Rashka, K., y Bliss, F. A. (1986). Phaseolin-protein variability in wild forms and landraces of the common bean (*Phaseolus vulgaris*): Evidence for multiple centers of domestication. *Econ. Bot.* 40, 451-468. doi:10.1007/BF02859659.

González García, V., Portal Onco, M. A., y Rubio Susan, V. (2006). Review. Biology and systematics of the form genus *Rhizoctonia*. *Spanish J. Agric. Res.* 4, 55. doi:10.5424/sjar/2006041-178.

Graham, P. H., y Ranalli, P. (1997). Common bean (*Phaseolus vulgaris* L.). *F. Crop. Res.* 53, 131-146. doi:10.1016/S0378-4290(97)00112-3.

Grotewold, E. (2005). Plant metabolic diversity: a regulatory perspective. *Trends Plant Sci.* 10, 57-62. doi:10.1016/j.tplants.2004.12.009.

Gupta, S., Mathew, K., Shyam, K., y Sharma, A. (1999). Fungicidal management of root rot (*Rhizoctonia solani*) of French bean. *Plant Dis. Res.* 14, 20-24.

Hagedorn, D. J. (1991). «Rhizoctonia Root Rot», en *Compendium of Bean Diseases*, ed. R. Hall (APS, American Phyopathological Society), 13.

Harman, G. E. (2000). Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzinum* T-22. *Plant Dis.* 84, 377-393. doi:10.1094/PDIS.2000.84.4.377.

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., y Lorito, M. (2004). *Trichoderma* species--opportunistic, avirulent plant symbionts. *Nat. Rev.* 2, 43-56. doi:10.1038/nrmicro797.

Harman, G. E., y Kubicek, C. P. (1998). Trichoderma *and* Gliocladium*. Volume 2: Enzymes, Biological Control and commercial applications*. United Kindong: CRC Press.

Harman, G. E., y Kubicek, C. P. (2002). Trichoderma *and* Gliocladium*. Volume 1: Basic biology, taxonomy and genetics*. United Kindong: CRC Press.

Henis, Y. (1978). Integrated control of *Rhizoctonia solani* damping-off of radish: Effect of successive plantings, PCNB, and *Trichoderma harzianum* on pathogen and disease. *Phytopathology* 68, 900. doi:10.1094/Phyto-68-900.

Hermosa, R., Belén Rubio, M., Cardoza, R. E., Nicolás, C., Monte, E., y Gutiérrez, S. (2013). The contribution of *Trichoderma* to balancing the costs of plant growth and defense. *Int. Microbiol.* 16, 69-80.

Hjeljord, L., y Tronsmo, A. (1998). «*Trichoderma* and *Gliocladium* in biological control: an overview», en Trichoderma *and* Gliocladium *Vol 2*, eds. G. E. Harman y C. P. Kubicek (Taylor and Francis London), 131-151.

Howell, C. R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.* 87, 4-10.

Index Fungorum (2017). *Index Fungorum*. Available at: http://www.indexfungorum.org/.

International Mycological Association (2016). Mycobank database. Fungal databases, nomenclature & species banks. *Mycobank database*. Available at: http://www.mycobank.org.

Jiménez-Díaz, R. M. (1996). «Interacciones planta-hongo: Mecanismos de infección, patogénesis y resistencia», en *Patología vegetal* (Valencia, Spain: Mundi-Prensa, Phytoma, Sociedad Española de Fitopatología), 739-769.

Junta de Castilla y León (2015). Superficies de cultivos herbáceos por provincias, comarcas y municipio. Available at: http://www.agriculturaganaderia.jcyl.es/web/jcyl/AgriculturaGanaderia/es/Plantilla100/1284675455995/_/_/_ [Accedido diciembre 30, 2016].

Junta de Castilla y León (2016). Anuario de estadistica agraria. Anuario 2014. Leguminosas. Available at: http://www.agriculturaganaderia.jcyl.es/web/jcyl/AgriculturaGanaderia/es/Plantilla100/1284495917557/_/_/_ [Accedido diciembre 30, 2016].

Kami, J., Velásquez, V. B., Debouck, D. G., y Gepts, P. (1995). Identification of presumed ancestral DNA sequences of phaseolin in *Phaseolus vulgaris*. *Proc. Natl. Acad. Sci.* 94, 1101-1104.

Keszler, A., Forgács, E., Kótai, L., Vizcaíno, J. A., Monte, E., y García-Acha, I. (2000). Separation and identification of volatile components in the fermentation broth of *Trichoderma atroviride*

by solid-phase extraction and gas chromatography-mass spectrometry. *J. Chromatogr. Sci.* 38, 421-4. doi:10.1093/CHROMSCI/38.10.421.

Lioi, L., y Piergiovanni, A. R. (2013). «European Common Bean», en *Genetic and genomic resources of grain legume improvement*, eds. M. Singh, H. D. Upadhyaya, y I. S. Bisht (Elsevier), 11-40. doi:10.1016/B978-0-12-397935-3.00002-5.

Liu, S., y Baker, R. (1980). Mechanism of biological control in soil suppressive to *Rhizoctonia solani*. *Phytopathology* 70, 404-412. doi:10.1094/Phyto-70-404.

Lorito, M., Harman, G. E., Hayes, C. K., Broadway, R. M., Tronsmo, A., Woo, S. L., et al. (1993). Chitinolytic enzymes produced by *Trichoderma harzianum* : antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology* 83, 302-307.

Lorito, M., Hayes, C. K., Di Pietro, A., Woo, S. L., y Harman, G. E. (1994). Purification, characterization, and synergistic activity of a glucan 1,3-β-glucosidase and an N-acetyl-β-glucosaminidase from *Trichoderma harzianum*. *Phytopathology* 84, 398-405.

Lorito, M., Woo, S. L., Harman, G. E., y Monte, E. (2010). Translational research on *Trichoderma*: From 'omics to the field. *Annu. Rev. Phytopathol.* 48, 395-417. doi:10.1146/annurev-phyto-073009-114314.

Lu, Z., Tombolini, R., Woo, S., Zeilinger, S., Lorito, M., y Jansson, J. K. (2004). *In vivo* study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Appl. Environ. Microbiol.* 70, 3073-81. doi:10.1128/AEM.70.5.3073-3081.2004.

Mamidi, S., Rossi, M., Annam, D., Moghaddam, S., Lee, R., Papa, R., et al. (2011). Investigation of the domestication of common bean (*Phaseolus vulgaris*) using multilocus sequence data. *Funct. Plant Biol.* 38, 953. doi:10.1071/FP11124.

Mayo, S., Izquierdo, H., González-López, Ó., Rodríguez-González, Á., Lorenzana, A., Gutiérrez, S., et al. (2016). Effect of farnesol , a compound produced by *Trichoderma* when growing on bean (*Phaseolus vulgaris* L.). *Planta Med.* 82, S1-S381. doi:10.1055/s-0036-1596823.

Mazzola, M. (2002). Mechanisms of natural soil suppressiveness to soilborne diseases. *Antonie Van Leeuwenhoek* 81, 557-564. doi:10.1023/A:1020557523557.

Mazzola, M. (2004). Assessment and management of soil microbial community structure for disease suppression. *Annu. Rev. Phytopathol.* 42, 35-59. doi:10.1146/annurev.phyto.42.040803.140408.

McClean, P. E., Lee, R. K., Otto, C., Gepts, P., y Bassett, M. J. (2002). Molecular and phenotypic mapping of genes controlling seed coat pattern and color in common bean (*Phaseolus vulgaris* L.). *J. Hered.* 93, 148-52.

Ministerio de Agricultura Pesca Alimentación y Medio Ambiente (2016). Leguminosas grano y oleaginosas - Leguminosas y oleaginosas - Cultivos herbáceos e industriales - Producciones agrícolas - Agricultura - mapama.es. Available at: http://www.mapama.gob.es/es/agricultura/temas/producciones-agricolas/cultivos-herbaceos/leguminosas-y-oleaginosas/default.aspx#para1 [Accedido diciembre 30, 2016].

Ministerio de Agricultura y Pesca Alimentación y Medio Ambiente (2015). Estadística anual de consumo de productos fitosanitarios y Estadística quinquenal de utilización de productos fitosanitarios en la agricultura. Available at: http://www.mapama.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura/estadisticas-medios-produccion/fitosanitarios.aspx [Accedido enero 6, 2017].

Moënne-Loccoz, Y., Mavingui, P., Combes, C., Normand, P., y Steinberg, C. (2015). «Microorganisms and Biotic Interactions», en *Environmental Microbiology: Fundamentals and Applications*, eds. J. C. Bertrand, P. Caumette, P. Lebaron, Matheron. R., y T. Normand,

P. Sime-Ngando (Dordrecht: Springer Netherlands), 395-444. doi:10.1007/978-94-017-9118-2_11.

Monte, E., y Suárez, M. B. (2010). «Muerte de plántulas», en *Enfermedades de las plantas causadas por hongos y oomicetos: naturaleza y control integrado*, eds. R. M. Jiménez Díaz y E. Montesinos Seguí (Phytoma), 115-133.

Mukherjee, P. K., Horwitz, B. A., Herrera-Estrella, A., Schmoll, M., y Kenerley, C. M. (2013). *Trichoderma* research in the genome era. *Annu. Rev. Phytopathol.* 51, 105-129.

Ogoshi, A. (1996). «Introduction - The genus *Rhizoctonia*», en Rhizoctonia *Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control*, eds. B. Sneh, S. Jabaji-Hare, S. Neate, y G. Dijst (Dordrecht: Springer Netherlands), 1-9. doi:10.1007/978-94-017-2901-7_1.

Öğüt, M., y Er, F. (2006). Micronutrient composition of field-grown dry bean and wheat inoculated with *Azospirillum* and *Trichoderma*. *J. Plant Nutr. Soil Sci.* 169, 699-703. doi:10.1002/jpln.200520597.

Piotrowski, M., y Volmer, J. J. (2006). Cyanide metabolism in higher plants: Cyanoalanine hydratase is a NIT4 homolog. *Plant Mol. Biol.* 61, 111-122. doi:10.1007/s11103-005-6217-9.

Reino, J. L., Guerrero, R. F., Hernández-Galán, R., y Collado, I. G. (2007). Secondary metabolites from species of the biocontrol agent *Trichoderma*. *Phytochem. Rev.* 7, 89-123. doi:10.1007/s11101-006-9032-2.

Reinoso, B. (2001). Prospección, caracterización y evaluación de variedades locales de judía grano (*Phaseolus vulgaris* L.) de la provincia de León. Tesis doctoral, Universidad de León, España.

Rennie, R. J., y Kemp, G. A. (1983). N2-fixation in field beans quantified by 15N isotope dilution. II. Effect of cultivars of beans. *Agron. J.* 75, 645-649.

Rivera, A., Casquero, P. A., Mayo, S., Almirall, A., Plans, M., Simó, J., et al. (2016). Culinary and sensory traits diversity in the Spanish core collection of common beans (*Phaseolus vulgaris* L.). *Spanish J. Agric. Res.* 14, e0701. doi:10.5424/sjar/2016141-7726.

Rodiño, A. P., Santalla, M., Montero, I., Casquero, P. A., y De Ron, A. M. (2001). Diversity of common bean (*Phaseolus vulgaris* L.) germplasm from Portugal. *Genet. Resour. Crop Evol.* 48, 409-417. doi:10.1023/A:1012248002436.

Rubio, M., Hermosa, R., Reino, J., Collado, I., y Monte, E. (2009). Thctf1 transcription factor of *Trichoderma harzianum* is involved in 6-pentyl-2H-pyran-2-one production and antifungal activity. *Fungal Genet. Biol.* 46, 17-27. doi:10.1016/j.fgb.2008.10.008.

Ruocco, M., Lanzuise, S., Vinale, F., Marra, R., Turrà, D., Woo, S. L., et al. (2009). Identification of a new biocontrol gene in *Trichoderma atroviride*: the role of an ABC transporter membrane pump in the interaction with different plant-pathogenic fungi. *Mol. Plant-Microbe Interact.* 22, 291-301. doi:10.1094/MPMI -22-3-0291.

Samuels, G. J. (1996). *Trichoderma*: a review of biology and systematics of the genus. *Mycol. Res.* 100, 923-935. doi:10.1016/S0953-7562(96)80043-8.

Santalla, M., Rodiño, A. P., y De Ron, A. (2002). Allozyme evidence supporting southwestern Europe as a secondary center of genetic diversity for the common bean. *Theor. Appl. Genet.* 104, 934-944. doi:10.1007/s00122-001-0844-6.

Sanz, L., Montero, M., Grondona, I., Vizcaíno, J. A., Llobell, A., Hermosa, R., et al. (2004). Cell wall-degrading isoenzyme profiles of *Trichoderma* biocontrol strains show correlation with rDNA taxonomic species. *Curr. Genet.* 46, 277-286. doi:10.1007/s00294-004-0532-6.

van Schoonhoven, A., y Pastor-Corrales, M. A. (1987). *Standard system for the evaluation of bean germplasm*. Cali (Colombia): Centro Internacional de Agricultura Tropical.

Shoresh, M., Harman, G. E., y Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 48, 21-43. doi:10.1146/annurev-phyto-

073009-114450.

Singh, S. P., Gepts, P., y Debouck, D. G. (1991). Races of common bean (*Phaseolus vulgaris*, Fabaceae). *Econ. Bot.* 45, 379-396. doi:10.1007/BF02887079.

Stavely, J. R. (1984). Pathogenic specialitation in *Uromyces phaseoli* in the United States and rust resistance in beans. *Plant Dis.* 68, 95-99.

Tadeo, F. R., y Gómez-Cadenas, A. (2008). «Fisiología de las plantas y el estrés», en *Fundamentos de fisología vegetal*, eds. J. Azcón-Bieto y M. Talón (Barcelona, Spain: McGraw-Hill), 577-597.

Tello, J. C., Lacasa, A., y Molina, R. (1985). Una nota fitopatológica sobre el complejo parasitario del pie de la judía (*Phaseolus vulgaris*). *ITEA* 61, 57-69.

Valenciano, J. B., Casquero, P. A., y Boto, J. A. (2004). Influence of sowing techniques and pesticide application on the emergence and the establishment of bean plants (*Phaseolus vulgaris* L.). *Agronomie* 24, 113-118. doi:10.1051/agro:2004007.

Valenciano, J. B., Casquero, P. A., Boto, J. A., y Guerra, M. (2006a). Effect of sowing techniques and seed pesticide application on dry bean yield and harvest components. *F. Crop. Res.* 96, 2-12. doi:10.1016/j.fcr.2005.04.016.

Valenciano, J. B., Casquero, P. A., Boto, J. A., y Marcelo, V. (2006b). Evaluation of the occurrence of root rots on bean plants (*Phaseolus vulgaris*) using different sowing methods and with different techniques of pesticide application. *New Zeal. J. Crop Hortic. Sci.* 34, 291-298. doi:10.1080/01140671.2006.9514419.

Vargas, W. A., Crutcher, F. K., y Kenerley, C. M. (2011). Functional characterization of a plant-like sucrose transporter from the beneficial fungus *Trichoderma virens*. Regulation of the symbiotic association with plants by sucrose metabolism inside the fungal cells. *New Phytol.* 189, 777-789. doi:10.1111/j.1469-8137.2010.03517.x.

Veitch, N. C. (2009). Isoflavonoids of the Leguminosae. *Nat. Prod. Rep.* 26, 776. doi:10.1039/b616809b.

Vinale, F., Flematti, G., Sivasithamparam, K., Lorito, M., Marra, R., Skelton, B. W., et al. (2009). Harzianic acid, an antifungal and plant growth promoting metabolite from *Trichoderma harzianum*. *J. Nat. Prod.* 72, 2032-2035. doi:10.1021/np900548p.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Woo, S. L., y Lorito, M. (2008). *Trichoderma*–plant–pathogen interactions. *Soil Biol. Biochem.* 40, 1-10. doi:10.1016/j.soilbio.2007.07.002.

Viterbo, A., Landau, U., Kim, S., Chernin, L., y Chet, I. (2010). Characterization of ACC deaminase from the biocontrol and plant growth-promoting agent *Trichoderma asperellum* T203. *FEMS Microbiol. Lett.* 305, 42-48. doi:10.1111/j.1574-6968.2010.01910.x.

Vizcaino, J. A., Sanz, L., Basilio, A., Vicente, F., Gutiérrez, S., Hermosa, M. R., et al. (2005). Screening of antimicrobial activities in *Trichoderma* isolates representing three *Trichoderma* sections. *Mycol. Res.* 109, 1397-1406. doi:10.1017/S0953756205003898.

Wang, K. L.-C., Li, H., y Ecker, J. R. (2002). Ethylene biosynthesis and signaling networks. *Plant Cell* 14, S131-S151. doi:10.1105/TPC.001768.

Weindling, R. (1932). *Trichoderma lignorum* as a parasite of other soil fungi. *Phytopathology* 22, 837-845.

Weindling, R. (1934). Studies on a lethal principle effective in the parasitic action of *Trichoderma lignorum* on *Rhizoctonia solani* and other soil fungi. *Phytopathology* 24, 151-153.

Woo, S. L., Scala, F., Ruocco, M., y Lorito, M. (2006). The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. *Phytopathology* 96, 181-185.

Zizumbo-Villarreal, D., y Colunga-GarcíaMarín, P. (2010). Origin of agriculture and plant domestication in West Mesoamerica. *Genet. Resour. Crop Evol.* 57, 813-825. doi:10.1007/s10722-009-9521-4.

CAPÍTULO I

Trichoderma species as biocontrol agents in legumes

ÍNDICE CAPÍTULO I

FIGURAS

Chapter

Trichoderma species as biocontrol agents in legumes.

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ABSTRACT

Trichoderma spp. (Teleomorph: *Hypocrea*) is a fungal genus that is found in the soil, and it is a secondary fast growing opportunistic invasive producer of chitinases, glucanases and proteases, and metabolites with antimicrobial activity. Many *Trichoderma* species are also well known as biocontrol agents of important phytopathogenic fungi. The primary mechanisms of biocontrol used by *Trichoderma* in direct confrontation with pathogenic fungi are the mycoparasitism, antibiosis, and competition for nutrients with the pathogen. *Trichoderma* species have developed opportunistic mechanisms for their adaptation to abiotic stresses as well as for nutrient uptake and solute transport. *Trichoderma*spp. is able to promote the growth of legumes. In addition, *Trichoderma* produces some organic acids such as citric or fumaric acids that reduces soil pH and allows the solubilization of phosphates and other micronutrients such as iron, manganese and magnesium in legume crops. In the legumes, *Trichoderma* spp. produce several changes in the defence response of the plant, as the accumulation of phytoalexins and down- or up-regulation of defence-related genes expression.

Keywords: Biocontrol; Defence response; Induction of resistance; Mycoparasitism; Nutrients; Rhizosphere; *Trichoderma*;

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1. INTRODUCTION

Pulses belong to the Fabaceae or Leguminosae family; these plants are the world's third largest group of plant life. They are thought to have originated some 90 million years ago, with a diversification process beginning in the early Tertiary era. The Fabaceae family contains over 20,000 species and 700 genera, of which only some are categorised as leguminous plants, such as the genus *Phaseolus*, *Vicia*, *Cicer*, *Lens,…* (Food and Agriculture Organization, 2016).

A plant is diseased when it is not functioning normally. The abnormal functioning of plant generally leads to reduction in quantity and quality of yield. Disease is the result of an interaction among the plant and its environmental and it is often affected by biotic and abiotic factors by (microorganisms, humidity, temperature…) that are detected as signals for the activation of plant response mechanisms (American Phytopathological Society, 2005). These crops are affected by a wide diversity of fungal pathogens for example *Sclerotinia* spp., *Fusarium* spp., *Pythium* spp., *Botrytis* spp, *Rhizoctonia* spp… causing some important economic losses in these crops.

A form of control to diseased is the application of chemical synthesis fungicides. Its application on the seed or directly to the soil can be effective against fungi that affect the crops during or shortly after germination (Beebe, Corrales, Schoonhoven, & Voysest, 1991) because they reduce its incidence and improve the emergence of plants (Valenciano, Casquero, & Boto, 2004). However, applications with fungicides aimed at avoiding damage caused by fungi that cause root rot or yellowing and wilting are often ineffective and usually impracticable due to the large volume of soil to which they should be directed.

Actually, the number of authorized products by protection plant has been reduced in order to ensure food safety and its sustainable in the long term. It is therefore proposed to prioritize non-chemical methods in integrated production, organic farming, and others.

As a strategy to control legume infectious diseases, mainly those caused by fungi, the use of biocontrol agents can reduce the negative effects of plant pathogens and they also can promote positive responses in the plant (Shoresh, Harman, & Mastouri, 2010). Biocontrol agents are perceived to have specific advantages over synthetic fungicides, including fewer non-target and environmental effects, efficacy against fungicide-resistant pathogens, reduced probability of resistance development and use in organic farming situations where synthetic fungicides are restricted (Brimner & Boland, 2003).

The genera including strains belonging to bacterial genera such as *Agrobacterium*, *Pseudomonas*, *Streptomyces* and *Bacillus*, and fungal genera such as *Gliocladium*, *Trichoderma*, *Ampelomyces*, *Candida* and *Coniothyrium*., are beneficial organisms that have shown good efficiency as biocontrol agents against pathogenic microorganisms (Vinale et al., 2008).

2. *TRICHODERMA* SPP.

Trichoderma spp. (Teleomorph: *Hypocrea*) is a fungal genus that is found in the soil, and it is a secondary fast growing opportunistic invasive (Mayo, Cominelli, et al., 2016) producer of chitinases, glucanases and proteases, and metabolites with antimicrobial activity (Lorito, Woo, Harman, & Monte, 2010). Many *Trichoderma* species are also well known as biocontrol agents of important phytopathogenic fungi. The primary mechanisms of biocontrol used by *Trichoderma* in direct confrontation with pathogenic fungi are the mycoparasitism, antibiosis, and competition for

nutrients with the pathogen (Harman, Howell, Viterbo, Chet, & Lorito, 2004). *Trichoderma* species colonize the root surface and cause substantial changes in plant metabolism (Shoresh et al., 2010). The physical interaction between *Trichoderma* and plants is limited to the first cell layer of the epidermis and the root bark. In addition, *Trichoderma* biocontrol strains are able to induce the expression of genes involved in defence response and also to promote plant growth, root development and nutrient uptake (Hermosa, Viterbo, Chet, & Monte, 2012) (Figure 1).

Trichoderma spp. is recognized for their important benefits to agriculture such as its ability to protect crops against diseases (Benítez, Rincón, Limón, & Codón, 2004) and increase crop yield under field conditions (Harman et al., 2004). Most species of *Trichoderma* have been linked to biocontrol and biotechnological applications (Monte, 2001), and the versatility of *Trichoderma* strains to suppress diseases caused by pathogens (Howell, 2003). Since *Trichoderma* strains grow and proliferate best when there are abundant healthy roots, they have evolved numerous mechanisms of action both to attack other fungi and to enhance plant and root growth (Benítez et al., 2004).

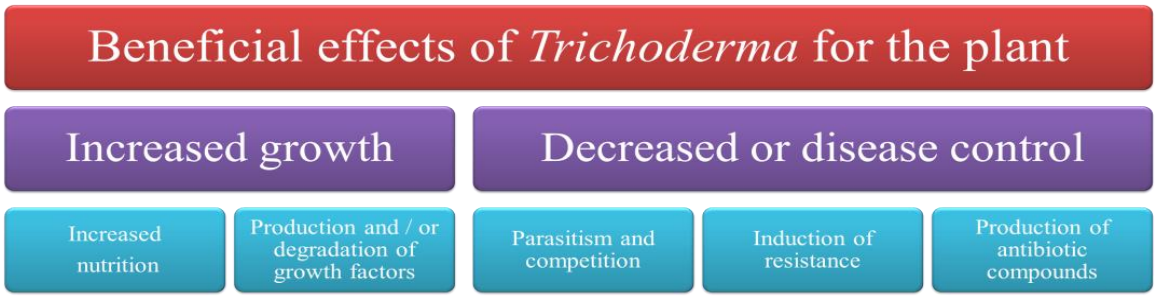

Figure 1: Beneficial effect of *Trichoderma* for the plant.

2.1. Promotion of plant growth

As rhizosphere colonizers, *Trichoderma* spp. have developed opportunistic mechanisms for their adaptation to abiotic stresses as well as for nutrient uptake and solute transport. In the plant, these processes are facilitated by the induction of cell wall extension and expansion, secondary root development, lateral root hair production and a higher photosynthetic rate (Hermosa et al., 2013; Shoresh et al., 2010)

In the culture of peanut, *Arachis hypogaea*, it has been checked the effect of *Trichoderma* inoculum concentration on plant emergence with and without a fungicide that could be used together with the antagonist species to improve seed protection and emergence. Considering that the fungicide alone is effective only for a short time and it does not spread through the soil with the root system, the use of both chemical and biological agents could increase the protection of seeds and roots (Rojo, Reynoso, Ferez, Chulze, & Torres, 2007).

In other work, when the peanuts were inoculated with four *Trichoderma* (*T. harzianum, T. viride, T. virens, T. atroviride*), length of shoot was increased between 51.78 % by *T. harzianum* and 10.48 % by *T. atroviride* respect to control plant; weight of the shoot was increased among 80.38 % (*T. harzianum*) and 14.04 % (*T. atroviride*); the weight of the roots was enhanced 64.42 % and the number of nodules per plant was 30.97 % respect to control plant when they inoculated with *T. harzianum. Trichoderma* spp. can be a useful tool that improves the microbial community in the rhizosphere, which result in an enhancement of plant growth and development. In this study

Trichoderma stimulated early growth in peanut plants, increasing the yield (Kamaruzzaman, Rahman, Islam, Ahmad, & Ahmad, 2016).

Bean plants, *P. vulgaris*, in contact with some *Trichoderma*, showed an increased size when the pathogen was not present. Other authors also observed that *T. harzianum* was able to promote the growth of common bean plants in comparison to plants grown in its absence (Pereira et al., 2014). However, plants treated with *T. harzianum* did not decrease their size in the presence of *R. solani* (Mayo et al., 2015). In other work, when bean plants were in contact with *T. velutinum* isolate, they increased respect to control plants 4.75 % their diameter of hypocotyl, 10.75 % their root system length, 4.27 % and 5.51 % in dry weight of aerial parts and root system, respectively. When plants were infected with the pathogen *R. solani*, the action of *T. velutinum* T028 caused an increased of the diameter of the hypocotyl in 8.76 %, 21.15 % in the length of root system, and 11.05 % and 3.43 % in dry weight of aerial parts and root system, respectively, respect to the control plant with the pathogen (Mayo et al., 2016). These data are in agreement with those from Yedidia and coworkers reporting a much stronger effect on cucumber plants treated with *T. harzianum*, which increased by a 75.0% the root length, 95.0% aerial parts, 80.0% dry weight and 80.0% the size of the blade relative to the untreated control (Yedidia, Srivastva, Kapulnik, & Chet, 2001). *T. harzianum* was able to promote the growth of common bean plants in comparison to plants grown in its absence (Pereira et al., 2014). In other study, it was evaluated the effect of farnesol on the development of bean plants. This compound that in fungi is presumably produced from farnesyl pyrophosphate, is a signalling molecule for self-regulation that by accumulating in the extracellular space generates a response across local fungal population. The plants were grown under controlled conditions in hydroponic culture for 14 days with different concentrations of farnesol (10 μM, 100 μM, 1000 μM, 2000 μM and 5000 μM) in the nutrition solution and they were evaluated by assessing the wet weight and dry weights of the aerial parts and root systems. The results showed a negative effect on growth at concentrations of 10 μM and 100 μM farnesol, which could be related to abscisic acid synthesis. However, with 2 mM of farnesol, bean plants showed increased development of aerial parts and root systems (Mayo, Izquierdo, et al., 2016).

In other study, Dubey and co-authors (2007) *Trichoderma* spp. signicantly reduced the wilt incidence in chickpea plants, *Cicer arietinum*, that were inoculated with *Fusarium oxysporum* f. sp. *Ciceris*. The incedence was reduced a 65.85% respect to control plants. Also, the presence of *Trichoderma* increased the germination of this culture respect to control plants with values between 13.36 % and 8.95 %, the weight of the shoot was increased 31.31 % and the weight of the root was a 41.30 % higher that in control plants.

Trichoderma produces some organic acids such as citric or fumaric acids that reduces soil pH and allows the solubilization of phosphates and other micronutrients such as iron, manganese and magnesium (Benítez et al., 2004; Harman et al., 2004). It has been observed that *T. harzianum* reduces the amount of iron, manganese, zinc and copper in the leaf biomass of beans, but increases the content of copper in its seeds (Öğüt & Er, 2006). On the other hand, there are some *in vitro* studies which indicate that *T. harzianum* and other isolates of *Trichoderma* could solubilize iron (III) oxide, manganese (IV) oxide, zinc and phosphates, which are highly insoluble compounds or with low solubility, owing to chelation processes and oxidation-reduction activity (Altomare, Norvell, Björkman, & Harman, 1999). The increment of all those nutrients, in particular phosphorus, could favor the growth of the plants, which are in contact with soils where this fungal species is present. It has been shown that *T. atroviride* produces and degrades indoleacetic acid, which when is produced in combination with ethylene by the microorganisms present in the rhizosphere causes an increase in plant growth (Gravel, Antoun, & Tweddell, 2007).

Chagas and co-authors (2016) inoculated cowpea, *Vigna unguiculata*, with some *Trichoderma* isolates. An increase in dry matter and phosphorous amounts in the aerial parts was observed in comparison to the control treatment. This effect might be explained by the phosphate solubilization ability of the biological control agents. Various factors might influence the *Trichoderma* phosphate solubilization ability, including the availability of carbon and nitrogen, the type of cultivated plants (Grayston, Vaughan, & Jones, 1997), and the type of phosphate that is solubilized (Barroso & Nahas, 2005). Similar results were also reported by Kapri and Tewari (2010), who inoculated *Trichoderma* into chickpea cultures and reported an increase in the phosphate solubilization ability *in vitro*, with a significant effect on biomass production. These works highlighted the potential of *Trichoderma* spp. as plant growth promoters in association with their phosphate solubilizing and indole acetic acid synthesis abilities.

According to other study the increase in biomass production related to growth hormones or analogs production is another mechanism by which strains of *Trichoderma* spp. can improve the growth of plants (Hoyos-Carvajal, Orduz, & Bissett, 2009).

2.2. Mycoparasitism

Mycoparasitism consists in the recognition of the fungus, attacking it and penetrating it with the purpose to cause its death. This process involves some different phases. First of all, *Trichoderma* locates the pathogen without previous contact, beginning to enlarge towards the pathogen by tropism (Chet, Harman, & Baker, 1981; Lu et al., 2004). During this process, *Trichoderma* secretes some enzymes that hydrolyze the cell wall of the pathogen (Howell, 2003; Woo, Scala, Ruocco, & Lorito, 2006). It has been studied that *Trichoderma* releases an extracellular exochitinase (Brunner et al., 2003) that might cause the liberation of some oligomers from the fungus, which could induce the expression of endochitinases that would diffuse and would start to attack to the pathogen even before the physical contact had happened. Some enzymes belonging to these fungi have been purified and used for biocontrol. When they have been assessed, they have showed antifungal activity and have controlled a large number of pathogens, such as *Fusarium, Rhizoctonia, Alternaria, Ustilago, Venturia* and *Colletotrichum* (M. Lorito et al., 1993; M. Lorito, Hayes, Di Pietro, Woo, & Harman, 1994).

A major part of the *Trichoderma* antifungal system consists of a number of genes encoding an astonishing variety of secreted lytic enzymes (Sanz et al., 2004) including endochitinases, N-acetyl-β-glucosaminidases, chitin 1,4-β-chitobiosidases, proteases, glucan β-1,3-glucosidases, glucan β-1,6-glucosidases, glucan α-1,3-glucosidases, lipases, xylanases, mannanases, pectinases, pectin lyases, amylases, phospholipases, RNAses, DNAses, etc. Some of these proteins have been purified and their corresponding genes have been cloned and characterized by our group: protease PRA1 (Suarez, Rey, Castillo, Monte, & Llobell, 2004), chitinases CHIT36 and CHIT37 (Viterbo et al., 2002), α-glucanases AGN13.1 (Ait-Lahsen et al., 2001) and AGN13.2 (Sanz, Montero, Redondo, Llobell, & Monte, 2005), and β-1,6-glucanases BGN16.2 and BGN16.3 (Montero, Sanz, Rey, Llobell, & Monte, 2007; Montero, Sanz, Rey, Monte, & Llobell, 2005).

The direct confrontation assays were used to verify the ability of *Trichoderma* spp. to overgrow the pathogen and its f mycoparasitim potential. In a study, the percentage of overgrow oscillated between 72.77 and 14.63%, according to the species of *Trichoderma* (Mayo et al., 2015).

Some *Trichoderma* spp. are selected because of their mycoparasitic mechanism but the most efficient biocontrol strains display, simultaneous or sequentially, more than one biocontrol strategy

(Howell, 2003). *Trichoderma* spp. can also exert marked antimicrobial activity (Vizcaino et al., 2005) due to the production of blends of secondary metabolites (Cardoza et al., 2005; Reino, Guerrero, Hernández-Galán, & Collado, 2007) of which we have studied the pyrones [6-pentyl-2H-pyran-2-one (6-PP) derivatives] (Keszler et al., 2000) and peptaibols (Vizcaino et al., 2005). Currently, better knowledge about *Trichoderma* has facilitated its use in biocontrol as whole microorganisms, able to be monitorized in natural environments (Hermosa, Grondona, Díaz-Mínguez, Iturriaga, & Monte, 2001; Rubio, Hermosa, Keck, & Monte, 2005), as enzyme formulations (Benítez et al., 2004) or as sources of genes for transgenic plant development (M Lorito et al., 1998). Since the early description of the capacity of *Trichoderma* to increase plant biomass production (Chang, Chang, Baker, Kleifeld, & Chet, 1986), several new general mechanisms for both biocontrol and plant growth increase have been demonstrated and it is now clear that there must be hundreds of separate genes and gene products involved in these processes.

2.3. Competition for nutrients

Competition between *Trichoderma* and pathogens would be established with the purpose to get more nutrients, oxygen, light, (Paulitz, 1990). *Trichoderma* is an excellent competitor for space and nutritional resources. It appears in almost all soils and in habitats that contains high amounts of organic matter. In those places, it would be an excellent decomposer of plant and fungal material. Moreover, some species of the genus *Trichoderma* show great metabolomic versatility that allows them to grow using a wide range of nitrogen and carbon sources. Furthermore, *Trichoderma* has the ability to colonize the rhizosphere, and this skill might be essential for being used as an excellent biological control agent (Howell, 2003).

Genes related to the capacity of *Trichoderma* to compete in soil and rhizosphere colonization (Chacón et al., 2007) were also described: the first oligopeptide transporter analyzed functionally in filamentous fungi (Vizcaíno et al., 2006) and two heat shock proteins that have been overexpressed in a biocontrol strain of *Trichoderma* and in plant, improving their resistance to abiotic stresses (Montero, Sanz, Rey, Monte, & Llobell, 2005; Montero, Sanz, Rey, Llobell, & Monte, 2007).

2.4. Production of antibiosis compounds

Trichoderma produces a large number of secondary metabolites with biological activity (Ghisalberti & Sivasithamparam, 1991; Sivasithamparam & Ghisalberti, 1998) that includes some natural chemical compounds related with its survival, its competition ability against organisms, as well as its symbiosis, its transport, its differentiation,... (Demain & Fang, 2000). Furthermore, antibiotics, which could inhibit microbial growth, are also included in this group.

In the antifungal assay on membranes, carried out by Mayo and co-authors (2015), where it was determined the ability of *Trichoderma* isolates to produce metabolites with inhibitory activity against *R. solan*i the percentages of inhibition ranged between 86% and 58%. In others assays the percentage of inhibition in membrane assays using *T. virens* T59 and *T. atroviride* T11 range between 100% and 84.7%, respectively (Campelo et al., 2010).

2.5. Induction of resistance

The relationships established between plant and microorganisms are very diverse, when a plant is exposed to a pathogenic microorganism, the production of molecules associated to salicylic acid is increased, being this a systemic acquired resistance (SAR) response. The response of plants against non-pathogenic microorganisms is different, resulting in activation of signalling cascades that are dependent on jasmonic acid and ethylene, such as hydroperoxide lyase, peroxidase and phenylalanine ammonia lyase, all of which belong to an induced systemic resistance (ISR) response (Druzhinina et al., 2011). Other responses result in a rapid cell death in infected tissues, then plants activate the hypersensitive response that involves the accumulation of salicylic acid, reactive oxygen species and an increased the influx of Ca^{2+} (Guerrero-González et al., 2011).

In the tripartite interaction of legume plants with a pathogen and a biocontrol *Trichoderma* species, several changes are produced in the plant, such as the increase in phenolic acid and lignin, accumulation of phytoalexins and down- or up-regulation of defence-related genes expression (Guerrero-González et al., 2011; Mayo et al., 2015) Different categories of defence-related genes whose expression is modulated by biotic stresses have been described in bean plants interacting with pathogen and non-pathogenic microorganisms (Mayo et al., 2015). Several works have studied the expression of defence-related genes in the plants when they have in contact with *Trichoderma* and/or pathogen.

WRKY transcription factors have been involved in the regulation of plant defence gene expression (Rushton & Somssich, 1998; Singh, Foley, & Oñate-Sánchez, 2002). Thus, *WRKY33* has a role in biotic stress defence, where it regulates the balance between necrotrophic and biotrophic pathogen responses (Birkenbihl, Diezel, & Somssich, 2012; Lippok et al., 2007; Pandey & Somssich, 2009). Previous studies have pointed out the involvement of *Arabidopsis* WRKY transcription factors in regulating the expression of *PR* genes by direct binding (W. Chen et al., 2002; Kim, Fan, & Chen, 2006). A rapid pathogen-induced *WKRY33* expression did not require salicylic acid signalling but a down-regulation of this gene involved a direct activation of jasmonic acid (Bakshi & Oelmüller, 2014). In the work by Mayo and co-workers (2016), when bean plants were in contact *T. velutinum* T028 without pathogen, *WRKY33* expression was significantly up-regulated while the *PR* genes expression (*PR2*, *PR3* and *PR4*) was significantly down-regulated compared to expression levels in plants without *Trichoderma* treatment. However, in the same work, when the pathogen *Rhizoctonia solani* was added to the substrate, expression of *WRKY33* was significantly down-regulated in plants with *Trichoderma* inoculation, while *PR2*, *PR3* and *PR4* were down-regulated.

WRKY33 is also involved in the regulation of the expression of genes modulated by components of the ethylene-signalling pathway. Expression of the *ERF1* and *ERF5* reached similar significant values either with or without *Trichoderma* and or *R. solani* in the substrate. *WRKY33* would act as a represor of *ERF1* and *ERF5* expression. Thus, when the expression of *WRKY33* is increased, expression of *ERF1* and *ERF5* is down-regulated (Mayo et al., 2016).

Other gene is *CH5b* encodes an endochitinase precursor and it is also related with the ethylene-signalling pathway. In previous works, it has been shown that, when this gene was over-expressed the *R. solani* symptoms were reduced in crops like *Nicotiana tabacum* and *Brassica napus* (Broglie et al., 1991). However, in the study by Mayo et al. (2016), when *P. vulgaris* plants were in contact with *R. solani,* the expression of this gene was down-regulated but not significantly, while treatment of these infected plants with *T. velutinum* resulted in its significant up-regulation. These results are in agreement with previous data, showing that the pathogen represses its expression, and the presence of *Trichoderma* induced it (Mayo et al., 2015).

PAL plays an important role in plant defence; it is involved in the biosynthesis of salicylic acid, which is related to plant systemic resistance (Chaman, Copaja, & Argandoña, 2003; Mauch-Mani & Slusarenko, 1996; Nugroho, Verberne, & Verpoorte, 2002). *PAL* gene expression is also regulated in response to pathogen infection. The presence of *T. velutinum* and *R. solani* in the soil resulted in a significant down-regulation of this gene compared with control plants (Mayo et al., 2016).

The *CNGC* genes can be related to early plant defence responses due to changes in ion flux, including H^+ and Ca^{2+} influx and K^+ and Cl^- efflux (Atkinson, Midland, Sims, & Keen, 1996). The up-regulation of *CNGC2* can confirm the importance of ion channels for the plant resistance response (Borges, Melotto, Tsai, & Caldas, 2012). *CNGC2* was down-regulated in plants treated with *T. velutinum* (Mayo et al., 2016).

HPL (hydroperoxide lyase) is involved in the production of antimicrobial and defence signalling oxylipins (Huang, Studart Witkowski, & Schwab, 2010; Noordermeer, Veldink, & Vliegenthart, 2001). In this study, the presence of *T. velutinum* and *R. solani*, resulted in a down-regulation of this gene expression respect to control plants. In the present case, after 45 days in contact with the fungus *T. velutinum* and/or *R. solani*, its expression was down-regulated, indicating that the plant identifies *Trichoderma* and *Rhizoctonia* as two invader organisms, and some of the mechanisms activated against the presence of both are similar, independently of the final response specifically activated in the plant by each one (Mayo et al., 2016).

hGS encodes a homoglutathione synthetase that is involved in response to oxidative stress. There is not much information about the behaviour of this gene in the plant. In the study of Mayo and coworkers (2016), when bean plants were in contact with *T. velutinum* and/or *R. solani*, expression of this gene was significantly up-regulated compared to control plants. In other studies, treatment of *Medicago truncatula* plants with compounds that release nitric oxide, a key signalling molecule in plants, induced expression of *GST* but not *hGS* in roots (Innocenti et al., 2007). Similarly, common bean plants treated with H_2O_2 showed up-regulation of *hGS* in nodules, whereas treatments with cadmium, sodium chloride, or jasmonic acid had no effect (Loscos, Matamoros, & Becana, 2008).

2.6. Production of antibiotic compounds: Changes in plant metabolism as defence response

When a plant is induced by exposure to a microorganism, starts the production of the biochemical products or physiological changes and activation of various metabolic pathways depends on the type and origin of these signalling natural products. Different secondary metabolites are synthesized after perception and recognition of the signals originating from plant or pathogenic microorganism elicitors created during the first steps of plant defence reactions (Boller & Felix, 2009; Grotewold, 2005; Veitch, 2009). Plants respond after the invasion of a phytopathogen or a biocontrol agent that activates disease resistance response to the invasion, by inducing the expression of defence genes (Mayo et al., 2016) and by the production of some secondary metabolites with antibacterial activity, such as phytoalexins (phenols, isoflavones, terpenes), and some substances that can block pathogen invasion and spread, such as lignin and callose (X. Chen, Qi, & Duan, 2015). Some plants do not produce phytoalexins when are in contact with pathogens, but release toxins that are normally stored as less toxic glycosides (Grayer & Kokubun, 2001).

Trichoderma species also produce plant hormones and solubilize minerals in the soil which help to promote plant growth and suppress the disease (Kim et al., 2006).

During the *Trichoderma*-plant interaction various classes of metabolites could induce resistance such as proteins with enzymatic activity, low molecular weight compounds, related to the fungal or the plant cell wall, originated by the enzymatic activity of *Trichoderma* (Woo et al., 2006; Woo & Lorito, 2007) and other secondary metabolites that trigger plant defence mechanisms against the pathogen (Hermosa et al., 2012; Malmierca et al., 2014), by inducing the expression of pathogenesis–related (PR) proteins that reduce the diseases symptoms.

During the plant-*Trichoderma* interactions, the fungus participates actively in protecting and improving its ecological niche. Leucine-rich repeat (LRR)-containing proteins are signal receptors regulating plant development and defence (Afzal, Wood, & Lightfoot, 2008). In the work of Marra and co-authors (2006) observed that LRR proteins increased in bean leaves (*Phaseolus vulgaris*)interacting with *T. atroviride*, and that hydrophobins and ABC transporters were accumulated in the proteome of the fungus. Hydrophobins (Rosado et al., 2007) and ABC transporters (Ruocco et al., 2007) support the biocontrol activity of *Trichoderma* and its ability to colonize the roots. In a similar way, a *Trichoderma*-secreted swollenin (an expansin-like 5 protein) remarkably increased fungus plant root colonization efficiency and, due to a cellulose-binding domain was able to trigger defence responses in the plant and afforded pathogen protection, indicating that this domain might therefore be recognized by the plant as a microbe-associated molecular pattern (MAMP) in the *Trichoderma*-plant interaction (Brotman, Briff, Viterbo, & Chet, 2008). At least four classes of substances that elicit plant defence responses have been identified in *Trichoderma*: polysaccharide oligomers, enzymes, low molecular weight proteins and peptaibols. Some cell wall oligomers may act as elicitor molecules released by plants following pathogen attack (Woo et al., 2006), the overexpression of *Trichoderma* chitinase genes in tobacco plants generates innate defence responses and enhanced stress tolerance (Dana, Pintor-Toro, & Cubero, 2006), hydrophobin-like cysteine-rich low molecular weight secreted proteins Sm1 from *T. virens* and Epl1 from *T. atroviride* (Djonović, Pozo, Dangott, Howell, & Kenerley, 2006; Seidl, Marchetti, Schandl, Allmaier, & Kubicek, 2006) can also trigger ISR but, with the exception of peptaibols as elicitors of plant defence responses (Viterbo, Wiest, Brotman, Chet, & Kenerley, 2007), the role of secondary metabolites in this task remains unexplored.

REFERENCES

Afzal, A. J., Wood, A. J., & Lightfoot, D. A. (2008). Plant receptor-like serine threonine kinases: Roles in signaling and plant defense. *Molecular Plant-Microbe Interactions*, *21(5),* 507–517. http://doi.org/10.1094/MPMI-21-5-0507.

Ait-Lahsen, H., Soler, A., Rey, M., de La Cruz, J., Monte, E., & Llobell, A. (2001). An antifungal exo-alpha-1,3-glucanase (AGN13.1) from the biocontrol fungus *Trichoderma harzianum*. *Applied and Environmental Microbiology, 67(12)*, 5833–5839. http://doi.org/10.1128/AEM.67.12.5833-5839.2001.

Altomare, C., Norvell, W. A., Björkman, T., & Harman, G. E. (1999). Solubilization of phosphates and micronutrients by the plant-growth- promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Applied and Environmental Microbiology, 65(7)*, 2926–2933.

American Phytopathological Society. (2005). Compendium of bean diseases. (Schwartz HF, Steadman J R, Hall R, & Forster R L, Eds.) (2nd ed.). APS Press, American Phytopathological Society.

Atkinson, M. M., Midland, S. L., Sims, J. J., & Keen, N. T. (1996). Syringolide 1 triggers Ca2 influx, K efflux, and extracellular alkalization in soybean cells carrying the disease-resistance gene *Rpg4*. *Plant Physiology, 112(1),* 297–302. http://doi.org/10.1104/pp.112.1.297.

Bakshi, M., & Oelmüller, R. (2014). WRKY transcription factors. *Plant Signaling & Behavior, 9(2),* e27700. http://doi.org/10.4161/psb.27700.

Barroso, C. B., & Nahas, E. (2005). The status of soil phosphate fractions and the ability of fungi to dissolve hardly soluble phosphates. *Applied Soil Ecology, 29(1),* 73–83. http://doi.org/10.1016/j.apsoil.2004.09.005.

Beebe, S. E., Corrales, M. P., Schoonhoven, A. van, & Voysest, O. (1991). Breeding for disease resistance. In O. Schoonhoven, A. van;Voysest (Ed.), *Common beans: research for crop improvemen*t. (pp. 561–617). Wallingford: CAB International.

Benítez, T., Rincón, A. M., Limón, M. C., & Codón, A. C. (2004). Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology, 7(4)*, 249–260. http://doi.org/10.2436/IM.V7I4.9480.

Birkenbihl, R. P., Diezel, C., & Somssich, I. E. (2012). *Arabidopsis* WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea* infection. *Plant Physiology, 159(1)*, 266–285. http://doi.org/10.1104/pp.111.192641.

Boller, T., & Felix, G. (2009). A Renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology, 60(1),* 379–406. http://doi.org/10.1146/annurev.arplant.57.032905.105346.

Borges, A., Melotto, M., Tsai, S. M., & Caldas, D. G. G. (2012). Changes in spatial and temporal gene expression during incompatible interaction between common bean and Anthracnose pathogen. *Journal of Plant Physiology, 169(12)*, 1216–1220. http://doi.org/10.1016/j.jplph.2012.04.003.

Brimner, T. A., & Boland, G. J. (2003). A review of the non-target effects of fungi used to biologically control plant diseases. *Agriculture, Ecosystems & Environment, 100(1),* 3–16. http://doi.org/http://dx.doi.org/10.1016/S0167-8809(03)00200-7.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., … Broglie, R. (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science, 254(5035)*, 1194–1197. http://doi.org/10.1126/science.254.5035.1194.

Brotman, Y., Briff, E., Viterbo, A., & Chet, I. (2008). Role of swollenin, an expansin-like protein from *Trichoderma*, in plant root colonization. *Plant Physiology, 147*, 779–789. http://doi.org/10.1104/pp.108.116293.

Brunner, K., Peterbauer, C. K., Mach, R. L., Lorito, M., Zeilinger, S., & Kubicek, C. P. (2003). The Nag1 N-acetylglucosaminidase of *Trichoderma atroviride* is essential for chitinase induction by chitin and of major relevance to biocontrol. *Current Genetics, 43(4),* 289–295.

Campelo, M. P., Cardoza, R. E., Lorenzana, A., Hermosa, M. R., Monte, E., Reinoso, B., … Casquero, P. A. (2010). Biological control of phytopathogenic fungi in bean (*Phaseolus vulgaris*, L.) with *Trichoderma atroviride* and *Trichoderma virens*. In P. Miklas & P. Gepts (Eds.), *Bean Improvement Cooperative Fort Collins* (Vol. 53, pp. 114–115). Colorado, USA.

Cardoza, R. E., Vizcaíno, J. A., Hermosa, M. R., Sousa, S., González, F. J., Llobell, A., … Gutiérrez, S. (2006). Cloning and characterization of the *erg1* gene of *Trichoderma harzianum*: Effect of the erg1 silencing on ergosterol biosynthesis and resistance to terbinafine. *Fungal Genetics and Biology, 43(3),* 164–178. http://doi.org/10.1016/j.fgb.2005.11.002.

Chacón, M. R., Rodríguez Galán, O., Benítez Fernández, C. T., Sousa, S., Rey, M., Llobell González, A., & Delgado Jarana, J. (2007). Microscopic and transcriptome analyses of early colonization of tomato roots by *Trichoderma harzianum. International Microbiology 10(1),* 19–27. http://doi.org/10.2436/20.1501.01.4.

Chagas, L. F. B., De Castro, H. G., Colonia, B. S. O., De Carvalho Filho, M. R., Miller, L. D. O., & Chagas, A. F. J. (2016). Efficiency of *Trichoderma* spp. as a growth promoter of cowpea (*Vigna unguiculata*) and analysis of phosphate solubilization and indole acetic acid synthesis. *Brazilian Journal of Botany, 39(2),* 437–445. http://doi.org/10.1007/s40415-015-0247-6.

Chaman, M. E., Copaja, S. V, & Argandoña, V. H. (2003). Relationships between salicylic acid content, Phenylalanine Ammonia-Lyase (PAL) activity, and resistance of barley to aphid infestation. *Journal of Agricultural and Food Chemistry, 51(8),* 2227–2231. http://doi.org/10.1021/jf020953b.

Chang, Y.-C., Chang, Y.-C., Baker, R., Kleifeld, O., & Chet, I. (1986). Increased growth of plants in the presence of the biological control agent *Trichoderma harzianum. Plant Disease, 70(2),* 145–148.

Chen, W., Provart, N. J., Glazebrook, J., Katagiri, F., Chang, H. S., Eulgem, T., … Zhu, T. (2002). Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *The Plant Cell, 14(3)*, 559–574. http://doi.org/10.1105/tpc.010410.

Chen, X., Qi, X., & Duan, L.-X. (2015). Overview. In X. Qi, X. Chen, & Y. Wang (Eds.), *Plant Metabolomics* (pp. 1–24). Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-94-017-9291-2_1.

Chet, I., Harman, G. E., & Baker, R. (1981). *Trichoderma hamatum*: Its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microbial Ecology, 7(1),* 29–38. http://doi.org/10.1007/BF02010476.

Dana, M. de las M., Pintor-Toro, J. A., & Cubero, B. (2006). Transgenic tobacco plants overexpressing chitinases of fungal origin show enhanced resistance to biotic and abiotic stress agents. *Plant Physiology, 142*, 722–730. http://doi.org/10.1104/pp.106.086140.

Demain, A. L., & Fang, A. (2000). The natural functions of secondary metabolites. In A. Fiechter (Ed.), *History of Modern Biotechnology I* (pp. 1–39). Springer Berlin Heidelberg. http://doi.org/10.1007/3-540-44964-7_1.

Djonović, S., Pozo, M. J., Dangott, L. J., Howell, C. R., & Kenerley, C. M. (2006). Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. *Molecular Plant-Microbe Interactions, 838(8),* 838–853. http://doi.org/10.1094/MPMI-19-0838.

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., … Kubicek, C. P. (2011). *Trichoderma*: The genomics of opportunistic success. *Nature Reviews Microbiology, 9(10),* 749–759.

Dubey, S. C., Suresh, M., & Singh, B. (2007). Evaluation of *Trichoderma* species against *Fusarium oxysporum* f. sp. *ciceris* for integrated management of chickpea wilt. *Biological Control, 40(1),* 118–127. http://doi.org/10.1016/j.biocontrol.2006.06.006.

Food and Agriculture Organization. (2016). Pulses: Nutritious seeds for a sustainable future. (P. Javaloyes & S. O'Broin, Eds.). *Food and Agriculture Organization of the United Nations*.

Ghisalberti, E. L., & Sivasithamparam, K. (1991). Antifungal antibiotics produced by *Trichoderma* spp. *Soil Biology and Biochemistry, 23(11),* 1011–1020. http://doi.org/10.1016/0038-0717(91)90036-J.

Gravel, V., Antoun, H., & Tweddell, R. J. (2007). Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: Possible role of indole acetic acid (IAA). *Soil Biology and Biochemistry, 39(8),* 1968–1977. http://doi.org/10.1016/j.soilbio.2007.02.015.

Grayer, R. J., & Kokubun, T. (2001). Plant–fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants. *Phytochemistry, 56(3),* 253–263. http://doi.org/10.1016/S0031-9422(00)00450-7.

Grayston, S. J., Vaughan, D., & Jones, D. (1997). Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability. *Applied Soil Ecology, 5(1)*, 29–56. http://doi.org/10.1016/S0929-1393(96)00126-6.

Grotewold, E. (2005). Plant metabolic diversity: a regulatory perspective. *Trends in Plant Science, 10(2),* 57–62. http://doi.org/10.1016/j.tplants.2004.12.009.

Guerrero-González, M. L., Rodríguez-Kessler, M., Rodríguez-Guerra, R., González-Chavira, M., Simpson, J., Sanchez, F., & Jiménez-Bremont, J. F. (2011). Differential expression of *Phaseolus vulgaris* genes induced during the interaction with *Rhizoctonia solani*. *Plant Cell Reports, 30(8),* 1465–1473. http://doi.org/10.1007/s00299-011-1055-5.

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., & Lorito, M. (2004). *Trichoderma* species--opportunistic, avirulent plant symbionts. *Nature Reviews in Microbiology*, 2(1), 43–56. http://doi.org/10.1038/nrmicro797.

Hermosa, M. R., Grondona, I., Díaz-Mínguez, J. M., Iturriaga, E. A., & Monte, E. (2001). Development of a strain-specific SCAR marker for the detection of *Trichoderma atroviride* 11, a biological control agent against soilborne fungal plant pathogens. *Current Genetics, 38(6),* 343–350. http://doi.org/10.1007/s002940000173.

Hermosa, M. R., Viterbo, A., Chet, I., & Monte, E. (2012). Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiology, 158(1),* 17–25. http://doi.org/10.1099/mic.0.052274-0.

Hermosa, R., Rubio, M. B., Cardoza, R. E., Nicolás, C., Monte, E., & Gutiérrez, S. (2013). The contribution of Trichoderma to balancing the costs of plant growth and defense. International Microbiology, 16(2), 69–80.

Howell, C. R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Disease, 87(1)*, 4–10.

Hoyos-Carvajal, L., Orduz, S., & Bissett, J. (2009). Growth stimulation in bean (*Phaseolus vulgaris* L.) by *Trichoderma*. *Biological Control, 51(3),* 409–416. http://doi.org/10.1016/j.biocontrol.2009.07.018.

Huang, F. C., Studart Witkowski, C., & Schwab, W. (2010). Overexpression of hydroperoxide lyase gene in *Nicotiana benthamiana* using a viral vector system. *Plant Biotechnology Journal, 8(7),* 783–795. http://doi.org/10.1111/j.1467-7652.2010.00508.x.

Innocenti, G., Pucciariello, C., Le Gleuher, M., Hopkins, J., de Stefano, M., Delledonne, M., … Frendo, P. (2007). Glutathione synthesis is regulated by nitric oxide in *Medicago truncatula* roots. *Planta, 225(6),* 1597–1602. http://doi.org/10.1007/s00425-006-0461-3.

Kamaruzzaman, M., Rahman, M. M., Islam, M. S., Ahmad, M. U., & Ahmad, M. U. (2016). Efficacy of four selective *Trichoderma* isolates as plant growth promoters in two peanut varieties. *International Journal of Biological Research, 4(2),* 152. http://doi.org/10.14419/ijbr.v4i2.6468.

Kapri, A., & Tewari, L. (2010). Phosphate solubilization potential and phosphatase activity of rhizospheric *Trichoderma* spp. *Brazilian Journal of Microbiology, 41(3),* 787–795. http://doi.org/10.1590/S1517-83822010005000001.

Keszler, A., Forgács, E., Kótai, L., Vizcaíno, J. A., Monte, E., & García-Acha, I. (2000). Separation and identification of volatile components in the fermentation broth of *Trichoderma atroviride* by solid-phase extraction and gas chromatography-mass spectrometry. *Journal of Chromatographic Science, 38(10),* 421–4. http://doi.org/10.1093/CHROMSCI/38.10.421.

Kim, K. C., Fan, B., & Chen, Z. (2006). Pathogen-induced *Arabidopsis* WRKY7 is a transcriptional repressor and enhances plant susceptibility to *Pseudomonas syringae*. *Plant Physiology, 142(3)*, 1180–1192. http://doi.org/10.1104/pp.106.082487.

Lippok, B., Birkenbihl, R. P., Rivory, G., Brümmer, J., Schmelzer, E., Logemann, E., & Somssich, I. E. (2007). Expression of *AtWRKY33* encoding a pathogen-or PAMP-responsive WRKY transcription factor is regulated by a composite DNA motif containing W box elements. *Molecular Plant-Microbe Interactions, 20(4),* 420–429. http://doi.org/10.1094/MPMI-20-4-0420.

Lorito, M., Harman, G. E., Hayes, C. K., Broadway, R. M., Tronsmo, A., Woo, S. L., & Di Pietro, A. (1993). Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology, 83(3),* 302–307.

Lorito, M., Hayes, C. K., Di Pietro, A., Woo, S. L., & Harman, G. E. (1994). Purification, characterization, and synergistic activity of a glucan 1,3-β-glucosidase and an N-acetyl-β-glucosaminidase from *Trichoderma harzianum. Phytopathology, 84(4),* 398–405.

Lorito, M., Woo, S. L., Garcia, I., Colucci, G., Harman, G. E., Pintor-Toro, J. A., … Fernandez, I. G. (1998). Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. *Proceedings of the National Academy of Sciences of the United States of America, 95(14),* 7860–7865.

Lorito, M., Woo, S. L., Harman, G. E., & Monte, E. (2010). Translational research on *Trichoderma* : From *'Omics to the Field. Annual Review of Phytopathology, 48(1),* 395–417. http://doi.org/10.1146/annurev-phyto-073009-114314.

Loscos, J., Matamoros, M. A., & Becana, M. (2008). Ascorbate and homoglutathione metabolism in common bean nodules under stress conditions and during natural senescence. *Plant Physiology, 146(3),* 1282–1292. http://doi.org/10.1104/pp.107.114066.

Lu, Z., Tombolini, R., Woo, S., Zeilinger, S., Lorito, M., & Jansson, J. K. (2004). *In vivo* study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Applied and Environmental Microbiology, 70(5),* 3073–81. http://doi.org/10.1128/AEM.70.5.3073-3081.2004.

Malmierca, M. G., Barua, J., Mccormick, S. P., Izquierdo-Bueno, I., Cardoza, R. E., Alexander, N. J., … Gutiérrez, S. (2014). Novel aspinolide production by *Trichoderma arundinaceum* with a potential role in *Botrytis cinerea* antagonistic activity and plant defence priming. *Environmental Microbiology, 17(4),* 1103–1118. http://doi.org/10.1111/1462-2920.12514.

Marra, R., Ambrosino, P., Carbone, V., Vinale, F., Woo, S. L., Ruocco, M., … Lorito, M. (2006). Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach. *Current Genetics, 50(5),* 307–321. http://doi.org/10.1007/s00294-006-0091-0.

Mauch-Mani, B., & Slusarenko, A. J. (1996). Production of salicylic acid precursors is a major function of Phenylalanine Ammonia-Lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. *The Plant Cell, 8(2),* 203–212. http://doi.org/10.1105/tpc.8.2.203.

Mayo, S., Cominelli, E., Sparvoli, F., González-López, O., Rodríguez-González, A., Gutiérrez, S., & Casquero, P. A. (2016). Development of a qPCR strategy to select bean genes involved in plant defense response and regulated by the *Trichoderma velutinum – Rhizoctonia solani* interaction. *Frontiers in Plant Science, 7,* 1109. http://doi.org/10.3389/fpls.2016.01109.

Mayo, S., Gutierrez, S., Malmierca, M. G., Lorenzana, A., Campelo, M. P., Hermosa, R., … Casquero, P. A. (2015). Influence of *Rhizoctonia solani* and *Trichoderma* spp. in growth of bean (*Phaseolus vulgaris* L.) and in the induction of plant defense-related genes. *Frontiers in Plant Science, 6*, 685. http://doi.org/10.3389/fpls.2015.00685.

Mayo, S., Izquierdo, H., González-López, Ó., Rodríguez-González, Á., Lorenzana, A., Gutiérrez, S., & Casquero, P. A. (2016). Effect of farnesol, a compound produced by *Trichoderma* when growing on bean (*Phaseolus vulgaris* L.). In *Joint Natural Products Conference. Copenhague*.

Monte, E. (2001). Understanding *Trichoderma* : between biotechnology and microbial ecology. *International Microbiology, 4*, 1–4. http://doi.org/10.1007/s101230100001.

Montero, M., Sanz, L., Rey, M., Llobell, A., & Monte, E. (2007). Cloning and characterization of bgn16·3, coding for a β-1,6-glucanase expressed during *Trichoderma harzianum* mycoparasitism. *Journal of Applied Microbiology*, *103(4),* 1291–1300. http://doi.org/10.1111/j.1365-2672.2007.03371.x.

Montero, M., Sanz, L., Rey, M., Monte, E., & Llobell, A. (2005). BGN16.3, a novel acidic β-1,6-glucanase from mycoparasitic fungus *Trichoderma harzianum* CECT 2413. *FEBS Journal, 272(13),* 3441–3448. http://doi.org/10.1111/j.1742-4658.2005.04762.x.

Noordermeer, M. A., Veldink, G. A., & Vliegenthart, J. F. G. (2001). Fatty acid hydroperoxide lyase: a plant cytochrome P450 enzyme involved in wound healing and pest resistance. *Chembiochem, 2(7-8),* 494–504. http://doi.org/10.1002/1439-7633(20010803)2:7.

Nugroho, L. H., Verberne, M. C., & Verpoorte, R. (2002). Activities of enzymes involved in the phenylpropanoid pathway in constitutively salicylic acid-producing tobacco plants. *Plant Physiology and Biochemistry, 40(9),* 755–760. http://doi.org/10.1016/S0981-9428(02)01437-7.

Öğüt, M., & Er, F. (2006). Micronutrient composition of field-grown dry bean and wheat inoculated with *Azospirillum* and *Trichoderma*. *Journal of Plant Nutrition and Soil Science, 169(5),* 699–703. http://doi.org/10.1002/jpln.200520597.

Pandey, S. P., & Somssich, I. E. (2009). The role of WRKY transcription factors in plant immunity. *Plant Physiology, 150(4)*, 1648–1655. http://doi.org/10.1104/pp.109.138990.

Paulitz, T. C. (1990). Biochemical and ecological aspect of competition in biological control. In R. R. Baker & P. E. Dunn (Eds.), *New directions in biological control. Alternatives for suppressing agricultural pests and diseases* (p. 837). New York (USA): Wiley-Liss Inc.

Pereira, J. L., Queiroz, R. M. L., Charneau, S. O., Felix, C. R., Ricart, C. A. O., Lopes Da Silva, F., … Noronha, E. F. (2014). Analysis of *Phaseolus vulgaris* response to its association with *Trichoderma harzianum* (ALL-42) in the presence or absence of the phytopathogenic fungi *Rhizoctonia solani* and *Fusarium solani. PLoS ONE, 9(5),* e98234. http://doi.org/10.1371/journal.pone.0098234.

Reino, J. L., Guerrero, R. F., Hernández-Galán, R., & Collado, I. G. (2007). Secondary metabolites from species of the biocontrol agent *Trichoderma*. *Phytochemistry Reviews, 7(1),* 89–123. http://doi.org/10.1007/s11101-006-9032-2.

Rojo, F. G., Reynoso, M. M., Ferez, M., Chulze, S. N., & Torres, A. M. (2007). Biological control by *Trichoderma* species of *Fusarium solani* causing peanut brown root rot under field conditions. *Crop Protection, 26(4),* 549–555. http://doi.org/10.1016/j.cropro.2006.05.006.

Rosado, I. V., Rey, M., Codón, A. C., Govantes, J., Moreno-Mateos, M. A., & Benítez, T. (2007). QID74 Cell wall protein of *Trichoderma harzianum* is involved in cell protection and adherence to hydrophobic surfaces. *Fungal Genetics and Biology, 44(10),* 950–964. http://doi.org/10.1016/j.fgb.2007.01.001.

Rubio, M. B., Hermosa, M. R., Keck, E., & Monte, E. (2005). Specific PCR assays for the detection and quantification of DNA from the biocontrol strain *Trichoderma harzianum* 2413 in soil. *Microbial Ecology, 49(1),* 25–33. http://doi.org/10.1007/s00248-003-0171-3.

Ruocco, M., Lanzuise, S., Vinale, F., Marra, R., Turrà, D., Woo, S. L., … Benítez, T. (2007). Identification of a new biocontrol gene in *Trichoderma atroviride*: the role of an ABC transporter membrane pump in the interaction with different plant-pathogenic fungi. *Molecular Plant-Microbe Interactions, 44(3)*, 950–964. http://doi.org/10.1016/j.fgb.2007.01.001.

Rushton, P. J., & Somssich, I. E. (1998). Transcriptional control of plant genes responsive to pathogens. *Current Opinion in Plant Biology, 1(4),* 311–315. http://doi.org/10.1016/1369-5266(88)80052-9.

Sanz, L., Montero, M., Grondona, I., Vizcaíno, J. A., Llobell, A., Hermosa, R., & Monte, E. (2004). Cell wall-degrading isoenzyme profiles of *Trichoderma* biocontrol strains show correlation with rDNA taxonomic species. *Current Genetics, 46(5)*, 277–286. http://doi.org/10.1007/s00294-004-0532-6.

Sanz, L., Montero, M., Redondo, J., Llobell, A., & Monte, E. (2005). Expression of an α-1,3-glucanase during mycoparasitic interaction of *Trichoderma asperellum. FEBS Journal, 272(2),* 493–499. http://doi.org/10.1111/j.1742-4658.2004.04491.x.

Seidl, V., Marchetti, M., Schandl, R., Allmaier, G., & Kubicek, C. P. (2006). Epl1, the major secreted protein of *Hypocrea atroviridis* on glucose, is a member of a strongly conserved protein family comprising plant defense response elicitors. *FEBS Journal, 273(18),* 4346–4359. http://doi.org/10.1111/j.1742-4658.2006.05435.x.

Shoresh, M., Harman, G. E., & Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annual Review of Phytopathology, 48,* 21–43. http://doi.org/10.1146/annurev-phyto-073009-114450.

Singh, K. B., Foley, R. C., & Oñate-Sánchez, L. (2002). Transcription factors in plant defense and stress responses. *Current Opinion in Plant Biology, 5(5),* 430–436. http://doi.org/10.1016/S1369-5266(02)00289-3.

Sivasithamparam, K., & Ghisalberti, E. L. (1998). Secondary metabolism in *Trichoderma* and *Gliocladium*. In C. P. Kubicek & G. E. Harman (Eds.), *Trichoderma and Gliocladium: Basic biology, taxonomy and genetics* (pp. 139–191). London, United Kingdon: Taylor and Francis Ltd.

Suarez, B., Rey, M., Castillo, P., Monte, E., & Llobell, A. (2004). Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity. *Applied Microbiology and Biotechnology, 65(1),* 46–55. http://doi.org/10.1007/s00253-004-1610-x.

Valenciano, J. B., Casquero, P. A., & Boto, J. A. (2004). Influence of sowing techniques and pesticide application on the emergence and the establishment of bean plants (*Phaseolus vulgaris* L.). *Agronomie, 24(2),* 113–118. http://doi.org/10.1051/agro:2004007.

Veitch, N. C. (2009). Isoflavonoids of the Leguminosae. *Natural Product Reports, 26(6),* 776. http://doi.org/10.1039/b616809b.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Woo, S. L., & Lorito, M. (2008). *Trichoderma*–plant–pathogen interactions. *Soil Biology and Biochemistry, 40(1)*, 1–10. http://doi.org/10.1016/j.soilbio.2007.07.002.

Viterbo, A., Montero, M., Ramot, O., Friesem, D., Monte, E., Llobell, A., & Chet, I. (2002). Expression regulation of the endochitinase chit36 from *Trichoderma asperellum (T. harzianum* T-203). *Current Genetics, 42(2),* 114–122. http://doi.org/10.1007/s00294-002-0345-4.

Viterbo, A., Wiest, A., Brotman, Y., Chet, I., & Kenerley, C. (2007). The 18mer peptaibols from *Trichoderma virens* elicit plant defence responses. *Molecular Plant Pathology, 8(6)*, 737–746. http://doi.org/10.1111/j.1364-3703.2007.00430.x.

Vizcaíno, J. A., Cardoza, R. E., Hauser, M., Hermosa, R., Rey, M., Llobell, A., … Monte, E. (2006). ThPTR2, a di/tri-peptide transporter gene from *Trichoderma harzianum*. *Fungal Genetics and Biology, 43(4),* 234–246. http://doi.org/10.1016/j.fgb.2005.12.003.

Vizcaino, J. A., Sanz, L., Basilio, A., Vicente, F., Gutierrez, S., Hermosa, M. R., & Monte, E. (2005). Screening of antimicrobial activities in *Trichoderma* isolates representing three *Trichoderma* sections. *Mycological Research, 109(12),* 1397–1406. http://doi.org/10.1017/S0953756205003898.

Woo, S. L., & Lorito, M. (2007). Exploiting the interactions between fungal antagonists, pathogens and the plant for biocontrol. In M. Vurro & J. Gressel (Eds.), *Novel Biotechnologies for Biocontrol Agent Enhancement and Management* (pp. 107–130). The Netherlands: Springer.

Woo, S. L., Scala, F., Ruocco, M., & Lorito, M. (2006). The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. *Phytopathology, 96(2),* 181–185.

Yedidia, I., Srivastva, A. K., Kapulnik, Y., & Chet, I. (2001). Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant and Soil, 235(2),* 235–242.

A quien corresponda la presente

Certificado de participación en el libro "**Legumes for Global Feed and Food Future"**.

Estimado comité científico de tesis,

Por la presente y como editores científicos del libro titulado "Legumes for Global Feed and Food Future**"**, que será publicado en 2017 por la editoral Nova Science Publisher, informamos de que todos los autores abajo presentes han contribuido a la consecución del capítulo de libro que consta de los siguientes datos:

Título: **Trichoderma species as biocontrol agents in legumes**

Autores: **Sara Mayo[1]; Santiago Gutiérrez[1]; Rosa E. Cardoza[1]; Rosa Hermosa[2]; Enrique Monte[2]; Pedro A. Casquero[1]**

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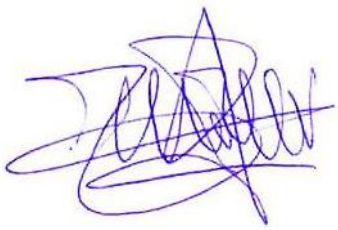

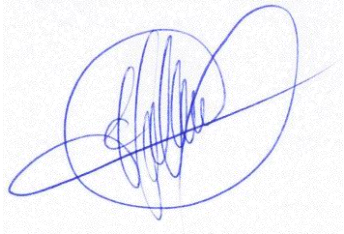

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CAPÍTULO II

Influence of *Rhizoctonia solani* and *Trichoderma* spp. in growth of bean (*Phaseolus vulgaris L.*) and in the induction of plant defense-related genes

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 16 September 2015
doi: 10.3389/fpls.2015.00685

Influence of *Rhizoctonia solani* and *Trichoderma* spp. in growth of bean (*Phaseolus vulgaris* L.) and in the induction of plant defense-related genes

Sara Mayo[1], *Santiago Gutiérrez*[2], *Monica G. Malmierca*[2], *Alicia Lorenzana*[1], *M. Piedad Campelo*[1], *Rosa Hermosa*[3] *and Pedro A. Casquero*[1]*

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Many *Trichoderma* species are well-known for their ability to promote plant growth and defense. We study how the interaction of bean plants with *R. solani* and/or *Trichoderma* affect the plants growth and the level of expression of defense-related genes. *Trichoderma* isolates were evaluated *in vitro* for their potential to antagonize *R. solani*. Bioassays were performed in climatic chambers and development of the plants was evaluated. The effect of *Trichoderma* treatment and/or *R. solani* infection on the expression of bean defense-related genes was analyzed by real-time PCR and the production of ergosterol and squalene was quantified. *In vitro* growth inhibition of *R. solani* was between 86 and 58%. In *in vivo* assays, the bean plants treated with *Trichoderma harzianum* T019 always had an increased size respect to control and the plants treated with this isolate did not decrease their size in presence of *R. solani*. The interaction of plants with *R. solani* and/or *Trichoderma* affects the level of expression of seven defense-related genes. Squalene and ergosterol production differences were found among the *Trichoderma* isolates, T019 showing the highest values for both compounds. *T. harzianum* T019 shows a positive effect on the level of resistance of bean plants to *R. solani*. This strain induces the expression of plant defense-related genes and produces a higher level of ergosterol, indicating its ability to grow at a higher rate in the soil, which would explain its positive effects on plant growth and defense in the presence of the pathogen.

Keywords: antifungal activity, defense-related genes, qPCR, ergosterol, squalene

OPEN ACCESS

Edited by:
Antonio M. De Ron, Spanish National Research Council - Misión Biológica de Galicia, Spain

Reviewed by:
Cristina Cabaleiro Sobrino, Universidade de Santiago de Compostela, Spain
Eleonora Cominelli, Istituto di Biologia e Biotecnologia Agraria, Consiglio Nazionale delle Ricerche, Italy

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Specialty section:
This article was submitted to Crop Science and Horticulture, a section of the journal Frontiers in Plant Science

Received: *29 June 2015*
Accepted: *18 August 2015*
Published: *16 September 2015*

Citation:
Mayo S, Gutiérrez S, Malmierca MG, Lorenzana A, Campelo MP, Hermosa R and Casquero PA (2015) Influence of Rhizoctonia solani and Trichoderma spp. in growth of bean (Phaseolus vulgaris L.) and in the induction of plant defense-related genes. Front. Plant Sci. 6:685. doi: 10.3389/fpls.2015.00685

Introduction

The common bean (*Phaseolus vulgaris* L.) is the third most important food legume crop worldwide, surpassed only by the soybean [*Glycine max* (L.) Merr.] and peanut (*Arachis hypogea* L.). Among the southern countries of the European Union, Spain together with Italy and Greece are the main common bean producers. León, a province located at the northwest of Spain, is

the main producer province by quantity and quality, with almost 45% of Spanish production in 2014. Socio-economic conditions of León province enabled possible the maintenance of local varieties in traditional cropping systems, which are based in small-scale farms (Casquero et al., 2006). The high quality of this legume has been awarded with a Protected Geographic Indication (PGI) (EC Reg. n.256/2010 published on 26 March 2010, OJEU L880/17). In the last few years, however dry bean production has gone through difficulties due to relatively low yields (mainly caused by fungus, virus, and bacteria) and insufficient income for growers.

Root rots are the main diseases caused by soil fungi having their incidence on bean yield. *Rhizoctonia solani* JG Kühn [Teleomorph: *Thanatephorus cucumeris* (AB Frank) Donk] is the main root rot in León, being detected in 91.8% of affected plants in an evaluation of its occurrence in bean plants (Valenciano et al., 2006). Plant infection occurs through wounds or by a coating of an organ with mycelium, which tears the cuticle and penetrates the epidermis. This pathogen is more aggressive at temperatures between 15 and 18°C and in moist soils. It is a necrotrophic pathogen, distributed worldwide (Guerrero-González et al., 2011). *R. solani* is one of the root and hypocotyl pathogen that causes most economic losses worldwide.

Trichoderma (Teleomorph: *Hypocrea*) is a fungal genus that is found in the soil. It is a secondary fast growing opportunistic invasive, which produces large numbers of spores, enzymes able to degrade the fungal cell wall (chitinases, glucanases, and proteases) and compounds with antimicrobial activity. Many *Trichoderma* species are also well known as biocontrol agents (BCA) of important phytopathogenic fungi. The primary mechanisms of biocontrol used by *Trichoderma* in direct confrontation with pathogenic fungi are the mycoparasitism (Papavizas, 1985) antibiosis, and competition for nutrients with the pathogen (Harman and Kubicek, 1998).

Many *Trichoderma* species colonize the root surface and cause substantial changes in plant metabolism (Harman et al., 2004). The physical interaction between *Trichoderma* and plants is limited to the first cell layer of the epidermis and the root bark. This symbiotic relationship would thus protect plants against pathogens. *Trichoderma* induces the expression of genes involved in defense response and promotes plant growth, root development and nutrient availability (Hermosa et al., 2012). During the *Trichoderma*-plant interaction various classes of metabolites could induce resistance such as proteins with enzymatic activity, low molecular weight compounds, related to the fungal or the plant cell wall, originated by the enzymatic activity of *Trichoderma* (Woo et al., 2006; Woo and Lorito, 2007) and other secondary metabolites that trigger plant defense mechanisms against the pathogen (Hermosa et al., 2012; Malmierca et al., 2014), by inducing the expression of pathogenesis–related (PR) proteins that reduce the diseases symptoms. Thus, when the plant contacts with a pathogen it is activated a mechanism of systemic acquired resistance (SAR). However, when they interact with a non-pathogen organism the plants activated a mechanism for induced systemic resistance (ISR) (Hermosa et al., 2013; Mukherjee et al., 2013).

Squalene is a polyunsaturated terpene that is an intermediate in the ergosterol biosynthetic pathway, which has an essential function in the fungal cell structure. The levels of squalene will influence the level of ergosterol biosynthesis (Garaiová et al., 2013). In addition to its structural function, and as a result of its importance in fungal development, ergosterol is also able to activate the expression of a number of defense genes and could increase the resistance of plants against pathogens (Lochman and Mikeš, 2005).

In this work 23 *Trichoderma* isolates were collected from bean fields. These isolates were used to study their effect on the growth of bean plants, and also in the defense response of plants against the phytopathogen *R. solani*. Thus, parameters as plant growth in the presence of the pathogen and/or the different *Trichoderma* isolates were evaluated, also analyzing the level of expression of defense-related genes in plants treated with the selected *Trichoderma* isolate.

Materials and Methods

Trichoderma and *R. solani* Isolates and Culture Collections

The present study was conducted with twenty-three isolates of *Trichoderma* (**Table 1**) collected from the production area of the Protected Geographical Indication (PGI), called "Alubia La Bañeza—León," without any genetic manipulation and three isolates from other collections. The *Trichoderma* isolates were stored in the collection "Pathogens and Antagonists of the Laboratory Diagnosis of Pests and Diseases" (PALDPD, University of León, León, Spain). *R. solani* R43 was also collected from plants of the same PGI and selected by its high virulence (**Table 1**).

In vitro Antifungal Assays

Trichoderma isolates were evaluated for their *in vitro* potential to antagonize the plant pathogenic fungus *R. solani* using two different tests. For all tests, plugs of 7 mm diameter collected from the edge of growing fungal colonies were used to inoculate potato dextrose agar medium (PDA) in sterile Petri dishes of 9 cm diameter. The dishes were incubated in the dark at 22°C for 7 days.

The aim of these tests was to study the percentage of *R. solani* growth inhibition caused by the different *Trichoderma* isolates.

The antifungal assay on membranes was used to quantify the ability of the *Trichoderma* isolates to produce metabolites and/or enzymes with inhibitory activity against *R. solani*. The surface of Petri dishes containing PDA medium was overlaid with a sterile cellophane membrane. *Trichoderma* plugs, extracted from PDA dishes grown for 7 days at 22°C, were placed in the center of the dish with the cellophane sheet, containing PDA medium, and incubated for 48 h at 22°C. Then, the cellophane membranes along with the mycelia of *Trichoderma* isolates were removed and *R. solani* plugs were placed in the same plates. Growth of *R. solani* was recorded after 72 h to calculate the percentage of pathogen growth inhibition (**Figure 1A**). Control PDA plates of *R. solani*, where *Trichoderma* spp. had not been previously grown, were also prepared in the same conditions as above.

TABLE 1 | *Trichoderma* and *Rhizoctonia* strains used in this study.

Lab. Code	Culture collection***	Localization/Received as	Identified as/References
T001*	PAULET20	Bustillo del Páramo (León)	*T. harzianum*
T002*	PAULET21	Roperuelos del Páramo (León)	*Trichoderma* spp.
T003*	PAULET22	Bercianos del Páramo (León)	*Trichoderma* spp.
T004*	PAULET23	Riego de la Vega (León)	*T. gamsii*
T005*	PAULET24	Valderrey (León)	*T. longibrachiatum*
T006*	PAULET25	San Esteban de Nogales (León)	*Trichoderma* spp.
T007*	PAULET26	Villamejil (León)	*Trichoderma* spp.
T008*	PAULET27	Fresno de la Vega (León)	*T. citrinoviride*
T009	PAULET28	Bustillo del Páramo (León)	*Trichoderma* spp.
T010	PAULET29	Bustillo del Páramo (León)	*T. harzianum*
T011*	PAULET30	San Pedro de Bercianos (León)	*T. harzianum*
T012*	PAULET31	Quintana del Castillo (León)	*T. harzianum*
T013*	PAULET32	Quintana del Castillo (León)	*T. atroviride*
T014	PAULET33	Santa Marina del Rey (León)	*Trichoderma* spp.
T015*	PAULET34	San Cristobal de la Polantera (León)	*T. harzianum*
T016	PAULET35	Bustillo del Páramo (León)	*Trichoderma* spp.
T017	PAULET36	Bustillo del Páramo (León)	*Trichoderma* spp.
T018	PAULET37	Urdiales del Páramo (León)	*Trichoderma* spp.
T019*	PAULET38	Carrizo de la Ribera (León)	*T. harzianum*
T020	PAULET39	Soto de la Vega (León)	*T. harzianum*
T021	PAULET40	Pozuelo del Páramo (León)	*T. harzianum*
T022	PAULET41	Villaornate (León)	*Trichoderma* spp.
T023	PAULET42	Cabreros del Río (León)	*Trichoderma* spp.
T024*	IMI 352941	*T. atroviride*	Hermosa et al., 2000
T025*	NBT 59	*T. virens*	Hermosa et al., 2004
T34**	CECT 2413	*T. harzianum*	Kullnig et al., 2001
R43	PAULER006	Santa María del Páramo (León)	*Rhizoctonia solani*

**Strains included in in vivo assays.*

***Strain only used in ergosterol and squalene assays.*

****All PAULE strains are in Pathogens and Antagonists of the Laboratory Diagnosis of Pests and Diseases (PALDPD) Collection, University of León, León, Spain; IMI, CABI Bioscience (Egham); NBT, Newbiotechnic S.A. (Seville); CECT, Spanish Type Culture Collection, Burjassot, Spain.*

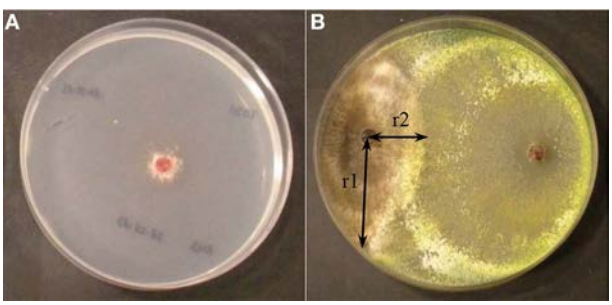


FIGURE 1 | (A) Inhibition of *R. solani* growth by metabolites of Trichoderma spp in assay on membranes **(B)** Growth of Trichoderma (right) and *R. solani* (left) in direct confrontation assays. Parameters to calculate the percentage of inhibition of growth in direct confrontation (ID) % ID = [(r1−r2)/r1] x 100.

The percentage of inhibition (IM) was calculated after 3 days of growth of *R. solani* in this medium using the formula %IM = [(C–T)/C]x100 (C: diameter of the *R. solani* control; T: diameter of *R. solani* after being exposed to the metabolites of *Trichoderma* spp.). Experiments were performed with four replicates. The results were compared by analysis of variance (ANOVA) and Fisher least significant difference (LSD) tests using SAS (SAS Institute Inc., 2004, Cary, NC, USA).

The direct confrontation assays were used to verify the ability of *Trichoderma* spp. to overgrow the pathogen. Each *Trichoderma* isolate was grown in dual culture with *R. solani* R43. The isolates were placed 5.5 cm apart on the same plate and incubated at 22°C for 5 days. Experiments were performed with four replicates. The parameters were measured after 5 days: r1 (distance between the pathogen sowing point and furthest point of the colony) and r2 (distance between the pathogen sowing point and the edge of the colony) from where *R. solani* and *Trichoderma* mycelia came into contact. Thus, the percentage of inhibition in the direct confrontation assay (ID) was calculated by the formula: %ID =[(r1–r2)/r1]x100 (**Figure 1B**). Inhibition of *R. solani* growth was compared by analysis of variance (ANOVA) and Fisher least significant difference (LSD) tests using SAS. (SAS Institute Inc., 2004, Cary, NC, USA).

In vivo Assay of the Antifungal Activity

These assays were only performed with those *Trichoderma* isolates that gave percentages of inhibition greater that 40% in membrane assays or 20% in direct confrontation assays, and that were able to sporulate on PDA medium.

The bioassays were performed in climatic chambers with 32 treatments as follow: 15 *in vitro* selected *Trichoderma* isolates against *R. solani* (R43) in order to test the antagonistic activity (RT0-number of *Trichoderma* isolate); 15 *in vitro* selected *Trichoderma* isolates in order to test their effect on plant (CT0-number of *Trichoderma* isolate); one control with *R. solani* (RC); and one control without fungi (CC). Thirty pots were used per treatment, with polypropylene pots (1 liter capacity) with substrate (80% white peat, 20% black peat and 5.5 pH). Each pot was watered with 250 ml of water prior inoculation. *R. solani* R43 was inoculated by surface irrigation with 50 ml per pot of a suspension of triturated micromicete culture of this pathogen using five Petri dishes (18 ml of PDA per dish) per liter of water. For control inoculation, only PDA medium was used without any pathogen. Pots were kept in a growth chamber for 8 days at 25°C (16 h) and 16°C (8 h), 60% relative humidity (RH) in the dark.

Trichoderma isolates were inoculated on PDA medium to grow in dark conditions (25°C) for 1 week. After that, they were exposed to light in order to induce the formation of spores. Spores suspensions were prepared at a final concentration of 2×10^7 spores/ml. Bean seeds of "Canela" variety were surface sterilized (sodium hypochlorite 1% for 3 min and distilled water for 6 min). Then, they were coated with a spore suspension of each *Trichoderma* isolate. The seeds were submerged in the spore suspension (45 seeds per 20 ml spores suspension) and they were dried in a flow chamber for 12 h. Coated seeds were sown after 8 days of the inoculation of *R. solani* R43. The culture was maintained for 45 days with a photoperiod of 16 h light, 25°C/16°C (day/night), 60% RH and brightness of 3500 lux. Irrigations were performed every 4 days with tap water (about 250 ml/pot). On the 2nd–4th week a nutrient solution was added (Rigaud and Puppo, 1975). Plants were removed after 45 days from sowing, tissues with symptoms were placed in PDA medium, incubating the plates at 22°C for 5 days and identifying the fungus for fulfill Koch's postulates. The next parameters were evaluated in removed plants after 45 days from sowing: wet weight and dry weight (72 h in an oven, 82°C) of the aerial part and root system.

The data were transformed by the formula $\sqrt{x + 0.5}$ and they were compared by analysis of variance (ANOVA) and Fisher least significant difference (LSD) tests using SAS (SAS Institute Inc., 2004, Cary, NC, USA).

Nucleic Acid Extraction and Manipulation

Genomic DNA from those isolates showing positive and negative phenotypic effects on bean plants, were extracted by growing the *Trichoderma* isolates in PDB medium (potato dextrose broth). Mycelia were then recovered by filtration, washed with 0.9% NaCl and dried on absorbent filter paper. The procedure for fungal genomic DNA isolation was performed as previously described (Cardoza et al., 2006).

PCR Amplification, Sequencing and DNA Analysis

The amplification of the ITS regions of the nuclear rDNA gene cluster and an approximately 0.56 kb fragment of the *tef1* (translation elongation factor 1-α) gene were carried out with the primer pairs ITS1/ITS4 and EF1-728F/EF1-LLErev, respectively, as described previously (Hermosa et al., 2004). The PCR products were purified from agarose gels using the NucleoSpin Extract II Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol. PCR fragments were sequenced in an ABI 377 Prism Sequencer (Applied Biosystems, Foster City, CA).

The *Trichoderma* ITS and *tef1* sequences obtained in this work were analyzed using the online interactive key (available from http://www.isth.info/tools/blast/index.php) (Druzhinina et al., 2005).

Analysis of Expression of Bean Defense-related Genes

Three bean leaves from 45 day-old plants of each treatment were randomly collected and stored at −80°C until use. Leaves were detached from plants inoculated with *Trichoderma* isolate showing positive phenotypic results in the *in vivo* test. Leaves were then reduced to a fine powder in a mortar under liquid nitrogen. Plant RNA isolation were performed as previously described (Malmierca et al., 2013).

cDNA were synthesized using 1 μg total RNA and a Reverse Transcription System with an $Oligo(dT)_{15}$ as the primer (Promega, Madison, WT). cDNA were quantified using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE) and used for further studies.

Real Time-PCR Analysis

In order to analyze the effect of *Trichoderma* treatment and/or *R. solani* infection of bean plants, oligonucleotides corresponding to seven defense-related genes were designed based on their available sequences (**Table 2**). *PR1, PR2, PR3,* and *PR4*, which encode for pathogenesis related proteins related to the salicylate (SA) pathway; *CH5b, CH1* encoding for related to the jasmonate/ethylene pathway (JA/ET), and *PAL* involved in the phenylpropanoid pathway, were selected to be analyzed in the present study. α-*actin* (Upchurch and Ramirez, 2010; Guerrero-González et al., 2011) and *PvEF1α* (this work) encoding genes were used as reference (housekeeping genes) for comparative analysis. The qPCR reactions were carried out using Step One Plus™ (Applied Biosystems, Foster City, CA). The reactions were performed in a total volume of 20 μl: 10 μl Power SYBR® Green PCR Master Mix (Applied Biosystems, USA), 0.4 μl Forward Primer 10 μM, 0.4 μl Reverse Primer 10 μM, 5 μl cDNA, and H_2O to 20 μl. The REST 2009© software (Pfaffl et al., 2002) was used to calculate the relative expression ratio and the significance of the differences between the gene expression levels. For each primer pair used in this work, we performed a standard curve with 320, 160, 80, 40, 20, and 10 ng cDNA to determine the PCR amplification efficiency (E value). Each measurement was made in triplicate (Malmierca et al., 2013).

TABLE 2 | Oligonucleotides designed for Real-Time PCR analysis.

Gene	Function gene	GenBank Accession number	Oligonucleotide name	Oligonucleotide sequence (5′-3′)	References
PvEF1α	Elongation Factor 1	EF660340.1	EF1α-F	CGGGTATGCTGGTGACTTTT	This work
			EF1α-R	CACGCTTGAGATCCTTGACA	
α-actin	Actine	U60500.1	α-actin-F	GAGCTATGAATTGCCTGATGG	This work
			α-actin-R	CGTTTCATGAATTCCAGTAGC	
PvCH5b	Chitinase	FE897014.1	CH5b-F	CAGCCAAAGGCTTCTACACC	This work
			CH5b-R	TTGTTTCGTGAGACGTTTGC	
PvPR1	Pathogenesis related 1	HO864272.1	PR1-F	TGGTCCTAACGGAGGATCAC	This work
			PR1-R	TGGCTTTTCCAGCTTTGAGT	
PvPR2	Pathogenesis related 2	HO864270.1	PR2-F	GTGAAGGACGCCGATAACAT	This work
			PR2-R	ACTGAGTTTGGGGTCGATTG	
PvPR4	Pathogenesis related 4	HO864354.1	PR4-F	CGCAGTGAGTGCATATTGCT	This work
			PR4-R	TGTTTGTCACCCTCAAGCAC	
PvCH1	Chitinase				Pereira et al., 2014
PvPR3	Pathogenesis related 3				Pereira et al., 2014
PvPAL	Phenylalanine ammonia-lyase				Pereira et al., 2014

Quantification of Ergosterol and Squalene

The *Trichoderma* selected strain was inoculated in 100 ml of CM medium (0.5% malt extract, 0.5% yeast extract, and 0.5% glucose) with 10^6 spores/ml, and incubated 24 h at 28°C. Then 20 ml from the previous cultures were inoculated on 100 ml potato dextrose broth (PDB medium) and were incubated as before during 24–96 h. The mycelia were filtered through nytal filters (30 μm diameter) and the liquid removed by drying between filter papers. The dry weight of the fungal pellet was calculated. Total intracellular sterols were extracted and ergosterol and squalene content were quantified as previously reported other authors (Cardoza et al., 2007; Ghimire et al., 2009). All measurements were made in duplicate in the *Trichoderma* selected isolate and a strain used as control of the same species of the selected isolate. The results were compared by analysis of variance (ANOVA) and Fisher least significant difference (LSD) tests using SAS (SAS Institute Inc., 2004, Cary, NC, USA).

Results

Analysis of the *in vitro* Antagonistic Activity of *Trichoderma* Isolates with *R. solani*

The first test to determine the *in vitro* antifungal ability of the different *Trichoderma* isolates was based on their ability to produce metabolites that may inhibit the growth of *R. solani* (**Table 3**, **Figure 1A**). *Trichoderma* isolates T003, T004, T006, T020, T022, T012, T013, T025, T016, T007, T024, T005, and T010 inhibited *R. solani* growth by more than 75%, with the highest inhibition produced by T003, T004, T006, T020, and T022 (86.70%). T019, T008, T002, T021, T001, T018, and T023 showed a remarkable inhibition (75–40%). Finally, T015, T014, T017, T011, and T009 inhibited *R. solani* growth by less than 40%, and T009 showed the lowest percentage (15.82%).

T021 was the *Trichoderma* isolate showing the highest percentage of inhibition (72.77%) in the direct confrontation assays (**Table 3**, **Figure 1B**), whereas T009 showed the lowest inhibition values (14.63%). The inhibition percentages detected for the other *Trichoderma* isolates ranged from 47.13 to 30.55%.

Analysis of the *in vivo* Antagonistic Activity of *Trichoderma* Isolates with *R. solani*

The results of the *in vitro* membrane assays and direct confrontation assays against *R. solani*, showed above, were used to select the isolates that would be used for the *in vivo* analysis. Thus, fifteen *Trichoderma* isolates (marked with asterisk in **Tables 1**, **3**) were tested since they were able to sporulate in PDA medium and showed a percentage of inhibition of *R. solani* growth higher than 40% in the membrane assays, and/or 20% in the direct confrontation assays.

On plants removed after 45 days from sowing for Koch's postulates, *R. solani* was found and *Trichoderma* isolates were also present in the medium.

When dry aerial parts (**Figure 2**) were analyzed, plants treated with CT019 had the greatest weight of the aerial part, being significantly different from the control (CC). In the RT019 treatment, plants did not show significant differences in comparison with the control (CC), what is indicative of a biocontrol effect by T019 isolate.

In the case of the root system (**Figure 3**), the situation was similar to that observed in the aerial parts. CT019 treated plants were not significantly different in dry weight, although it was always greater than the weight of the control plants (CC). If the pathogen was present in the soil with same *Trichoderma* isolate, RT019 treatment, in the case of wet weight, there were no significant differences between control (CC) and control pathogen (RC) (data not shown).

Based on these results, the *Trichoderma* isolate T019 was selected for further studies since it showed the best positive effects on plant phenotype among all the analyzed isolates (**Figure 4**).

Molecular Identification of *Trichoderma* Isolates

Those *Trichoderma* isolates able to sporulate in PDA medium, showing a percentage of inhibition higher than

TABLE 3 | *In vitro* antifungal activity of *Trichoderma* strains against *R. solani* R43.

Strain	Inhibition in growth assay on membranes (% ± Standard error) (1)		Strain	Inhibition in growth assay on direct confrontation (% ± Standard error) (2)	
T003*	86.70 ± 0.15	a	T021	72.77 ± 4.49	a
T004*	86.70 ± 0.15	a	T004*	47.13 ± 0.91	b
T006*	86.70 ± 0.15	a	T013*	43.49 ± 1.41	bc
T020	86.70 ± 0.15	a	T011*	42.57 ± 2.00	bcd
T022	86.70 ± 0.15	a	T014	41.99 ± 1.33	cde
T012*	86.67 ± 0.14	a	T023	41.57 ± 1.40	cdef
T013*	85.04 ± 1.04	a	T018	41.01 ± 1.93	cdefg
T025*	83.97 ± 1.97	a	T020	39.21 ± 1.41	cdefgh
T016	82.29 ± 2.33	a	T006*	38.68 ± 0.59	defghi
T007*	76.77 ± 2.52	b	T017	37.42 ± 1.61	efghij
T024*	76.22 ± 2.32	bc	T016	37.10 ± 0.63	fghij
T005*	75.65 ± 2.23	bc	T002*	36.44 ± 0.29	ghij
T010	75.14 ± 3.30	bc	T024*	36.43 ± 3.71	hij
T019*	72.36 ± 2.01	bcd	T007*	36.34 ± 1.30	hij
T008*	70.61 ± 1.82	cde	T005*	36.16 ± 0.67	hij
T002*	66.75 ± 1.18	def	T019*	35.09 ± 1.01	hijk
T021	65.98 ± 0.77	ef	T022	35.05 ± 0.34	hijk
T001*	65.51 ± 1.75	f	T008*	35.04 ± 0.89	hijk
T018	54.12 ± 2.35	g	T012*	34.59 ± 0.98	ijk
T023	48.26 ± 3.10	h	T025*	33.28 ± 4.24	kjl
T015*	39.99 ± 0.82	i	T010	30.55 ± 1.32	kl
T014	38.82 ± 2.96	ij	T015*	28.84 ± 0.85	lm
T017	34.10 ± 0.88	j	T001*	24.66 ± 0.74	m
T011*	21.40 ± 2.66	k	T003*	19.85 ± 2.97	n
T009	15.82 ± 1.40	l	T009	14.63 ± 0.83	o

Values in the same column followed by different letters are significantly different (Fisher's LSD. $p < 0.05$).

(1) Growth assay on membranes (effect of Trichoderma isolates on percent growth inhibition of R. solani. using cellophane membranes. (2) in direct confrontation assays (percentage of inhibition of R43 growth when grown confronted with Trichoderma strains during 5 days in PDA medium plates).

**Trichoderma isolates selected for in vivo experiments because they were able to sporulate in PDA medium and showed a percentage of inhibition higher than 40% in the membrane assays. and/or 20% in the direct confrontation assays.*

40% in the membrane assays, and/or 20% in the direct confrontation assays, were identified. ITS1 region of rDNA and a fragment of the translation elongation factor 1 (*tef1*) were amplified and sequenced for 12 isolates, and both nucleotide sequences were used for identification at species level. Eight out of these 12 isolates were identified as *T. harzianum*, and *T. atroviride*, *T. gamsii*, *T. longibrachiatum* and *T. citrinoviride* species were represented by one isolate (**Table 1**).

Effect of *Trichoderma* Treatment and/or *R. solani* Infection in the Expression of Bean Defense Related Genes

The amplification efficiencies of the oligo-pairs (**Table 2**) were: *α-actin* 1.150, *PvEF1α* 0.903, *CH5b* 0.883, *CH1* 1.098, *PR1* 1.094, *PR2* 1.048, *PR3* 0.947, *PR4* 0.922, and *PAL* 0.962.

α-actin and *PvEF1α* were used as housekeeping genes to determine the relative expression level of the other genes analyzed in the present work. *Trichoderma* T019 strain was selected, based on its positive effects on bean phenotype with and without *R. solani* infection. The results included in **Figure 5** showed that: (i) *R. solani* down-regulated the expression of all the *P. vulgaris* defense-related genes analyzed (**Figure 5A**), raising values of expression ranging between 0.099 ($p = 0.029$) for *PR2* and 0.397 ($p = 0.045$) for *CH1*; (ii) In plants treated with T019 compared with control plants (bean plants not treated with *Trichoderma* nor infected with *R. solani*) only the *CH5b* and *PR2* were significantly up-regulated, raising comparative expression values of 1.495 ($p = 0.000$) and 24.492 ($p = 0.000$), respectively (**Figure 5B**). Finally, (iii) treatment with T019 and infection with *R. solani* significantly up-regulated the expression ratio of the analyzed genes, except *PAL*, in comparison with plants only infected with *R. solani*, with values ranging from 1.420 ($p = 0.000$) to 42.975 ($p = 0.000$) for *CH1* and *PR4* respectively (**Figure 5C**).

A non-remarkable significant up-regulation of the defense-related gene expression was observed when expression ratios of the selected genes in plants treated with *T. harzianum* T019 and infected with *R. solani* were compared with those from plants untreated or uninfected (data not shown).

Production of Ergosterol and Squalene by the *Trichoderma* Selected Strains

Strain T019 (*T. harzianum*) was selected for this analysis because it was those producing the most remarkable positive phenotypic effect on beans (see above). *T. harzianum* T34 (**Table 1**) was used as control strain for comparative purposes because it is a well-known strain, widely characterized (Kullnig et al., 2001).

Production of ergosterol at 24 h of growth did not show significant differences between the strains *T. harzianum* (T34 and T019) (**Table 4**). When the production of ergosterol was analyzed at 96 h, T019 produced significantly higher amounts than its respective control (T34).

Regarding the squalene production at 24 h, *T. harzianum* (T34 and T019) strains did not show significant differences. However, at 96 h of growth, T019 produced amounts significantly higher than its control.

Discussion

Most of the *Trichoderma* isolates studied in the present work have been able to control the growth of *R. solani*. The percentage of growth inhibition in the direct confrontation assays raised values up to 72.77% for T021, but most of the isolates were between 47 and 24%. By contrast, in the antifungal assays on membranes, those percentages ranged mostly between 86 and 58%. In the assays conducted by Campelo et al. (2010) the percentage of inhibition in membrane assays using *T. virens* T59 (NBT59) and *T. atroviride* T11 (IMI352941) range between 100 and 84.7%, respectively. The differences observed in the different *in vitro* assays might be due to the variability of genotypes, with differences in growth, sporulation and in their environmental

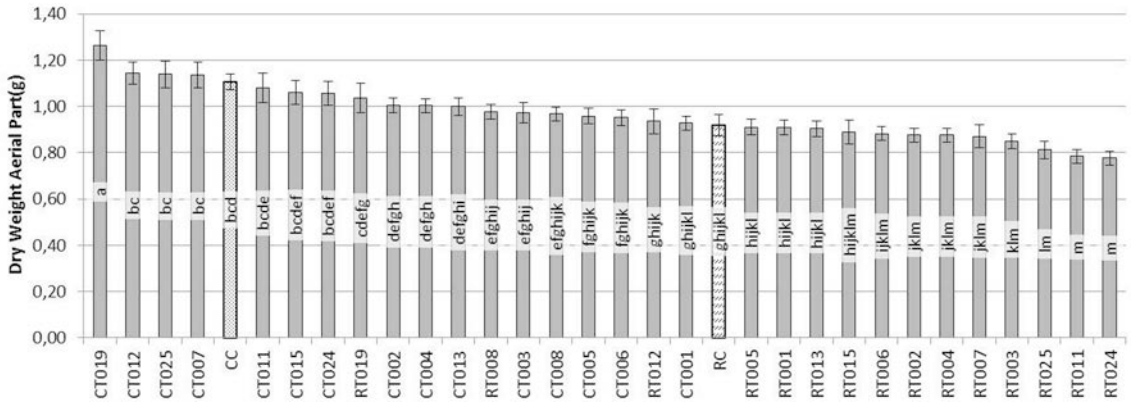


FIGURE 2 | Evaluation of the dry weight (g) of the aerial parts of bean plants grown during 45 days after sowing with 60 replicates. [*Trichoderma* isolates without pathogen (CT0-number of *Trichoderma* isolate), *Trichoderma* isolates with *R. solani* (RT0-number of *Trichoderma* isolate), *R. solani* control (RC) and control without fungus (CC)].

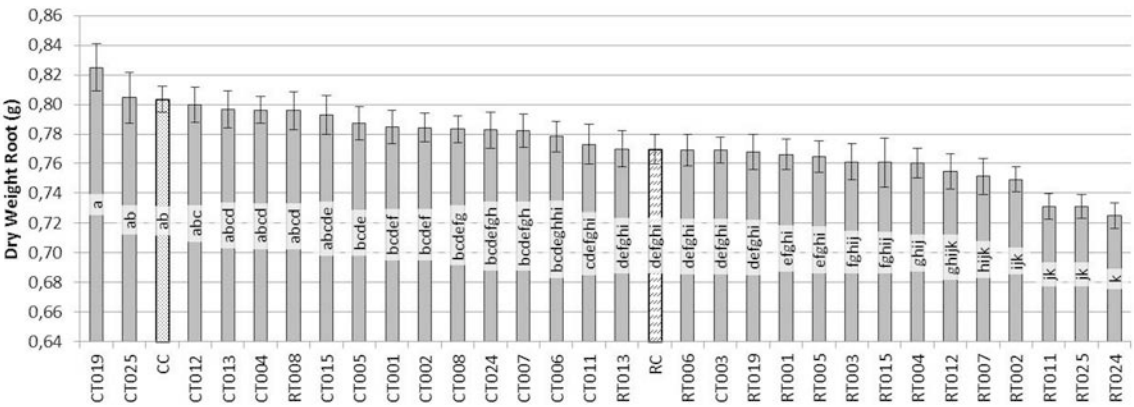


FIGURE 3 | Evaluation of the dry weight (g) of the root system of bean plants grown for 45 days after sowing with 60 replicates. [*Trichoderma* isolates without pathogen (CT0-number of *Trichoderma* isolate), *Trichoderma* isolates with *R. solani* (RT0-number of *Trichoderma* isolate), *R. solani* control (RC) and control without fungus (CC)].

biological interactions as a consequence of the prevalence of different modes of action on each of the analyzed strains (Ruano-Rosa et al., 2010).

The *in vitro* antagonistic activity of the *Trichoderma* isolates is an indication of their *in vivo* biological activity against phytopathogenic fungi. However, it has been found that the *in vitro* antifungal activity of *Trichoderma* does not correlate in a direct way with its *in vivo* ability to control diseases caused by phytopathogenic fungi, since many other factors influence that activity (Anees et al., 2010).

Regarding the *in vivo* assays, bean plants that had been in contact with some *Trichoderma* isolate had an increased size when the pathogen was not present. Pereira et al. (2014) also observed that *T. harzianum* was able to promote the growth of common bean plants in comparison to plants grown in its absence. However, in the present work only T019 treated plants did not decrease their size in the presence of *R. solani*. These data are in agreement with those from Yedidia and coworkers reporting a much stronger effect on cucumber plants treated with *T. harzianum*, which increased by 75% the length of the root, 95% aerial parts, 80% dry weight and 80% the size of the blade relative to the untreated control (Yedidia et al., 2001). *T. harzianum* was able to promote the growth of common bean plants in comparison to plants grown in its absence (Pereira et al., 2014).

However, in the present work opposite results were observed to those previously reported (Tello et al., 1985), indicating that infection with *R. solani* not always resulted in a reduction of the bean plant size, which was explained as a result of the activation of the natural plant-defense mechanisms (Cardoso and Echandi, 1987) that would lead to an enhanced development of the plants when this pathogen was present in the soil. In the present study we have observed that treatment with *R. solani* resulted in a down-regulation of all the bean defense-related genes analyzed, indicating a certain compensation effect between the intensity of plant defense response and plant growth (Hermosa et al., 2013).

FIGURE 4 | Differences in growth of bean plants. CC plant control; RC plant with *R. solani*, CT019 plant with *Trichoderma* T019; RT019 plant with *R. solani* and *Trichoderma* T019.

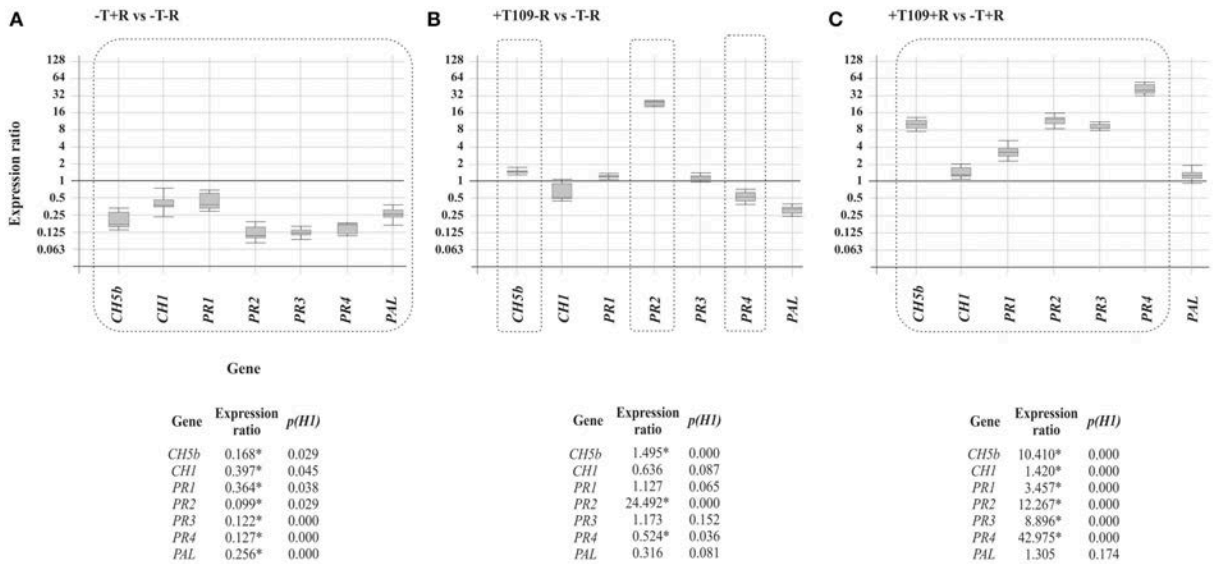
Little is known about the effect of *Trichoderma* treatment and/or infection with *R. solani* in the expression of bean defense-related gene expression. As indicated above, interaction of the plant with *R. solani* during 45 days of growth caused the repression of the seven defense-related genes studied (*CH5b, CH1, PR1, PR2, PR3, PR4*, and *PAL1*), as a mechanism to overcome the plant defense response and thus facilitating the progression of the infection process within the plant. Other authors have observed similar results in assays with tomato plants infected with *Pseudomonas syringae* pv. *tomato*, where a repression of *PR1* and *PR4* was shown, as an indication of the decrease in the plant self-defense mechanism, then facilitating disease progression (Zhao et al., 2003).

Expression of *P. vulgaris* defense-related genes was analyzed in leaves, even when the interaction with the pathogen *R. solani* is initially produced at the root level, to determine if the signals generated in roots as result of this interaction are able to systematically stimulate the bean defense along long distance from seed to leaf. When the interaction of plant with T019 was analyzed, *PR2* was up-regulated, *PR4* was slightly restrained but *PR1* and *PR3* were not affected. Pereira and coworkers observed that *T. harzianum* also seems to potentiate common bean response against the phytopathogenic fungus *R. solani,* as shown by the increase in the levels of *glu1* and *pod3* in the double treatment in comparison to that obtained for plants in the presence of *R. solani* alone (Pereira et al., 2014). Other studies showed that there was an increase in expression *PR1* at 16 h of interaction (Guerrero-González et al., 2011). Rivière and coworkers suggested that *PR1* was down-regulated by the β-1,3-glucanases (PR2 and PR3) (Rivière et al., 2008). In the present study, after 45 days of the inoculation of the biocontrol isolate we observed a higher expression of *PR2*, which could be due to an alteration of plant defense responses at these longer times in comparison with shorter ones assayed in the previous reported works. This would result in a higher β-1,3-glucanase activity in the cell wall that would increase oligosaccharides released by its action, then acting as elicitors of the plant defense response and/or of the fungal secondary metabolism (Druzhinina et al., 2011).

In the case of plants inoculated with *Trichoderma* and seeded in soil infected with *R. solani*, when compared with plants only infected with *R. solani*, thus excluding the effect due exclusively to this pathogen, a significant up-regulation of all the analyzed genes was observed, except *PAL*, indicating that in presence of *R. solani* several mechanisms are induced in *Trichoderma* that potentiate its ability to elicit plant defense-responses. In previous works, during the interaction of *Solanum tuberosum* with *R. solani* it was observed that after 120 h from the initial infection and damage to the first floor, new outbreaks produce less symptoms, suggesting that the plant might be prepared to defend and avoid disease progression (Lehtonen et al., 2008). Other studies have shown that the *PR4* was overexpressed in eggplants treated with BCAs suggesting that these agents promoted defensive reactions within the plant (Angelopoulou et al., 2014). In other assays with pepper plants an increase in the accumulation of PR1 and chitinases was also observed when these plants were previously inoculated with a strain of *Fusarium oxysporum* and subsequently exposed to *Verticillium dahliae* (Veloso and Díaz, 2012), emphasizing their importance in the response to pathogen infection and to abiotic stresses (Jung and Hwang, 2000). Thus, it could be inferred that the effect of *Trichoderma* would therefore modulate the response of the plant and prevent the suppression of defense genes caused by *R. solani.* However, the effect of the different *Trichoderma* strains in the different plants would indicate the existence of great differences between them.

In the case of *CH5b*, related to JA pathway, when *R. solani* was in contact with the plant for 45 days of development, the expression of this gene was down-regulated in comparison with non-infected plants. When bean plants were inoculated with *Trichoderma*, its expression was slightly induced. However, if the antagonist and the pathogen were present in the medium, the effect caused by *R. solani* was the overexpression of this gene. In previous works (Broglie et al., 1991) it was shown that *CH5b*, responsible for chitinase production, was overexpressed in *Nicotiana tabacum* and *Brassica napus* infected with *R. solani*, and their disease symptoms were reduced. Benhamou et al. (1993) found that the expression of this gene caused a reduction of disease in *B. napus* infected with the same pathogen, which correlates with similar results observed in strawberry plants inoculated with *Botrytis cinerea* (Vellicce et al., 2006). In the present study we observed that the contact of the plant with the pathogen during a period of 45 days resulted in a significant repression of this gene, similarly to what it was observed for the expression of *CH1*, which thereby facilitates disease progression. However, in the presence of *Trichoderma* a similar effect to that previously described for SA-related genes was observed, so facilitating a prevention of disease progression.

In the case of the phenylalanine ammonia lyase (PAL), this is a key enzyme in the metabolic pathway of phenylpropanoid compounds by catalyzing the amino acid L-phenylalanine



Gene	Expression ratio	p(H1)
CH5b	0.168*	0.029
CH1	0.397*	0.045
PR1	0.364*	0.038
PR2	0.099*	0.029
PR3	0.122*	0.000
PR4	0.127*	0.000
PAL	0.256*	0.000

Gene	Expression ratio	p(H1)
CH5b	1.495*	0.000
CH1	0.636	0.087
PR1	1.127	0.065
PR2	24.492*	0.000
PR3	1.173	0.152
PR4	0.524*	0.036
PAL	0.316	0.081

Gene	Expression ratio	p(H1)
CH5b	10.410*	0.000
CH1	1.420*	0.000
PR1	3.457*	0.000
PR2	12.267*	0.000
PR3	8.896*	0.000
PR4	42.975*	0.000
PAL	1.305	0.174

FIGURE 5 | Expression of *CH5b, CH1, PR1, PR2, PR3, PR4*, and *PAL* genes in comparison with *α-actin* and *PvEF-1α* reference genes. The comparisons and statistical analysis were performed using the REST2009© program (Pfaffl et al., 2002). The numeric data are illustrated at the bottom of the figure and those showing differences statistically significant ($p < 0.05$) are indicated with an asterisk and they are boxed in the graphic representation. Comparison of the gene expression of the bean defense-related genes **(A)** in plants infected with *R. solani* R43 vs. control plants. **(B)** In plants treated with *Trichoderma* T019 vs. control plants **(C)** In plants infected with *R. solani* R43 and treated with *Trichoderma* T019 vs. plants infected with *R. solani* R43.

TABLE 4 | Ergosterol and squalene quantification from 24 and 96 h samples of the selected *Trichoderma* strains selected for this study.

Trichoderma spp.	Ergosterol (mg E/g d wt)		Squalene (mg S/g d wt)	
	24 h	96 h	24 h	96 h
T34	16.197 ± 1.019 a	8.970 ± 0.388 b	0.317 ± 0.020 a	0.052 ± 0.049 b
T019	14.336 ± 2.459 a	12.502 ± 3.568 a	0.303 ± 0.041 a	0.348 ± 0.103 a

Values in the same column followed by different letters are significantly different (Fisher's LSD, $p < 0.05$).

deamination, giving rise to trans-cinnamic acid and ammonia. The trans-cinnamic acid is used for the synthesis of various phenolic compounds, which are precursors in the synthesis of esters, coumarins, flavonoids, and lignin. The production of this enzyme is controlled during plant growth, but is also induced in cells neighboring the infection site and, besides infection, various environmental stimuli such as injury, heavy metal contamination, light and growth regulators (Rahman and Punja, 2005). In the present work, we observed that this gene was down-regulated or not affected in all the conditions assayed, indicating that in the analyzed strain, PAL activity would be more focused in the plant response against environmental stress than in the response against pathogens, which contrast with the role of this enzyme described in pepper, where it is involved in the positive regulation of SA-dependent defense signaling (Kim and Hwang, 2014).

Regarding squalene and ergosterol production, in this study it was found that there were differences among the isolates. Ergosterol is a sterol found in the fungal membrane, which although considered by the plant as a PAMP (pathogen associated molecular patterns) (Nürnberger et al., 2004) triggers a series of reactions (Cervone et al., 1997) which would cause an activation of the genes of plant defense (Rossard et al., 2010). Squalene is a precursor of the ergosterol biosynthetic pathway (Malmierca et al., 2013) located in the cellular membranes or accumulated as droplets in the cytoplasm whose physiological function, apart from acting as an ergosterol precursor, still remains unclear, but it might play some role in the elicitation of plant defense responses. Thus, in this assay, an increased production of these compounds by *Trichoderma* would result in the induction of defense genes in the bean, then the plant could grow better under a pathogen presence in the soil.

Conclusions

Trichoderma isolates inhibit the development of *R. solani* under *in vitro* conditions, when they grow in Petri dishes. *In vivo* conditions, the bean plants that had been in contact with the *Trichoderma* isolates always had an increased size when the

pathogen was not present. When *R. solani* was present in the soil, the development of the bean plants was significantly reduced. However, bean plants treated with strain T019 did not decrease their size in the presence of *R. solani*.

The interaction of bean plants with *R. solani* caused, after 45 days of growth, the down-regulation of the seven defense-related genes studied (*CH5b, CH1, PR1, PR2, PR3, PR4, PAL*) as a mechanism to overcome the plant defense response and thus facilitating the progression of the infection process within the plant.

An increased production of ergosterol and squalene by *Trichoderma* resulted in the induction of defense genes in the bean plants. In this way, the plant would grow better under a pathogen presence in the soil.

T. harzianum T019 shows a positive effect on the level of resistance of bean plants to *R. solani*. This strain induces the expression of plant defense-related genes and produces a higher level of ergosterol, indicating its ability to grow at a higher rate in the soil, which would explain its positive effects on plant growth and defense in the presence of the pathogen.

Acknowledgments

The grant awarded to Sara Mayo (FPU12/00677) by the Ministry of Education, Culture and Sport (Spain) according the Resolution of April 25, 2012 (BOE of 10 May), as amended by Resolution of May 18, 2012 (BOE of 22 May), and by Resolution of October 31, 2012 (BOE of 12 November) and granted in the Resolution of December 20, 2012. Ministry of Economy and Competitiveness for National project "Farnesol as self-regulatory molecule in *Trichoderma*. Tyrosol and farnesol signaling in *Trichoderma*-bean interaction" (AGL2012-40041-C02-02). Junta de Castilla y León, Consejería de Educación for the project "Effect of terpenes and physiologically related compounds produced by *Trichoderma parareesei* in the development of common bean (*Phaseolus vulgaris* L.) and in defensive responses of this plant" (LE228U14). J. Alvarez for his technical support.

References

Anees, M., Tronsmo, A., Edel-Hermann, V., Hjeljord, L. G., Héraud, C., and Steinberg, C. (2010). Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani*. *Fungal Biol.* 114, 691–701. doi: 10.1016/j.funbio.2010.05.007

Angelopoulou, D. J., Naska, E. J., Paplomatas, E. J., and Tjamos, S. E. (2014). Biological control agents (BCAs) of verticillium wilt: influence of application rates and delivery method on plant protection, triggering of host defence mechanisms and rhizosphere populations of BCAs. *Plant Pathol.* 63, 1062–1069. doi: 10.1111/ppa.12198

Benhamou, N., Broglie, K., Chet, I., and Broglie, R. (1993). Cytology of infection of 35S-bean chitinase transgenic canola plants by *Rhizoctonia solani*: cytochemical aspects of chitin breakdown *in vivo*. *Plant J.* 4, 295–305. doi: 10.1046/j.1365-313X.1993.04020295.x

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., et al. (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* 254, 1194–1197.

Campelo, P., Cardoza, R., Lorenzana, A., Hermosa, M., Monte, E., Reinoso, B., et al. (2010). Biological control of phytopathogenic fungi in bean (*Phaseolus vulgaris* L.) with *Trichoderma atroviride* and *Trichoderma virens*. [Abstract]. *Bean Improv. Coop. Colo.* 53, 114–115.

Cardoso, J., and Echandi, E. (1987). Biological control of Rhizoctonia root rot of snap bean with binucleate *Rhizoctonia*-like fungi. *Plant Dis.* 71, 167–170. doi: 10.1094/PD-71-0167

Cardoza, R. E., Hermosa, M. R., Vizcaíno, J. A., González, F., Llobell, A., Monte, E., et al. (2007). Partial silencing of a hydroxy-methylglutaryl-CoA reductase-encoding gene in *Trichoderma harzianum* CECT 2413 results in a lower level of resistance to lovastatin and lower antifungal activity. *Fungal Genet. Biol.* 44, 269–283. doi: 10.1016/j.fgb.2006.11.013

Cardoza, R. E., Vizcaíno, J. A., Hermosa, M. R., Sousa, S., González, F. J., Llobell, A., et al. (2006). Cloning and characterization of the erg1 gene of *Trichoderma harzianum*: effect of the erg1 silencing on ergosterol biosynthesis and resistance to terbinafine. *Fungal Genet. Biol.* 43, 164–178. doi: 10.1016/j.frb.2005.11.002

Casquero, P. A., Lema, M., Santalla, M., and De Ron, A. M. (2006). Performance of common bean (*Phaseolus vulgaris* L.) landraces from Spain in the Atlantic and Mediterranean environments. *Genet. Res. Crop Evol.* 53, 1021–1032. doi: 10.1007/s10722-004-7794-1

Cervone, F., Castoria, R., Leckie, F., and De Lorenzo, G. (1997). "Perception of fungal elicitors and signal transduction," in *Signal Transduction in Plants*, ed P. Aducci (Basel, Boston, Berlin: Birkhäuser), 153–177.

Druzhinina, I. S., Kopchinskiy, A. G., Komon, M., Bissett, J., Szakacs, G., and Kubicek, C. P. (2005). An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet. Biol.* 42, 813–828. doi: 10.1016/j.fgb.2005.06.007

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., et al. (2011). *Trichoderma*: the genomics of opportunistic success. *Nat. Rev. Microbiol.* 9, 749–759. doi: 10.1038/nrmicro2637

Garaiová, M., Zambojová, V., Šimová, Z., Griac, P., and Hapala, I. (2013). Squalene epoxidase as a target for manipulation of squalene levels in the yeast *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 14, 310–323. doi: 10.1111/1567-1364.12107

Ghimire, G. P., Hei, C. L., and Jae, K. S. (2009). Improved squalene production via modulation of the methylerythritol 4-phosphate pathway and heterologous expression of genes from *Streptomyces peucetius* ATCC 27952 in *Escherichia coli*. *Appl. Environ. Microbiol.* 75, 7291–7293. doi: 10.1128/AEM.01402-09

Guerrero-González, M. L., Rodríguez-Kessler, M., Rodríguez-Guerra, R., González-Chavira, M., Simpson, J., Sanchez, F., et al. (2011). Differential expression of *Phaseolus vulgaris* genes induced during the interaction with *Rhizoctonia solani*. *Plant Cell Rep.* 30, 1465–1473. doi: 10.1007/s00299-011-1055-5

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. (2004). *Trichoderma* species–opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2, 43–56. doi: 10.1038/nrmicro797

Harman, G. E., and Kubicek, C. P. (1998). *Trichoderma and Gliocladium: Enzymes, Biological Control and Commercial Applications*. Vol. 2, London: CRC Press.

Hermosa, M. R., Grondona, I., Iturriaga, E. A., Díaz-Mínguez, J. M., Castro, C., Monte, E., et al. (2000). Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. *Appl. Environ. Microbiol.* 66, 1890–1898. doi: 10.1128/AEM.66.5.1890-1898.2000

Hermosa, M. R., Keck, E., Chamorro, I., Rubio, B., Sanz, L., Vizcaíno, J. A., et al. (2004). Genetic diversity shown in *Trichoderma* biocontrol isolates. *Mycol. Res.* 108, 897–906. doi: 10.1017/S0953756204000358

Hermosa, R., Belén Rubio, M., Cardoza, R. E., Nicolás, C., Monte, E., and Gutiérrez, S. (2013). The contribution of *Trichoderma* to balancing the costs of plant growth and defense. *Int. Microbiol.* 16, 69–80. doi: 10.2436/20.1501.01.181

Hermosa, R., Viterbo, A., Chet, I., and Monte, E. (2012). Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiol* 158, 17–25. doi: 10.1099/mic.0.052274-0

Jung, H. W., and Hwang, B. K. (2000). Pepper gene encoding a basic ß-1,3-glucanase is differentially expressed in pepper tissues upon pathogen infection

and ethephon or methyl jasmonate treatment. *Plant Sci.* 159, 97–106. doi: 10.1016/S0168-9452(00)00334-4

Kim, D. S., and Hwang, B. K. (2014). An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signalling of the defence response to microbial pathogens. *J. Exp. Bot.* 65, 2295–2306. doi: 10.1093/jxb/eru109

Kullnig, C., Krupica, T., Woo, S. L., Mach, R. L., Rey, M., Benítez, T., et al. (2001). Confusion abounds over identities of *Trichoderma* biocontrol isolates. *Mycol. Res.* 105, 770–772. doi: 10.1017/S0953756201229967

Lehtonen, M. J., Somervuo, P., and Valkonen, J. P. T. (2008). Infection with *Rhizoctonia solani* induces defense genes and systemic resistance in potato sprouts grown without light. *Phytopathol* 98, 1190–1198. doi: 10.1094/PHYTO-98-11-1190

Lochman, J., and Mikeš, V. (2005). Activation of different defence-related genes expression by ergosterol. *FEBS J.* 272, 470. doi: 10.1111/j.1742-4658.2005.4739_12.x

Malmierca, M. G., Barua, J., Mccormick, S. P., Izquierdo-Bueno, I., Cardoza, R. E., Alexander, N. J., et al. (2014). Novel aspinolide production by *Trichoderma arundinaceum* with a potential role in *Botrytis cinerea* antagonistic activity and plant defence priming. *Environ. Microbiol.* 17, 1103–1118. doi: 10.1111/1462-2920.12514

Malmierca, M. G., Cardoza, R. E., Alexander, N. J., McCormick, S. P., Collado, I. G., Hermosa, R., et al. (2013). Relevance of trichothecenes in fungal physiology: disruption of tri5 in *Trichoderma arundinaceum*. *Fungal Genet. Biol.* 53, 22–33. doi: 10.1016/j.fgb.2013.02.001

Mukherjee, P. K., Horwitz, B. A., Herrera-Estrella, A., Schmoll, M., and Kenerley, C. M. (2013). *Trichoderma* research in the genome era. *Annu. Rev. Phytopathol.* 51, 105–129. doi: 10.1146/annurev-phyto-082712-102353

Nürnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: Striking similarities and obvious differences. *Immunol. Rev.* 198, 249–266. doi: 10.1111/j.0105-2896.2004.0119.x

Papavizas, G. (1985). *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. *Annu. Rev. Phytopathol.* 23, 23–54. doi: 10.1146/annurev.py.23.090185.000323

Pereira, J. L., Queiroz, R. M. L., Charneaum, S. O., Felix, C. R., Ricart, C. A. O., Lopes Da Silva, F., et al. (2014). Analysis of *Phaseolus vulgaris* response to its association with *Trichoderma harzianum* (ALL-42) in the presence or absence of the phytopathogenic fungi *Rhizoctonia solani* and *Fusarium solani*. *PLoS ONE* 9:e98234. doi: 10.1371/journal.pone.0098234

Pfaffl, M. W., Horgan, G. W., and Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 30:e36. doi: 10.1093/nar/30.9.e36

Rahman, M., and Punja, Z. K. (2005). Biochemistry of ginseng root tissues affected by rusty root symptoms. *Plant Physiol. Biochem.* 43, 1103–1114. doi: 10.1016/j.plaphy.2005.09.004

Rigaud, J. R., and Puppo, A. (1975). Indole 3 acetic acid catabolism by soybean bacteroids. *J. Gen. Microbiol.* 88, 223–228. doi: 10.1099/00221287-88-2-223

Rivière, M., Marais, A., Ponchet, M., Willats, W., and Galiana, E. (2008). Silencing of acidic pathogenesis-related PR-1 genes increases extracellular β-(1→3)-glucanase activity at the onset of tobacco defence reactions. *J. Exp. Bot.* 59, 1225–1239. doi: 10.1093/jxb/ern044

Rossard, S., Roblin, G., and Atanassova, R. (2010). Ergosterol triggers characteristic elicitation steps in *Beta vulgaris* leaf tissues. *J. Exp. Bot.* 61, 1807–1816. doi: 10.1093/jxb/erq047

Ruano-Rosa, D., del Moral-Navarrete, L., and Lopez-Herrera, C. J. (2010). Selection of *Trichoderma* spp. isolates antagonistic to Rosellinia necatrix. *Span. J. Agric. Res.* 8, 1084–1097. doi: 10.5424/sjar/2010084-1403

Tello, J., Lacasa, A., and Molina, R. (1985). Una nota fitopatológica sobre el complejo parasitario del pie de la judía (*Phaseolus vulgaris*). *ITEA* 61, 57–69.

Upchurch, R. G., and Ramirez, M. E. (2010). Defense-related gene expression in soybean leaves and seeds inoculated with *Cercospora kikuchii* and *Diaporthe phaseolorum* var. meridionalis. *Physiol. Mol. Plant Pathol.* 75, 64–70. doi: 10.1016/j.pmpp.2010.08.007

Valenciano, J. B., Casquero, P. A., Boto, J. A., and Marcelo, V. (2006). Evaluation of the occurrence of root rots on bean plants (*Phaseolus vulgaris*) using different sowing methods and with different techniques of pesticide application. *N.Z. J. Crop. Horticul. Sci.* 34, 291–298. doi: 10.1080/01140671.2006.9514419

Vellicce, G. R., Ricci, J. C. D., Hernández, L., and Castagnaro, A. P. (2006). Enhanced resistance to *Botrytis cinerea* mediated by the transgenic expression of the chitinase gene ch5B in strawberry. *Transgenic Res.* 15, 57–68. doi: 10.1007/s11248-005-2543-6

Veloso, J., and Díaz, J. (2012). *Fusarium oxysporum* Fo47 confers protection to pepper plants against *Verticillium dahliae* and *Phytophthora capsici*, and induces the expression of defence genes. *Plant Pathol.* 61, 281–288. doi: 10.1111/j.1365-3059.2011.02516.x

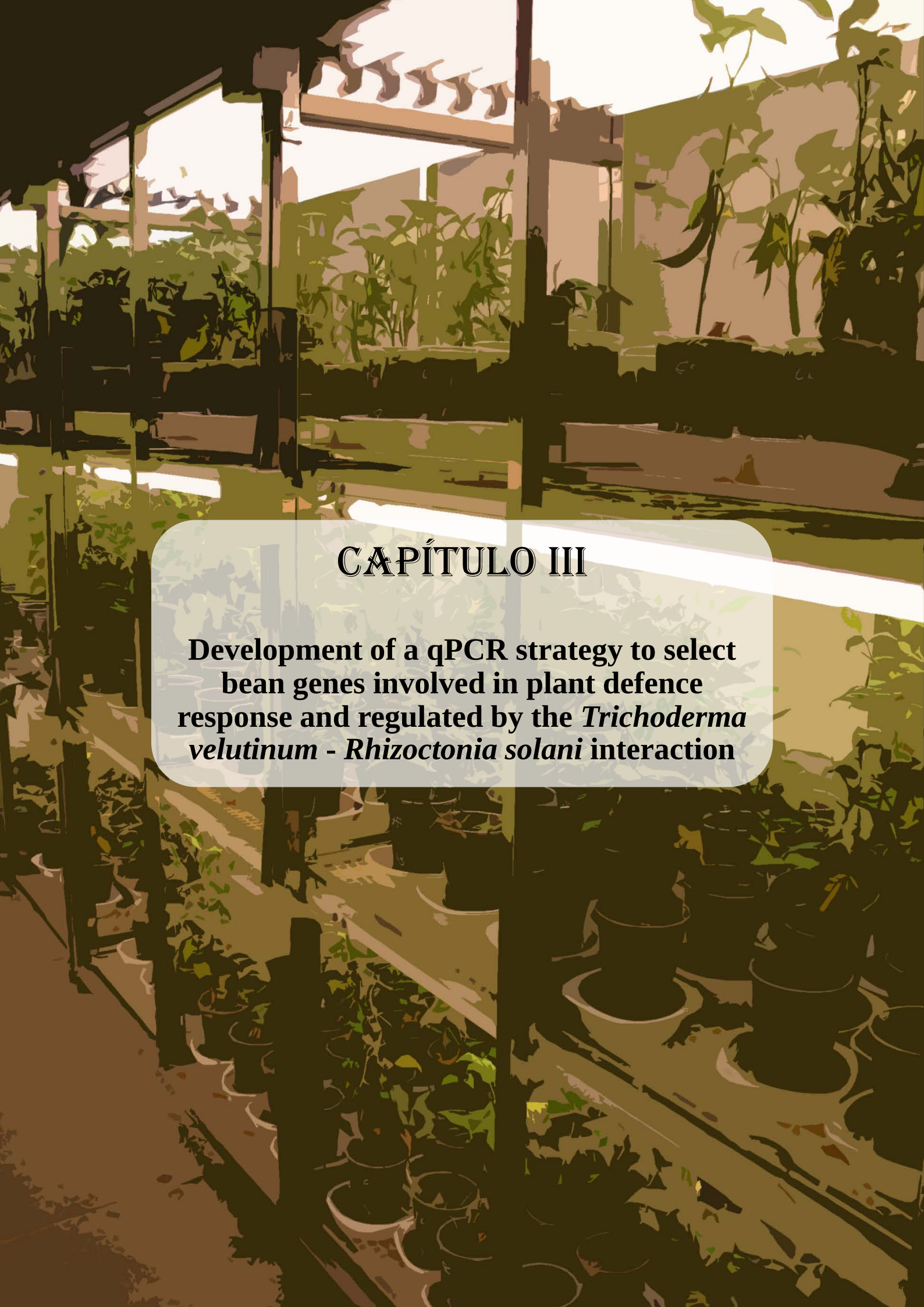
Woo, S. L., and Lorito, M. (2007). "Exploiting the interactions between fungal antagonists, pathogens and the plant for biocontrol," in *Novel Biotechnologies for Biocontrol Agent Enhancement and Management,* eds M. Vurro and J. Gressel (Dordrecht: Springer), 107–130.

Woo, S., Scala, F., Ruocco, M., and Lorito, M. (2006). The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. *Phytopathol* 96, 181–185. doi: 10.1094/PHYTO-96-018

Yedidia, I., Srivastva, A. K., Kapulnik, Y., and Chet, I. (2001). Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant Soil* 235, 235–242. doi: 10.1023/A:1011990013955

Zhao, Y., Thilmony, R., Bender, C. L., Schaller, A., He, S. Y., and Howe, G. A. (2003). Virulence systems of *Pseudomonas syringae* pv. tomato promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. *Plant J.* 36, 485–499. doi: 10.1046/j.1365-313X.2003.01895.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



CAPÍTULO III

Development of a qPCR strategy to select bean genes involved in plant defence response and regulated by the *Trichoderma velutinum* - *Rhizoctonia solani* interaction

ÍNDICE CAPÍTULO III

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 August 2016
doi: 10.3389/fpls.2016.01109

Development of a qPCR Strategy to Select Bean Genes Involved in Plant Defense Response and Regulated by the *Trichoderma velutinum* – *Rhizoctonia solani* Interaction

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OPEN ACCESS

Edited by:
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Reviewed by:
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Eriston Vieira Gomes, University of São Paulo, Brazil

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Specialty section:
This article was submitted to Crop Science and Horticulture, a section of the journal Frontiers in Plant Science

Received: *30 May 2016*
Accepted: *12 May 2016*
Published: *04 August 2016*

Citation:
Mayo S, Cominelli E, Sparvoli F, González-López O, Rodríguez-González A, Gutiérrez S and Casquero PA (2016) Development of a qPCR Strategy to Select Bean Genes Involved in Plant Defense Response and Regulated by the Trichoderma velutinum – Rhizoctonia solani Interaction. Front. Plant Sci. 7:1109. doi: 10.3389/fpls.2016.01109

Bean production is affected by a wide diversity of fungal pathogens, among them *Rhizoctonia solani* is one of the most important. A strategy to control bean infectious diseases, mainly those caused by fungi, is based on the use of biocontrol agents (BCAs) that can reduce the negative effects of plant pathogens and also can promote positive responses in the plant. *Trichoderma* is a fungal genus that is able to induce the expression of genes involved in plant defense response and also to promote plant growth, root development and nutrient uptake. In this article, a strategy that combines *in silico* analysis and real time PCR to detect additional bean defense-related genes, regulated by the presence of *Trichoderma velutinum* and/or *R. solani* has been applied. Based in this strategy, from the 48 bean genes initially analyzed, 14 were selected, and only *WRKY33*, *CH5b* and *hGS* showed an up-regulatory response in the presence of *T. velutinum*. The other genes were or not affected (*OSM34*) or down-regulated by the presence of this fungus. *R. solani* infection resulted in a down-regulation of most of the genes analyzed, except *PR1*, *OSM34* and *CNGC2* that were not affected, and the presence of both, *T. velutinum* and *R. solani*, up-regulates *hGS* and down-regulates all the other genes analyzed, except *CH5b* which was not significantly affected. As conclusion, the strategy described in the present work has been shown to be effective to detect genes involved in plant defense, which respond to the presence of a BCA or to a pathogen and also to the presence of both. The selected genes show significant homology with previously described plant defense genes and they are expressed in bean leaves of plants treated with *T. velutinum* and/or infected with *R. solani*.

Keywords: biotic stress, systemic acquired resistance, induced systemic resistance, hypersensitive response, defense genes, biocontrol agent, *Phaseolus vulgaris*

INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is the most important food legume crop worldwide. Bean production is often affected by biotic and abiotic factors (Guerrero-González et al., 2011) by microorganisms, humidity, temperature. . . that are detected as signals for the activation of plant response mechanisms. This crop is affected by a wide diversity of fungal pathogens (*Sclerotinia* spp., *Fusarium* spp., *Phytium* spp., *Botrytis* spp.,...) among them *Rhizoctonia solani* JG Kühn [Teleomorph: *Thanatephorus cucumeris* (AB Frank) Donk] has a remarkable importance as responsible of important economic losses in this crop (Valenciano et al., 2006). *R. solani* is a necrotrophic pathogen responsible for the root and hypocotyl diseases. Plant infection occurs through wounds or by the direct action of the fungal mycelium, which tears the cuticle and penetrates the epidermis (Guerrero-González et al., 2011).

As a strategy to control bean infectious diseases, mainly those caused by fungi, the use of biocontrol agents (BCA) can reduce the negative effects of plant pathogens and they also can promote positive responses in the plant (Shoresh et al., 2010). The genera *Trichoderma*, *Gliocladium*, *Rhizobium*, *Pseudomonas*, are beneficial organisms that have shown good efficiency as BCAs against pathogenic microorganisms. *Trichoderma* (Teleomorph: *Hypocrea*) is a fungal genus that is found in the soil, and it is a secondary fast growing opportunistic invasive. In addition, *Trichoderma* biocontrol strains are able to induce the expression of genes involved in defense response and also to promote plant growth, root development, and nutrient uptake (Hermosa et al., 2012).

The relationships established between plant and microorganisms are very diverse. When a plant is exposed to a pathogenic microorganism, the production of molecules associated to salicylic acid is increased, being this a systemic acquired resistance (SAR) response. The response of plants against non-pathogenic microorganisms is different, resulting in activation of signaling cascades that are dependent on jasmonic acid and ethylene, such as hydroperoxide lyase, peroxidase, and phenylalanine ammonia lyase, all of which belong to an induced systemic resistance (ISR) response (Druzhinina et al., 2011). Other responses result in a rapid cell death in infected tissues, then plants activate the hypersensitive response that involves the accumulation of salicylic acid, reactive oxygen species and an increased the influx of Ca^{2+} (Guerrero-González et al., 2011).

In the tripartite interaction of bean plants with the pathogen *R. solani* and a biocontrol *Trichoderma* species, several changes are produced in the plant, such as the increase in phenolic acid and lignin, accumulation of phytoalexins (Guerrero-González et al., 2011), and down- or up-regulation of defense-related genes expression (Mayo et al., 2015). Different categories of defense-related genes whose expression is modulated by biotic stresses have been described in bean plant interacting with pathogen and non-pathogenic microorganisms (Mayo et al., 2015).

Our hypothesis is that the combination of real time PCR with "*in silico*" analysis is a valid strategy to identify bean defense-related genes regulated by BCAs and/or plant pathogens. The aim is develop a systematic strategy to detect bean defense-related genes regulated by the presence of *Trichoderma velutinum* and/or *R. solani*. Finally, the procedure has been validated by the analysis of expression of the selected genes in the presence or absence of these two fungi.

MATERIALS AND METHODS

Trichoderma and *Rhizoctonia solani* Isolates and Culture Collections

Trichoderma velutinum T028, was collected from the bean traditional production area (Protected Geographical Indication, PGI), called "Alubia La Bañeza - León" (EC Reg. n.256/2010 published on March 26th, 2010, OJEU L880/17), from a High Quality variety of beans (**Figure 1**) without any genetic manipulation. It was isolated from soil plot bean in the Astorga region (León, Spain). This isolate gave percentages of inhibition greater that 60% in membrane assays and 40% in direct confrontation assays with *R. solani*, and that was able to sporulate on potato-dextrose-agar (PDA) medium.

Rhizoctonia solani R43 was isolated from bean plants of the same PGI and selected based on its high virulence. The isolated strains were stored in the collection "Pathogens and Antagonists of the Laboratory Diagnosis of Pests and Diseases" (PALDPD, University of León, León, Spain).

Isolates were inoculated on PDA (Becton Dickinson, Germany) medium and grown at 25°C in the dark for 1 week. After this incubation time *T. velutinum* T028 was exposed to light in order to induce the spore's formation.

FIGURE 1 | Bean seeds of "Canela" variety of the Protected Geographical Indication "Alubia La Bañeza – León" (Spain).

Plant Materials and Growth Conditions

Sixty bean seeds (Canela landrace, PGI "Alubia de la Bañeza – León," **Figure 1**) per treatment were germinated and cultured in presence or absence of the fungi in four conditions according to the procedure previously described by Mayo et al. (2015): (i) *T. velutinum* (T028) isolate plus *R. solani* (R43) (RT028); (ii) *T. velutinum* isolate (T028) without pathogen (C = control) (CT028); (iii) control (without *T. velutinum*) with *R. solani* (RC) and (iv) control without fungi (CC). The culture was carried out in climatic chamber and growth conditions were performed as previously described (Mayo et al., 2015). Six bean leaves from 45 day-old plants of each treatment were randomly collected and stored at −80°C until use.

RNA Extraction and Purification

The procedures for RNA extraction were performed as described previously (Reid et al., 2006). Bean leaves were lyophilized and were ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was mixed with 20 ml of extraction buffer/g of sample (extraction buffer: 0.1% SDS, 100 mM LiCl, 10 mM EDTA, 100 mM Tris-HCl, pH9) pre-warmed at 65°C, and 20 ml/g of phenol-chloroform-isoamyl alcohol 25:24:1 (Sigma–Aldrich, St. Louis, MO, USA). Then, the mixtures, in eppendorf tubes, were centrifuged at 13,000 rpm for 10 min at 4°C. The aqueous layer was transferred to a new tube. This step was repeated twice. Nucleic acids were precipitated with 1 volume of LiCl 4 M, mixed and kept overnight at 4°C. Tubes were then centrifuged at 13,000 rpm for 30 min at 4°C, and the resulting pellets were washed with ice cold ethanol 70%-DEPC, centrifuged again at 13,000 rpm for 10 min at 4°C and air dried. Finally, the pellets were dissolved in 50–200 μl H_2O-DEPC and stored at −20°C until use.

RNA concentrations and its purity were estimated from the A260/280 absorbance ratio with a NanoDrop (Thermo Scientific, Wilmington, DE, USA), considering the ideal absorbance ratio ($1.8 \leq$ A260/280 ≤ 2.0) and 1% agarose gel was run to visualize the integrity of the RNA.

cDNA Synthesis

Approximately 5 μg of RNA were treated with DNase using the TURBO DNAfree™ Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer instructions. cDNA was synthesized using High-Capacity cDNA Reverse Transcription kit (Applied-Biosystems, Foster City, CA, USA) according to the manufacture's manual.

qPCR Conditions and Analysis

qPCR reactions were performed with 7300 System (Applied Biosystems, Foster City, CA, USA) using SYBR® Green. Each reaction was performed in 20 μl containing 10 μl of 2 X Power SYBR Green PCR Master Mix (Life Technologies), 0.2–0.3 μM primers and cDNA samples diluted 1:20. Each qPCR reaction was performed in triplicate. Reactions were run using the cycling parameter described previously (Reid et al., 2006) and the qPCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). In order to analyze the qPCR data, *Act11* gene was used as housekeeping to determine the relative expression level of the other genes analyzed in this work (Borges et al., 2012). *T. velutinum* T028 strain was selected as reference strain in this study based on its positive effects on bean phenotype with and without *R. solani* infection (data no published). For the determination of qPCR efficiency of each primer pairs, a standard curve was performed using the following cDNA dilutions: 1:4, 1:16, 1:64; 1:256 and 1:1024. Every measurement was made in triplicate. The corresponding qPCR efficiencies (E) were calculated for every primer pair with the software 7300 System SDS software (Applied Biosystems, Foster City, CA, USA) according to the equation $E = (10^{-1/slope} - 1) \times 100$ (Rutledge and Stewart, 2008).

The significance of the differences between the gene expressions levels were compared by the Student's *t*-test using SAS (SAS Institute Inc., 2004, Cary, NC, USA).

RESULTS

Selection of Putative Bean Defense-Related Genes

Following an exhaustive and systematic analysis, summarized in the **Figure 2**, several bean genes were selected for their expression analysis in leaves from bean plants grown in interaction with *T. velutinum* and infected or not with *Rhizoctonia solani*. Thus, as result of the search in the literature, 48 genes were firstly found, showing stress and/or defense response (**Table 1**). Only those genes that resulted to be expressed in *P. vulgaris* leaves, based on transcriptomic data reported in the Phytozome database[1], were considered for qPCR expression analysis in leaves. The genes for which we confirmed expression in leaves were considered for further analyses.

As result, from the 48 genes selected for their involvement in bean stress and/or defense responses, only 19 were selected which showed a detectable level of expression in bean leaves.

The selected genes can be included in nine different groups (**Table 2**): (i) involved in the regulation of the balance between necrotrophic and biotrophic pathogen responses: *WRKY33* (WRKY transcription factor) (NM129404.3) (Bakshi and Oelmüller, 2014); (ii) pathogenesis related genes: *PR1* (pathogenesis related 1) (HO864272) (Guerrero-González et al., 2011), *PR2* (β 1-3 endoglucanase) (HO864270) (Guerrero-González et al., 2011), *PR3* (chitinase class I) (TC18606) (Pereira et al., 2014), *PR4* (pathogenesis related 4) (HO864354) (Guerrero-González et al., 2011), *PR16a* (germin.like protein 8) (CB540239) (Borges et al., 2012), *IPER* (basic peroxidase) (AF007211) (Upchurch and Ramirez, 2010), *PPO* (polyphenol oxidase) (EF158428) (Upchurch and Ramirez, 2010); (iii) related with the ethylene signaling pathway: *ERF1* (ethylene-responsive transcription factor 1) (AF076277) (Lorenzo et al., 2003), *ERF5* (ethylene-responsive transcription factor 5) (At5g47230) (Moffat et al., 2012), and *CH5b* (endochitinase precursor) (FE897014.1) (Vellicce et al., 2006); (iv) involved in phytoalexin

[1]http://phytozome.jgi.doe.gov/pz/portal.html

biosynthesis: *PAL1* (phenylalanine and histidine ammonia-lyase) (KF279696) (Kim and Hwang, 2014); (v) related in osmotin biosynthesis: *OSM34* (osmitin-like protein) (At4g11650) (Sharma et al., 2013); (vi) involved in Ca^{2+} signaling: *CNGC2* (cyclic nucleotide-gated ion channel 2) (CB542582) (Borges et al., 2012); (vii) needed for antimicrobials and oxylipins (defense signaling molecules): *HPL* (hydroperoxide lyase) (AW733791) (Upchurch and Ramirez, 2010), *Lox2* (lipoxygenase 2) (D13949) (Upchurch and Ramirez, 2010), *Lox7* (lipoxygenase 2) (Upchurch and Ramirez, 2010); (viii) *GSTa* (2,4-D inducible glutathione *S*-transferase) (HO864392) (Guerrero-González et al., 2011); and (ix) *hGS* (homoglutathione synthetase) (HO864377) both related with oxidative stress (Guerrero-González et al., 2011).

However, only 14 genes were selected to the study of the expression genes because *PR16a, IPER, PPO, Lox2,* and *Lox7*, showing negative qPCR results, were finally discarded.

Selection of a *Trichoderma* Strain to Validate the Gene Selection Strategy

Trichoderma velutinum T028 was the selected isolate, based on its positive effect on bean growth. Thus, plants inoculated with this strain showed a significant increase in dry weight of both aerial parts and root system, including when *R. solani* was present in the substrate (**Figure 3**). Thus, when bean plants were treated with *T. velutinum* T028 they increased respect to control plants (CC) 4.75% their diameter of hypocotyl, 10.75% their length of root system, 4.27 and 5.51% in dry weight of aerial parts and root system, respectively. When plants were infected with *R. solani*, the action of *T. velutinum* T028 caused an increased respect to the control plant with the pathogen (RC) of the diameter of hypocotyl in 8.76, 21.15% in the length of root system, and 11.05 and 3.43% in dry weight of aerial parts and root system respectively.

Based on these results, this isolate was used for further studies. In addition, this is the first report in which the effects of this strain on bean phenotype and plant gene regulation are studied.

Effect of *R. solani* Infection on Expression of the Selected Genes. Validation of the Procedure Used to Select Bean Genes Involved in Defense Responses (Strategy Validation I)

A significant down-regulation of expression of *PR2*, *PR3*, *PR4, ERF1, ERF5, PAL1, HPL*, and *GTSa* genes with ratios of expression ranging from 0.149 fold for *PAL1* and 0.763 fold for *PR3* was observed in bean plants grown in the presence of *R. solani* (RC) compared to control plants (CC). Conversely, expression of *PR1*, *OSM34*, *CNGC2,* and *hGS* genes was up-regulated, but with non-statistically significant differences with a ratios between 1.289 and 1.193 for *PR1* and *hGS*, respectively (**Figure 4**).

Effect of *Trichoderma* on Expression of the Selected Genes (Strategy Validation II)

Trichoderma treatment also down-regulates expression of most of the bean defense-related genes, but at a lower level than *R. solani*. Thus, when *T. velutinum* T028 was in the substrate (CT028), *PR2, PR3, PR4, ERF1, ERF5, PAL1, CNGC2, HPL,* and *GSTa*

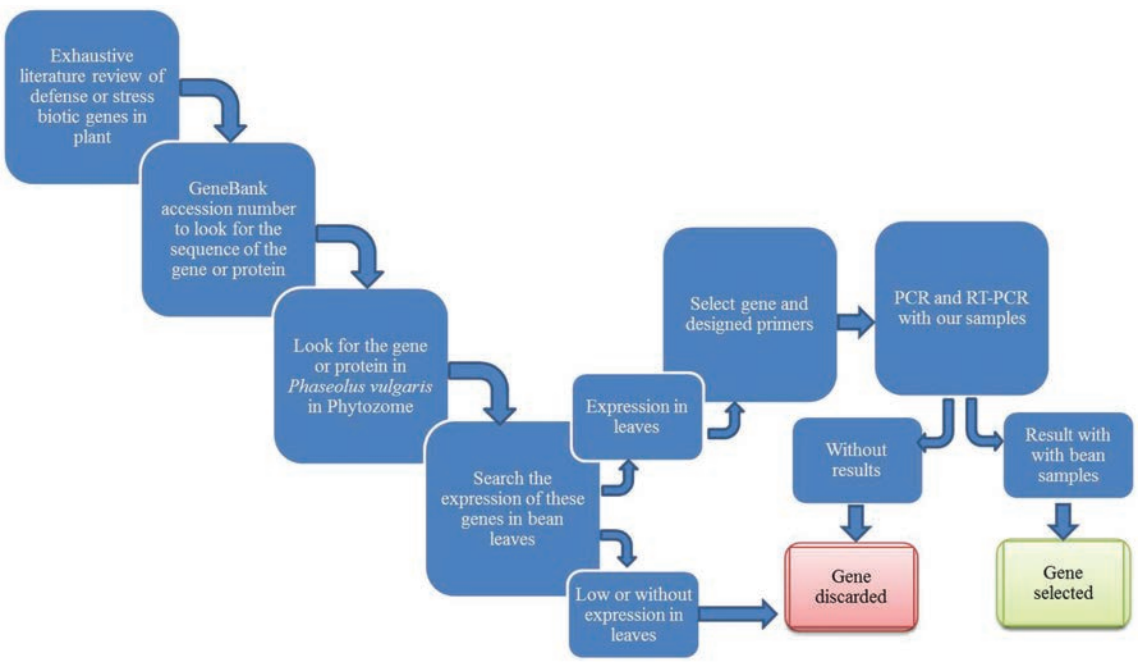


FIGURE 2 | Schematic representation of the work flow set up in the present work to select bean genes involved in plant defense.

TABLE 1 | Genes selected for stress and/or defense response and their empirical expression in *Phaseolus vulgaris* leaves.

Id	Gene	Accession number	Functional annotation	NCBI Phytozome
Pereira et al., 2014				
1	*Chit*	AY357300.2	Chitanase	
2	*Glu1*	DQ093563.1	β-1,3-glucanase	
3	*Pod3*	AF485265.1	Peroxidase	
4	*PR3*	TC18606	Chitinase class I	Phvul.009G116600
5	*Lox1*	U76687.2	Lipoxygenase	
Upchurch and Ramirez, 2010				
6	*PPO*	EF158428	Polyphenol oxidase	
7	*PR10*	AJ289155	Stress-induced ribonuclease-like protein	
8	*PR12*	BU964598	Defensin precursor	
9	*MMP2*	AY057902	Matrix metalloproteinase 2	
10	*CHS*	X53958	Chalcone synthase	
11	*AOS*	DQ288260	Allene oxide synthase	
12	*HPL*	AW733791	Hydroperoxide lyase	Phvul.005G116800
13	*LOX2*	D13949	Lipoxygenase 2	Phvul.005G156700
14	*LOX7*	U36191	Lipoxygenase 2	Phvul.005G156900
15	*IPER*	AF007211	Basic peroxidase	Phvul.009G215000
Borges et al., 2012				
16	*PR16a*	CB540239	Germin-like protein 8	Phvul.010G129900
17	*PGla*	CB542106	Polygalacturonase-inhibitor-like protein	
18	*MAPKK*	CB543156	MEK map kinase kinase	
19	*PROF*	CB543496	Profilin	
20	*CNGC2*	CB542582	Cyclin nucleotide-gated ion channel 2	Phvul.008G036200
Guerrero-González et al., 2011				
21	*PR1*	HO864272	Pathogenesis related protein 1	Phvul.003G109100
22	*PR2*	HO864270	Pathogenesis related protein 2	Phvul.003G109200
23	*PR4*	HO864354	Pathogenesis related protein 4	Phvul.006G102300
24	*PR10*	HO864271	Pathogenesis related protein 10)	
25	*LTP2*	HO864366	Lipid-transfer protein 2	
26	*SIP*	HO864290	Syringolide-induced protein B13-1-9	
27	*DAAP*	HO864358	Defense associated acid phosphatase	
28	*CHI*	HO864289	Chalcone isomerase	
29	*hGS*	HO864377	Homoglutathione synthetase	Phvul.006G094500
30	*aDO1*	HO864351	Alpha- dioxygenase 1	
31	*CPRD14*	HO864341	CPRD14 protein	
32	*OPR5*	HO864304	12-oxophytodienoic acid 10, 11-reductase	
33	*GST22*	HO864275	Glutathione S-transferase 22	
34	*CPRD8*	HO864396	CPRD8 protein	
35	*UDPGT*	HO864301	UDP-glucosyl transferase 72E1	
36	*ERD15*	HO864375	ERD15 protein	
37	*GTSa*	HO864392	2,4-D inducible glutathione S-transferase	Phvul.002G241400
38	*GST15*	HO864369	Glutathione S-transferase 15	
Gallou et al., 2009				
39	*GST1*	J03679	Gluthatione-S-transferase 1	
Lehtonen et al., 2008				
40	*TSI-1*	BQ121547	TSI-1 protein	
41	*Lip*	BQ112158	Lipase-like protein	
42	*Amintransf2*	BQ517030	Aminotransferase 2	Phvul.006G029100
Bakshi and Oelmüller, 2014				
43	*WRKY33*	NM129404.3	WRKY transcription factors	Phvul.008G090300
Vellicce et al., 2006				
44	*CH5b*	FE897014.1	Endochitinase precursor	Phvul.009G116500
Lorenzo et al., 2003				

(Continued)

TABLE 1 | Continued

Id	Gene	Accession number	Functional annotation	NCBI Phytozome
45	*ERF1*	AF076277	Ethylene-Responsive Transcription Factor 1	Phvul.007G127800
Moffat et al., 2012				
46	*ERF5*	At5g47230	Ethylene-Responsive Transcription Factor 5	Phvul.002G055700
Kim and Hwang, 2014				
47	*PAL1*	KF279696	Phenylalanine and histidine ammonia-lyase	Phvul.001G177800
Sharma et al., 2013				
48	*OSM34*	At4g11650	Osmotin-like protein	Phvul.002G155500

were significantly down-regulated with expression ratios ranging from 0.168 for *PR4* to 0.754 for *ERF1*. However, *WRKY 33, CH5b*, and *hGS* were up-regulated when compared with the levels of expression in control plants, with relative expression levels between 2.462 for *CH5b* and 1.576 for *hGS* (**Figure 5**). *OSM34* was slightly but not significantly up-regulated.

Effect of Interaction of *T. velutinum* and *R. solani* on Expression of the Selected Genes (Strategy Validation III)

When *T. velutinum* T028 and *R. solani* (RT028) were in the substrate, the genes *WRKY33, PR2, PR3, PR4, ERF1, ERF5, PAL1, OSM34, HPL and GSTa* were significantly down-regulated with values between 0.179 for *PAL1* and 0.631 for *WRKY33.* In the case of *PR1* and *CNGC2*, they were also down-regulated but not significantly respect to control plant (C). Conversely, *hGS* was up-regulated with a significant ratio of 1.589 respect to control plants, while *CH5b* was not significantly up-regulated with a ratio of 1.613 (**Figure 6**).

DISCUSSION

Plants have developed some defensive strategies to perceive pathogen attack and to translate this perception into an appropriate adaptive response. During attack, plants are able to enhance their resistance (induced, acquired, hypersensitive) (Lodha and Basak, 2012). Contact with pathogenic and non-pathogenic microorganisms triggers two mechanisms: (i) SAR that is usually triggered by local infections, it provides long-term systemic resistance to pathogen attack and requires the involvement of the signal molecule salicylic acid (Durrant and Dong, 2004), and (ii) ISR that is known to result from colonization of roots by certain non-pathogenic microorganisms and is dependent on components of the jasmonic acid and ethylene signaling pathways (Shoresh et al., 2010). Then, the combination of both types of induced resistance response can protect the plant against pathogens and can even result in additive level of induced protection against pathogens through both the jasmonic acid/ethylene and salicylic acid pathways (Verhagen et al., 2006).

In the present work we developed a strategy to select genes involved in bean defense response, which would belong to those pathways, but also genes that can contribute to plant defense by other mechanisms. In this sense several previous works have described genes involved in bean defense response (Guerrero-González et al., 2011; Mayo et al., 2015). However, in the present work, by a systematic approach, 48 genes were initially considered, and 14 finally selected, which match with the criteria set up in this work: (i) they showed significant homology with previously described plant defense genes, and (ii) were expressed in bean leaves of plants treated with *Trichoderma* and/or infected with *R. solani*.

The expression of *P. vulgaris* defense-related genes was analyzed in leaves, although the interaction with *Trichoderma* and/or *R. solani* is initially produced at the root level, to determine if the signals generated in roots as result of this interaction are able to systematically stimulate the bean defense along long distance from roots to the leaves. The isolate *T. velutinum* T028 was selected following a similar strategy to that previously described (Mayo et al., 2015), and based on its positive effect on bean growth. In this work, to select a *Trichoderma* isolate, the results of the *in vitro* membrane assays and direct confrontation assays against *R. solani* were analyzed. Isolate *Trichoderma* T019 was then selected, showing a percentage of inhibition higher than 40% in the membrane assays, and/or 20% in the direct confrontation assays. This isolate also showed the best positive effects on plant phenotype among all the analyzed isolates.

WRKY transcription factors have been involved in the regulation of plant defense gene expression (Rushton and Somssich, 1998; Singh et al., 2002). Thus, WRKY33 has a role in biotic stress defense, where it regulates the balance between necrotrophic and biotrophic pathogen responses (Lippok et al., 2007; Pandey and Somssich, 2009; Birkenbihl et al., 2012). Previous studies have pointed out the involvement of *Arabidopsis* WRKY transcription factors in regulating the expression of *PR* genes by direct binding (Chen et al., 2002; Kim et al., 2006). A rapid pathogen-induced *WKRY33* expression did not require salicylic acid signaling but a downregulation of this gene involved a direct activation of jasmonic acid (Bakshi and Oelmüller, 2014). In the present case, when bean plants were in contact *T. velutinum* T028 without pathogen, the *WRKY33* gene expression was significantly up-regulated while the *PR* genes expression (*PR2*, *PR3* and *PR4*) was significantly down-regulated compared to expression levels in plants without *Trichoderma* treatment. In the present work, when *R. solani* was added to the substrate, expression of *WRKY33* was significantly down-regulated in plants with *Trichoderma* inoculation, while *PR2*, *PR3* and *PR4* were down-regulated. In the study by Mayo et al. (2015),

TABLE 2 | Common bean sequences used for primer design for RT-PCR analysis.

Gene	Functional annotation	NCBI Phytozome	Forward/Reverse	Efficiency Reference
Reference genes				
Act11	Actin-11	Phvul.008G011000	TGCATACGTTGGTGATGAGG	1.084
			AGCCTTGGGGTTAAGAGGAG	
Ukn1	Unknown	Phvul.011G023200	ATTCCCATCATGCAGCAAAG	0.937
			AGATCCCTCCAGGTCAATCC	
Balance between necrotrophic and biotrophic pathogen responses				
WRKY33	WRKY transcription factors	Phvul.008G090300	TTTCACAGGACAGGTTCCAGC	0.938
			CCTTTGACAGAAATGACTGAAGGA	
Pathogenesis related genes				
PR1	Pathogenesis Related 1	Phvul.003G109100	TGGTCCTAACGGAGGATCAC	1.094
			TGGCTTTTCCAGCTTTGAGT	Mayo et al., 2015
PR2	Beta 1-3 Endoglucanase	Phvul.003G109200	GTGAAGGACGCCGATAACAT	1.048
			ACTGAGTTTGGGGTCGATTG	Mayo et al., 2015
PR3	Chitinase class I	Phvul.009G116600	TGGAGTTGGTTATGGCAACAA	1.034
			ATTCTGATGGGATGGCAGTGT	
PR4	Pathogenesis-related 4	Phvul.006G102300	CGCAGTGAGTGCATATTGCT	0.922
			TGTTTGTCACCCTCAAGCAC	Mayo et al., 2015
PR16a	Germin-like protein 8	Phvul.010G129900	GGCAGTCTCATGGTTATGGTTT	–
			GCATGCTCAAGTCTCAACACAT	
IPER	Peroxidase precursor	Phvul.009G215000	GGCAAGCATTATATGGTTGAAA	–
			GATGGCAACATCCATCACTTTA	
PPO	Polyphenol oxidase	Phvul.008G073200	GAAGACGATGATTTGCTGGTTA	–
			AAGAAACATTTTCCTTTGTGAAA	
Ethylene signaling pathway				
ERF1	Ethylene-Responsive Transcription Factor 1	Phvul.007G127800	CGCTCTCAAGAGGAAACACTCC	0.937
			TGAATCAGAAGGAGGAGGGAAT	
ERF5	Ethylene-Responsive Transcription Factor 5	Phvul.002G055700	GGCTCCAAGTGGATTGAGAAC	0.932
			TCAGAATCAGATAACTACAAAGCACAA	
CH5b	Endochitinase precursor	Phvul.009G116500	CAGCCAAAGGCTTCTACACC	0.883
			TTGTTTCGTGAGACGTTTGC	Mayo et al., 2015
Phytoalexins biosynthesis				
PAL1	Phenylalanine and histidine ammonia-lyase	Phvul.001G177800	TGAGAGAGGAGTTGGGCACT	1.034
			TTCCACTCTCCAAGGCATTC	
Osmotin biosynthesis				
OSM34	Osmotin-like protein	Phvul.002G155500	GAACGGAGGGTGTCACAAAATC	0.927
			CGTAGTGGGTCCACAAGTTCCT	
Involved in Ca^{2+} signaling				
CNGC2	Cyclic nucleotide-gated ion channel 2	Phvul.008G036200	ATTCAATTTGCTTGGAGACGTT	0.98
			ACAGTTTTATTGAAGGCCAGGA	
Antimicrobials and oxylipins (defense signaling molecules)				
HPL	Hydroperoxide lyase	Phvul.005G116800	TCAAGGCTACATTTGTATTTCCA	0.984
			TGGTGCACATTTCTTAGTAGCAA	
Lox2	Lipoxygenase 2	Phvul.005G156700	ATGCAAGGCTAAAGAGATCCAA	–
			ATGGTGACAGGAGCTAAACACA	
Lox7	Lipoxygenase 2	Phvul.005G156900	GAAGGCTTGACTTTCAGAGGAA	–
			AACACACGAGAAGATTCAACCA	
Oxidative stress				
GSTa	2.4-D inducible glutathione S-transferase	Phvul.002G241400	AGGGAGTCACACTGGCTATGTT	1.013
			ATGTGCCATTTGCATTTTAGTG	
hGS	Homoglutathione synthetase	Phvul.006G094500	GTGGCTATATGGTGCGTACAAA	1.023
			GAAACAAGAATGCATCTCCTCA	
Amintransf2	Aminotransferase 2	Phvul.006G029100	TTCTTCCTTTTCTGCTCTTTCAA	–
			AGATGACAAGATGCAATGATTTTT	

(–) Genes that empirically showing expression but showing negative qPCR results.

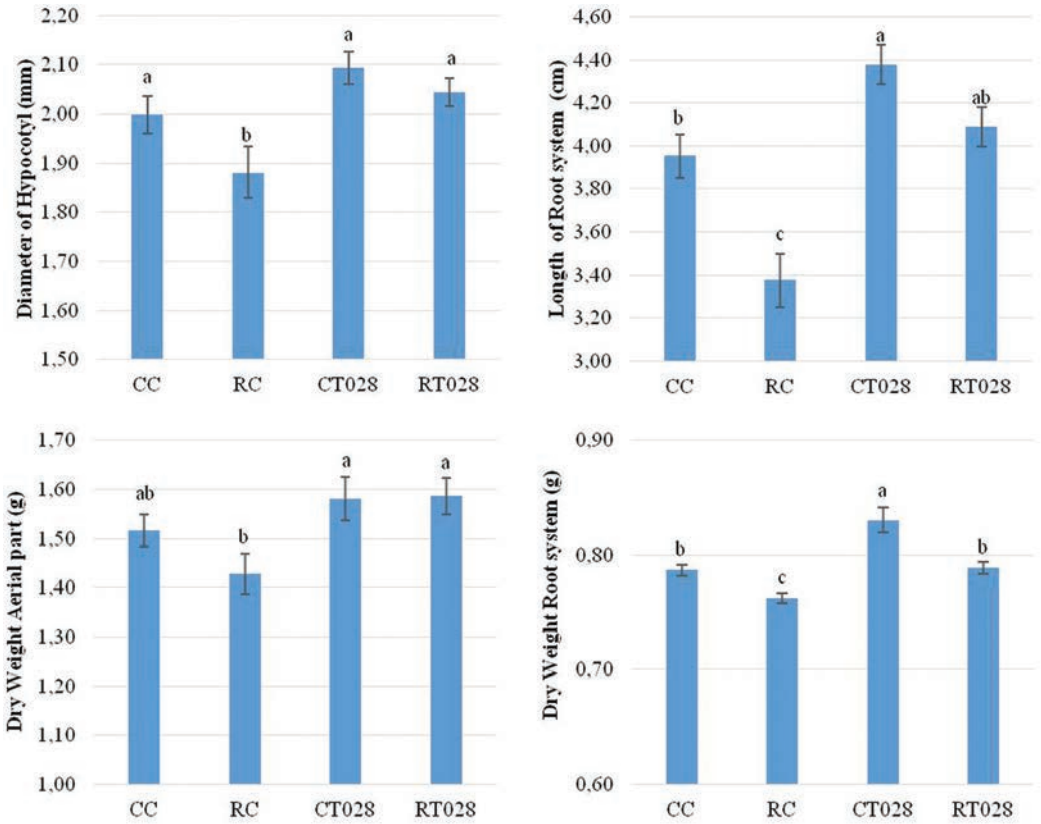


FIGURE 3 | Evaluation of the diameter of the hypocotyl (above left), length of root system (above right) dry weight of the aerial part (below left) and root system (below right) of bean plants grown during 45 days after sowing. [*Trichoderma velutinum* T028 without pathogen (CT028), *T. velutinum* T028 with *Rhizoctonia solani* (RT028), *R. solani* control (RC) and control without fungus (CC)]. Differences statistically significant respect to control plants ($p < 0.05$) are indicated with different letters.

the expression of *PR1*, *PR2*, *PR3,* and *PR4* was down-regulated when beans were inoculated with *R. solani*.

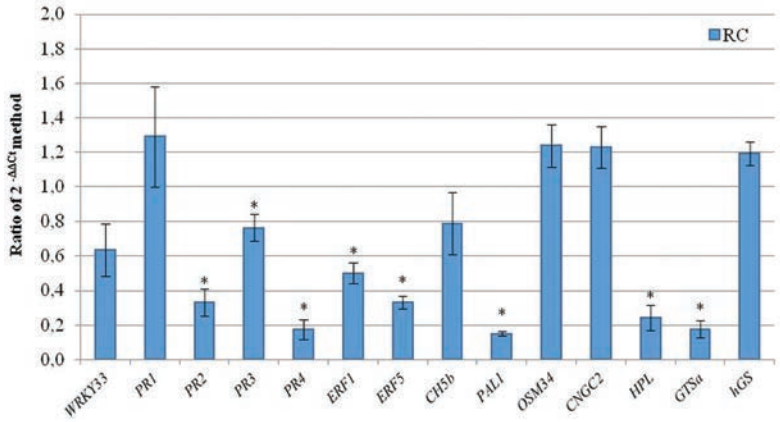
WRKY33 is also involved in the regulation of the expression of genes modulated by components of the ethylene signaling pathway. In this work, expression of the *ERF1* and *ERF5* reached similar significant values either with or without *Trichoderma* and or *R. solani* in the substrate. This result contrasts with previous reports showing that *ERF5* was up-regulated and *WRKY33* was down-regulated in *Arabidopsis* infected with *Alternaria brassicicola* (Son et al., 2012). *WRKY33* would act as a represor of *ERF1* and *ERF5* expression. Thus, when the expression of *WRKY33* is increased, expression of *ERF1* and *ERF5* is down-regulated.

CH5b encodes an endochitinase precursor and it is also related with the ethylene signaling pathway. In previous works, it has been shown that, when this gene was over-expressed the *R. solani* symptoms were reduced in crops like *Nicotiana tabacum* and *Brassica napus* (Broglie et al., 1991). However, in this study, when bean plants were in contact with *R. solani,* the expression of this gene was down-regulated but not significantly, while treatment of these infected plants with *T. velutinum* resulted in its significant up-regulation. These results are in agreement with previous data, showing that the pathogen represses its expression, and the presence of *Trichoderma* induced it (Mayo et al., 2015).

PAL plays an important role in plant defense; it is involved in the biosynthesis of salicylic acid, which is related to plant systemic resistance (Nugroho et al., 2002; Chaman et al., 2003). *PAL* gene expression is also regulated in response to pathogen infection. In this work, the presence of *T. velutinum* and *R. solani* in the soil resulted in a significant down-regulation of this gene compared with control plants.

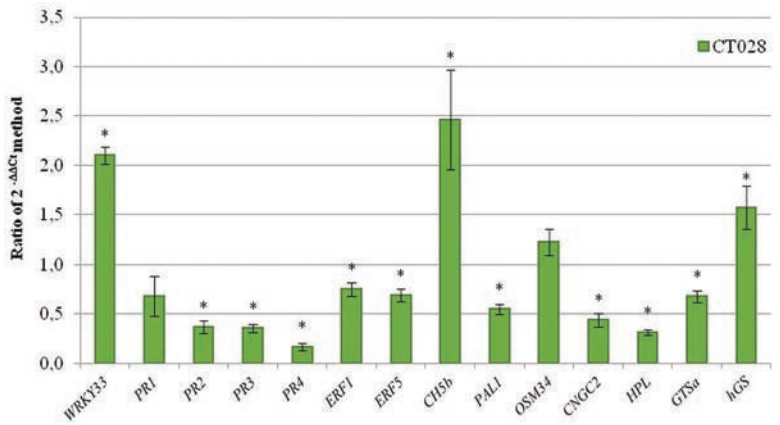
Osmotins have plant protective effects against pathogen infection (Narasimhan et al., 2009). In this study, when *T. velutinum* or *R. solani* were present in the soil, the expression of *OSM34* was not significantly up-regulated respect to control plants, but when both fungi were in the soil at the same time, *OSM34* was slightly but significantly down-regulated.

The *CNGC* genes can be related to early plant defense responses due to changes in ion flux, including H^+ and Ca^{2+} influx and K^+ and Cl^- efflux (Atkinson et al., 1996). The up-regulation of *CNGC2* can confirm the importance of ion channels for the plant resistance response (Borges et al., 2012). In this work, this gene was up-regulated when *R. solani* was present in the soil not significant. Conversely, *CNGC2* was down-regulated



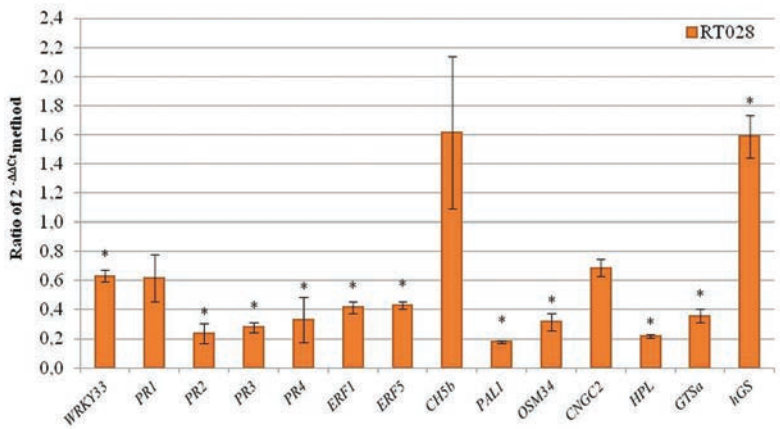
Gene	CC	RC
WRKY33	1.000 ± 0.190	0.634 ± 0.152
PR1	1.000 ± 0.266	1.289 ± 0.292
PR2	1.000 ± 0.085	0.331* ± 0.079
PR3	1.000 ± 0.127	0.763* ± 0.077
PR4	1.000 ± 0.197	0.176* ± 0.057
ERF1	1.000 ± 0.149	0.502* ± 0.061
ERF5	1.000 ± 0.134	0.333* ± 0.037
CH5b	1.000 ± 0.272	0.790 ± 0.179
PAL1	1.000 ± 0.116	0.149* ± 0.013
OSM34	1.000 ± 0.264	1.237 ± 0.125
CNGC2	1.000 ± 0.235	1.228 ± 0.122
HPL	1.000 ± 0.279	0.245* ± 0.073
GTSa	1.000 ± 0.158	0.177* ± 0.051
hGS	1.000 ± 0.115	1.193 ± 0.068

FIGURE 4 | Analysis of relative expression levels of the bean defense genes selected in the present work in bean plants infected with *R. solani versus* their levels of expression in control plants. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method. The differences statistically significant respect to control plants ($p < 0.05$) are indicated with an asterisk.



Gene	CC	CT028
WRKY33	1.000 ± 0.190	2.102* ± 0.086
PR1	1.000 ± 0.266	0.679 ± 0.201
PR2	1.000 ± 0.085	0.368* ± 0.065
PR3	1.000 ± 0.127	0.357* ± 0.041
PR4	1.000 ± 0.197	0.168* ± 0.038
ERF1	1.000 ± 0.149	0.754* ± 0.067
ERF5	1.000 ± 0.134	0.689* ± 0.064
CH5b	1.000 ± 0.272	2.462* ± 0.503
PAL1	1.000 ± 0.116	0.550* ± 0.052
OSM34	1.000 ± 0.264	1.230 ± 0.132
CNGC2	1.000 ± 0.235	0.441* ± 0.069
HPL	1.000 ± 0.279	0.316* ± 0.026
GTSa	1.000 ± 0.158	0.679* ± 0.059
hGS	1.000 ± 0.115	1.576* ± 0.215

FIGURE 5 | Analysis of relative expression levels of the bean defense genes selected in the present work in bean plants treated with *T. velutinum versus* their levels of expression in control plants. The data were analyzed as indicated in the legend to the **Figure 4**.



Gene	CC	RT028
WRKY33	1.000 ± 0.190	0.631* ± 0.042
PR1	1.000 ± 0.266	0.617 ± 0.164
PR2	1.000 ± 0.085	0.238* ± 0.068
PR3	1.000 ± 0.127	0.278* ± 0.037
PR4	1.000 ± 0.197	0.330* ± 0.156
ERF1	1.000 ± 0.149	0.416* ± 0.041
ERF5	1.000 ± 0.134	0.429* ± 0.026
CH5b	1.000 ± 0.272	1.613 ± 0.522
PAL1	1.000 ± 0.116	0.179* ± 0.011
OSM34	1.000 ± 0.264	0.316* ± 0.060
CNGC2	1.000 ± 0.235	0.689 ± 0.060
HPL	1.000 ± 0.279	0.219* ± 0.014
GTSa	1.000 ± 0.158	0.357* ± 0.046
hGS	1.000 ± 0.115	1.589* ± 0.147

FIGURE 6 | Analysis of relative expression levels of the bean defense genes selected in the present work in bean plants infected with *R. solani* and treated with *T. velutinum versus* their levels of expression in control plants. The data were analyzed as indicated in the legend to the **Figure 4**.

in plants treated with *T. velutinum*. Then, the pathogen would induce an activation of hypersensitive defense mechanisms.

Hydroperoxide lyase (*HPL*) is involved in the production of antimicrobial and defense signaling oxylipins (Noordermeer et al., 2001; Huang et al., 2010). In this study, the presence of *T. velutinum* and *R. solani*, resulted in a down-regulation of this gene expression respect to control plants. In previous works, when tomato plants were in contact with *Botrytis cinerea*, *HPL* expression increased 24 h after gray mold infection, but after that time the expression of this gene decreased gradually (Wan et al., 2013). In the present case, after 45 days in contact with the fungus *T. velutinum* and/or *R. solani*, its expression was down-regulated, indicating that the plant identifies *Trichoderma* and *Rhizoctonia* as two invader organisms, and some of the mechanisms activated against the presence of both are similar, independently of the final response specifically activated in the plant by each one.

GSTa (2,4-D inducible glutathione *S*-transferase) expression also responds to pathogen attack (Mauch and Dudler, 1993) and can be induced by molecules such as salicylic acid, methyl jasmonate, abscisic acid and H_2O_2 (Dixon et al., 2002; Moons, 2005). In *Gossypium arboretum*, GST provides resistance to fungal pathogens and oxidative stress (Barthelson et al., 2010). *GST* expression was up-regulated during fungal infection in barley, *Arabidopsis*, and cotton (Dowd et al., 2004; Durrant and Dong, 2004; Lu et al., 2005). However, in banana *GST* was down-regulated following *Fusarium oxysporum* f *specialis* (f. sp.) *cubense* infection (Wang et al., 2013), which is in agreement with the present case, where the expression of *GSTa* was down-regulated when *T. velutinum* and/or *R. solani* were present in the soil.

hGS encodes a homoglutathione synthetase that is involved in response to oxidative stress. There is not much information about the behavior of this gene in the plant. In the present study, when bean plants were in contact with *T. velutinum* and/or *R. solani*, expression of this gene was significantly up-regulated compared to control plants. In other studies, treatment of *Medicago truncatula* plants with compounds that release nitric oxide, a key signaling molecule in plants, induced expression of *GST* but not *hGS* in roots (Innocenti et al., 2007). Similarly, common bean plants treated with H_2O_2 showed up-regulation of *hGS* in nodules, whereas treatments with cadmium, sodium chloride, or jasmonic acid had no effect (Loscos et al., 2008).

CONCLUSION

From 48 genes initially analyzed, 14 bean genes were selected in the present work and only *WRKY33*, *CH5b* and *hGS* showed an up-regulatory response in the presence of *T. velutinum*, the other genes were or not affected (*OSM34*) or down-regulated by the presence of this fungus. *R. solani* infection resulted in a down-regulation of most of the genes analyzed, except *PR1*, *OSM34* and *CNGC2* that were not affected, and the presence of both, *T. velutinum* and *R. solani*, up-regulates *hGS* and down-regulates all the other genes analyzed, except *CH5b* which was not significantly affected.

As conclusion, the strategy described in the present work has been shown to be effective to detect genes involved in plant defense, which respond to the presence of a BCA or to a pathogen and also to the presence of both. The selected genes showed significant homology with described plant defense genes and they are expressed in bean leaves of plants treated with *T. velutinum* and/or infected with *R. solani*. The proposed strategy will be very useful in studies about the interaction of bean with pathogens and biocontrol fungi.

AUTHOR CONTRIBUTIONS

PC and SG conceived the research. SM, OG-L, and AR-G designed the research. SM, OG-L, and AR-G conducted the experiments. SM, PC, and SG analyzed data. SM, PC, SG, EC, and FS interpreted the data. SM, PC, SG, EC, and FS wrote the manuscript. All authors were agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors critically revised the manuscript. All authors approved the final version to be published.

ACKNOWLEDGMENTS

The grant awarded to Sara Mayo Prieto (FPU12/00677) by the Ministry of Education, Culture and Sport (Spain) according the Resolution of April 25, 2012 (BOE of 10 May), as amended by Resolution of May 18, 2012 (BOE of 22 May), and by Resolution of October 31, 2012 (BOE of 12 November) and granted in the Resolution of December 20, 2012. Ministry of Economy and Competitiveness for National project "Farnesol as self-regulatory molecule in *Trichoderma*. Tyrosol and farnesol signaling in *Trichoderma*-bean interaction" (AGL2012-40041-C02-02). Junta de Castilla y León, Consejería de Educación for the project "Effect of terpenes and physiologically related compounds produced by *Trichoderma parareesei* in the development of common bean (*Phaseolus vulgaris* L.) and in defensive responses of this plant" (LE228U14).

REFERENCES

Atkinson, M. M., Midland, S. L., Sims, J. J., and Keen, N. T. (1996). Syringolide 1 triggers Ca2 influx, K efflux, and extracellular alkalization in soybean cells carrying the disease-resistance gene Rpg4. *Plant Physiol.* 112, 297–302. doi: 10.1104/pp.112.1.297

Bakshi, M., and Oelmüller, R. (2014). WRKY transcription factors. *Plant Signal. Behav.* 9:e27700. doi: 10.4161/psb.27700

Barthelson, R. A., Qaisar, U., and Galbraith, D. W. (2010). Functional analysis of the *Gossypium arboreum* genome. *Plant Mol. Biol. Rep.* 28, 334–343. doi: 10.1007/s11105-009-0157-5

Birkenbihl, R. P., Diezel, C., and Somssich, I. E. (2012). *Arabidopsis* WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea* infection. *Plant Physiol.* 159, 266–285. doi: 10.1104/pp.111.192641

Borges, A., Melotto, M., Tsai, S. M., and Caldas, D. G. G. (2012). Changes in spatial and temporal gene expression during incompatible interaction between common bean and Anthracnose pathogen. *J. Plant Physiol.* 169, 1216–1220. doi: 10.1016/j.jplph.2012.04.003

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., et al. (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* 254, 1194–1197. doi: 10.1126/science.254.5035.1194

Chaman, M. E., Copaja, S. V., and Argandoña, V. H. (2003). Relationships between salicylic acid content, Phenylalanine Ammonia-Lyase (PAL) activity, and resistance of barley to aphid infestation. *J. Agric. Food Chem.* 51, 2227–2231. doi: 10.1021/jf020953b

Chen, W., Provart, N. J., Glazebrook, J., Katagiri, F., Chang, H. S., Eulgem, T., et al. (2002). Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell* 14, 559–574. doi: 10.1105/tpc.010410

Dixon, D. P., Lapthorn, A., and Edwards, R. (2002). Plant glutathione transferases. *Genome Biol.* 3, 3004.1–3004.10. doi: 10.1186/gb-2002-3-3-reviews3004

Dowd, C., Wilson, I. W., and McFadden, H. (2004). Gene expression profile changes in cotton root and hypocotyl tissues in response to infection with *Fusarium oxysporum* f. sp. vasinfectum. *Mol. Plant-Microbe Interact.* 17, 654–667. doi: 10.1094/MPMI.2004.17.6.654

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., et al. (2011). Trichoderma: the genomics of opportunistic success. *Nat. Rev. Microbiol.* 9, 749–759. doi: 10.1038/nrmicro2637

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209. doi: 10.1146/annurev.phyto.42.040803.140421

Gallou, A., Cranenbrouck, S., and Declerck, S. (2009). Trichoderma harzianum elicits defence response genes in roots of potato plantlets challenged by *Rhizoctonia solani*. *Eur. J. Plant Pathol.* 124, 219–230. doi: 10.1007/s10658-008-9407-x

Guerrero-González, M. L., Rodríguez-Kessler, M., Rodríguez-Guerra, R., González-Chavira, M., Simpson, J., Sanchez, F., et al. (2011). Differential expression of *Phaseolus vulgaris* genes induced during the interaction with *Rhizoctonia solani*. *Plant Cell Rep.* 30, 1465–1473. doi: 10.1007/s00299-011-1055-5

Hermosa, R., Viterbo, A., Chet, I., and Monte, E. (2012). Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiology* 158, 17–25. doi: 10.1099/mic.0.052274-0

Huang, F. C., Studart Witkowski, C., and Schwab, W. (2010). Overexpression of hydroperoxide lyase gene in *Nicotiana benthamiana* using a viral vector system. *Plant Biotechnol. J.* 8, 783–795. doi: 10.1111/j.1467-7652.2010.00508.x

Innocenti, G., Pucciariello, C., Le Gleuher, M., Hopkins, J., de Stefano, M., Delledonne, M., et al. (2007). Glutathione synthesis is regulated by nitric oxide in *Medicago truncatula* roots. *Planta* 225, 1597–1602. doi: 10.1007/s00425-006-0461-3

Kim, D. S., and Hwang, B. K. (2014). An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signalling of the defence response to microbial pathogens. *J. Exp. Bot.* 65, 2295–2306. doi: 10.1093/jxb/eru109

Kim, K. C., Fan, B., and Chen, Z. (2006). Pathogen-induced *Arabidopsis* WRKY7 is a transcriptional repressor and enhances plant susceptibility to *Pseudomonas syringae*. *Plant Physiol.* 142, 1180–1192. doi: 10.1104/pp.106.082487

Lehtonen, M. J., Somervuo, P., and Valkonen, J. P. T. (2008). Infection with *Rhizoctonia solani* induces defense genes and systemic resistance in potato sprouts grown without light. *Phytopathology* 98, 1190–1198. doi: 10.1094/PHYTO-98-11-1190

Lippok, B., Birkenbihl, R. P., Rivory, G., Brümmer, J., Schmelzer, E., Logemann, E., et al. (2007). Expression of AtWRKY33 encoding a pathogen-or PAMP-responsive WRKY transcription factor is regulated by a composite DNA motif containing W box elements. *Mol. Plant-Microbe Interact.* 20, 420–429. doi: 10.1094/MPMI-20-4-0420

Lodha, T. D., and Basak, J. (2012). Plant-pathogen interactions: what microarray tells about it? *Mol. Biotechnol.* 50, 87–97. doi: 10.1007/s12033-011-9418-2

Lorenzo, O., Piqueras, R., Sánchez-Serrano, J. J., and Solano, R. (2003). Ethylene response factor 1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell* 15, 165–178. doi: 10.1105/tpc.007468

Loscos, J., Matamoros, M. A., and Becana, M. (2008). Ascorbate and homoglutathione metabolism in common bean nodules under stress conditions and during natural senescence. *Plant Physiol.* 146, 1282–1292. doi: 10.1104/pp.107.114066

Lu, Z.-X., Gaudet, D. A., Frick, M., Puchalski, B., Genswein, B., and Laroche, A. (2005). Identification and characterization of genes differentially expressed in the resistance reaction in wheat infected with *Tilletia tritici*, the common bunt pathogen. *BMB Rep.* 38, 420–431. doi: 10.5483/BMBRep.2005.38.4.420

Mauch, F., and Dudler, R. (1993). Differential induction of distinct glutathione-S-transferases of wheat by xenobiotics and by pathogen attack. *Plant Physiol.* 102, 1193–1201. doi: 10.1104/pp.102.4.1193

Mayo, S., Gutiérrez, S., Malmierca, M. G., Lorenzana, A., Campelo, M. P., Hermosa, R., et al. (2015). Influence of *Rhizoctonia solani* and *Trichoderma* spp. in growth of bean (*Phaseolus vulgaris* L.) and in the induction of plant defense-related genes. *Front. Plant Sci.* 6:685. doi: 10.3389/fpls.2015.00685

Moffat, C. S., Ingle, R. A., Wathugala, D. L., Saunders, N. J., Knight, H., and Knight, M. R. (2012). ERF5 and ERF6 play redundant roles as positive regulators of JA/Et-mediated defense against *Botrytis cinerea* in *Arabidopsis*. *PLoS ONE* 7:e35995. doi: 10.1371/journal.pone.0035995

Moons, A. (2005). Regulatory and functional interactions of plant growth regulators and plant glutathione S-transferases (GSTs). *Vitam. Horm.* 72, 155–202. doi: 10.1016/S0083-6729(05)72005-7

Narasimhan, M. L., Bressan, R. A., D'Urzo, M. P., Jenks, M. A., and Mengiste, T. (2009). Unexpected turns and twists in structure/function of PR-proteins that connect energy metabolism and immunity. *Adv. Bot. Res.* 51, 439–489. doi: 10.1016/S0065-2296(09)51011-7

Noordermeer, M. A., Veldink, G. A., and Vliegenthart, J. F. G. (2001). Fatty acid hydroperoxide lyase: a plant cytochrome P450 enzyme involved in wound healing and pest resistance. *Chembiochem* 2, 494–504. doi: 10.1002/1439-7633(20010803)2:7/8<494::AID-CBIC494>3.0.CO;2-1

Nugroho, L. H., Verberne, M. C., and Verpoorte, R. (2002). Activities of enzymes involved in the phenylpropanoid pathway in constitutively salicylic acid-producing tobacco plants. *Plant Physiol. Biochem.* 40, 755–760. doi: 10.1016/S0981-9428(02)01437-7

Pandey, S. P., and Somssich, I. E. (2009). The role of WRKY transcription factors in plant immunity. *Plant Physiol.* 150, 1648–1655. doi: 10.1104/pp.109.138990

Pereira, J. L., Queiroz, R. M. L., Charneau, S. O., Felix, C. R., Ricart, C. A. O., Lopes Da Silva, F., et al. (2014). Analysis of *Phaseolus vulgaris* response to its association with Trichoderma harzianum (ALL-42) in the presence or absence of the phytopathogenic fungi *Rhizoctonia solani* and *Fusarium solani*. *PLoS ONE* 9:e98234. doi: 10.1371/journal.pone.0098234

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45–e45. doi: 10.1093/nar/29.9.e45

Reid, K. E., Olsson, N., Schlosser, J., Peng, F., and Lund, S. T. (2006). An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biol.* 6:27. doi: 10.1186/1471-2229-6-27

Rushton, P. J., and Somssich, I. E. (1998). Transcriptional control of plant genes responsive to pathogens. *Curr. Opin. Plant Biol.* 1, 311–315. doi: 10.1016/1369-5266(88)80052-9

Rutledge, R. G., and Stewart, D. (2008). Critical evaluation of methods used to determine amplification efficiency refutes the exponential character of real-time PCR. *BMC Mol. Biol.* 9:96. doi: 10.1186/1471-2199-9-96

Sharma, S., Lin, W., Villamor, J. G., and Verslues, P. E. (2013). Divergent low water potential response in *Arabidopsis* thaliana accessions *Landsberg erecta* and Shahdara. *Plant. Cell Environ.* 36, 994–1008. doi: 10.1111/pce.12032

Shoresh, M., Harman, G. E., and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 48, 21–43. doi: 10.1146/annurev-phyto-073009-114450

Singh, K. B., Foley, R. C., and Oñate-Sánchez, L. (2002). Transcription factors in plant defense and stress responses. *Curr. Opin. Plant Biol.* 5, 430–436. doi: 10.1016/S1369-5266(02)00289-3

Son, G. H., Wan, J., Kim, H. J., Nguyen, X. C., Chung, W. S., Hong, J. C., et al. (2012). Ethylene-responsive element-binding factor 5, ERF5, is involved

in chitin-induced innate immunity response. *Mol. Plant-Microbe Interact.* 25, 48–60. doi: 10.1094/MPMI-06-11-0165

Upchurch, R. G., and Ramirez, M. E. (2010). Defense-related gene expression in soybean leaves and seeds inoculated with *Cercospora kikuchii* and *Diaporthe phaseolorum* var. meridionalis. *Physiol. Mol. Plant Pathol.* 75, 64–70. doi: 10.1016/j.pmpp.2010.08.007

Valenciano, J. B., Casquero, P. A., Boto, J. A., and Marcelo, V. (2006). Evaluation of the occurrence of root rots on bean plants (*Phaseolus vulgaris*) using different sowing methods and with different techniques of pesticide application. *New Zeal. J. Crop Hortic. Sci.* 34, 291–298. doi: 10.1080/01140671.2006.9514419

Vellicce, G. R., Ricci, J. C. D., Hernández, L., and Castagnaro, A. P. (2006). Enhanced resistance to *Botrytis cinerea* mediated by the transgenic expression of the chitinase gene CH5b in strawberry. *Transgenic Res.* 15, 57–68. doi: 10.1007/s11248-005-2543-6

Verhagen, B. W. M., Van Loon, L. C., and Pieterse, C. M. J. (2006). "Induced disease resistance signaling in plants," in *Floriculture, Ornamental and Plant Biotechnology*, ed. J. A. Teixeira da Silva (Utrecht: Global Science Books), 334–343.

Wan, X.-H., Chen, S.-X., Wang, C.-Y., Zhang, R.-R., Cheng, S.-Q., Meng, H.-W., et al. (2013). Isolation, expression, and characterization of a hydroperoxide lyase gene from cucumber. *Int. J. Mol. Sci.* 14, 22082–22101. doi: 10.3390/ijms141122082

Wang, Z., Huang, S., Jia, C., Liu, J., Zhang, J., Xu, B., et al. (2013). Molecular cloning and expression of five glutathione S-transferase (GST) genes from Banana (*Musa acuminata* L. AAA group, cv. cavendish). *Plant Cell Rep.* 32, 1373–1380. doi: 10.1007/s00299-013-1449-7

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer EVG declared a past co-authorship with one of the authors SG to the handling Editor, who ensured that the process met the standards of a fair and objective review.

CAPÍTULO IV

Metabolomic response of bean plants (*Phaseolus vulgaris* L.) to the presence of *Trichoderma velutinum* and *Rhizoctonia solani*

ÍNDICE CAPÍTULO IV

TABLAS

FIGURAS

"METABOLOMIC RESPONSE OF BEAN PLANTS (*PHASEOLUS VULGARIS* L.) TO THE PRESENCE OF *TRICHODERMA VELUTINUM* AND *RHIZOCTONIA SOLANI*"

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ABSTRACT: The common bean (*Phaseolus vulgaris* L.) is a legume crop worldwide that is affected by diversity fungal pathogen, as *Rhizoctonia solani*. Biocontrol represents an alternative against phytopathogenic agents, and fungi as *Trichoderma* are effectively used to control fungal diseases and to promote plant growth. After the invasion of a pathogen or a biocontrol agent, the plant activates disease resistance responses, by inducing the expression of defence genes and by producing some bioactive secondary metabolites such as phytoalexins and some substances that could regulate defence reactions. The purpose of this article was to study the metabolomic response of the bean plants in the presence of *Trichoderma velutinum* and/or *R. solani,* including the characterization of the phytochemical compounds. Hydromethanol extracts from bean leaves were analysed by HPLC-DAD-TOF-MS and compared with information from the literature. In this work, metabolomics analysis of bean samples revealed an increased in the production of 36 compounds (amino acids, flavonoids, phenols, terpenes, etc), which are specific of the Fabaceae family that were differentially accumulated in leaves inoculated with biocontrol or pathogen fungi compared to controls. As conclusion, the presence of *T. velutinum* and/or *R. solani* modified the production of bean metabolites.

KEY WORDS: Defence responses, phytoalexins, biocontrol agent, phytopathogen, HPLC.

1. INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is one of the most important food legume crops worldwide. Bean production can be affected by biotic and abiotic stresses, such as pathogen attack, drought, cold, etc., and plants activate a plethora of mechanisms to respond to them (Guerrero-González et al., 2011).

Rhizoctonia solani JG Kühn [Teleomorph: *Thanatephorus cucumeris* (AB Frank) Donk] is a necrotrophic plant pathogen causing root and hypocotyl diseases. Rhizoctonia root rot of bean is a common disease throughout the world and is one of the most devastating root and hypocotyl diseases of bean in large and small plantings (Hagedorn, 1991).

Biological control represents an alternative against phytopathogenic agents, and fungi of the genus *Trichoderma* (Teleomorph: *Hypocrea*) are effectively used worldwide to both, control fungal diseases and promote plant growth. *Trichoderma* spp. represent a fundamental part of the soil microbiota, the rhizosphere microbiome, which helps plant to overcome numerous environmental constraints by stimulating plant defense responses and improving fitness and development (Hermosa et al., 2013). These abilities have supported the application of *Trichoderma* strains as biocontrol agents (BCA) or plant biofertilizer in agriculture and forestry.

When a plant is induced by exposure to a microorganism, the production of several metabolites is started, and the physiological changes and activation of various plant metabolic pathways depends on the type of these signalling natural metabolites. It is assumed that the plant recognition of *Trichoderma* triggers the activation of a cascade signal, including the secretion of antimicrobial reactive oxygen species, production of a plethora of secondary metabolites such as phytoalexins and pathogenesis-related proteins and callose depositions (Shoresh et al., 2010).

Plants respond after the invasion of a pathogen or a biocontrol agent that activates disease resistance response to the invasion, by inducing the expression of defence genes (Mayo et al., 2016), and by the production of bioactive secondary metabolites such as phytoalexins (phenols, isoflavones, terpenes), and some substances that can block the invasion and spread of the pathogen, such as lignin and callose (Chen et al., 2015). Biosynthesis of phytoalexins by plants from the Fabaceae family is of interest because they are produced in higher or lower amounts depending if the plant is interacting with a pathogen or with a biocontrol agent. It is also known that some plants do not produce phytoalexins when they are in contact with pathogens, but release toxins that are normally stored in the plant as less toxic glycosides. If the plant cell is broken when penetrated by fungal hyphae, the glycoside comes into contact with hydrolysing enzymes present in other cell compartments, releasing the toxic aglycone. Although this compound is released after fungal attack, it is not present in the intact plant and is newly produced when compromised by a fungal invader, it is not a true phytoalexin, because the glycosidases were already present in the plant and were not formed *de novo* (Grayer and Kokubun, 2001).

Polyphenols are one type of phytoalexins that are widely distributed in plants and can be classified into three groups: i) simple phenols, ii) phenolic acids (hydroxycinnamic- and benzoic-acid derivatives), and iii) flavonoids (e.g. flavones, flavanones, flavanonols, flavanols, isoflavons and lignans) (Abu-Reidah et al., 2013). Flavonoids and their derivatives comprise a large group of secondary metabolites whose production is specifically induced by symbionts and pathogens (Hassan and Mathesius, 2012). One of their roles inside the root could be to regulate defence reactions, and it has been proposed that mycorrhiza invasion triggers a temporary defence response in the root that involves induction of phytoalexins production (Harrison and Dixon, 1994).

Isoflavonoids are a subclass of flavonoids, which are thought to represent the majority of the phytoalexins produced by legume plants (Hassan and Mathesius, 2012).

Other phytoalexins, of terpene nature, are toxins that act as feeding repellents against many plant feeding insects and mammals. Then, playing important defensive roles in the plant (Taiz et al., 2015). This sort of compounds have been found in legumes, such as triterpenes and saponins, whose activity as antimicrobial defence compounds has been previously reported (Wink, 2003).

In this work, and based in the huge variety of compounds produced by plants in response to the interaction with pathogens and/or biocontrol agents, we have evaluated the pattern of production of polyphenols and others metabolites in bean plants in interaction with *Trichoderma velutinum* and/or *R. solani*.

2. MATERIAL AND METHODS

2.1. Fungal isolates and culture conditions

The present study was carried out using the isolate of *Trichoderma velutinum* T028, previously collected from the production area of the Protected Geographical Indication (PGI), called "Alubia La Bañeza - León" (EC Reg. n.256/2010 published on 26 March 2010, OJEU L880/17).

Rhizoctonia solani isolate R43 is a high virulence strain that was isolated from plants of the same PGI. This strain was stored in the collection "Pathogens and Antagonists of the Laboratory Diagnosis of Pests and Diseases" (PALDPD) at the University of León, Spain.

The fungal isolates were grown on potato-dextrose-agar (PDA, Sigma-Aldrich) plates in the dark at 25 °C for one week. *Trichoderma* cultures were then exposed to light for 3-4 days in order to induce the spore formation. Spores were maintained at -80ºC in 50% glycerol suspensions.

2.2. Plant materials and growth conditions

Bean (*P. vulgaris* L. cv. Canela) seeds were germinated and cultured in four conditions: inoculated with *T. velutinum* (T028) and with *R. solani* (R43) present in the substrate [RT028]; inoculated with T028 without the pathogen (CT028); seeds not inoculated with *T. velutinum* by with *R. solani* (CR) in the substrate; and control without fungi (CC) (Figure 1)

Cultures were carried out in climatic chamber and grown under the conditions previously described (Mayo et al., 2015). Thirty pots were used per treatment with two seeds per pot. Each pot was watered with 250 ml of water prior inoculation. *R. solani* R43 was inoculated by surface irrigation with 50 ml per pot of a suspension of triturated micromicete culture of this pathogen using five Petri dishes (18 ml of PDA per dish) per litre of water. For control inoculation, only PDA medium was used without any pathogen. Pots were kept in a growth chamber for 8 days at 25 °C (16h) and 16 °C (8h), 60% relative humidity in the dark. Spores suspension of *T. velutinum* (T028) was prepared at a final concentration of 2×10^7 spores/ml. Bean seeds were surface sterilized (sodium hypochlorite 1% for 3 minutes and distilled water for 6 minutes). Then, they were coated with the spore suspension. Coated seeds were sown after 8 days of the inoculation of *R. solani* R43 (Figure 1). The culture was maintained for 45 days with a photoperiod of 16 hours light, 25 °C/16 °C

(day/night), 60% relative humidity and brightness of 3,500 lux. Plants were removed after 45 days from sowing.

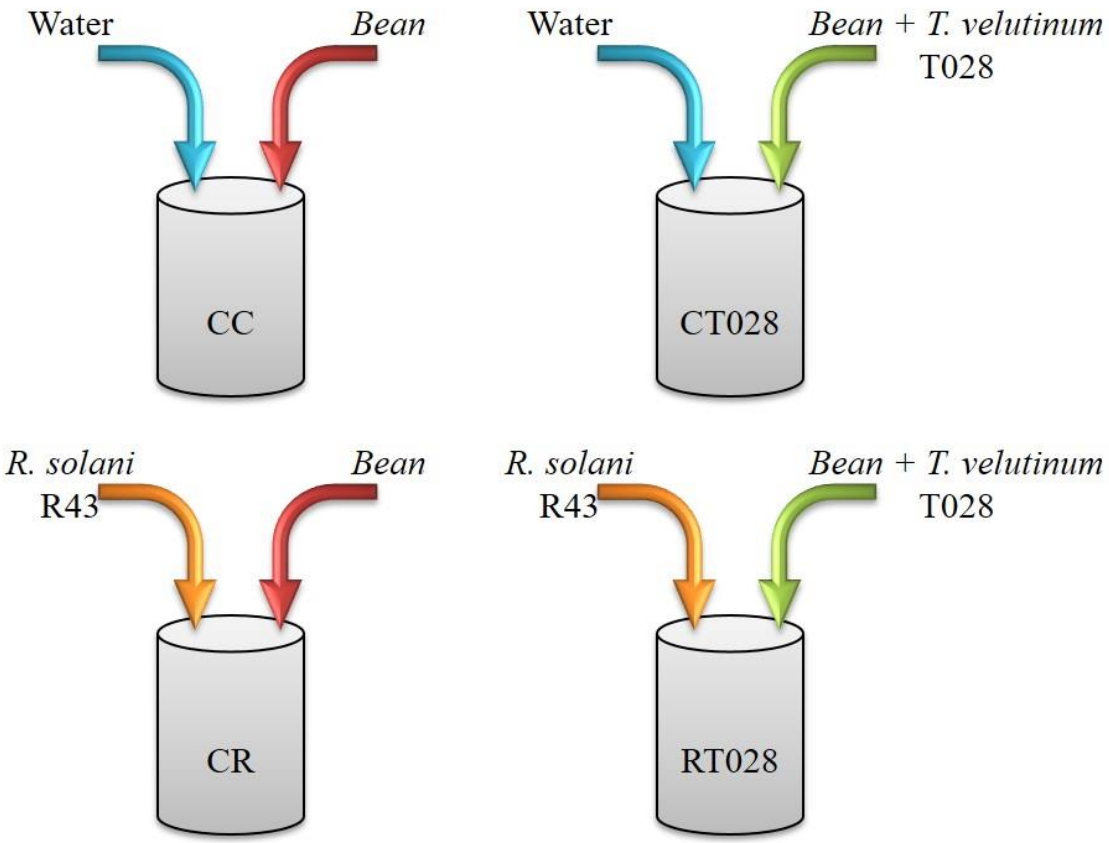


Figure 1: Scheme of the treatments used in this work: RT028, Coated seeds with *T. velutinum* (T028) against *R. solani* (R43); CT028, Coated seeds with *T. velutinum* without pathogen; CR, control with *R. solani*; and CC, control without fungi.

2.3. Preparation of bean leaf extracts

To extract the secondary plant metabolites from all treatments, bean leaves from 45 day-old plants were collected and lyophilized. Sample extraction was performed as described by Talhaoui et al. (2015) with some modifications. Briefly, dry leaves (250 mg) were crushed and extracted twice via Ultra-Turrax IKA T18 basic with 10 mL of MeOH/H_2O (80/20 v/v). Afterwards, samples were placed in an ultrasonic liquid processor (Sonics & Materials, Inc., Newtown, Ct, USA) for 10 min and centrifuged at 4000 rpm for 10 min at 4° C. After solvent evaporation, the extracts were reconstituted with 4 mL of MeOH:H_2O (50:50 v/v) and filtered using a 0.2 µm membrane (Minisart®, Sartorius stedim Biotech CA 0.2). Three replicates of each sample were processed.

2.4. HPLC-DAD-TOF-MS conditions

The compounds of the leaf extracts were separated on an Agilent 1200 series Rapid Resolution LC (Agilent Technologies, Santa Clara, CA, USA). The chromatographic separation was performed by a Zorbax Eclipse Plus C_{18} analytical column (4.6 x 100 mm, 3.5 µm particle size) from Agilent

Technologies. The conditions were performed as previously described (Abu-Reidah et al., 2013) with slight modifications.

The mobile phases were acidified water (0.1 % formic acid, FA) and acetonitrile (plus 0.1 % FA) as eluents A and B, respectively. The chromatographic method consisted in the following linear gradient with a flow rate of 0.80 ml/min: 0 min, 0% B; 10 min, 20 % B; 15 min, 30 % B; 20 min, 50 % B; 25 min, 75 % B; 30 min, 100 % B; 35 min, 100 % B; 35.10 min, 0 % B; and finally, a 5 min post-run was used after each analysis. The injection volume was 5.0 µl and the column temperature was maintained at 25 ºC.

The HPLC system was coupled to a time-of-flight mass spectrometer (TOF-MS) equipped with the model G6540B Dual ESI (Agilent Technologies) operating in negative ion mode. The optimum values of source parameters were: drying-gas temperature 300 ºC; drying-flow 9 l/min; nebulising-gas pressure 3.06 atm. The spectra were recorded in the targeted mode within the *m/z* mass range of 50-1100.

The MS data were processed through Agilent MassHunter Qualitative Analysis B.05.00 software (Agilent Technologies), which provide a list of possible elemental formula by using a generate molecular formula editor. According to the literature, the phytochemical compounds were identified based on the accurate mass measurements of the pseudomolecular $[M-H]^-$ as compared to a database containing more than 15.000 natural secondary metabolites, as well as literature on *Fabaceae* species, because no commercial standard was available for the compounds detected in bean leaves.

The statistical analyses were carried out using the Mass Profiler Professional Software 13.0 (G3835AA, Agilent Technologies). Significant statistical differences among treatments ($p < 0.05$) were assessed by one-way ANOVA and principal component analysis (PCA). All assays were run in triplicate.

3. RESULTS

3.1. Selection of *Trichoderma* strain

T. velutinum T028 was the selected isolated based on its positive effect on bean growth. Thus, plants inoculated with this strain showed a significant increase in dry weight of both aerial parts and root system, including when *R. solani* was present in the substrate. It was isolated from soil plot bean in the Astorga region (León, Spain). This isolate gave percentages of inhibition 61.73±0.61 (% ± standard error) in membrane assays and 47.08±1.78 (% ± standard error) in direct confrontation assays with *R. solani*, and was able to sporulate on PDA medium. Furthermore, when bean plants were treated with *T. velutinum* T028 they increased respect to control plants (CC) 4.75 % their diameter of hypocotyl, 10.75 % their length of root system, 4.27 % and 5.51 % in dry weight of aerial parts and root system, respectively. When plants were infected with *R. solani*, the action of *T. velutinum* T028 caused an increase of the diameter of hypocotyl in 8.76 %, 21.15 % in the length of the root system, and 11.05 % and 3.43 % in dry weight of aerial parts and root system respectively respect to the control plant with the pathogen (CR) (Mayo et al., 2016). Based on these results, this isolate was used for further studies. In addition, this is the first report in which the effects of this strain on bean phenotype and plant metabolite production are studied.

3.2. Characterization of compounds in bean leaf extracts

The base peak chromatogram of bean leaf resulted from the optimal-gradient-elution program and the optimal MS conditions in negative ionization mode (Figure 2). The compounds were identified by interpreting their mass spectra determined via TOFMS and taking into account the data reported in the literature.

Metabolomics analysis of bean leaf samples revealed up to 36 compounds, which are specific of the Fabaceae family that accumulated differentially in leaves inoculated with fungal microbes compared to controls (inoculated plants). The compounds detected in uninoculated bean leaves were used as a control for the comparisons with the two-way (CT028-CC and CR-CC) and the three-way interactions (RT028-CC) (Figure 1). The compounds were considered to be differentially accumulated when increase (UP) or decrease (DOWN) production was observed, compared to uninoculated controls (CC). The semi-quantification of compounds was performed by determining the peak areas of the base peak chromatogram of bean leaf samples. The following categories of compounds were found.

Table 1: Phytochemical compounds detected in bean leaves by negative ionization mode, including retention time, m/z, molecular formula, together with their proposed identities and the references.

Id	m/z	RT (min)	Formula	Tentative assignment	Reference
Amino acid					
8	204.09	8.52	$C_{11}H_{12}N_2O_2$	L-Tryptophan	PubChem ID number - 6305 (Lu et al., 2013)
15	246.10	14.20	$C_{13}H_{14}N_2O_3$	N-acetyltryptophan	PubChem ID number - 2002 (Lu et al., 2013)
Peptide					
7	230.16	8.42	$C_{11}H_{22}N_2O_3$	Valyl-Leucine	Pubchem ID number - 107487
11	372.24	10.00	$C_{17}H_{32}N_4O_5$	Ile Gln Ile	NA
29	402.19	19.94	$C_{15}H_{26}N_6O_7$	Gln Gln Gln	NA
Carbohydrate					
2	342.12	1.43	$C_{12}H_{22}O_{11}$	Sucrose	PubChem ID number - 5988 (Lu et al., 2013)
Glycoside					
4	338.08	4.96	$C_{12}H_{18}O_{11}$	L-Ascorbic acid-2-glucoside	Pubchem ID number - 54693473
Fatty acids					
35	278.22	30.59	$C_{18}H_{30}O_2$	γ-Linolenic acid	PubChem ID number - 5280933 (Brechenmacher et al., 2010; Kanehisa Laboratories, 2016; Lu et al., 2013)

Table 1: Phytochemical compounds detected in bean leaves by negative ionization mode, including retention time, m/z, molecular formula, together with their proposed identities and the references (continuation).

Id	m/z	RT (min)	Formula	Tentative assignment	Reference
Lipid					
	• **Fatty acyls - Octadecanoids**				
34	294.21	26.29	$C_{18}H_{30}O_3$	13(S)-HOTrE	Pubchem ID number - 47205624
	• **Glycerophosphates**				
36	578.42	30.59	$C_{31}H_{63}O_7P$	PA(O-16:0/12:0)	Pubchem ID number - 52929565
	• **Fatty acyls glycoside**				
30	334.20	19.94	$C_{16}H_{30}O_7$	3-O-α-L-rhamnopyranosyl-3-hydroxydecanoic acid	Pubchem ID number - 56936287 (Buckingham and Munasinghe, 2015; Yannai, 2012)
Flavonoids					
	• **Flavone**				
22	374.10	16.90	$C_{19}H_{18}O_8$	3´,5-Dihydroxy-3,4´,6,7-tetramethoxyflavone	(Buckingham and Munasinghe, 2015)
27	390.09	18.32	$C_{19}H_{18}O_9$	5,2',4'-Trihydroxy-3,7,8,5'-tetramethoxyflavone	Pubchem ID number - 85296959
	• **Flavonol**				
12	596.17	10.27	$C_{26}H_{28}O_{16}$	Quercetin 3-vicianoside	Pubchem Id number - 44259139 (Abu-Reidah et al., 2013)
26	454.24	18.05	$C_{20}H_{38}O_{11}$	n-Octyl-β-D-maltopyranoside	NA
	• **Flavonol**				
21	414.22	16.25	$C_{29}H_{50}O$	Sitosterol	PubChem ID number - 86821 (Buckingham and Munasinghe, 2015; Lu et al., 2013)
	• **Flavonone**				
3	580.18	1.47	$C_{27}H_{32}O_{14}$	Naringin	PubChem ID number - 442428 (Lu et al., 2013)
	• **Flavonoids glycoconjugate**				
1	400.10	1.31	$C_{17}H_{20}O_{11}$	5-Hydroxy-6,8-dimethoxy-2-oxo-2H-chromen-7-yl β-D-glucopyranoside	NA
10	612.17	9.37	$C_{27}H_{32}O_{16}$	3,4´,5,7-Tetrahydroxyflavanone 3,7-Di-O-β-D-glucopyranoside	CAS Number - 80212-10-8 (Buckingham and Munasinghe, 2015)
14	552.18	11.13	$C_{26}H_{32}O_{13}$	(Z)-Resveratrol 3,4'-diglucoside	PubChem ID number - 22298557 (Yannai, 2012)
16	642.12	14.34	$C_{30}H_{26}O_{16}$	Quercetagetin 7-(6"-(E)-caffeoylglucoside)	PubChem Id number - 44259848
18	654.18	16.11	$C_{29}H_{34}O_{17}$	Isopyrenin 7-O-glucoside	CAS Number - 61252-86-6 (Buckingham and Munasinghe, 2015).

Table 1: Phytochemical compounds detected in bean leaves by negative ionization mode, including retention time, m/z, molecular formula, together with their proposed identities and the references (continuation).

Id	m/z	RT (min)	Formula	Tentative assignment	Reference
	• **Flavonoids glycoconjugate (continuation)**				
19	540.18	16.11	$C_{25}H_{32}O_{13}$	12-Hydroxy, O-[3,4,5-trihydroxybenzoyl-(06)-β-D-glucopyranoside]	(Yannai, 2012)
20	678.29	16.24	$C_{32}H_{39}O_{16}$	Luteone 4,7-O-diglucoside	(Wojakowska et al., 2013, 2015)
24	470.24	17.61	$C_{20}H_{38}O_{12}$	(R)-1-O-[β-D-Glucopyranosyl-(1-6)-β-D-glucopyranoside]-1,3-octanediol	HMDB Id number - 32799
	• **Isoflavanoid**				
28	338.19	18.61	$C_{21}H_{22}O_4$ $C_{20}H_{18}O_5$	2′-O-Methylphaseollinisoflavan Wighteone	CAS Number - 49594-01-6 PubChem Id number - 5281814 (Rizk et al., 1980; Southon et al., 1994; Wojakowska et al., 2015; Yannai, 2012)
	• **Isoflavanone**				
25	424.23	17.62	$C_{25}H_{28}O_6$	2′,4′,5,7-Tetrahydroxy-3′,8-diprenylisoflavanone	CAS Number - 64280-18-8 (Southon et al., 1994)
	• **Isoflavans**				
6	324.14	8.13	$C_{20}H_{20}O_4$	Phaseollinisoflavan	Pubchem ID number - 4484952 (Rizk et al., 1980)
Phenol					
	• **Tyrosols // Flavonoid**				
5	316.11	7.29	$C_{14}H_{20}O_8$ $C_{16}H_{12}O_7$	Hydroxytyrosol 1-O-glucoside Isorhamnetin	Pubchem ID number - 13845930 (Lu et al., 2013)
	• **Phenylpropanoids**				
13	386.12	10.71	$C_{17}H_{22}O_{10}$	1-O-Sinapoylglucose	Pubchem ID number - 5280406
	• **Xanthonoid**				
31	392.11	21.10	$C_{19}H_{20}O_9$	Garcimangosone D	Pubchem ID number – 11003703 (Yannai, 2012)
33	628.31	25.77	$C_{38}H_{44}O_8$	Gambogic acid	Pubchem ID number - 5281632
Terpene					
	• **Terpenoid**				
9	444.20	8.88	$C_{21}H_{32}O_{10}$	Dihydrophaseic acid 4-O-β-D-glucoside	CHEBI Id number - 23758 (Pushpa et al., 2014)
	• **Triterpenoid**				
32	896.51	24.97	$C_{47}H_{76}O_{16}$	Akeboside Ste	Pubchem ID number - 46173935

Table 1: Phytochemical compounds detected in bean leaves by negative ionization mode, including retention time, m/z, molecular formula, together with their proposed identities and the references (continuation).

Id	m/z	RT (min)	Formula	Tentative assignment	Reference
	• **Terpene glycosides**				
17	416.20	14.78	$C_{20}H_{32}O_9$	Ethyl 7-epi-12-hydroxyjasmonate glucoside	HMDB Id number - 36340 (Lu et al., 2013)
	• **Terpene // Flavonoids - Methoxyisoflavones**				
23	268.13	17.52	$C_{14}H_{20}O_5$ $C_{16}H_{12}O_4$	Teucrein Formononetin	Pubchem ID number - 101288297 PubChem ID number - 5280378 (Lu et al., 2013)

RT: retention time; NA: not available

a) Amino acids and peptides

Statistical analysis revealed the different accumulation of two aminoacids and three peptides (Table 1). Compound #**8** (*m/z* 204.0896; Retention Time, RT = 8.52 min) and compound #**15** (*m/z* 246.1003; RT = 14.20 min), corresponding to the molecular formula $C_{11}H_{12}N_2O_2$, and $C_{13}H_{14}N_2O_3$, respectively, were putatively identified as L-tryptophan and N-acetyltryptophan. Both compounds were produced in lower amounts when *R. solani* was present (CR) (Table 2). However, in the presence of *T. velutinum* they were more produced at higher levels (CT028 and RT028) (Table 2).

Furthermore, three peptides (Table 1) were detected: valyl-leucine [compound #**7**, *m/z* 230.1627 at 8.42 min ($C_{11}H_{22}N_2O_3$)] was produced in the presence of *T. velutinum* (CT028 and RT028) at higher level than in control plants and at a lower level when only with *R. solani* was present (CR) (Table 2). Ile-Gln-Ile [compound #**11**, *m/z* 372.2370 at 10.00 min ($C_{17}H_{32}N_4O_5$)], whose production was reduced in the presence of *R. solani* (CR and RT028), and Gln-Gln-Gln [compound #**29**, *m/z* 402.1860 at 19.94 min ($C_{15}H_{26}N_6O_7$)] that whose amount decreased in the presence of *T. velutinum* (CT028 and RT028) (Table 2).

b) Carbohydrates and glycosides

Production of sucrose (Table 1) [compound #**2**, *m/z* of 342.1157 at 1.43 min ($C_{12}H_{22}O_{11}$)] shown a higher level in the presence of *R. solani* or *T. velutinum* (CT028 and CR) but not when both fungi were together (RT028) (Table 2).

L-ascorbic acid-2-glucoside (Table 1) was also identified, [compound #**4**, *m/z* 338.048, at 4.96 min ($C_{12}H_{18}O_{11}$)], at an increased level only in presence of *R. solani* (CR) without *T. velutinum* (Table 2*)*.

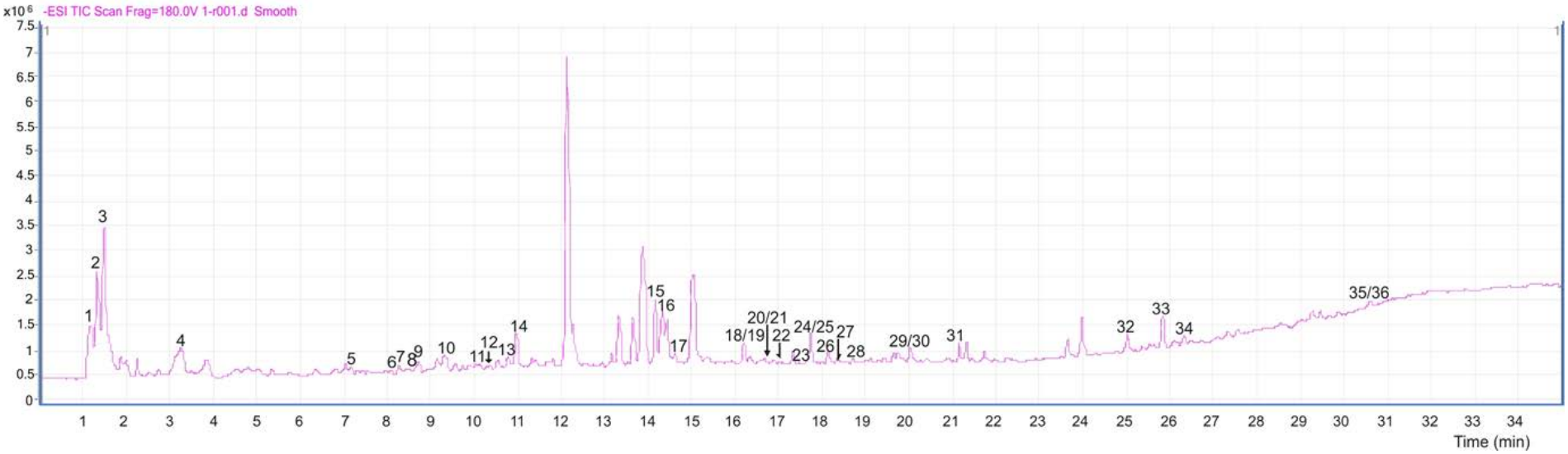


Figure 2: Base peak chromatogram of control plants (CC), obtained by HPLC-DAD-TOF-MS. See Table 1 for identification number.

c) Fatty acids and lipids

Three compounds were detected belonging to this group (Table 1): i) compound #**35**, with an *m/z* 278.2238 at 30.59 min ($C_{18}H_{30}O_2$); ii) #**34**, *m/z* 294.2193 at 26.29 min ($C_{18}H_{30}O_3$), and iii) #**36**, *m/z* 578.4296 at 30.59 min ($C_{31}H_{63}O_7P$). These compounds were γ-linolenic acid, 13(S)-HOTrE and PA(O-16:0/12:0), respectively. All of them were produced at a significantly lower amount respect to control plants when the pathogen *R. solani* was present (CR and RT028) (Table 2). 3-O-α-L-rhamnopyranosyl-3-hydroxydecanoic acid was also identified [#**30**, *m/z* 334.1990 at 19.94 min ($C_{16}H_{30}O_7$)], and was produced at a significantly lower amount when *T. velutinum* was present (CT028 and RT028), compared in all cases to control plants (Table 2).

d) Polyphenols

A huge number of compounds belonging to this group has been detected:

i) Two compounds, #**22** and #**27**, were determined as flavones (Table 1), one with *m/z* 374.0998 at 16.90 min ($C_{19}H_{18}O_8$), and other with *m/z* 390.0946 at 18.32 min ($C_{19}H_{18}O_9$), which were identified as 3´,5-dihydroxy-3,4´,6,7-tetramethoxyflavone and 5,2',4'-trihydroxy-3,7,8,5'-tetramethoxyflavone, respectively. Both compounds were produced at significantly lower amounts in plants with any of the fungi analyzed (CT028, CR, and RT028) (Table 2).

ii) Flavonols (Table 1). quercetin 3-vicianoside (#**12**) with an *m/z* 596.1736, at 10.27 min ($C_{26}H_{28}O_{16}$); n-octyl-β-D-maltopyranoside (#**26**) with an *m/z* 454.2409, at 18.05 min ($C_{20}H_{38}O_{11}$) and sitosterol (#**21**) with *m/z* 414.2248, at 16.25 min ($C_{29}H_{50}O$). These compounds showed different responses. In the case of compounds #**12** and #**21** they were produced at higher amounts in the presence of *T. velutinum* (CT028 and RT028) (Table 2). However, compound #26 was produced at lower level in presence of this fungus (CT028).

iii) Flavonones (Table 1). Naringin, (#**3**), *m/z* 580.1849, at 1.47 min ($C_{27}H_{32}O_{14}$), was produced at higher levels in the presence of *T. velutinum* (CT028) (Table 2).

iv) Flavonoid glycoconjugates (Table 1). 5-Hydroxy-6,8-dimethoxy-2-oxo-2H-chromen-7-yl β-D-glucopyranoside (#**1**), m/z 400.1024, at 1.31 min ($C_{17}H_{20}O_{11}$), which was in low amount in the presence of the two fungi (Table 2); 3,4´,5,7-Tetrahydroxyflavanone 3,7-Di-O-β-D-glucopyranoside (#**10**), *m/z* 612.1685, at 9.37 min ($C_{27}H_{32}O_{16}$) that was produced at higher level with *T. velutinum* (CT028 and RT028); (Z)-resveratrol 3,4'-diglucoside, (#**14**), *m/z* 552.1841, at 11.13 min ($C_{26}H_{32}O_{13}$) which was produced in higher amount in the presence of both *T. velutinum* and *R. solani* (RT028) (Table 2); quercetagetin 7-(6"-(E)-caffeoylglucoside) (#**16),** *m/z* 642.1192, at 14.34 min ($C_{30}H_{26}O_{16}$), produced at lower levels in plants infected with *R. solani* (CR) (Table 2); 12-hydroxy,O-[3,4,5-trihydroxybenzoyl-(06)-β-D-glucopyranoside] (#**19**), *m/z* 540.1836, at 16.11 min ($C_{25}H_{32}O_{13}$) whose production was lower in the presence of both fungi (CT028, CR and RT028) (Table 2); isopyrenin 7-O-glucoside (#**18**), *m/z* 654.1767, at 16.11 min ($C_{29}H_{34}O_{17}$), which was detected in lower amounts in the presence of both fungi (CT028, CR and RT028) (Table 2); luteone 4,7-O-diglucoside (#**20**), *m/z* 678.2894, at 16.24 min ($C_{32}H_{39}O_{16}$), which was produced at higher levels in the presence of *T. velutinum* (CT028 and RT028), while the infection with *R. solani* (CR) reduced its level (Table 2); and (R)-1-O-[β-D-glucopyranosyl-(1-6)-β-D-glucopyranoside]-1,3-octanediol (#**24**), *m/z* 470.2359, at 17.61 min ($C_{20}H_{38}O_{11}$) produced at lower level with *T. velutinum* (CT028 and RT028) (Table 2).

v) Others polyphenols. Isoflavanoids (Table 1): 2′-O-methylphaseollinisoflavan [#**28**, *m/z* 338.1939, at 18.61 min ($C_{21}H_{22}O_4$)], which could be also identified as wighteone ($C_{20}H_{18}O_5$). Isoflavanones (Table 1): 2′,4′,5,7-tetrahydroxy-3′,8-diprenylisoflavanone [#**25**, *m/z* 424.2304, at 17.62 min ($C_{25}H_{28}O_6$). Isoflavans: phaseollinisoflavan [#**6**, *m/z* 324.1419, at 8.13 min ($C_{20}H_{20}O_4$). All these compounds were produced at lower level in the presence of *T. velutinum* (CT028 and RT028), except in the case of phaseollinisoflavan, whose production was lower only if *R. solani* was present with this biocontrol agent (RT028) (Table 2). Phenols (Table 1): hydroxytyrosol 1-O-glucoside [#**5**, *m/z* 316.1152, at 7.29 min ($C_{14}H_{20}O_8$) or isorhamnetin, both with that same molecular weight, and produced at lower level in the presence of *T. velutinum* (CT028) (Table 2); 1-O-sinapoylglucose [#**13**, *m/z* 386.1202, at 10.71 min ($C_{17}H_{22}O_{10}$) that is produced at higher level in the presence of *T. velutinum* (CT028 and RT028) (Table 2); garcimangosone D [#**31**, *m/z* 392.1105, at 21.10 min ($C_{19}H_{20}O_9$), and produced in lower amount when both fungi were present (CT028, CR and RT028) (Table 2); gambogic acid [#**33**, *m/z* 628.3061, at 25.77 min ($C_{38}H_{44}O_8$)] also produced at lower level in the presence of *T. velutinum* (CT028 and RT028) (Table 2).

e) Terpenes

T. velutinum and *R. solani* affect to the level of terpenes production (Table 1). Thus, dihydrophaseic acid 4-O-β-D-glucoside [#**9**, *m/z* 444.1989, at 8.88 min ($C_{21}H_{32}O_{10}$)]; teucrein [#**23**, *m/z* 268.1305, at 17.52 min ($C_{14}H_{20}O_5$)] or it could be formononetin, with the formula $C_{16}H_{12}O_4$ that is a methoxyisoflavone with this same molecular weight. Both compounds were over produced with *T. velutinum* (CT028) (Table 2). Ethyl 7-epi-12-hydroxyjasmonate glucoside [#**17**, *m/z* 416.2043, at 14.78 min ($C_{20}H_{32}O_9$)] was produced in higher amount in presence of the fungi (CT028, CR and RT028) (Table 2). Akebosideste [#**32**, *m/z* 896.5127, at 24.97 min ($C_{47}H_{76}O_{16}$)] was produced in lower amounts when any of both fungi were present (CT028, CR and RT028) (Table 2).

Table 2: Compounds detected that were produced at significantly among different levels respect to the control plants when there were present or absence *Trichoderma* and/or *R. solani*.

Id	Formula	Tentative assignment	CT028-CC	CR-CC	RT028-CC
		Amino acid			
8	$C_{11}H_{12}N_2O_2$	L-Tryptophan	Up	Down	--
15	$C_{13}H_{14}N_2O_3$	N-acetyltryptophan	--	Down	Up
		Carbohydrate			
2	$C_{12}H_{22}O_{11}$	Sucrose	Up	Up	--
		Fatty acids			
35	$C_{18}H_{30}O_2$	γ-Linolenic acid	--	Down	Down

Table 2: Compounds detected that were produced at significantly among different levels respect to the control plants when there were present or absence *Trichoderma* and/or *R. solani* (continuation).

Id	Formula	Tentative assignment	CT028-CC	CR-CC	RT028-CC
		Flavonoids			
1	$C_{17}H_{20}O_{11}$	5-Hydroxy-6,8-dimethoxy-2-oxo-2H-chromen-7-yl β-D-glucopyranoside	Down	Down	Down
3	$C_{27}H_{32}O_{14}$	Naringin	Up	--	--
6	$C_{20}H_{20}O_4$	Phaseollinisoflavan	--	--	Down
10	$C_{27}H_{32}O_{16}$	3,4´,5,7-Tetrahydroxyflavanone 3,7-Di-O-β-D-glucopyranoside	Up	--	Up
12	$C_{26}H_{28}O_{16}$	Quercetin 3-vicianoside	--	--	Up
14	$C_{26}H_{32}O_{13}$	(Z)-Resveratrol 3,4'-diglucoside	--	--	Up
16	$C_{30}H_{26}O_{16}$	Quercetagetin 7-(6"-(E)-caffeoylglucoside)	--	Down	--
18	$C_{29}H_{34}O_{17}$	Isopyrenin 7-O-glucoside	Down	Down	Down
19	$C_{25}H_{32}O_{13}$	12-Hydroxy, O-[3,4,5-trihydroxybenzoyl-(06)-β-D-glucopyranoside]	Down	Down	Down
20	$C_{32}H_{39}O_{16}$	Luteone 4,7-O-diglucoside	Up	Down	Up
21	$C_{29}H_{50}O$	Sitosterol	--	--	Up
22	$C_{19}H_{18}O_8$	3´,5-Dihydroxy-3,4´,6,7-tetramethoxyflavone	Down	Down	Down
24	$C_{20}H_{38}O_{12}$	(R)-1-O-[β-D-Glucopyranosyl-(1-6)-β-D-glucopyranoside]-1,3-octanediol	Down	--	Down
25	$C_{25}H_{28}O_6$	2´,4´,5,7-Tetrahydroxy-3´,8-diprenylisoflavanone	Down	--	Down
26	$C_{20}H_{38}O_{11}$	n-Octyl-β-D-maltopyranoside	Down	--	--
27	$C_{19}H_{18}O_9$	5,2',4'-Trihydroxy-3,7,8,5'-tetramethoxyflavone	Down	Down	Down
28	$C_{21}H_{22}O_4$ $C_{20}H_{18}O_5$	2´-O-Methylphaseollinisoflavan Wighteone	Down	--	Down
		Glycoside			
4	$C_{12}H_{18}O_{11}$	L-Ascorbic acid-2-glucoside	--	Up	--
		Lipid			
30	$C_{16}H_{30}O_7$	3-O-α-L-rhamnopyranosyl-3-hydroxydecanoic acid	Down	--	Down
34	$C_{18}H_{30}O_3$	13(S)-HOTrE	--	Down	--
36	$C_{31}H_{63}O_7P$	PA(O-16:0/12:0)	--	Down	Down
		Peptide			
7	$C_{11}H_{22}N_2O_3$	Valyl-Leucine	Up	Down	Up
11	$C_{17}H_{32}N_4O_5$	Ile Gln Ile	--	Down	Down
29	$C_{15}H_{26}N_6O_7$	Gln Gln Gln	Down	--	Down
		Phenol			
5	$C_{14}H_{20}O_8$ $C_{16}H_{12}O_7$	Hydroxytyrosol 1-O-glucoside Isorhamnetin	Down	--	--
13	$C_{17}H_{22}O_{10}$	1-O-Sinapoylglucose	Up	--	Up
31	$C_{19}H_{20}O_9$	Garcimangosone D	Down	Down	Down
33	$C_{38}H_{44}O_8$	Gambogic acid	Down	--	Down

Table 2: Compounds detected that were produced at significantly among different levels respect to the control plants when there were present or absence *Trichoderma* and/or *R. solani* (continuation).

Id	Formula	Tentative assignment	CT028-CC	CR-CC	RT028-CC
		Terpene			
9	$C_{21}H_{32}O_{10}$	Dihydrophaseic acid 4-O-β-D-glucoside	Up	--	--
17	$C_{20}H_{32}O_9$	Ethyl 7-epi-12-hydroxyjasmonate glucoside	Up	Up	Up
23	$C_{14}H_{20}O_5$	Teucrein	Up	--	--
	$C_{16}H_{12}O_4$	Formononetin			
32	$C_{47}H_{76}O_{16}$	Akeboside Ste	Down	Down	Down

3.3. Comparison of metabolites whose production in induced in response to presence or absence of *Trichoderma* and/or *R. solani*

After the statistical analysis, several compounds were produced at significantly different levels compared with the control plants (Table 2). In the interaction with *T. velutinum* (CT028), twenty-five compounds were produced at significantly different levels respect to control plants, among them ten were increased in the presence of this fungus, and fifteen decreased their amount (Figure 3).

In the interaction with *R. solani* (CR), nineteen compounds, increased in their production level and sixteen compounds were decreased respect to control plants (Figure 3).

When bean plants were in contact with *T. velutinum* and *R. solani*, (RT028), twenty-six compounds were produced at significantly different levels respect to control plants, among them nine compounds were increased and seventeen were decreased (Figure 3).

Analysing the interaction of *R. solani* (Table 2), there were two compounds, a flavonoid (#**20**) and a peptide (#**7**), that were deceased in their level of production (CR), but when *T. velutinum* was present (CT028 and RT028), their amounts were increased. In the case of RT028, the presence of *Trichoderma* abolished the effect of the pathogen in the bean plants. However, a contrasting result was observed with other three compounds, a fatty acid (#**35**), a lipid (#**36**), and a peptide (#**11**), whose production decreased in the presence of *R. solani*, but the presence of *T. velutinum* did not counteract this effect. It was also a carbohydrate (#**2**) increased in presence of both fungi.

In the interaction with *T. velutinum* (Table 2) there were six compounds, three flavonoids (#**24**, #**25**, #**28**), a lipid (#**30**), a peptide (#**29**) and a phenol (#**33**) whose production was reduced and two compounds, a flavonoid (#**10**) and a phenol (#**13**) that were increased. *R. solani* did not cause any effect in the level of production of these compounds.

Analysing the interaction between bean plants – *T. velutinum* – *R. solani* (Table 2), seven compounds, five flavonoids (#**1**, #**18**, #**19**, #**22**, #**27**), a phenol (#**31**) and a terpene (#**32**) were reduced in their level of production respect to control plants and only one compound (Figure 3), a terpene, (#**17**) was increased in the three conditions (CT028, CR, RT028). This response could be an effect of the presence of the fungus independently if it was a pathogen or a biocontrol agent. Thus, the plant responds against invader organism using in some extend similar strategies.

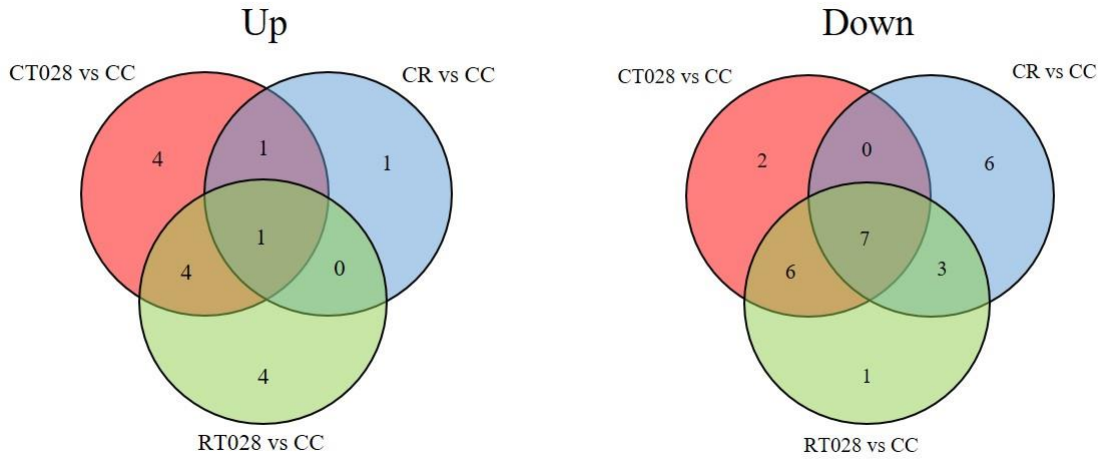


Figure 3: Venn´s diagram with the compounds with an Up-production and Down-production. See Table 2 for identification compounds.

3.4. Principal component analysis (PCA) of the compounds contents

HPLC separations of bean leaves extracts revealed differences in the accumulation pattern of compounds according to the interaction performed *in vitro* (Figure 1). Differences in compound accumulation among treatments were analyzed by principal component analysis (PCA). The compounds for each sample were grouped in a region of the graph (Figure 4). In the left top, there are grouped the control samples (CC), in the left bottom the samples from plants inoculated with *R. solani* (CR). Similarly, in the right side of the graph there are grouped the samples from plants inoculated with *Trichoderma*, being the top those corresponding to plants without pathogen (CT028) and in the bottom those infected with *R. solani* (RT028).

The MS data were subjected to PCA (Figure 4) aiming at exploring similarities or differences in metabolite profiles between the treatments in the bean plants with T. *velutinum* and *R. solani*. A four-component model, the first two components were explained accounting for 73.96 % of the variance. In the figure 2, the component 1 and 2 was able to separate the samples into four groups, when the two groups of the right were inoculated with *T. velutinum* and those of the left were without the biocontrol agent. In addition, it was possible to divide the bean plant inoculated with the pathogen, when the plants without R. solani were in the top of the graph and those with the pathogen were the bottom.

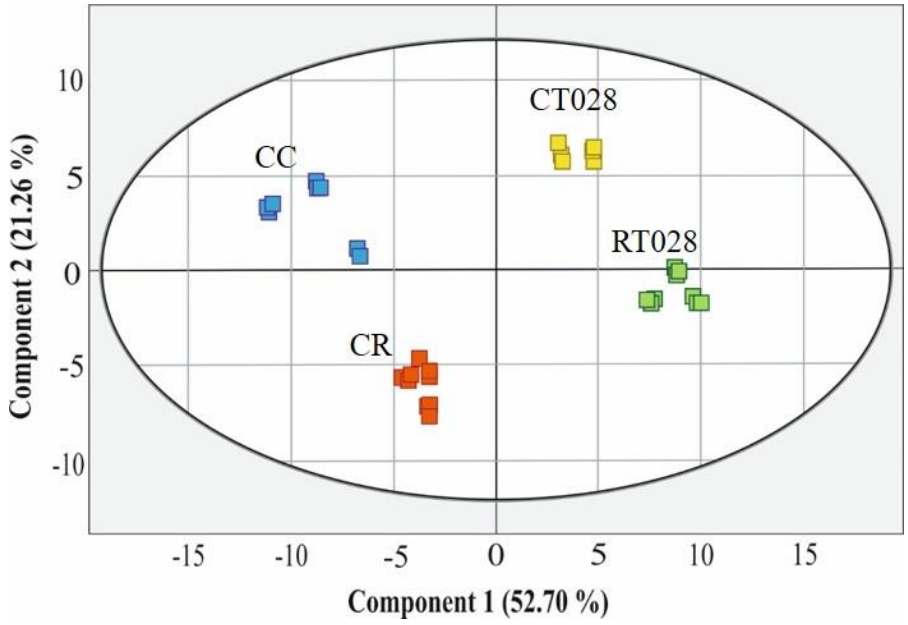


Figure 4: Principal components analysis of 36 compounds extracted by bean leaves subjected to different treatments. (Blue) control beans without pathogen and biocontrol agent (CC); (Red) beans that were infected with *R. solani* (CR); (Yellow) beans inoculated with *T. velutinum* (CT028); (Green) beans inoculated with *R. solani* and *T. velutinum* (RT028).

4. DISCUSSION

T. velutinum T028 isolate was the selected, based on its positive effect on bean growth. Thus, plants inoculated with this strain showed a significant increase in dry weight of both aerial parts and root system, including when *R. solani* was present in the substrate. Also, T028 and *R. solani* R43 isolates have been shown to be affect the expression of genes involved in plant defence. These genes showed significant homology with plant defence related genes and they are expressed in bean leaves of plants treated with *T. velutinum* and/or infected with *R. solani* (Mayo et al., 2016).

The plants-phytopathogen interaction is complex and would be very specific to a given partners combination. The defence strategies of plants against their pathogens are diverse and include the production of antifungal compounds. In this work, we are deseing an experimental strategy to know the changes in the metabolic profiles of beans plants when inoculated with *T. velutinum*, in the presence or absence of *R. solani*, during forty-five days of growth. In order to do that a metabolomics analysis of aqueous methanol extracts was applied when bean plants were in contact with these organisms, respect to control plants. This analysis had enabled the comparison of samples for the relative abundance of several metabolites, including flavonoids, phenols, terpenes, amino acids, carbohydrates, fatty acids, lipids, glycosides and peptides.

Phytoalexins are a heterogeneous group of compounds that show biological activity against a variety of pathogens and are considered as molecular markers of disease resistance (Ahuja et al., 2012) . They are synthesized in response to pathogen attack and they would not be present in healthy plants (Morrissey and Osbourn, 1999). Thus, one way to improve the plant resistance to disease would be to stimulate plants to produce their own phytoalexins, which might be carried out by inoculation with a non-pathogenic microorganism, as *Trichoderma*, or a pathogen hipovirulent strain (Grayer and Kokubun, 2001). This would cause a systemic resistance: after any subsequent

infection with a pathogen the plant responds rapidly to accumulate phytoalexins at the site of infection and produces compounds to stop the growth of the pathogen (Kuc, 1991). It had been found on Solanaceae, Leguminosae and Gramineae that the biotic and abiotic factors can induce the generation of phytoalexins (Hammerschmidt and Becker, 1997).

Many classes of polyphenolic metabolites have an antibiotic activity during plant interactions with phytopathogens and they play the roles of phytoalexins in plant tissues (VanEtten et al., 1994). There are several groups in the polyphenols, as phenols, phenolic acids and flavonoids (Abu-Reidah et al., 2013). Flavonoids, protect plants against various biotic and abiotic stresses and exhibit a diverse spectrum of biological functions, playing an important role in the interaction between the plant and their environment (Pourcel et al., 2007). The majority of flavonoids exist naturally as glycosides and the presence of sugars and hydroxyl groups make them water soluble whereas methyl groups and isopentyl units make flavonoids lipophilic (Samanta et al., 2011). In this work, there are more flavonoides produced in lower amounts when plants were grown in the presence of *T. velutinum*, as 3′,5-dihydroxy-3,4′,6,7-tetramethoxyflavone, 5,2',4'-trihydroxy-3,7,8,5'-tetramethoxyflavone, n-octyl-β-D-maltopyranoside, (R)-1-O-[β-D-glucopyranosyl-(1-6)-β-D-glucopyranoside]-1,3-octanediol, 5-hydroxy-6,8-dimethoxy-2-oxo-2H-chromen-7-yl-β-D-glucopyranoside, isopyrenin 7-O-glucoside, 12-hydroxy, O-[3,4,5-trihydroxybenzoyl-(06)-β-D-glucopyranoside], and 2′,4′,5,7-tetrahydroxy-3′,8-diprenylisoflavanone. However, naringin (flavonone) and 3,4′,5,7-tetrahydroxyflavanone 3,7-Di-O-β-D-glucopyranoside and luteone 4,7-O-diglucoside (flavonoid glyconjugates), were increased in presence of *T. velutinum*. When *R. solani* was present, normally, a minor production of this type of compounds was detected respect to control plants, as quercetagetin 7-[6"-(E)-caffeoylglucoside] and luteone 4,7-O-diglucoside (flavonoid glycoconjugates).

It is unclear whether the accumulation of different but structurally related phytoalexins in a plant is important for the resistance against pathogen, e.g. phaseollin, phaseollidin in beans (Kuc, 1991), but in this work, compounds such as 2′-O-methylphaseollinisoflavan and phaseollinisoflavan, which are phytoalexins derivatives, were produced in lower amount if *T. velutinum* was present. In a previous study in which bean plants were inoculated with *R. solani*, it was found that a considerable amount of phaseollin accumulated in the lesions within 36 hours after inoculation and continued to accumulate for at least 12 days after the lesions (Rizk et al., 1980). In the present case, *T. velutinum* reduced its production in presence de *R. solani* and 45 days after inoculation its production was not significant altered respect control plants.

In a plant's immune system, the plant hormone jasmonic acid and its derivatives have been recognized as key regulators that play crucial roles in plant defense responses to pathogens (Pieterse et al., 2012). There are some studies, based in the use genetically modified *Arabidopsis* plants in which jasmonic acid synthesis or signaling is modulated; consequently, the functions of jasmonic acid derivatives in plant resistance to both biotrophic and necrotrophic pathogens have been demonstrated (Okada et al., 2015). In the case of terpenes, ethyl 7-epi-12-hydroxyjasmonate glucoside was produced at a significantly higher level respect to control plants in the presence of this fungus *T. velutinum* and/or *R. solani*. The importance of this compound is remarkable, since it is a product of jasmonate acid that is synthesized when plants detected a microorganism. Then, they activate the signalling cascades dependent of this compound. In the case of potato, this compound was also detected when potato plants were in contact with the pathogen *Phytophthora infestans* (Yogendra et al., 2015). Similarly, in the case of 12-hydroxy-O-[3,4,5-trihydroxybenzoyl-(06)-β-D-glucopyranoside], a steroisomer of jasmonic acid, and akeboside ste which was produced at a

lower level in the presence of both fungi respect to control plants, bean plant could contemplate to *T. velutinum* and *R. solani* as a same type of organism causing the same response in the plant.

γ-Linolenic acid is the main precursor of the jasmonates (Heitz et al., 2016), whose production decreased in the presence of *R. solani*, compared to control plants. Similarly, 13(S)-HOTrE, a derivative of γ-Linolenic acid, was also produced at lower levels in the presence of the pathogen. This respond is possible because these compounds, depend of the jasmonate or theirs precursors, are responsible of the production of signalling cascade by respond to a non-pathogenic microorganism (Druzhinina et al., 2011). However, akeboside ste, was produced in a lower amount when both fungi were present. Bean plant could perceive *T. velutinum* and *R. solani* as a same kind of organism causing the same response in the plant.

In the case of the amino acids, there is a different response if the pathogen and/or the biocontrol agent were present in the soil. In the presence of *T. velutinum* (CT028), L-Tryptophan increase its amount, but if when *R. solani* (CR) was present the levels of this compound were reduced, compared with the control plants. This response was also observed for N-acetyltryptophan. However, in the presence of both fungi (RT028) the production was increased. This result can be explained because L-tryptophan is a physiological precursor of auxins, and it has been found as responsible for biosynthesis of auxins in the rhizosphere. Thus, it stimulates synthesis of auxins and triggers the plant growth. Then, when *T. velutinum* is present in the soil, the concentration of this compound will be increased respect to control plants. In other study, the inoculation in *Vigna mungo* with other biocontrol agents as *Rhizobium* spp. and *Bacillus* spp., also resulted in an increase of 23.36% in the yield when this compound was present (Qureshi et al., 2012).

Antifungal peptides derived from common bean have exerted an inhibitory activity over a number of plant pathogens such as *Mycosphaerella arachidicola*, *Rhizoctonia solani*, *Verticillium dahlia, Setosphaeria turcica* and *Fusarium oxysporum* (Luna-Vital et al., 2015). Thus, it is possible that the effectiveness of the antifungal activity against phytopathogens depends on the fungi and the cultivar, which are interacting. Several antifungal activities of common bean peptides have been previously described: induction of morphological changes in hyphae, membrane cell disruption, membrane permeabilization and induction of chitin accumulation at hyphal tips (Luna-Vital et al., 2015). In the present work, when the pathogen *Rhizoctonia* (CR) was present, the peptides Valyl-Leucine and Ile Gln Ile were producer in a lower amount respect to control plants. When only *T. velutinum* (CT028) the peptide Valyl-Leucine was produced in greater amount respect to control plant. When *T. velutinum* and *R. solani* (RT028) were both present, all the detected peptides, except Valyl-Leucine, were produced at a lower level respect to control plants. These results were possible because these peptides have a defence role in the plant, and those increased in the presence of *Trichoderma* could have an important function in the metabolic antifungal activity of the plant.

5. CONCLUSIONS

The presence of *T. velutinum* and/or *R. solani* modified the bean metabolome. They cause the activation of defence mechanisms, which is reflected in the production of polyphenols, terpenes and other compounds. *Trichoderma* and *Rhizoctonia* caused an increase in the production of a terpene and a lower production of five flavonoids, a phenol and a terpene. When *T. velutinum* is present two amino acids, six flavonoids, a peptide, a phenol and two terpenes increased their level of production and a fatty acid, five flavonoids, two lipids, two peptides and two phenols were produced in lower amounts. When *R. solani* was present, two amino acids, a fatty acid, two

flavonoids and two lipids were produced in minor amount and a carbohydrate and a glycoside were produced in major amount respect to control plant.

6. ACKNOWLEDGMENTS

The grant awarded to Sara Mayo Prieto (FPU12/00677) by the Ministry of Education, Culture and Sport (Spain) according the Resolution of April 25, 2012 (BOE of 10 May), as amended by Resolution of May 18, 2012 (BOE of 22 May), and by Resolution of October 31, 2012 (BOE of 12 November), granted in the Resolution of December 20, 2012 and the Resolution of Mobility for Short Term of December 9, 2014 (BOE of 22 December). Ministry of Economy and Competitiveness for National project "Farnesol as self-regulatory molecule in *Trichoderma*. Tyrosol and farnesol signaling in *Trichoderma*-bean interaction" (AGL2012-40041-C02-02). Junta de Castilla y León, Consejería de Educación for the project "Effect of terpenes and physiologically related compounds produced by *Trichoderma parareesei* in the development of common bean (*Phaseolus vulgaris* L.) and in defensive responses of this plant" (LE228U14).

7. REFERENCE LIST

Abu-Reidah, I. M., Arráez-Román, D., Lozano-Sánchez, J., Segura-Carretero, A., and Fernández-Gutiérrez, A. (2013). Phytochemical characterisation of green beans (*Phaseolus vulgaris* L.) by using high-performance liquid chromatography coupled with time-of-flight mass spectrometry. *Phytochem. Anal.* 24, 105–16. doi:10.1002/pca.2385.

Ahuja, I., Kissen, R., and Bones, A. M. (2012). Phytoalexins in defense against pathogens. *Trends Plant Sci.* 17, 73–90. doi:10.1016/j.tplants.2011.11.002.

Boller, T., and Felix, G. (2009). A Renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi:10.1146/annurev.arplant.57.032905.105346.

Brechenmacher, L., Lei, Z., Libault, M., Findley, S., Sugawara, M., Sadowsky, M. J., et al. (2010). Soybean metabolites regulated in root hairs in response to the symbiotic bacterium *Bradyrhizobium japonicum*. *Plant Physiol.* 153, 1808–1822. doi:10.1104/pp.110.157800.

Buckingham, J., and Munasinghe, V. R. N. (2015). *Dictionary of flavonoids*. , eds. J. Buckingham and V. R. N. Munasinghe Boca Raton, Florida: Taylor & Francis Group.

Chen, X., Qi, X., and Duan, L.-X. (2015). "Overview," in *Plant Metabolomics*, eds. X. Qi, X. Chen, and Y. Wang (Dordrecht: Springer Netherlands), 1–24. doi:10.1007/978-94-017-9291-2_1.

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., et al. (2011). *Trichoderma*: The genomics of opportunistic success. *Nat. Rev. Microbiol.* 9, 749–759.

Grayer, R. J., and Kokubun, T. (2001). Plant–fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants. *Phytochemistry* 56, 253–263. doi:10.1016/S0031-9422(00)00450-7.

Grotewold, E. (2005). Plant metabolic diversity: a regulatory perspective. *Trends Plant Sci.* 10, 57–62. doi:10.1016/j.tplants.2004.12.009.

Guerrero-González, M. L., Rodríguez-Kessler, M., Rodríguez-Guerra, R., González-Chavira, M., Simpson, J., Sanchez, F., et al. (2011). Differential expression of *Phaseolus vulgaris* genes induced during the interaction with *Rhizoctonia solani*. *Plant Cell Rep.* 30, 1465–1473.

doi:10.1007/s00299-011-1055-5.

Hagedorn, D. J. (1991). "Rhizoctonia Root Rot," in *Compendium of Bean Diseases*, ed. R. Hall (APS, American Phyopathological Society), 13.

Hammerschmidt, R., and Becker, J. S. (1997). "Acquired resistance to disease in plants," in *Horticultural Reviews*, ed. J. Janick (New York (USA): John Wiley & Sons), 247–280.

Harrison, M. J., and Dixon, R. A. (1994). Spatial patterns of expression of flavonoid/isoflavonoid pathway genes during interactions between roots of *Medicago truncatula* and the mycorrhizal fungus *Glomus versiforme*. *Plant J.* 6, 9–20. doi:10.1046/j.1365-313X.1994.6010009.x.

Hassan, S., and Mathesius, U. (2012). The role of flavonoids in root-rhizosphere signalling: opportunities and challenges for improving plant-microbe interactions. *J. Exp. Bot.* 63, 3429–44. doi:10.1093/jxb/err430.

Heitz, T., Smirnova, E., Widemann, E., Aubert, Y., Pinot, F., and Ménard, R. (2016). "The rise and fall of jasmonate biological activities," in *Lipids in plant and algae development* (Springer International Publishing), 405–426. doi:10.1007/978-3-319-25979-6_16.

Hermosa, R., Belén Rubio, M., Cardoza, R. E., Nicolás, C., Monte, E., and Gutiérrez, S. (2013). The contribution of *Trichoderma* to balancing the costs of plant growth and defense. *Int. Microbiol.* 16, 69–80.

Kanehisa Laboratories (2016). KEGG PATHWAY: alpha-Linolenic acid metabolism - *Phaseolus vulgaris* (common bean). 592. Available at: http://www.kegg.jp/kegg-bin/show_pathway?org_name=pvu&mapno=00592&mapscale=&show_description=hide.

Kuc, J. (1991). Phytoalexins, stress metabolism, and disease resistance in plants. *Annu. Rev. Phytopathol.*, 275–297. doi:10.1146/annurev.py.33.090195.001423.

Lu, Y., Lam, H., Pi, E., Zhan, Q., Tsai, S., Wang, C., et al. (2013). Comparative metabolomics in *Glycine max* and *Glycine soja* under salt stress to reveal the phenotypes of their offspring. *J. Agric. Food Chem.* 61, 8711–21. doi:10.1021/jf402043m.

Luna-Vital, D. A., Mojica, L., González de Mejía, E., Mendoza, S., and Loarca-Piña, G. (2015). Biological potential of protein hydrolysates and peptides from common bean (*Phaseolus vulgaris* L.): A review. *Food Res. Int.* 76, 39–50. doi:10.1016/j.foodres.2014.11.024.

Mayo, S., Cominelli, E., Sparvoli, F., González-López, O., Rodríguez-González, A., Gutiérrez, S., et al. (2016). Development of a qPCR strategy to select bean genes involved in plant defense response and regulated by the *Trichoderma velutinum – Rhizoctonia solani* interaction. *Front. Plant Sci.* 7, 1109. doi:10.3389/fpls.2016.01109.

Mayo, S., Gutierrez, S., Malmierca, M. G., Lorenzana, A., Campelo, M. P., Hermosa, R., et al. (2015). Influence of *Rhizoctonia solani* and *Trichoderma* spp. in growth of bean (*Phaseolus vulgaris* L.) and in the induction of plant defense-related genes. *Front. Plant Sci.* 6, 685. doi:10.3389/fpls.2015.00685.

Morrissey, J. P., and Osbourn, A. E. (1999). Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiol. Mol. Biol. Rev.* 63, 708–724.

Okada, K., Abe, H., and Arimura, G. -i. (2015). Jasmonates induce both defense responses and communication in monocotyledonous and dicotyledonous plants. *Plant Cell Physiol.* 56, 16–27. doi:10.1093/pcp/pcu158.

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521. doi:10.1146/annurev-cellbio-092910-154055.

Pourcel, L., Routaboul, J., Cheynier, V., Lepiniec, L., and Debeaujon, I. (2007). Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends Plant Sci.* 12, 29–36. doi:10.1016/j.tplants.2006.11.006.

Pushpa, D., Yogendra, K. N., Gunnaiah, R., Kushalappa, A. C., and Murphy, A. (2014). Identification of late blight resistance-related metabolites and genes in potato through nontargeted metabolomics. *Plant Mol. Biol. Report.* 32, 584–595. doi:10.1007/s11105-013-0665-1.

Qureshi, M. A., Iqbal, A., Akhtar, N., Shakir, M. A., and Khan, A. (2012). Co-inoculation of phosphate solubilizing bacteria and rhizobia in the presence of L-tryptophan for the promotion of mash bean (*Vigna mungo* L.). *Soil Environ.* 31, 47–54.

Rizk, A.-F., Wood, G. E., and Salunkhe, D. K. (1980). Phytoalexins of leguminous plants. *C R C Crit. Rev. Food Sci. Nutr.* 13, 245–295. doi:10.1080/10408398009527291.

Samanta, A., Das, G., and Das, S. (2011). Roles of flavonoids in plants. *J. Pharm. Pharm. Sci.* 6, 12–35.

Shoresh, M., Harman, G. E., and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 48, 21–43. doi:10.1146/annurev-phyto-073009-114450.

Southon, I. W., Bisby, F. A., Buckingham, J., Harborne, J. B., and Zarucchi, J. L. (1994). *Phytochemical dictionary of the Leguminosae*. First edit. , eds. F. A. Bisby, J. Buckingham, and J. B. Harbome Springer-Science + Business Media, B.V. doi:10.1007/978-1-4899-3047-7.

Taiz, L., Zeiger, E., Møller, I. M., and Murphy, A. S. (2015). *Plant physiology and development*. Sixth. , eds. L. Taiz, I. M. Møller, A. Murphy, and E. Zeiger Sinauer Associates, Incorporated.

Talhaoui, N., Gómez-Caravaca, A. M., Roldán, C., León, L., De la Rosa, R., Fernández-Gutiérrez, A., et al. (2015). Chemometric analysis for the evaluation of phenolic patterns in olive leaves from six cultivars at different growth stages. *J. Agric. Food Chem.* 63, 1722–1729. doi:10.1021/jf5058205.

VanEtten, H. D., Mansfield, J. W., Bailey, J. A., and Farmer, E. E. (1994). Two Classes of Plant Antibiotics: Phytoalexins versus "Phytoanticipins." *Plant Cell* 6, 1191–1192. doi:10.1105/tpc.6.9.1191.

Veitch, N. C. (2009). Isoflavonoids of the Leguminosae. *Nat. Prod. Rep.* 26, 776. doi:10.1039/b616809b.

Wink, M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* 64, 3–19. doi:10.1016/S0031-9422(03)00300-5.

Wojakowska, A., Kułak, K., Jasiński, M., Kachlicki, P., Stawiński, S., and Stobiecki, M. (2015). Metabolic response of narrow leaf lupine (*Lupinus angustifolius*) plants to elicitation and infection with *Colletotrichum lupini* under field conditions. *Acta Physiol. Plant.* 37, 152. doi:10.1007/s11738-015-1896-6.

Wojakowska, A., Muth, D., Narożna, D., Mądrzak, C., Stobiecki, M., and Kachlicki, P. (2013). Changes of phenolic secondary metabolite profiles in the reaction of narrow leaf lupin (*Lupinus angustifolius*) plants to infections with *Colletotrichum lupini* fungus or treatment with its toxin. *Metabolomics* 9, 575–589. doi:10.1007/s11306-012-0475-8.

Yannai, S. (2012). *Dictionay of food compounds with CD-ROM*. Second. , ed. S. Yannai Boca Raton, Florida: CRC Press Taylor & Francis Group.

Yogendra, K. N., Kushalappa, A. C., Sarmiento, F., Rodriguez, E., and Mosquera, T. (2015). Metabolomics deciphers quantitative resistance mechanisms in diploid potato clones against late blight. *Funct. Plant Biol.* 42, 284–298. doi:10.1071/FP14177.

RESULTADOS Y DISCUSIÓN

ÍNDICE RESULTADOS Y DISCUSIÓN

TABLAS

FIGURAS

RESULTADOS Y DISCUSIÓN GENERALES

1. AISLAMIENTOS DE HONGOS EMPLEADOS

Todos los ensayos realizados en el desarrollo de esta tesis se han llevado a cabo con aislamientos obtenidos de muestras vegetales de alubia y de muestras de suelo tomadas de parcelas en las que dicho cultivo formaba parte de la rotación de la parcela. En total se ha trabajado con un aislamiento de *R. solani,* R43, y con 60 aislamientos de *Trichoderma* (Tabla 1 y Anexos Tabla A.1).

Tabla 1: Aislamientos y fuente de aislamiento de *Trichoderma* spp. empleados en los ensayos según la zona de producción de la IGP "Alubia La Bañeza-León".

Especie	Aislamiento	Especie	Aislamiento
Maragatería - Cepeda			
Semilla		**Suelo**	
T. longibrachiatum	T005	*T. velutinum*	T027; T028; T029
T. harzianum	T007; T012	*T. viridescens*	T030
T. atroviride	T013	*T. koningiopsis*	T031
T. harzianum	T019		
Esla - Campos			
Semilla		**Suelo**	
T. citrinoviride	T008	*T. harzianum*	T044; T045
T. harzianum	T022	*T. spirale*	T048
Trichoderma sp.	T023	*T. virens*	T043; T046; T047
La Bañeza			
Semilla		**Suelo**	
T. gamsii	T004	*T. harzianum*	T033; T034; T035
T. harzianum	T020	*T. velutinum*	T036; T037
Trichoderma sp.	T006	*T. virens*	T032
Páramo			
Semilla		**Suelo**	
T. harzianum	T001; T002; T003; T010; T011; T015; T016; T018; T021	*T. brevicompactum*	T054
T. koningiopsis	T009	*T. gamsii*	T057
T. virens	T014	*T. hamatum*	T056
Trichoderma sp.	T017	*T. harzianum*	T050; T052; T055; T058; T059; T060; T061
		T. velutinum	T051
		T. virens	T049; T053
La Cabrera		**Laboratorio**	
Suelo		*T. atroviride*	T024
T. gamsii	T042	*T. virens*	T025
T. koningiopsis	T040		
T. virens	T039		
T. viridescens	T038; T041		

De los aislamientos muestreados, tras su identificación mediante la amplificación por PCR de las regiones adyacentes al gen que codifica el rRNA 5,8S con los cebadores ITS1, ITS4 e ITS5 , se han obtenido 12 especies diferentes distribuidas en las siguientes comarcas de la I.G.P. Alubia de la Bañeza-León: Maragatería-Cepeda, Esla-Campos, La Bañeza, La Cabrera y el Páramo (Tabla 1 y Figura 1).

Se ha observado que la especie más abundante ha sido *T. harzianum*, que estaba presente en todas las comarcas, excepto en la Cabrera, obteniéndose 26 aislamientos (Tabla 1 y Figura 1). El Páramo ha sido donde se han obtenido el mayor número de aislamientos de dicha especie. *T. virens* ha sido la segunda especie más abundante, siendo 8 aislamientos en las comarcas de Esla-Campos, la Bañeza, La Cabrera y el Páramo (Tabla 1 y Figura 1). Ambas especies son las más empleadas en el control biológico de fitopatógenos como *Uromyces appendiculatus*, (Burmeister y Hau, 2009), *R. solani, Fusarium oxysporum* o *Verticillium dahliae, Pythium* spp. (Hanson y Howell, 2004; Larkin, 2016; Vinale et al., 2009).

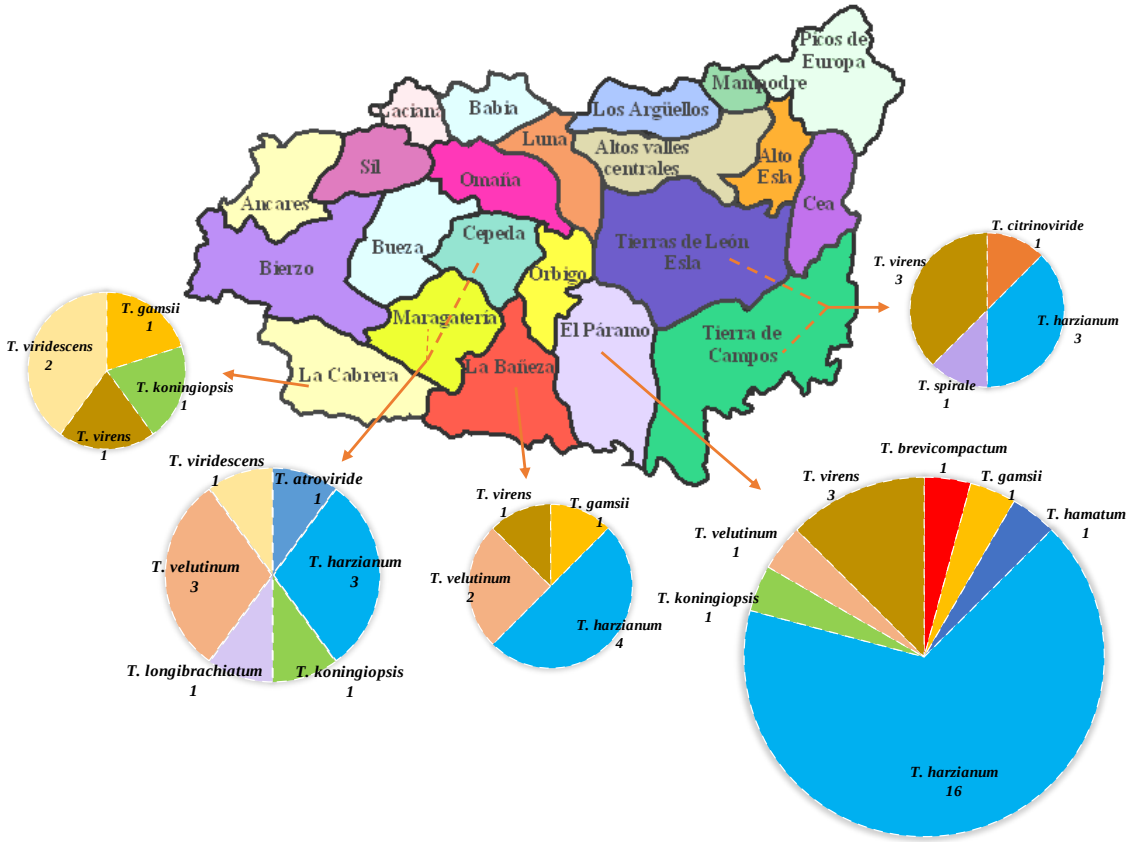


Figura 1: Número de aislamientos de *Trichoderma* según su especie y comarcas agrícolas pertenecientes a la I.G.P. Alubia la Bañeza-León donde se ha realizado el muestreo.

T. atroviride sólo ha sido obtenido en la zona productiva de la Maragatería-Cepeda, mientras que el único aislamiento de *T. citrinoviride* ha sido aislado en la zona de Esla-Campos. *T. gamsii* ha sido extraído en La Bañeza, La Cabrera y el Páramo. *T. hamatum* y *T. brevicompactum* han sido solamente aislados en el Páramo y *T. koningiopsis* lo ha sido de la zona de la Maragatería-Cepeda. *T. spirale* ha sido obtenido en la zona de Esla-Campos y *T. velutinum* en Maragatería-Cepeda y el Páramo. *T. viridescens* ha sido aislado en la Maragatería-Cepeda, La Bañeza y La Cabrera (Tabla 1 y Figura 1).

Aunque las especies de *T. harzianum* y *T. virens* han sido las más empleadas en el control de fitopatógenos, también las especies *T. atroviride, T. longibragiatum, T. koningiopsis, T. spirale, T. hamatum* se han estudiado en el control de enfermedades (Chet et al., 1981; Daryaei et al., 2016; Durak, 2016; Hermosa et al., 2001; Marra et al., 2006) y están comenzando a emplearse como agentes de biocontrol eficaces.

2. ENSAYOS *IN VITRO*

Tras la obtención de los aislamientos de *Trichoderma*, se han realizado ensayos *in vitro* para comprobar su capacidad de inhibición del crecimiento de *R. solani*. Para ello se han llevado a cabo dos ensayos, mediante el uso de membranas y por confrontación directa o cultivo dual (Figura 2).

Observando los resultados estadísticos de ambos ensayos se ha visto que hay diferencias significativas entre los aislamientos. Sin embargo, cuando se ha analizado la fuente de extracción de los aislamientos, no ha tenido diferencias significativas en los ensayos *in vitro* entre los *Trichoderma* obtenidos de suelo y los de semilla. Por lo tanto, los distintos aislamientos se han comportado de igual manera en condiciones *in vitro* independientemente de si han sido aislados de la parte aérea o si lo han sido del suelo.

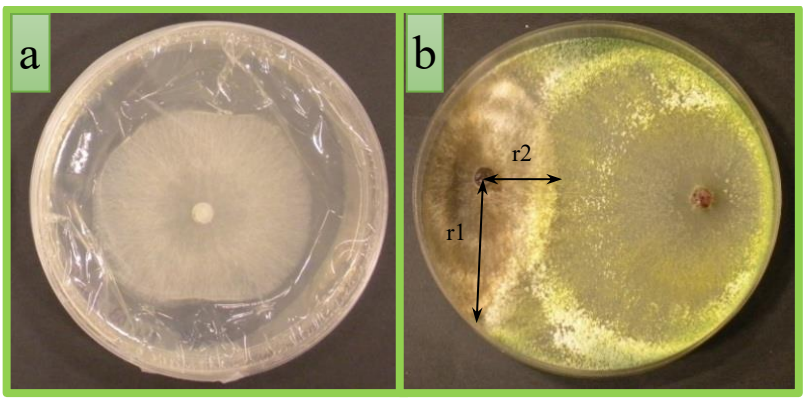


Figura 2: Ensayos *in vitro.* a) Ensayo mediante el empleo de membranas, en el que se observa la membrana en una placa Petri con medio PDA y sobre ella el aislamiento de *Trichoderma* 48 h después de su siembra. b) Ensayo de confrontación directa o cultivo dual en el que aparece a la izquierda *R. solani* y a la derecha un aislamiento de *Trichoderma* separados 5,5 cm. Se ha tomado la distancia entre el punto de siembra del patógeno y el punto más alejado de desarrollo del mismo (r1) y la distancia entre el punto de siembra y el punto más cercano de contacto con *Trichoderma* (r2).

En el caso de los aislamientos de *Trichoderma* obtenidos a partir de semillas, en el primer ensayo para determinar la capacidad antifúngica mediante la producción de metabolitos secundarios empleando membranas (Figura 3 y Figura 5), los aislamientos *T. atroviride*, T013 y T024; *T. gamsii*, T004; *T. harzianum*, T003, T007, T010, T012, T016, T020, y T022; *T. longibrachiatum*, T005; *T. virens*, T025 y el aislamiento T006 han mostrado un porcentaje de inhibición en el desarrollo de *R. solani* superior al 75 %, siendo los aislamientos T003, T004, T006, T020 y T022 los que mayor porcentaje presentaron (86,70 %). *T. harzianum*, T001, T002, T018, T019, T021; *T. citrinoviride* T008 y el aislamiento T023 han mostrado una inhibición entre 40 y 75 %. Por último, los aislamientos *T. harzianum* T011 y T015; *T. virens* T014, T017, y *T. longibrachiatum* T009 han

mostrado un porcentaje inferior al 40 %, siendo T009 el que menor porcentaje ha presentado (15,82%).

En el caso del ensayo de cultivo dual o confrontación directa (Figura 5), el aislamiento *T. harzianum* T021 ha sido el que mayor porcentaje de inhibición ha presentado (72,77 %) y *T. koningiopsis* T009 (14,63 %) el que menor. Los porcentajes de inhibición del resto de aislamientos han oscilado entre 47,13 y 30,55%.

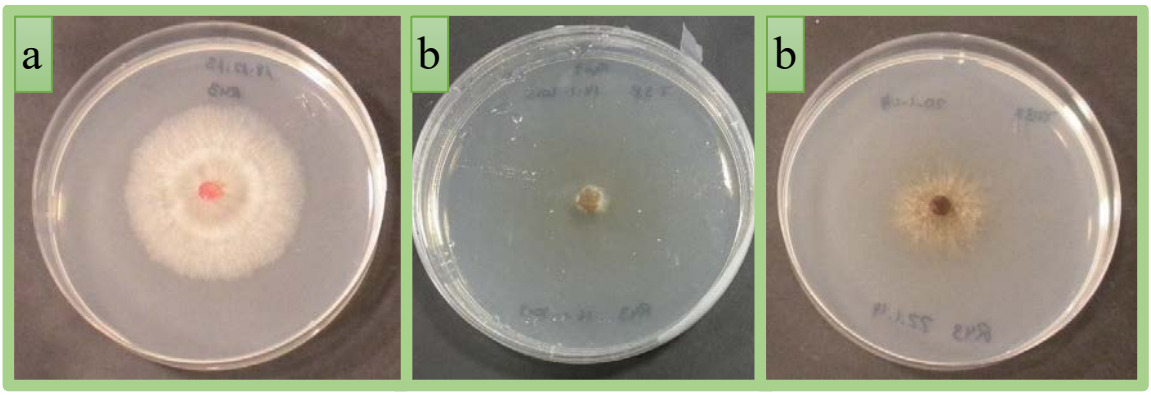


Figura 3: Ensayo *in vitro* mediante el empleo de membranas en placas Petri con medio PDA a los 3 días de desarrollo. a) Control de *R. solani*, b) Placas con *R. solani* tras haber crecido el aislamiento de *Trichoderma.*

En cuanto a los aislamientos obtenidos a partir de muestras de suelo (Figura 6), en el ensayo mediante el empleo de membranas los aislamientos *T. koningiopsis*, T040; *T. virens*, T043, T046, T047, T049, y T053; *T. brevicompactum,* T054; *T. harzianum*, T044, T050 y T055; *T. gamsii*, T042 y T057; *T. viridescens* T030 y T041 y *T. hamatum* T056 han mostrado porcentajes de inhibición entre 86 y 75 %, siendo T040 y T049 los que mayor porcentaje alcanzaron, 86,00 y 85,45% respectivamente. Los aislamientos que han tenido una inhibición entre 75 y 50 % han sido *T. virens* T032; *T. velutinum* T028, T029, T036, T037 y T051; *T. viridescens* T038; *T. harzianum*, T034, T035, T045, T052, T059, T060 y T061 y *T. koningiopsis* T031, siendo T032 con un valor de 73,91 % de inhibición el aislamiento que mayor porcentaje en este rango y T031 con 52,67 % el menor. Los tres aislamientos que han mostrado un porcentaje de inhibición inferior a 20 % han sido *T. velutinum* T027 con 14,57 % y *T. harzianum* T058 y T033 con 9,00 y -5,01 % respectivamente. El signo negativo del último valor indica que este aislamiento ha favorecido el desarrollo de *R. solani* respecto al control.

En el caso del ensayo por confrontación directa (Figura 4 y Figura 6), la mayoría de los aislamientos han presentado un porcentaje de inhibición entre 50 y 40 %. Los aislamientos T034, T035, T058 y T059, todos ellos pertenecientes a *T. harzianum* han tenido valores inferiores a 40 %, llegando hasta el 29,29 % del aislamiento T035.

La mayoría de los aislamientos empleados en los ensayos *in vitro* han obtenido unos porcentajes de inhibición del desarrollo de *R. solani* lo suficientemente elevados como para frenar completamente su desarrollo, bien sea por la producción de metabolitos secundarios con actividad antibiótica, o bien por la confrontación directa con *Trichoderma* (micoparasitismo y competición). Por ejemplo, no todos los aislamientos de la especie *T. harzianum* han sido capaces de impedir el desarrollo de *R. solani*, lo mismo que en el resto de especies. Las diferencias observadas en los diferentes ensayos *in vitro* podrían deberse al genotipo, diferencias de desarrollo, esporulación, interacciones con el medio y los distintos modos de acción en el control del fitopatógeno (Ruano-Rosa et al., 2010).

Existen trabajos en los que se ha evaluado la capacidad inhibidora de *Trichoderma* frente a *R. solani*. En los trabajos utilizando los aislamientos *T. virens* T59 (NBT59) y *T. atroviride* T11 (IMI352941) llevados a cabo por Campelo et al. (2010) el porcentaje de inhibición de este patógeno en los ensayos mediante el empleo de membranas osciló entre el 100 % y el 85,70% respectivamente.

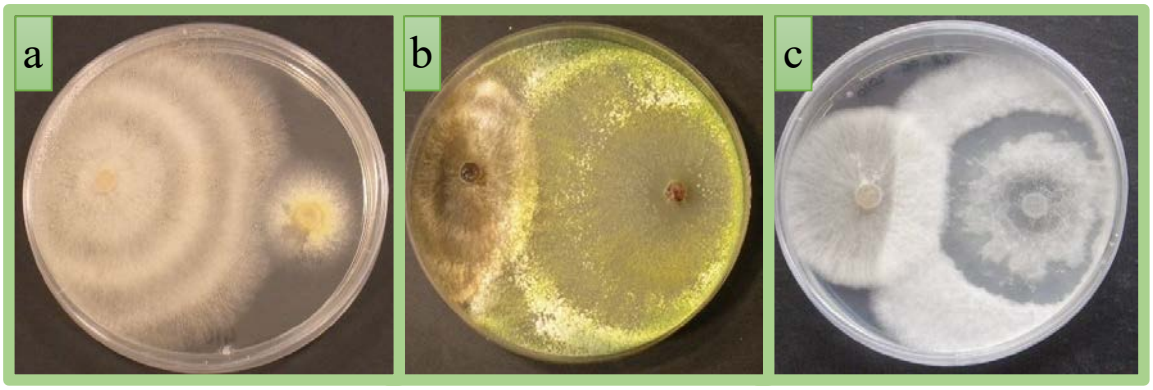


Figura 4: Ensayo *in vitro* de algunos enfrentamientos de distintos aislamientos *Trichoderma* (derecha) y *R. solani*(izquierda) en el ensayo de confrontación directa o cultivo dual en placas Petri con medio PDA a los 5 días de crecimiento.

La actividad antagonista *in vitro* de los aislados de *Trichoderma* podría ser un indicativo de la capacidad biológica *in vivo* frente a hongos fitopatógenos. Sin embargo se ha comprobado que no siempre existe tal correlación entre los resultados en condiciones *in vitro* con los llevado a cabo *in vivo* ya que en este último entran en juego otros factores que pueden interactuar y variar la actividad antibiótica (Anees et al., 2010).

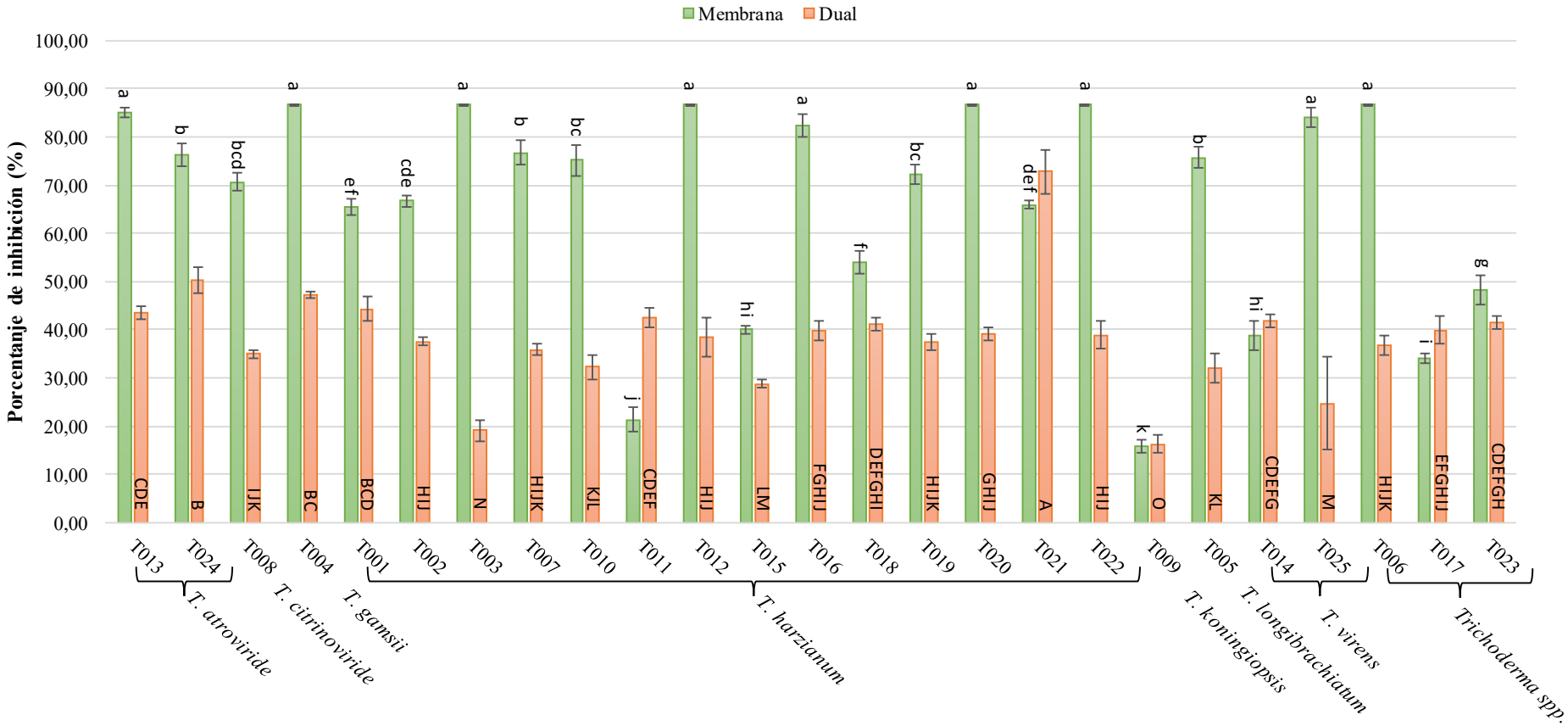


Figura 5: Porcentaje de inhibición del desarrollo de *R. solani* en los ensayos *in vitro* por parte de los aislamientos de *Trichoderma* obtenidos a partir de semilla. En color verde, son los resultados del ensayo mediante membranas y en color naranja son los resultados del ensayo por confrontación directa o dual. Los valores con letras diferentes presentan diferencias significativas (test DMS, $p < 0,05$). Las letras minúsculas corresponden al análisis estadístico del ensayo mediante el uso de membranas y las mayúsculas al ensayo por confrontación directa o cultivo dual.

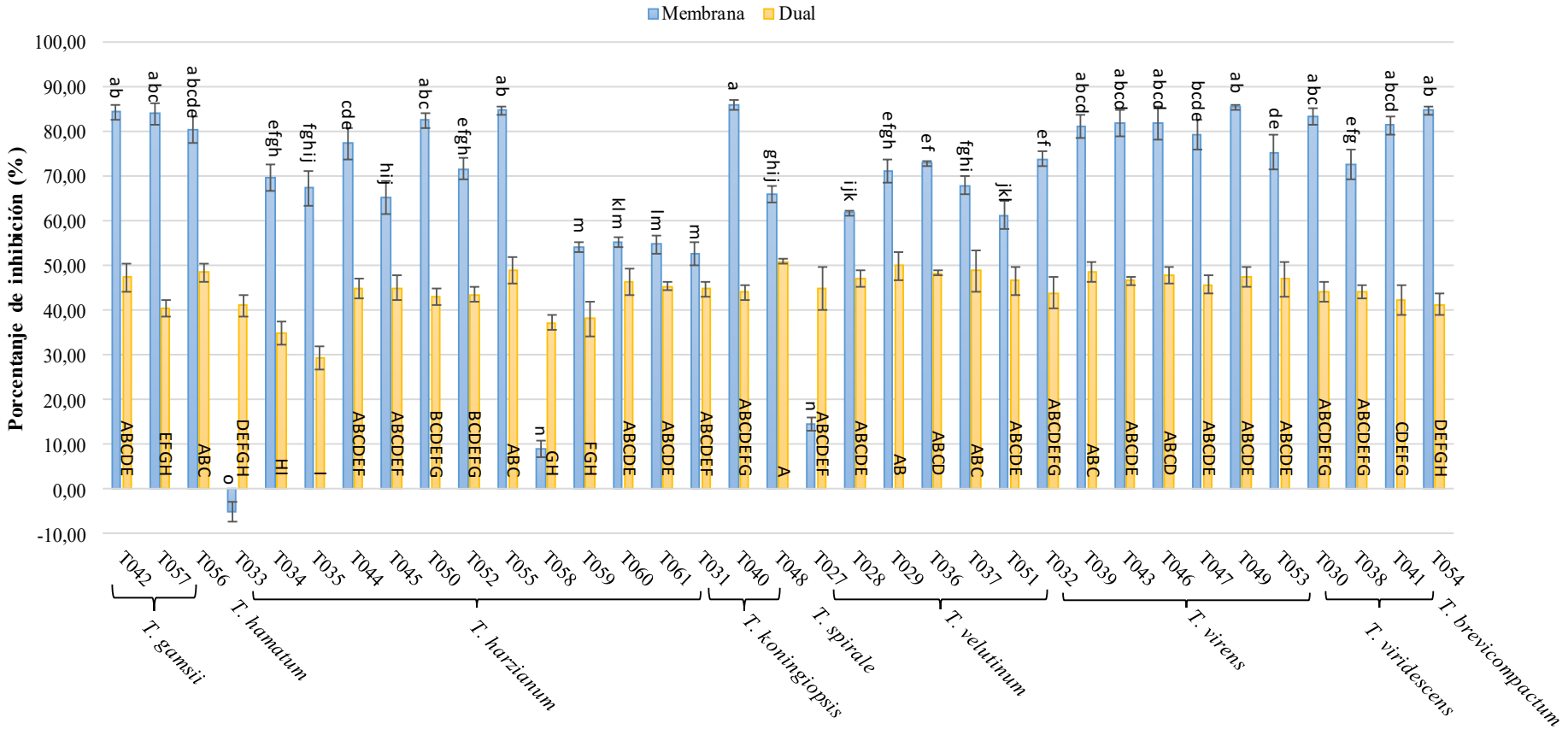


Figura 6: Porcentaje de inhibición del desarrollo de *R. solani* en los ensayos *in vitro* por parte de los aislamientos de *Trichoderma* obtenidos a partir de muestras de suelo. En color azul, son los resultados del ensayo mediante membranas y en color amarillo son los resultados del ensayo por confrontación directa o dual. Los valores con letras diferentes presentan diferencias significativas (test DMS, $p < 0,05$). Las letras minúsculas corresponden al análisis estadístico del ensayo mediante el uso de membranas y las mayúsculas al ensayo por confrontación directa o cultivo dual.

3. ENSAYOS *IN VIVO*

Tras los ensayos *in vitro*, se han probado 45 aislamientos de *Trichoderma* spp. que han tenido un porcentaje de inhibición en ensayo de membranas superior al 40 %, en cultivo dual superior al 30 % y que esporulaban en medio PDA. De esos aislamientos, 15 han sido extraídos de semilla y 30 han sido obtenidos de muestras de suelo.

Para la realización de los ensayos *in vivo* se ha seleccionado, la variedad de alubia "Canela" perteneciente a la IGP "Alubia de la Bañeza-León". Aunque en esta marca de calidad se producen otras variedades locales como "Riñón", "Plancheta" o "Pinta", se ha elegido la variedad "Canela" por su elevada sensibilidad a *R. solani*.

3.1. Evaluación de la germinación

Analizando la germinación según la fuente de extracción de los aislamientos de *Trichoderma*, se ha observado que existen diferencias significativas entre los que proceden de semilla y los que proceden de suelo tanto en la geminación como en el desarrollo de las plantas evaluadas a los 45 días tras la siembra.

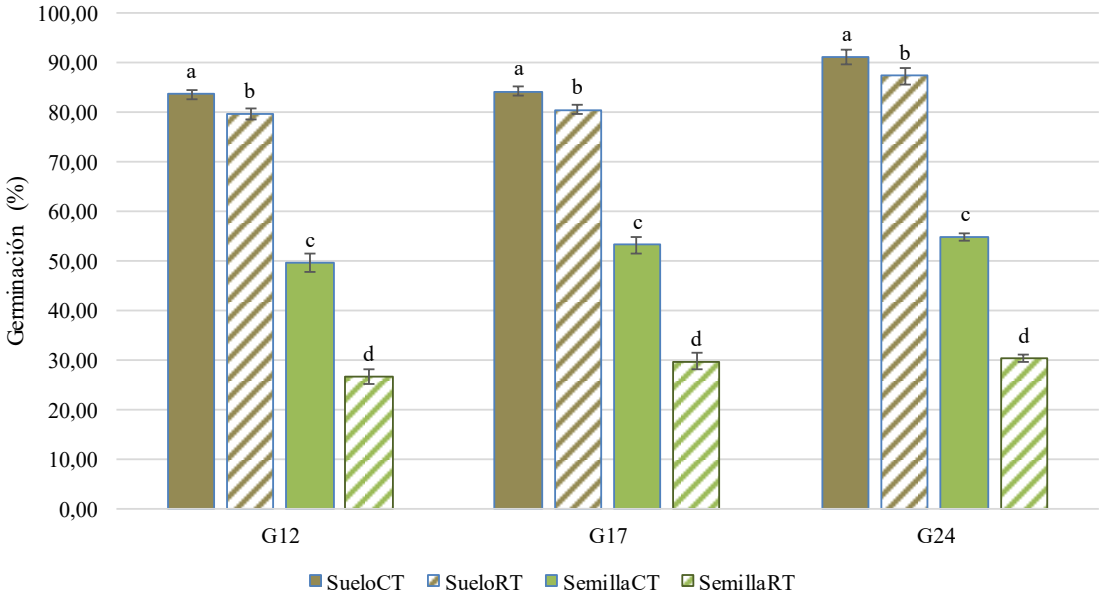


Figura 7: Porcentaje de germinación de las semillas en función de la fuente de extracción de *Trichoderma* spp., realizada a los 12 días (G12), 17 días (G17) y 24 días (G24) tras la siembra. Los valores con letras diferentes presentan diferencias significativas (test DMS, $p < 0,05$). [Plantas inoculadas con los aislamientos de *Trichoderma* de muestras de suelo (SueloCT); plantas inoculadas con los aislamientos de *Trichoderma* de muestras de suelo y con *R. solani* (SueloRT); plantas inoculadas con los aislamientos de *Trichoderma* de muestras de semilla (SemillaCT); plantas inoculadas con los aislamientos de *Trichoderma* de muestras de semilla y con *R. solani* (SemillaRT)].

En el caso de la germinación (Figura 7), se ha comprobado que aquellas alubias que han sido pildoradas con esporas de los aislamientos extraídos de muestras de suelos (SueloCT y SueloRT)

tuvieron un mayor número de semillas por maceta germinadas que aquellas que han sido recubiertas con esporas de *Trichoderma* aislados de semillas (SemillaCT y SemillaRT).

Se ha visto que estando presente el patógeno (RT), ha disminuido la germinación en ambos casos en los que se encuentra *R. solani* en el sustrato (SueloRT y SemillaRT) pero siendo más acusada cuando las semillas han sido pildoradas con *Trichoderma* aislado de semillas.

Posteriormente se ha analizado la germinación a los 24 días, momento en el que se ha considerado que la germinación de todas las semillas ha terminado teniendo en cuenta que la germinación en esta variedad local se produce entre los 7 y 14 días tras la siembra.

Tabla 2: Evaluación de la germinación a los 24 días tras la siembra en función de los distintos tratamientos estudiados.

G24 (%)[(1)]	**Origen[(2)]**	**Tratamientos[(3)]**
100,00	Semilla	--
	Suelo	CT028; CT037; CT038; CT040; CT041 RT036
99,99-90,00	Semilla	--
	Suelo	CT035; CT036; CT056; CT058; CT059; CT061; CT032; CT054; CT039; CT045; CT050; CT053; CT031; RT028; RT041; RT038; RT040; RT061; RT035; RT058; RT037; RT050; RT057; RT059; RT060; RT039;
89,99-80,00	Control	CC
	Semilla	--
	Suelo	CT029; CT030; CT046; CT048; CT060; CT047; CT057; CT044; CT043 RT044; RT048; RT054; RT056; RT030; RT053; RT043; RT029; RT034; RT043; RT046; RT055
79,99-60,00	Control	RC
	Semilla	CT004; CT012; CT019; CT002 RT008
	Suelo	CT033; CT034; CT055 RT047; RT031; RT045; RT032; RT033
59,99-40,00	Semilla	CT008; CT007; CT005; CT013; CT025; CT001; CT003; CT006; CT015; CT024; CT011 RT001; RT013; RT005; RT006
	Suelo	--
39,99-20,00	Semilla	RT002; RT004; RT019; RT003; RT012; RT007
	Suelo	--
<20,00	Semilla	RT015; RT011; RT025; RT024
	Suelo	--

(1) Porcentaje de germinación obtenido a los 24 días tras la siembra

(2) Origen de los aislamientos estudiados, bien de semilla o bien de suelo. El control está representado por aquellas semillas en las que no se ha inoculado ningún hongo (CC) o bien sólo *R. solani* (RC).

(3) Los tratamientos están ordenados de mayor a menor porcentaje de semillas germinadas por maceta y separados si han sido inoculados sólo con el aislamiento correspondiente de *Trichoderma* (CT-#) o si han sido inoculado tanto con el aislamiento de *Trichoderma* como con *R. solani* (RT-#).

Se ha determinado que la mayor germinación se ha dado en los tratamientos con *Trichoderma* extraído de muestras de suelo (Tabla 2 y Anexos Tabla A.2). Los tratamientos en los que germinaron el 100,00 % de las semillas han sido CT028, CT037, CT038, CT040, CT041 y RT036. El resto de los tratamientos con aislamientos de *Trichoderma* obtenidos de suelo, han tenido una germinación hasta del 70,00 %, mientras que los de *Trichoderma* extraídos de semilla han sido inferiores, incluso en algunos casos inferiores al tratamiento del control del patógeno (RC), como son el caso de CT008, CT007, CT005, CT013, CT025, CT001, CT003, CT006, CT015, CT024, CT011, RT001, RT013, RT005, RT006, RT002, RT004, RT019, RT003, RT012, RT007, RT015, RT011, RT025 y RT024.

3.2. Evaluación a los 45 días tras la siembra

Para la evaluación de las plantas a los 45 días tras la siembra, se han tenido en cuenta los parámetros de diámetro del hipocotilo, la longitud del sistema radicular, los pesos húmedos y secos tanto de la parte aérea como del sistema radicular. Al tratarse de hongos que se desarrollan en contacto directo con las raíces y el hipocotilo de la planta se ha considerado importante la toma de datos tanto del diámetro del hipocotilo como de la longitud del sistema radicular, sin dejar de lado los pesos que han alcanzado ambas zonas ya que están íntimamente relacionados con la producción. Al igual que en el apartado anterior, se ha realizado un análisis estadístico de los parámetros de estudio teniendo en cuenta la fuente de extracción de los aislamientos, viéndose que en todas las variables hay diferencias significativas (Figura 8).

En el caso de aquellas plantas inoculadas con aislamientos de *Trichoderma* extraídos de suelo (SueloCT y SueloRT), han presentado un mayor desarrollo en todos los parámetros de estudio siendo significativamente diferentes de aquellas que habían sido inoculadas con *Trichoderma* aislados a partir de semilla (SemillaCT y SemillaRT). La presencia del patógeno también ha reducido el desarrollo de las plantas. Sin embargo, aquellas plantas donde se han empleado los *Trichoderma* de suelo (SueloRT), su desarrollo ha sido significativamente superior a las plantas tratadas con *Trichoderma* procedente de semilla (SemillaRT) (Figura 8).

Hasta ahora no se ha publicado la influencia de la fuente de extracción de los aislados de *Trichoderma*. Se ha observado que aquellos aislamientos que han sido aislados a partir de semilla han protegido de forma menos efectiva a la planta frente a *R. solani* que aquellos que han sido obtenidos a partir de muestras de suelo de campos en los que la alubia formaba parte de la rotación. Los aislamientos extraídos de semilla tampoco han incrementado el desarrollo de la alubia, a excepción del aislamiento *T. harzianum* T019, como sí lo han hecho aquellos extraídos de suelo.

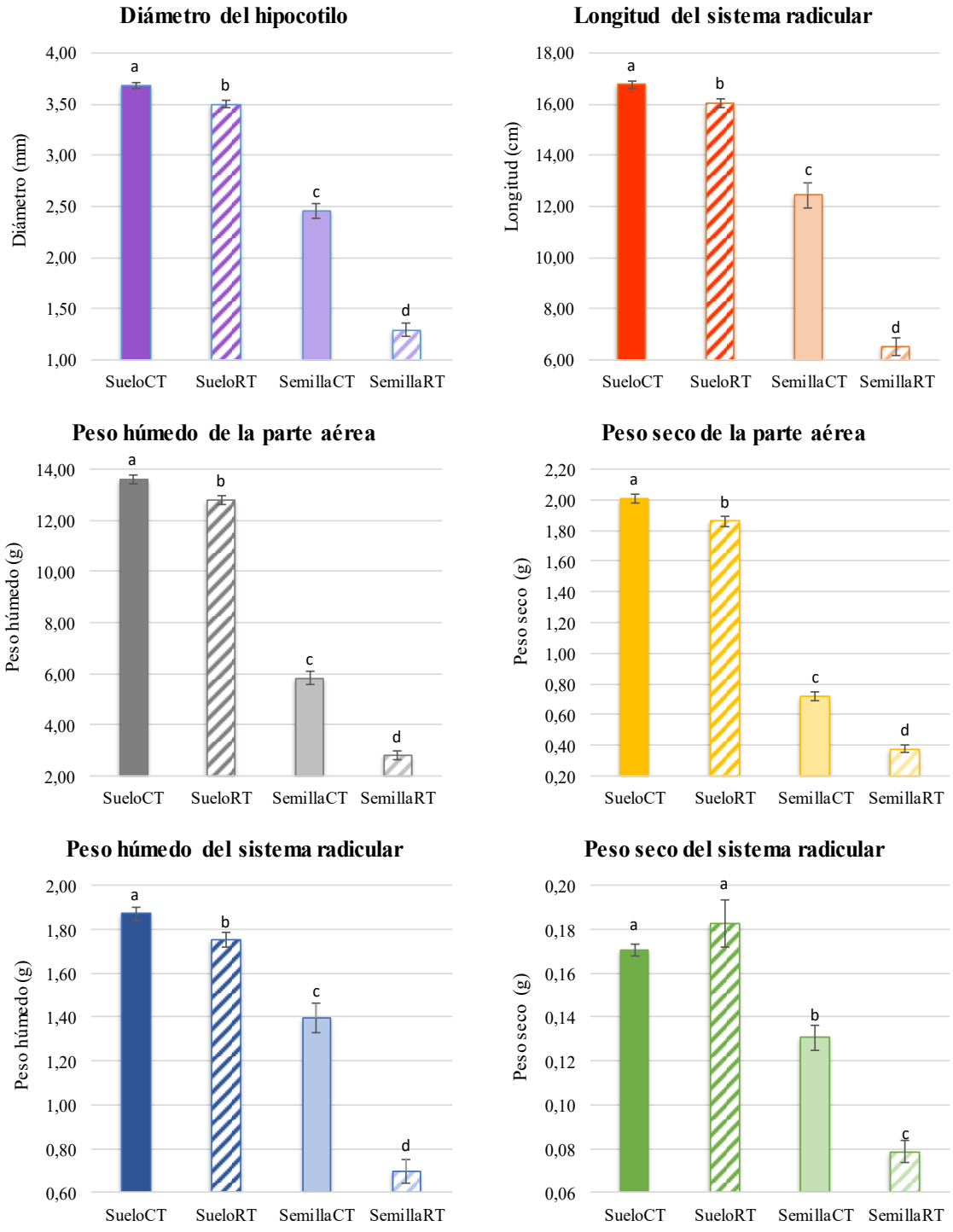


Figura 8: Evaluación a los 45 días tras la siembra de los aislamientos según su fuente de extracción. Se ha analizado el diámetro del hipocotilo (mm), la longitud de la raíz (cm), los pesos húmedos (g) y secos (g) de la parte aérea y del sistema radicular. Los valores con letras diferentes presentan diferencias significativas (test DMS, $p < 0,05$). [Plantas inoculadas con los aislamientos de *Trichoderma* de muestras de suelo (SueloCT); plantas inoculadas con los aislamientos de *Trichoderma* de muestras de suelo y con *R. solani* (SueloRT); plantas inoculadas con los aislamientos de *Trichoderma* de muestras de semilla (SemillaCT); plantas inoculadas con los aislamientos de *Trichoderma* de muestras de semilla y con *R. solani* (SemillaRT)]

La alubia, desde su domesticación, ha sido afectada por diferentes factores bióticos y abióticos. Con el paso del tiempo tanto el cultivo como todos los microorganismos asociados a él han coevolucionado, adaptándose a los cambios ambientales. Por ello, para la realización de los ensayos se ha seleccionado tanto el material vegetal (variedad local Canela) como el material fúngico (aislamientos del *R. solani* y *Trichoderma* spp.) extraídos de la zona donde se produce alubia bajo el sello de calidad I.G.P. "Alubia de La Bañeza-León". La evolución que han sufrido todas las partes ha sido para la adaptación a ese ambiente y sus cambios. Cualquier adaptación que haya sufrido el patógeno para poder "dañar" a la alubia también la habrá sufrido el agente de biocontrol, haciéndolo más eficiente para el control de la enfermedad, así como para su relación con la planta. Tanto *R. solani* como los aislamientos de *Trichoderma* spp. obtenidos del suelo han estado desarrollándose y evolucionando conjuntamente en él, adaptándose a los factores bióticos y abióticos a los que se hayan visto sometidos. Sin embargo, aquellos *Trichoderma* que han sido obtenidos a partir de muestras de semilla, en su coevolución, podrían haber desarrollado menor capacidad para el control de fitopatógenos del suelo. Por ello se podría decir que es más importante la selección de la parcela de cultivo que una selección de la semilla ya que es mucho más eficaz para el control de fitopatógenos de suelo la presencia de *Trichoderma* en el propio suelo que el que pudiera tener la semilla.

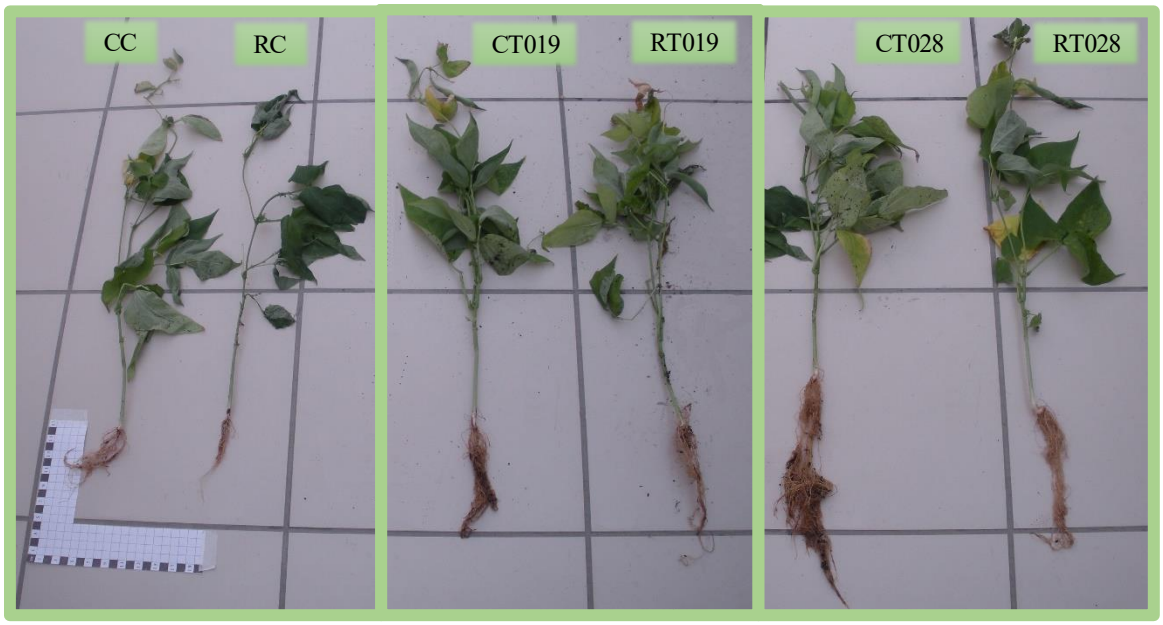


Figura 9: Plantas de alubia a los 45 días tras la siembra. CC, plantas a las cuales no se les ha inoculado ningún hongo. RC, plantas inoculadas *con R. solani*. CT-# plantas inoculadas con los aislamientos *T. harzianum*, T019 o *T. velutinum*, T028. RT-# plantas inoculadas con los aislamientos *T. harzianum*, T019 o *T. velutinum*, T028 y *R. solani*. Ambos aislamientos de *Trichoderma* han sido los empleados para la realización de los artículos que aparecen en el desarrollo de esta tesis.

Para la realización del resto de análisis presentados en esta tesis (Anexos Tablas A.3, A.4, A.5) se han seleccionado un aislamiento de *Trichoderma* obtenido a partir de semilla, *T. harzianum* T019, y otro extraído a partir de muestra de suelo, *T. velutinum* T028 (Figura 9). Para el estudio de respuesta de los genes de defensa de alubia se han empleado ambos aislamientos. Sin embargo, para el resto de los análisis sólo se ha empleado el aislamiento *T. velutinum* T028 ya que hasta el momento de la realización de los ensayos de respuesta defensiva, tanto genética como

metabolómica, ha sido el que mejor resultado ha mostrado en los ensayos *in vivo* tanto en la germinación como en la evaluación a los 45 días tras la siembra (Anexos Tabla A.2, A.3, A.4, A.5).

En el caso de *T. harzianum* T019 (Figura 9), cuando las plantas han sido tratadas sólo con el aislamiento (CT019) han tenido el mayor peso de la parte aérea (incremento del 63,84 % del peso húmedo y 54,11 % del seco respecto al control), siendo significativamente diferente de las plantas control (CC). En el caso del sistema radicular, se ha incrementado un 96,95 % en peso húmedo, pero se ha reducido un 20,00 % en el peso seco respecto a las plantas control. Cuando las plantas de alubia han estado en presencia de *R. solani* y T019 (RT019) tampoco han presentado diferencias significativas respecto al control (CC) en la parte aérea ni respecto al control del patógeno (RC) en el caso del sistema radicular (Mayo et al., 2015). Sin embargo, en este tratamiento, RT019, se ha reducido un 6,82 % y un 5,80 % el peso húmedo y seco de la parte aérea respectivamente y en el sistema radicular se ha disminuido el peso húmedo en 25,61 % y el seco en 33,33 %, todo ello respecto al control (CC).

En cuanto a *T. velutinum* T028 (Figura 9), las plantas inoculadas con este aislamiento (CT028 y RT028) han mostrado un incremento significativo del peso tanto seco como húmedo de la parte aérea como del sistema radicular, incluso cuando ha estado presente *R. solani*. Las plantas tratadas con T028 (CT028) han incrementado un 4,75 % el diámetro del hipocotilo, un 10,75 % la longitud del sistema radicular, y un 4,27 % y 5,51 % del peso seco de la parte aérea y del sistema radicular respectivamente, en todos los casos respecto a las plantas control (CC). Cuando han sido infectadas con *R. solani* (RT028), la acción de *T. velutinum* ha provocado un incremento respecto a las plantas control del patógeno (RC) de 8,76 % en el diámetro del hipocotilo, un 21,15 % de la longitud del sistema radicular y un 11,05 y 3,43 % del peso seco de la parte aérea y del sistema radicular respectivamente (Mayo et al., 2016).

4. RESPUESTA DEFENSIVA DE *P. VULGARIS* FRENTE A *TRICHODERMA* Y A *R. SOLANI*

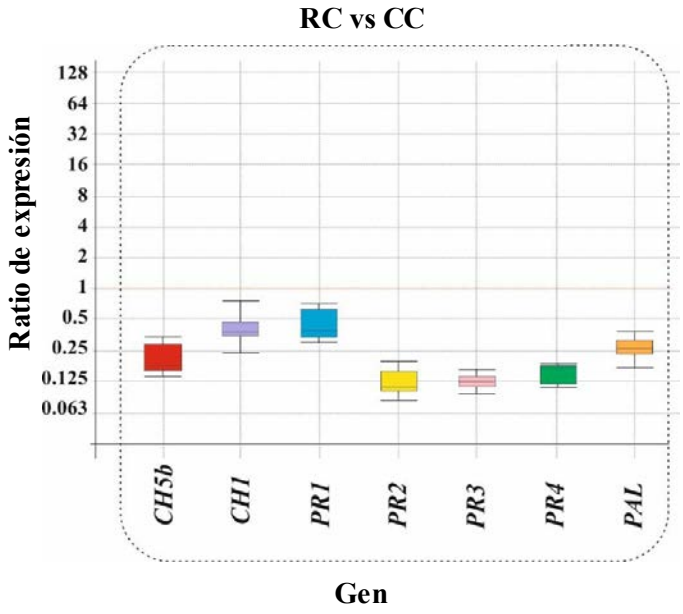
Las plantas desarrollan diversas estrategias para su defensa frente a la interacción con un organismo que puede ser patógeno o no. Las plantas pueden presentar diferentes respuestas (inducida, adquirida, hipersensibilidad) (Lodha y Basak, 2012) con mecanismos que varían según se encuentre frente a un microrganismo patógeno o uno no patógeno. El primero es la respuesta SAR (*Systemic Adquired Response*) que normalmente se debe a una infección local causada por un organismo patógeno involucrando señales dependientes del ácido salicílico (Durrant y Dong, 2004). La otra respuesta se conoce como ISR (*Induced Systemic Response*) que se produce como resultado de la colonización de la planta por parte de un microorganismo no patógeno desencadenado señales dependientes del etileno y del ácido jasmónico (Shoresh et al., 2010). La combinación de ambas respuestas pueden proteger a la planta frente al ataque de microorganismos (Verhagen et al., 2006). Otra respuesta que tiene la planta para defenderse de otros microorganismos es la hipersensibilidad (Tadeo y Gómez-Cadenas, 2008), lo que conlleva cambios en la permeabilidad de las membranas celulares, en la respiración y en la producción de metabolitos como fitoalexinas.

4.1. *P. vulgaris* frente a *R. solani*

a) Expresión de genes de defensa

El estudio de la expresión de los genes de defensa se ha realizado en dos ensayos en los que se ha inoculado el mismo aislamiento de *R. solani* (Anexos Tabla A.1). Sin embargo, a la hora de interpretarlos se han empleado dos métodos diferentes. En uno de ellos, los datos han sido analizados mediante el programa REST2009® (Pfaffl et al., 2002) y tomando como genes de referencia *actina* y *EF1α* (Mayo et al., 2015). En el otro caso se han estudiado los resultados mediante el método $2^{-\Delta\Delta Ct}$ y tomando como gen de referencia *Act11* (Mayo et al., 2016) ya que se ha estudiado la incorporación de otros genes de referencia como *UKN1* y *EF1α*, pero se ha comprobado que *Act11* ha sido el más estable.

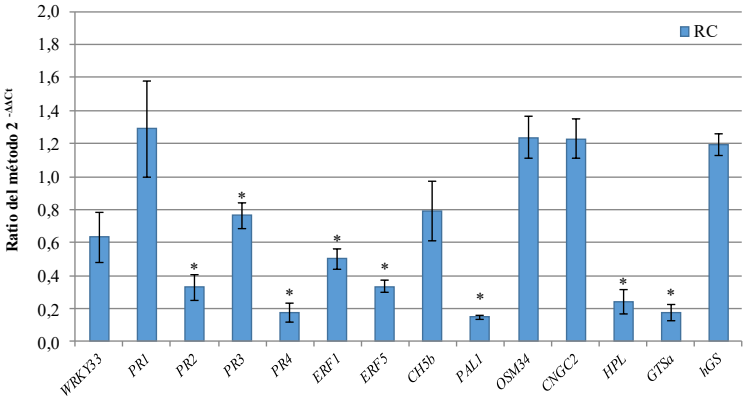
R. solani ha provocado una regulación negativa de varios genes de defensa siendo significativa en los genes *CH1*, *PR2, PR3, PR4, ERF1, ERF5, PAL, PAL1, HPL* y *GTSa* respecto a las plantas control (Figura 10 y Figura 11). Los genes *PR1* y *CH5b*, en el análisis con el programa REST2009, han presentado una regulación negativa significativa respecto al control (Figura 10). En el caso del resto de los genes, *OSM34*, *CNGC2* y *hGS* han sufrido una regulación positiva pero no ha sido estadísticamente significativa (Figura 11).



Gen	Ratio	*p(H1)*
CH5b	0,168*	0,029
CH1	0,397*	0,045
PR1	0,364*	0,038
PR2	0,099*	0,029
PR3	0,122*	0,000
PR4	0,127*	0,000
PAL	0,256*	0,000

Figura 10: Comparación de la expresión de los genes *CH5b, CH1, PR1, PR2, PR3, PR4* y *PAL* en plantas que han sido inoculadas con *R. solani* (RC) frente a plantas control (CC) en comparación con los genes de referencia *α-actin* y *EF1α*. La comparación y el análisis estadístico han sido realizados con el programa REST2009® (Pfaffl et al., 2002). Aquellos datos numéricos que presentan diferencias estadísticas ($p < 0,05$) están indicados con un asterisco en la tabla y rodeados en el gráfico.

R. solani durante 45 días en contacto con la planta ha causado la regulación negativa de la mayoría de los genes de defensa estudiados como un mecanismo para poder superar las barreras defensivas de la alubia y así poder invadirla y facilitar el proceso infeccioso en el interior de la misma (Mayo et al., 2015). En otros trabajos se ha observado un resultado similar, donde plantas de tomate infectadas por *Pseudomonas siringe* pv. *tomato* presentaron los genes *PR1* y *PR4* reprimidos, indicando un descenso de los mecanismos de defensa de la planta (Zhao et al., 2003).



Gene	CC		RC	
WRKY33	1.000	± 0.190	0.634	0.152
PR1	1.000	± 0.266	1.289	0.292
PR2	1.000	± 0.085	0.331*	0.079
PR3	1.000	± 0.127	0.763*	0.077
PR4	1.000	± 0.197	0.176*	0.057
ERF1	1.000	± 0.149	0.502*	0.061
ERF5	1.000	± 0.134	0.333*	0.037
CH5b	1.000	± 0.272	0.790	0.179
PAL1	1.000	± 0.116	0.149*	0.013
OSM34	1.000	± 0.264	1.237	0.125
CNGC2	1.000	± 0.235	1.228	0.122
HPL	1.000	± 0.279	0.245*	0.073
GTSa	1.000	± 0.158	0.177*	0.051
hGS	1.000	± 0.115	1.193	0.068

Figura 11: Comparación de la expresión de los genes *WRKY33*, *PR1, PR2, PR3, PR4, ERF1, ERF5, CH5b, PAL1, OSM34, CNGC2, HPL, GTSa* y *hGS* en plantas que han sido inoculadas con *R. solani* (RC) frente a plantas control (CC) en comparación con el gen de referencia *Act11*. Los datos han sido analizados por el método $2^{-\Delta\Delta Ct}$. Aquellos datos numéricos que presentan diferencias estadísticas ($p < 0,05$) están indicados con un asterisco.

b) Producción de metabolitos

La mayoría de los metabolitos significativamente diferentes que ha producido la alubia infectada con *R. solani* han descendido respecto a las plantas control (Tabla 3). Se considera que estos compuestos al actuar como fitoalexinas (Ahuja et al., 2012), reducen su producción en presencia de *R. solani* con lo que disminuye la respuesta defensiva de la planta, favoreciendo así la invasión de la planta por parte del patógeno.

Los únicos compuestos que han aumentado su producción han sido un carbohidrato (#**2**, $C_{12}H_{22}O_{11}$, sacarosa), un glicósido (#**4**, $C_{12}H_{18}O_{11}$, ácido L-ascórbico-2-glucósido), y un terpeno (#**17**, $C_{20}H_{32}O_9$, etil 7-epi-12-hidroxijasmonato glucósido) (Tabla 3). La producción de sacarosa se ha podido deber a que el patógeno ha establecido una relación con la planta a lo que ha demandado una mayor cantidad de este compuesto por lo que se ha detectado este aumento (Vargas et al., 2011). En cuando al terpeno, etil 7-epi-12-hidroxijasmonato glucósido, se trata de un derivado del ácido jasmónico y está muy ligado a la respuesta defensiva de la alubia (Pieterse et al., 2012). Por ello, este compuesto ha sido producido en una cantidad significativamente mayor que en las plantas control, cuando éstas han estado en contacto tanto con *R. solani* como con *T. velutinum* como respuesta a la presencia de ambos hongos.

Tabla 3: Compuestos detectados que se han producido en cantidades significativamente diferentes respecto a las plantas control (CC) cuando han estado en presencia de *R. solani* (RC).

Id[(1)]	Fórmula	Compuesto asignado	RC-CC
		Ácido graso	
35	$C_{18}H_{30}O_2$	Ácido γ-Linolénico	Reducido
		Aminoácido	
8	$C_{11}H_{12}N_2O_2$	L-Triptófano	Reducido
15	$C_{13}H_{14}N_2O_3$	N-Acetiltriptófano	Reducido

Tabla 3: Compuestos detectados que se han producido en cantidades significativamente diferentes respecto a las plantas control (CC) cuando han estado en presencia de *R. solani* (RC) (continuación).

Id[(1)]	Fórmula	Compuesto asignado	RC-CC
		Carbohidrato	
2	$C_{12}H_{22}O_{11}$	Sacarosa	Aumentado
		Flavonoide	
1	$C_{17}H_{20}O_{11}$	5-Hidroxi-6,8-dimethoxi-2-oxo-2H-chromen-7-il β-D-glucopiranósido	Reducido
16	$C_{30}H_{26}O_{16}$	Quercetagetin 7-(6"-(E)-caffeoilglucósido)	Reducido
18	$C_{29}H_{34}O_{17}$	Isopirenin 7-O-glucósido	Reducido
19	$C_{25}H_{32}O_{13}$	12-Hidroxi, O-[3,4,5-trihidroxibenzoil-(06)-β-D-glucopiranósido]	Reducido
20	$C_{32}H_{39}O_{16}$	Luteona 4,7-O-diglucósido	Reducido
22	$C_{19}H_{18}O_8$	3´,5-Dihidroxi-3,4´,6,7-tetramethoxiflavona	Reducido
27	$C_{19}H_{18}O_9$	5,2',4'-Trihidroxi-3,7,8,5'-tetramethoxiflavona	Reducido
		Fenol	
31	$C_{19}H_{20}O_9$	Garcimangosona D	Reducido
		Glicósido	
4	$C_{12}H_{18}O_{11}$	Ácido L-ascórbico-2-glucósido	Aumentado
		Lípido	
34	$C_{18}H_{30}O_3$	13(S)-HOTrE	Reducido
36	$C_{31}H_{63}O_7P$	PA(O-16:0/12:0)	Reducido
		Péptido	
7	$C_{11}H_{22}N_2O_3$	Valyl-Leucina	Reducido
11	$C_{17}H_{32}N_4O_5$	Ile Gln Ile	Reducido
		Terpeno	
17	$C_{20}H_{32}O_9$	Etil 7-epi-12-hidroxijasmonato glucósido	Aumentado
32	$C_{47}H_{76}O_{16}$	Akeboside Ste	Reducido

(1) Id: Número correspondiente al pico del cromatograma obtenido por HPLC-DAD-TOF-MS del capítulo IV.

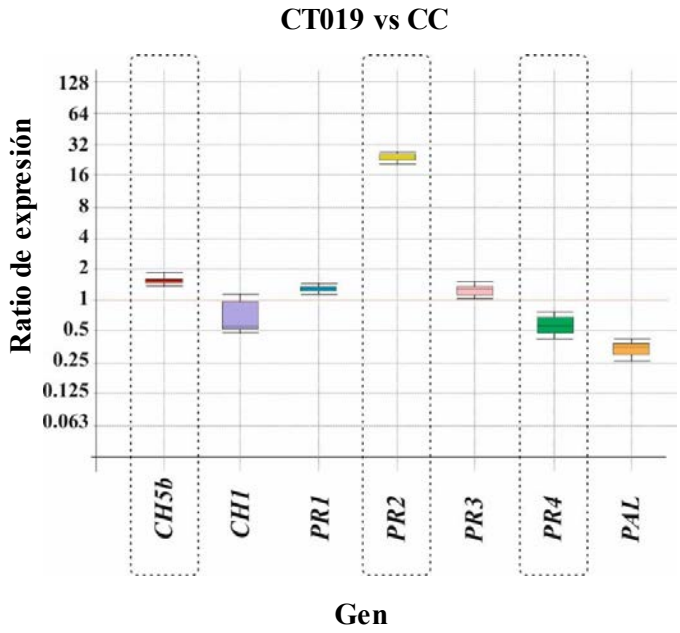
4.2. *P. vulgaris* frente a *T. harzianum* T019

a) Expresión de los genes de defensa

Cuando las plantas han sido tratadas con el aislamiento *T. harzianum* T019 (CT019), se ha observado que los genes *CH5b* y *PR2* han sido regulados positivamente de forma significativa respecto a las plantas donde no se ha inoculado ningún hongo (CC), con ratios de 1,495 y 24,492

respectivamente (Figura 12). El alto nivel de expresión de *PR2*, se debe a que existe una alteración de la respuesta defensiva de la alubia tras haber estado en contacto con *Trichoderma* durante un largo periodo en comparación con los datos aportados en otros trabajos en los que esta interacción dura unas pocas horas. Este resultado se podría deber a la alta actividad de las β-1,3-glucanasas en la pared celular, lo que incrementaría los oligosacáridos que actúan como moléculas elicitoras desencadenantes de la respuesta defensiva en la planta y/o en los metabolitos secundarios de los hongos (Druzhinina et al., 2011). Sin embargo, el gen *PR4* se ha regulado negativamente con una ratio significativa de 0,524 respecto al control (Figura 12). En un estudio llevado a cabo en berenjena, este gen incrementa su expresión cuando ha estado en contacto con agentes de biocontrol, sugiriendo con ello que promueven la respuesta defensiva de la planta (Angelopoulou et al., 2014).

Los genes *CH1, PR1*, *PR3* y *PAL* no se han visto afectados de forma significativa. En otros trabajos, se ha observado que *T. harzianum* potencia la respuesta de la alubia frente a fitopatógenos como *R. solani*. Rivière et al. (2008) han sugerido que *PR1* se regula negativamente debido a las β-1,3-glucanasas (PR2 y PR3) y en el trabajo de Guerrero-González et al. (2011) este gen se regula positivamente. En nuestro caso, este gen no presenta una respuesta significativa respecto al control.



Gen	Ratio	*p(H1)*
CH5b	1,495*	0,000
CH1	0,636	0,087
PR1	1,127	0,065
PR2	24,492*	0,000
PR3	1,173	0,152
PR4	0,524*	0,036
PAL	0,316	0,081

Figura 12: Comparación de la expresión de los genes *CH5b, CH1, PR1, PR2, PR3, PR4* y *PAL* en plantas que han sido inoculadas con *T. harzianum* T019 (CT019) frente a plantas control (CC) en comparación con los genes de referencia *α-actin* y *EF1α*. La comparación y el análisis estadístico han sido realizados con el programa REST2009® (Pfaffl et al., 2002). Aquellos datos numéricos que presentan diferencias estadísticas ($p < 0,05$) están indicados con un asterisco en la tabla y rodeados en el gráfico.

b) Producción de metabolitos de T. harzianum *T019*

La producción de metabolitos por parte de *Trichoderma* provoca respuestas en la planta que pueden incrementar su desarrollo o activar su respuesta defensiva.

La producción de escualeno por parte de un aislamiento fúngico puede ser importante para comprobar su eficiencia en el control de enfermedades vegetales. Este compuesto es un terpeno poliinsaturado que parece que tiene una función importante en la membrana celular de los hongos (Malmierca et al., 2013) y que es precursor del ergosterol. Este compuesto es un esterol que la planta puede reconocer como un PAMP (*Pathogen Associated Molecular Patterns*) (Nürnberger et al., 2004) ya que desencadena una serie de reacciones (Cervone et al., 1997) que podrían activar la respuesta defensiva de la planta. Por tanto, los niveles de producción de escualeno influirán en la síntesis de ergosterol (Garaiová et al., 2013). Además, al tratarse de un compuesto estructural resultante del desarrollo del hongo, el ergosterol podría activar la expresión de numerosos genes de defensa e incrementar la resistencia de la planta a fitopatógenos (Lochman y Mikeš, 2005).

En este caso se ha evaluado la producción de ergosterol y escualeno, en el aislamiento T019 comparándolo con otro aislamiento de la misma especie (Anexos Tabla A.1). La producción de ergosterol y de escualeno a las 24 h no ha presentado diferencias significativas respecto a la otra cepa de *T. harzianum* T34, pero a las 96 h el aislamiento T019 ha producido una cantidad significativamente mayor de ambos compuestos respecto a la cepa de referencia (Tabla 4). Así, un incremento de la producción de ambos compuestos puede inducir la respuesta defensiva de la planta, aumentar su desarrollo incluso en presencia de un fitopatógeno.

Tabla 4: Cuantificación de ergosterol y escualeno a las 24 h y 96 h de los aislamientos de *Trichoderma* seleccionados para este estudio.

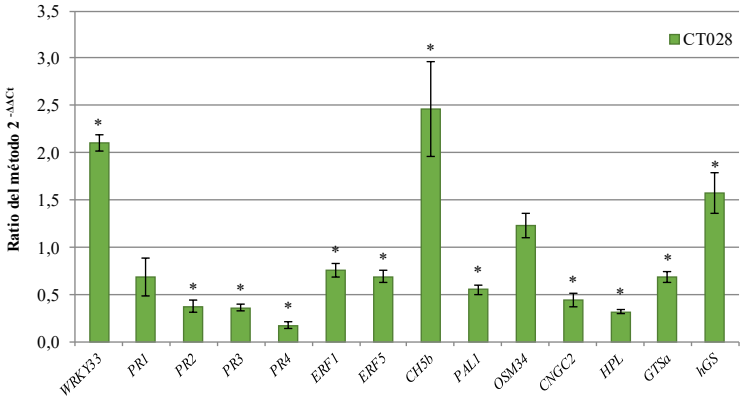
Aislamiento de T. harzianum	**Ergosterol (mg E/g peso seco)**		**Escualeno (mg S/g peso seco)**	
	24 h	**96 h**	**24 h**	**96 h**
T34	16,197 ± 1,019 a	8,970 ± 0,388 b	0,317 ± 0,020 a	0,052 ± 0,049 b
T019	14,336 ± 2,459 a	12,502 ± 3,568 a	0,303 ± 0,041 a	0,348 ± 0,103 a

Los valores con letras diferentes presentan diferencias significativas (test DMS, $p < 0,05$)

4.3. *P. vulgaris* frente a *T. velutinum* T028

a) Expresión de los genes de defensa

Las plantas de alubia cuando han estado desarrollándose durante 45 días en contacto con *T. velutinum*, T028, (CT028) la mayoría de los genes de estudio han sido regulados negativamente, aunque en menor medida que cuando han estado desarrollándose con *R. solani* (RC) (Figura 13). Así, la expresión de los genes *PR2, PR3, PR4, ERF1, ERF5, PAL1, CNGC2, HPL* y *GTSa* ha sido significativamente inferior respecto a la de las plantas control, ratios que han oscilado entre 0,168 de *PR4* y 0,754 de *ERF1*. En el caso de *WRKY33*, *CH5b* y *hGS*, han sido regulados positivamente respecto al control con valores que varían entre 2,462 de *CH5b* y 1,579 de *hGS*. *PR1* ha sido regulado negativamente y *OSM34* regulado positivamente, pero en estos dos últimos casos las diferencias no han sido significativas respecto al control.



Gene	CC		CT028	
WRKY33	1.000	± 0.190	2.102*	0.086
PR1	1.000	± 0.266	0.679	0.201
PR2	1.000	± 0.085	0.368*	0.065
PR3	1.000	± 0.127	0.357*	0.041
PR4	1.000	± 0.197	0.168*	0.038
ERF1	1.000	± 0.149	0.754*	0.067
ERF5	1.000	± 0.134	0.689*	0.064
CH5b	1.000	± 0.272	2.462*	0.503
PAL1	1.000	± 0.116	0.550*	0.052
OSM34	1.000	± 0.264	1.230	0.132
CNGC2	1.000	± 0.235	0.441*	0.069
HPL	1.000	± 0.279	0.316*	0.026
GTSa	1.000	± 0.158	0.679*	0.059
hGS	1.000	± 0.115	1.576*	0.215

Figura 13: Comparación de la expresión de los genes *WRKY33*, *PR1, PR2, PR3, PR4, ERF1, ERF5, CH5b, PAL1, OSM34, CNGC2, HPL, GTSa* y *hGS* en plantas que han sido inoculadas con *T. velutinum*, T028 (CT028) frente a plantas control (CC) en comparación con el gen de referencia *Act11*. Los datos han sido analizados por el método $2^{-\Delta\Delta Ct}$. Aquellos datos numéricos que presentan diferencias estadísticas ($p < 0,05$) están indicados con un asterisco.

b) Producción de metabolitos

En la interacción de *T. velutinum* T028 y la alubia (CT028), se han detectado 25 compuestos con diferencias significativas respecto a las plantas control, de los cuales 10 han incrementado su producción y 15 la han reducido (Tabla 5).

Entre los compuestos que han aumentado su producción está un aminoácido (#**8**, $C_{11}H_{12}N_2O_2$, L-Triptófano) (Tabla 5). Este incremento se debe a que el L-Triptófano es un precursor de auxinas que son las responsables de la biosíntesis de estas hormonas en la rizosfera con lo que estimulan el desarrollo de la planta, aumentado así su desarrollo. Por lo que cuando está presente *T. velutinum* en el suelo, la concentración de este compuesto aumenta. En otros estudios llevados a cabo en *Vigna mungo* en la que se inocularon otros agentes de biocontrol como *Rhizobium* spp. y *Bacillus* spp, se incrementó la producción un 23,36 % cuando estaba presente este compuesto en la rizosfera (Qureshi et al., 2012).

Otro compuesto que ha aumentado su producción ha sido un carbohidrato (compuesto #**2**, $C_{12}H_{22}O_{11}$, sacarosa) (Tabla 5). Este incremento se debe a la relación simbiótica que existe entre *Trichoderma* y la planta de alubia, de esta forma favorecería la expansión de éste en la rizosfera así como la penetración en la raíz de la planta (Vargas et al., 2011).

Muchos polifenoles (fenoles, flavoniodes ácidos fenólicos) juegan un papel importante en la interacción de las plantas con los fitopatógenos (VanEtten et al., 1994). En este trabajo, la mayoría de los flavonoides ha sufrido una reducción de su producción salvo los compuestos #**3**, ($C_{27}H_{32}O_{14}$, Naringin), #**10**, ($C_{27}H_{32}O_{16}$, 3,4´,5,7-Tetrahidroxiflavanona 3,7-Di-O-β-D-glucopiranósido) y #**20**, ($C_{32}H_{39}O_{16}$, Luteona 4,7-O-diglucósido) (Tabla 5). Un resultado similar, han presentado los fenoles, donde la mayoría reducen su nivel de producción salvo el #**13** ($C_{17}H_{22}O_{10}$, 1-O-Sinapoilglucoso) (Tabla 5). En el caso del lípido (#**30**, $C_{16}H_{30}O_7$, Ácido 3-O-α-L-rhamnopiranosil-3-hidroxidecanoico) se ha producido en una cantidad significativamente menor (Tabla 5).

En los péptidos, el #**29** ($C_{15}H_{26}N_6O_7$, Gln Gln Gln) ha tenido la misma respuesta que cuando ha estado presente *R. solani*. Sin embargo, el #**7** ($C_{11}H_{22}N_2O_3$, Valil-Leucine) ha aumentado su

cantidad cuando está *T. velutinum* (Tabla 5). La actividad de los péptidos en la alubia está descrita como indicadores por ejemplo de cambios en la permeabilización de membranas celulares, o inducción de la acumulación de chitinasas. Por lo que están relacionados con la respuesta defensiva de la planta (Luna-Vital et al., 2015).

En el caso de los terpenos, la mayoría ha incrementado significativamente su producción a excepción del #**32** ($C_{47}H_{76}O_{16}$, Akeboside Ste) que ha sido reducida (Tabla 5). Cabe destacar el #**17** ($C_{20}H_{32}O_9$, Etil 7-epi-12-hidroxijasmonato glucósido) que ha incrementado su producción al igual que lo hiciera en presencia del patógeno, con lo que este derivado del ácido jasmónico puede estar muy ligado a la respuesta defensiva de la alubia (Pieterse et al., 2012).

Tabla 5: Compuestos detectados que se han producido en cantidades significativamente diferentes respecto a las plantas control (CC) cuando han estado en presencia de *T. velutinum* T028 (CT028).

Id[(1)]	Fórmula	Compuesto asignado	CT028-CC
		Aminoácido	
8	$C_{11}H_{12}N_2O_2$	L-Triptófano	Aumentado
		Carbohidrato	
2	$C_{12}H_{22}O_{11}$	Sacarosa	Aumentado
		Flavonoide	
1	$C_{17}H_{20}O_{11}$	5-Hidroxi-6,8-dimethoxi-2-oxo-2H-chromen-7-il β-D-glucopiranósido	Reducido
3	$C_{27}H_{32}O_{14}$	Naringin	Aumentado
10	$C_{27}H_{32}O_{16}$	3,4′,5,7-Tetrahidroxiflavanona 3,7-Di-O-β-D-glucopiranósido	Aumentado
18	$C_{29}H_{34}O_{17}$	Isopirenin 7-O-glucósido	Reducido
19	$C_{25}H_{32}O_{13}$	12-Hidroxi, O-[3,4,5-trihidroxibenzoil-(06)-β-D-glucopiranósido]	Reducido
20	$C_{32}H_{39}O_{16}$	Luteona 4,7-O-diglucósido	Aumentado
22	$C_{19}H_{18}O_8$	3′,5-Dihidroxi-3,4′,6,7-tetramethoxiflavona	Reducido
24	$C_{20}H_{38}O_{12}$	(R)-1-O-[β-D-Glucopiranosil-(1-6)-β-D-glucopiranósido]-1,3-octanediol	Reducido
25	$C_{25}H_{28}O_6$	2′,4′,5,7-Tetrahidroxi-3′,8-diprenilisoflavanona	Reducido
26	$C_{20}H_{38}O_{11}$	n-Octil-β-D-maltopiranosido	Reducido
27	$C_{19}H_{18}O_9$	5,2',4'-Trihidroxi-3,7,8,5'-tetramethoxiflavona	Reducido
28	$C_{21}H_{22}O_4$ $C_{20}H_{18}O_5$	2′-O-Methilphaseollinisoflavona Wighteone	Reducido
		Fenol	
5	$C_{14}H_{20}O_8$ $C_{16}H_{12}O_7$	Hidroxitirosol 1-O-glucósido Isorhamnetin	Reducido
13	$C_{17}H_{22}O_{10}$	1-O-Sinapoilglucoso	Aumentado

Tabla 5: Compuestos detectados que se han producido en cantidades significativamente diferentes respecto a las plantas control (CC) cuando han estado en presencia de *T. velutinum* T028 (CT028) (continuación).

Id[(1)]	Fórmula	Compuesto asignado	CT028-CC
		Fenol (continuación)	
31	$C_{19}H_{20}O_9$	Garcimangosone D	Reducido
33	$C_{38}H_{44}O_8$	Ácido Gambogico	Reducido
		Lípido	
30	$C_{16}H_{30}O_7$	Ácido 3-O-α-L-rhamnopiranosil-3-hidroxidecanoico	Reducido
		Péptido	
7	$C_{11}H_{22}N_2O_3$	Valil-Leucine	Aumentado
29	$C_{15}H_{26}N_6O_7$	Gln Gln Gln	Reducido
		Terpeno	
9	$C_{21}H_{32}O_{10}$	Ácido Dihidrofaseico 4-O-β-D-glucósido	Aumentado
17	$C_{20}H_{32}O_9$	Etil 7-epi-12-hidroxijasmonato glucósido	Aumentado
23	$C_{14}H_{20}O_5$ $C_{16}H_{12}O_4$	Teucrein Formononetin	Aumentado
32	$C_{47}H_{76}O_{16}$	Akeboside Ste	Reducido

(1) Id: Número correspondiente al pico del cromatograma obtenido por HPLC-DAD-TOF-MS del capítulo IV.

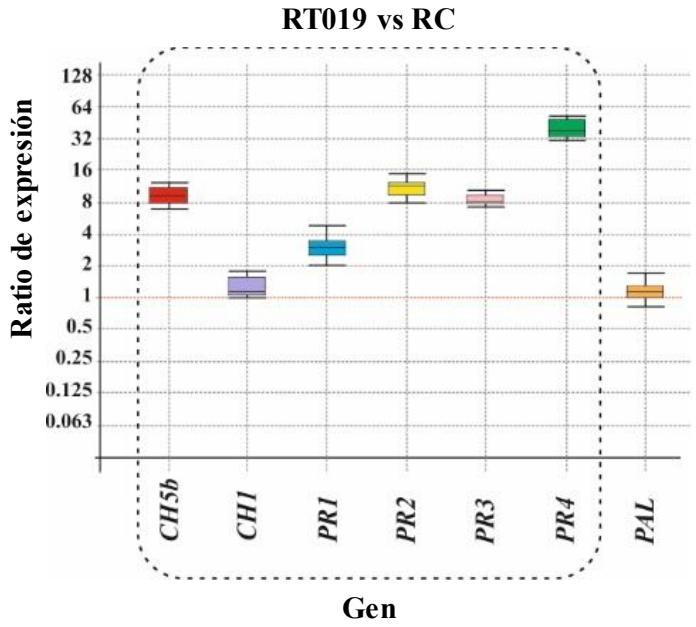
4.4. *P. vulgaris* frente a *Trichoderma* y *R. solani*

a) Expresión de los genes de defensa

Se ha realizado la expresión de los genes de estudio cuando las plantas de alubia han sido inoculadas con *R. solani* y con los aislamientos de *T. harzianum* T019 (RT019) y *T. velutinum* T028 (RT028).

Analizando la respuesta genética de *P. vulgaris* cuando ha sido inoculada con el patógeno y con *T. harzianum* (RT019), se ha visto que respecto a las plantas control (CC) no ha mostrado resultados significativos de regulación positiva en ninguno de los genes. Pero analizando la respuesta que tienen estas mismas plantas respecto al control de *R. solani* (RC) (Figura 14) se ha observado que los genes *CH5b, CH1, PR1, PR2, PR3* y *PR4* han sido regulados positivamente. Es decir, la presencia de *T. harzianum* en plantas con el patógeno hace que el agente de biocontrol desencadene la respuesta defensiva de la planta.

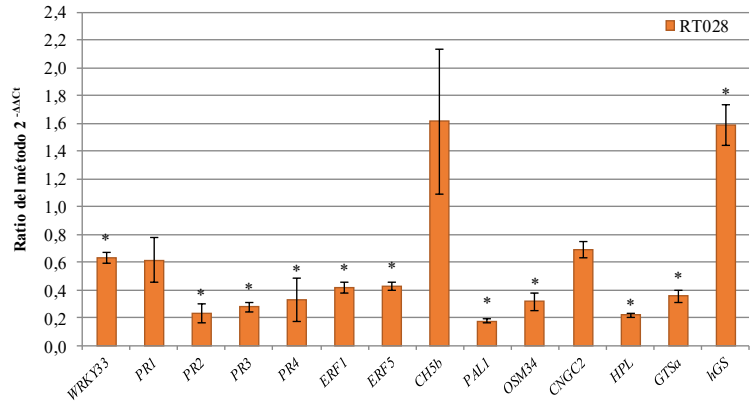
En otros trabajos, durante la interacción de *Solanum tuberosum* con *R. solani*, se ha observado que tras 120 h desde la infección inicial y los primeros síntomas de la enfermedad, han aparecido nuevos brotes con una reducción de la sintomatología lo que sugiere que la planta podría estar preparada para su defensa y así evitar el avance de la enfermedad (Lehtonen et al., 2008). En el presente trabajo, la presencia de *T. harzianum* hace que se module la respuesta de la planta y prevenga la supresión de los genes defensivos por parte de *R. solani*, con lo que ayuda a la alubia a resistir a dicho ataque.



Gen	Ratio	*p(H1)*
CH5b	10,410*	0,000
CH1	1,420*	0,000
PR1	3,457*	0,000
PR2	12,267*	0,000
PR3	8,896*	0,000
PR4	42,975*	0,000
PAL	1,305	0,174

Figura 14: Comparación de la expresión de los genes *CH5b, CH1, PR1, PR2, PR3, PR4* y *PAL* en plantas que han sido inoculadas con *R. solani* y con *T. harzianum*, T019 (RT019) frente a plantas control de *R. solani* (RC) en comparación con los genes de referencia *α-actina* y *EF1α*. La comparación y el análisis estadístico han sido realizados con el programa REST2009 ® (Pfaffl et al., 2002). Aquellos datos numéricos que presentan diferencias estadísticas ($p < 0,05$) están indicados con un asterisco en la tabla y rodeados en el gráfico.

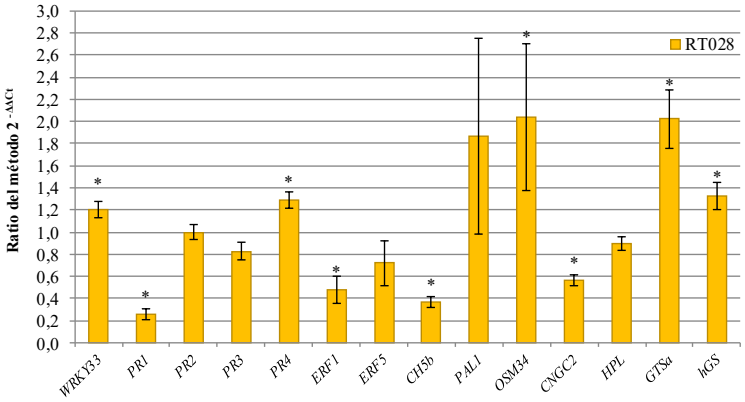
Cuando *R. solani* y *T. velutinum* T028 han estado en contacto con la alubia durante 45 días (RT028), la respuesta de la planta respecto al control (CC) ha sido significativa para la mayoría de los genes de estudio salvo para *PR1*, *CH5b* y *CNGC2* (Figura 15). Los genes regulados negativamente respecto a las plantas control han sido *WRKY33*, *PR2*, *PR3*, *PR4*, *ERF1*, *ERF5*, *PAL1*, *OSM34*, *HPL* y *GTSa*, con ratios que han variado desde 0,179 de *PAL1* hasta 0,631 de *WRKY33*. Los regulados positivamente han sido *CH5b* y *hGS*, aunque sólo en este último la diferencia de expresión ha sido significativa.



Gene	CC		RT028	
WRKY33	1.000	± 0.190	0.631*	0.042
PR1	1.000	± 0.266	0.617	0.164
PR2	1.000	± 0.085	0.238*	0.068
PR3	1.000	± 0.127	0.278*	0.037
PR4	1.000	± 0.197	0.330*	0.156
ERF1	1.000	± 0.149	0.416*	0.041
ERF5	1.000	± 0.134	0.429*	0.026
CH5b	1.000	± 0.272	1.613	0.522
PAL1	1.000	± 0.116	0.179*	0.011
OSM34	1.000	± 0.264	0.316*	0.060
CNGC2	1.000	± 0.235	0.689	0.060
HPL	1.000	± 0.279	0.219*	0.014
GTSa	1.000	± 0.158	0.357*	0.046
hGS	1.000	± 0.115	1.589*	0.147

Figura 15: Comparación de la expresión de los genes *WRKY33*, *PR1, PR2, PR3, PR4, ERF1, ERF5, CH5b, PAL1, OSM34, CNGC2, HPL, GTSa* y *hGS* en plantas que han sido inoculadas con *R. solani* y *T. velutinum*, T028 (RT028) frente a plantas control (CC) en comparación con el gen de referencia *Act11*. Los datos han sido analizados por el método $2^{-\Delta\Delta Ct}$. Aquellos datos numéricos que presentan diferencias estadísticas ($p < 0,05$) están indicados con un asterisco.

Al igual que en el caso del aislamiento T019, se ha evaluado la respuesta de las alubias cuando han estado inoculadas con *T. velutinum* T028 y *R. solani* (RT028) respecto a las que se han desarrollado en presencia de *R. solani* (Figura 16). Los genes *PR1*, *ERF1*, *CH5b* y *CNGC2* han sido regulados negativamente mientras que *WRKY33*, *PR4*, *OSM34*, *GTSa* y *hGS* han sido regulados positivamente, en todos los casos de forma significativa, respecto a las plantas control de *R. solani* (RC) con ratios que han variado entre 0,256 de *PR1* y 2,042 de *OSM34*.



Gene	RC		RT028	
WRKY33	1,000	0,104	1,202*	0,074
PR1	1,000	0,101	0,256*	0,048
PR2	1,000	0,240	0,997	0,066
PR3	1,000	0,122	0,828	0,081
PR4	1,000	0,111	1,288*	0,077
ERF1	1,000	0,227	0,479*	0,127
ERF5	1,000	0,239	0,719	0,205
CH5b	1,000	0,037	0,365*	0,048
PAL1	1,000	0,325	1,870	0,885
OSM34	1,000	0,226	2,042*	0,661
CNGC2	1,000	0,099	0,561*	0,049
HPL	1,000	0,300	0,896	0,057
GTSa	1,000	0,290	2,021*	0,259
hGS	1,000	0,057	1,332	0,123

Figura 16: Comparación de la expresión de los genes *WRKY33*, *PR1, PR2, PR3, PR4, ERF1, ERF5, CH5b, PAL1, OSM34, CNGC2, HPL, GTSa* y *hGS* en plantas que han sido inoculadas con *R. solani* y *T. velutinum*, T028 (RT028) frente a control de *R. solani* (RC) en comparación con el gen de referencia *Act11*. Los datos han sido analizados por el método $2^{-\Delta\Delta Ct}$. Aquellos datos numéricos que presentan diferencias estadísticas ($p < 0,05$) están indicados con un asterisco.

b) Producción de metabolitos

En la interacción de las plantas de alubia con *R. solani* y *T. velutinum* T028 (RT028) se han detectado 26 compuestos con diferencias significativas de producción, de los cuales 9 han aumentado y 17 han reducido respecto a las plantas control (CC) (Tabla 6).

La producción del ácido graso (#**35**, $C_{18}H_{30}O_2$, ácido γ-Linolénico) cuando ha estado en presencia de ambos hongos se ha reducido al igual que lo hiciera estando en presencia de *R. solani* (Tabla 3 y Tabla 6). En este caso, la presencia del patógeno ha provocado que este compuesto se haya reducido y ni la presencia del agente de biocontrol ha hecho que restablezca a niveles del control. El ácido γ-Linolénico es un precursor de jasmonato con lo que está ligado a una respuesta ISR, por lo que el patógeno reduciría la defensa de la planta dependiente de este compuesto.

El aminoácido (#**15**, $C_{13}H_{14}N_2O_3$, N-acetiltriptófano) ha aumentado respecto al control (Tabla 6). Al contrario que el anterior compuesto, en presencia de *R. solani* (RC), ha reducido su producción (Tabla 3). Sin embargo, en la combinación de ambos hongos se ha incrementado. Por ello, la presencia de *T. velutinum* y *R. solani*, ha aumentado su producción, con lo que el agente de biocontrol inhibe el efecto del patógeno en la producción de este compuesto.

Tabla 6: Compuestos detectados que se han producido en cantidades significativamente diferentes respecto a las plantas control (CC) cuando han estado en presencia de *R. solani* y *T. velutinum* T028 (RT028).

Id[(1)]	Fórmula	Compuesto asignado	RT028-CC
		Ácido graso	
35	$C_{18}H_{30}O_2$	Ácido γ-Linolénico	Reducido
		Aminoácido	
15	$C_{13}H_{14}N_2O_3$	N-acetiltriptófano	Aumentado
		Flavonoide	
1	$C_{17}H_{20}O_{11}$	5-Hidroxi-6,8-dimethoxi-2-oxo-2H-chromen-7-il β-D-glucopiranósido	Reducido
6	$C_{20}H_{20}O_4$	Phaseollinisoflavana	Reducido
10	$C_{27}H_{32}O_{16}$	3,4′,5,7-Tetrahidroxiflavanona 3,7-Di-O-β-D-glucopiranósido	Aumentado
12	$C_{26}H_{28}O_{16}$	Quercetin 3-vicianósido	Aumentado
14	$C_{26}H_{32}O_{13}$	(Z)-Resveratrol 3,4'-diglucósido	Aumentado
18	$C_{29}H_{34}O_{17}$	Isopirenin 7-O-glucósido	Reducido
19	$C_{25}H_{32}O_{13}$	12-Hidroxi, O-[3,4,5-trihidroxibenzoil-(06)-β-D-glucopiranósido]	Reducido
20	$C_{32}H_{39}O_{16}$	Luteona 4,7-O-diglucósido	Aumentado
21	$C_{29}H_{50}O$	Sitosterol	Aumentado
22	$C_{19}H_{18}O_8$	3′,5-Dihidroxi-3,4′,6,7-tetramethoxiflavona	Reducido
24	$C_{20}H_{38}O_{12}$	(R)-1-O-[β-D-Glucopiranosil-(1-6)-β-D-glucopiranósido]-1,3-octanediol	Reducido
25	$C_{25}H_{28}O_6$	2′,4′,5,7-Tetrahidroxi-3′,8-diprenilisoflavanona	Reducido
27	$C_{19}H_{18}O_9$	5,2',4'-Trihidroxi-3,7,8,5'-tetramethoxiflavone	Reducido
28	$C_{21}H_{22}O_4$ $C_{20}H_{18}O_5$	2′-O-Methilphaseollinisoflavana Wighteone	Reducido
		Fenol	
13	$C_{17}H_{22}O_{10}$	1-O-Sinapoilglucoso	Aumentado
31	$C_{19}H_{20}O_9$	Garcimangosone D	Reducido
33	$C_{38}H_{44}O_8$	Ácido Gambógico	Reducido
		Lípido	
30	$C_{16}H_{30}O_7$	Ácido 3-O-α-L-rhamnopiranosil-3-hidroxidecanoico	Reducido
36	$C_{31}H_{63}O_7P$	PA(O-16:0/12:0)	Reducido

Tabla 6: Compuestos detectados que se han producido en cantidades significativamente diferentes respecto a las plantas control (CC) cuando han estado en presencia de *R. solani* y *T. velutinum* T028 (RT028) (continuación).

Id[(1)]	Fórmula	Compuesto asignado	RT028-CC
		Péptido	
7	$C_{11}H_{22}N_2O_3$	Valil-Leucine	Aumentado
11	$C_{17}H_{32}N_4O_5$	Ile Gln Ile	Reducido
29	$C_{15}H_{26}N_6O_7$	Gln Gln Gln	Reducido
		Terpeno	
17	$C_{20}H_{32}O_9$	Etil 7-epi-12-hidroxijasmonato glucósido	Aumentado
32	$C_{47}H_{76}O_{16}$	Akeboside Ste	Reducido

(1) Id: Número correspondiente al pico del cromatograma obtenido por HPLC-DAD-TOF-MS del capítulo IV.

En cuanto a los flavonoides ha habido 9 compuestos (Tabla 6) (#**1**, $C_{17}H_{20}O_{11}$, 5-Hidroxi-6,8-dimethoxi-2-oxo-2H-chromen-7-il β-D-glucopiranósido; #**6**, $C_{20}H_{20}O_4$, Phaseollinisoflavana; #**18**, $C_{29}H_{34}O_{17}$, Isopirenin 7-O-glucósido; #**19**, $C_{25}H_{32}O_{13}$, 12-Hidroxi, O-[3,4,5-trihidroxibenzoil-(06)-β-D-glucopiranósido]; #**22**, $C_{19}H_{18}O_8$, 3´,5-Dihidroxi-3,4´,6,7-tetramethoxiflavona; #**24**, $C_{20}H_{38}O_{12}$, (R)-1-O-[β-D-Glucopiranosil-(1-6)-β-D-glucopiranósido]-1,3-octanediol; #**25**, $C_{25}H_{28}O_6$, 2´,4´,5,7-Tetrahidroxi-3´,8-diprenilisoflavanona; #**27**, $C_{19}H_{18}O_9$, 5,2',4'-Trihidroxi-3,7,8,5'-tetramethoxiflavone; #**28**, $C_{21}H_{22}O_4$, 2´-O-Methilphaseollinisoflavana o $C_{20}H_{18}O_5$, Wighteone) que han reducido significativamente su producción frente a 5 que la han incrementado (Tabla 6) (#**10**, $C_{27}H_{32}O_{16}$, 3,4´,5,7-Tetrahidroxiflavanona 3,7-Di-O-β-D-glucopiranósido; #**12**, $C_{26}H_{28}O_{16}$, Quercetin 3-vicianósido; #**14**, $C_{26}H_{32}O_{13}$, (Z)-Resveratrol 3,4'-diglucósido; #**20**, $C_{32}H_{39}O_{16}$, Luteona 4,7-O-diglucósido; #**21**, $C_{29}H_{50}O$, Sitosterol). De los productos que han reducido su cantidad, los compuestos #**24**, #**25** y #**28** también lo han hecho en presencia de *T. velutinum* (CT028) (Tabla 5) por lo que su merma se debe a este hongo y no al patógeno. Sucede lo contrario en los compuestos #**10** y #**20**, que han aumentado su producción con *Trichoderma* (CT028 y RT028) (Tabla 5).

En el caso de los fenoles, se ha producido una reducción significativa de los compuestos #**31** ($C_{19}H_{20}O_9$, Garcimangosone D) y #**33** ($C_{38}H_{44}O_8$, Ácido Gambógico), mientras que se ha incrementado la cantidad significativa del #**13** ($C_{17}H_{22}O_{10}$, 1-O-Sinapoilglucoso) (Tabla 6). Como en los compuestos anteriores, la variación de las cantidades producidas ha variado según la presencia de un hongo u otro. En el caso del #**13** ha aumentado su producción en presencia de *Trichoderma* y en #**33** la ha reducido en la misma situación (Tabla 5 y Tabla 6). Por lo que la presencia del agente de biocontrol va a modificar la producción de estos compuestos.

En cuanto a los lípidos, su producción ha sido significativamente reducida en la interacción con *Trichoderma* y *Rhizoctonia* (RT028) [#**30**, $C_{16}H_{30}O_7$, Ácido 3-O-α-L-rhamnopiranosil-3-hidroxidecanoico; #**36**, $C_{31}H_{63}O_7P$, PA(O-16:0/12:0)] (Tabla 6). Sin embargo, esa reducción se ha debido a diferentes hongos. En el caso de #**30**, ha reducido su cantidad de la misma manera que lo hiciera en presencia sólo de *T. velutinum* (CT028) (Tabla 5) por lo que esta menor producción se ha debido al agente de biocontrol. En cuanto al #**36**, ha disminuido su cantidad de igual modo que lo hiciera en presencia de sólo *R. solani* (RC) (Tabla 3), así que esta reducción la ha causado el patógeno.

Los péptidos, #**11** ($C_{17}H_{32}N_4O_5$, Ile Gln Ile) y #**29** ($C_{15}H_{26}N_6O_7$, Gln Gln Gln) han reducido su cantidad en presencia de *T. velutinum* y *R. solani* (RT028) (Tabla 6). El #**11** ha mermado su cantidad si estaba presente el patógeno (RC y RT028) (Tabla 3 y Tabla 6) y el #**29** lo ha hecho si estaba el agente de biocontrol (CT028 y RT028) (Tabla 5 y Tabla 6). Sin embargo el #**7** ($C_{11}H_{22}N_2O_3$, Valil-Leucine) ha aumentado su cantidad cuando ha estado presente *T. velutinum* (CT028 y RT028) y la ha reducido en presencia sólo de *R. solani* (RC) (Tabla 3, Tabla 5 y Tabla 6).

Los flavonoides #**1**, #**18**, #**19**, #**22** y #**27**, el fenol #**31** y el terpeno #**32** que han reducido su producción en cualquiera de las interacción estudiadas (RC, CT028 y RT028) (Tabla 3, Tabla 5 y Tabla 6). El único compuesto que ha incrementado significativamente su producción en las tres interacciones ha sido el terpeno #**17** (Tabla 3, Tabla 5 y Tabla 6). Esta respuesta por parte de la alubia se puede deber a que considere a *T. velutinum* y *R. solani* como un mismo tipo de microorganismo con lo que la planta responde de la misma forma en cuanto a la producción de estos compuestos.

5. BIBLIOGRAFÍA

Ahuja, I., Kissen, R., y Bones, A. M. (2012). Phytoalexins in defense against pathogens. *Trends Plant Sci.* 17, 73-90. doi:10.1016/j.tplants.2011.11.002.

Anees, M., Tronsmo, A., Edel-Hermann, V., Hjeljord, L. G., Héraud, C., y Steinberg, C. (2010). Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani*. *Fungal Biol.* 114, 691-701. doi:10.1016/j.funbio.2010.05.007.

Angelopoulou, D. J., Naska, E. J., Paplomatas, E. J., y Tjamos, S. E. (2014). Biological control agents (BCAs) of verticillium wilt: Influence of application rates and delivery method on plant protection, triggering of host defence mechanisms and rhizosphere populations of BCAs. *Plant Pathol.* 63, 1062-1069. doi:10.1111/ppa.12198.

Burmeister, L., y Hau, B. (2009). Control of the bean rust fungus *Uromyces appendiculatus* by means of *Trichoderma harzianum*: leaf disc assays on the antibiotic effect of spore suspensions and culture filtrates. *Biocontrol* 54, 575-585. doi:10.1007/s10526-008-9202-9.

Campelo, M. P., Cardoza, R. E., Lorenzana, A., Hermosa, M. R., Monte, E., Reinoso, B., et al. (2010). Biological control of phytopathogenic fungi in bean (*Phaseolus vulgaris*, L.) with *Trichoderma atroviride* and *Trichoderma virens*. en *Bean Improvement Cooperative Fort Collins*, eds. P. Miklas y P. Gepts (Colorado,USA), 114-115.

Cervone, F., Castoria, R., Leckie, F., y De Lorenzo, G. (1997). Perception of fungal elicitors and signal transduction. *Signal Transduct. Plants*, 153-177.

Chet, I., Harman, G. E., y Baker, R. (1981). *Trichoderma hamatum*: Its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microb. Ecol.* 7, 29-38. doi:10.1007/BF02010476.

Daryaei, A., Jones, E. E., Ghazalibiglar, H., Glare, T. R., y Falloon, R. E. (2016). Culturing conditions affect biological control activity of *Trichoderma atroviride* against *Rhizoctonia solani* in ryegrass. *J. Appl. Microbiol.* 121, 461-472. doi:10.1111/jam.13163.

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., et al. (2011). *Trichoderma*: The genomics of opportunistic success. *Nat. Rev. Microbiol.* 9, 749-759.

Durak, E. D. (2016). Biological control of *Rhizoctonia solani* on potato by using indigenous *Trichoderma* spp. en *International Conference on Advances in Natural and Applied Sciences* (Antalya (Turkey): American Institute of Physics Inc.), 20020. doi:10.1063/1.4945846.

Durrant, W. E., y Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185-

209. doi:10.1146/annurev.phyto.42.040803.140421.

Garaiová, M., Zambojová, V., Šimová, Z., Griac, P., y Hapala, I. (2013). Squalene epoxidase as a target for manipulation of squalene levels in the yeast *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 14, 310-323. doi:10.1111/1567-1364.12107.

Guerrero-González, M. L., Rodríguez-Kessler, M., Rodríguez-Guerra, R., González-Chavira, M., Simpson, J., Sanchez, F., et al. (2011). Differential expression of *Phaseolus vulgaris* genes induced during the interaction with *Rhizoctonia solani*. *Plant Cell Rep.* 30, 1465-1473. doi:10.1007/s00299-011-1055-5.

Hanson, L. E., y Howell, C. R. (2004). Elicitors of plant defense responses from biocontrol strains of *Trichoderma virens*. *Phytopathology* 94, 171-176.

Hermosa, M. R., Grondona, I., Díaz-Mínguez, J. M., Iturriaga, E. A., y Monte, E. (2001). Development of a strain-specific SCAR marker for the detection of *Trichoderma atroviride* 11, a biological control agent against soilborne fungal plant pathogens. *Curr. Genet.* 38, 343-350. doi:10.1007/s002940000173.

Larkin, R. P. (2016). Impacts of biocontrol products on *Rhizoctonia* disease of potato and soil microbial communities, and their persistence in soil. *Crop Prot.* 90, 96-105. doi:10.1016/j.cropro.2016.08.012.

Lehtonen, M. J., Somervuo, P., y Valkonen, J. P. T. (2008). Infection with *Rhizoctonia solani* induces defense genes and systemic resistance in potato sprouts grown without light. *Phytopathology* 98, 1190-1198. doi:10.1094/PHYTO-98-11-1190.

Lochman, J., y Mikeš, V. (2005). Activation of different defence-related genes expression by ergosterol. en *FEBS Journal, Abstract of 30th FEBS Congress* (Budapest), 470. Available at: http://www.muni.cz/research/publications/586702.

Lodha, T. D., y Basak, J. (2012). Plant-pathogen interactions: what microarray tells about it? *Mol. Biotechnol.* 50, 87-97. doi:10.1007/s12033-011-9418-2.

Luna-Vital, D. A., Mojica, L., González de Mejía, E., Mendoza, S., y Loarca-Piña, G. (2015). Biological potential of protein hydrolysates and peptides from common bean (*Phaseolus vulgaris* L.): A review. *Food Res. Int.* 76, 39-50. doi:10.1016/j.foodres.2014.11.024.

Malmierca, M. G., Cardoza, R. E., Alexander, N. J., McCormick, S. P., Collado, I. G., Hermosa, R., et al. (2013). Relevance of trichothecenes in fungal physiology: Disruption of tri5 in *Trichoderma arundinaceum*. *Fungal Genet. Biol.* 53, 22-33.

Marra, R., Ambrosino, P., Carbone, V., Vinale, F., Woo, S. L., Ruocco, M., et al. (2006). Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach. *Curr. Genet.* 50, 307-321. doi:10.1007/s00294-006-0091-0.

Mayo, S., Cominelli, E., Sparvoli, F., González-López, O., Rodríguez-González, A., Gutiérrez, S., et al. (2016). Development of a qPCR strategy to select bean genes involved in plant defense response and regulated by the *Trichoderma velutinum – Rhizoctonia solani* interaction. *Front. Plant Sci.* 7, 1109. doi:10.3389/fpls.2016.01109.

Mayo, S., Gutierrez, S., Malmierca, M. G., Lorenzana, A., Campelo, M. P., Hermosa, R., et al. (2015). Influence of *Rhizoctonia solani* and *Trichoderma* spp. in growth of bean (*Phaseolus vulgaris* L.) and in the induction of plant defense-related genes. *Front. Plant Sci.* 6, 685. doi:10.3389/fpls.2015.00685.

Nürnberger, T., Brunner, F., Kemmerling, B., y Piater, L. (2004). Innate immunity in plants and animals: Striking similarities and obvious differences. *Immunol. Rev.* 198, 249-266.

Pfaffl, M. W., Horgan, G. W., y Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 30, e36. doi:10.1093/nar/30.9.e36.

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., y Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489-521. doi:10.1146/annurev-cellbio-092910-154055.

Qureshi, M. A., Iqbal, A., Akhtar, N., Shakir, M. A., y Khan, A. (2012). Co-inoculation of phosphate solubilizing bacteria and rhizobia in the presence of L-tryptophan for the promotion of mash bean (*Vigna mungo* L.). *Soil Environ.* 31, 47-54.

Rivière, M.-P., Marais, A., Ponchet, M., Willats, W., y Galiana, E. (2008). Silencing of acidic pathogenesis-related *PR-1* genes increases extracellular β-(1→3)-glucanase activity at the onset of tobacco defence reactions. *J. Exp. Bot.* 59, 1225-1239. doi:10.1093/jxb/ern044.

Ruano-Rosa, D., del Moral-Navarrete, L., y Lopez-Herrera, C. J. (2010). Selection of *Trichoderma* spp. isolates antagonistic to *Rosellinia necatrix*. *Spanish J. Agric. Res.* 8, 1084-1097.

Shoresh, M., Harman, G. E., y Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 48, 21-43. doi:10.1146/annurev-phyto-073009-114450.

Tadeo, F. R., y Gómez-Cadenas, A. (2008). «Fisiología de las plantas y el estrés», en *Fundamentos de fisología vegetal*, eds. J. Azcón-Bieto y M. Talón (Barcelona, Spain: McGraw-Hill), 577-597.

VanEtten, H. D., Mansfield, J. W., Bailey, J. A., y Farmer, E. E. (1994). Two Classes of Plant Antibiotics: Phytoalexins versus «Phytoanticipins». *Plant Cell* 6, 1191-1192. doi:10.1105/tpc.6.9.1191.

Vargas, W. A., Crutcher, F. K., y Kenerley, C. M. (2011). Functional characterization of a plant-like sucrose transporter from the beneficial fungus *Trichoderma virens*. Regulation of the symbiotic association with plants by sucrose metabolism inside the fungal cells. *New Phytol.* 189, 777-789. doi:10.1111/j.1469-8137.2010.03517.x.

Verhagen, B. W. M., Van Loon, L. C., y Pieterse, C. M. J. (2006). «Induced disease resistance signaling in plants», en *Floriculture, Ornamental and Plant Biotechnology* (Utrecht (Holande): Global Science Books), 334-343.

Vinale, F., Flematti, G., Sivasithamparam, K., Lorito, M., Marra, R., Skelton, B. W., et al. (2009). Harzianic acid, an antifungal and plant growth promoting metabolite from *Trichoderma harzianum*. *J. Nat. Prod.* 72, 2032-2035. doi:10.1021/np900548p.

Zhao, Y., Thilmony, R., Bender, C. L., Schaller, A., He, S. Y., y Howe, G. A. (2003). Virulence systems of *Pseudomonas syringae* pv. *tomato* promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. *Plant J.* 36, 485-499. doi:10.1046/j.1365-313X.2003.01895.x.

CONCLUSIONES

CONCLUSIONES

- Existe una alta diversidad de especies de *Trichoderma* donde la alubia forma parte de la rotación de cultivos en zonas pertenecientes a la IGP "Alubia de La Bañeza-León", siendo *T. harzianum* la especie más frecuente.
- Los aislamientos de *Trichoderma* inhiben el desarrollo de *Rhizoctonia solani* en condiciones *in vitro*, sin diferencias entre los extraídos de semilla y de suelo.
- En condiciones *in vivo*, las plantas de alubia en contacto con *R. solani* reducen su desarrollo. Sin embargo, en contacto con algunos aislamientos de *Trichoderma*, incrementan su desarrollo.
- Las plantas de alubia muestran un desarrollo y una protección frente al patógeno mayor con aquellos asilamientos de *Trichoderma* extraídos de muestras de suelo que los procedentes de semilla. Su eficacia se debe a que tanto *R. solani* como *Trichoderma* han sufrido una evolución conjunta en la rizosfera haciendo que sea más eficiente el biocontrol de este fitopatógeno presente en los suelos de cultivo.
- Los aislamientos *T. harzianum* T019 y *T. velutinum* T028, extraídos de semilla y de suelo respectivamente, incrementan el desarrollo de la alubia y en presencia de *R. solani* controlan su poder patógeno.
- Un aumento de la producción de ergosterol y escualeno en *Trichoderma* produce la inducción de los genes de defensa de la planta de alubia, desarrollándose mejor en presencia del patógeno.
- De los 48 genes analizados inicialmente, se seleccionan 16 genes. Sólo *WRKY33*, *CH5b* y *hGS* muestran una regulación positiva en la alubia en presencia de *T. velutinum* mientras que los otros genes son regulados negativamente. La interacción con *R. solani* provoca una regulación negativa de la mayoría de los genes, excepto *PR1*, *OSM34* y *CNGC2*. La interacción de ambos hongos regula positivamente *hGS* y negativamente el resto de genes a excepción de *CH5b* que no es afectado.
- La interacción de la alubia con *Trichoderma* y/o *R. solani* influye en la producción de metabolitos por parte de la planta. La presencia del patógeno provoca una menor producción de metabolitos. *T. velutinum* modifica algunos compuestos de la alubia, produciéndolos en menor o mayor cantidad según sea la función de los mismos. Hay compuestos que son producidos por la planta de modo similar en presencia de cualquiera de los dos hongos provocando la misma respuesta.

CONCLUSIONS

- There is a high diversity of *Trichoderma* spp. where bean is a rotation crop in areas belonging to the PGI "Alubia de La Bañeza-León", being *T. harzianum* the most frequent species.
- *Trichoderma* isolates inhibite the development of *R. solani* under *in vitro* conditions, without differences between *Trichoderma* extracted from bean seed and from soil.
- On *in vivo* conditions, bean plants in contact with *R. solani* reduce the development. However, when plants are in contact with the *Trichoderma* isolates always show an increased size.
- Bean plants show higher development and protection against the pathogen when *Trichoderma* isolates is extracted from soil samples in comparison with those obtained from bean seed. Their effectiveness can be explained because *R. solani* and *Trichoderma* have co-evolved in the rhizosphere, making biocontrol of the phytopathogens more efficient.
- Isolates *T. harzianum* T019 and *T. velutinum* T028, from bean seed and soil, respectively, increase their development and in the presence of *R. solani* control their pathogen power.
- An increased production of ergosterol and squalene by *Trichoderma* resulte in the induction of defence genes in the bean plants. In this way plants would grow better under a pathogen presence in the soil.
- From 48 genes initially analysed, 16 bean genes are selected, only *WRKY33*, *CH5b* and *hGS* show an up-regulatory response in the bean plant in the presence of *T. velutinum* and the other genes were down-regulated. *R. solani* interaction resulte in a down-regulation of most of the genes analysed, except *PR1*, *OSM34* and *CNGC2*. The presence of both, *T. velutinum* and *R. solani*, up-regulated *hGS* and down-regulated all the other genes analysed, except *CH5b* is not affected.
- The interaction of bean plants with *Trichoderma* and/or *R. solani* influenced the production of metabolites by the plant. The presence of the pathogen caused a minor production of the metabolites. *T. velutinum* modifies some compounds of the bean plants, in an increase or decrease amount depending on their function. Some products is detected which modified their production in the presence of either of the two fungi causing the same response in the bean plants.

ANEXOS

ÍNDICE ANEXOS

TABLAS

ANEXOS

1. ENSAYOS *IN VIVO*

Tabla A. 1: Aislamientos de *R. solani* y de *Trichoderma* empleados en los ensayos

Código colección[(1)]	Código laboratorio	Especie	Cultivo	Localidad	Zona productiva
Origen: Semilla					
Patógeno					
PAULER006	R43	*R. solani*	Alubia	Santa María del Páramo	El Páramo
Trichoderma spp.					
PAULET20	T001	*T. harzianum*	Alubia	Antoñanes del Páramo	El Páramo
PAULET21	T002	*T. harzianum*	Alubia	Moscas del Páramo	El Páramo
PAULET22	T003	*T. harzianum*	Alubia	Bercianos del Páramo	El Páramo
PAULET23	T004	*T. gamsii*	Alubia	Riego de la Vega	La Bañeza
PAULET24	T005	*T. longibrachiatum*	Alubia	Carral	Maragatería-Cepeda
PAULET25	T006	*Trichoderma* sp.	Alubia	San Esteban de Nogales	La Bañeza
PAULET26	T007	*T. harzianum*	Alubia	Sueros de Cepeda	Maragatería-Cepeda
PAULET27	T008	*T. citrinoviride*	Alubia	Fresno de la Vega	Esla - Campos
PAULET28	T009	*T. koningiopsis*	Alubia	Acebes del Páramo	El Páramo
PAULET29	T010	*T. harzianum*	Alubia	Acebes del Páramo	El Páramo
PAULET30	T011	*T. harzianum*	Alubia	La Mata del Páramo	El Páramo
PAULET31	T012	*T. harzianum*	Alubia	Donillas	Maragatería-Cepeda
PAULET32	T013	*T. atroviride*	Alubia	Donillas	Maragatería-Cepeda

Tabla A. 1: Aislamientos de *R. solani* y de *Trichoderma* empleados en los ensayos (continuación).

Código colección	Código laboratorio	Especie	Cultivo	Localidad	Zona productiva
PAULET33	T014	*T. virens*	Alubia	Villavante	El Páramo
PAULET34	T015	*T. harzianum*	Alubia	Veguellina de Fondo	El Páramo
PAULET35	T016	*T. harzianum*	Alubia	Bustillo del Páramo	El Páramo
PAULET36	T017	*Trichoderma* sp.	Alubia	Bustillo del Páramo	El Páramo
PAULET37	T018	*T. harzianum*	Alubia	Urdiales del Páramo	El Páramo
PAULET38	T019	*T. harzianum*	Alubia	Villanueva de Carrizo	Maragatería-Cepeda
PAULET39	T020	*T. harzianum*	Alubia	Soto de la Vega	La Bañeza
PAULET40	T021	*T. harzianum*	Alubia	Altobar de la Encomienda	El Páramo
PAULET41	T022	*T. harzianum*	Alubia	Villaornate	Esla - Campos
PAULET42	T023	*Trichoderma* sp.	Alubia	Jabares de los Oteros	Esla - Campos
Origen: Laboratorio					
IMI 352941	T024	*T. atroviride*		(Hermosa et al., 2000)	
NBT59	T025	*T. virens*		(Hermosa et al., 2004)	
CECT2413[(2)]	T34	*T. harzianum*		(Kullnig et al., 2001)	
Origen: Suelo					
IASULE1	T027	*T. velutinum*	Alubia	Otero de Escarpizo	Maragatería-Cepeda
IASULE2	T028	*T. velutinum*	Alubia	Otero de Escarpizo	Maragatería-Cepeda
IASULE3	T029	*T. velutinum*	Alubia	Otero de Escarpizo	Maragatería-Cepeda
IASULE4	T030	*T. viridescens*	Trigo	Otero de Escarpizo	Maragatería-Cepeda

Tabla A. 1: Aislamientos de *R. solani* y de *Trichoderma* empleados en los ensayos (continuación).

Código colección	Código laboratorio	Especie	Cultivo	Localidad	Zona productiva
IASULE5	T031	*T. koningiopsis*	Alubia	Sueros de Cepeda	Maragatería-Cepeda
IASULE6	T032	*T. virens*	Trigo	San Juan de Torres	La Bañeza
IASULE7	T033	*T. harzianum*	Trigo	San Juan de Torres	La Bañeza
IASULE8	T034	*T. harzianum*	Trigo	San Juan de Torres	La Bañeza
IASULE9	T035	*T. harzianum*	Alubia	San Juan de Torres	La Bañeza
IASULE10	T036	*T. velutinum*	Alubia	San Juan de Torres	La Bañeza
IASULE11	T037	*T. velutinum*	Alubia	San Juan de Torres	La Bañeza
IASULE12	T038	*T. viridescens*	Alubia	Castrocontrigo	La Cabrera
IASULE13	T039	*T. virens*	Alubia	Castrocontrigo	La Cabrera
IASULE14	T040	*T. koningiopsis*	Alubia	Castrocontrigo	La Cabrera
IASULE15	T041	*T. viridescens*	Patata	Castrocontrigo	La Cabrera
IASULE16	T042	*T. gamsii*	Patata	Castrocontrigo	La Cabrera
IASULE17	T043	*T. virens*	Alubia	Javares de los Oteros	Esla-Campos
IASULE18	T044	*T. harzianum*	Alubia	Javares de los Oteros	Esla-Campos
IASULE19	T045	*T. harzianum*	Alubia	Javares de los Oteros	Esla-Campos
IASULE20	T046	*T. virens*	Soja	Javares de los Oteros	Esla-Campos
IASULE21	T047	*T. virens*	Maíz	Cubillas	Esla-Campos
IASULE22	T048	*T. spirale.*	Maíz	Cubillas	Esla-Campos

Tabla A. 1: Aislamientos de *R. solani* y de *Trichoderma* empleados en los ensayos (continuación).

Código colección	Código laboratorio	Especie	Cultivo	Localidad	Zona productiva
IASULE23	T049	*T. virens*	Alubia	Bercianos del Páramo	Páramo
IASULE24	T050	*T. harzianum*	Alubia	Bercianos del Páramo	Páramo
IASULE25	T051	*T. velutinum*	Alubia	Bercianos del Páramo	Páramo
IASULE26	T052	*T. harzianum*	Alubia	Bercianos del Páramo	Páramo
IASULE27	T053	*T. virens*	Alubia	Bercianos del Páramo	Páramo
IASULE28	T054	*T. brevicompactum*	Girasol	Bercianos del Páramo	Páramo
IASULE29	T055	*T. harzianum*	Remolacha	La Milla del Páramo	Páramo
IASULE30	T056	*T. hamatum*	Remolacha	La Milla del Páramo	Páramo
IASULE31	T057	*T. gamsii*	Remolacha	La Milla del Páramo	Páramo
IASULE32	T058	*T. harzianum*	Alubia	La Milla del Páramo	Páramo
IASULE33	T059	*T. harzianum*	Alubia	La Milla del Páramo	Páramo
IASULE34	T060	*T. harzianum*	Alubia	La Milla del Páramo	Páramo
IASULE35	T061	*T. harzianum*	Alubia	La Milla del Páramo	Páramo

(1) PAULE: aislamientos de la colección de Patógenos y Antagonistas del Laboratorio de Diagnóstico de Plagas y Enfermedades de Vegetales de la Universidad de León. IASULE: aislamientos de la colección de Patógenos y Antagonistas del Grupo Universitario de Investigación en Ingeniería y Agricultura Sostenible de la Universidad de León. IMI, CABI Bioscience (Egham, Reino Unido); NBT, Newbiotechnic S.A. (Sevilla, España); CECT, Colección Española de Cultivo Tipo, (Burjasot, España).

(2) Aislamiento empleado en el ensayo de ergosterol y escualeno.

Tabla A. 2: Germinación por maceta (Media ± Error estándar y porcentaje) de cada tratamiento medida a los 24 días tras la siembra.

Tratamiento	Germinación día 24	%	Tratamiento	Germinación día 24	%
CC	1,606 ± 0,044	80,28	RC	1,389 ± 0,055	69,44
CT001	1,033 ± 0,140	51,67	RT001	0,900 ± 0,121	45,00
CT002	1,233 ± 0,149	61,67	RT002	0,700 ± 0,119	35,00
CT003	1,033 ± 0,131	51,67	RT003	0,567 ± 0,141	28,33
CT004	1,333 ± 0,111	66,67	RT004	0,667 ± 0,130	33,33
CT005	1,067 ± 0,117	53,33	RT005	0,833 ± 0,128	41,67
CT006	1,000 ± 0,117	50,00	RT006	0,833 ± 0,136	41,67
CT007	1,100 ± 0,139	55,00	RT007	0,400 ± 0,123	20,00
CT008	1,167 ± 0,108	58,33	RT008	1,200 ± 0,130	60,00
CT011	0,800 ± 0,139	40,00	RT011	0,233 ± 0,079	11,67
CT012	1,333 ± 0,111	66,67	RT012	0,467 ± 0,115	23,33
CT013	1,067 ± 0,117	53,33	RT013	0,867 ± 0,124	43,33
CT015	0,967 ± 0,112	48,33	RT015	0,367 ± 0,102	18,33
CT019	1,267 ± 0,117	63,33	RT019	0,667 ± 0,130	33,33
CT024	0,967 ± 0,140	48,33	RT024	0,167 ± 0,084	8,33
CT025	1,067 ± 0,143	53,33	RT025	0,233 ± 0,079	11,67
CT028	2,000 ± 0,000	100,00	RT028	1,967 ± 0,033	98,33
CT029	1,767 ± 0,079	88,33	RT029	1,633 ± 0,102	81,67
CT030	1,767 ± 0,079	88,33	RT030	1,700 ± 0,085	85,00
CT031	1,800 ± 0,074	90,00	RT031	1,500 ± 0,115	75,00
CT032	1,867 ± 0,063	93,33	RT032	1,400 ± 0,113	70,00
CT033	1,567 ± 0,104	78,33	RT033	1,400 ± 0,113	70,00
CT034	1,533 ± 0,115	76,67	RT034	1,633 ± 0,112	81,67
CT035	1,967 ± 0,033	98,33	RT035	1,900 ± 0,074	95,00
CT036	1,967 ± 0,033	98,33	RT036	2,000 ± 0,000	100,00
CT037	2,000 ± 0,000	100,00	RT037	1,867 ± 0,063	93,33
CT038	2,000 ± 0,000	100,00	RT038	1,933 ± 0,046	96,67
CT039	1,833 ± 0,069	91,67	RT039	1,800 ± 0,088	90,00
CT040	2,000 ± 0,000	100,00	RT040	1,933 ± 0,046	96,67
CT041	2,000 ± 0,000	100,00	RT041	1,967 ± 0,033	98,33
CT043	1,600 ± 0,123	80,00	RT043	1,667 ± 0,111	83,33
CT044	1,633 ± 0,112	81,67	RT044	1,767 ± 0,079	88,33
CT045	1,833 ± 0,069	91,67	RT045	1,433 ± 0,114	71,67
CT046	1,767 ± 0,092	88,33	RT046	1,600 ± 0,103	80,00
CT047	1,733 ± 0,095	86,67	RT047	1,567 ± 0,104	78,33
CT048	1,767 ± 0,092	88,33	RT048	1,767 ± 0,092	88,33
CT050	1,833 ± 0,069	91,67	RT050	1,867 ± 0,063	93,33
CT053	1,833 ± 0,069	91,67	RT053	1,700 ± 0,098	85,00
CT054	1,867 ± 0,063	93,33	RT054	1,733 ± 0,095	86,67
CT055	1,467 ± 0,115	73,33	RT055	1,600 ± 0,103	80,00
CT056	1,967 ± 0,033	98,33	RT056	1,733 ± 0,082	86,67
CT057	1,733 ± 0,082	86,67	RT057	1,833 ± 0,069	91,67
CT058	1,933 ± 0,046	96,67	RT058	1,900 ± 0,056	95,00

Tabla A. 2: Germinación por maceta (Media ± Error estándar) de cada tratamiento medida a los 24 días tras la siembra (continuación).

Tratamiento	Germinación día 24	%	Tratamiento	Germinación día 24	%
CT059	1,933 ± 0,046	96,67	RT059	1,833 ± 0,084	91,67
CT060	1,767 ± 0,092	88,33	RT060	1,833 ± 0,069	91,67
CT061	1,933 ± 0,046	96,67	RT061	1,933 ± 0,046	96,67

Tabla A. 3: Evaluación a los 45 días tras la siembra del diámetro del hipocotilo (Media ± Error estándar) (mm) y de la longitud del sistema radicular (Media ± Error estándar) (cm).

Trat. (1)	Diámetro del hipocotilo (mm)			Trat. (1)	Longitud del sistema radicular (cm)		
CT035	4,11	± 0,07	a	RT050	20,87	± 0,86	a
RT057	4,05	± 0,14	ab	CT045	19,94	± 0,80	ab
CT041	3,96	± 0,06	abc	RT048	19,91	± 0,77	abc
RT048	3,95	± 0,13	abc	RT043	19,27	± 1,20	abcd
CT028	3,94	± 0,11	abc	CT050	19,24	± 0,90	abcd
CT031	3,93	± 0,22	abc	CT028	19,18	± 0,65	abcde
CT040	3,92	± 0,07	abcd	RT036	18,82	± 2,63	abcdef
CT037	3,92	± 0,07	abcd	RT046	18,70	± 1,20	abcdef
RT035	3,90	± 0,13	abcde	CT059	18,57	± 0,66	abcdefg
CT038	3,87	± 0,07	abcdef	CT047	18,32	± 0,84	abcdefgh
CT053	3,87	± 0,16	abcdef	RT059	18,29	± 0,70	abcdefgh
CT036	3,85	± 0,10	abcdef	RT060	18,15	± 0,79	abcdefghi
CT059	3,85	± 0,12	abcdef	CT053	18,01	± 0,84	abcdefghij
RT041	3,82	± 0,11	abcdefg	RT044	17,91	± 0,97	abcdefghij
RT059	3,81	± 0,13	abcdefgh	RT057	17,89	± 0,74	abcdefghij
CT061	3,81	± 0,08	abcdefgh	CT048	17,79	± 0,78	abcdefghijk
RT034	3,78	± 0,25	abcdefghi	CT043	17,73	± 1,14	abcdefghijkl
CT045	3,78	± 0,13	abcdefghi	RT053	17,34	± 1,07	bcdefghijklm
RT060	3,78	± 0,14	abcdefghi	CT046	17,25	± 0,98	bcdefghijklm
CT039	3,77	± 0,14	abcdefghij	CT032	17,20	± 0,76	bcdefghijklm
CT030	3,76	± 0,20	abcdefghij	CT035	17,20	± 0,44	bcdefghijklm
CT048	3,75	± 0,16	abcdefghij	RT040	17,13	± 0,63	bcdefghijklmn
RT036	3,75	± 0,12	abcdefghij	RT054	17,10	± 0,99	bcdefghijklmn
CT032	3,73	± 0,15	abcdefghij	CT060	16,98	± 0,84	bcdefghijklmn
RT028	3,73	± 0,10	abcdefghij	CT058	16,94	± 0,60	bcdefghijklmn
RT040	3,69	± 0,11	abcdefghij	CT057	16,80	± 0,85	bcdefghijklmno
CT047	3,68	± 0,16	abcdefghijk	RT028	16,70	± 0,61	bcdefghijklmno

Tabla A. 3: Evaluación a los 45 días tras la siembra del diámetro del hipocotilo (Media ± Error estándar) (mm) y de la longitud del sistema radicular (Media ± Error estándar) (cm) (continuación).

Trat. (1)	Diámetro del hipocotilo (mm)			Trat.(1)	Longitud del sistema radicular (cm)		
CT029	3,67	± 0,20	abcdefghijk	CT036	16,69	± 0,53	cdefghijklmnop
RT044	3,66	± 0,18	abcdefghijk	CT041	16,61	± 0,60	defghijklmnopq
RT037	3,65	± 0,16	abcdefghijkl	CT039	16,52	± 0,68	defghijklmnopq
RT061	3,64	± 0,12	abcdefghijkl	RT041	16,52	± 0,58	defghijklmnopq
RT050	3,64	± 0,14	abcdefghijkl	RT047	16,49	± 1,15	defghijklmnopq
CT046	3,62	± 0,18	abcdefghijkl	RT035	16,48	± 0,66	defghijklmnopq
RT038	3,62	± 0,11	abcdefghijkl	CT044	16,37	± 1,16	defghijklmnopq
CT058	3,60	± 0,11	abcdefghijkl	CT061	16,35	± 0,50	defghijklmnopq
CT050	3,59	± 0,17	abcdefghijkl	CT054	16,35	± 0,66	defghijklmnopq
CT054	3,58	± 0,13	abcdefghijkl	CT056	16,31	± 0,53	defghijklmnopqr
RT030	3,57	± 0,21	abcdefghijkl	CT038	16,24	± 0,58	defghijklmnopqr
CT056	3,57	± 0,10	abcdefghijkl	RT061	16,22	± 0,60	defghijklmnopqr
CT033	3,57	± 0,23	abcdefghijkl	CC	16,14	± 0,47	defghijklmnopqr
CT057	3,56	± 0,15	abcdefghijklm	CT005	15,97	± 5,00	efghijklmnopqr
RT053	3,48	± 0,19	bcdefghijklmn	RT045	15,85	± 1,34	fghijklmnopqrs
RT029	3,45	± 0,24	cdefghijklmno	CT029	15,82	± 0,85	fghijklmnopqrst
RT039	3,43	± 0,16	cdefghijklmno	CT030	15,68	± 0,83	fghijklmnopqrst
RT058	3,42	± 0,11	cdefghijklmno	CT037	15,67	± 0,51	fghijklmnopqrst
CT034	3,39	± 0,26	cdefghijklmnop	RT056	15,46	± 0,91	ghijklmnopqrstu
CT044	3,34	± 0,21	defghijklmnopq	CT034	15,17	± 1,21	hijklmnopqrstuv
CT060	3,34	± 0,15	defghijklmnopq	CT019	15,16	± 1,55	hijklmnopqrstuv
RT046	3,34	± 0,19	efghijklmnopq	CT040	15,14	± 0,56	hijklmnopqrstuvw
CT043	3,33	± 0,20	fghijklmnopq	RT058	15,14	± 0,58	hijklmnopqrstuvw
CC	3,31	± 0,09	fghijklmnopq	RT039	15,03	± 0,83	ijklmnopqrstuvwx
RT056	3,30	± 0,18	fghijklmnopq	CT031	14,91	± 0,77	jklmnopqrstuvwx
RT047	3,26	± 0,22	ghijklmnopq	RT038	14,63	± 0,60	klmnopqrstuvwxy
CT012	3,24	± 0,31	hijklmnopq	CT012	14,59	± 1,40	klmnopqrstuvwxy

Tabla A. 3: Evaluación a los 45 días tras la siembra del diámetro del hipocotilo (Media ± Error estándar) (mm) y de la longitud del sistema radicular (Media ± Error estándar) (cm) (continuación).

Trat. [1]	Diámetro del hipocotilo (mm)			Trat. [1]	Longitud del sistema radicular (cm)		
RT043	3,21	± 0,20	ijklmnopq	RT055	14,53	± 1,03	lmnopqrstuvwxy
RT054	3,19	± 0,18	jklmnopq	CT033	14,18	± 1,00	mnopqrstuvwxy
RT032	3,10	± 0,31	klmnopqr	CT004	13,95	± 1,34	nopqrstuvwxyz
RT031	3,08	± 0,25	lmnopqr	CT055	13,70	± 1,12	opqrstuvwxyz
CT019	2,98	± 0,31	mnopqrs	RT030	13,61	± 0,88	opqrstuvwxyz
RT055	2,91	± 0,20	nopqrst	RT029	13,46	± 0,99	pqrstuvwxyzA
RC	2,89	± 0,11	opqrst	CT008	13,41	± 1,45	qrstuvwxyzA
RT033	2,88	± 0,27	pqrst	RT008	13,01	± 1,40	rstuvwxyzA
CT004	2,82	± 0,27	pqrstu	RT037	12,68	± 0,76	stuvwxyzAB
RT045	2,81	± 0,23	qrstu	RC	12,61	± 0,49	stuvwxyzAB
CT055	2,59	± 0,20	rstuv	CT002	12,61	± 1,28	tuvwxyzAB
CT025	2,56	± 0,32	rstuvw	CT025	12,31	± 1,50	uvwxyzAB
CT007	2,55	± 0,32	rstuvw	RT034	12,24	± 0,88	uvwxyzABC
RT008	2,53	± 0,28	rstuvw	RT032	11,93	± 1,05	vwxyzABC
CT015	2,46	± 0,33	stuvw	CT013	11,91	± 1,42	wxyzABC
CT002	2,45	± 0,25	tuvw	RT031	11,88	± 1,05	xyzABC
CT013	2,37	± 0,29	tuvwx	CT003	11,59	± 1,43	yzABC
CT024	2,35	± 0,33	tuvwx	CT001	11,56	± 1,46	yzABC
CT008	2,29	± 0,26	uvwx	CT015	11,56	± 1,56	yzABC
CT001	2,22	± 0,28	vwxy	CT007	11,49	± 1,46	yzABCD
CT006	2,15	± 0,27	vwxy	RT033	10,88	± 1,08	zABCD
CT003	2,13	± 0,28	vwxyz	CT006	10,76	± 1,33	zABCD
CT005	2,12	± 0,27	vwxyz	CT024	10,25	± 1,41	ABCDE
CT011	2,08	± 0,32	vwxyz	CT011	9,62	± 1,52	BCDEF
RT001	1,99	± 0,30	wxyz	RT001	9,46	± 1,38	BCDEF
RT005	1,87	± 0,29	xyzA	RT005	9,07	± 1,39	CDEF
RT013	1,80	± 0,28	xyzAB	RT006	8,31	± 1,31	DEFG

Tabla A. 3: Evaluación a los 45 días tras la siembra del diámetro del hipocotilo (Media ± Error estándar) (mm) y de la longitud del sistema radicular (Media ± Error estándar) (cm) (continuación).

Trat. (1)	Diámetro del hipocotilo (mm)			Trat. (1)	Longitud del sistema radicular (cm)		
RT006	1,65	± 0,26	yzAB	RT013	8,28	± 1,29	DEFG
RT019	1,56	± 0,29	zABC	RT002	7,42	± 1,34	EFGH
RT004	1,31	± 0,24	ABCD	RT019	7,33	± 1,38	EFGH
RT002	1,28	± 0,23	BCD	RT004	6,96	± 1,26	FGHI
RT003	1,23	± 0,25	BCD	RT003	6,56	± 1,33	FGHI
RT012	1,07	± 0,26	CDE	RT012	5,60	± 1,34	GHIJ
RT015	0,88	± 0,24	DEF	RT015	4,77	± 1,27	HIJK
RT007	0,83	± 0,24	DEF	RT007	3,98	± 1,12	IJK
RT011	0,55	± 0,20	EF	RT011	2,70	± 0,98	JK
RT025	0,49	± 0,18	F	RT025	2,63	± 0,95	JK
RT024	0,35	± 0,15	F	RT024	1,76	± 0,76	K

En la comparación de medias, los valores con letras diferentes presentan diferencias significativas (test DMS, $p < 0,05$).

(1) Trat.: Tratamientos, El control representa aquellas semillas que no se ha inoculado ningún hongo (CC) o bien sólo *R. solani* (RC) y si han sido inoculados sólo con el aislamiento correspondiente de *Trichoderma* (CT-#) o si han sido inoculado tanto con el aislamiento de *Trichoderma* como con *R. solani* (RT-#).

Tabla A. 4: Evaluación a los 45 días tras la siembra del peso húmedo (Media ± Error estándar) (g) y del peso seco (Media ± Error estándar) (g) de la parte aérea.

Trat.[(1)]	Peso húmedo de la parte aérea (g)		Trat.[(1)]	Peso seco de la parte aérea (g)	
CT033	17,89 ± 1,56	a	CT032	3,96 ± 0,17	a
CT028	17,53 ± 1,08	ab	CT033	3,84 ± 0,27	ab
CT034	17,28 ± 1,58	abc	RT029	3,75 ± 0,27	abc
CT035	17,24 ± 0,98	abc	CT034	3,71 ± 0,28	abc
CT039	16,99 ± 1,07	abc	CT029	3,71 ± 0,20	abc
RT029	16,95 ± 1,47	abc	CT031	3,60 ± 0,19	abcd
CT032	16,28 ± 0,97	abcd	RT030	3,56 ± 0,22	bcd
RT035	16,28 ± 1,11	abcd	CT030	3,47 ± 0,19	dc
RT038	16,24 ± 1,18	abcd	RT031	3,32 ± 0,28	de
CT037	15,85 ± 0,90	abcde	RT034	3,29 ± 0,24	def
RT028	15,56 ± 0,86	abcdef	RT032	2,97 ± 0,27	ef
CT036	15,41 ± 0,86	abcdefg	RT033	2,94 ± 0,28	f
CT041	15,33 ± 0,87	bcdefg	CT028	2,12 ± 0,13	g
RT040	15,22 ± 1,00	bcdefgh	RT028	2,10 ± 0,11	gh
CT038	15,10 ± 0,87	bcdefgh	RT038	1,87 ± 0,12	ghi
CT029	15,09 ± 1,03	bcdefgh	CT039	1,86 ± 0,12	ghij
CT040	15,02 ± 0,99	bcdefghi	CT038	1,85 ± 0,10	ghij
RT041	14,86 ± 0,81	cdefghij	CT037	1,84 ± 0,09	ghij
RT031	14,36 ± 1,39	defghijk	CT035	1,84 ± 0,10	ghijk
RT039	14,36 ± 1,27	defghijk	RT040	1,83 ± 0,12	ghijk
RT030	14,35 ± 1,08	defghijk	CT061	1,81 ± 0,08	ghijkl
CT031	13,89 ± 0,97	defghijkl	RT041	1,79 ± 0,10	ghijkl
CT061	13,79 ± 0,61	defghijklm	CC	1,76 ± 0,08	ghijkl
RT036	13,78 ± 1,17	defghijklm	CT041	1,75 ± 0,11	hijklm
RT059	13,58 ± 0,67	efghijklmn	RT035	1,71 ± 0,11	ijklmn
CT054	13,44 ± 0,72	efghijklmno	RT057	1,66 ± 0,08	ijklmno
RT048	13,44 ± 0,62	efghijklmno	RT059	1,66 ± 0,09	ijklmno

Tabla A. 4: Evaluación a los 45 días tras la siembra del peso húmedo (Media ± Error estándar) (g) y del peso seco (Media ± Error estándar) (g) de la parte aérea (continuación).

Trat.[(1)]	Peso húmedo de la parte aérea (g)			Trat.[(1)]	Peso seco de la parte aérea (g)		
RT057	13,40	± 0,63	efghijklmno	CT040	1,66	± 0,10	ijklmno
RT034	13,22	± 1,29	fghijklmnop	CT058	1,66	± 0,18	ijklmnop
CT030	13,15	± 0,92	fghijklmnop	CT059	1,65	± 0,08	ijklmnop
CT019	12,96	± 1,62	ghijklmnopq	RT036	1,63	± 0,12	ijklmnopq
CT059	12,70	± 0,55	hijklmnopqr	RT060	1,59	± 0,07	ijklmnopqr
CT048	12,69	± 0,71	hijklmnopqr	CT036	1,59	± 0,09	ijklmnopqr
CC	12,52	± 0,55	ijklmnopqrs	CT054	1,58	± 0,08	ijklmnopqr
RT033	12,48	± 1,38	ijklmnopqrs	RT039	1,58	± 0,13	ijklmnopqr
RT032	12,43	± 1,33	jklmnopqrs	RC	1,57	± 0,08	ijklmnopqrs
RT060	12,42	± 0,63	jklmnopqrst	CT057	1,56	± 0,09	ijklmnopqrs
CT060	12,23	± 0,77	klmnopqrst	CT050	1,55	± 0,08	ijklmnopqrs
RT044	12,07	± 0,75	klmnopqrstu	CT060	1,54	± 0,09	ijklmnopqrs
CT050	11,93	± 0,65	klmnopqrstu	RT048	1,49	± 0,07	jklmnopqrst
CT045	11,86	± 0,59	klmnopqrstu	CT048	1,47	± 0,09	klmnopqrst
RT061	11,84	± 0,54	klmnopqrstu	RT061	1,47	± 0,07	klmnopqrst
RT050	11,68	± 0,58	lmnopqrstuv	CT045	1,46	± 0,07	lmnopqrst
RT055	11,65	± 0,92	lmnopqrstuv	RT055	1,40	± 0,11	mnopqrst
RT037	11,56	± 0,91	lmnopqrstuv	CT056	1,39	± 0,06	nopqrst
CT057	11,52	± 0,67	lmnopqrstuv	CT053	1,38	± 0,08	nopqrst
CT056	11,41	± 0,49	lmnopqrstuvw	RT037	1,38	± 0,11	nopqrst
CT044	11,37	± 0,80	lmnopqrstuvw	RT050	1,37	± 0,07	nopqrst
CT053	11,32	± 0,60	mnopqrstuvw	CT044	1,35	± 0,10	nopqrstu
RT046	11,25	± 0,73	mnopqrstuvw	RT046	1,33	± 0,09	opqrstuv
CT058	11,19	± 0,54	nopqrstuvwx	CT019	1,33	± 0,16	opqrstuv
CT047	11,03	± 0,60	nopqrstuvwx	RT044	1,33	± 0,08	opqrstuv
CT046	11,01	± 0,71	opqrstuvwxy	CT046	1,32	± 0,08	opqrstuv
RC	10,72	± 0,50	pqrstuvwxy	CT047	1,31	± 0,07	opqrstuv

Tabla A. 4: Evaluación a los 45 días tras la siembra del peso húmedo (Media ± Error estándar) (g) y del peso seco (Media ± Error estándar) (g) de la parte aérea (continuación).

Trat.[(1)]	Peso húmedo de la parte aérea (g)			Trat.[(1)]	Peso seco de la parte aérea (g)		
RT056	10,68	± 0,71	pqrstuvwxy	RT058	1,30	± 0,07	pqrstuvw
RT043	10,54	± 0,71	qrstuvwxy	RT043	1,29	± 0,09	qrstuvw
RT058	10,48	± 0,54	qrstuvwxy	RT056	1,29	± 0,08	qrstuvw
RT054	10,42	± 0,78	qrstuvwxy	RT053	1,27	± 0,08	rstuvw
RT053	10,37	± 0,70	rstuvwxy	RT047	1,26	± 0,09	rstuvwx
RT047	10,00	± 0,77	stuvwxy	RT054	1,25	± 0,09	rstuvwx
CT043	9,88	± 0,69	tuvwxyz	CT043	1,24	± 0,08	rstuvwx
CT025	9,64	± 1,36	uvwxyzA	CT055	1,21	± 0,11	stuvwx
CT055	9,20	± 0,81	vwxyzAB	RT045	1,15	± 0,10	tuvwxy
CT012	8,89	± 1,04	wxyzAB	CT025	0,99	± 0,14	uvwxyz
CT007	8,88	± 1,27	wxyzAB	CT007	0,98	± 0,14	vwxyz
RT045	8,64	± 0,80	xyzAB	CT012	0,94	± 0,11	wxyz
CT011	8,51	± 1,56	yzAB	CT011	0,90	± 0,16	xyzA
RT019	7,37	± 1,48	zAB	RT019	0,81	± 0,16	yzAB
CT024	7,26	± 1,12	AB	CT015	0,77	± 0,12	zABC
CT015	7,05	± 1,03	B	CT024	0,77	± 0,12	zABC
RT012	4,12	± 1,04	C	CT013	0,58	± 0,08	ABCD
CT008	3,90	± 0,51	CD	CT002	0,57	± 0,07	ABCD
RT015	3,81	± 1,10	CD	CT003	0,56	± 0,13	ABCD
CT004	3,54	± 0,39	CDE	CT004	0,56	± 0,06	ABCD
CT013	3,54	± 0,49	CDE	RT012	0,55	± 0,13	ABCD
RT007	3,50	± 1,11	CDE	RT008	0,51	± 0,06	BCDE
RT008	3,43	± 0,42	CDE	CT008	0,49	± 0,06	BCDE
CT002	3,17	± 0,38	CDE	CT005	0,49	± 0,07	BCDE
CT006	3,04	± 0,42	CDE	CT006	0,47	± 0,07	BCDE
RT005	2,97	± 0,52	CDE	RT015	0,45	± 0,13	BCDE
CT005	2,65	± 0,37	CDE	CT001	0,41	± 0,06	CDE

Tabla A. 4: Evaluación a los 45 días tras la siembra del peso húmedo (Media ± Error estándar) (g) y del peso seco (Media ± Error estándar) (g) de la parte aérea (continuación).

Trat.(1)	Peso húmedo de la parte aérea (g)			Trat.(1)	Peso seco de la parte aérea (g)		
RT006	2,50	± 0,45	CDE	RT007	0,40	± 0,13	DE
RT013	2,43	± 0,46	CDE	RT005	0,40	± 0,07	DE
CT003	2,33	± 0,32	CDE	RT013	0,39	± 0,07	DE
RT001	2,29	± 0,37	CDE	RT001	0,38	± 0,06	DE
CT001	2,27	± 0,32	CDE	RT006	0,33	± 0,06	DE
RT025	1,85	± 0,67	CDE	RT002	0,32	± 0,06	DE
RT004	1,83	± 0,36	CDE	RT004	0,32	± 0,06	DE
RT002	1,81	± 0,36	CDE	RT003	0,28	± 0,07	DE
RT003	1,72	± 0,41	CDE	RT025	0,24	± 0,08	DE
RT011	1,42	± 0,55	DE	RT011	0,16	± 0,06	E
RT024	1,23	± 0,61	E	RT024	0,16	± 0,08	E

En la comparación de medias, los valores con letras diferentes presentan diferencias significativas (test DMS, $p < 0,05$).

(1) Trat.: Tratamientos, El control representa aquellas semillas que no se ha inoculado ningún hongo (CC) o bien sólo *R. solani* (RC) y si han sido inoculados sólo con el aislamiento correspondiente de *Trichoderma* (CT-#) o si han sido inoculado tanto con el aislamiento de *Trichoderma* como con *R. solani* (RT-#).

Tabla A. 5: Evaluación a los 45 días tras la siembra del peso húmedo (Media ± Error estándar) (g) y del peso seco (Media ± Error estándar) (g) del sistema radicular.

Trat.[(1)]	Peso húmedo del sistema radicular (g)			Trat.[(1)]	Peso seco del sistema radicular (g)		
CT019	3,23	± 0,49	a	RT059	0,51	± 0,25	a
CT061	2,96	± 0,15	ab	RT056	0,41	± 0,18	b
CT060	2,78	± 0,20	ab	RT060	0,27	± 0,02	c
RT043	2,66	± 0,20	bcd	CT061	0,27	± 0,01	c
RT050	2,61	± 0,18	bcde	RT043	0,27	± 0,02	cd
RT061	2,61	± 0,21	bcde	RT050	0,25	± 0,02	cde
RT048	2,57	± 0,15	bcde	RT061	0,25	± 0,02	cde
CT059	2,53	± 0,21	bcdef	RT048	0,25	± 0,01	cde
CT057	2,51	± 0,23	bcdefg	CT043	0,25	± 0,02	cde
CT054	2,50	± 0,20	bcdefg	CT057	0,24	± 0,02	cde
CT044	2,48	± 0,24	bcdefgh	RT057	0,24	± 0,01	cde
RT060	2,46	± 0,16	bcdefghi	CT044	0,24	± 0,02	cde
CT028	2,45	± 0,22	bcdefghij	CT060	0,24	± 0,02	cdef
RT047	2,44	± 0,21	bcdefghij	CT050	0,24	± 0,02	cdef
CT012	2,43	± 0,30	bcdefghij	CT059	0,24	± 0,01	cdefg
CT025	2,40	± 0,39	cdefghij	RT047	0,23	± 0,02	cdefgh
RT057	2,39	± 0,14	cdefghij	CT054	0,23	± 0,02	cdefgh
RT059	2,37	± 0,18	cdefghij	RT053	0,22	± 0,01	cdefghi
CT046	2,32	± 0,18	cdefghij	RT045	0,21	± 0,02	cdefghij
RT053	2,32	± 0,16	cdefghij	CT045	0,20	± 0,02	cdefghijk
RT056	2,27	± 0,18	cdefghijk	CT047	0,20	± 0,01	cdefghijkl
CT050	2,27	± 0,15	cdefghijk	RT046	0,20	± 0,02	cdefghijkl
RT046	2,22	± 0,21	defghijkl	CT028	0,20	± 0,02	cdefghijkl
CT032	2,18	± 0,18	defghijkl	RT044	0,20	± 0,02	cdefghijkl
RT045	2,13	± 0,23	efghijklm	CT058	0,20	± 0,01	cdefghijkl
CT045	2,13	± 0,16	efghijklm	CT019	0,20	± 0,03	cdefghijkl
RT044	2,12	± 0,20	efghijklm	RT058	0,19	± 0,02	cdefghijklm

Tabla A. 5: Evaluación a los 45 días tras la siembra del peso húmedo (Media ± Error estándar) (g) y del peso seco (Media ± Error estándar) (g) del sistema radicular (continuación).

Trat.[(1)]	Peso húmedo del sistema radicular (g)			Trat.[(1)]	Peso seco del sistema radicular (g)		
CT043	2,10	± 0,17	efghijklmn	CT046	0,19	± 0,02	cdefghijklmn
CT015	2,01	± 0,30	fghijklmno	RT040	0,19	± 0,02	cdefghijklmno
CC	2,01	± 0,10	fghijklmno	RT054	0,18	± 0,01	cdefghijklmnop
CT047	1,99	± 0,15	ghijklmnop	CT056	0,17	± 0,01	defghijklmnopq
CT034	1,97	± 0,19	hijklmnopq	CC	0,17	± 0,01	efghijklmnopqr
RT040	1,94	± 0,19	ijklmnopq	CT055	0,17	± 0,02	efghijklmnopqr
CT058	1,94	± 0,12	ijklmnopq	RT055	0,17	± 0,01	efghijklmnopqr
CT029	1,93	± 0,15	jklmnopq	CT025	0,16	± 0,03	efghijklmnopqrs
RT054	1,78	± 0,14	klmnopqr	CT053	0,16	± 0,01	efghijklmnopqrst
CT030	1,78	± 0,14	klmnopqr	RC	0,15	± 0,01	fghijklmnopqrst
CT053	1,76	± 0,12	klmnopqrs	CT012	0,15	± 0,02	fghijklmnopqrst
CT007	1,74	± 0,26	lmnopqrst	CT048	0,15	± 0,01	fghijklmnopqrst
CT048	1,66	± 0,15	mnopqrstu	CT013	0,14	± 0,02	ghijklmnopqrstu
CT011	1,62	± 0,31	mnopqrstuv	RT008	0,14	± 0,02	hijklmnopqrstu
RT058	1,61	± 0,24	mnopqrstuv	RT035	0,14	± 0,01	hijklmnopqrstu
CT056	1,61	± 0,11	mnopqrstuv	CT038	0,14	± 0,01	hijklmnopqrstu
CT055	1,58	± 0,17	nopqrstuvw	CT004	0,14	± 0,01	hijklmnopqrstu
RT035	1,57	± 0,18	opqrstuvwx	CT015	0,14	± 0,02	hijklmnopqrstu
CT033	1,53	± 0,16	opqrstuvwx	CT032	0,14	± 0,01	hijklmnopqrstu
CT024	1,49	± 0,23	opqrstuvwx	CT034	0,14	± 0,01	hijklmnopqrstu
RT029	1,48	± 0,15	pqrstuvwx	CT036	0,13	± 0,02	ijklmnopqrstu
RC	1,48	± 0,10	pqrstuvwxz	CT005	0,13	± 0,02	jklmnopqrstuv
RT055	1,46	± 0,13	qrstuvwxzA	CT039	0,12	± 0,01	jklmnopqrstuv
RT008	1,40	± 0,23	rstuvwxzAB	RT028	0,12	± 0,01	jklmnopqrstuv
CT038	1,40	± 0,11	rstuvwxzAB	CT001	0,12	± 0,02	jklmnopqrstuv
CT035	1,39	± 0,09	rstuvwxzABC	RT036	0,12	± 0,01	jklmnopqrstuvw
RT030	1,31	± 0,12	rstuvwxzABCD	RT041	0,12	± 0,01	jklmnopqrstuvw

Tabla A. 5: Evaluación a los 45 días tras la siembra del peso húmedo (Media ± Error estándar) (g) y del peso seco (Media ± Error estándar) (g) del sistema radicular (continuación).

Trat.[(1)]	Peso húmedo del sistema radicular (g)			Trat.[(1)]	Peso seco del sistema radicular (g)		
RT032	1,29	± 0,16	rstuvwxzABCD	CT037	0,12	± 0,01	jklmnopqrstuvw
CT041	1,28	± 0,10	rstuvwxzABCD	CT002	0,12	± 0,02	jklmnopqrstuvw
CT031	1,24	± 0,16	stuvwxzABCD	CT024	0,12	± 0,02	jklmnopqrstuvw
RT019	1,22	± 0,25	tuvwxzABCD	CT007	0,12	± 0,02	jklmnopqrstuvw
RT036	1,21	± 0,11	tuvwxzABCD	CT030	0,12	± 0,01	jklmnopqrstuvw
RT041	1,17	± 0,08	uvwxzABCDE	CT035	0,12	± 0,01	jklmnopqrstuvw
RT034	1,15	± 0,09	uvwxzABCDE	CT008	0,12	± 0,01	jklmnopqrstuvw
CT039	1,15	± 0,09	uvwxzABCDE	CT041	0,11	± 0,01	klmnopqrstuvw
RT007	1,12	± 0,36	vwxzABCDE	CT040	0,11	± 0,01	klmnopqrstuvw
RT012	1,12	± 0,33	vwxzABCDE	CT029	0,11	± 0,01	klmnopqrstuvw
RT031	1,08	± 0,12	wxzABCDEF	CT006	0,11	± 0,02	klmnopqrstuvw
RT015	1,06	± 0,33	wxzABCDEFG	CT011	0,11	± 0,02	lmnopqrstuvw
CT008	1,04	± 0,14	xzABCDEFGH	RT013	0,10	± 0,02	mnopqrstuvw
CT036	1,02	± 0,07	zABCDEFGHI	RT032	0,10	± 0,01	mnopqrstuvw
RT039	1,02	± 0,10	zABCDEFGHI	RT039	0,10	± 0,01	mnopqrstuvw
CT013	0,96	± 0,16	zABCDEFGHIJ	RT006	0,10	± 0,02	mnopqrstuvw
CT004	0,95	± 0,13	ABCDEFGHIJ	CT033	0,10	± 0,01	mnopqrstuvw
RT028	0,94	± 0,07	ABCDEFGHIJ	RT019	0,10	± 0,02	mnopqrstuvw
CT040	0,88	± 0,05	BCDEFGHIJK	CT003	0,10	± 0,01	mnopqrstuvw
CT037	0,87	± 0,07	CDEFGHIJKL	RT038	0,09	± 0,01	mnopqrstuvw
RT038	0,85	± 0,07	DEFGHIJKL	RT029	0,09	± 0,01	nopqrstuvw
CT005	0,85	± 0,14	DEFGHIJKL	RT001	0,09	± 0,02	opqrstuvw
RT037	0,81	± 0,07	DEFGHIJKLM	RT015	0,09	± 0,03	opqrstuvw
RT005	0,81	± 0,15	DEFGHIJKLM	RT005	0,09	± 0,02	pqrstuvw
RT033	0,67	± 0,07	EFGHIJKLMN	RT031	0,09	± 0,01	qrstuvw
RT006	0,66	± 0,12	EFGHIJKLMN	RT003	0,09	± 0,02	qrstuvw
CT006	0,66	± 0,10	EFGHIJKLMN	CT031	0,08	± 0,01	qrstuvw

Tabla A. 5: Evaluación a los 45 días tras la siembra del peso húmedo (Media ± Error estándar) (g) y del peso seco (Media ± Error estándar) (g) del sistema radicular (continuación).

Trat.(1)	Peso húmedo del sistema radicular (g)			Trat.(1)	Peso seco del sistema radicular (g)		
RT013	0,56	± 0,15	FGHIJKLMN	RT004	0,08	± 0,02	qrstuvw
CT002	0,53	± 0,08	GHIJKLMN	RT030	0,08	± 0,01	qrstuvw
CT003	0,52	± 0,08	HIJKLMN	RT037	0,08	± 0,01	rstuvw
CT001	0,51	± 0,07	IJKLMN	RT012	0,08	± 0,02	rstuvw
RT001	0,46	± 0,09	JKLMN	RT034	0,07	± 0,01	stuvw
RT003	0,40	± 0,10	KLMN	RT007	0,07	± 0,02	stuvw
RT011	0,38	± 0,16	KLMN	RT002	0,06	± 0,01	tuvw
RT002	0,36	± 0,08	LMN	RT033	0,05	± 0,01	uvw
RT004	0,31	± 0,07	MN	RT011	0,04	± 0,01	vw
RT025	0,28	± 0,11	N	RT025	0,04	± 0,01	vw
RT024	0,27	± 0,18	N	RT024	0,03	± 0,02	w

En la comparación de medias, los valores con letras diferentes presentan diferencias significativas (test DMS, $p < 0,05$).

(1) Trat.: Tratamientos, El control representa aquellas semillas que no se ha inoculado ningún hongo (CC) o bien sólo *R. solani* (RC) y si han sido inoculados sólo con el aislamiento correspondiente de *Trichoderma* (CT-#) o si han sido inoculado tanto con el aislamiento de *Trichoderma* como con *R. solani* (RT-#).

2. SECUENCIA DEL TRANSCRIPTOMA DE LOS GENES DE ESTUDIO

2.1. α-*actin* chr15

Localización: Glyma.15G050200 cromosoma 15

5´UTR CDS 3´UTR Primers

TTTTCTTTTCCAACATTTTCCACAATAGTCTCGCGTTGCTTGGAATAGATTTATCTCGAAACCAAACACTCA
AAACTGCGTTGTCCAAGTCGAAAGTTAATTTGCATTTGAGTTTCATTTCACTCTCATTTAGTTCTCTTCAAT
GAATATAAAAGCTTCCTTCTTCAGTGAAGCTCTTTCGCGAAACACTCCCCTTTGCCGCTGTTGTTGTTGCTG
CACCACCGAACAACTTCCCCTGTAAATCGCGTTTCGTTTGTGCAAGGTTTTAAAAGATGGCTGATGCTGAGG
ACATTCAACCTCTTGTTGTTGATAATGGAACTGGAATGGTCAAGGCTGGTTTTGCAGGTGATGATGCTCCTA
GGGCCGTGTTCCCCAGCATTATTGGGAGACCACGCCATACTGGTGTAATGGTTGGGATGGGTCAGAAGGATG
CTTACGTGGGTGATGAAGCCCAATCAAAAAGGGGTATACTCACCTTGAAGTATCCTATAGAGCATGGCATAG
TCAGCAACTGGGACGATATGGAGAAGATTTGGCATCACACATTTTACAATGAGCTACGTGTTGCCCCTGAAG
AACACCCCGTACTTCTTACCGAGGCACCACTCAATCCCAAGGCCAACAGAGAGAAGATGACCCAAATTATGT
TTGAGACTTTCAATGTGCCTGCCATGTATGTTGCAATTCAGGCTGTCTTGTCTCTGTATGCCAGTGGACGTA
CTACAGGTATTGTGCTTGATTCTGGTGATGGTGTGAGCCACACGGTGCCCATATATGAAGGGTATGCTCTTC
CTCATGCAATTCTTCGGTTGGACCTTGCTGGCCGTGATTTAACAGAGTACTTGGTGAAAATCCTTACCGAGA
GAGGATACTCCTTCAGCACCTCTGCCGAAAAGGAAATTGTCCGTGATGTGAAGGAGAAATTGGCATACGTGG
CCCTTGATTTTGAACAAGAAATGGAGACTACTAAGAGTAGTTCTGCAGTGGAAAAGAGCTATGAATTGCCTG
ATGGACAGGTCATTACAATAGGTTCTGAGAGATTCCGTTGCCCAGAAGTACTGTTCCAGCCTTCTCTGATTG
GTATGGAAGCTACTGGAATTCATGAAACGACATACAATTCCATCATGAAATGTGATGTTGATATTAGGAAGG
ATCTGTATGGAAACATCGTGCTTAGTGGAGGGTCTACTATGTTTCCGGGAATTGCTGATCGCATGAGCAAGG
AAATTAGTGCTCTTGCACCCAGTAGCATGAAAATCAAGGTGGTTGCACCTCCTGAGAGAAAGTACAGTGTCT
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AATCTGGGCCAGCCATCGTTCATCGGAAGTGCTTCTAAAGTTGGGTCAAGTCGAGCTGGATGGTGATCAAGT
TCTTCATGCACCCAGTCCTGTGCCATATTTCAGTAAAGTTTGGTTGACTTATTTGGTATTTGAAGCTTTGGG
TTTTTAATAGATATATCTAGTTTAATGAATGGATTTGTAAGTTGAGTATGTTATAAGTTTCTCCGTTAGGTC
ACCTTGGAAGCCAGACTTTTTATGCAATTGGCGGATGGTATTGGAACTTTGTAGGTTCTGCTTGGTTTGGTT
TTTCCTTTTATTCGATTTTCATGTTGTGGCCAAGAATTAGGTGCCTCGAGTTAGGAGGACGATAGTCTAAGC
ATCTTTTCTCACAGGGATTTTTGAGTCAGTTTCTGTTTATATTTATTATATGCATAATTGGTTTTTATGTTT
GCAGTACACTCAGAAATCAGAATGTTTTTATTGTTTGAGGTGATTAATCGTTTATACACCATATGATTAAAT
TT

2.2. *Act11* chr8

Localización: Phvul.008G011000 cromosoma 8

5´UTR CDS 3´UTR Primers

TAAAACGAGAAATCAACGGTTGATATTGCATCGTATGCTTGTTATATTGAATACCAGTTTCCATTTTTTCTT
GGTACCTCTCTCTCTCTCTCTCGTTTCCCTCATCACTAGCTCTGCATTTCTCTCCCTCTTTGCCTTCCATCA
CCCTCGCCATTTCCCTTCTCAAGAAAGTAGAAGATGGCAGACGCTGAGGATATCCAACCTCTTGTTTGTGAC
AATGGAACAGGAATGGTTAAGGCTGGATTTGCTGGAGATGATGCCCCACGAGCTGTGTTTCCCAGCATTGTA
GGTCGTCCCCGTCACACTGGTGTGATGGTTGGCATGGGTCAAAAAGATGCATACGTTGGTGATGAGGCACAG
TCTAAGAGGGGTATTTTGACTCTTAAGTACCCTATTGAACATGGAATTGTGAGCAATTGGGATGATATGGAA
AAGATTTGGCATCATACCTTCTATAATGAACTTCGTGTCGCCCCTGAAGAGCATCCAGTTCTGTTAACTGAG
GCTCCTCTTAACCCCAAGGCTAACCGTGAAAAGATGACCCAGATCATGTTTGAGACATTTAACACCCCTGCC
ATGTATGTTGCCATCCAGGCCGTTCTTTCACTGTATGCAAGTGGTCGTACAACTGGTATTGTGTTGGATTCT
GGTGATGGTGTCAGCCACACAGTCCCCATCTATGAGGGTTATGCTCTCCCCCATGCCATCCTTCGCCTCGAT
CTGGCTGGTCGCGACCTCACTGATTTCTTGATGAAAATTTTGACTGAACGTGGTTATTCCTTCACCACCTCA
GCAGAGCGGGAAATTGTAAGGGATGTTAAAGAAAAGCTGGCCTACATTGCCCTTGACTACGAGCAAGAACTC
GAGACAGCCAGGACCAGTTCATCTGTGGAGAAGAGCTACGAGTTGCCTGATGGACAGGTCATCACCATTGGT
GATGAGCGTTTCAGATGTCCAGAGGTTCTGTTCCAACCATCCATGATAGGAATGGAAGCTGCCGGCATTCAC
GAGACCACATACAACTCCATAATGAAGTGTGATGTTGATATCAGGAAGGACCTCTATGGCAACATTGTCCTT

TCAGGAGGAACAACCATGTTCCCTGGCATTGCTGACAGAATGAGCAAGGAAATTTCTGCCTTGGCACCCAGC
AGCATGAAGATCAAGGTTGTAGCACCACCTGAGAGGAAGTACAGTGTCTGGATTGGTGGTTCCATCTTGGCA
TCTCTCAGCACATTCCAGCAGATGTGGATTGCAAAGGCAGAATATGACGAGTCTGGTCCATCAATTGTTCAC
AGAAAGTGCTTTTAAAGTGTTTTAAGAAATAGGTTTATTGAAGGGAAAGACTACTATGATGATTTGTCTGGA
AGCAATGCATTGCAGATTGTAATCCTTTCATTCTAGCTTTTGTTATCCGTTCATGTTATTCTCTTGTTGGAG
TGGTCATTTATTTGCAGAAGGTGAGGAGAGTTGTTTTTGAAAATGGTCCAGTTCTTTTCATGTACTTTGACA
AGTTTTTGACTGGTTCATTCTGCCTTATGAGTTAGCTGGATTATTAAACTACGCTTGTACTCGTTGTATGCA
TAATTATAATCCATCATCCTTTTGAACTACAA

2.3. *UKN1* chr11

Localización: Phvul.011G023200 cromosoma 11

5´UTR CDS 3´UTR Primers

GTGAAGAGCGAGGTGTGCATCGCAAATTCTAATTTTCTGATAAAACTAATAATAATAACAACAAAACAGACA
CACCTGTGTAAGAAGGAGGTGAGTTACGTACGATTAGGTTTTCTTCTTCTCTTTCTCTCTGATTCGATCCAT
TTCGATTGCTGAATTCATCCCATCGCAGAGCGTTTGAATTTGTAGTTGAACAACGATGGTGGTGCCGCTGCT
TAGTGTTTTCATTCTTTTCGCTTTTATTCCAAACGGATTGTTAGCAGTTCCTTCAACCGTCCCCGCATTCCT
TTGGTCATCACATTATGAACTGGCCTCTGAAACTGGATTGAAGGAATCTGTAAATTACCAAGTCATTTCTCC
TTATGATCTTGCAAAGTCTGTTATATCTGAAGCGGGCTGGTCAAATTTTCTGTGCAAAGGAAAGAAAACTCA
CAAGCCTCTGGATCTGGCACTTTTGTTCGTTGGTGGAGAGCTGCAATCTTCAGATTTAAGCATGAACAAACA
TGCAGACCCAGCCCTTTTAGATCTGCTCAAGATCTCTTTTGCCAGATCCAACACTTCTATGGCTTTTCCCTA
TGTTTCTGCATCAGAGGATTTGCTTTTGGAAAACACTTTGGTTTCAGGATTTTCTGAAGCCTGTGGAGATGA
TACGGCAATTGCCAATGTTGCGTTCCATGGATCTTGCTCCATGGGTGGTACAAATGAAGAAGTCACAGCTTT
GCATTCAGTACAAGACTATTTGACAAAGAGGATGAAAGATAGTCACAGGGGGAAAACAGATTTGGTTGTGTT
CTGCAATGGAGGCTCTCAAGATCTTAAAAATGTTGACAGGACACAATCTGAAGGGGAAACTTTATCGAAGCT
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GTATCCTTCTTACAGAGAACTGCAAAGGTTTCTTGAAGAAGGTGCTGAATCAACCAATTCCACAGGTTGTGA
TAAAGTCTGCATGCTTAAATCATCTCTTTTGGAGGGAATTTTTGTGGGTTTAGTTTTGCTAATCATTTTGAT
ATCGGGCCTTTGCTGCATGATGGGAATTGACACCCCAACAAGATTTGAGACGCCACAAGAGTAATGATCCGT
TTCTCGAAGCTAAAATTTGTTGTGATGCTCGGTCTAATGATTAAAATATTGCTGTCGTATTATTTGCGATTC
TTCCGGAGGCTCTGATAAGTATTCTGTAGTTGATGACTTATCTCAGGGACTTTTAATATTGAATATTAAAAT
AATTTGTATCAGTATATAAAAATAAAACTTCAATGTTAGGTTTATTTTTGATCCCGCTACCACTAGTGTAAA
ATTTGAAGAAATTTCAGATTGTGATGTATTATTGTTATTACTGTCTGCAAATTTGATCCATTCGTTTTGCAT
AACTGCTGATCGATGCAGTTCCAAGT

2.4. *EF1a* chr4

Localización: Phvul. 004G060000 cromosoma 4

5´UTR CDS 3´UTR Primers

ACGAAAAGTAGCTCCCACAATATAAAACAATTGGAACCCTAGTTCATTATCACTCCTTGCTTCCTCGCTCTA
TTTTTGCGGCTGAGGATTCTGAACCAGCGACCTTCGTTTCTCTCTAAGTTTAACTCGTCATGGGTAAGGAAA
AGGTTCATATCAACATTGTGGTCATTGGACATGTCGACTCTGGGAAGTCAACTACCACCGGCCACTTGATCT
ACAAGCTTGGAGGTATTGACAAGCGTGTGATTGAAAGGTTCGAGAAGGAAGCTGCTGAGATGAACAAGAGGT
CATTCAAGTATGCCTGGGTGCTTGACAAGCTCAAGGCTGAGCGTGAAAGAGGAATTACCATTGATATTGCTT
TGTGGAAATTCGAAACCACCAAGTACTACTGCACAGTCATCGATGCCCCTGGACATCGTGACTTTATCAAGA
ACATGATCACTGGAACCTCCCAGGCTGATTGCGCTGTCCTTATTATTGATTCCACAACTGGTGGTTTTGAAG
CCGGTATTTCCAAGGATGGACAAACCCGTGAGCATGCTCTTCTTGCTTTCACCCTTGGTGTGAAGCAGATGA
TCTGTTGCTGTAACAAGATGGATGCCACTACCCCTAAGTACTCCAAGGCTAGGTATGATGAAATCATCAAGG
AAGTTTCATCATACTTGAAGAAGGTTGGTTACAACCCAGACAAAATTCCATTTGTCCCAATCTCTGGATTTG
AGGGTGACAACATGATTGAGAGGTCCACCAACCTTGACTGGTACAAGGGACCCACTCTCCTTGAAGCCCTTG
ACCAAATCAATGAGCCCAAGAGGCCCTCAGACAAGCCTCTCAGGCTCCCCTTGCAGGATGTCTACAAGATTG
GTGGTATTGGTACCGTGCCAGTGGGACGTGTTGAAACCGGTGTCATCAAGCCGGGTATGCTGGTGACTTTTG

CTCCCACAGGTTTGACAACTGAAGTTAAGTCTGTTGAGATGCACCACGAGGCTCTCCTAGAGGCTCTTCCAG
GTGACAATGTCGGCTTCAACGTGAAGAACGTTGCTGTCAAGGATCTCAAGCGTGGTTTCGTTGCTTCCAACT
CCAAGGATGACCCTGCCAAGGAAGCTGCCAACTTCACATCCCAAGTCATCATCATGAATCACCCTGGCCAGA
TCGGAAATGGCTATGCACCAGTGCTTGATTGCCACACCTCCCACATTGCAGTCAAGTTCGCAGAACTCGTGA
CCAAGATCGACAGGCGATCAGGTAAGGAGATCGAGAAGGAGCCCAAATTTTTGAAGAATGGTGATGCAGGTT
ATGTTAAGATGATTCCAACCAAGCCCATGGTTGTTGAAACCTTCTCTGAGTATCCTCCCCTTGGTCGTTTTG
CCGTGAGGGACATGCGTCAGACCGTGGCTGTCGGAGTCATCAAGAGTGTTGAGAAGAAAGACCCCACTGGAG
CCAAGGTCACCAAGGCTGCTCAGAAGAAGAAGTGATCATAAAAATCTTGTTCCTTTATAGTCTTAGTCTTAA
AACCCGGGTTCATGGTTCAATTTTCGTTGTTGTTTATTCCTGAGAGGACTATTTTTGTTTTTTTGTTTTTAT
TTTTGATGTAAGAGTCTGAGTTTTACATTTTCACAAAGCAGACATAGGGTTTTAGCCCTTGCTGAGAGATGT
TTTAAATGCTTTTAATTTATAAGAAAGTTTTGTCAATGGTTTTATCGGTTTAACGTAAGCCTATTTTATAAT
CC

2.5. WRKY33 chr8

Localización: Phvul.008G090300 cromosoma 8

5´UTR CDS 3´UTR Primers

TGTGATACAAAATGATCCAATAATTTGTGTTATAATGGAACAAGGGTGGTAGCAACGAGCTGGCAGGCTGAG
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AAGCACCTTCTTCCAAGACTTTTTGCTGGTGATTTCATGCTTTGCCACAAAATCATGCACCTCTGCATCACT
ATTCACTTCTGTCTCCTCTCCTTATATCACGCCATACACCACTTTCTGTCTTTCCAATATAAACTCAAAACC
AACCAACCTTTTCTTCATCCAAGAGGATATCTTTCACTCACCTTCTTTCCTTTCCAACAACAGTAACAAACA
ACAATACTAGTAACATGACCATGGGTGATCATAATAACAACTGGGGAACTTCTGAATTTAATCATCATCAAG
TTGGACTTCTTGATGTTCCAAAATTCAAGTCTGCTCAACCTCCCTCACTACCCCTCTCTCCCTCTCCAGCCT
CCCCTTCTTCTTACTTGGCCTTTTCGTCTGCTTTCAGCCCATCTGAGTTCTTCAACTCTTCTTTGTTTTCTC
CTTCTCCAAATATTTTTGCATCTCCTACTACTGAGCCTTTGGCTGGCCAGACCTTCAACTGGAGGAACGGTT
CAGGAGAAGAACAACAACGTGACAAGGAGGATGAGAAAAACTACTCGGACTTCTCTTTCCAAACTCAAATTC
AATCTTCCTCAGGCATGTTTCAAGTGCAGGAACCACTTAAGAAACAAGATATATGGAAATTCAATGAACCTT
CAAAGCGAACTGATTTTTCATCTGATAGGACAGCAACAAAATCTGAATACCCTTCAATTCAGAGCTTTTCCT
CAGAAATGGCATCGGATAAATCAGAATTACAAAGCAATTCAGTTCCTGGATCTAGTCGTTTTGATTACCCCA
ATGCATCTCAGTCTGTTAGAGAACAAAAGAGATCAGAAGATGGGTTCAACTGGAGAAAATATGGACAGAAAC
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CTACTAGAAGAACAAACTCTCAATCAATTCATCAACCTTCTTCATCTTGCACAAACTCGGGGATTTCAGATC
AATCAGTGGTGACCCTAGGCAATCCACAAATGGATCATTTCTCCATGCAGGAAGATTCTTCAGCTTCCGTAG
GAGAGGAAGAGTTTGAGCAAACATCCCAAACCAGTTATTCTGGGGGAGATCAAGATAACCTTGGACCAGAGG
CCAAACGATGGAAAGGAGATAATGAAAATGATGACTATTCCGCCTCAGGGAGTAGAACTGTGAGAGAGCCTA
GAGTTGTAGTTCAAACCACAAGCGAAATTGATATACTTGATGATGGCTTTAGGTGGAGGAAATATGGACAGA
AAGTAGTGAAAGGAAATCCAAATGCAAGGAGCTATTACAAATGCACAGCCCCTGGTTGTTCAGTGAGAAAAC
ATGTTGAGAGAGCTGCACATGATATCAAAGCTGTGATCACAACTTATGAAGGGAAACACAACCATGATGTGC
CTGCTGCACGTGGTAGCGCAAGTTATAACATGAACAGAAATTCCCTGAACAGCAACATATCCGCTCCTATAA
GACCCTCGGCAGTGAACTGTTATTCTAGCTCATCAGGTTTCAAAAATTCTCTCTACGATAATACCAGGCTTC
CAGCAACCGGAAATCAAGAATCGTTTTCACAGGACAGGTTCCAGCACCCAGGAAGTTTTGGCCATTCGGCTC
TTAACAAATCAATGGGTTCATATGCTGATCATGCACTGTACTCAGATGCTGCACACTCAAATGTCAAGGATG
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AATTCTGTTATTCTTTTGTTGTATTTGTAGGGAAAAAGGTTTTCTTAGAAGAATCTTCATTATTTAATCTTG
TAAGAATGTTTGTGCTGGCAATTTG

2.6. *PR1* chr3

Localización: Phvul.003G109100 cromosoma 3

GCAGCTTCATTCACACTATAGCATGACTCTCGAGTCATGTACATGGGTAGATGCAGATTGATACCAGACTCA
TTGCTTCCTTATAAATACATGCCACTCCTCTGCTAAACACCCACACAGCACAGCAAAAACATCTTCTTCTCC
TTCAATTCTCAAATACATTTCCATCTCTTTAAGGTTCTATCATGGGTGTTTTCACATTCGAGGATCAAACCA
CTTCTCCTGTAGCTCCTGCTACCCTCTACAAAGCTGTTGCTAAAGACGCCGATACCATCTTCCCAAAGGCTC
TTCCTGATTCCTTCAAGAGTGTTGAAATCGTTGAGGGCAACGGTGGCCCTGGAACCATCAAGAAGATCTCTT
TCGTTGAGGATGGAGAGACAAAATTTGTGTTGCACAAAATAGAATCAATAGATGAGGCCAATTTGGGATACA
GCTACAGCATAGTTGGAGGTGTTGCCTTGCCAGAAACTGCAGAGAAGATCACTTTCGACTCCAAACTCAGTG
ATGGTCCTAACGGAGGATCACTCATTAAGCTGAGTATAACATACCACAGCAAAGGAGATGCTCCACCCAATG
AAGATGAACTCAAAGCTGGAAAAGCCAAGAGTGATTCTCTTTTCAAGGCCGTAGAGGCATACCTTCTGGCCA
ATCCCTGATTACAACTGATTCAATTGAGTTATCGATATCAGTCAGTAACAAGGTTGCTTATATGATATACAA
GAGTTTTATTGGCTTGCATATGAAGTGTTATCAAATCAGAGTGTTCTTAAGTTAAGTTGGCTTCCATTTTCA
GGAGTGTTTTCTTTCTTATTTCCCTTTGCTGCATAATCGTGAGAGCTCGTGAGTATCTTTGTATCTTTCTTC
ATCAATAAATGATAATAAAGAAAATAATCACACTTTAACTTTTCTCA

2.7. *PR2* chr3

Localización: Phvul.003G109200 cromosoma 3

GGTTATAAATAGAAGGCAGTCCTCTGGCAAATACCCACACAGCAAACATCTTCTCCTTCTCTTCTGATATTA
TATATTCTTCCATTTCATTAGATTCCATTCATCATGGCTGTTTTCACCTTCGAAGACCAAACCACTTCTCCC
GTGGCTCCAGCTACCCTTTACAAAGCTCTTGTGAAGGACGCCGATAACATCGTCCCAAAGGCCGTTGATTCC
TTCAAGAGTGTTGAAATCGTTGAGGGCAACGGTGGCCCCGGAACCATCAAGAAGATCTCTTTCCTCGAGGAT
GGGGAGACAAAGTTTGTGTTGCACAAAATAGAAGGAATAGATGAGGCAAACTTGGGATACAGCTACAGCATA
GTTGGAGGTGCTGCCTTGCCAGAGACTGCAGAGAAGATCACAATCGACTCCAAACTCAGTGATGGGCCCAAC
GGAGGATCAGTTGTAAAGTTGAGTATAAAATACCACAGCAAAGGAGATGCTCCACCCAATGAAGATGAGCTC
AAAACTGGCAAAGCCAAGAGTGATGCTCTTTTCAAGGTCATCGAGGCTTACCTTTTGGCCAACGCTTGATCA
CAATTGAGTTATTAGCGACATCTTTGCTTATATTGTACACAAAAGAGTTCTCTTGTGTGGAGTGTGTTTAAG
TTATGTTTGCTTCCAATGTCACAGAGTGTTTCCTTTTACTGCAGAATTGTGAGAGACATAATTCATGTGCTT
TGTATCAGAGTTCAAAAATAAATTATAATAAAGGAAAGTATTATGTTAATGTATGGCAATTTCTAGAATTTG
TACAGTGACCACATATAAACACTCATATACGTTGTGTTGTGTTAAAATATTAATGGAAAGAGG

2.8. *PR3* chr9

Localización: Phvul. 009G116600 cromosoma 9

GATTATGATATGCAGTGTAGGAGTGGTGTGGATGCTGTTAGTTGGAGGAAGCTACGGAGAGCAGTGTGGAAG
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CTACTGCGGCAAGGATTGCCAGAGCCAGTGCGGGGGACCGTCTCCTGCTCCTACTGATCTCAGCGCCCTCAT
ATCCAGGTCCACCTTCGACCAGGTGCTCAAACATCGCAACGACGGAGCATGCCCAGCCAAAGGCTTCTACAC
CTACGATGCCTTCATCGCCGCCGCCAAGGCTTACCCCAGCTTCGGAAACACCGGAGACACGGCCACTCGCAA
GAGGGAGATTGCGGCCTTCTTGGGGCAAACGTCTCACGAAACAACCGGGGGATGGGCCACTGCGCCCGACGG
ACCATACGCATGGGGATACTGCTTCGTGAGGGAGCGGAACCCCAGTGCGTACTGCTCCGCCACTCCCCAGTT
CCCCTGCGCCCCTGGGCAGCAGTACTACGGCAGGGGTCCCATCCAGATATCCTGGAACTACAACTATGGTCA
GTGCGGAAGGGCCATTGGGGTTGACTTGCTCAACAAACCTGATCTAGTCGCCACTGACTCTGTCATCTCCTT
CAAGTCCGCCCTCTGGTTCTGGATGACCGCACAGTCCCCCAAACCTTCCTCCCACGACGTCATCACCTCTCG

ATGGACCCCCTCCTCTGCCGACGTCGCCGCCCGCCGGCTTCCCGGCTACGGCACTGTGACGAACATCATCAA
CGGAGGCCTGGAGTGCGGGCGAGGACAGGACAGCAGGGTTCAAGACCGCATCGGATTCTTCAAGAGATACTG
TGATCTGCTTGGAGTTGGTTATGGCAACAACCTTGACTGCTACTCTCAGACTCCATTTGGAAATTCACTCTT
CCTCTCTGACCTTGTCACCTCTCAGTGACACTGCCATCCCATCAGAATAAATAAACTCATAAATCTGTGTTT
CCCTTTCTGATCACAACTTTCCAATAACACTTTTCCCACCATCTATCAATAAATTCACTACTTCTATATACC

2.9. *PR4* chr6

Localización: Phvul.006G102300 cromosoma 6

5´UTR CDS 3´UTR Primers

AAGAAAGAAAGGGCATTTTCATAATTTTGTGATGCTAGAGAGGCCCTATAAATGTGGAAGAGGAGAAGCGAG
TGAATGAAGTGAAATGCAAAGAAGGATAATGGGAAAAGTAATTGTGTTTGTTGTGTGCGTGTTTTGTGTTGT
GGCTTTGGCCTCAGCTCAGAGTGCGAGCAATGTGAGAGCTACGTATCATTTGTACCAGCCTGAGCAGCATAA
CTGGGACTTACTCGCAGTGAGTGCATATTGCTCCACTTGGGATGCTGGCAAATCCTTGGCATGGCGCAGCAA
ATATCCTTGGACAGCTTTCTGTGGACCCTCTGGCCCTCGGGGCCAAGAATCTTGTGGCAAGTGCTTGAGGGT
GACAAACACAAGAACAAATGCTCAGATAACGGTGAGAATTGTTGACCAGTGCCGCAACGGGGGCTTGGACTT
GGATATTAGTCCATTCCAAAAGATAGACACGGATGGGAATGGCTATGCTCAAGGCCATCTTACTGTTAACTA
TGATTTTGTGAACTGCGGTGACTAAACACTACATATAATTAATTACCTTATGTGTGCTCTACTCAGTCACAA
GAAATCGAATAAAGAAAGATGTACAAGTGTGTTACTTCTTATAACAGTAATAAAAATAAATAAAGCCTTGTT
ATTATAAAAAAAACTGTTCTGGATTTGAAAGGAGTAATTATTAGTATTACC

2.10. *PR16a* chr10

Localización: Phvul.010G129900 cromosoma 10

5´UTR CDS 3´UTR Primers

CTAAAGAATCCATAAAGTGTAACTCTGGCTCTAATTTTGCATTTGTCATTATTAGTTGCATGGTATATGTTC
TGCCTCCAACTTGCAAGAAACTTTGTATATATATAGCACCATCTTGTAGCCTAGAATTCTCACATTCCCATT
CACAGTTTCACACTACCAAGTTAAGATGTTTCTCCAAATTGTCTTCCTCCTTTCCTTTCTCTTATCCACTTC
CCATGCTACTGTTCAAGACTTCTGTGTGGCAGACCTCAAAAGTGCAGATGGCCCTGCAGGCTTTCCCTGCGT
GCCACCTGCCAAAGTTACTTCAGATGACTTTGTGTTTGCTGGCTTATCTGAAGCTGCAAATGTCACAAACAT
AATCAATGCTGCTGTGTCCCCTGCATTTGTTGGTCAATTTCCAGGTGTTAATGGTCTTGATCTCTCTGCAGC
AAGGTTAGACCTTGGTCCTGCAGGAGTTATACCACTGCACACTCATCCTGGTGCCAATGAACTTCTAATAGT
GAACCAGGGTCACATCCTCGCTGGATTCATATCATCAAGTAACGTTGTTTATCAGAAGGTACTCAAAAGGGG
AGAGCTTATGGTCTTCCCACAAGGATTGTTGCACTTTCAAATAGCAGCTCACAAGAGGAAGGCCATTGCTTT
TGCTGTTTTCAGCAGTGCAAACCCTGGCCTCCAAATCCTTGACTTTGCACTCTTTGCCAGCAACTTCTCCAC
ACCTTTGATCGCACAGACTACTTTCCTTGACCCTGACCTAGTCAAGAAGCTTAAGAGTGTCCTTGGGGGCAG
TGGCTAAGCTAGGGCAGTCTCATGGTTATGGTTTATGCATTGTTTATTATTTTGCTTAATTCATTCCTTGTG
TTGTTTCAATTCCTTATACTTTTGCAGACTGTAATTGACTTTGTTTTTCCATTTCTGCTCCAAAGCTGAATG
TGTTGAGACTTGAGCATGCTGTGTTTAGAAGATTACATTTGTCTTGTAAGGATTACATTGTTACATTTCATT
TGATGTGAATGCAATTTTCTTACCCTAAAAGTACATATCTATGGAGTGTATACTATATGGATGA

2.11. *IPER* chr9

Localización: Phvul.009G215000 cromosoma 9

5´UTR CDS 3´UTR Primers

TTTCTCTTCTCTTTCATGTAAACCCTAATTCTCTCGTTTCCCTCCTCACTTCCCTCTCCCTCCAATCCTTCC
ACGTCGTAACCATTCGCTCCTCCAACCTTAACGCCATCCTCCGCCTCCCGCTTCAGACGCCACCCTCCGTCT
CCTGCTCCAACGTCACTACACTCTCTCGCTGCGCCGCTCTATTCCACAATGCTAACAAGTACGCCTCCAATT
GGACCTCCATTCTCGAGAACACCAGTGTGTGCGATTCTTCCAAAGTTGTTCTAATTGGAGAGTCTGAAATTG
GGAAATCAAATCTGCTCTTGCAAAAGATGTATTCCGGTAGGATTTCAAACCCACAATACGGGTAGAATTTGC
TTACCGGAACATCAAGGTTGGAGAATAACTCATCAAAGCTCAAATATGGGACACTGCAGGCCAAGAGAGGGT
TGTGAGGCATCAATTTTACTGAACAACACTGCTATTCTAGAAAGCAAGCAAGATGCAATTCCAAATAACAAT
TCAATAAGGGGTTTGGATGTGGTCAAATAATATCAAGACAGCGGTGGAAAATACTTGTCCCGGGACAGTTTC
TTGTGCTGATATTCTTGCTATTGAAGCTGAAATAGGTTCTATTCTGGTTCAACCAAATGTTCCACTCTCACC
ACCGTTTGTTACATATTCCCAGAATCTTCCAAACAAGGTTTTCCAGCACTTCCTCTTCTATGTTAGATCTCT
GCACCAAACCCCAGCACCTTCAACAACTCCATGCAAGGTTCTTCCTCCATGGCCTTCACCAAAACCAATCTC
TTTCCTCCAAACTCATGGATTGCTACGCCAAATTTGGACTCCTGGACTTGTCGCAGAAGCTTTTTTACTACA
CGCAAAACCCAGATATCGTTCTCTATAGAGCAATCTTAAGAACCTTGTACCAGTTTGGTGAATATGACAATA
CCCTCTTTTTGTACAAAGCGATGGTTGGGAAGTCCATGTACCCAGATGAAGAAAGTTGCTCCTTTGTTTTGA
GATCTTGCTTTTGTTTGTCTCATGAACAAGGGAAGATGATTCATGCGCAGATAGTGAGGCTGGGTTTGGATG
CATTTGACTTGGTGGGAAGAACTTTGGTAGAGTTGTATGACATGAATGGTTTTTTTAATGTGGATGAACCAG
TTGAAGGAAAATATGTAATGGAATTGAATTATTGGAATAACTTGATTTCAGAGGCATCTGAAAATGGAAAAA
TGGAGGAAAGCTTTAAACATTTTTGTAGGATGAGAAAGGAGAATATCCAACCTAATTCAATTACTGTGATTG
GATTGTTAAGGTCCACTGTTGAGTTGAACTCACTGAAGACGGGACAGGCCCTACACTCTTTTGTTGTTGTGA
GCTGCCTGTATGGAAAACTAACTGTGAATACTGCACTGTTGTCAATGTATGCAAAGTTGGGTAGTCTAGAAG
ATGCAAGAATGGTGTTTGAGAAAATGCCTGACAAGGACCTTGTGGTCTGGAACATAATGATTTCAGCATATT
CAGGGAATGGATTCCCCAAGGAATCCTTAGAACTTGTATATTGTATGGTTAGATCAGGTTTTAGACCTGACT
TGTTCACGGCAATTCCTGCTATTTCTTCTATCACACAGTTAAAGTATAATGAATGGGGAAAGCAAATGCATG
CTCATGTGATAAGAAATGGTTCAGATTATCAGGTATCCATTCACAATTCTCTTATTGACATGTATTCTGCAT
GTGATGACTTAAATTTGGCACAGAAGATTTTTGGCTTCATAATGAACAAGACTGTGGTTTCATGGAGTGCAA
TGATCAAAGGATATACAATGCATGATCGGCCTCTTGAGGCTCTGTCTCTTTTCTTGGAAATGAAACGGAGTG
GTACTAGACTTGACTTCATTATAGTCATTAACATCTTGCCAGCCTTTGCAAAGATAGGGGCATTGCATTATG
TAAGTTACTTACATGGTTACTCGTTGAAAACCAGCCTTGATTCCCTTAAATCCCTTAAGACATCCTTTCTTA
GTAGCTATGCAAAATGTGGTTGCATAGAAATGGCCAGAAAGCTTTTTGATGAAGAGAAAAGTATCCATAGAG
ATATAATTGCGTGGAACTCAATGATCAGTGCGTATTCTAAACATGGGGAGTGGTTTAGATGTTTTCATTTGT
ACAGCCAAATGAAACTATCGAGCATTGTACCGGACCAAGTGACATTTCTTGGGCTACTCACAGCTTGTGTTA
ATTCAGGCCTTGTTAGCAAAGGCAAGGAGATTTTCAAGGAAATGGTGGAAATATATGGATACCAACCTTGTA
AGGAACATCATACTTGTATGGTTGATCTACTCGGACGTTCTGGCCAAATCCATGAAGCCAATGAAATAATAA
AAACTATTCCATTGGAGTCAGATGCCAGGGTTTATGGCCCTTTGTTGAGTGCCTGTAAGATGCACTCAGAGA
CCCGTTTAGCAGAACTTGCTGCTCAGAAGCTTATAAATATGGAACCTAAAAATGCTGGAAACTATGTACTGC
TCTCCAATATATATGCTGCAGCAGGAAATTGGGACAAAGTTGCTAAAATGAGGAGCTTTCTTAGAGATAGAG
GACTAAAGAAAACACCAGGTTGTAGCTGGCTTGAATTAAATGGACAAGTGCACGAGTTTCGTGTTGCGGATA
AATCTCATCAAAGATGGGAAGATATATACTCAATATTAAATGTACTGGAGTTGGAAGCAGGGGATATGGAAG
ATGATGATCTTGAACTCTTTGACCCTTTTGTCATTAAGGGGTGTGAATTGCCCATGAATCAACCCAACCTAA
GACATAACACCTTATAAGTTAAGAGAAGAGAGGTGGAAATGAAGGTTGCTCCGGTGGTGGTGTTCTTGTTCA
GAGACTCAGAGGGTTTCGCCGCCGCTATCTTACAAGCTCTCCACCCCAACACGTCATCCTCCTTCACCCGCC
GAGAGGACACATTCGACCACTCCCTGGAGCCTTATGGAATCAAACACCTCAAAGCATCTGGGAGCGTTTCGC
ACTTCGTTGATGAGCACGGCGCTTATATGGTTTCAATTGTGGCTATGGAGCATTATAAGCCACCAGTACTAG
CTTGTGCTCTTAATGAAGTCCTCAATAAAATTACTGCAGATAAATCATCCCTGCCTACACTTTTGGTACCAT
TTTTGGTGGAATCTTCCAAGGTTAAAGGGAAAATTAAACACCTAGGATCAGATGAAAGCAAACCTCTGATAT
TTGGTATACCGATTGGTCAAAATACAGACATAATGCAGGCCTTACTCAACAAAACCCAGGAGCTACCATCTT
CGTCATGGATTCAACATGAAATTTTTGCATCTTTTCTTCACTTTGTTCGTGTAATGCAGTTACCAACCTTTT
TTCTAATTGGACAAACTAGTTAATATTTGGACGATCAATCCACCAAACAACATGAGATAATTCATGCTATAG
GAGAGATTTTGGCTAGTACTACAGGTCTGCAGTTTTCAGAAGACAGAGTGGTATGGAATCCAAAAAAGAAAT
CAGTTCAAAGCAAAGAGCCATGGCAAGCATTATATGGTTGAAACAGGATCATTTACGTACTGACTTGGAACC
AGTATTTTCATGGATCTGATTCACCGAACCTTTGGTCTTATGTATCAACTATTTCCTTCAAAAGTTATTATT
AATTTCCCCCCATATTTTCCTTACATCTTGTTCAAGTATCTTAGTATTGTGTAAAGTGATGGATGTTGCCAT
CACATGTAGCTGCATTTTCTTTTGAGTATTTGTTGGTTTTATTTTGATCAACCTATTCGACGTATTGCATAG
AGTAGATTTAACCGTTTATGTTATGCACCACACTGTATACTCACTGTCGCTTTGGTGCCTTGACCTCTTTTG

AAGGGAGGTCAAGATAAGAGCCCCAGATTTTTAGATTTTGCTTCTGTAAAAGAAACAATTAAAGATGAGGAT
G

2.12. *PPO* chr8

Localización: Phvul.008G073200 cromosoma 8

5´UTR CDS 3´UTR Primers

CTACGTCACTCTATTAATGGCTACCATTCAAAGCTTATCGCACTCACTCTTCTCTTCGCACTCACTCTATTA
ATGTGCAAATTTTGAGAGCATGAAAAAGTGCAGCAATGACCACTCCTTCTAAGCCTCTACCTTTATTCTTTG
CTCTTACTATTATCTTCCTCATTCCCTTCATTTCTCTACTCAACAATCACTTCTCTGTCATCACCGCCATAA
AACTCATTTGCAGACAAACATATAAACCCCCTCATGCCAAACAAAATGGCCTCGTCAAAAGTTCCAATGAAA
GAGACAAATCTCGTCTTTGGAGGGAACCCTTCATTGGCTTCAAAAATATAGATGACTCATCTTCCATTTCTA
GACCAATATCTCCTAATATAAGCGATTGTTTTCCTGTTGAGTTACCTTCTAATGCAATTACACCTGCCCATT
GCTGTCCATCTAAACCATCTTTCTCCAAGATCTTAGATTTCAAAGATTTTGCTTCTCCCAGTACCACACTGA
GAGTAAGAAAACCATTCCACTTGCTAGATAATGAGGATATAGCCAAATTTGAAAAGGGCATTGCACTTATGA
AAGCCCTCCCCGATGATGACCCTCGTAGCTTTATTCAACAAGCTAAGGTCCATTGCGCTTATTGTAATGGAG
CCTATCACCTGTCTCATCCTTTCCAAGACACAAAACTCGACATTCACAGGTCTTGGTTTTTCTTTCCCTTCC
ACCGTTGGTACATTTACTTCTTTGAACGAATTTTGGGGAACTTGATTGGTGACCCTAACTTTGCTTTACCAT
TTTGGAGTTGGGATTCTATAGAGGGCATGCAAATGCCATCGTATTTCGCAAACCCTAACTCGTCACTTTATC
ACAAACTCCGACACCAGAAACACCTGCCACCCCACGTGGTGGACCTGAACTATGCAAGTGAAAGTAGCTATG
TCCCTTCTCATCAACAAGTTTCGTATAATTTAGCCACCATGTACAGACAAATGGTGCTAGCAAGTACCACGG
AATTGTTCATGGGAGGCCCTTTTCGACTAGGGGATAACCCTCGTCCTGGTCCTGGTTCTGTGGAGGCTGCTC
CACATAACACCGTTCATACATGGGTTGGTGGAGCCGAAACTCCAAACCATGAGGACATGGGAACGTTCTACA
CAGCTGCTAGAGACCCCATTTTCTATGGTCATCACTCGAACTTGGATCGAATGTGGGCGATATGGAAAACAC
TGGGAGAAGGAAGAAAGGACTATAGTGATGAAGATTGGTTAGATTCTGAGTTTTTCTTCTACGATGAGAATG
CTAATTTTGTTCGTGTTAAGGTAAGAGATTGCGTTGATACTAAAAGCTTGGGGTATGTGTACCAAGATGTTG
ATCTTGCATGGTTGCGTACGCCACCCACATCACGAAAAAGTAAGCTACTGAGACAAAGGAAGAAGGCTAAAG
TTTGGAGTTCGAAGCCAAGAAAGTTTCCTCTGGTTTTGGATTCCGTAACAAGTGTAATTGTTAAGAGGGCGA
AGAAATGGAGGAGCAAGGAAGAGAAAGAAGAAGAGGAAGAGGTTTTGGTGATAGAAGGGATTGAGTTTGGAA
GTGATAAATTTGTTAAGTTTGATATTCATGTTGATGATGATGAAGACAGGTTGAGTAATCCAGATGAGACAG
AATTTGTGGGAAGTTTTGTGAGTCTGTACCATGGACGTGGCCATAAAATCAGCACTAGCTTTAAAGTAGGAA
TTTCAAAAGTTCTGGAGAATTTAGAAGCTGAAGAAGACGATGATTTGCTGGTTACTTTGGTACCTAAGCTGG
GAAAAGGAGATGTAATCATAGGAAGCATCAAAATTCAGTTTATTCCAAAACAGTAGACTTATATTATATTTT
CTGTGTATTAAAATTATTCTTTTCACAAAGGAAAATGTTTCTTTTGT

2.13. *ERF1* chr7

Localización: Phvul.007G127800 cromosoma 7

5´UTR CDS 3´UTR Primers

AAAGAGACGAGAAAAACAGAGCAACTCAACACCTTACTTCTCCTTTTCACTATTCACTCTGTTTGTTTTCTA
AACATGGATATAGATTCATCCTCCTTCTTCAACTCCCCCTCTTCCGATTTCTCCTCCGAATCTTCCTCTCCG
GATGCCTTCTCCTGGGAGGGATACCTCCCTTTCAACGAGAACGACCCGGAGGAGATGCTCCTCTACGGCATG
ATCGCCGGCGCCACCACAGCCGAGCGCACCGCCGACCGAGCGGGCTCTGAGGAGAGTGCGGCGCCGCAAAAG
GAGAAGTCCTACCGCGGAGTGAGGCGACGGCCGTGGGGGAAGTTTGCGGCGGAGATTAGGGACTCCACACGG
CACGGGATGAGGGTGTGGCTTGGGACATTCGACAGCGCGGAAGCCGCGGCTTTGGCTTACGACCAAGCGGCG
TTTTCCATGCGCGGGTCCGCCGCGATTCTGAACTTCCCCGTTGATATCGTTAGAGAATCACTTAGGGAGATG
AACTACGCGGTGGATAGCAGTGAAGAAGGGTGCTCCCCTGTTGTCGCTCTCAAGAGGAAACACTCCTTGCGG
AGGAAAATTGGTGTAAAGAAAAACAACAAACAGAGTACCGTAGACAATGCGGTCGTGTTTGAAGACCTTGGT
CCTGACTACTTGGAACAGTTGTTGATGTCCTCTGATCATATTCCCTCCTCCTTCTGATTCATTCATTCCTCA
ATTATAATCCAAACCTCGAAAGCCTTCACGCTGCGGCTCAAGCACCAATTCCGCAACGTTTTTTATAACCTT
ATAAATATCCCACGCTGCATAGTTTCTACCTAGGCTTTTTTATGTTAGTGTTAATTCCTTTTTCTTCTTTGT

TCTTTGGTTCTTGTATGGCTGAGATGCTTGTGTCATGTTTTGATGACAGAGTTCAACATCTTCTGAATCTGT
ATCTTTTTATCTCTTC

2.14. *ERF5* chr2

Localización: Phvul.002G055700 cromosoma 2

5´UTR CDS 3´UTR Primers

CTTAAGTTTCTCATCCAAGTTCATGTACCGTCCCTTTTATAAAAACCCTCGTCCACTTTCCCCTTCCAACTT
CCAACATTCATTTCACACCCAAATCCTTCTCTCAACACAAACACAAATATCAGGGTTACAAAATGTACGAAG
AAACTACCTACGACTCCGATTTGGCTCTTTTGGATTCCATTCGCCGCCACTTACTCGGGGACTTCGAGGATC
TCAAATTCGGAGCCCCGATTGTTAACTCCGGTAACACTCCCCTTTACTCTCGGAGCACCAGTTTCAGTAGGT
TGTACCCGTGTTTGAGTGACGATTGGGGAGAGCTTCCTCTTAAGGAAGATGATTCAGAGGACATGGTGCTCT
GCGGTGTTCTTCGCGATGCCATCAACGTAGGCTGGGTCCCATCTCTCAACGCAGCCTCACCGAACACCTTTT
CCTCGGGTTTCATCCCGGAAGTTACCGTGAAACCCGAACCGGATTTATTTCCGGCACCGGAACCTGTTTTCA
CCGCTTCGGTGGTTCAGAAGGATGCACCGGCGGTTGTTCCGGCCAAGGGGAAGCACTACCGCGGCGTGAGGC
AACGGCCGTGGGGAAAATTCGCGGCGGAGATCCGCGACCCGGCTAAAAACGGGGCTCGGGTCTGGCTCGGAA
CGTTCGAGACGGCTGAGGACGCCGCCCTGGCATACGATAGAGCCGCGTATCGAATGCGCGGCTCGCGGGCTT
TGTTGAATTTCCCGCTTCGGATTAATTCGGGCGAGCCCGAACCGGTTCGGGTGACGTCGAAGCGAGCGTCGC
CGGAGCCTTCGTCCTCGTCGGAGAGTGGATCGTCGGCGAAGCGGCGGAAAAAAGTGGTGGGGTCGACGGTGA
CGGCTCCAAGTGGATTGAGAACGGGGCAAGTGGGAAGTCAAGTGTCTCAGTGCACACGTGCGGAACAGTTGT
TGGTTAACTAATGCGTTATGCTGACGTGGATTTCACTGTTCTTTTTGTGTAAATTGTGCTTTGTAGTTATCT
GATTCTGAGATGCGTGTGACAGTGGCGGAAACTGTTATTAAAAGAATGAAAATAATAAGCCAACAAGGGAAG
GACAGTGGCACAGTGTAAAAAATGGATTGATACCTCAACTCCATCAAGTCGGAGTCATAAAATCTAAACCAG
AAAAAATTAAGAAAACCAAAACAAAGTTGCAAAAAGCAATTAGGGTGGAGGTTTTGTTTGGGTCTCTGAATA
CAGTCCCAATATTTGTTATTTTTTGGTTCAATATTTATGAAATTTGTGAATTGGGTTGCATCAACTGAAGCC
TCTACTTTATTCTCTC

2.15. *Ch5b* chr9

Localización: Phvul.009G116500 cromosoma 9

5´UTR CDS 3´UTR Primers

GGAAAGAGAGAGAGAAATGAAGAAGAATAGGATGATGATGATGATATGGAGCGTAGGAGTGGTGTGGATGCT
GTTGTTGGTTGGAGGAAGCTACGGAGAGCAGTGTGGAAGGCAAGCAGGAGGTGCACTCTGCCCAGGGGGCAA
CTGTTGCAGCCAGTTCGGGTGGTGCGGCTCCACCACCGACTACTGCGGCCCGGGTTGCCAGAGCCAGTGCGG
GGGACCGTCTCCTGCTCCTACTGATCTCAGCGCCCTCATATCCAGGTCCACCTTCGACCAGATGCTCAAACA
TCGCAACGACGGAGCCTGCCCAGCCAAAGGCTTCTACACCTACGATGCCTTCATCGCCGCCGCCAAGGCTTA
CCCCAGCTTCGGAAACACCGGAGACACGGCCACTCGCAAGAGGGAGATTGCGGCCTTCTTGGGGCAAACGTC
TCACGAAACAACCGGGGGATGGGCCACTGCGCCCGACGGACCATACGCATGGGGATACTGCTTCGTGAGGGA
GCGGAACCCCAGCACGTACTGCTCCGCCACTCCCCAGTTCCCCTGCGCCCCTGGGCAGCAGTACTACGGCAG
GGGTCCCATCCAGATATCCTGGAACTACAACTACGGTCAGTGCGGAAGGGCCATTGGGGTTGACTTGCTCAA
CAAACCTGATCTAGTCGCCACTGACTCTGTCATCTCCTTCAAGTCCGCCCTCTGGTTCTGGATGACCGCACA
GTCCCCCAAGCCTTCCTCCCACGACGTCATCACCTCTCGATGGACCCCCTCCTCTGCCGACGTCGCCGCCCG
CCGGCTTCCCGGCTACGGCACTGTGACGAACATCATCAACGGAGGCCTGGAGTGCGGGCGAGGACAGGACAG
CAGGGTTCAAGACCGCATCGGATTCTTCAAGAGATACTGTGATCTGCTTGGAGTCGGTTATGGCAACAACCT
TGACTGCTACTCTCAGACTCCATTTGGAAATTCACTCTTACTCTCTGACCTTGTCACCTCTCAGTGACACTG
TCATCCCATCAGAATAAATAAACTCATAGATCTGTGTTTCACTTTCTGATCACAACTTTTCCAATAACACTT
TTCCCACCATCTATCAATAAATTCACTACTTCTATATACCATATGCACA

2.16. *PAL1* chr1

Localización: Phvul. 001G177800 cromosoma 1

5´UTR CDS 3´UTR Primers

ACTCTATTCTTCTCAGGAAATCCTCAACACACATCAACATTTCCTCACTCTCTCTCTCTCTCTCTACA
CAAAACTCACCTCCATTTTTTCATTCTCTTCATTTTATCACGATGGAAGCAACTACCCTTACCAACGCCCAA
AACGGTTCCTTCTGCCTCCCATCCGCCAAGACCACTGATCCGCTCAATTGGGGAACGGCCGCGGAGGCGCTC
AAGGGGAGCCACCTCGACGAGGTCAAGCGCATGGTGGCCGAATACCGCCAGCCGCTGGTCCGCCTCGGCGGC
GAGACGCTCACCATCGCTCAGGTGGCGGCGGTGTCTGGCCACGACCAAGGCGTGAGGGTTGAGCTGTCGGAG
TCCGCTCGGGAAGGTGTTAAGGCGAGCAGTGAGTGGGTGATGAACAGCATGAACAATGGTACTGACAGTTAC
GGCGTCACCACCGGCTTCGGTGCCACCTCCCACCGCCGAACCAAACAAGGTGGTGCTCTGCAGAAAGAACTC
ATCAGGTTTTTAAACGCTGGAATATTTGGAAATGGGACTGAATCATCCCACACACTGCCACACACTGCAACC
AGAGCAGCCATGTTAGTGAGGATCAACACTCTTCTTCAAGGCTACTCAGGCATCAGATTTGAAATCTTAGAA
TCCATCACCAAGCTCCTTAACAACAACGTTACCCCATGTTTGCCACTTCGTGGTACAATCACAGCTTCTGGA
GATCTTGTTCCACTTTCCTATATTGCTGGTTTGCTCACTGGCAGACCTAACTCCAAAGCTGTTGGACCTTCT
GGAGAAGTACTTACTGCTAAGCAAGCTTTTGAATTGGCTAACATCAATTCTGAGTTCTATGAATTGCAACCC
AAGGAAGGCCTTGCCCTTGTTAATGGCACGGCTGTCGGTTCTGGCATGGCTTCTATAGTTCTCTTTGATGCA
AATATACTAGCTGTGTTGTCTGAAGTTCTATCAGCTATTTTTGCCGAAGTGATGCAAGGGAAACCTGAGTTT
ACTGATCATTTGACACACAAGTTGAAGCACCACCCTGGTCAAATTGAGGCTGCTGCCATTATGGAGCACATT
TTGGATGGAAGTTCCTACATGAAGGATGCTAAGAAGCTGCATGAAATAGATCCGTTGCAAAAGCCAAAACAA
GATAGATATGCCCTCAGAACTTCACCACAGTGGCTTGGTCCTCTCATTGAAGTCATTCGTTTCTCCACCAAG
TCAATTGAGAGAGAGATTAACTCCGTGAATGACAACCCTCTTATTGATGTTTCAAGGAACAAGGCATTGCAT
GGTGGTAATTTCCAAGGAACCCCAATTGGAGTCTCCATGGACAACACTCGTTTGGCTCTTGCATCTATCGGC
AAACTCATGTTTGCTCAATTCTCCGAGCTTGTCAATGATTTCTACAACAACGGTCTGCCTTCAAATCTCACT
GCTAGCAGAAATCCTAGCTTGGATTATGGTTTCAAGGGAGCTGAAATTGCCATGGCTTCCTATTGCTCTGAA
CTCCAGTATCTGGCAAATCCAGTAACAAGCCATGTCCAAAGTGCTGAGCAACACAACCAAGATGTGAACTCT
TTGGGTTTGATTTCAGCTAGGAAGACAAATGAATCTATTGAGATCCTTAAGCTCATGTCTTCCACGTTCTTG
ATGGGACTTTGCCAAGCAATTGACTTGAGGCATTTGGAGGAGAATTTGAAAAGCTCGGTCAAGAACACTGTG
AGTCAAGTTTCCAAGAGGACTCTTACCACAGGTGGCAACGGAGAACTCCATCCTTCAAGATTTTGCGAAAAG
GATCTGCTGAAAGTGGTTGACAGGGAGTATGTATTTTCCTACATAGATGACCCATGCAGTGGTACATACCCA
CTGATGCAAAAACTTAGGCAAGTGCTTGTAGACCATGCCTTGATAAATGCAGAGAATGAGAAGGATGTCAAC
ACATCAATCTTTCAAAAGATTGCAACCTTTGAGGAGGAGTTGAAGACCATCTTGCCAAAGGAGGTTGAAAGT
ACAAGGGCTGCTTATGAGAGTGGGAAAGCTGCAATTCCAAACAAGATAAAAGAATGCAGATCTTACCCACTC
TACAAGTTTGTGAGAGAGGAGTTGGGCACTGGGTTGCTAACTGGAGAAAAGGTGAAGTCACCAGGTGAAGAG
TTTGACAAGTTATTCACAGCAATATGCCAGGGCAAAATTATTGATCCTCTTCTCGAATGCCTTGGAGAGTGG
AATGGAGCTCCTCTTCCAATTTGTTGATTTTACTGTCATTTTTATAAGTATTTTTCTCTGTATCTACGCAAG
TGAAACCATAATCATTGAGTTTGGTAAGCCTTTTAACAAATCTACATGGAAAGCCGGATTCTAATGTTTCCT
CCATGTCAGATAGGACCTTGTAATTTAATATTATAGTGAAATTTCAGTTGATTAATGATTCGTACT

2.17. *OSM34* chr4

Localización: Phvul. 002G155500 cromosoma 4

5´UTR CDS 3´UTR Primers

ATAAAAATTTGATTTCTCAACGTATCATTACCATTCATATAAATTGTTAAAATCTCTCTACACTCAACCATG
TAAGGCTATATAAACACCAAACACCAGTTTTGGAATCCACACTGAACACGATAAACATGGCTCACTCACTCT
CCCTAACGCCACTTTGCGCCCTCCTCATGCTCGCCTCATTATCTCTCGCCACCGCTGCAAACTTCGAGATCG
TCAACAACTGCCCCTACACGGTGTGGGCGGCTGCCAGCCCCGGCGGCGGGCGGCGCCTGGACCGCGGGCAGA
CGTGGAACCTGTGGGTGAACGCGGGCACTGCCATGGCCCGCATATGGGGTCGCACCGGGTGCAACTTCGACG
GCAGTGGGCGAGGGCGGTGCCAGACAGGTGACTGCACCGGCGGCCTGAACTGCCAGGGCTGGGGCGTTCCTC
CGAACACCCTGGCGGAGTTCGCCCTGAACCAATTCGGCAACTTGGACTTCTACGACATCTCCCTGGTGGACG
GTTTCAACATCCCTATGGACTTCTACCCCGTGAACGGAGGGTGTCACAAAATCAGTTGCACCGCCGACATTA
ATGGGCAGTGCCCTGGGCCATTAAGGGCCCAAGGGGGATGCAACAACCCCTGCACCGTGTATAAGACGAACG
AGTATTGCTGCACCAATGGGCAAGGAACTTGTGGACCCACTACGTACTCTAGGTTCTTCAAAGATAGGTGCC
GTGATGCTTACAGTTACCCTCAGGATGACCCTACCAGCACCTTCACTTGCCCTGCTGGTTCTAACTACAAGG

TTCTCTTCTGTCCTCTCGGAGATCCTCTTCACATGCTTCCTAATACCACCACCAATCAATATTAAAATAACC
ATACCAACGCTGTGTAGATCCTTCGATGGGCCTACCTACAAGGAAATGAAGTTTTTTCACAACTTCATTTTT
TATCATCAATAAAATTGGATCTCCTTTCTGTTTCATTTGATCTCTTCTACTGCCATCAAATTAGAAAATGAA
ATCTCTCTCACTCAACTTTCTATGTCGCGCTAGTTTCTGTAAGGAAAAAGTTT

2.18. *CNGC2* chr6

Localización: Phvul.008G036200 cromosoma 6

5´UTR CDS 3´UTR Primers

AGAACAACGTATAGCAACCAACCCCACCATCACTCCCTCAACGTTGCGTAACCGTAAGCTCCAATCCCTACA
CTCCTGATTCACATGCAAATTCATCTTCTTCTTCCAATCCTCTTTCTTATATACTCTTCTTCCATGTGCATA
CCCTGACACACCTAAACCCCACTTTTCCTCCATCTAACTCCCCTCCTCCCATGGTTTCTCTCCATCATCAAC
CATGCCCAACACCTTCTCCCTCCTCTGGTGGATTGGCAAAAAGTTGCGAAGAAGGAACACAGTTAGTAATGG
AGAGAGTGGGAGCAACGAGAACGTCGTCGGTGCCGTTGTTGTGGATGACAATCCATTTGGCAGCACGGTAGA
GTGTTACGCTTGCACGCAGGTTGGCGTGCCGGTGTTTCACTCCACCAGCTGCGACAGTTTTAACCAGCCGCA
ATGGGAGGCTTCGGCCGGCTCCTCCCTTGTTCCGATCCAGAATCGGCCGAACAAGGTTCTCGGGTTTCGGAC
CGTATCCGGTTCGAATATTGGACCGTTTGGGAGGGTTTTGGATCCGCGGAGCAAGCGCGTGCAAAAGTGGAA
CCGCGCGCTGCTGCTGGCGCGTGGGGTGGCGTTGGCGATTGACCCGCTGTTCTTCTACTCGCTGTCGATCGG
GAGGGAGGGGTCGCCGTGCCTTTACATGGACGGAGGGCTGGCGGCGATGGTGACGGTGGCGCGGACGTGCGT
GGACGCGGTGCATCTCTTGCACGTGTGGCTGCAGTTCCGGCTGGCGTACGTGTCGCGCGAGTCGCTGGTAGT
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2.19. *HPL* chr5

Localización: Phvul.005G116800 cromosoma 5

5´UTR CDS 3´UTR Primers

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G

2.20. *Lox2* chr5

Localización: Phvul.005G156700 cromosoma 5

5´UTR CDS 3´UTR Primers

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2.21. *Lox2* chr10

Localización: Phvul.010G134800 cromosoma 10

5´UTR CDS 3´UTR Primers

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2.22. *Lox7* chr5

Localización: Phvul.005G156900 cromosoma 5

5´UTR CDS 3´UTR Primers

GGTGTATATAAATAGGAGCATGGCCATGCTCACTCACACACAGAAGAAGAACAACAACAGTGTTCGTTGAAA
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2.23. *GSTa* chr2

Localización: Phvul.002G241400 cromosoma 2

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2.24. *hGS* chr6

Localización: Phvul.006G094500 cromosoma 6

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2.25. *Aminotransf 2* Clase V chr6

Localización: Phvul.006G029100 cromosoma 6

5´UTR CDS 3´UTR Primers

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TCTTATTTCTGACTATCTCATGCTC

3. BIBLIOGRAFÍA

Hermosa, M. R., Grondona, I., Iturriaga, E. A., Diaz-Minguez, J. M., Castro, C., Monte, E., et al. (2000). Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. *Appl. Environ. Microbiol.* 66, 1890-1898.

Hermosa, M. R., Keck, E., Chamorro, I., Rubio, B., Sanz, L., Vizcaíno, J. A., et al. (2004). Genetic diversity shown in *Trichoderma* biocontrol isolates. *Mycol. Res.* 108, 897-906.

Kullnig, C. M., Krupica, T., Woo, S. L., Mach, R. L., Rey, M., Benítez, T., et al. (2001). Confusion abounds over identities of *Trichoderma* biocontrol isolates. *Mycol. Res.* 105, 770-772.