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Polifenoles como respuesta a hongos micorrícicos arbusculares en vid (*Vitis vinifera* L.)

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Los viñedos de *Vitis vinifera* son un antiguo, extenso e importante cultivo en Chile y el mundo. Actualmente, buscan desarrollar una agricultura sustentable y resolver problemáticas que atañen a toda la agricultura, como la escasez de agua, pérdida de fertilidad de los suelos, mal uso de agroquímicos, entre otros. En el caso de la vid esto se vuelve fundamental, pues estos factores afectan la producción, calidad de la uva y calidad de los vinos, en un mercado que se mueve por la diferenciación, altos estándares de calidad y por el impacto generado en el medioambiente. La micorriza arbuscular en *Vitis vinifera*, es una asociación benéfica que perdura tras los años, actualmente las investigaciones reportan los múltiples beneficios de esta simbiosis, entre los que se destaca el desarrollo y estimulación de crecimiento del hospedero, así como, la estimulación en la producción de metabolitos secundarios del tipo polifenoles, terpenos y alcaloides, compuestos con importantes aplicaciones en la alimentación y salud humana. Sin embargo, en nuestro país no se ha reportado ningún estudio sobre la diversidad de los hongos micorrícicos arbusculares (HMA) presentes en los viñedos, esta información es fundamental para el adecuado manejo de los cultivos y las aplicaciones de nuevas tecnologías relacionadas con los biofertilizantes en base a HMA. Por otro lado, actualmente existe la interrogante de si los HMA modifican la producción de polifenoles, propiedad importante para las vides, que se caracterizan por sus altas concentraciones de estos metabolitos. Lo anteriormente expuesto generó la hipótesis: la formación de las micorrizas arbusculares nativas aumenta la concentración de compuestos fenólicos en vides (*V. vinifera*) de Chile. El objetivo principal de este estudio es estudiar los hongos micorrícicos arbusculares nativos y si modifican la concentración de polifenoles en plantas micorrizadas de vides (*V. vinifera*) de Chile. Se obtuvo el material biológico a partir de cinco zonas seleccionadas con viñedos de distinto manejo. Se realizaron ensayos a diferentes niveles (*in vitro* y en terreno), y análisis preliminares de suelo y porcentajes de micorrización. Posteriormente se efectuó un análisis de diversidad, utilizando la taxonomía clásica y técnicas de biología molecular. A partir de los ensayos, se evalúo la micorrización y sus efectos en los polifenoles utilizando técnicas cromatograficas HPLC y LC-MS. Se encontró una alta diversidad de hongos micorrícicos en los viñedos chilenos de distintos manejos, se encontró que el manejo orgánico favorece considerablemente la formación de micorrizas arbusculares, aprovechando los servicios ecosistémicos que esta entrega. A su vez se comprobó que la simbiosis modifica y aumenta la

concentración de metabolitos secundarios principalmente la quercitina. Esta investigación puede ser la base tecnológica para la aplicación de los hongos micorrícicos arbusculares para desarrollar una agricultura sustentable o para estimular la producción de metabolitos de interés.

Palabras claves: Hongos micorrícicos arbusculares; *Vitis vinífera* L.; Viticulture; Agricultura sustentable; Polifenoles

ABSTRACT

The vineyards of *Vitis vinifera* are an ancient, extensive and important crop in Chile and the world. Currently, they seek to develop a sustainable agriculture and solve problems that affect all agriculture, such as water scarcity, loss of soil fertility, poor use of agrochemicals, among others. In the case of vine this becomes fundamental, as these factors affect the production, quality of the grape and quality of the wines, in a market that moves by the differentiation, high quality standards and by the impact generated in the environment. The arbuscular mycorrhiza in *Vitis vinifera*, is a beneficial association that lasts after the years, currently the investigations report the multiple benefits of this symbiosis, among which the development and stimulation of growth of the host, as well as the stimulation in the production Of secondary metabolites of the type polyphenols, terpenes and alkaloids, compounds with important applications in food and human health. However, in our country, no study has been reported on the diversity of arbuscular mycorrhizal fungi (AMF) present in the vineyards, this information is fundamental for proper crop management and applications of new technologies related to biofertilizers in To AMF. On the other hand, there is currently the question of whether AMFs modify the production of polyphenols, an important property for vines, which are characterized by their high concentrations of these metabolites. The above hypothesis generated the formation of the native arbuscular mycorrhizae increases the concentration of phenolic compounds in vines (*V. vinifera*) of Chile. The main objective of this study is to study native arbuscular mycorrhizal fungi and to modify the concentration of polyphenols in mycorrhizal vines (*V. vinifera*) plants in Chile. The biological material was obtained from five selected areas with different management vineyards. Tests were carried out at different levels (*in vitro* and in the field), preliminary soil analysis and mycorrhization rates. Subsequently a diversity analysis was carried out, using classical taxonomy and molecular biology techniques. From the tests, mycorrhization and its effects on polyphenols were evaluated using HPLC and LC-MS chromatographic techniques. It was found a high diversity of mycorrhizal fungi in Chilean vineyards of different managements, it was found that the organic management favors considerably the formation of arbuscular mycorrhiza, taking advantage of the ecosystemic services that this delivery. In turn, it was verified that the symbiosis modifies and increases the concentration of secondary metabolites mainly the quercitine. This research may be the

technological basis for the application of arbuscular mycorrhizal fungi to develop a sustainable agriculture or to stimulate the production of metabolites of interest.

Key words: Arbuscular mycorrhizal fungi; *Vitis vinifera* L.; Viticulture; Sustainable agriculture; Polyphenols

INTRODUCCIÓN

La vid (*Vitis vinifera* L.)

La vid (*Vitis vinifera* L.) es un cultivo antiguo de gran importancia económica y alimentaria en Chile y el mundo (Gil & Pszczólkowski, 2007). Chile es un país que por su clima mediterráneo templado y las particulares características geográficas del país, ha desarrollado una amplia viticultura con muchas variedades comerciales que generan distintos estilos de vino. Desde el siglo XVI se ha plantado *V. vinifera* en el país, y las variedades fueron traídas desde Europa por los colonizadores, siendo principalmente Cabernet Sauvignon, Merlot, Malbec, Carmenère y Sauvignon Blanc. Los viñedos europeos sufrieron la plaga de *Phylloxera*, entre otras enfermedades como *Plasmopara vitícola* y *Uncinula necátor*, etc., y que obliga actualmente a utilizar porta injertos resistentes a estas plagas y pestes, sin embargo, esta situación favoreció a los productores chilenos, donde no se encuentra Phylloxera y que permite el cultivo natural de la vid, logrando viñas de edades muy avanzadas, lo que favorece la calidad de los vinos. En 1994 se encontró en el país la variedad francesa Carmenère, que estaba presente en los viñedos de Merlot. Se pensaba que esta variedad se había perdido con el ataque de la Phylloxera, pero como había llegado a Chile antes de su brote, aquí se mantuvo. Las condiciones térmicas oscilantes durante el año y también durante el día, favorecen a la producción de frutos y vinos de alta calidad. La alta variedad de tipos de suelos, genera el cultivo de muchas variedades de vid y producción de vinos únicos. Actualmente hay viñedos plantados desde el valle del Elqui (30° latitud sur), por el norte, hasta el valle del Malleco (39° latitud sur), por el sur, con 210.000 ha aproximadamente plantadas al 2014, según información del SAG.

La vid aunque posee una alta plasticidad fenotípica, posee requerimientos nutricionales específicos para un adecuado desarrollo y producción de frutos. Los viñedos de secano, que solo reciben agua de las lluvias, representan un 25% de los viñedos del país, estos a menudo presentan bajas producciones y bajas calidades en el fruto para la posterior elaboración de vinos, lo que disminuye el precio en el mercado (Chávez *et al.*, 2010). Esto ocurre principalmente, donde por las condiciones climáticas y geográficas las plantas de vid tienen baja disponibilidad de agua. Esta problemática se aborda con cultivos intensivos con irrigación constante, lo que involucra el uso de modernas tecnologías y mayores costos de producción.

La productividad de las plantas está limitada en primer lugar por la disponibilidad de agua en los ecosistemas y el cambio climático puede aumentar la limitación de este recurso, por la mayor intensidad y frecuencia de sequias (Romero *et al.*, 2013).

El cultivo de vid tradicional, involucra el uso continuo de clones de variedades antiguas, con características que benefician la producción de vinos, pero esto no considera, que el uso de poca variabilidad genética en los cultivos, puede volver al cultivo más susceptible al ataque de patógenos, creando plagas, que pueden generar pérdidas millonarias. Esta costumbre se ve aumentada por los muchos años que se debe esperar para que una variedad cultivada pueda ser evaluada desde un punto de vista productivo y fitopatológico, por la difícil identificación de los síntomas de una enfermedad (Myles, 2013).

La nutrición es otro aspecto importante del manejo del viñedo. La fertilización de nitrógeno (N) aumenta el crecimiento y el rendimiento del cultivo, influenciando varios parámetros de la producción de vinos, como la formación y calidad del fruto y por lo tanto, la calidad del vino. Mayores cantidades de N aumentan el vigor, el rendimiento de la fruta y modifican la composición del jugo (pH y concentración de ácidos orgánicos y ésteres) pero también crean condiciones favorables para enfermedades como necrosis del racimo (Smith & Smith, 2011). La aplicación de N en el viñedo puede modificar la absorción de otros nutrientes, altos niveles de N puede reducir la disponibilidad de fósforo (P), boro (B), potasio (K) y cobre (Cu), del mismo modo, la aplicación de fertilizantes de N puede afectar la biodisponibilidad de elementos traza en el suelo y de metales. Los fertilizantes minerales de N contienen amonio que acidifica el suelo, disminuyendo el pH de la rizósfera y aumentando la concentración de metales pesados. (Domagała-Swiątkiewicz & Gąstoł, 2013). El P es un nutriente fundamental para el crecimiento y metabolismo de la planta, a pesar de estar presente en el suelo, mayoritariamente no está disponible o soluble para la absorción por parte de la planta, por lo que presenta bajos niveles de concentración y esto se ve aumentado por un pH ácido del suelo, producido principalmente por un exceso de fertilización nitrogenada, ya que el P forma compuestos minerales con aluminio, hierro y manganeso disminuyendo la disponibilidad para los cultivos (Smith *et al.*, 2011).

La vitivinicultura sustentable se ha convertido en una elección lógica, y las viñas orgánicas y biodinámicas están en alza, de hecho, algunos de los más grandes viñedos orgánicos del mundo están en Chile, dando origen a los vinos que piden los consumidores, enfocados en la ecología, el medioambiente y conscientes de una buena relación precio-calidad (SAG, 2014).

Al igual que otras plantas con bajas densidad de raíces o con pocas raíces finas, las vides parecen ser dependientes de hongos beneficos de raíz, llamados hongos micorrícicos arbusculares, para un favorable desarrollo y crecimiento, sobre todo cuando actualmente existen muchos viñedos en condiciones de estrés hídrico y con baja fertilización en sus suelos (Likar *et al.,* 2013). Así estos hongos forman parte del ecosistema del viñedo y de las aplicaciones para el desarrollo de una agricultura sustentable, que mantenga los niveles productivos y la calidad, mientras minimiza el impacto en el medioambiente (Nasim, 2012).

Los hongos micorrícicos arbusculares

Los hongos micorrícicos arbusculares forman una asociación simbiótica mutualista con las raíces de la mayoría de las plantas terrestres. En esta simbiosis la planta hospedera entrega azúcares provenientes de la fotosíntesis y el hongo deja a mayor disposición el agua y los nutrientes del suelo principalmente N y P (Smith & Read 2008). Las micorrizas arbusculares son el tipo más común de micorriza, se encuentran asociadas al 80% de las plantas, existiendo hace más de 460 millones de años (Redecker 2000). Son organismos biótrofos obligados de la división *Glomeromycota*, con 14 familias donde las mas comunes corresponden a las familia *Glomaceae, Acauolosporaceae, Gigasporaceae* (Leake *et al.,* 2004, Oehl et al. 2011). Carecen de reproducción sexual, pero poseen grandes esporas multinucleadas, hifas no septadas, y en las raíces forman estructuras características como son las vesículas de almacenamiento y los arbúsculos intracelulares, donde ocurre el intercambio de nutrientes, entre micobionte y fitobionte.

La simbiosis micorrícica se produce cuando las plantas y las hifas de los hongos reconocen moléculas señal en la rizosfera, realizando un intercambio de compuestos solubles en el suelo (Bonfante & Requena, 2011). Antes de ocurrir la simbiosis, las plantas producen grupos de estrigolactonas derivados de los carotenoides y cuando ya existe contacto físico, la percepción

de estas moléculas promueve la germinación esporal y la ramificación de la hifa, el hongo produce los factores micorrícicos (Factores Myc), incluyendo lipo-quito-oligosacáridos (LCOs) y posiblemente estos compuestos son reconocidos por la planta hospedera para activar la rutas de señales común de la simbiosis, promoviendo la invasión de la raíz y generando oscilaciones de calcio (Ca) (Smith & Read 2008). La invasión de los hongos micorrícicos arbusculares implica la formación de los apresorios que permiten el crecimiento de las hifas fúngicas en la célula epidérmica de la raíz. Luego que las hifas intercelulares invaden el córtex de la raíz, los arbúsculos son formados en el interior de las células corticales de raíz desde las hifas intercelulares, se mantienen las membranas, formándose la membrana peri fúngica alrededor de las hifas y de los arbusculos (Oldroyd, 2013). En la colonización MA se reconocen dos tipos anatómicos principales: Arum y Paris descritos por Gallaud (1905). En el tipo Arum, las hifas crecen intercelularmente en la corteza radical y forman arbúsculos finos y altamente ramificados dentro de las células. En el tipo Paris, las hifas forman lazos hifales y, ocasionalmente, se forman estructuras semejantes a arbúsculos e hifas arbusculares.

La colonización con hongos micorrícicos arbusculares otorga a la planta principalmente una mayor absorción de agua y macronutrientes como el N y el P, de baja disponibilidad en los suelos así como de algunos micronutrientes (Zn, Cu, etc.) lo que mejora su crecimiento y rendimiento (Smith & Smith, 2012). Además permite una mayor resistencia a estrés hídrico, estrés salino y a presencia de metales pesados. La presencia de micorrizas arbusculares, puede aumentar la tolerancia al déficit hídrico, pues las hifas pueden acceder a pequeños poros del suelo, aumentando el área de absorbancia de la raíz (Worchel *et al.,* 2013). Existe múltiple evidencia de la resistencia que generan ante el ataque de patógenos, desde bacterias, nematodos, hongos y algunos insectos que atacan la raíz (Jung *et al.*, 2013). La formación de micorrizas arbusculares modifica la rizosfera, influencia la estructura del suelo y aumenta la capacidad exploratoria del suelo de la raíces en búsqueda de nutrientes y agua. Se cree que la mayor resistencia a patógenos está relacionada con la competencia por espacio y nutrientes en la raíz, así como por la inducción de cambios en el metabolismo primario y secundario y la regulación de los mecanismos de defensa de la planta, afectando también las partes aéreas de las plantas (Toussaint, 2007). Esta capacidad de aumentar la tolerancia a distintos tipos de

estrés biótico y abiótico, han permitido la sobrevivencia a través de años de evolución de esta simbiosis entre las plantas y los hongos micorrícicos (Worchel *et al.*, 2013).

Con todas las ventajas para los cultivos y las propiedades fertilizantes, la simbiosis micorrícica se presenta como una alternativa para el desarrollo de una agricultura sustentable y el reemplazo de los fertilizantes tradicionales o plaguicidas, que muchas veces se traduce en contaminación de las napas freáticas y de la materia orgánica del suelo, disminuyendo de este modo, la fertilidad del suelo a través del tiempo (Duhamel & Vandenkoornhuyse, 2013). Pero para lograr este objetivo, se debe en primera instancia conocer la biodiversidad de los hongos micorrícicos arbusculares presentes en los tipos de viñedos del país en las distintas épocas del año, ya que, la formación de inóculos adecuados para el uso de los hongos micorrícicos arbusculares como biofertilizante o biorremediadores de suelos, a mayor escala en los viñedos, se debe evaluar en las diferentes especies encontradas o un consorcio de especies nativas que favorezcan al cultivo y analizar su efecto a largo plazo en un ciclo de cultivo, dada las condiciones específicas del lugar y variedad analizados, ya que, las diferentes variedades de vid poseen distinta afinidad con hongos micorrícicos arbusculares y puede ser más efectiva con ciertas especies de hongos *Glomeromycetes* , incluso con especies que corresponden a un sitio específico (Lee *et al.*, 2013). También se ha observado que las condiciones ambientales y geográficas, como el tipo de suelo o la altura, afectan directamente la diversidad de especies, la micorrización y los beneficios de esta en las plantas (Schreiner & Mihara, 2009).

En diferentes partes del mundo, se ha realizado la identificación de las especies de hongos micorrícicos presentes en los viñedos y otros cultivos (Öpik *et al.*, 2013), pero considerando las variaciones geográficas, clima, tipo de suelo, microbiota del suelo, prácticas culturales, variedades utilizadas, porta injertos, etc., que influyen en la diversidad de especies de hongos micorrícicos, se considera que el conocimiento de diversidad de hongos micorrícicos en viñedos está todavía muy incompleto (Schreiner *et al.*, 2007, Likar *et al.*, 2013). En el caso de Chile, a pesar de ser un país importante productor de uva, no se tiene ningún reporte de la diversidad de especies de hongos micorrícicos presente en los viñedos, y es necesario poseer esta información, tanto para un adecuado manejo del cultivo, conservación de la biodiversidad

de los microorganismos, como también, para poder diseñar aplicaciones agroecológicas que colaboren con la sustentabilidad del viñedo.

En las identificaciones actuales de biodiversidad de hongos micorrícicos se busca realizar estudios de meta genómica buscando el total de los genomas presentes en los consorcios microbianos en diferentes ambientes para ello se utilizan partidores previamente evaluados para amplificar y poder identificar géneros y especies de *Glomeromycetes*, a partir de muestras de raíces, luego se realiza secuenciación Sanger, pero generalmente se llega a identificar solamente el género *Glomus* en la mayoría de los estudios (Beck *et al.*, 2007, Ballestrini *et al.*, 2010, Shi *et al.*, 2012). Sin embargo en el estudio morfológico y esporal de los suelos, se observa la presencia de otras familias como *Gigaspora* y *Acauloespora*, por lo que estos estudios moleculares convencionales basados en los hongos presentes sólo en las raíces, poseen la limitante de no abarcar a cabalidad con la diversidad de especies que se puede encontrar en un viñedo (Lindahl *et al.*, 2013).

A pesar de la valiosa información que aportan otras técnicas moleculares su alcance es limitado, consumen tiempo y su costo es elevado. Nuevas aproximaciones moleculares como la pirosecuenciación, se ofrecen como alternativas más económicas, de alto rendimiento, menos engorrosas y con la capacidad de caracterizar muchas muestras ambientales de distinto origen en paralelo y develar mayor variabilidad de la diversidad intra e inter específica, sin la necesidad de aislar y cultivar a los organismos (Dumbrell *et al.*, 2010, Schnoor *et al.*, 2011, Unterseher *et al.*, 2011, Yu *et al.*, 2012, Orgiazzi *et al.*, 2013). Sin embargo un problema que surge al momento de utilizar la meta genómica para el estudio de los genomas microbianos, es que de la inmensa diversidad de microorganismos que se cree que existe, solo una pequeña porción ha sido identificada (Haas *et al.*, 2011). Actualmente se han creado bases de datos donde se incluyen los taxones virtuales que son grupos resueltos filogenéticamente de secuencias que dan una resolución taxonómica similar a las especies morfológicamente reconocidas, pero sólo para algunas familias (Öpik *et al*., 2013). En la actualidad, la base de datos más grande y actualizada con más frecuencia es MaarjAM que contiene secuencias de AMF SSU que pueden usarse para definir taxones virtuales (Öpik *et al*., 2010).

El estudio de estos hongos se dificulta debido a su carácter de biótrofos obligados, es decir, su incapacidad de completar su ciclo de vida en ausencia de una planta hospedante. El uso del cultivo monoxenico de raíces transformadas de zanahoria como hospedantes, permite el desarrollo de la simbiosis y la obtención de grandes cantidades de esporas y micelio en poco tiempo, asegurando la pureza del inóculo y la ausencia de microorganismos indeseados (Declerck *et al*., 2005). Debido al inmenso interés en el estudio de las HMA y la gran preocupación en conservar la diversidad nativa de estos hongos simbiontes, utilizar esta metodología es la más adecuada para la investigación de la simbiosis micorrícica (Fernández, 2012).

Se ha observado que la presencia de ciertas especies de hongos en la simbiosis micorrícica promueve la acumulación de distintos metabolitos específicos en las plantas, como los terpenos, fenoles y alcaloides, siendo muchos de estos compuestos de alto interés para la salud y el bienestar humano (Toussaint, 2007). Aún no está claro por qué o a través de qué rutas bioquímicas podrían aumentar la cantidad de metabolitos secundarios en las plantas micorrizadas, se cree que una razón sería la mayor absorción de nutrientes principalmente de fósforo, que actuaría como precursor de acetyl-CoA, ATP y NADPH, quienes participan en la biosíntesis de isoprenoides, del mismo modo, la mayor disponibilidad de N actuaría como precursor de diferentes aminoácidos originando alcaloides específicos, también el cambio en las hormonas vegetales podría inducir en la mayor o distinta producción de metabolitos, aunque esto no se explica en todos los casos (Zeng *et al.*, 2013).

Los compuestos fenólicos

Los compuestos fenólicos son los metabolitos secundarios más abundantes en las plantas. Poseen una estructura común que comprende un anillo de benceno aromático con uno o más sustituyentes hidroxilos. Ellos representan un amplio y diverso grupo de moléculas incluyendo dos familias principales; los flavonoides basados en el esqueleto común C6-C3-C6 y los no flavonoides. En las plantas tienen un rol en el crecimiento, fertilidad y reproducción y también en varias reacciones de defensa y protección frente estrés abiótico como la intensidad lumínica o en estrés biótico como depredadores o ataque de patógenos. También constituyen componentes básicos de los pigmentos, esencias y sabores. Muchos compuestos fenólicos

(resveratrol, quercetina, antocianinas) han sido reportados con múltiples actividades biológicas, incluyendo antinflamatorio, anticancerígeno y cardioprotector, atribuidos principalmente a su capacidad antioxidante y antiradicales libres (Lorrain *et al.,* 2013).

La vid, la uva y el vino poseen altas cantidades de polifenoles, que juegan un rol significativo en una adecuada salud humana, protegiendo sobre muchas enfermedades relevantes, como el cáncer o enfermedades al corazón, con propiedades terapéuticas, antioxidantes, antifúngicas y antibacterial (Artero *et al*., 2005). Para las plantas, estos metabolitos secundarios le otorgan protección frente a estrés biótico y abiótico. Estos compuestos se pueden encontrar en raíces, tallos, hojas, flores y frutos (Yu & Ahmedna, 2013). Los compuestos fenólicos en la vid dependen de la variedad, clima, condiciones del suelo, estrés biótico y abiótico y prácticas agrícolas. Ha sido reportado que los activadores de plantas Benzotiadizol (BTH), un análogo al ácido salicílico y el quitosano (CHT), son capaces de estimular el complejo sistema de resistencia adquirido, provocando la síntesis de polifenoles en la vid (Ruggiero *et al.,* 2013). Los compuestos fenólicos son esenciales para la calidad de los productos alimenticios derivados de plantas, por su contribución a la estabilidad oxidativa y características organolépticas. De hecho, las características de los vinos están definidas por varios compuestos fenólicos extraídos de la uva durante la elaboración del vino. Entre ellos los flavonoides, incluyendo las antocianinas (color rojo) y taninos (astringencia, amargura y estabilidad del color) son los más importantes en la calidad del vino (Xia *et al.,* 2010).

En la vid se ha evaluado la relación de polifenoles con la presencia de micorrizas arbusculares y se ha encontrado una mayor cantidad de estilbenos en la raíz de plantas micorrizadas, así como los polifenoles de las hojas y se ha concluido que existen diferencias significativas entre las plantas con micorrizas arbusculares y las no micorrizadas (Sbrana *et al*., 2014). Esto genera nuevos campos de aplicación de esta simbiosis en la agricultura, como sería la inducción de estos cambios metabólicos para poder extraer metabolitos de interés farmacéutico en los desechos de las podas de los viñedos, o aumentar la cantidad de polifenoles en los vinos producidos, sin embargo, esto depende de los genotipos de vid evaluados y de las cepas fúngicas utilizadas (Eftekharia *et al.,* 2012).

Un polifenol de gran interés es el resveratrol puesto que posee la mayor actividad biológica de los componentes del vino y se encuentra en una concentración de 1,9 mg/l llegando hasta 14,3 mg/l que dependen de múltiples factores como la variedad, la zona geográfica, las prácticas agrícolas y la exposición a estres biótico y abiótico se obtiene a partir de la piel y semillas de la uva, además posee múltiples propiedades en la salud humana (Fernández *et al.*, 2012).

Otros estudios realizados en la evaluación de cómo las micorrizas arbusculares afectan a los compuestos fenólicos en las vides muestran evidencia de que la simbiosis aumenta su producción, sin embargo, hasta el momento se desconocen las rutas por las cuales se produce este cambio metabólico o por qué la planta modifica la distribución de los recursos energéticos en una mayor producción de estos compuestos (Krishna *et al*., 2005). En estudios realizados en otras plantas se ha comprobado que bajo condiciones de estrés, como el déficit hídrico, las micorrizas arbusculares permiten una mayor tolerancia al estrés y aumenta la producción de polifenoles (Jurkiewicz *et al.*, 2010, Baslam & Goicoechea, 2012).

HIPOTESIS Y OBJETIVOS

Pregunta de investigación

¿Los hongos micorrícicos nativos modifican los polifenoles en *Vitis vinifera*?

Hipótesis

La formación de las micorrizas arbusculares nativas aumenta o modifica la concentración de compuestos fenólicos en vides (*Vitis vinifera*) de Chile.

Objetivo principal

Estudiar si los hongos micorrícicos arbusculares nativos modifican la concentración de polifenoles en plantas micorrizadas de vides (*Vitis vinifera*) de Chile.

Objetivos específicos

- Conocer la diversidad de los hongos micorrícicos arbusculares nativos presentes en viñedos representativos de Chile.
- Determinar si se modifica la concentración de polifenoles con la formación de micorrizas arbusculares en las vides chilenas.

CHAPTER I

Mycorrhizal arbuscular native fungi for sustainable Viticulture in Chile

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Abstract

In Chile viticulture is economic sector of great importance. It includes a crop area of 211.000 ha, wine production of 10 Mhl, and US $1.515 million in wine exports. Chilean viticulture has to adapt to new challenges as they arise, measures against such as pesticide reduction, and increased droughts. Nevertheless, these challenges need to be addressed with sustainable solutions. Viticulture adaptation can benefit from arbuscular mycorrhiza, a plant–fungus symbiosis. The following study analyzes the potential of mycorrhizal arbuscular native fungi for sustainable viticulture in Chile. The major points of this study are the following: (1) the unique characteristics of Chilean viticulture (2) The ecosystem benefits of arbuscular mycorrhiza for grapevine production (3) Vineyard agricultural practices impact on sustainability.

Keywords: Arbuscular mycorrhiza; Grapevine; Sustainable Viticulture

1. Introduction

The grapevine is a perennial crop grown in various geographic areas around the world crop. It is highly responsive to local environmental conditions and viticulture practices, yielding highly region specific wines (OIV, 2010). This feature gradually brought about the « terroir » concept, officially defined by the International Organization of Vine and Wine (OIV) as, "a concept which refers to an area in which collective knowledge of the interactions between the identifiable physical and biological environments and applied viticulture practices develops, providing distinctive characteristics for the products originating from this area" (Resolution OIV/Viti 333/2010). Bokulich et al. (2014) proposed the existence of a non-random microbial terroir as a determining factor of regional variation among vines. The soil microbiome is also undoubtedly of importance, but still remains less studied.

Viticulture is a sector of economic importance in Chile with a cultured area of 211.000 ha. Chilean vineyards extend over 2.000 Km from Elqui valley (IV Region) to Aysen valley (XI Region) hosting a wide variety of environmental conditions (SAG, 2014). The condition under which Chilean wine is grown varies, especially as vineyards continue to expand into new and uncharted territory. The majority of the vineyard soils tend to be poor in nutrients and the need of irrigation is increasing in Chilean viticulture. Chile's newer vineyards are often in areas with less access to water, especially in the arid north and hilly west of the country, where river water is scarce.

Arbuscular mycorrhiza (AM) is probably the most widespread beneficial interaction between plants and microorganisms (Smith and Read 2008; Parniske 2008). This association between plant and fungi from the *Glomeromycota* phylum dates back to the first appearance of land plants (about 400 million years ago) (Redecker 2000). Nowadays, the vast majority (80%) of land plant species form AM symbiosis, living in a wide range of terrestrial environments (Wang and Qiu 2006). The host plant supplies the biotrophic partner with carbon (C) while the AMF enhances the ability for the plant to receive water and nutrients from the soil (Smith and Read 2008). Since AMF constitutes a direct soil–plant link, investigating their occurrence and impact in vineyard conditions will allow the assessment of their contribution to the « terroir » an area of particular importance in viticulture.

The following study analyzes the potential ecosystem services provided by AMF with respect to adaptation to climate change and achieving sustainable viticulture. This paper presents highly relevant information about AMF, the impact of AMF on the grapevine plant species, and the influence of viticulture practices on these populations.

2. Unique characteristics of Chilean viticulture

Chile's unique combination of geography and climate make it ideal for winegrowing. The unusual geography of Chile, which presents a particularly long and slim territory compared to its width, is one of its many unique characteristics. Chile has clear natural boundaries, the Atacama Desert, one of the driest in the world to the north, and the Patagonia's glaciers to the south. To the east and all along the border is the great Andes mountain range, and to the west, the Pacific Ocean (Fig 1). The cool sea air is partially blocked by the Coastal Mountains, but navigates its way inland by following the course of the transversal river valleys. During the day, sea breezes carried by the cold Humboldt Current penetrate inland, and each night, cold air descends from the snow-covered peaks of the Andes. Chile's climate is highly influenced by the cooling effect of the Pacific Ocean and the Humboldt Current that begins in the icy waters near Antarctica and flows up the western coast of South America. Curiously, when the effect of the Humboldt's cold current hits Chile's northern coastline it produces clouds and fog, but little or no precipitation, which then contributes to making the Atacama Desert the driest on Earth. Together these geographic barriers help maintain healthy agricultural conditions, protecting vineyards against pests and disease. Chile's Mediterranean climate features the warm, dry summers and cold, rainy winters that vines love. Even better, the interaction between the effects of the sea and those of the Andes result in a growing season that revels in bright sunny days and temperatures that take a dramatic dip each night to create the broad daily temperature oscillation that wine grapes need to develop fresh fruit flavors and crisp acidity. In the case of red wines, ripe tannins, deep color, and high levels of antioxidants and flavonols are produced from these temperatures. The combination of beneficial natural barriers and a benevolent Mediterranean climate make sustainability and organics a logical choice in Chilean winegrowing. In fact, Chile has some of the largest organic vineyards in the world.

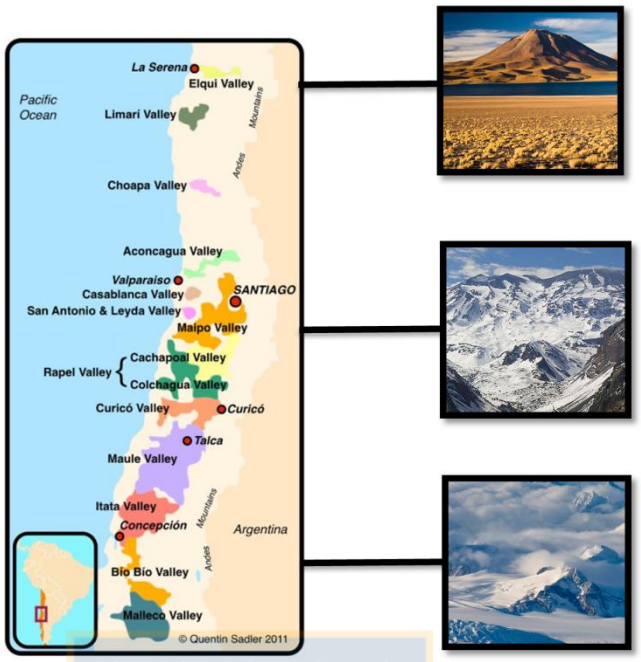

Figure 1. Unique characteristics of Chilean viticulture. Source: Self-elaboration.

In recent years, more vineyards have been established at higher altitudes closer to the peaks, where the sun is slow to appear over the eastern peaks. Currents of wind climb and descend over the course of the day to create a daily pendulum of temperatures between daytime highs and nighttime lows. It is these temperatures that red grapes, such as Cabernet Sauvignon thrive on. In central Chile we found just the type of conditions that cool-climate grapes such as Sauvignon Blanc, Chardonnay, and Pinot Noir thrive on due to the geographic variety, the Chilean landscape also offers a vast mosaic of terroirs and soil types (Table 1). Soils are healthy, well-drained, and have a variety of origins (alluvial, colluvial, fluvial, etc.) and textures (loam, clay, sand, silt). This mosaic of climates and 'terroirs' combined with a vinicultural industry with few restrictions in regards to cultivation and wine production, explain the success of Chilean wines around the world. Chile's highly diverse geography and beneficial climate makes Chilean wine a valid option for today's consumers who demand high quality and ecologically sound practices.

Table 1. Climate and soil condition representative viticulture zone of Chile. Source: Self-elaboration.

	ZONE 1	ZONE 2	ZONE 3	ZONE 4	ZONE 5
Valley	Elqui	Casablanca	Cachapoal	Maule	Itata
Kind of climate	Semiarid Cold	Ocean Mediterranean	Dry Mediterranean	Ocean Mediterranean	Continental Mediterranean
Kind of climate Köppen	BWk	Csb	BSh	Csb	Csa
Annual rainfall	60mm	412 mm	550 mm	780 mm	1077 mm
Temperature X	15.4	14.8	14.9	14.4	13.5
Temperature Max	24	23	22	20	20
Temperature Min	8	12	9	9	9
Kind of soil	Alfisol	Granitic	Alluvial-Coluvial	Granitic	Alluvial
Soil Texture	Clayey	Limo	Limo medium	Limo fine	Clayey

a. Carménère: a uniquely Chilean wine

Carménère, marked by a singular history, this stock, originally from France, is today only produced in Chile, a country that provides unique conditions for its development. Recognized as the most complex of grapes when it comes to achieving its maturity, this is because it suffered more than any strain from the effects of the phylloxera plague. A plague that affected wine grapes in Europe between 1860 and 1870, and France in particular, where the strain originates. As a result of the plague the French tired of the complexities of its cultivation, stopped cultivating Carménère, and it was later believed to have disappeared.

Ten years prior to the devastating phylloxera plague rootstock of this grape had been sent to Chile. As a result of the crisis that Europe was going through at the time, a large number of enologists emigrated from Europe to Chile, giving a vigorous boost to the already emerging Chilean winemaking industry. In 1991, French ampelographer Claude Vallat pointed out that some of the Merlot Chile was producing was not actually Merlot, but was unable to establish

what stock it belonged to. Two years later, Jean Michel Boursiquot, a pupil of Vallat's, determined that some of the varieties of Chilean Merlot belonged to the Carménère stock, which had long since disappeared in Europe.

Although the information gave rise to certain problems within the wine industry, these issues were ultimately overcome with the strong potential for the Chilean industry in terms of Carménère strain production. Today there are more than 8,000 Carménère hectares planted in the country. As stated by sommelier Ricardo Grellet, "Chile is the country that has been identified with this variety, distinguishing it from others in the industry". The potential of Carménère is outstanding, due to the fact that Chile is the only country to produce it. Chile is recognized among the international traders as the country for Carménère.

3. Potential of mycorrhizal diversity and ecosystem services

Key ecosystem processes are affected by a loss in soil biodiversity, and are greatly dependent on land use. AMF are an important functional group in soil ecosystems, and a reduction of their biodiversity will impact plant functionality and more generally global ecosystems (Jeffries et al. 2003). Differences in growth strategies between these fungi imply that soil management can greatly impact the diversity of AMF and as a consequence their ecosystem services. (Fig 2)

AMF in the vineyard communities are highly influenced by the soil characteristics, but also by the host plant development stage (Balestrini et al. 2010). Although, AMF, generally are not host specific, their effect on plants can vary among species and isolates. AMF develop an extensive mycelium in the soil, extending the prospective nutrient zone of the plant. The extent of the mycelium varies among species and also among isolates within the same species (Avio et al. 2006), implying that the beneficial effect of this fungal network against soil erosion or for improving plant nutrition varies depending on fungal diversity. When a mycorrhizal inoculum is used, the positive effect of mycorrhizal fungi on plant growth strongly depends on its composition, and a combination of mycorrhizal fungi is more effective than a monospecific inoculum (Gogoi and Singh 2011). Other important effect of noculation with AMF even increased total phenolic components in leaves by up to 800 % (Krishna et al. 2005).

The question as to which AMF are associated with grapevine has been addressed in several studies, initially using surveys based on spore morphology (Oehl et al. 2005). These studies highlighted the dominance of genera as *Glomus, Rhizophagus, Funneliformis, Claroideoglomus*, and *Paraglomus*. Schreiner and Mihara (2009) mainly detected that the majority of grapevine roots across vineyards were dominated by the same three or four phylotypes (out of a total of 6–11), with subtle differences across different soil types and vineyard ages. However, no seasonal effect was found.

Lumini et al. (2010) used a pyrosequencing approach in vineyards in Sardinia to characterize local AMF communities, where two differently managed types of vineyards, analyzed as part of a land-use gradient, harbored slightly different levels of AMF diversity. Significant differences were found in terms of sequence abundance after casting aside possible effects of soil chemistry. The presence of inter-row vegetation in vineyard had an effect on the overall vineyard's AMF community. A dominance of *Glomeraceae* in colonized roots has also been demonstrated for many other ecosystems.

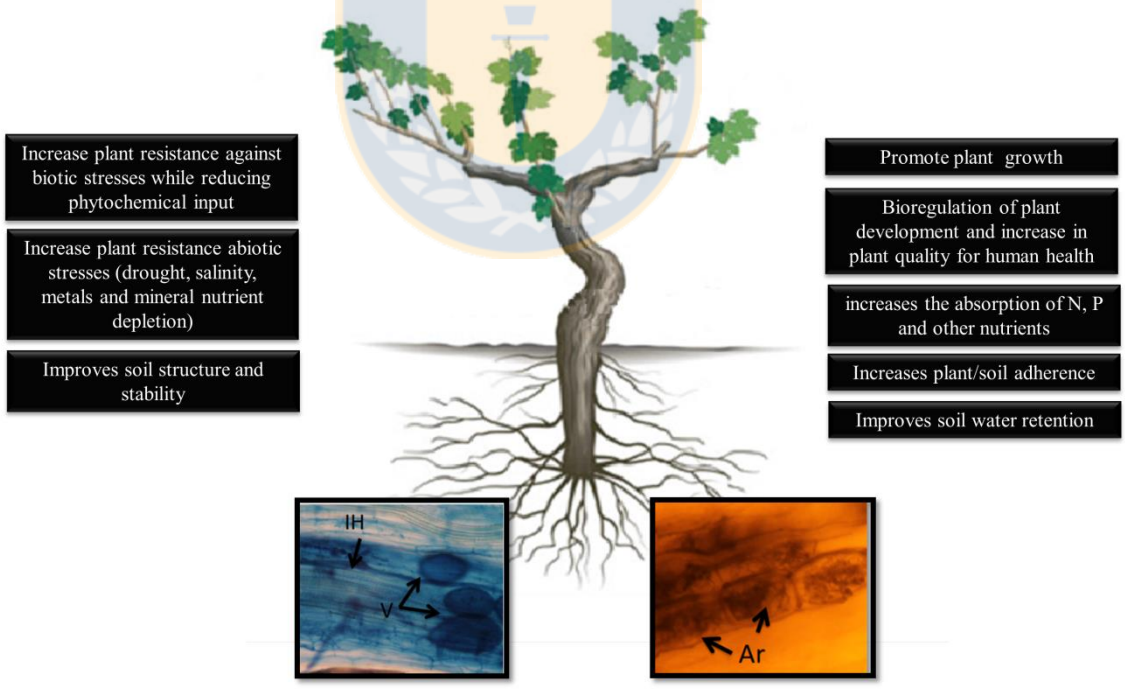

Figure 2. Ecosystem services provided by arbuscular mycorrhiza (modified from Trouvelot et al., 2015)

a. Enhancement of growth and nutrition

Vine nutrition is essential as it determines vine functioning and wine quality. Fertilization is complex and highly dependent on plant material, soil characteristics, and the type of production (table grapes, vines, etc.). Most of the vineyard soils are poor in nutrients. AM colonization occurs mainly under low levels of either plant nutrient status or low levels of soil fertility (mainly P) (Smith and Read 2008). Indeed, in grapevine, high levels of soil fertility (N or P) as well as a high plant nutrient status reduce arbuscular mycorrhiza colonization (Schreiner 2005b). Moreover, plants 'dependency on arbuscular mycorrhiza for nutrients could depend on root architecture and mainly root hairs. Grapevine root architecture was described as having a low density and a large diameter of fine roots. AM could therefore be highly beneficial by extending the volume of the explored soil allowing an adequate uptake of water and nutrients such as phosphate (Schreiner 2005b). Fungal mycelium increases the exploitable soil volume and since individual hyphae have diameters comparable to root hairs, they allow access to soil pores that would have been otherwise unexplorable (Smith and Read 2008). Mycorrhiza establishment changes the biological and physical-chemical properties of the rhizosphere, developing the socalled mycorrhizosphere (Azcón-Aguilar and Barea 2015).

Although nitrogen input is usually minimized to limit the growth of grapevine aerial parts and susceptibility to diseases, N capture from the soil is crucial for vine metabolism and building up of wood reserves (Wermelinger et al. 1991). Furthermore, among soil elements picked up by the roots, nitrogen is the one that most influences both vine development and grape composition (Reynolds, 2010). Besides inorganic N uptake, it is well established that AMF can draw substantial amounts of N from decomposing organic materials (Hodge and Fitter 2010). Organic nitrogen represents a large proportion of total soil N. In the absence of N fertilization only, total N uptake from the residue was significantly enhanced by the fungus (Smith et al. 2011).

Grapevine phosphorus deficiency is usually observed. Phosphorus deficiencies symptoms correspond to stunted shoot growth decrease in dry matter, and berry clusters. P deficiency mainly occurs in acidic soils that are favorable for fixing phosphorus as well as in higher rainfall areas or following fumigation of low phosphorus soils (Jackson, 2014). In such acidic and/or low phosphorus soils, applying fertilizers containing phosphorus is needed to avoid

deficiency symptoms. AMF can make a significant contribution to P uptake. The main form of P absorbed by plants is the soluble Pi, (HPO_4^{2-}), resulting from the slow breakdown of organic forms transformed by soil microorganisms. Pi availability is generally low because of its very slow diffusion through soil solution (Smith et al. 2011). Indeed, availability can be reduced by fixation in soils with high levels of calcium or by bounding to organic matter or soil particles. Moreover, once absorbed by roots, a depletion zone might appear around them, thus impairing plant nutrition and growth (Nye and Tinker 1977). In mycorrhizal roots, the extraradical mycelium forms an extensive network beyond the root depletion zone, thus shortening the distance for the diffusion of Pi in soil. Indeed, an increase in P levels in roots or leaves was observed in almost all grapevines studied in the presence of AMF (Schreiner 2005a).

Among the other chemical elements essential for grapevine growth, potassium (K), magnesium (Mg), zinc (Zn), and boron (B) are important for fertilization. Potassium deficiency occurs in high calcium (Ca) or Mg containing soils, as well as in high rainfall regions. However, certain cultural practices or soil properties can impair nutrient uptake. Indeed, it has been reported that nitrogen fertilization depressed the uptake of some elements such as B and Mn. Macro- and micronutrient uptake could depend not only on the fungal partner, but also on the host plant. Colonization levels would be a determining factor inmodulating AM-induced nutrient uptake and plant growth. It has also been reported that the content of most nutrients in grapevine cuttings (Pinot noir) was significantly increased depending on the soil type (Schreiner, 2005b). Indeed, P, K, Ca, Mg, Fe, and B contents were increased only in a low P soil. This effect would be linked to the primary stimulation of P uptake and resulting growth stimulation. Schreiner, (2005a) suggest that the selection of appropriate inoculant for specific functions would be the best strategy for inoculant producers. Plant growth stimulation mainly resulted from the arbuscular mycorrhiza-induced reduction of pseudomonads present in replant soil, rather than in nutrition enhancement.

The C costs of the fungal partner can be considerable in arbuscular mycorrhiza symbiosis since the fungus can receive 4 to 20 % of the plant's photosynthetically fixed C (Smith and Read 2008). Grapevine, as a deciduous plant, mainly relies on stored C for growth of new tissues, especially in spring. Since AMF growth and nutrient acquisition also rely on host plant reserves, C reserve mobilization and plant development could be significantly influenced by

the AMF (Smith et al. 2010). The C drains during rapid root colonization had a negative impact on host stem growth before colonization was maximum. Once symbiosis is functional, more C can be used for nutrient acquisition (Gilbert et al. 2014). Such a negative response following the first 2 months of arbuscular mycorrhiza colonization is thought to be transient in grapevine as a significant increase in growth (Lindermann and Davis 2001).

Rapid growth response following inoculation could also depend on the fungus. Moreover, AMF may induce changes in hormonal balance, leading to an increase in cytokinin content responsible for greater vegetative development, principaly in biomass (Harrison 1999). The arbuscular mycorrhiza-dependent enhancement of grapevine growth as a whole, or of grapevine roots only, varies with rootstock and fungus (Smith and Read 2008). Although plant growth was enhanced at the end of the first year after planting, plants could achieve optimal growth after being colonized by native fungal propagules and thus no longer benefit from artificial inoculation. Therefore, a preliminary study of the native inoculum present in the replant soil is essential before considering the suitability of arbuscular mycorrhiza inoculation.

b. Increase of tolerance to abiotic stresses

Abiotic stress causes extensive losses in agricultural productivity. Drought, salinity, or heavy metals are serious problems in many parts of the world.

Soil water status and plant water use play a key role in grapevine growth, harvest yield, and in grape quality. Vineyards are mainly located in areas of dry climate where during the growing season, water deficit frequently occurs. Shown to influence grape phenology, water stress during maturation has a positive effect on grape quality (Chaves et al. 2010). Prolonged water stress may have a strong negative effect on photosynthesis and yield. An effective development of external mycelium and/or arbuscules can be crucial in dry situations. Grapevines compensate for a lower density of fine roots by increasing arbuscular colonization (Schreiner et al. 2007). There is no current evidence of direct water transfer to plants via arbuscular mycorrhiza fungal hyphae. However, increased water use efficiency in arbuscular mycorrhiza plants has been reported in several studies, including studies on grapevine. Donkó et al. (2014) reported that the degree of mycorrhizal colonization is higher in drier soil areas. Arbuscular mycorrhiza-colonized grapevine roots can exhibit more efficient water uptake, allowing the grapevine to cope with water stress. This highlights “functional diversity” in

arbuscular mycorrhiza symbioses that should be taken into account when optimizing arbuscular mycorrhiza colonization protocols, especially in the field (van Rooyen et al. 2004).

Arbuscular mycorrhiza inoculation can enhance the plant water relations and the photosynthesis performance of transplanted grapevine rootstocks, thus potentially alleviating transplantation shock, and increasing survival. This improved drought tolerance is of special importance for transplanted plants in which root systems are less extended and more superficial during the first years of growth. It is established that while most roots are produced in spring when soil temperatures are favorable, root development is rootstock-dependent since some do produce roots during the warm dry season, at greater depths, thus improving water foraging and transport (Smart et al. 2006). In dry soils, the root extension rate is lower because of reduced turgor and increased soil strength, as well as reduced nutrient availability. In these situations, the development of extra radicular mycelium can be crucial for optimizing nutrient uptake. Improved drought tolerance is closely linked to improved nutrition (especially P) of arbuscular mycorrhiza plants.

Soil salinity refers to the amount of dissolved salts in soils and is one of the major abiotic factors limiting viticulture productivity, mainly in arid and semi-arid zones. Leading to limiting plant growth. Saline soil conditions occur naturally in some vineyards. It could be due to primary salinization that occurs naturally when the soil parent material is rich in soluble salts. Moreover, salt-affected soils often occur in irrigated vineyards, especially in arid and semi-arid regions, due to the insufficient leaching, improper irrigation, poor drainage, or irrigation water high in salts. Grapevine, in comparison to other crop types, is classified as moderately sensitive to salinity. High salinity considerably inhibits shoot growth and affects berry quality (Walker et al. 2010). AMF occur naturally in saline environments and alleviate salinity stress in many plant cultures, by improving uptake of nutrients such as P, N, Zn, Cu, and Fe. The improvement in the plant P status is probably the most important source of salinity stress tolerance in arbuscular mycorrhiza colonized plants (Giri and Mukerji 2004).

Copper-based fungicides have been used intensively since the end of the nineteenth century to control vine fungal diseases, especially downy mildew, and their long-term application and subsequent wash off from treated plants have led to considerable Cu accumulation up to toxic concentrations in vineyard surface soils. Although this increased Cu availability does not

appear to change grapevine nutritional status, it has a negative influence on the soil flora and fauna and may lead to phytotoxicity in acidic soils, leaf oxidative stress, yield losses, and poorer wine quality. Soil copper is not expected to induce phytotoxicity in mature grapevines as their root systems extend below the surface soils where most of the applied copper tends to remain. In contrast, young vines may be affected as their root systems are much shallower while establishing. In this context, Cu may have phytotoxicity implications for vine nurseries or when vineyards are replanted.

AMF inoculation has become a prospective tool for enhancing plant tolerance to environmental stress conditions in metal-contaminated soils. Immobilization of metals in the fungal biomass is proposed as a major mechanism whereby AMF may increase plant tolerance to heavy metals. Mycorrhizal roots may act as a barrier against metal transport, reducing transfer and enhancing root/shoot Cd ratios. This effect is attributed to metal adsorption onto hyphal walls because chitin has an important metal-binding capacity. In soil, glomalin, a glycoprotein produced by AMF, may have a metal-chelating function and thereby diminish metal availability for plants (González-Chávez et al. 2004). AMF play a significant ecological role in the phytostabilization of potentially toxic trace element-polluted soils through sequestration mechanisms and, in turn, help mycorrhizal plants to survive in polluted soils. Their potential roles in phytoremediation of heavy metal-contaminated soils are also emerging, and are expected to aid in Cu-contaminated vineyard soils. Moreover, since AMF are present on the plant roots growing on said soil types, isolation of indigenous Cu stress-adapted AMF could be a potential biotechnological tool for inoculating plants in disturbed ecosystems.

c. Protection against biotic stresses

Most grapevine varieties (*V. vinifera cvs*) are susceptible to diseases caused by fungisuch as downy mildew, causing great yield losses and require numerous fungicide treatments. Plants can develop an enhanced defensive capacity after infection by AMF. This “mycorrhiza-induced resistance” (MIR) could provide systemic protection against a wide range of biotrophic and necrotrophic pathogens, nematodes, and herbivorous arthropods (Cameron et al. 2013). Therefore, AMF can suppress or reduce plant pests and diseases through the induction of systemic resistance (Jung et al. 2012). Depending on the plant/pathogen/AMF

interaction, MIR could be associated with systemic acquired resistance (SAR)-like priming of salicylic acid (SA)-dependent genes, but more often coincides with priming of jasmonic acid (JA)-dependent defenses, especially in grapevine (Hao et al. 2012). Bioprotection of mycorrhizal plants can be active against a wide spectrum of pathogen. Therefore, management of AMF can be considered as an ecosystem service and could complement innovative protective strategies.

In the case of grapevines, MIR has been described mainly against root pathogens and especially against the fungi *Armillaria* and *Cylindrocarpon spp*. For example, local and systemic MIR was evidenced against the ectoparasitic nematode *Xiphinema index*, the vector of grapevine fan leaf virus (GFLV). This bioprotective effect was not linked to an improved P status of the plant, but directly involved an unknown induced systemic factor.

d. Increase of soil stability

A large number of vineyards are planted on hill slopes and are susceptible to risks of erosion. In these situations, soil structuration is essential. AMF symbionts grow out from the mycorrhizal root to develop a complex, ramifying network into the surrounding soil which can reach up to 30 m of fungal hyphae per gram of soil (Wilson et al. 2009). This mycelial network can have a binding action on soil particles and improve soil structure. Moreover, the secretion of hydrophobic, "sticky" proteinaceous substances by AMF, consisting of glomalin, also contributes to soil stability and water retention. All of these factors are considered important to help stabilize soil aggregates thus, leading to increased soil structural stability and quality (Bedini et al. 2009).

In this way, a reduction in fungal biomass will result in a negative effect on soil stability, consequently increasing the risk of soil erosion. Erosion of cultivated land soil by water is detrimental on site as well as off-site, posing problems globally. These problems include soil and nutrient loss, long-term productivity loss of degraded soils, and a wide range of environmental problems arising from sediment delivery to the drainage network and reservoirs. This irreversible soil degradation may cause major environmental and economic damage (Pimentel 1995). AMF may have a double beneficial effect, firstly by enhancing soil structural stability (due to the external hypha network and glomalin secretions) and secondly by enhancing N and P uptake from the soil, thereby limiting damage related to nutrient losses.

4. Impact of vineyard agricultural practices on sustainability

The several cultural practices used in grapevine include fertilization, soil management, weed vegetation cover (chemical or mechanical weed control), and pests (Fig 3). With an objective to achieve sustainable viticulture development, efforts have been made to reduce the use of pesticides, and agroecological initiatives such as green, organic, biologic, or biodynamic management have been developed (Altieri 2002). Many achievements have been reached with the application of microbial biotechnology in agriculture but many challenges as well as opportunities need to be explored for the future sustainable agricultural developments. (Barea 2015)

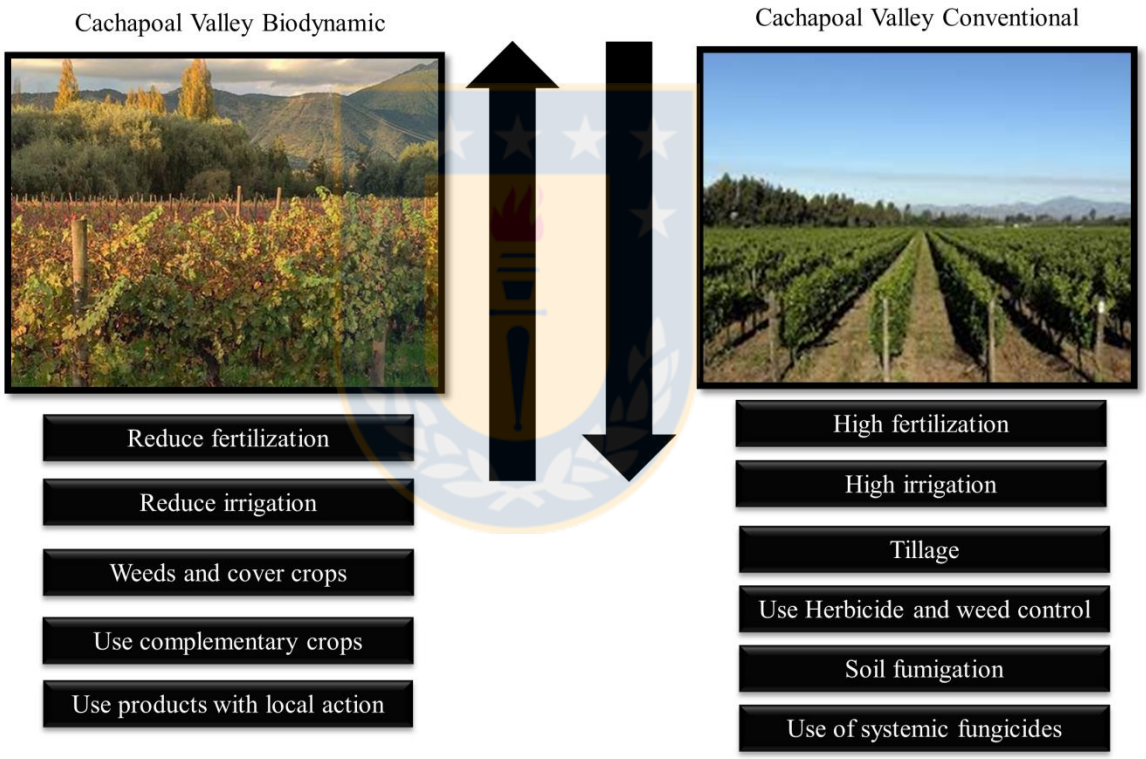

Figure 3. Impact of contrasting vineyard agricultural practices on grapevine mycorrhization. Source: Self-elaboration.

a. Impact of soil management practices

Tillage is arguably the most unique and strongest agricultural selection pressure for mycorrhizal symbionts. Although tillage practices can vary in intensity from ploughing

(lower-intensity) to ripping (higher-intensity). Tillage is arguably the most unique and strongest agricultural selection pressure for mycorrhizal symbionts. Tillage directly affects the integrity of the mycelial network (Verbruggen and Kiers 2010). Repeated destruction of this integral hyphal network in agricultural systems has the potential to radically alter the evolutionary trajectory of the organism. It is well-established that tillage decreases mycorrhizal diversity at the family level, and can lead to competitive dominance by only a few strains. AMF species have been found to differ in their tolerance to hyphal disruption (de la Providencia et al. 2007) and ecological shifts in AMF community composition are often noted when high and low tillage vineyards are compared. Tilled vineyard soil harbored a higher diversity than the covered vineyard soil. The question thus arises whether tillage at the soil surface would affect AMF communities active in the roots, which grow relatively deep in the soil. Considering this factor, it is suggested to reduce tillage for considerable periods of time in order to maintain the mycelial networks present in the vineyards.

b.. Impact of high fertilizer inputs

Conventional agriculture introduces large amounts of fertilizer into the vineyards, but high fertilizer inputs (especially P) are known to reduce AMF root colonization and propagules in many agrosystems, including vineyards. Furthermore, grapevine N fertilization affected AMF colonization and, in turn, affected berry composition (Karagiannidis et al. 2007). it is worthwhile to note that urea suppressed AMF root colonization and sporulation. For these reasons, new alternatives must be sought to maintain soil fertility and/or to enhance the native AMF inoculations in the vineyards, like organic amendments to maximize a recovery of soil fertility (Scotti et al. 2015).

c. Impact of weeds/cover crops

The composition and diversity of the plant community can influence the structure of the AMF community, AMF composition is influenced by neighboring plants that can result in beneficial, inhibitory or neutral effects. Weed control practices are often used in vineyards as alternatives to herbicides. Weed control and cover cropping has an impact on vineyard soil communities (Baumgartner et al. 2010), So it could be expected for AMF diversity and colonization of grapevine to increase with the increasing frequency and diversity of

mycorrhizal hosts on the vineyard floor. Mycorrhizal hosts on the vineyard floor do not significantly influence mycorrhizal colonization or mycorrhizal fungal communities of grapevines. Herbaceous weed species could promote a different set of dominant mycorrhizal fungi, potentially providing a wider spectrum of these fungi for colonizing grapevine roots (Radic et al., 2012). Encouraging host plant diversity in vineyards can be valuable, provided that it does not develop nutrient and/or water competition with the grapevine. The formation of grapevine mycorrhizal networks with neighboring plants depends on vineyard soil management practices; especially weed control and cover cropping.

d. Impact of pest management practices

The fumigation of the soils can generate a high impact in the AMF communities; in fact, soil pest management is complex, and the equilibrium between fighting pests and protecting/promoting beneficial microorganism such as AMF is difficult to achieve. Nematodes and AMF coexist and interact with grapevine roots and with the rhizosphere.

Fumigation of soils is known to kill the endemic AM fungi, and in the fumigated P deficient soils, vines are weaker due to the absence or the clear decrease of AM fungi. Even if soil fumigation does not suppress 100% of the AMF inoculum in the soil, it may decrease the diversity of AMF species to the extent that efficient colonizers of grapevine roots are eliminated from the AMF community (Cheng and Baumgartner 2004). It may also decrease the chances of grapevine roots encountering the most beneficial combination of AMF species. Therefore, if soils are fumigated before planting, then reintroducing AMF will likely greatly benefit plant establishment and growth.

5. Conclusion

The majority of Chilean vineyards are currently not taking advantage of all the ecosystem benefits provided by arbuscular mycorrhizal fungi, all of which have been sufficiently studied and have clear management proposals. Global trends indicate that viticulture must evolve into a more sustainable process, thus allowing better results in quality, production and environmental impacts to be obtained. There is currently a growing interest in organic and biodynamic management in Chile. These crop systems undoubtedly benefit the native microbial soil populations, and given the isolated geographic characteristics that Chile

possesses will be an opportunity to preserve the unique properties of microbial terroir present in the Chilean wine-growing valleys. Preserving this microbial terroir will further enhance the denominations of Chilean wines.

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7. References

Altieri, M. 2002. Agroecology: the science of natural resource management for poor farmers in marginal environments. Agr Ecosyst Environ 93:1–24

Avio L, Pellegrino E, Bonari E, Giovannetti M. 2006. Functional diversity of arbuscular mycorrhizal fungal isolates in relation to extraradical mycelial networks. New Phytol 172:347–357

Azcón-Aguilar C., Barea, J. 2015. Nutrient cycling in the mycorrhizosphere. Journal of Soil Science and Plant Nutrition 25(2): 372-396

Balestrini, R., Magurno, F., Walker, C., Lumini, E., Bianciotto, V. 2010. Cohorts of arbuscular mycorrhizal fungi (AMF) in *Vitis vinifera*, a typical Mediterranean fruit crop. Environ Microbiol Rep 2:594–604

Barea, J. 2015. Future challenges and perspectives for applying microbial biotechnology in sustainable agriculture based on a better understanding of plant-microbiome interactions. Journal of Soil Science and Plant Nutrition 15(2):261-282

Baumgartner, K., Fujiyoshi, P., Smith, R., Bettiga, L. 2010. Weed flora and dormant-season cover crops have no effects on arbuscular mycorrhizae of grapevine. Weed Res 50:456–466

Bedini, S., Pellegrino, E., Avio, L., Pellegrini, S., Bazzoffi, P., Argese, E., Giovannetti, M. 2009.Changes in soil aggregation and glomalin-related soil protein content as affected by the arbuscular mycorrhizal fungal species *Glomus mosseae* and *Glomus intraradices*. Soil Biol Biochem 41:1491–1496

Bokulich, N., Thorngate, J., Richardson, P., Mills, D. 2014. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc Natl Acad Sci U S A 111:E139–E148

Cameron, D., Neal, A., van Wees, S., Ton, J. 2013. Mycorrhiza-induced resistance: more than the sum of its parts? Trends Plant Sci 18:539–545

Chaves, M. M., Zarrouk, O., Francisco, R., Costa, J. M., Santos, T., Regalado, A. Rodrigues, M., Lopes, C. 2010. Grapevine under deficit irrigation: hints from physiological and molecular data. Annals of Botany 105(5):661–676

Cheng, X., Baumgartner, K. 2004. Arbuscular mycorrhizal fungi-mediated nitrogen transfer from vineyard cover crops to grapevines. Biol Fertil Soils 40:406–412

de la Providencia, I., Fernandez, F., Declerck, S. 2007. Hyphal healing mechanism in the arbuscular mycorrhizal fungi *Scutellospora reticulata* and *Glomus clarum* differs in response to severe physical stress. FEMS Microbiology Letters 268:120–125

Donkó, Á., Zanathy, G., Èros-Honti, Z., Villango, S., Denes, G.2014. Changes of mycorrhizal colonization along moist gradient in a vineyard of Eger (Hungary). Acta Univ Sap Agric Environ 6:13-26

Gilbert, JA., van der Lelie, D., Zarraonaindia, I. 2014. Microbial terroir for wine grapes. Proc Natl Acad Sci U S A 111:5–6

Giri, B., Mukerji, K. 2004. Mycorrhizal inoculant alleviates salt stress in *Sesbania aegyptiaca* and *Sesbania grandiflora* under field conditions: evidence for reduced sodium and improved magnesium uptake. Mycorrhiza 14:307–312

Gogoi, P., Singh, R. 2011. Differential effect of some arbuscular mycorrhizal fungi on growth of *Piper longum L. (Piperaceae).* Indian J Sci Tech 4:119–125

González-Chávez, M., Carrillo-González, R., Wright, S., Nichols, K. 2004. The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements. Environ Pollut 130:317–323

Hao, Z., Fayolle, L., van Tuinen, D., Chatagnier, O., Li, X., Gianinazzi, S., Gianinazzi-Pearson, V. 2012. Local and systemic mycorrhiza-induced protection against the ectoparasitic nematode *Xiphinema index* involves priming of defence gene responses in grapevine. J Exp Bot 63:3657–3672

Harrison, M. 1999. Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annu Rev Plant Physiol Plant Mol Biol 50:361–389

Hodge, A., Fitter, A. 2010. Substantial nitrogen acquisition by arbuscular mycorrhizal fungi from organic material has implications for N cycling. Proc Natl Acad Sci U S A 107:13754–13759

Holland, T., Bowen, P., Bogdanoff, C., Hart, M. 2014. How distinct are arbuscular mycorrhizal fungal communities associating with grapevines? Biol Fertil Soils 50:667–674

Jackson, RS. 2014. Wine science: principle and applications. Science, Elsevier

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., Barea, J. 2003. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 37:1–16

Jung, S., Garcia-Andrade, J., Verhage, A, et al. 2012. Arbuscular mycorrhiza induce systemic resistance against gray mold (*Botrytis cinerea*) in tomato through priming of JA-dependent defense responses. In: Schmitt A, Mauch-Mani B, Pozo MJ, et al (eds) Proceedings of the IOBC/WPRS Working group. Induced resistance in plants against insects and diseases. Granada 139–144

Karagiannidis, N., Nikolaou, N., Ipsilantis, I., Zioziou, E. 2007. Effects of different N fertilizers on the activity of *Glomus mosseae* and on grapevine nutrition and berry composition. Mycorrhiza 18:43–50

Krishna, H., Singh, S., Sharma, R., Khawale, R. 2005. Biochemical changes in micropropagated grape (*Vitis vinifera* L.) plantlets due to arbuscular-mycorrhizal fungi (AMF) inoculation during *ex vitro* acclimatization. Sci Hortic-Amsterdam 106:554–567

Lindermann, R., Davis, E. 2001. Comparative response of selected grapevine rootstocks and cultivars to inoculation with different mycorrhizal fungi. Am J Enol Vitic 52:8–11

Lumini, E., Orgiazzi, A., Borriello, R., Bonfante, P., Bianciotto V. 2010. Disclosing arbuscular mycorrhizal fungal biodiversity in soil through a land-use gradient using a pyrosequencing approach. Environ Microbiol 12:2165– 2179

Nye, P. and Tinker, P. 1977. Solute movement in the soil-root system: studies in ecology. Blackwell Scientific Publications, Oxford

Oehl, F., Sieverding, E., Ineichen, K., Ris, E., Boller, T., Wiemken, A. 2005. Community structure of arbuscular mycorrhizal fungi at different soil depths in extensively and intensively managed agroecosystems. New Phytol 165:273–283

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat. Rev. Microbiol 6:763-775

Pimentel, D. 1995. Amounts of pesticides reaching target pests: environmental impacts and ethics. J Agri Environ Ethic 81:17–29

Radic, T., Hancevic, K., LikarMet, A., Protega, I., Jug-Dujaković, M., Bogdanović, I. 2012. Neighbouring weeds influence the formation of arbuscular mycorrhiza in grapevine. Symbiosis 56: 111–120

Redecker, D. 2000. Glomalean fungi from the ordovician. Science 289: 1920–1921

Reynolds, A. 2010. Managing wine quality: viticulture and wine quality.Science, Elsevier

SAG. 2014. Servicio Agrícola y Ganadero. Subdepartamento de Viñas y Vinos. Informe Ejecutivo Producción de Vinos.

Schreiner, R. 2005a. Spatial and temporal variation of roots, arbuscular mycorrhizal fungi, and plant and soil nutrients in a mature pinot noir (*Vitis vinifera* l.) vineyard in Oregon, USA. Plant Soil 276:219–234

Schreiner, R. 2005b. Mycorrhizae and mineral acquisition in grapevines. In: Christensen, L. P., Smart D (eds) Proceedings of the Soil Environment and Vine Mineral Nutrition Symposium. JP Christensen and DR Smart (Eds.) 49–60

Schreiner, R., Mihara, K. 2009. The diversity of arbuscular mycorrhizal fungi amplified from grapevine roots (*Vitis vinifera* L.) in Oregon vineyards is seasonally stable and influenced by soil and vine age. Mycologia 101:599–611

Schreiner, R., Tarara, J., Smithyman, R. 2007. Deficit irrigation promotes arbuscular colonization of fine roots by mycorrhizal fungi in grapevines (*Vitis vinifera* L.) in an arid climate. Mycorrhiza 17:551–562

Scotti, R., Bonanomi, G., Scelza, R., Zoina, A., Rao, M. 2015. Organic amendments as sustainable tool to recovery fertility in intensive agricultural systems. Journal of Soil Science and Plant Nutrition 15(2):333-352

Smart, D., Schwass, E., Lakso, A., Morano, L. 2006. Grapevine rooting patterns: a comprehensive analysis and review. Am J Enol Vitic 57:89–104

Smith SE, Read DJ. 2008. Mycorrhizal Symbiosis, Third Edition, 3rd edn. Academic Press

Smith, SE., Facelli, E., Pope, S., Smith, F. 2010. Plant performance in stressful environments: interpreting new and established knowledge of the roles of arbuscular mycorrhizas. Plant Soil 326:3–20

Smith, SE., Jakobsen, I., Grønlund, M., Smith, F. 2011. Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. Plant Physiol 156:1050–1057

Trouvelot, S., Bonneau, L., Redecker, D., van Tuinen, D., Adrian, M., Wipf, D. 2015. Arbuscular mycorrhiza symbiosis in viticulture: A review. Agron. Sustain. Dev. 35: 0

Van Rooyen, M., Valentine, A., Archer, E. 2004. Arbuscular mycorrhizal colonisation modifies the water relations of young transplanted grapevines (*Vitis*). South Afr J Enol Vitic 25:37–42

Verbruggen, E., Kiers, T. 2010. Evolutionary ecology of mycorrhizal functional diversity in agricultural systems. Evol Appl 3:547–560

Walker, R., Blackmore, D., Clingeleffer, P. 2010. Impact of rootstock on yield and ion concentrations in petioles, juice and wine of Shiraz and Chardonnay in different viticultural environments with different irrigation water salinity. Aust J GrapeWine Res 16:243–257

Wang, B., Qiu, Y. 2006. Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza 16:299–363

Wermelinger, B., Baumgärtner, J., Gutierrez, A. 1991. A demographic model of assimilation and allocation of carbon and nitrogen in grapevines. Ecol Model 53:1–26

Wilson, G., Rice, C., Rillig, M., Springer, A., Hartnett, D. 2009. Soil aggregation and carbon sequestration are tightly correlated with the abundance of arbuscular mycorrhizal fungi: results from long-term field experiments. Ecol Lett 12:452–461

CHAPTER II

Mycorrhizal activity in conventional and organic vineyard from five representative wine regions of Chile

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Abstract

Chile produces wine, which is apreciated around the world; furthermore, its isolated geographic characteristics have favored the creation of viticulture with a unique terroir. In this report we will describe for the first time the presence and colonization of arbuscular mycorrhizal fungi (AMF) associated with grapevine. AMF were studied in *Vitis vinifera* roots and rhizosphere soil in five vineyards located in the north, center and south of Chile, covering an area of approximately 750 km. AMF presence on roots of grapevines was analyzed by observing fungal root colonization and typical structures. The AMF colonization of these grapevines roots was consistent along the whole of these five regions, at 5 to 45 % of fine roots. The potential of inoculum of AMF communities on the roots of these grapevines showed that AMF associated in trap culture seems to be relatively stable. It is observed that the form of AMF structures change seasonally probably due to the variation of root colonizing species of the vine plants. Some of the changes in the presence of AMF were attributed to environmental factors (plant-available P) and location of the vineyard, although the latter could also have been influenced by an unmeasured environmental factor. The management of the vineyards directly affects the formation of arbuscular mycorrhiza, for an organic or

biodynamic management favors the development of a healthy community of microorganisms in the rhizosphere. It can be observed that the native arbuscular mycorrhizal communities have a high potential, in plants inoculated with mycorrhizae shown in positive results like an increase in the length of roots and plants, as well as in the number of leaves.

Keywords: Organic agriculture, Arbuscular mycorrhizal fungi, *Vitis*, Vineyard

1. Introduction

Arbuscular mycorrhizal fungi (AMF, *Glomeromycota*) establish beneficial symbioses with the roots of nearly all land plants, including the most important food crops (Smith and Read 2008), such as grapevines (Karagiannidis and Nikolaou 1999; Schreiner and Mihara 2009; Schubert and Cravero 1985). AMF are the essential elements of soil fertility, plant nutrition and productivity, facilitating soil mineral nutrient uptake by means of an extensive extra-radical network of fungal hyphae spreading from colonized roots into the soil (Avio et al. 2006). Several experiments showed that AMF not only might protect plants from biotic and abiotic stresses, such as fungal pathogens and drought (Sikes et al. 2009), but also provide key agro-ecosystem services, including soil aggregation and carbon sequestration (Gianinazzi et al. 2010). Arbuscular mycorrhizal fungi (AMF) are considered the most important soil organisms for agro-ecosystem sustainability, as they establish root symbioses with most crop plants and, acting as a living interface between plant roots and soil, translocate mineral nutrients—mainly P, N, Zn, Ca and Cu—from soil to plants with the large extraradical mycelial network spreading from mycorrhizal roots into the surrounding environment (Giovannetti and Avio 2002). Nevertheless, data on AMF relevance in extensive cropping systems are still inconsistent. AMF play an increasingly important role in vineyard production systems, as many vineyards receive little water and are planted on less fertile soils (Schreiner 2005). AMF inoculation of grapevines has been associated with increased growth (Schubert et al. 1988), drought tolerance (Schreiner 2007a) and nutrient uptake (Schreiner 2007b), in comparison with non-inoculated grapevines. As such, AMF represent an integral and important component of the vineyard ecosystem and might provide significant applications for sustainable agricultural ecosystems (Schreiner and Bethlenfalvay 1995).

Organic agriculture, defined as "a production system that sustains the health of soils, ecosystems and people" by the International Federation of Organic Agriculture Movements (http://www.ifoam.org/growing_organic/definitions/doa/index.html), is a broad group of farming systems characterized by strict limitation of chemical fertilizers, herbicides and pesticides, by soil management through addition of organic materials and by the use of crop rotation (IFOAM 2006). As a consequence, soil fertility and productivity of organic farming systems largely rely on biological processes carried out by soil microbes, which represent the key elements of agro-ecosystem functionality, and therefore, are a critical factor for the success of organic agriculture (Gosling et al. 2006). Intensive agricultural practices, such as monocropping, deep ploughing, chemical fertilization and pesticide use, are detrimental to soil microbial linkages (Tilman et al. 2002), negatively affecting AMF populations in terms of biodiversity, activity evaluated as colonization ability (Ryan et al. 2000) On the other hand, several studies showed that AMF diversity (Oehl et al. 2003, 2004), root colonization (Mäder et al. 2000) were higher in organically managed soils, suggesting that AMF may play an essential functional role in the maintenance of soil biological fertility and structure, compensating for the reduced use of chemical fertilizers and pesticides (Ryan and Tibbet 2008). A number of studies have demonstrated that AMF diversity is adversely affected by the excessive use of chemical fertilizers and pesticides, as well as intensive agricultural managements (e.g. monocropping) or practices (e.g. tillage). The intensity of agricultural management may differentially affect AM fungal spore communities (Oehl et al. 2003, 2004). For example, low-input agricultural systems, such as organic farming, where the use of chemical fertilizers and pesticides is not allowed, tend to promote higher AM fungal diversity than conventional agriculture (Oehl et al. 2004), whilst fields where conventional practices like intensive tillage and monoculture prevail show a lower AM fungal diversity (Hijri et al. 2006). Some herbicide, like simazine is subjected to microbial degradation with negligible leaching in agricultural soils and point out the crucial role of native microbiota in the herbicide removal. (Morgante et al. 2010, 2012)

The conversion from conventional to organic management, with the resulting changes in AMF communities, is a slow and gradual process (Göllner et al. 2005); in particular, in Mediterranean and semi-arid soils, such a process may even take longer, because of the low content and high turn-over of organic matter (Raviv 2010). Investigations on the effects of

organic farming on AMF communities in arable soils could contribute to the understanding of the behavior of these symbiotic fungi and their role as a provider of ecological services in sustainable agriculture. However, little is known, regarding the species composition of the AMF communities that colonize grapevines in production vineyards, as most of the published studies have been carried out on small-scale experiments. As mentioned the AM symbiosis is one plant survival strategy in the face of mineral nutrient deficiencies, and this has also been demonstrated for grapevine (Schreiner 2007b).

The main aims of the study was to describe the AMF communities that colonize the roots of the grapevines in the production vineyards located in five regions of Chile and to evaluate their spatial distribution patterns across the selected geographical area. We utilized different parameters to evaluate AMF presence and activity in arable soils of Central Chile from conventional to organic farming, to elucidate the AMF state hosted by a vine grape root system and associated rhizosphere soil, to discover AMF presence and to assess the influence of latitude and plant management in Chilean vineyards.

2. Materials and methods

2.1 Study area

The study area is located in five regions of central Chile (Table 1). Climatic conditions of the experimental area are typical of the Mediterranean zone.

Table 1. Geographical and agricultural field properties of sampling sites from five Chilean regions. Source: Self-elaboration.

	SITE 1	SITE 2	SITE 3	SITE 4	SITE 5	SITE 6	SITE 7	SITE 8	SITE 9	SITE 10
Region	Coquimbo (IV)	Coquimbo (IV)	Valparaíso (V)	Valparaíso (V)	O'Higgins (VI)	O'Higgins (VI)	Maule (VII)	Maule (VII)	Biobío (VIII)	Biobío (VIII)
Province	Elqui	Elqui	Valparaíso	Valparaíso	Cachapoal	Cachapoal	Cauquenes	Cauquenes	Ñuble	Nuble
Community	Paihuano	Vicuña	Casablanca	Casablanca	Rosario	Rengo	Cauquenes	Cauquenes	Bulnes	Bulnes
Elevation (mamsl)	1077	671	286	283	384	355	138	138	128	69
Latitude south	30° 4′ 38.72 "	30° 2′ 19.72 "	33° 19′ 43.93 "	33° 19′ 30.82 "	34° 23′ 23.17 "	34° 24′ 19.26 "	35° 58′ 36.32 "	35° 58′ 36.32 "	36°46'49.0"	36°43' 5.13"
Longitude west	70° 29′ 49.79"	70° 41′ 30.51 "	71° 26′ 58.95 "	71° 26′ 26.19 "	70° 47′ 30.63 "	70° 50′ 5.12 "	72° 19′ 23.25 "	72° 19′ 23.25 "	72°12'58.0"	72°21' 21.92"
Farmer	Viña Cavas del Valle	INIA	Viñedos Casas del Bosque	Viñedos Casas del Bosque	Viña Tipaume	Viñedos Torreon de Paredes	Viñedos Lomas de cauquenes	Viñedos Lomas de cauquenes	Viña Chillan	Viña Casanueva
Field size (ha)	6,5	5	23	212	6	150	2533	2534	22	70
Field slope (%)	30	0	15	10	0	0	36	18	0	0
Agricultural practices	Organic	Traditional	Organic	Traditional	Biodynamic	Traditional	Organic	Traditional	Organic	Traditional
Planting date	1999	1994	2010	1993	1996	1979	1980	1980	1998	1980
Organic matter (%)	3,29	3,22	2,4	1,8	7,6	9	10	1,9	5,9	4,5
pH	7,69	8,12	7,41	8,22	6,49	6,34	7,4	5,12	6,03	6,55
N (mg/Kg)	39,6	13,7	26,01	6,15	35,61	20,22	61,79	11,34	24,08	7,73
P(mg/Kg)	50	12,8	54	8,3	68,4	65	197	10,1	23	34,9
K disp (mg/Kg)	269	148	106	82	1095	457	552	91	411	432
K int (mg/Kg)	0,69	0,38	0,27	0,21	2,8	1,17	1,41	0,23	1,05	1,11

2.2. Soil characteristics

Soil samples for chemical analyses were collected in July 2014. Chemical soil characteristics were analyzed as follows: pH in a 1:2.5 (w/v) soil water ratio, total organic C by the Walkley–Black method (Nelson and Sommers 1996), total N by Kjeldahl digestion (Bremner 1996), available P (Olsen P) by extracting soil with 0.5 M $NaHCO_3$ at pH 8.5 (Olsen and Sommers 1982), exchangeable K using ammonium nitrate method (Mehlich 1984), following official methods in an external laboratory.

2.3. Soil and root sampling

Soil samples and plant roots were collected in July of 2014 and January of 2016. Samples consisted of plant roots and rhizosphere soil (500 g) collected at 0 to 20 cm depth of five plants to ten plants covering the majority of each vineyard surface. Root systems with rhizosphere soil were carefully extracted. The samples were placed in polyethylene bags, and stored at 4 ºC until processed. Roots belonging to root systems of the sampled species were selected, washed and processed to assess mycorrhizal colonization.

2.4. AM fungal root colonization

Mycorrhizal colonization was assessed with roots, which were carefully washed in tap water to remove soil particles and organic matter, fixed in 70% ethanol and subsequently cleared and stained with Trypan blue or aniline blue according to Brundrett et al. (1996). Selected stained

root segments were mounted on glass slides in lactoglycerol and observed with a Polyvar light microscope at ×100 to ×1,000 magnification. Details of root and mycorrhizal structures were documented by digital photomicrographs using a digital microscope camera. Percentage mycorrhization (presence of AM fungal structures per cm root length) was calculated using the Trouvelot et al. (1986) method. Twenty 1 cm segments of stained fine roots were mounted on a slide and observed at ×40 magnification. Every root segment was assigned to a relative category of mycorrhization from 0 (0% of mycorrhization) to ~~five~~ 5 (>95% mycorrhization). The mycorrhization percentage was then calculated as follows:

$$\mathbf{\%\ M} = \mathbf{(}n1 + n2 \bullet 5 + n3 \bullet 30 + n4 \bullet 70 + n5 \bullet 95\mathbf{)} \,/\, n\ total$$

$M_{Trouvelot}$ (%) is symmetrical in the 5 - 95% range, N is the number of observed segments, n_1 to n_5 , represents the number of segments categorized as 1 to 5, respectively. The mycorrhization percentage was obtained for each plant. The presence of DSE was only qualitatively recorded.

2.5. Mycorrhizal inoculum potential bioassay

Mycorrhizal inoculum potential (MIP) of the experimental field soils was assessed using *Pisum sativum* L. and *Allium schoenoprasum* L. as host plant. *P. sativum* and A. *schoenoprasum* seeds were sown in 50 mL sterile plastic tubes filled with 40 mL of each soil sample. Three replicate tubes per soil sample were prepared. Five days after emergence, *P. sativum* and A. *schoenoprasum* plants were thinned to two individuals per tube. 60 days after emergence, plants were removed from tubes and root systems were washed, stained and mounted on microscope slides. Root length, plant height and number of leaves were measured. Root colonization was assessed on stained 60 days old roots at magnification of ×125 and verified at magnification of ×500 under a Polyvar light microscope was calculated using the Trouvelot et al. (1986) method.

2.6. Statistical analyses

Root colonization data were analyzed by two-way ANOVA in a split-plot design with cover crop as the main factor and mycorrhizal inoculation as the sub-plot factor. Before analyses, data on root colonization were arcsine-transformed. The data reported in tables and figures are

back-transformed values. Wherever necessary, Tukey's HSD post hoc comparison was done to test for pairwise mean differences at P=0.05. All ANOVAs were performed by using R.

3. Result

3.1. Soil characteristics

Soil chemical analyses showed that in the experimental area the pH was moderately alkaline in the North and acidic in the South, no significant differences were observed among the five micro agro-ecosystems. The level of organic matter, total N, available phosphate content and exchangeable potassium content were significantly higher in organic management. It is also possible to observe a trend among the different valleys that better soil conditions and fertility for the vineyards in Chile dominate in the central valleys (Table 1).

3.2. Mycorrhizal root colonization

Microscopic examination of the stained root segments revealed the presence of hyphae, arbuscules and vesicles of AMF (Figure 1). Total fungal colonization of roots ranged from 5 to 45 % (Figure 2). The fungal colonization showed statistically significant differences between the vineyards. Correlation analysis of soil parameters and colonization levels revealed a positive correlation between organic matter, N and P content and fungal colonization (Pearson's R0 0.52, $p<0.001$). No other correlation between soil parameters and colonization levels could be observed. An important significant difference between the two types of management is observed, showing how an organic management increases the colonization of arbuscular mycorrhizae, which is kept constant throughout the studied areas. A slight seasonal trend is observed although there are no significant differences.

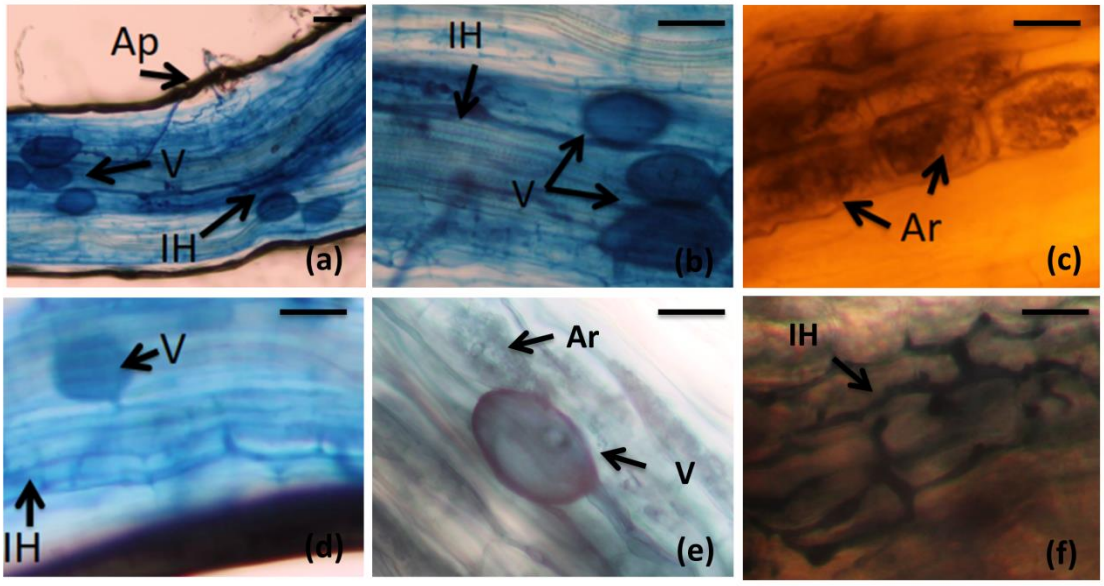

Figure 1. Typical structures of arbuscular mycorrhiza in Chilean grapevine. Light micrographs showing colonization pattern in cortex of grapevine (*Vitis vinifera* L) roots by AMF. To reveal mycorrhizal structures, roots are stained with Trypan blue. (a), (d) dense colonization of *Vitis*, showing intercellular hyphae running along the longitudinal root axis and forming many arbuscules and vesicles; (b) detail of arbuscules formed within adjacent root cells, showing dichotomous branching of hyphae; (c) Dense patches of arbuscules in contiguous cortical root cells of *Vitis*; (e) sparse root colonization, with rare arbuscules and vesicle; (f) intercellular hyphae running along the longitudinal root axis, bar = 100 lm. Source: Self-elaboration.

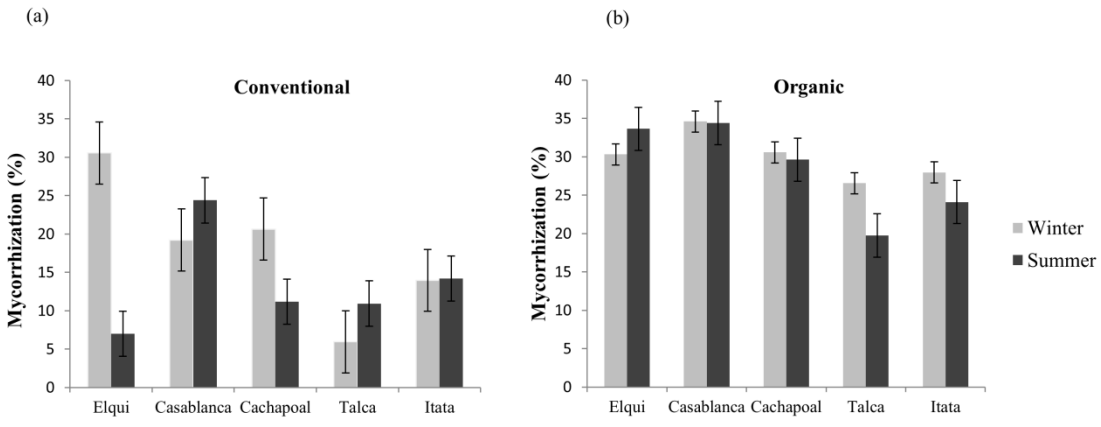

Figure 2. Root colonization of arbuscular mycorrhiza of five regions studied with management conventional and organic. (a) Total root colonization in winter and summer

with management conventional; (b) Total root colonization in winter and summer with management organic. (P<0.05). Source: Self-elaboration.

3.3. Mycorrhizal inoculum potential bioassay

Mycorrhizal inoculum potential (MIP) was not significantly different among the five micro agro-ecosystems. In mycorrhizal root colonization, it showed significant differences among management systems, 60 days after emergence. Root length, plant height and number of leaves were measured between two treatments (Figure 3), and showed large significant differences in the plants that had formed the symbiosis of mycorrhizal arbuscular fungi, with a longer root length, greater plant growth and a larger number of leaves.

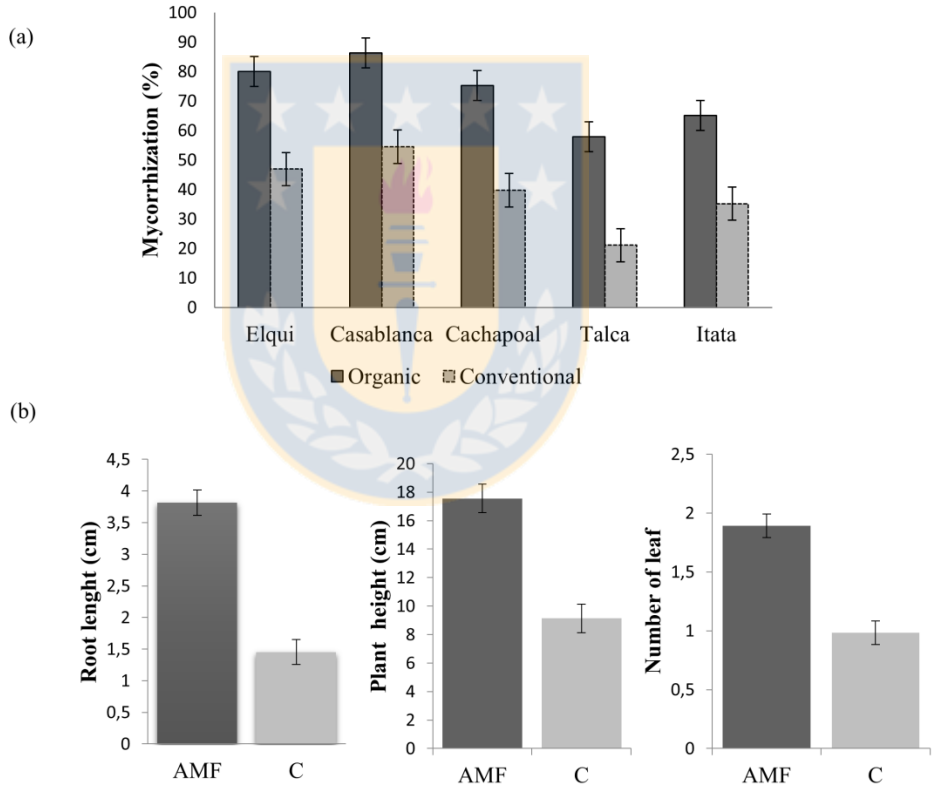

Figure 3. Root colonization of arbuscular mycorrhiza of inoculum potential bioassay. (a) Root colonization of arbuscular mycorrhiza in mycorrhizal inoculum potential bioassay of five zones studied with management conventional and organic; (b) Root length (RL), plant height (PH) and numbers of leaves (NL) were measured between two treatments with arbuscular mycorrhizal fungi and control. (P<0.05). Source: Self-elaboration.

4. Discussion

Grapevines are economically one of the most important crops in Chile. Their implementation and growth depends on the presence of symbiotic fungal soil fungi (Schreiner and Mihara 2009). To date, little has been published on the AMF symbionts in production vineyards, and no data are available for Chile. In this work, we assessed the differences in AMF activity among conventionally and organically managed soils of Chilean viticulture. Our work shows that: (a) AMF population activity and root colonization were higher in organic micro agro-ecosystems and (b) AMF presence was not affected by the different seasons of the year. In the present study, 5 to 45 % of the fine root length from grapevines in vineyards along these five Chilean regions was colonized by AMF. These levels are considerably lower compared to those reported for grapevine plants growing in typical soils with pH values as observed (Schreiner 2003, 2005). Although some studies have reported differences in colonization levels in soils with different pH values (Schreiner and Linderman 2005), others have failed to find significant effects of soil pH on AMF colonization levels (Nappi et al. 1985), as is in the present study.

AMF colonization is probably influenced by several factors other than soil pH, for example, specific rootstocks do not perform consistently across studies (Schreiner 2003). Also, some studies have found that AMF colonization of grapevines is negatively correlated to soil nitrogen and phosphate availability (Karagiannidis and Nikolaou 1999). It is possible to observe correlations, which were seen in the present study, between soil phosphate and nitrogen concentrations and AMF colonization of the grapevine roots, opposite to the findings of Schreiner and Linderman (2005). Also, although higher AMF colonization has been reported for agrosystems enriched in organic matter (Purin et al. 2006), only a positive correlation between total colonization and soil organic matter content in the grapevines from the studied regions was revealed. Various studies that have evaluated the influence of organic matter on AMF have produced very different results, which indicate the variable responses for plants and fungi (Joner and Jakobsen 1995; Gaur and Adholeya 2002; Carrenho et al. 2002). Accordingly, Purin et al. (2006) suggested that highest MIP values detected in native grassland surrounding conventional and organic orchards could be explained by the higher

amount of mycorrhizal plant species in grassland than under the canopy of the orchards, which could be an explanation of the significant differences found in the organic vineyards.

High AMF colonization in organic fields has been often attributed to low levels of available soil P (Ryan et al. 2000) which, at high concentrations, negatively affect mycorrhizal establishment (Kahiluoto et al. 2000). In our assessment available soil P concentration was different among the micro agro-ecosystems, but two other factors, such as crop rotation (Gavito and Miller 1998), cover crops and AMF population diversity (Scullion et al. 1998), could have contributed to enhance mycorrhizal colonization in organically managed fields. Some studies showed a higher AMF species richness in organically managed soils (Oehl et al. 2004; Hijri et al. 2006; Verbruggen et al. 2010), while others found non or only slight differences after 5 or 15 years of organic cultivation, compared with conventional farming (Bedini et al. 2008). Some species have been reported to occur in grasslands and to disappear in disturbed soils (Oehl et al. 2010), although sometimes retrieved in conventionally managed soils as spores (Bedini et al. 2007) or sequences (Turrini et al. 2008). The occurrence of such sensitive species in conventionally managed arable soils may be explained by the dispersal of AMF propagules from natural undisturbed sites close to cultivated soils, which dispersed by mammals and wind, may rapidly colonize crop plants growing nearby (Fracchia et al. 2011). In organic systems, AMF may provide ecosystem services in sustainable agro-ecosystems, in terms of maintaining soil structure, reducing erosion and contributing to belowground carbon pools.

Exploiting the interactions between soil microbial communities and crops is a relevant approach to increase food production for the growing world population at the lowest environmental costs, in the current scenario of global change (Barea 2015). In fact, the mycorrhizal inoculation is capable of alleviating the damage caused by salt stress conditions on pepper plants, to maintaining the membranes stability and plant growth, and this could be related to P nutrition, (Beltrano et al. 2013) the application efficient during seedling transplantation increase overall growth and yield performance of crop and could be considered as a sustainable substitute to higher phosphorus fertilizer cultivation, (Tanwar et al. 2013) and enhances both phenolics and tannins as well as stimulates antioxidant activity suggesting a

positive influence of AMF inoculation on yield and for the production of nutraceuticals (Jugran et al. 2015)

5. Conclusion

AMF colonization of fine roots of grapevines from vineyards along the five Chilean regions was withing the typical range reported for grapevines in other parts of the wordl. Comparison of the AMF colonization in the grapevine roots along the studied 700 km transects indicated that the fungal state associated with grapevine roots seems to be relatively stable. Some of the changes in the mycorrhization or structures of AMF could be attributed to environmental factors (plant-available P) and location of the vineyard, although the latter could also have influenced unmeasured environmental factors. It was observed that the type of management of the vineyard directly affects the formation of AMF; this is because an organic or biodynamic management promotes the development of a community in the rhizosphere of the plant. Further observations showed that the AMF structures change seasonally probably due to the variation of root colonizing species of the vine plants. The arbuscular mycorrhizal communities have a great potential, since the plants inoculated with mycorrhizae showed an increase in the length of the roots and plants, as well as in the amount of leaves. Further long-term studies should be performed in order to assess the population dynamics of AMF and the specific functional role played by these beneficial soil organisms in organic agriculture.

6. Acknowledgements

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7. References

Avio, L., Pellegrino, E., Bonari, E., Giovannetti, M. 2006. Functional diversity of arbuscular mycorrhizal fungal isolates in relation to extraradical mycelia networks. New Phytol 172:347–357

Barea, J. 2015. Future challenges and perspectives for applying microbial biotechnology in sustainable agriculture based on a better understanding of plant-microbiome interactions. Journal of Soil Science and Plant Nutrition, 15 (2), 261-282

Bavaresco, L., Fogher, C.1996. Lime-induced chlorosis of grapevine as affected by rootstock and root infection with arbuscular mycorrhiza and *Pseudomonas fluorescens*. Vitis 35:119–123

Bavaresco, L., Cantu, E., Trevisan, M. 2000. Chlorosis occurrence, natural arbuscular-mycorrhizal infection and stilbene root concentration of ungrafted grapevine rootstocks growing on calcareous soil. J Plant Nutr 23:1685–1697

Bedini, S., Avio, L., Argese, E., Giovannetti, M. 2007. Effects of long-term land use on arbuscular mycorrhizal fungi and glomalin-related soil protein. Agric Ecosyst Environ 120:463–466

Bedini, S., Cristani, C., Avio, L., Sbrana, C., Turrini, A., Giovannetti, M. 2008. Influence of organic farming on arbuscular mycorrhizal fungal populations in a Mediterranean agro-ecosystem. In: Neuhoff D et al (eds) Cultivating the future based on science, vol 1, Organic Crop Production Proceedings of the Second Scientific Conference of the International Society of Organic Agriculture Research (ISOFAR). ISOFAR, Bonn, FiBL, Frick, galpp 172–175

Beltrano, J., Ruscitti, M., Arango, M., Ronco, M. 2013. Effects of arbuscular mycorrhiza inoculation on plant growth, biological and physiological parameters and mineral nutrition in pepper grown under different salinity and P levels. Journal of Soil Science and Plant Nutrition, 13(1), 123-141

Bremner, J. 1996. Nitrogen-Total. In: Sparks DL (Ed) Methods of Soils Analysis. Part 3. Chemical Methods. SSSA and ASA, SSSA Book Ser 5, Madison, WI, pp 1085–1121

Brundrett, M., Bougher, N., Dell, B., Grove, T. and Malajczuk, N. 1996. Working with mycorrhizas in forestry and agriculture. ACIAR. Canberra.

Carrenho, R., Trufem, S., Bononi, V. 2002. Effects of using different host plants on the detected biodiversity of arbuscular mycorrhizal fungi from an agroecosystem. Rev Brasil Bot 25:93–101

de Oliveira, F., Mayumi, Y., da Silva, F., de Melo, N., Costa Maia, L. 2011. Soil biochemistry and microbial activity in vineyards under conventional and organic management at Northeast Brazil. Sci Agricola 68:223–229

Fracchia, S., Krapovickas, L., Aranda-Rickert, A., Valentinuzzi, V. 2011. Dispersal of arbuscular mycorrhizal fungi and dark septate endophytes by *Ctenomys cf. knighti* (Rodentia) in the northern Monte Desert of Argentina. J Arid Environ 75:1016–1023

Gaur, A., Adholeya, A. 2002. Arbuscular mycorrhizal inoculation of five tropical fodder crops and inoculum production in marginal soil amended with organic matter. Biol Fert Soil 35:214–218

Gavito, M., Miller, M. 1998. Changes in mycorrhiza development in maize induced by crop management practices. Plant Soil 198:185–192

Gianinazzi, S., Gollotte, A., Binet, M., van Tuinen, D., Redecker, D., Wipf, D. 2010. Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza 20:519–530

Giovannetti, M., Avio, L. 2002. Biotechnology of arbuscular mycorrhizas. In: Khachatourians GG, Arora DK (eds) Applied mycology and biotechnology. Agriculture and Food Production. Elsevier : 275–310

Göllner, M., Friedel, J., Freyer, B. 2005. Arbuscular mycorrhiza of winter wheat under different duration of organic farming. In: Researching sustainable systems. Proceedings of the 1st scientific conference of the international society of organic agriculture research (ISOFAR). Adelaide : 92–96

Gosling, P., Hodge, A., Goodlass, G., Bending, G. 2006. Arbuscular mycorrhizal fungi and organic farming. Agric Ecosyst Environ 113:17–35

Hijri, I., Sýkorová, Z., Oehl, F., Ineichen, K., Mäder, P., Wiemken, A., Redecker, D. 2006. Communities of arbuscular mycorrhizal fungi in arable soils are not necessarily low in diversity. Mol Ecol 15:2277–2289

IFOAM. 2006. The IFOAM basic standards for organic production and processing. Version 2005. IFOAM Publications, Germany

Joner, E., Jakobsen, I. 1995. Growth and extracellular phosphate activity of arbuscular mycorrhizal hyphae as influenced by soil organic matter. Soil Biol Biochem 27:1153–1159

Jugran, A., Bahukhandi, A., Dhyani, P., Bhatt, I., Rawal, R., Nandi, S., Palni, L. 2015. The effect of inoculation with mycorrhiza: AM on growth, phenolics, tannins, phenolic composition and antioxidant activity in *Valeriana jatamansi Jones*. Journal of Soil Science and Plant Nutrition 15(4):1036-1049

Kahiluoto, H., Ketoja, E., Vestberg, M. 2000. Promotion of AM utilization through reduced P fertilization. 1. Bioassays in a growth chamber. Plant Soil 227:191–206

Karagiannidis, N., Nikolaou, N. 1999. Arbuscular mycorrhizal root infection as an important factor of grapevine nutrition status multivariate analysis application for evaluation and characterization of the soil and leaf parameters. Agrochimica 43:151–165

Larimer, A., Bever, J., Clay, K. 2010. The interactive effects of plant microbial symbionts: A review and meta-analysis. Symbiosis 51:139–148

Mäder, P., Edenhofer, S., Boller, T., Wiemken, A., Niggli, U. 2000. Arbuscular mycorrhizae in a long-term field trial comparing low-input (organic, biological) and high-input (conventional) farming systems in a crop rotation. Biol Fert Soils 31:150–156

Mehlich, A. 1984. Mehlich 3 soil test extractant: a modification of the Mehlich 2 extractant. Comm Soil Sci Plant Anal 15:1409–1416

Morgante, V., López-López, A., Flores, C., González, M., González, B., Vásquez, M., Rosselló-Mora, R., Seeger, M. 2010. Bioaugmentation with Pseudomonas sp. strain MHP41 promotes simazine attenuation and bacterial community changes in agricultural soils. FEMS Microbiol Ecol. Jan 71(1):114-26

Morgante, V., Flores, C., Fadic, X., González, M., Hernández, M., Cereceda-Balic, F., Seeger, M. 2012. Influence of microorganisms and leaching on simazine attenuation in an agricultural soil. J Environ Manage 95:S300-5

Nappi, P., Jodice, R., Luzzati, A., Corino, L. 1985. Grapevine root systemand VA mycorrhizae in some soils of Piedmont (Italy). Plant Soil 85:205–210

Nelson, D. and Sommers, L. 1996. Total carbon, organic carbon, and organic matter. In: Methods of Soil Analysis, Part 2, 2nd Ed., A.L. Page et al., Am. Soc. of Agron., Inc. Madison, WI Ed. Agronomy. 9:961-1010

Oehl, F., Sieverding, E., Ineichen, K., Mäder, P., Boller, T., Wiemken, A. 2003. Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of central Europe. Appl Environ Microbiol 69:2816–2824

Oehl, F., Sieverding, E., Mäder, P., Dubois, D., Ineichen, K., Boller, T., Wiemken, A. 2004. Impact of long-term conventional and organic farming on the diversity or arbuscular mycorrhizal fungi. Oecologia 138:574–583

Oehl, F., Laczko, E., Bogenrieder, A., Stahr, K., Bösch, R., van der Heijden, M., Sieverding, E. 2010. Soil type and land use intensity determines the composition of arbuscular mycorrhizal fungal communities. Soil Biol Biochem 42:724–732

Olsen, S., Sommers, L. 1982. Phosphorus. In: Page AL (Ed) Methods of soil analysis. Part 2. Chemical and microbiological properties, 2nd edn, Agronomy Monograph 9:403–430

Purin, S., Filhoa, O., Sturmer, S. 2006. Mycorrhizae activity and diversity in conventional and organic apple orchards from Brazil. Soil Biol Biochem 38:1831–1839

Raviv, M. 2010. The use of mycorrhiza in organically-grown crops under semi arid conditions: A review of benefits, constraints and future challenges. Symbiosis 52:65–74

Ryan, M., Small, D., Ash, J. 2000. Phosphorus controls the level of colonisation by arbuscular mycorrhizal fungi in conventional and biodynamic irrigated dairy pastures. Austr J Exp Agric 40:663– 670

Ryan, M., Tibbet, M. 2008. The role of arbuscular mycorrhizas in organic farming. In: Kirchmann H, Bergström L (eds) Organic crop production: ambition and limitations. Springer 1:189–229

Schreiner, R., Bethlenfalvay, G. 1995. Mycorrhizal interactions in sustainable agriculture. Crit Rev Biotechnol 15:271–285

Schreiner, R. 2003. Mycorrhizal colonization of grapevine rootstocks under field conditions. Am J Enol Vitic 54:143–149

Schreiner, R. 2005. Mycorrhizas and mineral acquisition in grapevines. In: Christensen LP, Smart DR (eds) Proceedings of the soil environment and vine mineral nutrition symposium. 49-60

Schreiner, R., Linderman, R. 2005. Mycorrhizal colonisation in dryland vineyards of the Willamette Valley. Oregon, Small Fruits Rev 4:41–55

Schreiner, R., Tarara, J., Smithyman, R. 2007a. Deficit irrigation promotes arbuscular colonization of fine roots by mycorrhizal fungi in grapevines (*Vitis vinifera* L.) in an arid climate. Mycorrhiza 17:551–562

Schreiner, R. 2007b. Effects of native and nonnative arbuscular mycorrhizal fungi on growth and nutrient uptake of 'Pinot noir' (*Vitis vinifera* L.) in two soils with contrasting levels of phosphorus. App Soil Ecol 36:205–215

Schreiner, R., Mihara, K. 2009. The diversity of arbuscular mycorrhizal fungi amplified from grapevine roots (*Vitis vinifera* L.) in Oregon vineyards is seasonally stable and influenced by soil and vine age. Mycologia 101:599–611

Schubert, A., Cravero, M. 1985. Occurrence and infectivity of vesicular-arbuscular mycorrhizal fungi in north-western Italy vineyards. Vitis 24:129–138

Schubert, A., Cammarata, S., Eynard, I. 1988. Growth and colonization of grapevines inoculated with different mycorrhizal endophytes. Hort Sci 23:302–303

Scullion, J., Eason, W., Scott, E. 1998. The effectivity of arbuscular mycorrhizal fungi from high input conventional and organic grassland and grass–arable rotations. Plant Soil 204:243–254

Sikes, B., Kottenie, K., Klironomos, J. 2009. Plant and fungal identity determines pathogen protection of plant roots by arbuscular mycorrhizas. J Ecol 97:1274–1280

Smith, S., Read, D. 2008. Mycorrhizal symbiosis. Academic Press, London

Tanwar, A., A. Aggarwal, N. Kadian and A. Gupta. 2013. Arbuscular mycorrhizal inoculation and super phosphate application influence plant growth and yield of *Capsicum annuum*. Journal of Soil Science and Plant Nutrition 13(1):55-66

Tilman, D., Cassman, K., Matson, P., Naylor, R., Polasky, S. 2002. Agricultural sustainability and intensive production practices. Nature 418:671–677

Trouvelot, A., Kough, J., Gianinazzi-Pearson, V. 1986. Mesure du taux de mycorrhization VA d'un système radiculaire. Recherche de methods d'estimation ayant une signification fonctionelle. 'Physiological and genetical aspects of mycorrhizae'. Gianinazzi-Pearson V and Gianinazzi S eds., INRA 101-109

Turrini, A., Avio, L., Bedini, S., Giovannetti, M. 2008. In situ collection of endangered arbuscular mycorrhizal fungi in a Mediteranean UNESCO Biosphere Reserve. Biodivers Conserv 17:643–657

Vandenkoornhuyse, P., Baldauf, S., Leyval, C., Straczek, J., Young, J. 2002. Extensive fungal diversity in plant roots. Science 295:2051

Verbruggen, E., Roling, W., Gamper, H., Kowalchuk, G., Verhoef, H., van der Heijden, M. 2010. Positive effects of organic farming on below-ground mutualists: large-scale comparison of mycorrhizal fungal communities in agricultural soils. New Phytol 186:968–979

CHAPTER III

Grapevine associated arbuscular mycorrhizal fungal communities in Chilean vineyard

Article in preparation for: Mycorrhiza

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Abstract

Here, the community composition of arbuscular mycorrhizal fungi (AMF) associated with grapevine in Chile is described for the first time. AMF were studied in vine grape rhizosphere soil in ten vineyards located in the north, center and south of Chile, covering an area of approximately 750 km. AMF species were distinguished by morphological analysis and sequencing an approx. 1,500 bp nuclear rDNA region. Twelve species of AMF were identified, from grapevine rhizosphere soil. Interestingly, latitude and organic management affected species composition of different species diversity. The three most common colonizers of grapevine roots detected were *Funneliformis verruculosum, Septoglomus constricus* and an unknown *Septoglomus sp*. The grapevine-associated AMF diversity observed in this region is much higher than that reported for grapevine in other parts of the world. Grapevine plants were colonized by diverse species from 5 of the 14 *Glomeromycota* families. Identification of

the AMF species is important for their potential use in sustainable management practices to improve grapevine production in the Andean region.

Keywords: Viticulture, Arbuscular mycorrhizal fungi, *Vitis*, Vineyard

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are found as root symbionts in the majority of land-plant species, including the ten most important crops for human nutrition (FAO, 2012). *Vitis vinifera* (L.), cultivated for beverage, represents one of the most economically important fruit crop worldwide, with widespread cultivation and high commercial value. Grapes and their derivatives have a large and expanding worldwide market. They can be grown at latitudes from 50°N to 40°S and up to 3000 m above sea level (Vivier and Pretorius, 2002). *V. vinifera* is a crop whose market value depends largely on fruit quality. Grape quality is an important aspect of fruit quality and depends on local soil conditions (Seguin, 1986; van Leeuwen et al., 2004). *V. vinifera* is cultivated as a perennial crop, traditionally non-irrigated and with a diffused distribution over a wide area of dry or semi-dry ecosystems (Schreiner, 2007). Many viticulturists now aim to produce high quality wine, increasing profit from the land and reducing agronomic input through encouraging natural beneficial organisms. Wine grape production is a cropping system in which mineral fertilizer and cover crops can be combined to optimize soil nutrient content.

Arbuscular mycorrhizal (AM) fungi may be an important aspect of grape quality. These fungi (*Glomeromycota*) are root symbionts that have beneficial effects on host productivity (van der Heijden et al., 1998; Linderman and Davis, 2001), pathogen resistance (Li et al., 2006; Nogales et al., 2009), and nutrient uptake (Smith et al., 2003; Schreiner, 2007). The roots of grapevines (*Vitis* spp.) are often heavily colonized by arbuscular mycorrhizal fungi (AMF) under field conditions (Possingham and Obbink, 1971; Schreiner, 2003; Schubert and Cravero, 1985). In some cases, AMF appear to be necessary for normal growth and survival of field-planted grapevines (Menge et al., 1983). This makes sense, since AM fungi are very important for plants with low-density roots and few root hairs, like grapevines (Schreiner, 2005). Correspondingly, there is also evidence that AM fungi improve *Vitis* performance, including water use efficiency (Valentine et al., 2006), vine biomass (Linderman and Davis, 2001), and soil nutrient uptake (Schreiner, 2007). As these fungi provide the plant with water and soil

mineral nutrients (mainly phosphorus) and protection against biotic and abiotic stresses (Smith and Read 2008), they are regarded as a potential solution to increase crop yields sustainably without polluting the environment with high fertilizer and pesticide inputs.

Much of this work is from small scale experiments, but little is known about the species composition of AMF colonizing grapevines in production vineyards. The most common species reported from vineyard soils have been *Rhizoglomus intraradices, Funneliformis mosseae* (previously named *Glomus intraradices* and *Glomus mosseae*, respectively), *Glomus macrocarpum* and *Paraglomus occultum* (Menge et al., 1983, Schubert and Cravero, 1985, Oehl et al., 2005). It is not clear which of these fungi found as spores in soil actually colonize roots of grapevines. Some might be associated only with other plants in the vineyard, such as cover crops or weeds, which could play a role in maintaining AMF species diversity in the roots of grapevines. Molecular methods can show which organisms are actually present in roots of individual plants, but few such studies are published on the AM fungal composition in vineyards, which represent good candidates in studies devoted to verify the AMF communities in a land-use gradient from natural to conventionally managed ecosystems (Lumini et al., 2010).

Understanding of diversity and community composition of arbuscular mycorrhizal fungi (AMF) is a necessary prerequisite towards their effective utilization in improving biological soil fertility and crop production (Liu et al., 2014) and might be particularly relevant for organic systems (Oehl et al., 2004). Presently, the role of AMF in influencing soil fertility, crop productivity, yield quality and protection against environmental stresses is widely acknowledged (Smith and Read, 2008; Giovannetti et al., 2012). AMF contribution to crop growth and productivity can be influenced by both interspecific and intraspecific differences (Munkvold et al., 2004; Vogelsang et al., 2006). Consequently, through sampling or niche complementarity effects, increased AMF diversity may provide agroecological services directly affecting crop production (van der Heijden et al., 2008).

AMF are strongly affected by anthropogenic activities (Giovannetti and Gianinazzi-Pearson, 1994), and intensive agricultural practices, such as crop rotation fertilization pest control and tillage impact AMF, reducing population biodiversity (Daniell et al., 2001). Organic agriculture has been shown to increase AMF root colonization and propagule numbers (Galvez

et al., 2001; Oehl et al., 2003), although low input practices used in such management system do not always allow the level of biodiversity to increase, even after a long time (Bedini et al., 2007). Hence, understanding the structure and the dynamics of AMF populations as affected by diverse agricultural practices represents an important prerequisite for the success of organic farming.

This study was therefore performed to characterize AMF species diversity associated with grapevine in Chile. AMF communities in rhizosphere soil were analyzed at different regions using molecular-biological methods that allow monitoring of AMF at species level. The aim was to elucidate the AMF diversity hosted by a grapevine associated rhizosphere soil, to discover whether there are preferential associations between grapevine plants and certain AMF species, and to assess the influence of latitude and plant management on such preferences.

2. Materials and methods

2.1 Study area, soil characteristics and soil sampling

The study area is located in five zones of central region of Chile (Table 1). Climatic conditions of the experimental area are typical of the Mediterranean zone. Soil samples for chemical analyses were collected in July 2014.

Chemical soil characteristics were analysed as follows: pH in a 1:2.5 (w/v) soil water ratio, total organic C by the Walkley–Black method (Nelson and Sommers, 1996), total N by Kjeldahl digestion (Bremner, 1996), available P (Olsen P) by extracting soil with 0.5 M $NaHCO_3$ at pH 8.5 (Olsen and Sommers, 1982), exchangeable K using ammonium nitrate method (Mehlich, 1984), following official methods in an external laboratory.

Soil samples were collected in July 2014 and January 2015. Samples consisted of rhizosphere soil (500 g) collected at 0 and 20 cm depth of random ten plants covering the majority of each vineyard surface. The samples were placed in polyethylene bags, and stored at 4 ºC until processed.

Table 1. Geographical and agricultural field properties of sampling sites from ten Chilean vineyards. Source: Self-elaboration.

	Valley	Latitude south	Longitude west	Agricultural practices	Organic matter (%)	pH	N (mg/Kg)	P(mg/Kg)
SITE 1	Elqui (IV)	30° 4′ 38.72 "	70° 29′ 49.79"	Organic	3,29	7,69	39,6	50
SITE 2	Elqui (IV)	30° 2′ 19.72 "	70° 41′ 30.51 "	Conventional	3,22	8,12	13,7	12,8
SITE 3	Casablanca(V)	33° 19′ 43.93 "	71° 26′ 58.95 "	Organic	2,4	7,41	26,01	54
SITE 4	Casablanca(V)	33° 19′ 30.82 "	71° 26′ 26.19 "	Conventional	1,8	8,22	6,15	8,3
SITE 5	Cachapoal (VI)	34° 23′ 23.17 "	70° 47′ 30.63 "	Biodynamic	7,6	6,49	35,61	68,4
SITE 6	Cachapoal (VI)	34° 24′ 19.26 "	70° 50′ 5.12 "	Conventional	9	6,34	20,22	65
SITE 7	Talca(VII)	35° 58′ 36.32 "	72° 19′ 23.25 "	Organic	10	7,4	61,79	197
SITE 8	Talca (VII)	35° 58′ 36.32 "	72° 19′ 23.25 "	Conventional	1,9	5,12	11,34	10,1
SITE 9	Itata(VIII)	36°46'49.0"	72°12'58.0"	Organic	5,9	6,03	24,08	23
SITE 10	Itata (VIII)	36°43' 5.13"	72°21' 21.92"	Conventional	4,5	6,55	7,73	34,9

2.2 Fungal spore extraction and identification

Spores and sporocarps of AMF were extracted from duplicate sievings of 25 g of each sample, by wet-sieving and decanting, through a set of nested sieves (Gerdemann and Nicolson, 1963). Spores and sporocarps were retained on sieves of pore size 400, 250, 100 and 45 µm. After sieving, the material obtained from the 125 and 32-µm sieves was transferred to five 50-mL vials per sample constituting five 25-mL suspensions. The suspensions were under-layered with 25 mL of a 70 % w/v sucrose solution, and the water/sucrose solution density gradient was centrifuged at 2,000 rpm for 2 min. In the material from the 500-µm sieve, no spores or sporocarps were observed. After centrifugation, the supernatant was passed through a 45-µm sieve and washed with tap water. The trapped material, largely containing spores, spore clusters and sporocarps, was flushed into 9-cm diameter Petri dishes, examined under a dissecting microscope (Wild, Leica) at magnifications up to ×50 with illumination by incident light from a fibre-optic quartz-halogen light source. When present, sporocarps were dissected with forceps and the released spores were counted. Numbers and morphotypes of AM fungal spores were recorded. Only intact, healthy appearing spores were counted. Spores were manually isolated by using capillary pipettes, separated according to their morphology and colour, placed in Eppendorf tubes, submitted to sonication as needed in a B-1210 cleaner (Branson Ultrasonics, Soest, The Netherlands), washed three times in sterile distilled water, mounted on microscope slides in polyvinyl alcohol lactoglycerol (PVLG) and examined under

a Polyvar light microscope equipped with Nomarski differential interference contact optics (Reichert-Young, Vienna, Austria). For taxonomical identification, which was morphologically based, spores were mounted both in PVLG and in PVLG + Melzer's reagent (1:1, v/v) as media. Qualitative spore traits (spore shape, colour and size, spore wall ornamentation, wall structure and shape, colour and size of the subtending hypha) were examined on at least 20 spores using a micrometric eyepiece for each morphotype, except some spores, which were found in low number. Spore morphotypes were compared with original diagnoses of AMF species and with the reference culture descriptions at http://invam.caf.wvu.edu/fungi/taxonomy/speciesID.htm and http://www.agro.ar.szczecin.pl/~jblaszkowski/index.html. Since important changes of AMF nomenclature have been recently proposed by different authors (Oehl et al., 2011; Krüger et al., 2012), with some taxa differently named, we utilised the new binomials only for consistent species names and maintained the previous ones for the others.

Total spore densities were determined as spore number 100 g^{-1} soil.

2.3 DNA extraction from spores

Intact, healthy spores identified as different morphotype were manually collected with a capillary pipette under the dissecting microscope and cleaned by sonication (120 s) in the B-1210 cleaner. After three rinses in sterile distilled water (SDW), spores were surface sterilised with 2% chloramine T supplemented with streptomycin (400 µg/ml) for 20 min and rinsed five times in SDW. Intact single spores were selected under the dissecting microscope and transferred in Eppendorf tubes before DNA extraction. Spores were individually crushed into PCR tubes using a glass pestle immediately, and then the DNA was extracted by using MasterPure yeast DNA purification KIT (Epicentre, Madison, USA) according to manufacturer instructions.

2.4 PCR conditions of full-length SSU-ITS-LSU sequences

DNA extracts from single spores were used to analyse a DNA region covering partial SSU, ITS and partial LSU sequences by a nested PCR protocol (Kruger et al. 2009). In the first PCR reaction DNA extracts (1 µl) were amplified in 25 µl of PCR reaction mix using 0.125 U of GoTaq Flexi DNA Polymerase (Promega, Milan, Italy), 0.4µM each primer (Af1, Af2 and

Ar1, Ar2, Ar3, Krüger et al. 2009), 0.2 mM (each) dNTPs, 1.5 mM $MgCl_2$ and 1× manufacturer's reaction buffer. The thermal cycler was programmed as follows: a manual "hot start" at 95°C for 3 min, 35 cycles at 95°C for 30 s, 60°C for 1 min, 72°C for 2 min and a final extension step at 72°C for 10 min. The nested PCR reactions were performed by diluting (1:100) the first PCR amplicons and using 2 µl of dilutions as template for the second reaction in a final volume of 50 µl. Each primer pair,Cf1, Cf2, Cf3 and Br1, Br2, Br3, Br4 (Krüger et al., 2009; 0.4µM), were added to the PCR mix. Taq DNA polymerase, dNTPs, buffer and $MgCl_2$ concentrations were as described above. Amplification conditions were as follows: a manual "hot start" at 95°C for 3 min, 35 cycles at 95°C for 30 s, 63°C for 45 s, 72°C for 1,5 min and a final extension step at 72°C for 10 min. PCR products (10 µl) were separated on 0.8% agarose gels containing ethidium bromide (0.5 µg/ml).

2.5 Cloning and sequencing

Amplified DNA fragments of SSU-ITS-LSU regions were purified by Wizard SV Gel and PCR Clean-Up System according to the manufacturer's instructions (Promega), with a final elution volume of 20 µl and purified products (2 µl) were quantified by a BioPhotometer (Eppendorf). Purified products were cloned into pGem®-T Easy vector according to the manufacturer's instructions (Promega). Putative positive clones were screened by standard SP6/T7 amplifications, followed by a nested PCR using primer pairs by Krüger et al. 2009. Concentration of PCR mix components and PCR conditions were the same described above for PCR reactions. Amplification products (ten clones from each sample) were tested for restriction fragment length polymorphism patterns by digesting the PCR products with HinfI (Takara, Madison, Wi, USA) or HinfI/MboI restriction enzymes, respectively in order to recover sequence variability. Digested DNA was electrophoresed through 2% MetaPhor agarose (BMA, Rockland, ME USA) containing ethidium bromide (0.5 µg/ml) and 1 kb Plus DNA Ladder (Invitrogen, Milano, Italy) was used as a molecular weight marker. DNA profiles were analysed by Uvitec Cambridge, Essential v4 system. Fifteen clones from single spores (five clones/spore) containing recombinant plasmids with different HinfI patterns were purified by Wizard® Plus SV Minipreps (Promega). Recombinant plasmids were sequenced forward and reverse at GATC Biotech (AG European Custom Sequencing Centre, Cologne, Germany).

2.6 Sequence analyses and data analysis

Sequences were edited in MEGA6 and their similarities were determined using the Basic Local Alignment Search Tool (BLASTn) provided by NCBI. The detection of chimeric sequences was performed using USEARCH 6.0 (http://fungene. cme.msu.edu/FunGenePipeline/chimera_check/form.spr). Sequences were aligned with those corresponding to the closest matches from GenBank as well as with sequences from major clades of Glomeromycota using MUSCLE as implementedin MEGA6. Phylogenetic tree were inferred by Neighbour-Joining analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method. The confidence of branching was assessed using 1000 bootstrap resamplings.

3. Results and discussion

3.1. Physical and chemical analyses of the soil

Results of physical and chemical analyses of soils are shown in Table 1. Apart from differences in N and P content there were strong difference in pH the ten studied sites. Some evidence shows that pH in particular can affect the AMF species composition (Wang et al., 1993), and some works are suggesting that sporulation of particular AMF can be stimulated by increasing organic matter (Gryndler et al., 2004; Oehl et al., 2009).

3.2. AMF spores in soils

Extracted spores were identified morphologically, and species determined by reference to the literature and from experience. Several morphotypes were identified and described (Table 2). The three most common colonizers of grapevine roots detected were *Funneliformis verruculosum*, *Septoglomus constricus* and an unknown *Septoglomus sp*. The lack of certainty in species determination reflects the difficulties in identifying spores from soil extractions (Fig 1). The taxonomy of AM fungi is continuously updated, mainly based on molecular analysis. The analyses of AMF associated with grapevine in the Chilean Valleys show a relatively high fungal diversity. Twelve taxes, interpreted as species, were detected in rhizosphere soil at the ten study sites from a 700 km long transect. However, direct

comparisons with other works are difficult because different studies use variable means to describe the AMF communities.

Table 2. AMF Morphological characters of different morphotypes isolated from Chilean valleys. The species marked (*) were identified with morphological features. Source: Self-elaboration.

	Morphotype	Spore Number	Spore Colour	Spore Shape	Spore Size	Surface Texture	Subtending Hyphae	Subtending Hyphae Size	Contents	Wall Layers	Wall Layers Size (u)	Specie
1	GI1	18	Dark red brown	Globosa	370 u	smooth	Gigasporoide	Unb	Reticulate	2	12,5/10	**Scutelospora sp ***
2	GL1	326	Yellow-red-brown	Globosa	173 u	smooth	Glomoide	18,6 u	Reticulate	2	2/13,6	**Funneliformis verruculosum ***
3	GL2	153	Red brown	Globosa	163 u	smooth	Glomoide	15,5 u	Small droplets	2	15,5/12,4	**Uncultured Septoglomus**
4	GL3	134	Clear orange	Globose	175 u	Smooth	Glomoide	16,6 u	Large droplet	2	6/12,7	**Uncultured Septoglomus**
5	GL4	249	Dark-Clear orange	Globose	160,1 u	Smooth	Glomoide	15,5 u	Small droplets	2	9,3/6,2	**Uncultured Septoglomus**
6	GI2	9	Lemon yellow	Globose	240 u	pits	Gigasporoide	43,4 u	Reticulate	3	15,5/ 6,3	**Claroideoglomus etunicatum ***
7	AC1	13	Bright yellow	Globose	213 u	smooth	Glomoide	15,5 u	Small droplets	2	15,5/6,2	**Acaulospora sp**
8	GL5	314	Yellow-red-brown	Globose	107,5 u	Smooth	Glomoide	5,6 u	Large droplet	3	2,5/6,3/ 3,8	**Septoglomus constricus**
9	GI3	57	White	Globose	238 u	Smooth	Gigasporoide	46,5 u	Granules	1	13	**Cetrospora gilmorei**
10	PA1	26	White	Subglobosa	186 u	smooth	Glomoide	9,3 u	Large droplet	2	1,25/1,25	**Pacispora scintillans**
11	PAR1	29	Hyaline	Subglobosa	116 u	Reticulate	Glomoide	4,65 u	Granules	2	6,25/ 5	**Paraglomus sp ***
12	GL6	36	Orange Brown	Subglobosa	437,5 u	hyphae	Unb	Unb	Gores	Unb	Unb	**Sclerocystis sp ***

In the present study, AMF communities were not fully represented by the sampling strategy used. Although sampling density was comparable to that in many published studies, the species accumulation curves based on plant and soil sampling indicated that the sampling was not dense enough to cover all the diversity. Thus, the low number of samples and high variability of AMF species found in soil systems resulted in incomplete coverage of the root associated diversity. Consequently, the approach used did not cover all the grapevine-associated AMF diversity. The expected total number of grapevine-associated AMF for all studied samples would be in the range of 19±4 species in soil.

The AMF associated with grapevine varied in the different Chilean Valleys. Similar to many other studies, the *Glomeraceae* was a dominating family. This family is usually described to be ubiquitous (Sýkorová et al., 2007) and occurring frequently in, or dominating, agricultural systems. they were also reported as preferential colonizer of grapevine plants in an agricultural area (Oehl et al., 2005). These studies highlighted the dominance of species of the former genus *Glomus sp,* now classified in genera such as *Glomus sp, Rhizophagus, Funneliformis, Claroideoglomus* and *Paraglomus*. The result suggest a similar situation in Chilean fields, although the other parts of the world are very different from Chile in kind of climate, soil, variety, etc..

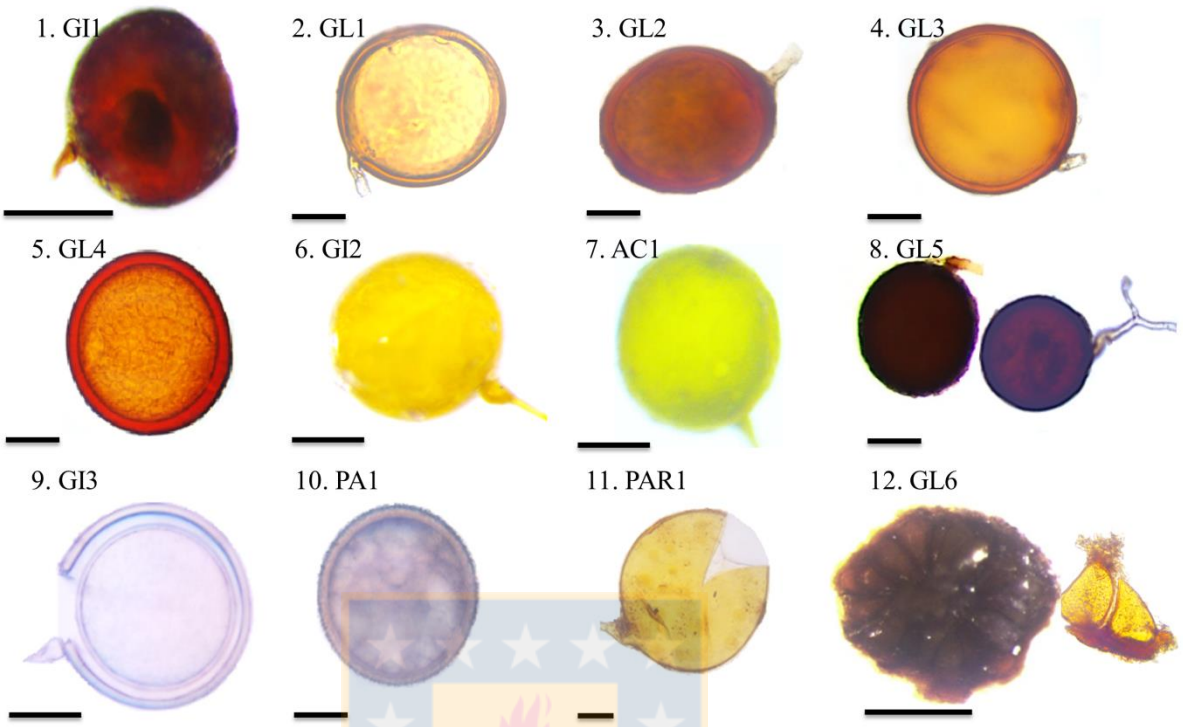

Figure 1. AMF Spore morphotypes from soil of five Chilean grapevine valleys. Spores were extracted from the soil by the wet-sieving, and sucrose centrifugation method, and then they were washed into watch glasses with water and examined first under a dissecting microscope, and, after mounting on microscope slides in polyvinyl alcohol lacto-glycerol (PVLG).**1.** GI1 *Scutellospora sp.* * **2**.GL1 *Funneliformis verruculosum* * **3**.GL2 Uncultured *Septoglomus* **4**.GL3 Uncultured *Septoglomus* **5**.GL4 Uncultured *Septoglomus* **6**.GI2 *Claroideoglomus etunicatum* * **7**.AC1 *Acaulospora sp* **8**.GL5 *Septoglomus constricus* **9.**GI3 *Cetrospora gilmorei* **10.**PA1 *Pacispora scintillans* **11**.PAR1 *Paraglomus sp* * **12**. GL6 *Sclerocystis sp* * . The species marked(*) were identified with morphological description. Source: Self-elaboration.

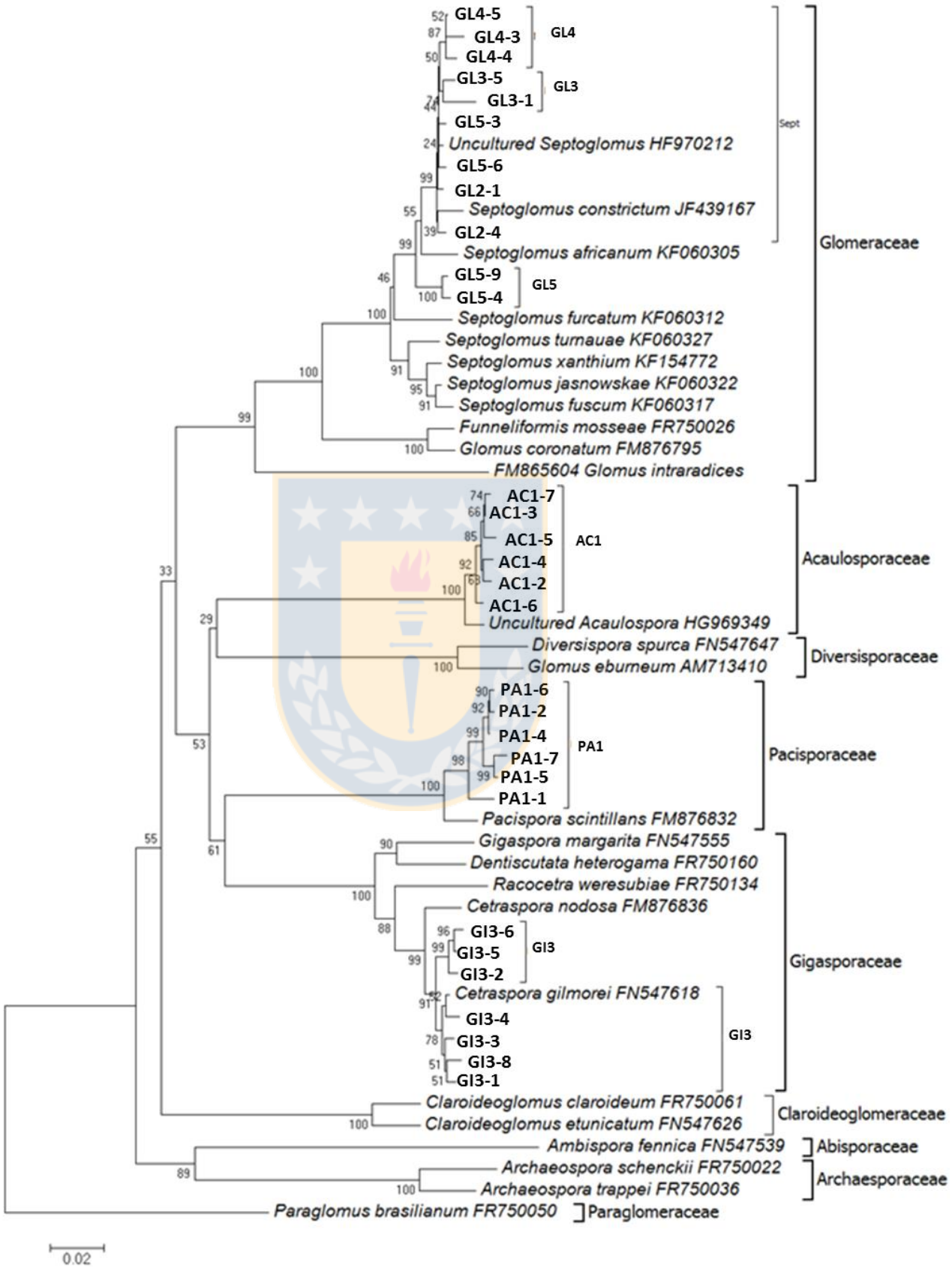
GL4-5
GL4-3
GL4-4
GL4
GL3-5
GL3-1
GL3
GL5-3
Uncultured Septoglomus HF970212
GL5-6
GL2-1
Septoglomus constrictum JF439167
GL2-4
Septoglomus africanum KF060305
GL5-9
GL5-4
GL5
Septoglomus furcatum KF060312
Septoglomus turnauae KF060327
Septoglomus xanthium KF154772
Septoglomus jasnowskae KF060322
Septoglomus fuscum KF060317
Funneliformis mosseae FR750026
Glomus coronatum FM876795
FM865604 Glomus intraradices
Sept
Glomeraceae
AC1-7
AC1-3
AC1-5
AC1-4
AC1-2
AC1-6
AC1
Uncultured Acaulospora HG969349
Acaulosporaceae
Diversispora spurca FN547647
Glomus eburneum AM713410
Diversisporaceae
PA1-6
PA1-2
PA1-4
PA1-7
PA1-5
PA1-1
PA1
Pacispora scintillans FM876832
Pacisporaceae
Gigaspora margarita FN547555
Dentiscutata heterogama FR750160
Racocetra weresubiae FR750134
Cetraspora nodosa FM876836
GI3-6
GI3-5
GI3-2
GI3
Cetraspora gilmorei FN547618
GI3-4
GI3-3
GI3-8
GI3-1
GI3
Gigasporaceae
Claroideoglomus claroideum FR750061
Claroideoglomus etunicatum FN547626
Claroideoglomeraceae
Ambispora fennica FN547539
Abisporaceae
Archaeospora schenckii FR750022
Archaeospora trappei FR750036
Archaesporaceae
Paraglomus brasilianum FR750050
Paraglomeraceae
0.02

Figure 2. Phylogenetic tree showing the AMF species detected in the Chilean grapevine valleys. Neighbour-joining phylogenetic tree based on the alignment of SSU-ITS-LSU sequences of different species belonging to *Glomeromycota*. Bootstrap values (>70%) were determined for neighbour joining (1,000 resamplings). A sequence of *Paraglomuus brasilianum* was used as outgroup. Sequences obtained in this study are shown in bold face. Source: Self-elaboration.

Detection at the species level is an important step towards characterization of functional aspects of AMF communities and individual species in the field. However, species identification in the kingdom Fungi is difficult due to the unexplored diversity and lack of reliable resonance sequences (Kõljalg et al., 2013). Usually, there exists no simple sequence similarity threshold that can be used for species delimitation, and this is particularly true for AMF with their enormous intraspecific ribosomal DNA (rDNA) sequence variability (Stockinger et al., 2009, 2010). Most molecular ecological studies targeting entire AMF communities analyze an undefined taxonomic level between genus and species (e.g., Öpik et al., 2013). This allows some important comparisons at global scales but is limited in terms of AMF species diversity and community analyses because a single taxonomic unit may cover more than one species while, at the same time, several distinct taxonomic units may represent sequence variants from a single species.

It has been shown that a 1.5-kb fragment covering the SSU-ITS-LSU rDNA region is suitable for members of the *Glomeromycota* as an extended DNA barcode providing species-level resolution, also in field studies (Krüger et al., 2009; Stockinger et al., 2010; Schoch et al., 2012).

In the present study, where obtained sequence types were characterized at species level, half of the species represent new ones or species previously not characterized by sequence data, which indicates the existence of a large number of unknown AMF species in the Chilean valleys. Although grapevine appears to be colonized by a wide variety of AMF in Chile (Fig 2). The present data on AMF in Chilean Valleys support the concept that different AMF species or taxa can be host or habitat generalists, or specialists (e.g., Oehl et al., 2010; Öpik and Moora, 2012). However, clearly more detailed information is needed to draw conclusions about the specialists and the drivers of their occurrence.

3.3. Influence of latitude and management on AMF communities

The highest AMF species diversity was found at the middle latitude for both soil management, but in the case of organics vineyard, we have more species presents (Fig 3). The results obtained suggest that the maintenance of a different AMF community seems to be affected by soil characteristics, and we can speak about microbial terroir. The relationship between vineyard soils and AMF assemblages found in this work indicate that the soil characteristics are important variable influencing AMF communities (Schreiner and Mihara, 2009). The results of this study focus on vineyard ecosystems, give us show some of the factors responsible for the differences in AMF assemblages in this economically important culture.

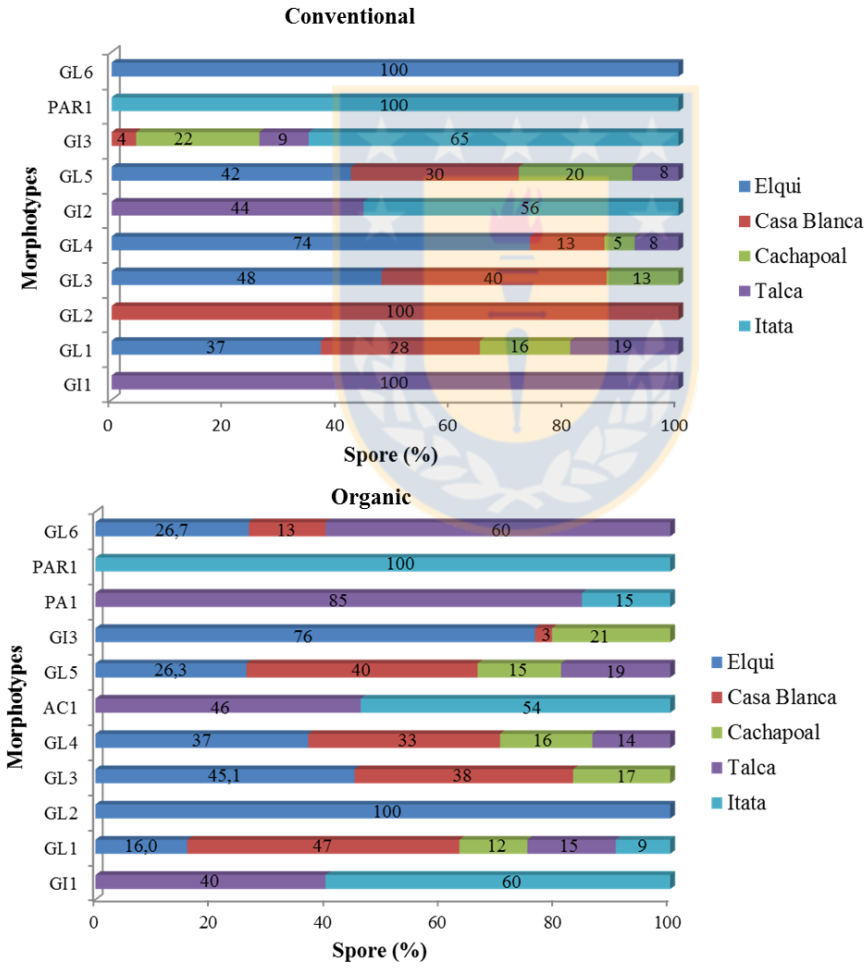

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Figure 3. Distribution of different AMF morphotypes in five Chilean grapevine valleys. This is based on the total number of spores of AMF found in each valley, using conventional and organic management. Source: Self-elaboration.

Notwithstanding the increasing practice of AMF inoculation and the rising global trade of AMF commercial inocula, it is still unclear how the introduction of exotic AMF strains either directly in the field or indirectly via transplanting of preinoculated plants affects the native AMF diversity and community composition. This may have great ecological consequences, leading to introduction of invasive strains that are deleterious to native AMF biodiversity (Janoušková et al. 2013; Schwartz et al. 2006). Knowledge of how native AMF communities in organic systems are affected by common agronomic practices including cover crop and crop management is a crucial step towards effective utilization of AMF in sustainable crop production

4. Conclusion

In conclusion, the number of AMF species found in Chilean grapevine valleys is high and similar to that found in some other vineyard that appear to harbor a high AMF diversity. The AMF community composition did vary among different sites and the type of management. A deeper analysis will be needed to monitor the AMF, including the active symbiosis on the root a using other molecular tools, like next generation sequencing.

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6. References

Bedini S., Avio L., Argese E., Giovanetti M. (2007). Effects of long-term land use on arbuscular mycorrhizal fungi and glomalin-related soil protein. Agr. Ecosys. Environ.120:463-466

Bremner, J. 1996. Nitrogen-Total. In: Sparks DL (Ed) Methods of Soils Analysis. Part 3. Chemical Methods. SSSA and ASA, SSSA Book Ser 5, Madison, WI, pp 1085–1121

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31:3497–3500

Daniell T.J., Husband R., Fitter A.H., Young J.P.W. (2001). Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. FEMS Microbiol. Ecol. 36:203-209.

FAO (2012) Food and Agriculture Organization of the United Nations, Land Resources. http://www.fao.org/nr/land/en/.Accessed07July2013

Galvez L, Douds D.D., Drinkwater L.E., Wagoner P. (2001). Effect of tillage and farming system upon VAM fungus populations and mycorrhizas and nutrient uptake of maize. Plant Soil 228:299–308

Gerdemann JW, Nicolson TH (1963) Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. Trans Br Mycol Soc 46:235–244

Giovannetti M., Gianinazzi-Pearson V. (1994). Biodiversity in arbuscular mycorrhizal fungi. Mycol. Res. 98:705-715

Giovannetti M, Avio L, Barale R, Ceccarelli N, Cristofani R, Iezzi A, Mignolli F, Picciarelli P, Pinto B, Reali D, Sbrana C, Scarpato R (2012) Nutraceutical value and safety of tomato fruits produced by mycorrhizal plants. Brit J Nutr 107:242–251

Janoušková M, Krak K, Wagg C, Štorchová H, Caklová P, Vosátka M (2013) Effects of inoculum additions in the presence of a preestablished arbuscular mycorrhizal fungal community. Appl Environ Microb 79:6507–6515

Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, Bates ST, Bruns TD, Bengtsson-Palme J, Callaghan TM, Douglas B, Drenkhan T, Eberhardt U, Dueñas M, Grebenc T, Griffith GW, Hartmann M, Kirk PM, Kohout P, Larsson E, Lindahl BD, Lücking R, Martín MP, Matheny PB, Nguyen NH, Niskanen T, Oja J, Peay KG, Peintner U, Peterson M, Põldmaa K, Saag L, Saar I, Schüßler A, Scott JA, Senés C, Smith ME, Suija A, TaylorDL,TeresaTelleriaM,WeißM,LarssonK-H(2013) Towards a unified paradigm for sequence-based identification of fungi. Mol Ecol 22:5271–5277

Krüger M, Stockinger H, Krüger C, Schüßler A (2009) DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytol 183:212–223

Li HY, Yang GD, Shu HR, Yang YT, Ye BX, Nishida I, Zheng CC (2006) Colonization by the arbuscular mycorrhizal fungus *Glomus versiforme* induces a defense response against the root-knot nematode *Meloidogyne incognita* in the grapevine (*Vitis amurensis* Rupr), which includes transcriptional activation of the class III chitinase gene VCH3. Plant Cell Physiol 47:154–163

Linderman RG, Davis AE (2001) Comparative response of selected grapevine rootstocks and cultivars to inoculation with different mycorrhizal fungi. Am J Enol Viticult 52:8–11

Liu W, Zheng C, Fu Z, Gai J, Zhang J, Christie P, Li X (2014) Facilitation of seedling growth and nutrient uptake by indigenous arbuscular mycorrhizal fungi in intensive agroecosytems. Biol Fertil Soils 50:381–394

Lumini E, Orgiazzi A, Borriello R, Bonfante P, Bianciotto V (2010) Disclosing arbuscular mycorrhizal fungal biodiversity in soil through a land-use gradient using a pytosequencing approach. Environ Microbiol 12:2165–2179

Menge JA, Raski DJ, Lider LA, Johnson ELV, Jones NO, Kissler JJ, Hemstreet CL (1983) Interactions between mycorrhizal fungi, soil fumigation and growth of grapes in California. Am J Enol Viticult 34:117–121

Mehlich, A. 1984. Mehlich 3 soil test extractant: a modification of the Mehlich 2 extractant. Comm Soil Sci Plant Anal 15:1409–1416

Munkvold L, Kjøller R, Vestberg M, Rosendahl S, Jakobsen I (2004) High functional diversity within species of arbuscular mycorrhizal fungi. New Phytol 164:357–364

Nelson, D. and Sommers, L. 1996. Total carbon, organic carbon, and organic matter. In: Methods of Soil Analysis, Part 2, 2nd Ed., A.L. Page et al., Ed. Agronomy. Am. Soc. of Agron., Inc. Madison, WI 9:961-1010.

Nogales A, Aguirreolea J, Maria ES, Camprubi A, Calvet C (2009) Response of mycorrhizal grapevine to Armillaria mellea inoculation:disease development and polyamines. Plant Soil 317:177–187

Oehl F., Sieverding E., Ineichen K., Mader P., Boller T., Wiemken A. (2003): Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of central Europe. Appl. Environ. Microbiol. 69:2816–2824

Oehl F, Sieverding E, Mäder P, Dubois D, Ineichen K, Boller T, Wiemken A (2004) Impact of long-term conventional and organic farming on the diversity of arbuscular mycorrhizal fungi. Oecologia 138:574–583

Oehl, F., Sieverding, E., Ineichen, K., Ris, E.-A., Boller, T., and Wiemken, A. (2005) Community structure of arbuscular mycorrhizal fungi at different soil depths in extensively and intensively managed agroecosystems. New Phytol 165: 273–283

Oehl, F., Sieverding, E., Ineichen, K., Mäder, P., Wiemken, A., and Boller, T. (2009) Distinct sporulation dynamics of arbuscular mycorrhizal fungal communities from different agroecosystems in long-term microcosms. Agric Ecosys Environ 134: 257–268

Oehl F, Sieverding E, Palenzuela J, Ineichen K, Silva GA (2011) Advances in *Glomeromycota* taxonomy and classification. IMA Fungus 2:191–199

Olsen, S., Sommers, L. 1982. Phosphorus. In: Page AL (Ed) Methods of soil analysis. Part 2. Chemical and microbiological properties, 2nd edn, Agronomy Monograph No. 9 ASA, Madison, WI, pp 403–430

Öpik M, Moora M (2012) Missing nodes and links in mycorrhizal networks. New Phytol 194:304–306

Öpik M, Zobel M, Cantero J, Davison J, Facelli J, Hiiesalu I, Jairus T et al (2013) Global sampling of plant roots expands the described molecular diversity of arbuscular mycorrhizal fungi. Mycorrhiza 23: 411–430

Possingham J V and Obbink J G 1971 Endotrophic mycorrhiza and the nutrition of grape vines. Vitis 10:120–130

Seguin G (1986) ‘Terroirs’ and pedology of wine growing. Experientia 42:861–873

Smith SE, Smith AF, Jakobsen I (2003) Mycorrhizal fungi can dominate phosphorous supply to plants irrespective of growth response. Plant Physiol 133(1):16–20

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, London

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Bolchacova E, Voigt K, Crous PW et al (2012) Nuclearibosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci U S A 109: 6241–6246

Schreiner PR (2007) Effects of native and nonnative arbuscular mycorrhizal fungi on growth and nutrient uptake of 'Pinot noir' (*Vitis vinifera* L) in two soils with contrasting levels of phosphorus. Appl Soil Ecology 36:205–215

Schreiner RP, Linderman RG (2005) Mycorrhizal colonization in dryland vineyards of the Willamette Valley, Oregon. Small Fruits Review 4:41–55

Schreiner PR, Mihara KL (2009) The diversity of arbuscular mycorrhizal fungi amplified from grapevine roots (*Vitis vinifera* L) in Oregon vineyards is seasonally stable and influenced by soil and vine age. Mycologia 101:599–611

Schreiner R P 2003 Mycorrhizal colonization of grapevine rootstocks under field conditions. Am. J. Enol. Vitic. 54:143–149.

Schubert A and Cravero M C 1985 Occurrence and infectivity of vesicular-arbuscular mycorrhizal fungi in north-western Italy vineyards. Vitis 24:129–138.

Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9:501–515

Stockinger H, Walker C, Schüßler A (2009) "*Glomus intraradices* DAOM197198", a model fungus in arbuscular mycorrhiza research, is not *Glomus intraradices*. New Phytol 183:1176–1187

Stockinger H, Krüger M, Schüßler A (2010) DNA barcoding of arbuscular mycorrhizal fungi. New Phytol 187:461–474

van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf- Engel R, Boller T, Wiemken A, Sanders IR (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69–72

van der Heijden MGA, Bardgett RD, van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol Lett 11:296–310

van Leeuwen C, Friant P, Chone X, Tregoat O, Koundouras S, Dubourdieu D (2004) Influence of climate, soil and cultivar on terroir. Am J Enol Vitic 55:207–217

Valentine AJ, Mortimer PE, Lintnaar M, Borgo R (2006) Drought responses of arbuscular mycorrhizal grapevines. Symbiosis 41(3):127–133

Vivier, M.A., and Pretorius, I.S. (2002) Genetically tailored grapevines for the wine industry. Trends Biotechnol 20: 472–478

Vogelsang KM, Reynolds HL, Bever JD (2006) Mycorrhizal fungal identity and richness determine the diversity and productivity of a tallgrass prairie system. New Phytol 172:554–562

Wang, G.M., Stribley, D.P., Tinker, P.B., and Walker, C. (1993) Effects of pH on arbuscular mycorrhiza. I. Field observations on the long-term liming experiments at Rothamsted and Woburn. New Phytol 124: 465–472

CHAPTER IV

Effect of arbuscular mycorrhizal fungi in polyphenols in *Vitis vinifera* L.

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Abstract

The aim of this study is to examine the effect of arbuscular mycorrhizal fungi (AMF) colonization, polyphenol profile and content of economically important grapevine. An *in vitro* and field experiment was performed with *Vitis vinifera* cv Cabernet Sauvignon applying a commercially available AMF mixture for inoculation. Major polyphenols were identified using TPC, TLC, HPLC and LC-MC we showed that AMF can provide different ecosistemic services for grapevine. AMF inoculation significantly increased the polyphenols in field and *in vitro* assay, principally from the quercitin family. Therefore the quantity and quality of plant material could be improved by the application of optimized AMF inoculum. Our data show that mycorrhizal inoculation may represent an efficient and sustainable strategy to improve productivity and enhance plant biosynthesis of secondary metabolites with health promoting activities.

Keywords: *In vitro* culture, polyphenols, Arbuscular mycorrhizal fungi, *Vitis*, Vineyard

1. Introduction

Phenolic compounds are a large group of phytochemicals, existing ubiquitously in plants as secondary metabolites. In human diet, the majority of them belong to phenolic acids, flavonoids, and tannins. Besides contribution to sensory properties of food, phenolic compounds also exhibit a wide range of biological and physiological functions, such as anti-allergenic, anti-inflammatory, antimicrobial and antioxidant activities, which are beneficial for human health (Crozier, et al. 2009; Balasundram et al. 2006;Claudine et al. 2005; Middleton et al. 2000; Shahidi and Naczk, 2004). Besides fruits and vegetables, which serve as excellent sources of phenolic compounds, it is widely recognized that grapevine are rich in phenolic compounds. (Okuda 1999)

Agronomic and environmental factors, inducing low increases of plant phytochemicals may "represent probably a good balance between effectiveness and safety" (Poiroux-Gonord et al. 2010). Accordingly, more attention should be paid to studies aimed at selecting the best performing plant genotypes, growth conditions, agronomic techniques and environmental factors to improve health-promoting properties of crop plants. Among such factors, soil health and biological fertility represent the most important ones, playing a key role in the production of safe plant foods, as a result of the action of beneficial soil microorganisms, which promote plant growth and health and reduce the need of chemical fertilizers and pesticides. Microbial populations colonizing soil, surfaces, and internal tissues of plants provide many ecosystem services, and represent important components to take into consideration in studies aimed at understanding the genetic and functional capacity of the host. Indeed, researches aiming at studying plant microbiome have shown that it represents a genomic resource for the host, regulating plant trait expression and affecting its productivity and interactions with the environment. In particular, a group of plant symbionts, arbuscular mycorrhizal fungi (AMF), living in intimate association with the roots of most plant species worldwide, have been shown not only to improve plant nutrition and health, but also to induce changes in plant secondary metabolism, leading to enhanced biosynthesis of health-promoting phytochemicals,such as polyphenols, carotenoids, flavonoids, phytoestrogens, and to a higher activity of antioxidant enzymes. (Sbrana et al. 2014)

With the development of new agricultural techniques, the yield and quality of plant material can be improved significantly to satisfy the increasing industrial and consumer demands. One of the promising possibilities for enhancing the biomass and increase the quality of grapevine can be the application of arbuscular mycorrhizal fungi (AMF) during their cultivation. AMF occur in all terrestrial ecosystems and form symbiotic associations with the majority of terrestrial plant species, including many agriculturally important crop species. (Brundrett, 2009) Botanically the arbuscular mycorrhiza (AM) is a mutualistic relationship between phytobionts and mycobionts. AMF have direct and indirect effects on plant nutrient uptake, root morphology, many physiological and developmental processes of plants (Marschner 1997). These fungi are obligate biotrophs, which must develop a symbiotic association with the host in order to grow and complete their life cycle (Parniske, et al. 2008). AM fungi take up products of host plant photosynthesis. Hexose transfer may occur through arbuscules as an intracellular interface of symbiotic association. In contrast to the diverse array of terrestrial plants that form AM, only ca. 200-250 *Glomeromycota* species have so far been described, suggesting that mycobiont specificity is low relative to their potential plant hosts (Heijden, et al. 2004). The root colonization by AMF has been found to increase the productivity of several crops due to a range of benefits provided to the host plants. Positive effects of AM symbiosis on plant growth, water uptake, nutrition uptake and tolerance to environmental stressors (drought, salt, heavy metal pollution, soil pathogens) have been proved in numerous studies (Gianinazzi, et al. 2010; Gosling, et al. 2006; Takács 2012). Apart from these beneficial effects, accumulation of secondary metabolites such as essential oils, alkaloids and phenolic compounds in medicinal plants can also be affected by this symbiosis inducing important changes in physiological processes of host (Gianinazzi, et al. 2010; Zeng, et al. 2013; Chen, et al. 2014) . However, the way of this influence on the active substances is not always evident. The presence of AMF might either enhance the accumulation of the secondary metabolites in the plant's organs but in some cases there is no detectable effect of it. Recent observations indicate that the influence of the AMF colonization on the level of secondary metabolites highly depends on the type of host plant and the fungi species. For example, the polyphenol content of *Ocimum basilicum L*. was increased as a result of the colonization by *Glomus caledonium*, while the colonization by *Glomus intraradices* had no effect (Toussaint, et al.

2007). At the same time, the inoculation with *Glomus intraradices* increased the polyphenol content of *Echinacea purpurea (L.) Moench.* (Araim, et al. 2009)

AMF colonization of root cortical cells produces several cytological and metabolic changes, such as a marked proliferation of plastids during arbuscule formation (Smith and Read 2008). The activation of plastid biosynthetic pathways and Krebs cycle leads to increased metabolic activity and higher production of amino acids, fatty acids and apocarotenoids, linked to the formation of symbiotic structures (Lohse et al. 2005). AM symbiosis influences primary and secondary metabolism of host plants: it induces important changes both in enzymatic activities (i.e. superoxide dismutase and catalase) (Marin et al. 2002) and in physiological mechanisms leading to the accumulation of secondary metabolites, such as carotenoids and polyphenols (Toussaint et al. 2007). Some authors reported higher levels of reactive oxygen species (ROS) in colonized roots, and suggested that mycorrhizal plants respond to oxidative stresses by the accumulation of antioxidative enzymes and carotenoids (Fester and Hause 2005).

Consequently, the use of appropiate AMF during the cultivation can improve the quantity as well as the quality of the obtained plant material. Although the chemical composition and pharmacological activities of grapevine have been well studied and earlier findings show that grapevine is usually colonized by AMF, no data are available about the influence of AMF on the polyphenol content of these important crop. Accordingly, polyphenolics can be considered important factors to improve the quality profile and the nutritional and nutraceutical value of grapevine. Thus, the aim of the present study is to examine the effect of a commercially available AMF mixture on the profile and content of phenolic compounds of grapevine. Our findings allow us to evaluate the possibility of eco-friendly agricultural application of AMF in order to improve the cultivation of grapevine.

2. Materials and methods

2.1 Chemicals and Reagents

All reagents were of analytical grade. Acetonitrile, methanol (LC-MS grade) and formic acid (~98%) were purchased from Merck (Merck, Darmstadt, Germany). FolinCiocalteu reagent and 1 ml semi-micro polystyrene cuvets were purchased from Sigma-Aldrich S.r.l.(Chile). A

Milli-Q ultrapure water system (Merck Millipore, Billerica, MA, USA) was used throughout the study to obtain high purity water for the HPLC (high performance liquid chromatography) analysis.

2.2 Plant material

The plants of *Vitis vinifera* L. cv Cabernet Sauvignon were obtained from an experimental vineyard in INIA (Institute of Agricultural Research) Santiago of Chile. The inoculum compound for *Glomus intraradices* was obtained from commercial product called MYCOSYM from the spain company MYCOSYM-TRITON S.L.

2.3 *In vitro* experiment

In vitro (V) micropropagated plantlets of Vitis *vinifera* were first propagated from woody cuttings, and the newly elongated shoots were cut and surface-sterilized. Nodal segments of the disinfected shoots were cultured on a modified Murashigue and Skoog (MMS) medium (Murashigue and Skoog, 1962) adapted to vine by Torregrosa and Bouquet (1996) and kept in a growth chamber set at 25 ± 2°C with 50 µmol m–2 s–1 photon flux density (PFD), provided by fluorescent lights (Sylvania cool-white) for a 16 hours-photoperiod. New shoots were formed from each segment and grapevine plantlets were sub-cultured by nodal cuttings every five weeks on MMS medium (Torregrosa et al., 2001). Shoots of 4.5-5.5 cm long with uniform root systems were used in the experiments.

Experimental setup

Two treatments were considered, with ten replicates each: non-inoculated plants and inoculation with commercial AM fungal inoculum (*G. intraradices).* Petri plates 15 cm in diameter were filled with MSR medium lacking sugar and vitamins (Voets et al., 2005) and two plugs of inoculum isolated from an AM fungal of *G. intraradices* containing approximately spores were placed in the centre of the plate. This inoculation method was aimed at achieving a exposure of AM fngal, as it occurs in the field. Approximately 5 cm long *in vitro* micropropagated plantlets of *Vitis*, were then transferred to the Petri plates and an autotrophic culture system was established. The roots remained in the Petri plate on the culture medium, devoid of sucrose and vitamins, while the shoots grew in open air conditions as

described for potato plants (Voets et al., 2005). Petri dishes were covered with opaque plastic strips in order to keep the root system in the dark, and plants were kept inside a plastic box at 100% of relative humidity in a growth chamber set at 25°C with 16h photoperiod and a PFD of 200 µmol m–2 s–1 development a culture *in vitro* autotrophic (VA) . Ten days later their acclimatization was induced by progressively opening the box. Sterilized (121°C for 15 min) MSR medium lacking sucrose and vitamins was periodically added to the Petri plates to maintain an adequate level of nutrients and liquid in the plates. After 13 weeks, the set-up of the experiment, the percentage of mycorrhizal intraradical colonization was estimated by the grid-line intersect method (Giovannetti and Mosse, 1980) on a 1 g portion of each root system which was cleared and stained following the method of Phillips and Hayman (1970).

2.4 Field experiment

This experiment was aimed at assessing phenols in HPLC of grapevine from a field grown plants, previously inoculated with commercial kit of inoculum of AMF.

Experimental setup

We work with plants of *Vitis vinifera L* cv Cabernet Sauvignon in experimental plot the field. of INIA La Platina. Chemical characteristics of the field soil were as follows: pH (H_2O), 7.72; N, 10,7 $mgkg^{-1}$; available P (P), 12,9 mg kg^{-1}; exchangeable K (K_2O), 198 mg kg^{-1};Organic matter (%), 3,7. All the plants of a single treatment were grouped together and followed by the plants of another treatment. Two g/plant of a ternary fertilizer (NPK: 11-22-16) was distributed after one month. Weed control was carried out manually. Off shoots were removed in spring and autumn.

2.4 Leaf extracts preparation

Leaf samples (2.5 g) were collected from 6 plants per treatment, frozen and hand crushed in a mortar, added with 50 ml of 80% MeOH, and extracted at 4°C for 48 h. The extract was filtered in N°1 Whatman paper and the solvent was evaporated at reduced pressure in a rotary evaporator at 40 °C (Heildolph Unimax 2010), the extracts were concentrated until dry and weighted in order to obtain total yield. The extraction was repeated three times, and stored at 4°C in darkness.

2.5 Total Polyphenol Content (TPC)

This method consists of determining the total polyphenol content through oxidation of phenolic compounds using a mix of phosphotungstic and phosphomolybdic acids in base medium, producing blue acids of tungsten and molybdenum. Absorbance of these acids was then read at 765 nm). Results were expressed in mg gallic acid/100 mL. Each assay was performed in triplicate. TPC was measured by means of the Folin-Ciocalteu method (Folin, Ciocalteu, 1927; Moyer et al., 2002). Different diluitions of the 80% MeOH extracts (0.25 ml) were mixed with 0.25 ml of Folin-Ciocalteu reagent (diluted with distilled H 2O1:1v/v), 0.5 ml of a saturated Na solution (20%) and 4 ml of HO. The reaction mixtures were left at room temperature for 30 min. Absorbance at 725 nm (A 725 2) was measured using a Hitachi U-3200 spectrophotometer. Each replicate was analysed twice. Extract dilutions corresponded to 10, 5, 2.5, 1.25, 0.625, 0.250, 0.0625 mg A calibration curve was obtained by mixing different quantities of chlorogenic acid (CA) standard (0, 1, 5, 10, 15, 20, 25, 30, 35, 40, 50, 75 and 100 µg) with 0.25 ml of Folin-Ciocalteu reagent.

2.6 Thin chromatography layer (TLC)

All thin-layer chromatographic analysis was performed using TLC plates pre-coated aluminium oxide F-254 (Type E) with layer thickness of 0.25 mm made in Germany by E. Merck, Darmstadt. TLC plate (F-254, type E) was used to separate the active compounds present in the extracts, using the mixture of mobile phase chloroform (90%)-methanol (10%). The TLC sheets after development are visualized by spraying with sulfuric acid (2%).

2.7 High performance liquid chromatography (HPLC)

A calibration curve was prepared with 7, 15, 30, 100, 175, and 250 mg L−1 from one standard deviation of quercetin at 98.9 % purity CAT# 500105 Calbiochem. HPLC was used with a YL 9111S binary pump, YL9160 PDA detector with manual injection, and 20-µL loop; Kromasil 100-5-C18 column, 250 × 4.6 mm Ø. As a mobile phase, 0.1 % formic acid was used in H2O Milli-Q and acetonitrile (80:20), at isocratic pressure at milliter per minute and 365 nm wavelength.

2.8 Liquid chromatography-Mass Spectrometry (LC-MS)

LC-MS was used with a LC-30AD quaternary pump, SPD-M30A PDA detector with automatic injection, and 10-µL sample volume; Kromasil 100-5-C18 column, 250 × 2.1 mm Ø. A mobile phase, 0.1 % formic acid was used in H2O Milli-Q and acetonitrile, gradient from 5%-60% in 20 minutes at isocratic pressure at 0.4 milliter per minute and 280-365 nm wavelength. Acquisition mode scan, positive ions, mass range (m/z) from 50-2000. We utilize electro spray ionization ESI with an interface of temperature 350 °C, DL temp (250). Nebulizng gas flow 1,5 L/min and heat block of 200°C. The acquisition time was 30 minutes with a flow of box 0,4 mL/min in a range of 200-700 nm, column oven 30°C.

2.9 Statistical analysis

Data analysis was performed using Statistica software (version 6.0, StatSoft, Inc, Tulsa, OK, USA). The data were treated with one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Differences were considered statistically significant when $p<0.05$.

3. Results

3.1 *In vitro* experiment

Root colonization and plant growth parameters: Root colonization did not show significant differences among different plants inoculated with AMF with values between 65% and 80% of colonized root length, in *in vitro* autotrophic culture. Significant differences were found for leaf area and root length in plants inoculated with AMF mix, compared with control plants ($P<0.05$) (data not shown).

Total phenolic compounds in vitro grapevine: The leaf samples collected from control and mycorrhizal plants, *in vitro* and *in vitro* autotrophic were examined and compared for their total phenolic content (TPC), (Fig. 1). The TPC content of plants inoculated with AMF mix showed values were significantly different ($P<0.05$) from those of control plants.

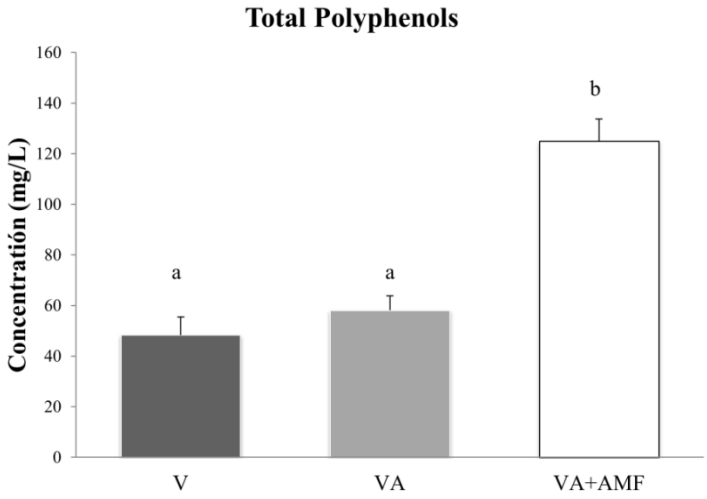

Figure 1. Effect of AMF in total polyphenols in leaves of *Vitis vinifera* L cv Cabernet Sauvignon.It is possible to observe the effect of the different growth systems, V (*in vitro* culture), VA (*in vitro* autotrophic culture) and VA+AMF(*in vitro* autotrophic culture with arbuscular mycorrhizal fungi). ($P<0.05$). Source: Self-elaboration.

3.2 Field experiment

Total polyphenols analyses The methanolic extracts from the grapevine was preliminary analysed using a TLC (Fig 2). In HPLC all inoculated plants showed significant differences compared to controls, it possible to observe the differences of concentration in the samples with the AMF (Table 1). In LC-MS all the chromatograms indicate the different patron and intensity of the molecules in inoculated plants with AMF showed significant differences compared to controls.(Fig3a) The tentative identification of phenolic compounds in grapevine by LC-MS, based on the m/z 465 and the elution order, compounds more common in the samples were identified as Quercitina3-O-Glucoronide, Quercitina 3-O-Galactoside, Quercitina 3-O-Glucoside, Kaempferol 3-o-glucoside. (Table 2) The principal compounds are Quercitina3-O-Glucoronide and Kaempferol 3-o-glucoside (Fig 3b).

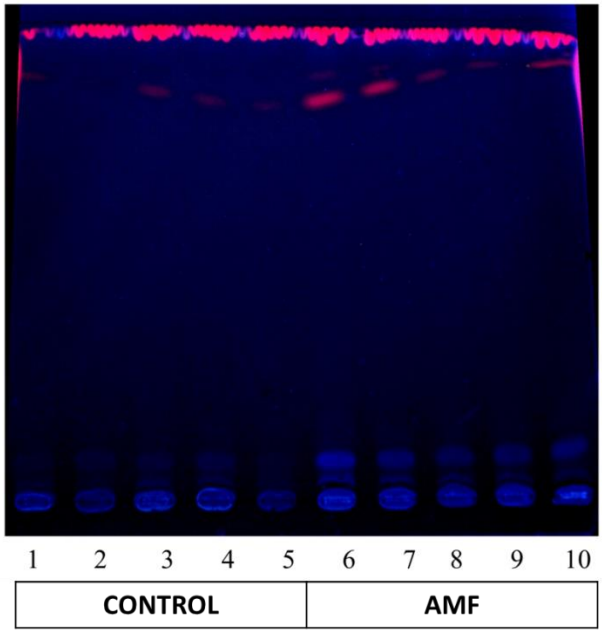

Figure 2. Effect of AMF in total polyphenols from grapevine leaf extract analized by TLC. Line 1-5; control plants; line 6-10; inoculated plant with commercial *Glomus intraradices*. Source: Self-elaboration.

Table 1. Quantification of phenolic compounds from leaf grapevine inoculated with commercial *Glomus intraradices* analized by HPLC. The treatments are with arbuscular mycorrhizal fungi and control. (*) indicated the higher concentration of compound in the total extract (ug/g); Rt: retention time. Source: Self-elaboration.

Treatments	Peack no.	Rt	ug/g extract
Control	1	2.7	4,05*
	2	3.2	0,22
	3	3.9	2,32*
	4	4.5	0,67
	5	6.1	6,60*
	6	7.1	0,35
	7	7.7	1,31*
	8	8.4	0,09
	9	11.7	0,44
AMF	1	2.7	15,24*
	2	3.0	0,31
	3	3.9	8,36*
	4	4.5	0,85
	5	5.0	0,11
	6	5.3	0,73
	7	5.9	0,32
	8	6.5	30,43*
	9	8.3	0,70
	10	9.0	3,95*

Table 2. Identification of tentative phenolic compounds in grapevine control and inoculated with *Glomus intraradices* analized by LC-MS.

Peak no.	Rt (min)	Tentative identification	Abbreviation	$UV_{\lambda max}$ (nm)	$[M+H]^+$ (m/z)	$[A+H]^+$and other ions(m/z)
1	10.108	Rutin*	Rut	280-330	611	465/303
2	10.913	Quercetin3-O-glucoronide*	Q-Glu	256-355	479	303
3	10.913	Quercetin 3-O-Galactoside*	Q-Gal	254-355	465	303
4	10.913	Quercetin 3-O-Glucoside*	Q-Gluc	254-355	465	303
5	11.515	Quercetin 3-O-rhamnoside*	K-Rham	267-346	449	303
6	11.883	Kaempferol 3-O-glucoside	K-Glu	266-346	449	287

* Confirmation of these compounds has been done by reference standards. All other peaks were tentatively identified compared with literature data wavelength; $[M-H]^+$: positive ion mode. Rt: retention time; λmax: maximal. Source: Self-elaboration.

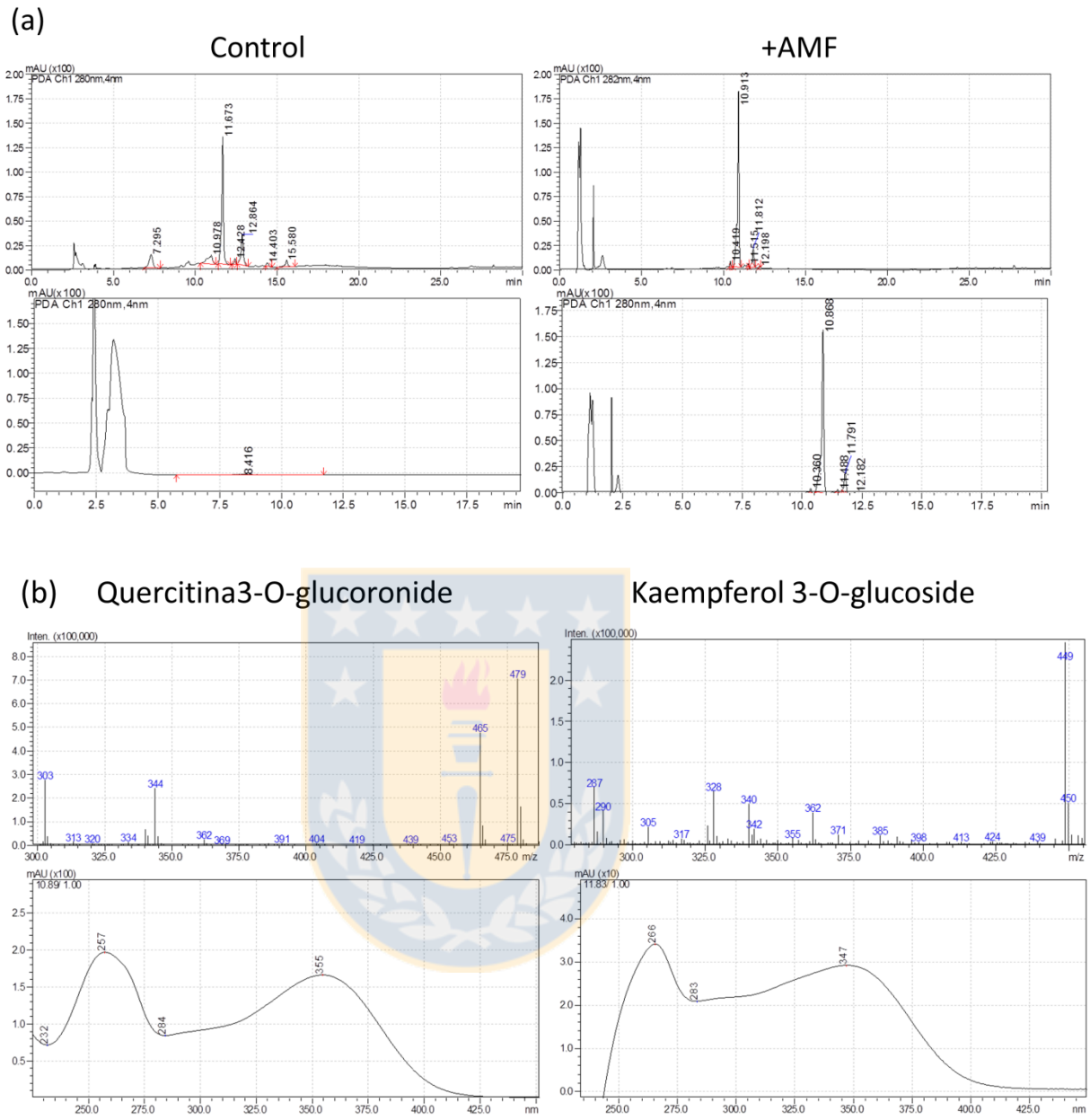

Figure 3. **LC-MS chromatographic profiles of leaf from *Vitis vinifera* L cv Cabernet Sauvignon control and inoculated with commercial *Glomus intraradices*.** (A) Chromatogram of leaf control and with AMF at 280 nm; (B) The MS spectrum and UV absorption spectra of corresponding chemical compounds are indicated at the top. Quercitin 3-O-glucoronide and Kaempferol 3-O-glucoside. Source: Self-elaboration.

Mycorrhizal root colonisation Root samples collected in the field from AMF inoculated artichoke plants showed significantly higher mycorrhizal colonization (45-55%) than uninoculated plants (15-18%) ($P < 0.05$).

4. Discussion

One of the most used techniques for separation and characterization of polyphenols and carotenoids is HPLC, a highly selective and sensitive technique ensuring the preservation of sample characteristics. HPLC separates each compound on the basis of its polarity, solubility, and size, and, when coupled with a photodiode-array detector (HPLC-DAD), allows the identification of compounds by their retention time and UV/visible spectral data, compared with standards (Tsao, 2010). Unfortunately, the lack of suitable standards, especially for some flavonoids and proanthocyanidines, limits the application of such kind of analysis. Our results are similar at works that indicate AMF symbiosis establishment induce modifications in plant metabolism and physiology as a result of multiple transcriptional changes (Fiorilli et al. 2009; Hohnjec et al. 2005). Genes that are differentially regulated by AM symbiosis were found to be involved in both primary—nitrogen, proteins, and carbohydrate pathways—and secondary metabolism of mycorrhizal tomato plants (Salvioli et al., 2012).

Physiological alterations in host plants during AM symbioses are partly due to a transient activation of host defense reactions, which involves the production of ROS in colonized roots and the consequent accumulation of antioxidant molecules.(Fig 4) Moreover, some defense-related alkaloids, including trigonelline, castanospermine, and camptothecin, are accumulated in mycorrhizal plants (Andrade et al., 2013). A reduced disease severity was found to be correlated with the enhanced phenolic content of *Vicia faba* and tomato mycorrhizal plants challenged with *Botrytis fabae* and *Phytophthora nicotianae*,respectively (Rabie, 1998; Cordier et al., 1996)

Nutritional improvement of plants by mycorrhizal symbionts plays an important role in inducing secondary metabolites accumulation. K rates were also reported to modulate total phenolic content, antioxidant capacity, rosmarinic and cichoric acids concentration in basil leaves. (Nguyen Phuong et al., 2010)

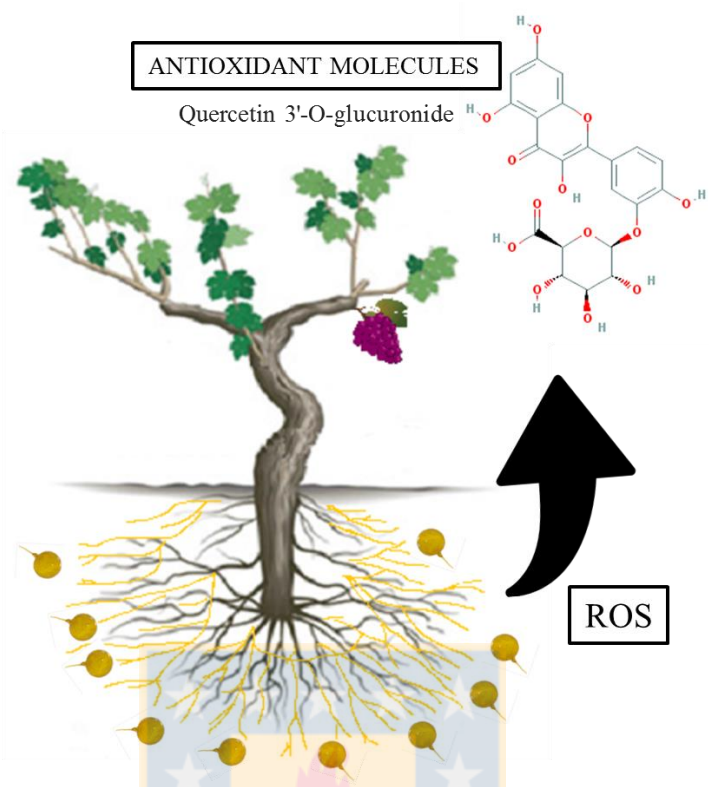

Figure 4. Model of the effect of the arbuscular mycorrhizal fungi in polyphenols of grapevine. Source: Self-elaboration.

Among plants used for human nutrition, mycorrhizal Allium cepa bulbs showed enhanced antioxidant capacity and quercetin-4-O monoglucoside concentration (Perner et al., 2008). In the greenhouse, *Vitis vinifera* plants inoculated with many different AM fungal species showed enhanced content of mineral nutrients, larger carotenoid concentration (up to 31%) and up to nine times increase in total phenolic content in leaves, with respect to non mycorrhizal plants (Krishna et al., 2005), very sit our result. Unfortunately, no information is available on quantitative or qualitative changes in nutraceutical compounds contained in grapes. Mycorrhizal lettuce showed higher concentration of anthocyanins, carotenoids, and, to a lesser extent, phenolics in the leaves, compared to nonmycorrhizal controls (Rasouli-Sadaghiani et al., 2010; Albrechtova et al., 2012). A 2-year field study reported that globe artichoke previously inoculated with AMF showed a larger flower heads production, accompanied by higher phenolic content and antioxidant activity in artichoke leaves and flower heads. (Ceccarelli et al., 2010)

Future agriculture should implement sustainable practices to reduce the environmental impact of intensive management, by using strategies involving the use and maintenance of fundamental ecosystem services provided by beneficial soil microrganisms (Sbrana et al. 2014; Aroca 2013; de Brujin 2013; Varma and Oelmuller 2007), such as AMF.

5. Conclusion

Grapevine may be regarded as a functional plant food for its high content of secondary metabolites with antioxidant properties. The quality of plant food is presently defined not only by its nutritional features, but also by its content in natural substances that prevent diseases induced by oxidative stress, such as atherosclerosis and carcinogenesis. Soil health and biological fertility play a key role in the production of safe plant foods, as a result of the action of beneficial soil microorganisms, in particular of the root symbionts arbuscular mycorrhizal fungi. They improve plant nutrition and health and induce changes in secondary metabolism leading to enhanced biosynthesis of health-promoting phytochemicals, such as polyphenols. Mycorrhizal inoculation with selected species of AMF represents a useful biotechnological application, affecting plant biosynthesis of secondary metabolites with health promoting activities. However, more information is needed on plant genetic variability concerning polyphenolic contents as affected by plant development and environmental and agronomic conditions; further research should investigate the mechanisms involved in the boost of plant secondary metabolism by AMF, by testing plant genotypes for their responsiveness to AM symbiosis in terms of phytochemicals content and by selecting AMF taxa for their activity as bioenhancers of the nutraceutical value of plant-based foods.

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7. References

Albrechtova, J., Latr, A., Nedorost, L., Pokluda, R., Posta, K., Vosatka, M. (2012). Dual inoculation with mycorrhizal and saprotrophic fungi applicable in sustainable cultivation improves the yield and nutritive value of onion. Sci. World J 2012: 1-8

Andrade, S., Malik, S., Sawaya, A., Bottcher, A., Mazzafera P. (2013) Association with arbuscular mycorrhizal fungi influences alkaloid synthesis and accumulation in *Catharanthus roseus* and *Nicotiana tabacum* plants, Acta Physiol. Plant 35:867–880

Araim, G., Saleem, A., Arnason, J., Charest, C. (2009). Root colonization by an arbuscular mycorrhizal (AM) fungus increases growth and secondary metabolism of purple coneflower, *Echinacea purpurea* (L.) Moench. J. Agric. Food Chem 57:2255-2258

Aroca, R. (Ed.), Symbiotic Endophytes. (2013) Soil Biology Series,Vol.37, Springer-Verlag, Heidelberg

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. Food Chemistry 99(1):191–203

Brundrett, M. C. (2009). Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant Soil. 320:37-77

Ceccarelli, N., Curadi, M., Martelloni, L., Sbrana, C., Picciarelli, P., Giovannetti, M. (2010). Mycorrhizal colonization impacts on phenolic content and antioxidant properties of artichoke leaves and flower heads two years after field transplant. Plant Soil 335:311–323

Chen, Y., Li, J., Guo, L., He, X., Huang, L. (2014). Application of am fungi to improve the value of medicinal plants. In mycorrhizal fungi: use in sustainable agriculture and land restoration, Solaiman, Z.; Abbott, L. K.; Varma, A., Eds. Springer-Verlag:1171-187

Claudine, M., Andrzej, M., & Augustin, S. (2005). Polyphenols and prevention of cardiovascular diseases. Current Opinion in Lipidology 16(1): 77–84

Cordier, C., Gianinazzi, S., Gianinazzi-Pearson, V. (1996). Colonisation patterns of root tissues byPhytophthora nicotianae var.parasitica related to reduced disease in mycorrhizal tomato, Plant Soil 185:223–232

Crozier, A., Jaganath, I., Clifford, M. (2009). Dietary phenolics: chemistry, bioavailability and effects on health. Nat. Prod. Rep. 26:1001-1043

de Bruijn, F. J. (Ed.), Molecular microbial ecology of the rhizosphere. (2013). Vol.1–2, Wiley-Blackwell, Hoboken, New Jersey

Fester T, Hause G (2005) Accumulation of reactive oxygen species in arbuscular mycorrhyzal roots. Mycorrhiza 15:373–379

Fiorilli, V., Catoni, M., Miozzi, L., Novero, M., Accotto, G. P., Lanfranco, L. (2009). Global and cell-type gene expression profiles in tomato plants colonized by an arbuscular mycorrhizal fungus, New Phytol. 184:975–987

Folin, C., Ciocalteu, V. Tyrosine and tryptophan determination in protein. (1927).J. Biol. Chem. 73:627-650

Gianinazzi, S., Gollotte, A., Binet, M., van Tuinen, D., Redecker, D., Wipf, D. (2010). Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza 20:519- 530

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vescicular arbuscular mycorrhizal infection in roots. New Phytol 84:489–500

Gosling, P. Hodge, A., Goodlass, G., Bending, G. (2006). Arbuscular mycorrhizal fungi and organic farming. Agric. Ecosyst. Environ. 113:17-35

Heijden, M., Scheublin, T., Brader, A. (2004). Taxonomic and functional diversity in arbuscular mycorrhizal fungi - is there any relationship? New Phytologist 164:201-204

Hohnjec, N., Vieweg, M. F., Puhler, A., Becker, A., Kuster, H. (2005). Overlaps in the transcriptional profiles of *Medicago truncatula* roots inoculated with two different *Glomus* fungi provide insights into the genetic program activated during arbuscular mycorrhiza, Plant Physiol. 137:1283–1301

Krishna, H., Singh, S., Sharma, R., Khawale, R. N., Grover, M., Patel, V. (2005). Biochemical changes in micropropagated grape (*Vitis vinifera L.*) plantlets due to arbuscular-mycorrhizal fungi (AMF) inoculation during *ex vitro* acclimatization, Sci. Hortic. 106:554–567

Lohse S, Schliemann W, Ammer C, Kopka J, Strack D, Fester T (2005) Organisation and metabolism of plastids and mitochondria in arbuscular mycorrhizal roots of *Medicago truncatula*. Plant Physiol 139:329–340

Marin M, Ybarra M, Garcia-Ferriz F, Garcia-Ferriz L (2002) Effect of arbuscular mycorrhizal fungi and pesticides on *Cynara cardunculus* growth. Agr Food Sci Finland 11:245–251

Marschner, H. (1997) The soil-root interface (rhizosphere) in relation to mineral nutrition In Mineral nutrition of higher plants, 2 Ed.; Marschner, H., Ed. Academic Press: London 537-594

Middleton, E., Kandaswami, C., & Theoharis, T. C. (2000). The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease and cancer. Pharmacological Reviews 52:673–751

Moyer, R.A.; Hummer, K.E.; Frei, B.; Wrolstad, R.E.(2002). Anthocyanins, phenolics, and Antioxidants capacity in diverse small fruits: Vaccinium, Rubus, and Ribes. J. Agric. Food Chem. 50: 519-525

Murashigue T., Skoog F.(1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497

Nguyen Phuong, M., Kwee Eileen, M., Niemeyer, E. (2010). Potassium rate alters the antioxidant capacity and phenolic concentration of basil (*Ocimum basilicum L.*) leaves. Food Chem. 123:1235–1241

Okuda, T. (1999) Antioxidants in Herbs: Polyphenols. In antioxidant food supplements in human health, Packer, L.; Hiramatsu, M.; Yoshikawa, T., Eds. Academic Press: California, USA :393-410

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat. Rev. Microbiol. 6:763-775

Perner, H., Rohn, S., Driemel, G., Batt, N., Schwarz, D., Kroh, L. W., George, E. (2008). Effect of nitrogen species supply and mycorrhizal colonization on organosulfur and phenolic compounds in onions, J. Agric. Food Chem. 56:3538–3545

Phillips, J. M.; Hayman, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55:158-161

Poiroux-Gonord, F., Bidel, L. P., Fanciullino, A. L., Gautier, H., Lauri-Lopez, F., Urban, L., J. (2010). Health benefits of vitamins and secondary metabolites of fruits and vegetables and prospects to increase their concentrations by agronomic approaches. Agric. Food Chem. 58: 12065–12082

Rabie, G. H. (1998). Induction of fungal disease resistance in *Vicia faba* by dual inoculation with *Rhizobium leguminosarum* and vesicular-arbuscular mycorrhizal fungi. Mycopathologia. 141:159–166

Rasouli-Sadaghiani, M., Hassani, A., Barin, M., Rezaee Danesh, Y., Sefidkon, F. (2010) Effects of arbuscular mycorrhizal (AM) fungi on growth, essential oil production and nutrients uptake in basil, J. Med. Plant Res. 4:2222–2228

Salvioli, A., Zouari, I., Chalet, M., Bonfante, P. (2012). The arbuscular mycorrhizal status has an impact on the transcriptome profile and amino acid composition of tomato, BMC Plant Biol. 1:12-44

Sbrana C, Avio L, Giovannetti M. (2014). Beneficial mycorrhizal symbionts affecting the production of health-promoting phytochemicals. Electrophoresis. 35(11):1535-46

Shahidi, F., and Naczk, M. (2004). Phenolics in food and nutraceuticals: Sources, applications and health effects. Boca Raton: CRC Press.

Takács, T. (2012). Site-specific optimization of arbuscular mycorrhizal fungi mediated phytoremediation. In Toxicity of heavy metals to legumes and bioremediation 1Ed.; Zaidi, A.; Ahmad, W. P.; Khan, M. S., Eds. Springer-Verlag: Berlin Heidelber :179-202

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. Nutrients 2:1231–1246

Torregrosa L., Bouquet A., Goussard P.G. (2001). *In vitro* culture and propagation of grapevine. In: Molecular biology and biotechnology of grapevine (Roubelakis-Angelakis K.A., Ed). Kluwer Academic Publ:281-326

Toussaint, J. P.; Smith, F. A.; Smith, S. E.(2007). Arbuscular mycorrhizal fungi can induce the production of phytochemicals in sweet basil irrespective of phosphorus nutrition. Mycorrhiza 17:291-297

Varma, A., Oelmuller, R. (Eds.). (2007). Advanced techniques in soil microbiology, Soil Biology Series,Vol.11,Springer-Verlag, Berlin Heidelberg.

Voets, L., Dupré de Boulois, H., Renard, L., Strullu, D.G., Declerck, S., 2005. Development of an autotrophic culture system for the in vitro mycorrhization of potato plantlets. FEMS Microbiology Letters 248: 111-118

Zeng, Y.; Guo, L. P.; Chen, B. D.; Hao, Z. P.; Wang, J. Y.; Huang, L. Q.; Yang, G.; Cui, X. M.; Yang, L.; Wu, Z. X.; Chen, M. L.; Zhang, Y. (2013) Arbuscular mycorrhizal symbiosis and active ingredients of medicinal plants: current research status and prospectives. Mycorrhiza 23:253-265

DISCUSIÓN

El desarrollo de una agricultura sustentable es una necesidad actual, debido al interés creciente de proteger al medio ambiente y consumir alimentos de alta calidad, además de mantener una alta producción debido a la creciente demanda alimentaria. El suelo representa un factor determinante en los cultivos, la perdida de fertilidad de los suelos, debido a la agricultura intensiva y al uso indiscriminado de agroquímicos, es un problema que afecta a los agricultores de todo el mundo (FAO, 2012). En el suelo, existen múltiples organismos que lo componen y que cumplen un rol determinante para mantener la fertilidad, y su composición fisicoquímica. Los hongos micorrícicos arbusculares (HMA), están presentes en los suelos formando una simbiosis con la mayoría de las plantas terrestres, incluyendo a los cultivos más importantes a nivel mundial. Forman parte de la rizósfera y otorgan múltiples servicios eco sistémicos como la mejor y mayor absorción de nutrientes, como N, P y microelementos, mayor absorción de agua, esto es debido al aumento en la zona radicular por el crecimiento del micelio del HMA alrededor de la raíz que permite alcanzar una mayor área, y por la liberación de moléculas que permiten una mayor solubilidad de los nutrientes, esto a su vez permite mantener la porosidad y fertilidad de los suelos. Los HMA además estimulan al metabolismo primario y secundario, estimulando las rutas de defensa de las plantas y la producción de antioxidantes, lo que mejora la protección ante patógenos y las respuestas de las plantas ante diversos tipos de estrés. (Smith & Smith, 2012)

En este estudio se ha recopilado la información presente en múltiples trabajos de HMA en diferentes cultivos, enfocándose principalmente en viñedos, que demuestran todos los servicios ecosistémicos de las HMA, así como también las prácticas culturales y los sistemas de producción de los cultivos (Trouvelot et al., 2015), y es totalmente aplicable a la viticultura en Chile. Estos factores pueden afectar directamente, la efectividad de estos servicios, sugiriendo desarrollar una agricultura sin una excesiva labranza, disminuyendo el consumo de agroquímicos, como fertilizantes, herbicidas, fungicidas y pesticidas, manteniendo una variabilidad de especies agrícolas en los cultivos y disminuyendo el consumo de riego del cultivo, todo en pos de aprovechar los servicios ecosistémicos de los HMA, lo que se traduce en una reducción de los costos asociados a agroquímicos y agua, protegiendo al

medioambiente, a la vez que permite desarrollar y mantener suelos fértiles, lo que a largo plazo también beneficia a los agricultores. Por otro lado también se mejoran los productos agrícolas, pues los HMA estimulan la producción de metabolitos primarios y secundarios, aumentando el crecimiento de las plantas y mejorando el perfil antioxidante, lo que se traduce en mejores características organolépticas. Por todo lo anteriormente expuesto, representan una alternativa real para desarrollar a pequeña o gran escala una agricultura sustentable.

En este estudio se trabajó con viñedos chilenos de un rango aproximado de 700 km, desde el Valle del Elqui (IV Región) a el Valle del Itata (VIII Región) con viñedos de diferentes tipos de manejo (tradicional y orgánico) y se realizó por primera vez, un catastro del estado micorricico de las mismas, encontrándose micorrizadas de manera indistinta del tipo de manejo, características de suelo o estación del año. Se observó que en los viñedos de manejo orgánico se encontró un porcentaje más alto de micorrización (Schreiner et al., 2007). Al analizar la composición de las comunidades de HMA en los suelos estudiados, se encontró una alta diversidad, que varía de acuerdo a la latitud y el tipo de manejo, existiendo mayor diversidad en el manejo orgánico. Varias de las especies encontradas, están siendo por primera vez reportadas y se cuenta con la identificación morfológica a partir de esporas y la identificación molecular, pero la descripción final de estas posibles nuevas especies requiere mayor investigación y el apoyo de especialistas taxónomos de HMA. Es muy relevante conocer la diversidad de especies de HMA en nuestro país, y como se comportan en el tiempo las comunidades, pues actualmente se ingresan, productos comerciales de HMA de especies cosmopolitas y generalistas, que pudiesen afectar las comunidades nativas y especialistas de estos hongos, que recién se comienzan a identificar y describir en Chile (Janoušková et al. 2013).

Por otro lado, también se pudo observar y comprobar que los HMA aumentan la producción de compuestos fenólicos totales, especialmente los del grupo de las quercitinas, compuestos que en la vid son de real importancia, tanto en el viñedo como en la producción de vinos, esto si bien requiere estudios complementarios, representa un beneficio potencial para la producción de ciertos metabolitos de interés para la industria farmacéutica o alimentaria, del mismo modo que han indicado otros autores (Krishna et al., 2005). Además estos resultados

refuerzan el concepto de ¨terroir¨ microbiano, que indica, que las características únicas de cierto¨terroir¨, están dadas principalmente por los microorganismos que componen el suelo, pues son ellos, lo que en definitiva estimulan una composición única de metabolitos secundarios (Gilbert et al. 2014).

Esta investigación puede ser utilizada como base tecnológica para el desarrollo de productos biotecnológicos basado en HMA para desarrollar una agricultura sustentable, como un bioestimulante o un biofertilizante, con este estudio se ha generado un conocimiento base que otorgará en el futuro próximo una directriz para la aplicación del trabajo con hongos micorrícicos arbusculares, además de poder tener acceso a algunas especies caracterizadas en este trabajo para comenzar a probar en terreno, en cultivos comerciales, un pool de especies y su efecto real en la agricultura, tanto de tipo intensiva como orgánica.

Finalmente, se sugiere que los resultados obtenidos en este estudio podrían servir de modelo para ser aplicado a otras especies de cultivo (por ejemplo: frutales y hortalizas) que, junto a los viñedos, constituyen importantes cultivos de la agricultura en Chile. Además, se espera que con esta tesis se haya estimulado el fortalecimiento de estudios en el contexto de la micología de hongos micorrícicos arbusculares, como una disciplina que indudablemente aportará hallazgos novedosos de importancia ecológica, económica y social. Entonces, podrán enfrentarse de mejor manera nuevas preguntas que han surgido al culminar la presente investigación, y referidas a los HMA mencionados con anterioridad ¿Qué otros compuestos estimulan en las diferentes plantas? ¿Cuáles son las rutas metabólicas que se estimulan a la producción de estos compuestos? ¿Qué ocurre con las micorrizas nativas al utilizarse micorrizas comerciales?¿Existen cambios estacionales en las comunidades de las micorrizas arbusculares nativas?

CONCLUSIONES

- Los hongos micorrícicos arbusculares pueden ofrecer multiples servicios ecosistémicos que entregan a los viñedos, lo que representa una gran oportunidad para la viticultura en chile.
- Las prácticas culturales de un viñedo como la fertilización, irrigación, labranza, uso de agroquímicos; afectan de distinto modo los servicios ecosistemicos que entregan los hongos micorrícicos arbusculares.
- Los viñedos chilenos se encuentran micorrizados, independiente de su tipo de manejo convencional u organico, sin embargo, el manejo organico potencia el establecimiento de micorrizas arbusculares y aumenta la diversidad de especies.
- Los viñedos chilenos presentan especies de hongos micorrícicos arbusculares principalmente, *Funneliformis verruculosum*, *Septoglomus constricus* y un desconocido *Septoglomus sp*, encontrándose también presentes las otras principales familias de los *Glomeromycota*.
- Los hongos micorrícicos arbusculares modifican el perfil de metabolitos secundarios en estudios *in vitro* y en campo, afectando principalmente, en este estudio los niveles de Quercetina.
- Este estudio representa un primer conocimiento de la diversidad de especies de hongos micorrícicos arbusculares en los viñedos de Chile, realizándose la descripción de posibles nuevas especies que podrian ser la base del primer banco de *Glomeromycota* en Chile.
- Se sugiere realizar futuros estudios en el conocimiento de la diversidad de especies de hongos micorrícicos arbusculares, tanto en otros cultivos comerciales de Chile como en bosque nativo.
- Se sugiere realizar futuros estudios en el efecto de los hongos micorrícicos arbusculares en otros metabolitos secundarios de interés.
- Este estudio tiene una aplicación importante en la agricultura, pues puede utilizarse como base tecnológica para la formulación de biofertilizantes, basado en hongos micorrícicos arbusculares nativos.

BIBLIOGRAFIA

Artero, A., Artero, A., Tarín, J., Cano, A. 2015.The impact of moderate wine consumption on health. Maturitas 80(1):3-13

Ballestrini, R., Magurno, F., Walker, C., Lumini, E. & Bianciotto, V.2010. Cohorts of arbuscular mycorrhizal fungi (AMF) in *Vitis vinifera*, a typical Mediterranean fruit crop. Environmental Microbiology Report 2:594–604

Baslam, M. & Goicoechea, N. 2012. Water deficit improved the capacity of arbuscular mycorrhizal fungi (AMF) for inducing the accumulation of antioxidant compounds in *lettuce* leaves. Mycorrhiza 22:347–359

Beck, A., Haug, I., Oberwinkler, F. & Kottke, I. 2007. Structural characterization and molecular identification of arbuscular mycorrhiza morphotypes of *Alzatea verticillata (Alzateaceae*), a prominent tree in the tropical mountain rain forest of South Ecuador. Mycorrhiza 17(7):607-25

Bonfante, P. & Requena, N. 2011. Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis. Current Opinion in Plant Biology 14:451–457

Chaves, M., Zarrouk, O., Francisco, R., Costa, M., Santos, T., Regalado, A., Rodrigues, M. & Lopes, C. 2010. Grapevine under deficit irrigation: hints from physiological and molecular data. Annals of Botany 105: 661–676

Declerck S., Strullu D.G and Fortin J.A. 2005. In vitro culture of mycorrhizas. Heidelberg, Springer-Verlag. 386 p.

Domagała-Swiątkiewicz, I. & Gąstoł, M. 2013. Effect of nitrogen fertilization on the content of trace elements in cv. Bianca grapevine (*vitis sp*.) Journal of Elementology 18(1):39

Duhamel M. & Vandenkoornhuyse P. 2013. Sustainable agriculture: possible trajectories from mutualistic symbiosis and plant neodomestication. Trends in Plant Science 18(11): 597-600

Dumbrell, A., Ashton, P., Aziz, N., Feng, G., Nelson, M., Dytham, C., Fitter, A. & Helgason, T. 2010. Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. New Phytologist 190(3):794-804

Eftekharia, M., Alizadeha, M. & Ebrahimib, P. 2012. Evaluation of the total phenolics and quercetin content of foliage in mycorrhizal grape *(Vitis vinifera L.)* varieties and effect of postharvest drying on quercetin yield. Industrial Crops and Products 38:160–165

FAO (2012) Food and Agriculture Organization of the United Nations, Land Resources. http://www.fao.org/nr/land/en/.Accessed07July2016

Fernández, K. 2012. *In Vitro* culture systems applied to AMF studies. Cultivos Tropicales 33(2):33-43

Fernández, M., Mateos, R., Garcí, M., Puertas, B. & Cantos, E. 2012. Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review. Food Chemistry 130:797–813

Gallaud, I. 1905. Etudes sur les mycorrhizes endotrophs. Revue Gen. Botanique 17: 5-48, 66-85, 123-136, 223-239, 313-325, 423-433, 479-500

Gil, G. & Pszczólkowski P. 2007. Viticultura, Fundamentos para optimizar la producción y calidad. Santiago, Chile, Ed. Universidad Católica de Chile, 535 p.

Gilbert, JA., van der Lelie, D., Zarraonaindia, I. 2014. Microbial terroir for wine grapes. Proc Natl Acad Sci U S A 111:5–6

Haas, B., D. Gevers, A. Earl, M. Feldgarden, D. Ward, G. Giannokous, D. Ciulla, D. Tabbaa, S. Highlander, E. Sodergren, B. Methe, T. Desantis, J. Petrosino, R. Knight & B. Birren. 2011. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Research 21: 494-504

Janoušková M, Krak K, Wagg C, Štorchová H, Caklová P, Vosátka M. 2013. Effects of inoculum additions in the presence of a preestablished arbuscular mycorrhizal fungal community. Appl Environ Microb 79:6507–6515

Jurkiewicz, A., Ryszka, P., Anielska, T., Waligórski, P., Białońska, D., Góralska, K., Michael ,M. & Turnau, K. 2010. Optimization of culture conditions of *Arnica montana* L.: effects of mycorrhizal fungi and competing plants. Mycorrhiza 20:293–306

Jung, S., Martínez-Medina, A., Lopez-Raez, J. & Pozo, M. 2012. Mycorrhiza-induced resistance and priming of plant defenses. Journal of Chemical Ecology 38(6):651-64

Krishna, H., Singh, S., Sharma, R., Khawale, R. 2005. Biochemical changes in micropropagated grape (*Vitis vinifera* L.) plantlets due to arbuscular-mycorrhizal fungi (AMF) inoculation during *ex vitro* acclimatization. Sci Hortic-Amsterdam 106:554–567

Lee, E., Ju-Kyeong, E., Kang-Hyeon, K. & Ahn-Heum, E. 2013. Diversity of arbuscular mycorrhizal fungi and their roles in ecosystems. Mycobiology 41(3): 121-125

Leake, J., Johnson, D., Donnelly, D., Muckle, G., Boddy, L. & Read, D. 2004. Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. Canadian Journal of Botany 82:1016–1045

Likar, M., Hančević, K., Radić, T. & Regvar, M. 2013. Distribution and diversity of arbuscular mycorrhizal fungi in grapevines from production vineyards along the eastern Adriatic coast. Mycorrhiza 23(3):209-19

Lindahl, B., Nilsson, R., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., Kõljalg, U., Pennanen, T., Rosendahl, S., Stenlid, J. & Kauserud, H. 2013. Fungal community analysis by high-throughput sequencing of amplified markers a user's guide. New Phytology 199(1):288-99

Lorrain, B., Ky, I., Pechamat, L. & Teissedre, P. 2013. Evolution of analysis of polyhenols from grapes, wines, and extracts. Molecules 18(1):1076-100

Lumini, E., Orgiazzi, A., Borriello, R., Bonfante, P. & Bianciotto, V. 2010. Disclosing arbuscular mycorrhizal fungal biodiversity in soil through a land-use gradient using a pyrosequencing approach. Environmental Microbiology 12(8):2165-79

Myles, S. 2013. Improving fruit and wine: what does genomics have to offer? Trends Genetics 29(4):190-6

Nasim, Ghazala. 2012. Arbuscular mycorrhizae for sustainable agriculture. Crop production for agricultural improvement. Springer Science. Chapter 23.

Oehl F, Sieverding E, Palenzuela J, Ineichen K, Silva GA. 2011. Advances in *Glomeromycota* taxonomy and classification. IMA Fungus 2:191–199

Oldroyd, G. 2013. Speak, friend, and enter: signaling systems that promote beneficial symbiotic associations in plants. 2013. Nature Reviews Microbiology 11(4):252-63

Öpik M, Vanatoa A, Vanatoa E, Moora M, Davison J, Kalwij JM et al. 2010. The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (*Glomeromycota*). New Phytol 188: 223–241

Öpik, M., Zobel, M., Cantero, J., Davison, J., Facelli, J., Hiiesalu, I., Jairus, T., Kalwij, J., Koorem, K., Leal, M., Liira, J., Metsis, M., Neshataeva, V., Paal, J., Phosri, C., Põlme, S., Reier, Ü., Saks, Ü., Schimann, H., Thiéry, O., Vasar, M. & Moora, M. 2013. Global sampling of plant roots expands the described molecular diversity of arbuscular mycorrhizal fungi. Mycorrhiza 23(5):411-30

Orgiazzi, A., Bianciotto, V., Bonfante, P., Daghino, S., Ghignone, S., Lazzari, A., Lumini, E., Mello, A., Napoli, C., Perotto, S., Vizzini, A., Bagella, S., Murat, C. & Girlanda, M. 2013. 454 pyrosequencing analysis of fungal assemblages from geographically distant, disparate soils reveals spatial patterning and a core mycobiome. Diversity 5(1):73-98

Redecker, D. 2000. *Glomalean* fungi from the ordovician. Science 289: 1920–1921

Romero, P., Gil-Muñoz, R., Del Amor, M., Valdés, F., Fernández, E., & Martinez-Cutillas, M. 2013. Regulated deficit irrigation based upon optimum water status improves phenolic composition in *Monastrell* grapes and wines. Agricultural Water Management 121(C):85-101

Ruggiero, A., Vitalini, S., Burlini, N., Bernasconi, S. & Iriti, M. 2013. Phytosterols in grapes and wine, and effects of agrochemicals on their levels, Food Chemistry 141(4):3473-9

SAG. 2014. Servicio Agrícola y Ganadero. Subdepartamento de Viñas y Vinos. Informe Ejecutivo Producción de Vinos.

Sbrana, C., Avio, L., Giovannetti, M. 2014. Beneficial mycorrhizal symbionts affecting the production of health-promoting phytochemicals. Electrophoresis. 35(11):1535-46

Schnoor, T., Lekberg, Y., Rosendahl, S. & Olsson, P. 2011. Mechanical soil disturbance as a determinant of arbuscular mycorrhizal fungal communities in semi-natural grassland. Mycorrhiza 21(3):211-20

Schreiner, R., Tarara J. & Smithyman, R. 2007. Deficit irrigation promotes arbuscular colonization of fine roots by mycorrhizal fungi in grapevines (*Vitis vinifera* L.) in an arid climate. Mycorrhiza 17:551–562

Schreiner, R. & Mihara, K. 2009. The diversity of arbuscular mycorrhizal fungi amplified from grapevine roots (*Vitis vinifera L.)* in Oregon vineyards is seasonally stable and influenced by soil and vine age. Mycologia 101(5):599-611

Shi, P., Abbott, L., Banning, N. & Zhao, B. 2012. Comparison of morphological and molecular genetic quantification of relative abundance of arbuscular mycorrhizal fungi within roots. Mycorrhiza 22(7):501-13

Smith, S., Jakobsen, I., Grønlund, M. & Smith, A. 2011. Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. Plant Physiology 156:1050–1057

Smith SE, Read DJ. 2008. Mycorrhizal Symbiosis, Third Edition, 3rd edn. Academic Press

Smith, S. & Smith, F. 2011. Roles of arbuscular mycorrhizas in plant nutrition and growth:new paradigms from cellular to ecosystem scales. Annuals Reviews Plant Biology 62:227-50

Smith, S. & Smith, F. 2012. Fresh perspectives on the roles of arbuscular mycorrhizal fungi in plant nutrition and growth. Mycologia 104(1):1-13

Toussaint, J. 2007. Investigating physiological changes in the aerial parts of AM plants: what do we know and where should we be heading. Mycorrhiza 17(4):349-53

Trouvelot, S., Bonneau, L., Redecker, D., van Tuinen, D., Adrian, M., Wipf, D. 2015. Arbuscular mycorrhiza symbiosis in viticulture: A review. Agron. Sustain. Dev. 35: 0

Unterseher, M., Jumpponen, A., Opik, M., Tedersoo, L., Moora, M., Dormann, C. & Schnittler, M. 2011. Species abundance distributions and richness estimations in fungal metagenomics--lessons learned from community ecology. Molecular Ecology 20(2):275–285

Worchel, E., Giauque, H. & Kivlin, S. 2013. Fungal symbionts alter plant drought response. Microbiology Ecology 65(3):671-8

Xia, E., Deng, G., Guo, Y. & Li, H. 2010. Biological activities of polyphenols from grapes. International Journals of Molecular Sciences 11(2):622-46

Yu, L., Nicolaisen, M., Larsen, J. & Ravnskov, S. 2012. Molecular characterization of root-associated fungal communities in relation to health status of *Pisum sativum* using barcoded pyrosequencing. Plant Soil 357:395–405

Yu, J. & Ahmedna, M. 2013. Functional components of grape pomace: their composition, biological properties and potential applications. International Journal of Food Science & Technology 48: 221–237

Zeng, Y., Guo, L., Chen, B., Hao, Z., Wang, J., Huang, L., Yang, G., Cui, X., Yang, L., Wu, Z., Chen, M. & Zhang, Y. 2013. Arbuscular mycorrhizal symbiosis and active ingredients of medicinal plants: current research status and prospectives. Mycorrhiza 23(4):253-65