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VINO

GRAPEVINE ROOT-ASSOCIATED
ACTINOBACTERIA AS NOVEL BIOCONTROL AGENTS
AGAINST FUNGI INVOLVED IN TRUNK DISEASES

Actinobacterias asociadas al sistema radicular de vid
como novedosos agentes de biocontrol frente a hongos
implicados en enfermidades de madera

Memoria presentada por la Licenciada en Biología

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A mi familia

*“Research is to see what everybody else has
seen, and to think what nobody else has thought”*

*“Investigar es ver lo que todo el mundo ha visto,
y pensar lo que nadie más ha pensado”*

Albert Szent-Györgyi

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SUMMARY **(RESUMEN)**

SUMMARY

Grapevines are frequently affected by the so-called 'trunk diseases'. They are one of the most worrisome problems to face by the wine sector causing significant economic losses. Grapevine trunk diseases (GTD) are produced by different fungi, affecting to both established and young vineyards. Within established vineyards, four diseases can be highlighted: Esca complex, Eutypa dieback, Botryosphaeria dieback and Phomopsis dieback. In young vineyards, GTD are responsible for young grapevine decline (YGD), a syndrome which has drastically increased worldwide since the early 1990s. The main fungal trunk diseases associated with YGD are black foot (associated to fungal species of *Cylindrocarpon*, *Campylocarpon*, *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Theonectria* genera) and Petri disease (whose main pathogens associated are *Phaeomoniella chlamydospora* and *Phaeoacremonium* species). The incidence of GTD has increased over the last decades. This increase is attributable to the lack of full effective strategies to fight these pathologies, and to changes in the production methods with an intensive mechanisation that led to a very high plant density in the vineyards.

For many years, the most common strategy to limit the growth of fungal pathogens has been the use of chemical pesticides. However, due to their toxicity and health risks, their use has been restricted or even banned, while emerging natural products as a possible tool to control fungal crop infections. Another eco-friendly alternative to ensure the crop quality, without environmental risks, is the replacement of chemical compounds by beneficial microorganisms inhabiting the plant. Actinobacteria, and particularly the *Streptomyces* genus, are widely distributed in soils representing a high proportion of the microbial community of the rhizosphere, but also colonise the inner plant tissues being found in nearly every plant worldwide. Moreover, they represent a major source of biological active compounds, making them very attractive candidates, in the field of agriculture, as biocontrol agents (BCAs).

This study is based on the actinobacteria isolation from both the rhizosphere and the root system of young grapevines and their application as novel BCAs to control GTD infections. Thus, 152 actinobacteria (58 endophytic and 94 rhizosphere strains) were isolated, mainly belonged to the *Streptomyces* genus. Eight of them were selected according to a bioassay against phytopathogenic fungi, and applied in field trials to analyse their effect on controlling the infection of fungal pathogens involved in YGD. In general, all of them showed a clear reduction in the rate of pathogens isolated, compared to untreated plants. However, the most promising strains according to field assays during three consecutive years (the endophytic *Streptomyces* sp. VV/E1 and the rhizosphere strain *Streptomyces* sp. VV/R4), were selected to analyse different colonization methods and to design molecular markers to their fast detection and/or quantification. Two different colonization methods were tested: direct plant immersion into an actinobacterial suspension and direct injection of a bacterial suspension into the rootstock. Actinobacteria

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detection in plant material was analysed by qPCR technique, using SCAR molecular markers, designed from poorly conserved regions of the genome for both strains. This technique also allowed to analyse the movement of the endophytic actinobacteria through the grapevine plant, observing that it could move through the rootstock and colonise tissues, reaching the second petiole without being detected on the leaves. Although there are no significant differences between both inoculation methods in terms of bacterial colonisation, immersion would be the preferred option ensuring a proper implementation with no additional steps in the industrial process of grafted vines production in nurseries. The application of these BCAs is a promising tool to reduce the incidence of YGD in new plantations around the world.

The genomes of VV/E1 and VV/R4 strains were sequenced. An *in silico* analysis led to the identification of several clusters involved in the synthesis of secondary metabolites that could be related to the biocontrol effect showed by these strains under field conditions. Genetic manipulation based on CRISPR, together with the analysis of extracellular enzymes and other secondary metabolites detected by HPLC-MS techniques in *Streptomyces* sp. VV/E1 strain allowed us to elucidate whether or not these compounds were involved in biocontrol activity. Thus, bioactive compounds described as antifungal agents, like antimycin, candicidin or maculosin, were not related to the antifungal effect showed by *Streptomyces* sp. VV/E1 under field conditions against fungal trunk pathogens. In a similar way, the production of chitinase extracellular enzymes, were also found not to be responsible for the biocontrol activity observed.

In summary, the results obtained in this Thesis project showed that actinobacteria inhabiting the rhizosphere and the plant inner tissues of grapevines are interesting sources of biocontrol agents to reduce the incidence of grapevine trunk pathogens and, therefore, to improve the plants quality in nurseries and vineyards worldwide. Moreover, the protocol to introduce the BCA into the plant is reliable to perform in the industrial production process in grapevine nurseries and even directly in vineyards. However, the bioactive compounds that are involved in the biocontrol process remains unidentified, so that additional research is necessary to shed light into this issue.

RESUMEN

Uno de los problemas más serios a los que se enfrenta el sector vitivinícola en todo el mundo, y que implica importantes pérdidas económicas, son las llamadas “enfermedades de madera de la vid”. Estas enfermedades son causadas por diferentes hongos y afectan tanto a viñedos establecidos como a viñedos jóvenes. Dentro de los viñedos establecidos se pueden destacar cuatro enfermedades: Yesca, Eutipiosis, decaimiento por *Botryosphaeria* y excoriosis de la vid causada por *Phomopsis viticola*. En viñedo joven, las enfermedades de madera son responsables del síndrome conocido como *decaimiento prematuro de la vid*, el cual se ha incrementado de forma drástica en todo el mundo desde principios de la década de 1990. Las principales enfermedades fúngicas asociadas al decaimiento prematuro de la vid son “Pie negro” (principalmente asociada hongos pertenecientes a los géneros *Cylindrocarpon*, *Campylocarpon*, *Dactylonectria*, *Ilyonectria*, *Neonectria* y *Theonectria*) y “la enfermedad de Petri” (cuyos principales patógenos asociados son *Phaeomoniella chlamydospora* y diferentes especies del género *Phaeoacremonium*). El aumento de la incidencia de las enfermedades de madera a lo largo de la última década se atribuye a la pérdida de estrategias efectivas de control y a los cambios en los métodos de producción, donde la mecanización ha provocado que la densidad de los viñedos haya aumentado considerablemente.

Durante muchos años, la estrategia empleada para limitar el crecimiento de hongos patógenos fue el uso de pesticidas de síntesis. Sin embargo, debido a su toxicidad, unido al riesgo para la salud, han visto limitado su uso, al mismo tiempo que han emergido y emergen productos naturales como posibles herramientas para controlar las infecciones fúngicas en campos de cultivo. Alternativamente, en línea con la estrategia anterior, existe la posibilidad de sustituir los compuestos químicos por microorganismos beneficiosos que habitan de forma natural en la planta y su entorno. Las actinobacterias, y en concreto el género *Streptomyces*, se encuentran ampliamente distribuidos no solo en suelo, donde representan una proporción elevada de los microorganismos presentes en la rizosfera, sino también colonizando los órganos internos de casi todas las plantas. Además, constituyen la mayor fuente de compuestos biológicos activos, lo que los convierte en unos interesantes candidatos como agentes de biocontrol (BCA, “*biocontrol agents*”) en agricultura.

En la presente Tesis Doctoral se ha realizado un aislamiento y caracterización de actinobacterias, tanto de la rizosfera como del sistema radicular de plantas jóvenes de vid, para su posterior aplicación, como alternativa novedosa, prometedora y respetuosa medioambientalmente para combatir las infecciones causadas por hongos patógenos de vid. Se aislaron un total de 152 actinobacterias (58 endofíticas y 94 rizosféricas), la mayoría pertenecientes al género *Streptomyces*. De todas ellas, ocho cepas fueron seleccionadas de acuerdo a ensayos *in vitro* realizados frente a hongos fitopatógenos, y posteriormente aplicados en ensayos de campo con el fin último de analizar su efecto en

el control de hongos patógenos causantes del decaimiento prematuro de la vid. En general, las cepas en estudio mostraron una clara disminución en la tasa de aislamiento de patógenos en comparación con las plantas no tratadas. Sin embargo, las cepas más prometedoras, de acuerdo a los resultados obtenidos de los ensayos en campo realizados durante tres anualidades consecutivas (la cepa endofítica *Streptomyces* sp. VV/E1 y la rizosférica *Streptomyces* sp. VV/R4), fueron seleccionadas para analizar en profundidad los métodos de colonización mediante el desarrollo de marcadores moleculares para su detección y/o cuantificación. Se estudiaron dos métodos de colonización diferentes: inmersión del sistema radicular en una suspensión de actinobacterias y la inyección directa de una suspensión de actinobacterias en el interior del portainjertos.

La detección de estas actinobacterias en el material vegetal se analizó mediante qPCR, desarrollando marcadores moleculares de tipo SCAR, diseñados a partir de regiones poco conservadas del genoma de ambas cepas. Ésta técnica también permitió el estudio del movimiento de la actinobacteria endofítica a lo largo de la planta, observando su capacidad de moverse a lo largo del portainjertos y alcanzar el segundo peciolo, sin llegar a detectarse en hoja. Aunque los dos métodos fueron efectivos para la colonización de la actinobacteria, la inoculación mediante inmersión es la modalidad más adecuada, ya que asegura una correcta implantación de la actinobacteria sin añadir pasos adicionales en el proceso industrial de obtención de injertos de vid en los viveros. Por tanto, la aplicación de estas actinobacterias como agentes de biocontrol se presenta como una estrategia muy prometedora para reducir el decaimiento de plantas jóvenes de vid en las nuevas plantaciones a lo largo de todo el mundo.

Los genomas de las cepas VV/E1 y VV/R4 fueron secuenciados. Su análisis *in silico* condujo a la identificación de varias agrupaciones génicas implicadas en la síntesis de metabolitos secundarios que podrían estar implicados en el efecto de biocontrol mostrado por estas cepas en campo. La manipulación genética basada en CRISPR, junto con el análisis de enzimas extracelulares y otros metabolitos secundarios detectados por HPLC-MS en la cepa *Streptomyces* sp. VV/E1 fueron los métodos empleados para dilucidar si estos compuestos estaban involucrados o no en la actividad de biocontrol. Así, compuestos descritos en bibliografía como compuestos antifúngicos, como la antimicina, la candicina o la maculosina, no pudieron ser relacionados con el efecto de biocontrol provocado por esta cepa en los ensayos en campo. De un modo similar, la producción de enzimas extracelulares del tipo de las quitinasas, tampoco podría ser considerada como responsable de la actividad de biocontrol.

A modo de resumen, los resultados obtenidos en la presente Tesis Doctoral indicaron que las actinobacterias que colonizan la rizosfera y el aparato radicular de las plantas de vid son una fuente importante de agentes de biocontrol para reducir la incidencia de patógenos de vid, y por tanto, capaces de mejorar la calidad de las plantas en vivero y viñedo en todo el mundo. Además, el protocolo de incorporación del agente de biocontrol

en las planta es operativamente posible en el proceso de producción industrial en vivero de planta de vid, así como en viñedo. Sin embargo, los principales compuestos bioactivos implicados en el proceso de biocontrol no han podido ser elucidados, y es necesario, por tanto, realizar investigaciones adicionales en esta dirección.

INTRODUCTION

1. A short overview of the wine sector in Spain

The genus *Vitis L.* (grapevines), with more than 100 species currently described, is the most widely planted crop worldwide (Gramaje *et al.*, 2018). Areas under vines in vineyards in the main production countries according to the International Organisation of Vine and Wine (OIV) are shown in Table 1.1.

Table 1.1. Total areas (thousands of hectares) under vines in vineyards in the main production countries (OIV, 2018).

Country	2014	2015	2016 ^a	2017 ^b
Spain	975	974	975	967
China	813	847	864	870
France	789	785	786	787
Italy	690	682	690	695
Turkey	502	497	468	448
United States	450	446	441	441
Portugal	224	204	195	194
Argentina	228	225	224	223
Chile	213	214	209	209
Romania	192	191	191	191
Australia	154	147	145	145
South Africa	132	130	129	125
Greece	110	107	105	106
Germany	102	103	102	102
Brazil	87	86	86	86
Hungary	62	68	68	68
Russia	63	87	85	85
Bulgaria	63	64	64	64
Austria	45	45	46	46
New Zealand	38	39	39	40
Switzerland	15	15	15	15

^a: Provisional data

^b: Forecasted data

The latest available data obtained from the OIV shows a trend to the stabilisation of the areas under vines in most of the countries. It is worth mentioning China, where there has been a rapid expansion of vineyards in the last four years. Moreover, it should be noted that Spain is the country with the largest area of vineyards.

This genus has a high commercial value for the production of fresh table grapes, dried fruit, wine production and nursery plants (commercial grafted plants) (Gramaje *et al.*, 2018). For instance, in terms of wine production, Spain is the third country behind Italy and France with a forecast production of 32.1 million of hectolitres (MhL) in 2017, while consumption is around 10.3 MhL. In terms of exportation, Spain remained the biggest exporter with 22.1 MhL and a global market share of 20.5%. Wine exportation (excluding juice and musts) in 2017 represented a profit of 2,814 million of euros (OIV, 2018).

However, grapevine cultivation generates substantial production costs, not only due to the high financial investment for the vineyard establishment, but also for the disease management programmes that include cultural practices and the cost of chemical and/or biological control products and their application (Gramaje *et al.*, 2018).

2. Brief introduction to grapevine trunk diseases

Grapevines, as well as other perennial crops, are affected by so-called 'trunk diseases', which cause damage in the trunk and other woody tissues (Morales-Cruz *et al.*, 2017). The term '**grapevine trunk diseases**' (GTD), established by Dr. Luigi Chiarappa along with other scientists in the late 1990s, refers to several diseases caused by pathogenic fungi, which are responsible for the deterioration of the perennial organs in grapevines (Hofstetter *et al.*, 2012). These complex disease occurs whenever grapes are grown and consists of the primary biotic cause of decline, with the subsequent mortality in both young and adult vineyards (Úrbez-Torres *et al.*, 2015). Besides, no grapevine taxa, either cultivated or wild, are known to be resistant to trunk diseases (Bertsch *et al.*, 2013).

There are several reasons that contribute to the **great complexity of GTD**. The following reasons can be cited as the most relevant:

- i. Up until some decades ago, trunk diseases usually mainly damaged old plants. Nevertheless, these diseases have been recently observed to be in rapid expansion and they also affect young plants (2-3 years old) (Fontaine *et al.*, 2016).
- ii. *V. vinifera* is known to host the widest pathogen variety among all wood agricultural plants (Gramaje *et al.*, 2018). Grapevine is susceptible to 29 fungal diseases (Wilcox *et al.*, 2016), including GTD, which are the most destructive. So far, up to 133 fungal species, which belong to 34 genera, have been associated with GTD around the world (Gramaje *et al.*, 2018).
- iii. Many of the fungi involved in GTD can infect plants, even at the same time, due to the possibility that different pathogens infect individual vines throughout a season and over the years (Morales-Cruz *et al.*, 2017; Gramaje *et al.*, 2018). Several taxonomically unrelated groups of Ascomycete fungi cause trunk diseases in grapevines (Rolshausen *et al.*, 2010), forming a complex and dynamic microbial community. This community may include many pathogenic species and other microorganisms, with negative, neutral or beneficial interactions, while colonising the same plant organ (Morales-Cruz *et al.*, 2017). Under field conditions, the interaction between microbes, or between them and plants, contribute to the development of the disease and can make it severe (Lamichhane and Venturi, 2015). As a result of the co-existence of different grapevine trunk pathogen species forming this complex

and dynamic community, the study of the molecular mechanisms underlying disease development, especially under vineyard conditions is a hard issue (Morales-Cruz *et al.*, 2017).

- iv. The expression of decline symptoms is erratic, being deeply influenced by environmental conditions and cultural practices (Oliveira *et al.*, 2004).
- v. Pathogenic fungi come into the plant through different routes, but mainly through pruning wounds (Cobos *et al.*, 2015) and/or the root system (Álvarez-Pérez *et al.*, 2017). Annual pruning wounds are the primary point of entry, providing many infections each growing season during the life of a vineyard (Gramaje *et al.*, 2018).
- vi. Global studies have reported that nurseries constitute an important source of infections, since fungal pathogens infect the plant at different stages of the nursery propagation process (Agustí-Brisach *et al.*, 2011; Weckert, 2012). Diminish, or even remove, the infection rate of fungal pathogens in nurseries is an important task to improve grapevine health.
- vii. The pathogenicity factors involved in GTD are poorly known. When colonizing the wood, some trunk pathogens present the ability to enzymatically digest the plant cell walls. Others are able to produce toxins to overcome the host's inducible defences (Morales-Cruz *et al.*, 2015). Toxic metabolites produced belong to different classes of natural compounds. For example, grapevine trunk pathogens *Phaeoacremonium minimum*, *Phaeomoniella chlamydospora* and Botryosphaeriaceae species produced naphthalenone pentaketides and polyphenols. All of this bioactive compounds are toxic to vines. These phytotoxic compounds have been, at least in part, chemically characterised and test for their toxicity on grapevines. However, their mode of action remains to be fully elucidated (Andolfi *et al.*, 2011).
- viii. Finally, but not least, once the fungi are established inside the plant and some damage is evident, there is no any curative treatment.

2.1. Main pathologies associated to GTD

GTD is a very complex group of pathologies, although it is traditionally considered that there are five major grapevine trunk diseases, all of them caused by different fungi.

- i. *Diseases affecting to established vineyards.* Within this group, up to four different diseases can be distinguished:

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Fig. 1 Esca complex in grapevine. Apoplectic phase of esca (Source: USDA-Agricultural Research Service, Crops Pathology and Genetics Research Unit, Davis, CA) (A) Foliar symptoms of Esca, starting as chlorosis and becoming to necrosis (Morales-Cruz *et al.*, 2017). (B). Cross-section showing a central white rot surrounded by black spots and sectorial necrosis of an esca infected vine (Gramaje *et al.*, 2018) (C).

Fig. 2 Eutypa dieback in grapevine. Foliar symptoms including stunted shoots (A). Wood cross-section showing a wedge of discoloured tissue characteristic of eutypiosis (Source: www.wineaustralia.com) (B).

a. **Esca complex** is also known as “*Grapevine Leaf Stripe and Apoplexy*”, which are two of the main symptoms of this pathology, as well as the discoloration of decaying wood tissues (Gramaje *et al.*, 2018) (Fig. 1). After infection, disease symptoms do not appear immediately, but usually take a few years, when biochemical, physiological, structural and morphological changes in different plant organs happen (Rusjan *et al.*, 2017). It is mainly associated with different species of the ascomycetes *Phaeoacremonium* sp. and *Phaeomoniella chlamydospora*, together with the basidiomycete *Fomitiporia mediterranea* (Bertsch *et al.*, 2013; Yacoub *et al.*, 2016). Other pathogens like *Stereum hirsutum* might also be involved although its role is less clear (Yacoub *et al.*, 2016).

b. **Eutypa dieback** is mainly produced by several ascomycetes belonging to the Diatrypaceae family. *Eutypa lata* is recognised as the main responsible pathogen (Bertsch *et al.*, 2013). Principal symptoms are shrivelling of shoots (fan leaf), which present chlorotic, wrinkled and ripped leaves with marginal necrosis, and can become widespread over the whole limb. Sometimes, “*eutypiosis*” can produce dried out inflorescences, and also the death of the shoot (dead arm). In the trunk, a brown and hard sectorial necrosis with dark stripes or scratches are usually detected (Fontaine *et al.*, 2016) (Fig. 2).

c. **Botryosphaeria dieback** is produced by species of the Botryosphaeriaceae family. For many years, Botryosphaeriaceae species have been mostly considered as saprophytes or secondary colonisers in grapevines. However, nowadays they are considered as one of the most widespread and diverse fungal group that contribute to trunk diseases (Baskarathevan *et al.*, 2012). Species of this family have been frequently

Fig 3 Botryosphaeria dieback. Dead arm affected by Botryosphaeriaceae species, together with the lack of spring wood and the dead of buds (Úrbez-Torres *et al.*, 2009) (A). Wedge-shaped wood necrosis in cross section (Arzanlou *et al.*, 2012) (B).

isolated from young declining vineyards in different areas worldwide (Andolfi *et al.*, 2011; Gramaje and Armengol, 2011). Up to now, 26 taxa belonging to the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, *Neoscytalidium*, *Phaeobotryosphaeria* and *Spencermartinsia* have been associated with Botryosphaeria dieback in grapevines, being species of *Lasiodiplodia* and *Neofusicoccum* the fastest wood-colonizing fungi and, consequently, the most virulent (Gramaje *et al.*, 2018). Symptoms expression caused by these fungi differ

from one region to another and between different grapevine cultivars (Gramaje and Armengol, 2011). Despite this, plants infected by this disease often show a lack of spring wood growth together with the ongoing death of buds and xylem necrosis. The main wood symptom of this disease is the presence of wedge-shaped perennial cankers (Úrbez-Torres *et al.*, 2009, Arzanlou *et al.*, 2012; Gramaje *et al.*, 2018), often indistinguishable to that of eutypiosis. Nevertheless, they can be distinguished in the field by the lack of foliar symptomatology (Gramaje *et al.*, 2018) (Fig. 3).

Fig. 4 Phomopsis dieback. Characteristic leaf symptoms (Source: SCRI Trunk Disease Project) (A). Small, round and dark spots symptoms in berries known as black measles (Gramaje *et al.*, 2018) (B). Phomopsis symptoms on green shoots and unripe berries (Barba *et al.*, 2018) (C).

d. **Phomopsis dieback.** This pathology, produced by *Phomopsis viticola* (Niekerk *et al.*, 2006, Barba *et al.*, 2018), is more severe in regions with a humid climate through the growing season (Úrbez-Torres *et al.*, 2013). Symptoms of this disease are present in all herbaceous organs (shoots, basal wood, leaves, stems or fruits). On the young shoots, the disease results in the first internodes with the presence of small black spots, that later develop into well-individualized blackish-brown crusts or brown lesions with strips of corky appearance like "chocolate". In branches, it could appear as a strangulation at their base, which can lead to breakage, especially with windy conditions. Blackish necrotic spots may also be encountered along the main and secondary veins, as well as the petioles. Some leaf portions can also turn yellow, pale green and/or brown colour. Severely infected leaves or leaves with heavily infected petioles may fall. On the other hand, fruits turn brown and wither, with shrivelled berries close to harvest (Fontaine *et al.*, 2016) (Fig. 4).

- ii. *Diseases affecting to young vineyards.* In young vineyards, GTD are responsible for a syndrome known as **young grapevine decline (YGD)** (Úrbez-Torres *et al.*, 2015) that is reviewed bellow.

2.2. Importance of GTD and their impact in young vineyards

GTD have been known since the end of the 19th century, but their significant and impact on vineyards has been recognised relatively recently. **GTD constitute a major threat for the wine sector**, causing devastating epidemics and important economic losses to the wine industry (Andolfi *et al.*, 2011; Cobos *et al.*, 2015; Álvarez-Pérez *et al.*, 2017; Gramaje *et al.*, 2018). **The overall economic impact of GTD was estimated to be in excess of € 1.132 billion in 2012 (US\$ 1.502 billion)** attributable to the replacement of only 1% of death plants every year (Hofstetter *et al.*, 2012). Nevertheless, **this impact looks like to be underestimated**. According to other studies, the major threat to the economic sustainability for the future of the sector are GTD and, especially, the decline and mortality of young vines (Úrbez-Torres *et al.*, 2015).

Several factors such as the grapevine planting 'boom' around the world during the 1990s, or the gradually elimination of chemical compounds have influenced the GTD incidence worldwide. Moreover, the radical change in the production methods with the mechanisation era, have promoted fungal infections (Gramaje *et al.*, 2018).

As a consequence, **decline symptoms in young vineyards have drastically increased around the world since the early 1990s**, when the vine industry entered a period of rapid expansion (Gramaje and Armengol, 2011; Álvarez-Pérez *et al.*, 2017). YGD affects vines in many young vineyards around the world with infection rates in 2015 of around 5 to 15%, causing significant economic losses (Úrbez-Torres *et al.*, 2015). Furthermore, over 50% infection rates have been reported to take place in France, Australia, Spain and, more recently, in Canada (Úrbez-Torres *et al.*, 2015).

YGD symptoms are first recognised in the field at all stages of growth (Andolfi *et al.*, 2011; Úrbez-Torres *et al.*, 2015) owing to the diminish in vineyard longevity and productivity (Rolshausen *et al.*, 2010). They include the death of the spurs, arms and cordons, the eventual death of the vines due to a progressive wood necrosis and decay of plant tissue, lack of spring growth and short shoot internodes (Andolfi *et al.*, 2011; Úrbez-Torres *et al.*, 2015). Moreover, most of the GTD also produce leaf symptoms including

Fig. 5 Schematic representation of the parts of a grapevine plant

chlorosis, necrosis, deformation and stunting (Andolfi *et al.*, 2011) (Fig. 5).

Evaluation of YGD has shown that many factors are involved in the poor performance of vines. Three major groups of factors have been identified (Gramaje and Armengol, 2011):

- i. One group consists of nursery induced stresses, including structural vine defects, extended cold storage, limited vine carbohydrates and pot-bound root system.
- ii. Other group is related to the vineyard establishment and management stresses that encompasses inadequate ground preparation, incorrect planting, inappropriate irrigation and nutritional deficiencies or excesses.
- iii. Finally, the last group contains biological stresses where we find nematodes, root-colonizing fungal pathogens, viruses, rootstock/scion incompatibilities, or fungal trunk pathogens.

Taking into account the different causes of this syndrome, fungal trunk pathogens are currently considered some of the most destructive (Gramaje and Armengol, 2011; Gramaje *et al.*, 2018).

2.3. Main phytopathogenic fungi involved in young grapevine decline

The main fungal trunk diseases associated with YGD are black foot and Petri disease.

2.3.1. Black-foot

Black-foot is an important disease in most wine and grape-producing regions all over the world, affecting specially to nurseries and young vineyards (Agustí-Brisach and Armengol, 2013). It was firstly reported to be caused by *Cylindrocarpon* and *Campylocarpon* species (Gramaje and Armengol, 2011), but nowadays it is known to be also associated with fungal species of *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Theonectria* genera (Fontaine *et al.*, 2016; Carlucci *et al.*, 2017; Gramaje *et al.*, 2018).

This disease can be recognised by black, sunken, necrotic lesion on roots, and reddish brown discoloration in the base of wood tissue that develops from the base of the rootstock, causing death of young vines (Fig. 6).

Fig. 6 Black foot disease in grapevines. Decline symptoms associated with this disease, including absence of budding and weak vegetation (Halleen *et al.*, 2006) (A). Black discoloration of wood tissues with necrotic lesions characteristic of black-foot disease (Gramaje and Armengol, 2011) (B).

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Fungal pathogens causing black-foot are able to infect the plant through either pruning wounds or the root system (Gramaje and Armengol, 2011; Bertsch *et al.*, 2013; Álvarez-Pérez *et al.*, 2017).

2.3.2. Petri disease

Petri disease, formerly named black goo decline, is found worldwide and, its incidence and severity have increased considerably during the last decade in Europe. Petri disease primarily affects young grapevines, **causing establishment problems** (Edwards and Pascoe, 2004).

This disease can be recognised by the presence of dark-coloured phenolic compounds in xylem vessels of the trunks, which exude out of the vessels when cut in cross sections and dark streaks in longitudinal sections (Gramaje *et al.*, 2018) (Fig. 7).

The ascomycetous fungi *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (formerly named as *P. aleophilum*) are known to be the main pathogens associated to Petri disease. However, up to other 24 species of *Phaeoacremonium* and several *Cadophora* species have also been reported to be involved in these disease (Úrbez-Torres *et al.*, 2014). Among *Cadophora* species, *C. luteo-olivacea* is the most relevant (Gramaje *et al.*, 2018). Nevertheless, the role of *Cadophora* in the decline of grapevine has been questioned since it has been isolated from both asymptomatic and symptomatic grapevine wood, in nursery and field plants showing black streaking of xylem vessels (Fontaine *et al.*, 2016).

Fig. 7 Petri disease in grapevines. Poor vigor grapevines showing chlorotic leaves affected by this disease (Gramaje *et al.*, 2018) (A). Dark coloured phenolic compounds in xylem vessels (Gramaje and Armengol, 2011) (B) and dark streaks in longitudinal sections characteristic of grapevine plants infected with Petri disease (Úrbez-Torres *et al.*, 2014) (C).

Until recently, black foot and Petri disease were considered as two separate GTD responsible for YGD, but nowadays it is well accepted that they constitute a disease complex in which several species can often coexist in the same symptomatic plant (Gramaje and Armengol, 2011; Úrbez-Torres *et al.*, 2015).

3. Methods for controlling young grapevine decline

The incidence of GTD has increased over the last decades, mainly as a result of the lack of effective strategies to fight back against these diseases (Álvarez-Pérez *et al.*, 2017), as well as for the drastic changes developed in the production methods, such as mechanical pruning, that produces a significantly higher number of pruning wounds, or the transformation from traditional low-density head-trained vines to more high density vineyards (Gramaje *et al.*, 2018).

The adoption of preventive practices starting in young and healthy vineyards is crucial to achieve adequate yields and, therefore, positive returns in the long term (Hillis *et al.*, 2017). Trend to eradication or prohibition of chemical products made that remedial surgery became as the only management strategy for the grape growing industries to combat GTD. However, the economic cost for this strategy can be very high and not always effective. As a consequence, **in recent years, the main priority**, not only for the vine industry, but also for researchers, **has been the discovery and study of novel active compounds, as well as cultural practices aimed to controlling or diminishing infection rates caused by GTD pathogens** (Álvarez-Pérez *et al.*, 2017; Gramaje *et al.*, 2018). Management of GTD is a complex task and it varies depending on both, the disease and/or pathogen, and the geographical region. This is one of the reasons why, nowadays, their complete eradication is not possible. Therefore, control programmes are essential for the proactive management of trunk diseases, and consequently, to reduce infections by fungal pathogens in nurseries (Andolfi *et al.*, 2011; Halleen and Fourie, 2016; Gramaje *et al.*, 2018). Several strategies, which include chemical, biological and physical treatments are being investigated with special emphasis on environmentally safe (eco-friendly) alternatives (Halleen and Fourie, 2016).

3.1. Chemical pesticides

For many years, the common strategy used for limiting fungal growth were contact fungicides (Halleen and Fourie, 2016). Several chemical products have been tested with different results, depending on the trunk pathogen among other factors. Some examples are detailed next:

- The effectiveness of several chemical compounds such as bemoyl, carbendazim, didecylmethylammonium chloride or chlorine dioxide to reduce fungal graft infections in nurseries were tested by Halleen and Fourie (2016). The obtained results showed that none of the treatments consistently affected the number of certifiable vines produced, although some of them reduced the incidence of some fungal pathogens. Thus, bemoyl, together with flusilazole are the most effective chemical compounds controlling *Eutypa lata* infections

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(Sosnowski *et al.*, 2013) and reducing natural *P. chlamydospora* infections of pruning wounds (Halleen *et al.*, 2010).

- Pruning wound protection by treatments based on carbendazim and bemoyl had also been used to control both eutypiosis and Botryosphaeria dieback (Mondello *et al.*, 2018).
- Thiophanate-methyl had been used for the treatment of Petri disease and several Botryosphaeriaceae species (Rolshausen *et al.*, 2010). Díaz and Latorre (2013) supported the use of two benzimidazole fungicides (bemoyl and thiophanate-methyl), with a similar mode of action, for the control of the devastating trunk diseases in all grape productions worldwide.
- Sodium arsenite was registered as an effective compound to control Esca and Botryosphaeriaceae dieback (Úrbez-Torres, 2011). In fact, in some European countries, such as France, Portugal and Spain, the sole product available to treat Esca symptoms was, for many decades, sodium arsenite (Mondello *et al.*, 2018).
- Methyl bromide was used as fungicide due to its strong fungicidal activity and also a weak effect against nematodes (Martin, 2003; Cabrera *et al.*, 2012).
- Benomyl, tebuconazole and mixed fungicides of carbendazim + flusilazole and cyprodinil + fludioxonil showed an improvement in plant growth and reduction of disease incidence in plants affected by *Cylindrocarpon* species (Rego *et al.*, 2006).
- Other fungicides such as captan, copper oxychloride, hydroxyquinoline sulphate or prochloraz also showed an effect in the decrease of root disease severity values in *Cylindrocarpon liriodendri* and *Dactylonectria macrodidyma* (Alaniz *et al.*, 2011).

As it has been told before, the industrial production of grafted grapevine plants in nurseries has been identified as a critical point where many infections can occur (Gramaje *et al.*, 2018). The application of chemical strategies to control fungal trunk pathogens in nurseries is difficult. On the one hand, traditional techniques such as chemical sprays and dips, used for the surface control of pathogens, do not penetrate dormant grapevines cuttings to control pathogens inhabiting the phloem and xylem tissues (Gramaje and Armengol, 2011). On the other hand, depending on the plant status and fungi that produce plant infection, chemical fungicides are effective or not. For instance, soaking propagation material before cold storage or grafting in benomyl, carbendazim, didecylmethylammonium chloride or captan reduce significantly *Ph. chlamydospora* and *Phaeoacremonium* infection levels in basal ends and graft unions of nursery plants, but they are not effective against black-foot pathogens (Gramaje and Armengol, 2011).

Moreover, many chemical fungicides present drawbacks in terms of toxicity and efficacy, and are responsible for environmental impact and healthy risks associated with their use (Cobos *et al.*, 2015). Owing to these public concerns, the most effective chemical compounds against GTD caused by pathogenic fungi, such as benzimidazole fungicide and methyl bromide were gradually eliminated in the early 2000s (Gramaje *et al.*, 2018), while bemoyl and carbendazim are also no longer available in most countries, especially in Europe (Halleen and Fourie, 2016; Mondello *et al.*, 2018). In the case of sodium arsenite, it was definitively banned in Europe in 2003 due to its high risks for humans and the environment (Mondello *et al.*, 2018).

As a result of this restriction in the use of chemical fungicides, alternative products, less aggressive for the environment, have been studied in recent years.

3.2. Natural antifungal compounds and related strategies

As fast as synthetic fungicides were reduced in agriculture, natural products have emerged as a possible tool for controlling fungal crop infections (Cobos *et al.*, 2015).

In this sense, chitosan is considered a fungicidal compound against several fungal species involved in grapevine decline, such as *Botryosphaeria* sp., *Phomopsis* sp., *Eutypa lata*, *C. liriodendri*, *Ph. chlamydospora* and *Fomitiporia* sp. Apart from its antifungal effect, this compound also improves plant growth and decreases disease incidence. Moreover, this polymer is non-toxic and biodegradable (Nascimento *et al.*, 2007).

Other natural products like garlic extract and lactoferrin (a globular protein firstly isolated from milk) reduced significantly colonization of wounds by *E. lata* and ascospore germination although they were required in high concentration (Sosnowski *et al.*, 2013).

Cobos *et al.* (2015) evaluated the efficacy of different natural antifungals which inhibit the *in vitro* growth of several fungi causing grapevine trunk diseases. Some natural compounds tested were chitosan, vanillin and garlic extract, which exhibited a high rate of protection against pruning wound infection, and reduction of mortality in field trials (Mateos *et al.*, 2013; Cobos *et al.*, 2015; Mateos *et al.*, 2015).

Some studies also showed that phenolic compounds in grapevines, such as *p*-coumaric acid, catechin, caffeic acid and tannins and other phenolic compounds present a reduction in Petri disease symptoms at the same time that promote plant growth, since these compounds inhibited enzyme activities involved in lignin degradation after fungal infection (Río *et al.*, 2004; Rusjan *et al.*, 2017).

Finally, the use of heat-water treatment has also been reported as a promising method to control black-foot and Petri disease pathogens in grapevine propagating material. Its application in dormant nursery vines has been recommended to control above mentioned pathogens (Rego *et al.*, 2009; Halleen and Fourie, 2016). However it only has a short-

term effect and could be involved in grapevine failure due to damage caused to sensitive grapevine cultivars and rootstocks (Rego *et al.*, 2009).

3.3. Biocontrol agents

As a result of the large array of pathogens causing diseases in crops, considerable amounts of pesticides have been used, and are still in use, in agriculture with the subsequent negative impact on the environment and health. A way to avoid this negative impact while ensuring product quality consists of the replacement of this chemical compounds by beneficial microorganisms inhabiting the plant rhizosphere and/or endosphere. Actually, biocontrol bacteria strains and their secondary metabolites can reduce pathogen diseases, either directly or indirectly, affecting pathogen mode of action by antibiosis, competition for niche and nutrients, interfering with pathogen signalling or stimulating host plant defence. Thus, the development of biocontrol strains has become an important tool for the future control of trunk disease in viticulture (Compant *et al.*, 2013). As a consequence, different biological control agents (BCAs) have been reported in the last years.

Regarding fungal biocontrol agents, several strains of *Trichoderma* have been tested. In this way, *T. harzianum* has been reported to have protective effects on natural infection by *Cylindrocarpon* species, *Phaeomoniella* species and *Ph. chlamydospora*, since they reduce the incidence of these fungi in roots of nursery grapevines (Fourie *et al.*, 2001). This fungi together with *T. longibrachiatum* have also been demonstrated to protect grapevine pruning wounds against *Ph. chlamydospora* and therefore, they could contribute to control Esca (Marco *et al.*, 2004). *T. atroviridae* and *T. harzianum* have also being showed to be responsible for the reduction in the artificial infection of several pathogen species that cause grapevine decline, such as, *Phomopsis viticola*, *E. lata*, *Ph. chlamydospora* and different species of Botryosphaeriaceae (Kotze *et al.*, 2011). *In vitro* tests have revealed that the supprehesive effect of *Trichoderma* sp. includes mycoparasitism, antibiosis by volatile and non-volatile compounds and competition for nutrient and niche (Mutawila *et al.*, 2011).

Antagonistic substances against the growth of grapevine pathogenic fungi have also found in bacteria. In particular, *in vitro* assays have reported that metabolites produced by *Bacillus subtilis* inhibit the growth of fungi involved in trunk diseases such as *Ph. chlamydospora*, *P. minimum* or *Lasioidiplodia theobromae* (Alfonzo *et al.*, 2009). Other bacteria, such as *Enterobacter* sp., *Paenibacillus* sp., *P. illinoisensis*, *Bacillus* sp., *B. pumilus* and *Brevibacillus reuszeri* have also been reported to control *Ph. chlamydospora* (Haidar *et al.*, 2016).

Likewise, **actinomycetes play an important role in terms of biocontrol in the field of agriculture** (Khucharoenphaisan *et al.*, 2016) according to their protective effect against plant pathogenic fungi (Couillerot *et al.*, 2014). *Streptomyces anulatus* has been reported to present the ability of colonizing *in vitro* grapevine plants, promote grapevine growth and induce natural defences against phytopathogenic fungi (Couillerot *et al.*, 2013, 2014). Loqman *et al.* (2009) have isolated *Actinobacteria* from grapevine plants which are effective in the control of different fungal pathogens not involved in GTD. Recently, our research group have isolated endophytic and rhizosphere *Actinobacteria*, mainly *Streptomyces* species from the root system of *V. vinifera* plants, which have shown a biocontrol effect against fungi that cause GTD, and therefore, represent a new and promising tool for controlling fungal trunk pathogens (Álvarez-Pérez *et al.*, 2017).

3.4. Genetic and genomic manipulation

Genotyping by sequencing is a relatively novel approach based on next generation sequencing technologies that could be very suitable for population studies of GTD pathogens. Having genomes of grapevine fungal pathogens completely sequenced improves the ability to locate, identify, compare, isolate and manipulate genes related to the mechanism of pathogenesis and virulence in the pathogens, and of resistance in their host plants (Gramaje *et al.*, 2018). This knowledge is important for the development of targeted disease management strategies (Grünwald and Goss, 2011), and also for the improvement of disease-resistant cultivars (Gramaje *et al.*, 2018). Currently some genomes of the GTD pathogens have been sequenced: *Botryosphaeria dothidea* (Joint Genomic Institute [JGI], <http://1000.fungalgenomes.org>), *Dactylonectria macrodidyma* (Malapi-Wight *et al.*, 2015), *Diplodia seriata* (Morales-Cruz *et al.*, 2015; Robert-Siegwald *et al.*, 2017), *Eutypa lata* (Blanco-Ulate *et al.*, 2013), *Neofusicoccum parvum* (Blanco-Ulate *et al.*, 2013a), *P. minimum* (Blanco-Ulate *et al.*, 2013b) or *Ph. chlamydospora* (Antonielli *et al.*, 2014; Morales-Cruz *et al.*, 2015).

4. Actinobacteria: a short introduction to the genus *Streptomyces*

The phylum *Actinobacteria* is one of the largest taxonomic units among the major lineages currently recognised within the *Bacteria* domain (Barka *et al.*, 2016). This phylum share some characteristic features among which are the following:

- Actinobacteria are Gram-positive bacteria with a high content in GC in their genome. In fact, the GC content in some genera like *Streptomyces* and *Frankia* is over 70% (Barka *et al.*, 2016)

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Fig. 8 Schematic overview of the life cycle of *Actinobacteria* (Barka *et al.* 2016).

Although it is undoubted that they are bacteria, they have been traditionally considered as a transitional form between fungi and bacteria due to their characteristic life cycle (Fig. 8). Alike to filamentous fungi, they produce a mycelium which reproduce by sporulation. In contrast, like all bacteria, their cells have a chromosome organized in a prokaryotic

nucleoid and a peptidoglycan cell wall. Moreover, they are susceptible to antibacterial agents (Barka *et al.*, 2016).

- Most of them are free-living organisms widely distributed, inhabiting in both aquatic and terrestrial ecosystems. Despite they have adapted to live in a wide range of ecological environments, they are more numerous in soil, where they play an important role within the microbial population. Soil population are dominated by the genus *Streptomyces*, accounting for over 95 % of the Actinomycetales strains isolated from soil (Barka *et al.*, 2016).

Domain: *Bacteria*

Phylum: *Actinobacteria*

Class: *Actinobacteria*

Order: *Actinomycetales*

Family: *Streptomycetaceae*

Genus: *Streptomyces*

Fig 9. Scientific classification of the genus *Streptomyces*.

Actinobacteria and especially *Streptomyces* (Fig. 9) have a large secondary metabolism, being responsible for the production of about two-thirds of all naturally derived antibiotics, as well as many anticancer, anthelmintic and

antifungal compounds (Barka *et al.*, 2016; Chater, 2016). The diversity of secondary metabolite production is due in part to their genomic structure. They have abundant transposable elements near the ends of the linear chromosome allowing for widespread movement and exchange of “accessory” genes (Kinkel *et al.*, 2012). Recent genomic analysis have revealed that any of this strains has the potential to make some of these secondary metabolites. Furthermore, metagenomic studies have described a wide number of biosynthetic gene clusters involved in such production (Chater, 2016). Consequently **these bacteria are of major importance for biotechnology, medicine and agriculture**. They are also important in soil ecology due to their ability to hydrolyse a vast

range of polysaccharides and other natural macromolecules (Barka *et al.*, 2016).

4.1. Importance of *Actinobacteria* in soil microbiota and their relationship with plants

The interaction between plant and bacteria has been studied for many decades. Nevertheless, the mechanisms developed by bacteria to interact with plants remains unknown (Santoyo *et al.*, 2016).

***Actinobacteria*, and particularly the *Streptomyces* genus, are extensively distributed in soils and represent a high proportion of the microbial community of the rhizosphere** (Loqman *et al.*, 2009; Barka *et al.*, 2016). The rhizosphere constitutes an interesting area in which interactions occur between plants and microorganisms. The specific structure and diversity of these bacterial communities varies between plant species and root zones (Himaman *et al.*, 2016). They have the ability to colonise the rhizosphere as a result of their antagonistic and competitive characteristics concerning other soil microorganisms. They can affect the plant growth either directly (supplying the plant a beneficial compound or nutrient, such as providing fixed nitrogen, phytohormones, ion chelators or soluble phosphate) or indirectly (by preventing the plant against the negative effect produce by plant pathogens, which includes biocontrol or antagonism toward soil pathogens) (Sadeghi *et al.*, 2012; Passari *et al.*, 2015; Qin *et al.*, 2015).

Despite *Actinobacteria* are known as soil and rhizosphere bacteria, several reports indicate that they **also ubiquitously colonise the internal tissues of plants, being found in nearly every plant worldwide, as both endophytic and mycorrhizal associated strains** (Li *et al.*, 2012; Santoyo *et al.*, 2016; Álvarez-Pérez *et al.*, 2017). In fact, endophytic *Actinobacteria* have been isolated from a high variety of plants, being the most frequent genera *Microbispora*, *Nocardia*, *Micromonospora* and *Streptomyces*. They promote plant growth by producing auxins that favour root growth and development (Barka *et al.*, 2016). They have also been shown to protect plants against different soil-borne plant pathogens (Qin *et al.*, 2011; Kinkel *et al.*, 2012; Cao *et al.*, 2016; Álvarez-Pérez *et al.*, 2017). **Streptomycetes can also be frequently associated to mycorrhiza** (Schrey *et al.*, 2012). In fact, mycorrhiza formation is promoted by so-called mycorrhization helper bacteria, including several actinomycete species (Schrey and Tarkka, 2008).

The interaction between plant and actinobacteria appears to be specific, even in a crop or cultivar (Loqman *et al.*, 2009). In the search for new natural resources for crop protection, streptomycetes are gaining interest (Kunova *et al.*, 2016). Several strains of the *Actinobacteria* phylum have been described as both, biocontrol and stimulating agents,

to use in agriculture without harmful effects to the environment, as a result of secondary metabolites produced with antimicrobial activity (Schrey *et al.*, 2012; Kunova *et al.*, 2016).

4.2. Actinobacteria as promising biocontrol agents for fungal pathogens

As previously mentioned, Actinomycetes act as agents that control soil-borne plant pathogens. Therefore, they play an important role on agriculture because of their immense potential to reduce the rate of agro-chemical usage for sustaining crop yields (Dinesh *et al.*, 2017), as well as in plant biotechnology due to their antagonistic activity against plant pathogens (Ranjani *et al.*, 2016). The effect of these microorganisms against an extensive variety of phytopathogenic fungi has been reported (Schrey *et al.*, 2012; Khucharoenphaisan *et al.*, 2016). Consequently, biologists are becoming interested in using actinomycetes as agents to influence plant growth and for biological control of soil-borne root diseases of crop plants (Loqman *et al.*, 2009). For instance, plant growth promoting *Actinobacteria* and their metabolites can be used for controlling efficiently pests and pathogens in many crops such as grain legumes (Mingma *et al.*, 2014; Sathya *et al.*, 2017), wheat, rice, potato, carrots, tomato (Qin *et al.*, 2015), rhododendron (Kunoh, 2002), banana (Cao *et al.*, 2005), lettuce (El-Tarabily *et al.*, 2000), paddy fields and orchards (Prapagdee *et al.*, 2008), and also in grapevines (Couillerot *et al.*, 2014; Andreolli *et al.*, 2016; Álvarez-Pérez *et al.*, 2017).

In summary, the worldwide efforts in the search of natural products for crop protection have found within *Actinobacteria*, and especially in *Streptomyces* genus, good candidates to control plant diseases (Ranjani *et al.*, 2016).

4.3. Actinobacteria as secondary metabolite producers

Actinobacteria, in particular the genus *Streptomyces*, represent one of the most prolific source of bioactive natural products with a wide range of biological activities (Donadio *et al.*, 2007; Couillerot *et al.*, 2013; Palazzotto and Weber, 2018). In fact, **streptomyces have been the major source of clinical antibiotic, being responsible for over the 80% of all antibiotics of microbial origin** (Barka *et al.*, 2016).

Many of these secondary metabolites are produced by two types of multi-domain enzymes: polyketide synthases (PKS) (Fig. 10A), and non-ribosomal peptide synthetases (NRPS) (Fig. 10B) (Donadio *et al.*, 2007). PKS synthesise polyketides, which are composed of simple carboxylic acid monomers assembled. There are three different types of PKSs depending on their architecture: type I (modular) and types II and III (iterative). NRPS synthesises peptide-derived compounds (Robertsen, 2017).

***Streptomyces* potential to produce a wide range of biological active compounds** including antibiotics and hydrolytic enzymes, together with their highly resistance against

desiccation and stresses, **make them very attractive candidates as biocontrol agents** (Verma *et al.*, 2011). The plant possesses its own mechanisms of resistance, but soil microorganisms could contribute to this fight either by excreting substances which limit the growth of pathogenic fungi or stimulating the natural defences of the plant (Loqman *et al.*, 2009). Among the compounds excreted by *Actinobacteria*, can be highlighted degrading-cell wall enzymes such as chitinases, the production of auxins like indole-3 acetic acid (IAA) or the production of siderophores (Loqman *et al.*, 2009; Tamreihao *et al.*, 2016).

Fig. 10 Schematic representation of the biosynthetic cycles of PKS and NRPS, representing both initiation and extension modules. Biosynthetic cycle of minimal PKS initiation and chain extension modules. The Acyltransferase (AT) domains initiate chain building by selection of an appropriate acyl-CoA (i) and transfer to the Acyl carrier protein (ACP) of the same module (ii). The ketosynthase (KS) of the first extension module self-acylates with the starter unit (iii), which it then condenses with the extender unit attached to the ACP within its own module. Following chain extension, the β -keto group can be reductively modified by ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains (A). Biosynthetic cycle of minimal NRPS initiation and chain extension modules. Biosynthesis begins with activation of a specific amino acid building block as its aminoacyl adenylate by an adenylation (A) domain (i) and transfer to a peptidyl carrier protein (PCP) through a thiolation reaction resulting in an amino-acid-PCP-thioester (ii). Peptide bond formation and further chain elongation are catalysed by the condensation (C) domain using the two aminoacyl-PCPs as substrates (B). In both cases, the sequence of reactions is repeated until off-loading of the full-length polypeptide by the terminal thioesterase (TE) domain. Chemical modification by tailoring domains can occur either before or following chain extension (Weissman, 2015).

4.3.1. Chitinase activity

Chitin, a β -(1-4)-linked polymer, is one of the most abundant and important source of nutrients and energy in nature (Taechowisan *et al.*, 2004). Some *Actinobacteria* possesses chitinases that carry out the decomposition of chitin in soil. This soil enzyme plays a critical role in maintaining soil ecology, fertility and health (Jog *et al.*, 2012). Furthermore, chitin is part of the fungi cell wall and therefore, chitinases from *Actinobacteria* can be involved in the fungal cell degradation (Taechowisan *et al.*, 2004), and hence in the control of many fungal pathogens.

4.3.2. Indole-3-acetic acid (IAA) production

It has been reported that those *Streptomyces* which produce IAA promote plant growth (Sadeghi *et al.*, 2012; Passari *et al.*, 2015). In *Streptomyces*, IAA biosynthesis occurs by indoleacetamide pathway, wherein tryptophan present in the medium act as a precursor (Jog *et al.*, 2012). This pathway take place in a two-step reaction (Fig. 11) (Duca *et al.*, 2014). Even, it has been reported that this auxin acts as a common regulator agent for both sporulation and production of secondary metabolites in actinomycetes (Jog *et al.*, 2012).

Fig. 11 Indole-3-acetic acid pathway. The production of this compound occurs in a two-step reaction from a tryptophan (Trp) precursor. The first reaction is catalysed by the tryptophan 2- monooxygenase enzyme. This enzyme converts tryptophan to the indole-3 acetamide (IAM) intermediate. The second reaction is catalysed by an IAM-specific hydrolase/amidase. This enzyme hydrolases the IAM to indole-3 acetic acid (IAA) [Adapted from Duca *et al.* (2014)].

4.3.3. Siderophores production

Some studies also reveal that those *Streptomyces* which produce siderophores also promote plant growth (Verma *et al.*, 2011; Sadeghi *et al.*, 2012), and improve nutrient uptake (Passari *et al.*, 2015). Siderophores consist of low molecular weight iron chelaters produced by microorganisms in order to transport iron into cells via active transport mechanisms. Among the different types of siderophores, *Streptomyces* species are known to produce siderophores that may have an inhibition effect against plant pathogens. This could be due to the fact that the *Streptomyces* strains inhibit the growth of the phytopathogen by limiting iron in the rhizosphere, and therefore, reducing the fungi growth and survival (Verma *et al.*, 2011; Jog *et al.*, 2012). This molecules can also be used by plants as an iron source (Jog *et al.*, 2012). The chemical structure of some siderophores with biocontrol activity is shown in Fig. 12.

Fig. 12 Chemical structure of some siderophores with biocontrol activity. [Adapted from Johnstone and Nolan (2015)].

5. Biocontrol agents. State of the art

For several years it has been known that there are microorganisms with biological activity, useful to control plant diseases (Lehman *et al.*, 2000). As example, some of the products patented for this purpose are listed below.

- A group of binocluate *Rhizoctonia* have been patented due to their ability to protect different kind of plants, such as tomatoes, wheat, rape, alfalfa, sugar, beet, carrot, garlic, onions, grass, or pine trees against pathogenic fungi like *Fusarium* or *Penicillium* (Rubio *et al.*, 2000).
- *Streptomyces* sp. NRRL No. B-30145 and antibiotics produced by this bacterium have been patented for their antifungal activity against some specific plant pathogens (*Alternaria*, *Botrytis*, *Phytophthora*, *Rhizoctonia* and *Sclerotinia*) (Lehman *et al.*, 2000).
- A combination of *Trichoderma harzianum* T22 and *Trichoderma virens* G41 has been commercialised due to its ability to control diseases in corn plants caused by *Phytophthora*, *Pythium*, *Fusarium*, *Rhizoctonia*, *Sclerotium* and/or *Thielaviopsis* species (Martin and Hayes, 2010).
- Another strain of the genus *Trichoderma*, *T. atroviridae*, has also been commercialised to inhibit the growth of the phytopathogen fungi *Verticillium dahliae* (Barroso-Albarracín *et al.*, 2014).
- *Cladosporium cladosporioides* H39 (CBS 122244) and *C. cladosporioides* R406 (CBS 122243) have been commercialised for their ability to control the fungal plant pathogen *Venturia inaequalis* on leaves and fruits, particularly in fruit crops such as apple (Jurgen, 2015).
- *Streptomyces* sp. VV/E1, *Streptomyces* sp. VV/R1 and *Streptomyces* sp. VV/R4, used in this study, have also been patented as a product to combat fungal trunk pathogens responsible for the grapevine decline (Álvarez-Pérez *et al.*, 2016a, Álvarez-Pérez *et al.*, 2016b). These strains have been selected according to their ability to inhibit *in vitro* growth of several pathogen fungi responsible for grapevine decline in young vineyard.

It is currently unknown whether any of the above products have been placed on the market (licensed). Its use is protected under this form of legal protection.

In terms of phytosanitary products to control GTD, at the beginning of 2018 had been registered the first product, known as “BLINDAR”, based on two different *Trichoderma* strains (*T. asperellum* ICC 012 and *T. gamsii* ICC 080) as a fungicide to use in agriculture after grapevine pruning (<http://www.isagro.es/fichas-seg/blindar.pdf>).

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OBJECTIVES

(OBJETIVOS)

OBJECTIVES

The **main goal** of this work **faces the problem of the decline or early death of young grapevine plants**. In order to shed light into this problem, endophytic and rhizosphere *Actinobacteria* strains associated to the root system of young grapevine plants with the potential to behave as biocontrol agents (BCAs) were isolated, selected and characterised. These BCAs should be able to decrease the incidence infection rate through the root system of pathogenic fungi involved in young grapevine decline.

The **specific objectives** established to reach this purpose were:

1. Isolation, identification and selection of actinobacteria with biocontrol activity against grapevine pathogenic fungi. Analysis of their effectiveness on field assays.
2. Development of molecular tools for the evaluation of different inoculation methods. Detection of biocontrol strains into grapevine plants.
3. *In silico* analysis of the genomes of the most promising strains to identify putative clusters encoding antifungals.
4. Analysis of the role of enzymes, putative antifungals and other compounds on the biocontrol activity.

OBJETIVOS

El **principal objetivo** de este trabajo **trata de solucionar el problema asociado al declive o muerte prematura de plantas en viñedo joven**. Para ello, se abordó el aislamiento, selección y caracterización de cepas de actinobacterias endofíticas y rizosféricas, asociadas al sistema radicular de plantas jóvenes de vid con un potencial comportamiento como agentes de biocontrol. Para ser consideradas como agentes de biocontrol deben ser capaces de disminuir la tasa de incidencia de infecciones a través del sistema radicular, causadas por hongos patógenos.

Para la consecución de este objetivo principal, se propusieron los siguientes **objetivos específicos**:

1. Aislamiento, selección y caracterización de actinobacterias con potencial efecto como agentes de biocontrol frente a los principales hongos patógenos de vid. Comprobación de la eficacia de las cepas más prometedoras en ensayos en campo.
2. Desarrollo de herramientas moleculares para analizar diferentes métodos de inoculación. Detección de las cepas seleccionadas con efecto de biocontrol en plantas de vid.
3. Análisis *in silico* de los genomas de las cepas más prometedoras para detectar agrupaciones génicas implicadas en la biosíntesis de antifúngicos.
4. Análisis del papel desarrollado por enzimas, posibles antifúngicos y otros compuestos en el efecto de biocontrol observado.

CHAPTER I: ISOLATION, IDENTIFICATION AND SELECTION OF ACTINOBACTERIA WITH BIOCONTROL ACTIVITY AGAINST GRAPEVINE PATHOGENIC FUNGI

Chapter corresponding to the publication: Álvarez-Pérez J.M., **González-García S.**, Cobos R., Olego M.A., Ibáñez A., Díez-Galán A., Garzón-Jimeno E., Coque J.J.R., 2017. Use of endophytic and rhizosphere actinobacteria from grapevine plants to reduce nursery fungal graft infections that lead to young grapevine decline. *Appl. Environ. Microbiol.* 83, e01564-17. <https://doi.org/10.1128/AEM.01564-17>

I.1. Abstract

Grapevine trunk diseases are a major threat to the wine and grape industry worldwide. They cause a significant reduction in yields as well as in grape quality, and they can even cause plant death. **Trunk diseases are caused by fungal pathogens that enter through pruning wounds and/or the root system.** Although different strategies have recently been developed to protect pruning wounds using antifungal compounds (natural or synthetic) or biocontrol agents, no tools are yet available for controlling **soil pathogens** that infect plants through their root system.

Endophytic and rhizosphere actinobacteria isolated from the root system of 1-year-old grafted *Vitis vinifera* plants were evaluated for their activities against fungi that cause grapevine trunk diseases. A total of 58 endophytic and 94 rhizosphere isolates were tested. According to an *in vitro* bioassay, 15.5% of the endophytic isolates and 30.8% of the rhizosphere isolates exhibited antifungal activity against the fungal pathogen *Diplodia seriata*, whereas 13.8% of the endophytic isolates and 16.0% of the rhizosphere isolates showed antifungal activity against *Dactylonectria macrodidyma* (formerly *Ilyonectria macrodidyma*). Those strains which showed the greatest *in vitro* efficacy against both pathogens were further analysed for their ability to inhibit the growth of *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (formerly *Phaeoacremonium aleophilum*). Based on their antifungal activity, three rhizosphere and three endophytic isolates were applied on grafts in an open-root field nursery in a 3-year trial. The field trial led to the selection of one endophytic strain (*Streptomyces* sp. VV/E1) and two rhizosphere isolates (*Streptomyces* sp. VV/R1 and *Streptomyces* sp. VV/R4) which significantly reduced the infection rates produced by the fungal pathogens *Dactylonectria* sp., *Ilyonectria* sp., *Ph. chlamydospora*, and *P. minimum*, all of which cause young grapevine decline (YGD). The VV/R1 and VV/R4 isolates also significantly reduced the mortality level of grafted plants in the nursery.

This study shows that different actinobacterial isolates, when applied to grafts in a nursery, can significantly reduce the infection rate caused by fungal pathogens that enter through the root system. This is a new, promising, and eco-friendly alternative for controlling fungal trunk pathogens that infect grapevine plants through the root system and preventing the decline of young grapevines in nurseries and vineyards.

I.2. Introduction

Grapevines are one of the most important economic crops worldwide (Luque *et al.*, 2014; Torregrosa *et al.*, 2015). **Grapevine trunk diseases (GTD) are a major threat for the wine sector, being responsible for significant economic losses to the wine industry** (Gramaje and Armengol, 2011). GTD include different fungal diseases, being the most relevant Botryosphaeria dieback, Esca, Eutypa dieback, Petri disease, and black-foot (Gramaje and Armengol, 2011; Bertsch *et al.*, 2013). **The incidence of GTD has increased over the last decades, primarily due to the lack of effective strategies for fighting these diseases** (Chiarappa, 2000; Graniti *et al.*, 2000). **Adult plant infections in mature vineyards can occur through the root system.** This is the primary mode of colonization for pathogens belonging to the *Dactylonectria* or *Ilyonectria* genus (Halleen *et al.*, 2006), which are responsible for black-foot disease. **Other pathogens**, such as species of the Botryosphaeriaceae family and *Eutypa lata*, **mainly infect the plant through pruning wounds** produced at the end of the growing season each year (Rolshausen *et al.*, 2010; Van Niekerk *et al.*, 2011; Luque *et al.*, 2014). Finally, other pathogens, like *Phaeoacremonium minimum* or *Phaeomoniella chlamydospora*, can use both pathways to penetrate the plant, and these two microorganisms are the primary causal agents of Petri disease (Gramaje and Armengol, 2011; Bertsch *et al.*, 2013). **Young grapevine plants can be infected in the field by the same mechanisms described for adult plants. It is well known that some planting material (grafted plants) produced in nurseries is frequently infected with fungal pathogens, especially those involved in black-foot and Petri diseases.** In fact, it has been widely reported that grafts can be infected at different stages of the propagation process that takes place in the nurseries (Gramaje and Armengol, 2011; Agustí-Brisach *et al.*, 2013). GTD, particularly black-foot and Petri disease, are also recognised as prevalent causes of YGD. Decline symptoms in young vineyards have dramatically increased all over the world since the early 1990s, and these plants are primarily infected through their root system (Gramaje and Armengol, 2011).

Accordingly, in recent years, there have been many different studies aimed at controlling or diminishing fungal pathogen infection rates (Marco and Osti, 2007; Halleen *et al.*, 2010; Hayes, 2010; Rolshausen *et al.*, 2010; Kotze *et al.*, 2011; Martin and Mutawila *et al.*, 2011; Díaz and Latorre, 2013; Sosnowski *et al.*, 2013; Cobos *et al.*, 2015). Most studies have focused on protecting pruning wounds by applying natural antifungal compounds (Cobos *et al.*, 2015) or different chemical fungicides, which have been detailed in the introduction (see sections 3.1 and 3.2). The efficacy of several biological control agents (BCAs), including natural epiphytes and colonisers of pruning wounds has been tested (McMahan *et al.*, 2001; Schmidt *et al.*, 2008), as well as the application of several *Trichoderma* strains (Marco and Osti, 2007; Halleen *et al.*, 2010; Kotze *et al.*, 2011; Mutawila *et al.*, 2011).

Unfortunately, up to date there is no treatment available to protect grafts in nurseries, or grapevines in vineyards, from phytopathogenic fungi that can infect plants through the root system. Nevertheless, some studies have been focused on the application of different *Trichoderma* strains to soils or the grapevine root system to prevent infection by fungi that cause GTD. The protective effect of this strains has been enumerated (section 3.3).

Actinobacteria, particularly streptomycetes, are a complex group of Gram-positive bacteria making up around 10% of the total soil microbiome (Janssen, 2006). Although they are known as soil and rhizosphere bacteria, several reports indicate that they are also more intimately associated with plants, either as endophytic strains (Cao *et al.*, 2004; Joseph *et al.*, 2012; Li *et al.*, 2012), or mycorrhiza associated strains (Schrey and Tarkka, 2008; Schrey *et al.*, 2012). They are well known as important secondary metabolite producers, including antibiotic and antifungal compounds (Robertsen *et al.*, 2018), and for their ability to control plant diseases (Doubou *et al.*, 2001; Davelos *et al.*, 2004; Schrey and Tarkka, 2008).

Recently, bacterial communities associated with *Vitis vinifera* rhizospheres and their variations, including factors like geographical features (Burns *et al.*, 2015) and vineyard management (Vega-Avila *et al.*, 2015; Burns *et al.*, 2016), have been characterised. Some reports indicate that grapevine rhizosphere-associated bacteria could have some beneficial effects on plants, particularly in protecting them from reactive oxygen species (Salomon *et al.*, 2016), Nevertheless, no special attention has been focused on actinobacteria and streptomycetes as a potential group from which to isolate strains to control phytopathogenic fungi that cause GTD (Loqman *et al.*, 2009).

This study further characterises the endophytic and rhizosphere microbiota associated with the grapevine root system, particularly as a potential source of strains of interest in controlling the incidence of GTD produced by fungi that penetrate plants through their root system and, which are therefore involved in YGD. The best-performing isolates were tested in a 3-year field trial with grafted vines in a commercial nursery in Spain.

I.3. Material and Methods

I.3.1. Isolation of culturable endophytic and rhizosphere actinobacteria from the grapevine root system

I.3.1.1. Sample collection

A total of four 7-month-old *Vitis vinifera* cv. Tempranillo plants grafted on Richter 110 (110R) rootstock were collected from an open-root field nursery (Viveros Villanueva Vides S.L.) located in Larraga (Navarra, Spain) at 350 m above sea level (42°32'12.9"N 01°48'45.8"W). Actinobacteria associated with the root system were isolated from both the root-adjacent soil (rhizosphere strains) and the root tissue (endophytic strains).

I.3.1.2. Isolation of culturable actinobacteria from the grapevine root system

Two different approaches were used for the isolation of actinobacteria population. The difference was based on the grapevine zone where the actinobacteria were isolated, either the root-adjacent soil or the root tissue.

I.3.1.2.1. Rizospheric actinobacteria

Rhizosphere actinobacteria were isolated from 1.0 g of root-adjacent soil samples from every plant collected performing the following protocol. The soil sample was re-suspended in 10 mL of dH₂O and homogenized using vortex for 1 min. Next, 100 mL of 10-fold serial dilutions from 10⁻² to 10⁻⁴ were spread on DPA^{I.1} (Li *et al.*, 2012), ISP2^{I.2} (Shirling and Gottlieb, 1966), SAA^{I.3} (Li *et al.*, 2012) and SC^{I.4} (Kuster and Williams, 1964) agar plates supplemented with 150 µg/mL cycloheximide and 25 µg/mL nalidixic acid to prevent fungal and Gram-negative bacterial growth, respectively. Plates were incubated at 28°C for 3-7 days until growth was observed.

^{I.1} **DPA (Dulcitol Proline Agar):** 2.0 g/L dulcitol; 0.5 g/L proline; 0.3 g/L K₂HPO₄; 0.3 g/L NaCl; 1.0 g/L MgSO₄ · 7H₂O; 1.0 g/L CaCl₂ · 2H₂O; 20.0 g/L agar

^{I.2} **ISP2 (Yeast extract-malt extract):** 4.0 g/L yeast extract; 10.0 g/L malt extract; 4.0 g/L dextrose; 20.0 g/L agar; pH 7.2 ± 0.2

^{I.3} **SAA (Sodium succinate Asparagine Agar):** 1.0 g/L sodium succinate; 0.2 g/L L-asparagine; 0.9 g/L KH₂PO₄; 0.6 g/L K₂HPO₄; 0.1 g/L MgSO₄ · 7H₂O; 0.2 g/L CaCl₂ · 2H₂O; 0.3 g/L KCl; 0.001 g/L FeSO₄ · 7H₂O; 20.0 g/L agar

^{I.4} **SC:** 2.0 g/L starch; 0.3 g/L casein; 2.0 g/L KNO₃; 2.0 g/L NaCl; 2.0 g/L K₂HPO₄; 0.05 g/L MgSO₄ · 7H₂O; 0.02 g/L CaCO₃; 0.01 g/L FeSO₄ · 7H₂O; 20.0 g/L agar

I.3.1.2.2. Endophytic actinobacteria

Endophytic actinobacteria were isolated from root tissue samples of collected grapevines. Two different sections of root tissue were used: 2-cm stubs corresponding to the root insertion point, and root fragments corresponding to the 6-cm apical ends. Both samples were washed in sterile phosphate-buffered saline (PBS) solution (Sigma) and sonicated (160W / 2 min) to remove soil and organic matter from the sample surface. After drying at RT, roots were cut into 2.0 cm long fragments. These fragments were surface-sterilized by rising them in 0.1% Tween 20 (v/v) for 30 s and 1% (v/v) sodium hypochlorite for 6 min. Residual chlorine was removed by washing fragments into 2.5% (w/v) sodium thiosulphate for 10 min. Then, samples were washed three times with dH₂O. Next, root fragments were submerged in 70% (v/v) ethanol for 6 min, followed by three washes with dH₂O and air-dried in a laminar flow bench. To check the effectiveness of the surface sterilization process, 200 µL water of the final washing step were spread on an ISP2 agar plate and incubated at 28°C.

Two different methods were performed for the isolation of endophytic actinobacteria from surface-sterilized fragments. In the first method, 5 g of root material were disrupted using a mortar and pestle and resultant material was dissolved in 20 mL of 0.9% (w/v) NaCl and incubated at 220 rpm / 28°C to favour microorganism extraction. In the second method, 0.1 g of sample were disrupted in 2 mL tubes containing glass beads (0.5 mm diameter) using a FastPrep-24 homogenizer (MP Biomedicals), 10 times at 4,000 oscillations per min for 8 s each.

In both cases, samples were 10-fold diluted and 100 µL from 10⁻² to 10⁻⁴ dilutions were plate on DPA, ISP2, SAA and SC agar plates supplemented with 150 µg/mL cycloheximide and 25 µg/mL nalidixic acid and incubated at 28°C for 3 to 7 days.

I.3.2. Identification of actinobacteria

I.3.2.1. Preliminary identification by 16S rRNA sequencing

Different isolates were selected according to their morphological and cultural characteristics, including colony appearance, presence/absence of aerial mycelia, spore mass colour, distinctive reverse colony colour, and production of diffusible pigments. Media used for sporulation and their composition are detailed in Table SI-1. Isolates were routinely cultivated on ISP2 medium at 4°C. Spore-producing isolates were maintained as spore suspensions at -20°C in 40% (w/v) glycerol.

Endophytic and rhizosphere strains exhibiting antifungal activity were identified by 16S rRNA sequencing. Genomic DNA isolation from actinobacteria strains was carried out from liquid cultures grown in TSB (Sigma-Aldrich) at 220 rpm / 3 days / 30°C following the protocol detailed by Hopwood *et al.* (1985) with some variations. Briefly, cell culture was centrifuged at 7,000 rpm

/ 10 min / RT and cell pellet was re-suspended in 3 mL TES lysis solution^{1.5} supplemented with 5 mg/mL lysozyme (Sigma-Aldrich) and incubate for 30 min / 37°C in a water bath. After, 10 mL of Kirby's solution 2x^{1.6} were added and mixed by vortex for 1 min. Next, 1 volume of phenol-CIA^{1.7} solution was added, intensively mixed by vortex and centrifuge at 7,000 rpm / 10 min / RT. The aqueous phase (upper phase) was collected in a new tube and 1 volume of CIA was added, mixed and centrifuged in the same conditions. Aqueous phase was collected.

Nucleic acid precipitation was carried out by adding 1/10 volume of sodium acetate^{1.8} and 1 volume of isopropanol. Sample was mixed thoroughly by inversion and incubate for 30 min / RT before centrifuge at 7,000 rpm / 10 min / RT. Supernatant was discarded and nucleic acids were washed by the addition of 1 mL 70% (v/v) ethanol, dried and re-suspended in 500 µL TE buffer^{1.9}.

In order to remove the RNA, nucleic acids were treated with 50 µg/mL RNase (Fermentas) and incubate for 45 min / 37°C. Phenol-CIA and CIA steps and precipitation of nucleic acids were performed as before and re-suspended genomic DNA in 500 µL TE buffer.

Extracted DNA was analysed by electrophoresis in a 1% (w/v) agarose gel in 1x TAE buffer^{1.10} containing 0.15 µg/mL ethidium bromide (Sigma-Aldrich) and the concentration was measured on NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific). Genomic DNA was stored at -20°C until use.

16S rRNA genes were amplified using the oligonucleotides 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') (Lane, 1991). Amplification reactions were developed in 1x paq5000 buffer (Agilent Technologies) with 1 µM of each primer (IDT), 0.1 mM dNTP mix (EURx), 1U Paq5000 DNA polymerase (Agilent Technologies) and 15 ng template DNA. ddH₂O was used to bring the total reaction volume to 20 µL.

PCR amplification was carried out in 0.2 mL sterile tubes using a Mastercycler Gradient Thermocycler (Eppendorf). PCR conditions were: 98°C for 1 min; 4 cycles of 97°C for 1 min, 50°C for 1 min, 72°C for 2 min; 24 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 2 min; and a final extension of 72°C for 5 min. Tubes were kept at 4°C until the reactions were analysed in 1% (w/v) agarose gels and photographed using VWR GenoSmart equipment (VWR).

^{1.5} **TES lysis solution:** 75.0 mM NaCl; 25.0 mM EDTA, pH 8.0; 20.0 mM Tris-HCl, pH 7.5

^{1.6} **Kirby's solution 2x:** 20.0 g/L SDS; 12.0 g/L sodium 4-aminosalicylate; 60.0 mL/L neutral phenol; 100.0 mL/L Tris-HCl 1 M, pH 8.0

^{1.7} **CIA:** chloroform: isoamyl alcohol (24:1, v/v)

^{1.8} **Sodium acetate:** 3 M sodium acetate, pH 6.0

^{1.9} **TE buffer:** 10.0 mM Tris-HCl; 1.0 mM EDTA, pH 8.0

^{1.10} **50x TAE buffer** 57.1 mL/L acetic acid; 100.0 mL/L EDTA 0.5 M, pH 8.0; 242.0 g/L Tris base

Isolates were identified by comparing them with type strains corresponding to sequences found on the EzTaxon-e database (Kim *et al.*, 2012) (<http://www.ezbiocloud.net/eztaxon/identify>). Sequence alignment and generation of phylogenetic trees were carried out using MEGA 6.0 software (<http://www.megasoftware.net/>) with a neighbour-joining algorithm. Evolutionary distance was calculated using the Kimura two-parameter model for nucleotide sequences (Kimura, 1980). The 16 rRNA gene sequence data have been deposited in GenBank, whose accession numbers are referenced in Álvarez-Pérez *et al.* (2017).

I.3.2.2. Identification of the actinobacteria selected for field assays using multilocus sequence analysis

Multilocus sequence analysis (MLSA) was performed using five housekeeping genes: *atpD* (ATP synthase F1 beta subunit), *gyrB* (DNA gyrase B subunit), *recA* (recombinase A), *rpoB* (RNA polymerase beta subunit) and *trpB* (tryptophan synthase beta subunit) (Rong and Huang, 2012). PCR amplification was performed in a final volume of 50 µL reaction mixture under the conditions described by Guo *et al.*, (2008) and Rong *et al.*, (2009). Primers used in this assay are shown in Table SI-2. The GenBank accession number of DNA sequences from the housekeeping genes are shown in Table SI-3.

Phylogenetic trees were constructed using a concatenation of the five housekeeping genes by joining those head to tail. DNA sequences were manually trimmed at the same position after being aligned using MEGA 6.0 software with sequences from type strains achieved from the ARS Microbial Genomic Sequence Database server (<http://199.133.98.43>). Phylogenetic trees were constructed using the maximum likelihood method with the Kimura two-parameter model (Kimura, 1980). MLSA evolutionary distances were calculated using MEGA 6.0 by calculating the Kimura two-parameter distance. Strain pairs having ≤ 0.007 MLSA evolutionary distance were considered conspecific (same specie) based on the guideline empirically determined by Rong and Huang (2012).

I.3.2.3. Amplification of the *groEL2* gene

In the case of those selected actinobacteria that belong to a taxonomic group, whose sequences are indistinguishable for the 16S rRNA region, the *groEL2* gene was amplified and subsequently sequenced. In order to amplify this gene, a PCR was performed using the oligonucleotides STGROF1 (5'-CCATCGCCAAGGAGATCGAGCT-3') and STGROR2 (5'- TGAAGGTG CCRCGGATCTTGTT-3') (Duchêne *et al.*, 1994; Kim *et al.*, 2008). Amplification reactions were developed in 1x paq5000 buffer with 500 nM of each primer, 0.1 mM dNTP mix, 1U Paq5000 DNA polymerase and 5 ng template DNA. ddH₂O was used to bring the total reaction volume to 25 µL.

PCR amplification was carried out in 0.2 mL sterile tubes using a Mastercycler Gradient Thermocycler (Eppendorf). PCR conditions were: 94°C for 2 min; 30 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 45 s; and a final extension of 72°C for 7 min. Tubes were kept at 4°C until the reactions were subjected to electrophoresis analysis in 1% (w/v) agarose gels, photographed and sequenced (Sanger technology).

I.3.3. Evaluation of antifungal activity

I.3.3.1. Antifungal activity against *Dactylonectria macrodidyma* and *Diplodia seriata*

All isolates were tested for antifungal activity using an *in vitro* antifungal assay, following a modified protocol of Lamsal *et al.*, (2012). In order to perform this assay, isolates were inoculated on both potato dextrose agar (PDA) (Conda Laboratories) and TSA agar plates in a 1.0 cm² area (4 isolates per plate inoculated at 1 cm from the edge of the plate), placing an agar plug of the fungi (*Da. macrodidyma* CBS 120170 or *D. seriata* CBS 112555) in the middle of the plate.

Plates were incubated at 25°C / up to 12 days to detect growth inhibition areas. Growth was quantified by calculating the inhibition index (I index): I index (%) = $[1 - (Ra - R) / (Rc - R)] \times 100$; where Ra is the radius of the fungal colony opposite the bacterial colony; Rc is the maximum radius of the fungal colony (farther away from the bacterial effect) and R is the radius of the agar plug containing the fungi (3.5 mm). This assay was performed in triplicate for each actinobacterium tested (Fig. I-1A).

Fig. I-1 *In vitro* bioassay for the evaluation of antifungal activity of isolated actinobacteria (Act) against the fungal pathogens *D. seriata* or *Da. macrodidyma*. Four isolated actinobacteria were inoculated on the periphery of the plate in a 1.0 cm² area. The fungal pathogen (FP) was inoculated in the centre of the plate using a 7.0 mm diameter agar plug. Plates were incubated up to 12 days at 25°C to detect growth inhibition areas. Growth was quantified by calculating the inhibition rate (I index %) (A). *In vitro* bioassay for the evaluation of antifungal activity of isolated actinobacteria against *Ph. chlamydospora* or *P. minimum* pathogens. Fungal pathogens were inoculated on the plate forming a circle 2 cm from the edge of the plate. Actinobacteria strains were inoculated in 1.5 cm² patches located 5 mm from the edge of the fungal strain. Plates were incubated at 25°C for up to 20 days to detect the effect of growth inhibition (B).

I.3.3.2. Antifungal activity against *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora*

Those strains which showed antifungal activity against both pathogens were also tested against *Ph. chlamydospora* CBS 239.74 and *P. minimum* CBS 246.91.

This assay was performed in 3 different media: Czapek^{I.11} (Czapek, 1902), ISP5^{I.12} (Shirling and Gottlieb, 1966), and PDA agar plates. Fungal pathogens were inoculated on an agar plate drawing a 2 cm circle from the periphery of the petri dish. Actinobacterial strains were inoculated in 2.25 cm² patches at 5 mm from the edge of the fungal strains (Fig. I-1B). Plates were incubated at 25°C / up to 20 days in order to detect the growth inhibitory effect.

I.3.3.3. Evaluation of antifungal activity in field assays

Field assays were performed in three experimental open-root field nurseries of grafted *Vitis vinifera* cv. Tempranillo plants that were grafted on Richter 110 (110R) rootstock for three consecutive years with a total of 525 plants in every experimental nursery.

Grafts were subdivided into 7 batches of 75 units (3 replicas of 25 units per batch). Each batch was treated with one of the 6 selected actinobacteria: 3 endophytic (VV/E1, VV/E2, and VV/E5) and 3 rhizosphere (VV/R1, VV/R4, and VV/R5) isolates. A negative-control batch (grafts not treated with actinobacteria) was also included in the study.

The biomass production of each strain was obtained from 200 mL liquid cultures of TSB medium incubated at 220 rpm / 3 days / 28°C in 500 mL Erlenmeyer flasks that were inoculated with 8x10⁸ spores (for *Streptomyces* sp. VV/E5, which do not sporulate in any tested media, the culture was inoculated with 5 mL of a glycerol stock).

^{I.11} **Czapek:** 30.0 g/L sucrose; 4.0 g/L NaNO₃; 1.0 g/L K₂HPO₄; 1.0 g/L MgSO₄·7H₂O; 0.01 g/L FeSO₄·7H₂O; 20.0 g/L agar; pH 6.5

^{I.12} **ISP5 (glycerol-asparagine agar):** 1.0 g/L L-asparagine (anhydrous basis); 10.0 g/L glycerol; 1.0 g/L K₂HPO₄ (anhydrous basis); 10.0 mL/L trace solution*; 20.0 g/L agar

* **Trace solution:** consists of a small amounts of different salts to improve the composition of several ISP media and, therefore, increase the actinobacteria growth. The composition of this solution is: 1.0 g/L FeSO₄·7H₂O; 0.1 g/L MnCl₂·4H₂O; 0.1 g/L ZnSO₄·7H₂O. It is sterilized on autoclave for 15 min / 121°C and added to the corresponding media when necessary.

I.3.3.3.1. Fungal isolation from wood

Plants were removed from the soil and analysed for the presence of pathogenic fungi. A 1-cm stub was cut 1 cm above the root insertion point. The wood was surface-sterilised by immersion in 70% ethanol for 1 min, next in 4% sodium hypochlorite for 2 min, and finally in 70% ethanol for 1 min. Seven wood chips (3 by 2 by 2 mm) were obtained from the inner tissues using a sterile scalpel and placed on PDA agar plates supplemented with 150 µg/mL chloramphenicol to prevent bacterial growth. Plates were incubated for up to 4 weeks at 25°C, subculturing when necessary.

I.3.3.3.2. Identification of fungal pathogens

I.3.3.3.2.a. *Dactylonectria macrodidyma* and *Ilyonectria* species

A pair of primers CylH3F (5'-AGGTCCACTGGTGGCAAG-3') and CylH3R (5'-AGCTGGATGTCCTTGGACTG-3') (Crous *et al.*, 2006) was used to amplify a fragment of around 500 bp of the histone H3 gene. Amplification reactions were developed in 1x paq5000 buffer with 200 nM of each primer, 0.2 mM dNTP mix, 1U paq5000 DNA polymerase and 15 ng template DNA. ddH₂O was used to bring the total reaction volume to 25 µL.

PCR amplification was carried out in 0.2 mL sterile tubes using a Mastercycler Gradient Thermocycler. PCR conditions were: 95°C for 2 min; 35 cycles of 95°C for 30 s, T_m = 52°C for 30 s, 72°C for 30 s; and a final extension of 72°C for 5 min.

PCR fragments were sequenced and compared to sequences of the HIS region found in the GenBank database.

I.3.3.3.2.b. *Phaeoacremonium minimum*

The pair of primers Pal 1N (5'-AGGTCGGGGCCAAC-3') and Pal 2 (5'-AGGTGTAACTACTGCGC-3') (Tegli *et al.*, 2000) targeting specifically the ITS region of this fungi were used for its identification. They generate a PCR band of 395 bp. Amplification reaction and PCR conditions were the same as those described for *Dactylonectria* and *Ilyonectria* species but using as T_m 65°C. Tubes were kept at 4°C until the reactions were analysed in 1% (w/v) agarose gels and photographed.

I.3.3.3.2.c. *Phaeomoniella chlamydospora*

The pair of primers Pch 1 (5'-CTCCAACCCTTTGTTTATC-3') and Pch 2 (5'-TGAAAGTTGATATGGACCC-3') (Tegli *et al.*, 2000) target specifically the ITS region of this fungi, amplifying a band of 380 bp. Amplification reaction and PCR conditions were the same as those described for *Dactylonectria* and *Ilyonectria* species but using a T_m of 62°C. Tubes were kept at 4°C until the reactions were analysed in 1% (w/v) agarose gels and photographed.

I.3.4. Statistical data analysis

Plant growth data were tested for univariate normality using the Shapiro-Wilk test. They were subjected to univariate analysis of variance using the general linear means procedure to determine if there were significant differences between treated and untreated plants. Previously, total height and length of the 7th internode data were tested for univariate normality using the Shapiro-Wilk test and then subjected to univariate analysis of variance using the general linear means procedure. In the case of grafted grapevine mortality data, a chi-square was used to test the null hypothesis of no differences in mortality rates against a two-tailed alternative hypothesis that the difference in mortality rates was not zero. When the expected frequencies were less than 5, Fisher's exact test was used. The chances of plant survival versus untreated control plants were calculated as the ratio between the odds of vine survival between a particular treatment and the control (the odds ratio for a particular treatment is the quotient between the number of live and dead plants). To research the treatment efficacy on the number of fungal pathogens isolated, the Poisson distribution (comparing the results achieved with nested models) was used to construct generalized linear models. The null hypothesis of the independence assumption between the frequency of fungal pathogen isolation and treatments was checked using either the chi-square or Fisher's exact test, depending on the nature of the data. Statistical analyses were performed using R Core Team (3.3.0) software (<https://www.r-project.org/>).

I.4. Results

I.4.1. Isolation and identification of culturable actinobacteria associated with the root system of *Vitis vinifera*.

I.4.1.1. Isolation of actinobacteria and identification according to the 16S rRNA gene

A total of 58 endophytic and 94 rhizosphere strains were isolated from four different young *V. vinifera* plants. All the endophytes and those rhizosphere strains exhibiting antifungal activity against *D. seriata* and/or *Da. macrodidyma* were analysed for their identification by sequencing the 16S rRNA gene (Table I-1).

Table I-1A Rhizosphere actinobacteria isolated from the root system of grafted grapevines.

Isolate	Identification	16S rRNA sequence accession no.	Isolation medium
VV/R1	<i>Streptomyces</i> sp.	KY978704	ISP2
VV/R2	<i>Streptomyces</i> sp.	KY978705	SC
VV/R3	<i>Streptomyces</i> sp.	KY978706	ISP2
VV/R4	<i>Streptomyces</i> sp.	KY978707	SC
VV/R5	<i>Streptomyces</i> sp.	KY978708	SC
VV/R6	<i>Streptomyces</i> sp.	KY978709	SC
VV/R7	<i>Streptomyces</i> sp.	KY978710	DPA
VV/R8	<i>Streptomyces</i> sp.	KY978711	SAA
VV/R9	<i>Streptomyces</i> sp.	KY978712	ISP2
VV/R10	<i>Streptomyces</i> sp.	KY978713	SC
VV/R11	<i>Streptomyces</i> sp.	KY978714	SC
VV/R12	<i>Streptomyces</i> sp.	KY978715	SC
VV/R13	<i>Streptomyces</i> sp.	KY978716	ISP2
VV/R14	<i>Streptomyces</i> sp.	KY978717	SC
VV/R15	<i>Streptomyces</i> sp.	KY978718	ISP2
VV/R16	<i>Streptomyces</i> sp.	KY978719	DPA
VV/R17	<i>Streptomyces</i> sp.	KY978720	SC
VV/R18	<i>Streptomyces</i> sp.	KY978721	SC
VV/R19	<i>Streptomyces</i> sp.	KY978722	SC
VV/R20	<i>Streptomyces</i> sp.	KY978723	SAA
VV/R21	<i>Streptomyces</i> sp.	KY978724	SAA
VV/R22	<i>Streptomyces</i> sp.	KY978725	ISP2
VV/R23	<i>Streptomyces</i> sp.	KY978726	SC
VV/R24	<i>Streptomyces</i> sp.	KY978727	ISP2
VV/R25	<i>Streptomyces</i> sp.	KY978728	ISP2
VV/R26	<i>Streptomyces</i> sp.	KY978729	DPA
VV/R27	<i>Streptomyces</i> sp.	KY978730	SC
VV/R28	<i>Streptomyces</i> sp.	KY978731	SC
VV/R29	<i>Streptomyces</i> sp.	KY978732	ISP2

Table I-1B Endophytic actinobacteria isolated from the root system of grafted grapevines.

Isolate	Identification	16S rRNA sequence accession no.	Isolation medium
VV/E1	<i>Streptomyces</i> sp.	KY978646	SC
VV/E2	<i>Streptomyces</i> sp.	KY978647	ISP2
VV/E3	<i>Saccharopolyspora</i> sp.	KY978648	SC
VV/E4	<i>Streptomyces</i> sp.	KY978649	SC
VV/E5	<i>Streptomyces</i> sp.	KY978650	SC
VV/E6	<i>Streptomyces</i> sp.	KY978651	DPA
VV/E7	<i>Saccharopolyspora pathumthaniensis</i>	KY978652	ISP2
VV/E8	<i>Streptomyces</i> sp.	KY978653	DPA
VV/E9	<i>Streptomyces</i> sp.	KY978654	DPA
VV/E10	<i>Saccharopolyspora</i> sp.	KY978655	ISP2
VV/E11	<i>Streptomyces</i> sp.	KY978656	SAA
VV/E12	<i>Streptomyces atrovirens</i>	KY978657	ISP2
VV/E13	<i>Streptomyces</i> sp.	KY978658	ISP2
VV/E14	<i>Streptomyces</i> sp.	KY978659	SAA
VV/E15	<i>Streptomyces</i> sp.	KY978660	SC
VV/E16	<i>Streptomyces</i> sp.	KY978661	SC
VV/E17	<i>Streptomyces</i> sp.	KY978662	ISP2
VV/E18	<i>Streptomyces</i> sp.	KY978663	SAA
VV/E19	<i>Micromonospora</i> sp.	KY978664	ISP2
VV/E20	<i>Micromonospora</i> sp.	KY978665	ISP2
VV/E21	<i>Micromonospora</i> sp.	KY978666	SC
VV/E22	<i>Streptomyces</i> sp.	KY978667	SC
VV/E23	<i>Streptomyces</i> sp.	KY978668	DPA
VV/E24	<i>Streptomyces</i> sp.	KY978669	SC
VV/E25	<i>Streptomyces</i> sp.	KY978670	SC
VV/E26	<i>Streptomyces</i> sp.	KY978671	DPA
VV/E27	<i>Micromonospora palomenae</i>	KY978672	ISP2
VV/E28	<i>S. pathumthaniensis</i>	KY978673	SC
VV/E29	<i>Streptomyces</i> sp.	KY978674	ISP2
VV/E30	<i>Streptomyces</i> sp.	KY978675	SC
VV/E31	<i>S. pathumthaniensis</i>	KY978676	ISP2
VV/E32	<i>Streptomyces</i> sp.	KY978677	SC
VV/E33	<i>S. pathumthaniensis</i>	KY978678	ISP2
VV/E34	<i>Rathayibacter caricis</i>	KY978679	ISP2
VV/E35	<i>Nonomuraea kuesteri</i>	KY978680	ISP2
VV/E36	<i>S. pathumthaniensis</i>	KY978681	SC
VV/E37	<i>N. kuesteri</i>	KY978682	SC
VV/E38	<i>S. pathumthaniensis</i>	KY978683	ISP2
VV/E39	<i>Micromonospora</i> sp.	KY978684	ISP2
VV/E40	<i>Micromonospora</i> sp.	KY978685	SC
VV/E41	<i>Micromonospora</i> sp.	KY978686	ISP2
VV/E42	<i>Micromonospora</i> sp.	KY978687	ISP2
VV/E43	<i>Micromonospora</i> sp.	KY978688	ISP2
VV/E44	<i>Streptomyces</i> sp.	KY978689	SAA
VV/E45	<i>Micromonospora</i> sp.	KY978690	SC
VV/E46	<i>Micromonospora</i> sp.	KY978691	ISP2
VV/E47	<i>Micromonospora</i> sp.	KY978692	ISP2
VV/E48	<i>Micromonospora</i> sp.	KY978693	SC
VV/E49	<i>S. pathumthaniensis</i>	KY978694	SC
VV/E50	<i>Micromonospora</i> sp.	KY978695	ISP2
VV/E51	<i>Nonomuraea</i> sp.	KY978696	SC
VV/E52	<i>S. pathumthaniensis</i>	KY978697	ISP2
VV/E53	<i>Streptomyces</i> sp.	KY978698	DPA
VV/E54	<i>Nonomuraea</i> sp.	KY978699	ISP2
VV/E55	<i>Kribbella</i> sp.	KY978700	SAA
VV/E56	<i>Corynebacterium</i> sp.	KY978701	ISP2
VV/E57	<i>Micromonospora</i> sp.	KY978702	ISP2
VV/E58	<i>Streptomyces</i> sp.	KY978703	SAA

16S rRNA gene sequencing of endophytic strains revealed that *Streptomyces* was the most abundant genus representing the 44.8% of the total isolates, followed by *Micromonospora* and *Saccharopolyspora* which represented the 25.9% and 17.2% respectively. Other taxa, including *Nonomuraea*, *Kribbella*, *Rathayibacter* and *Corynebacterium* were isolated in fewer percentages, representing, on the whole, the 12.1% of the total isolates. On the other hand all rhizosphere actinobacteria isolated belonged to the *Streptomyces* genus (Fig I-2). Phylogenetic tree based on 16S rRNA gene sequences of culturable endophytic and rhizosphere actinobacteria with a putative antifungal activity against grapevine trunk pathogens is shown in Fig. SI-1.

Fig I-2 Pie charts of the actinobacteria representation in analysed grapevine plants. Within actinobacteria isolated from the root system (endophytic strains) *Streptomyces* was the most representative genus (A) while within actinobacteria isolated from the rhizosphere was the only genus identified (B).

I.4.1.2. Multilocus sequence analysis (MLSA) of actinobacterial strains selected for field assays

Preliminary characterization of selected endophytic and rhizosphere *Actinobacteria* based on the 16S rRNA sequencing led, in most cases, to their identification as species of the genus *Streptomyces*. However, the use of the 16S rRNA as a phylogenetic marker did not provide sufficient resolution for species-level identification. With the aim of obtaining a further characterization and more precisely identification, a MLSA study of five housekeeping genes was performed. The analysed genes were *atpD*, *gyrB*, *recA*, *rpoB* and *trpB* genes.

MLSA analysis (Fig. SI-2) confirmed that isolates VV/E1 and VV/R4 were the same species since no MLSA distance was detected (Table I-2), although they exhibit macroscopic differences when grow and sporulate in different culture media (Fig. I-3). Besides, they were closely related to *Streptomyces albaduncus* with a MLSA distance of 0.028.

Table I-2. MLSA distances for strains phylogenetically near to our selected isolates.

No.	Strain ^a	MLSA (Kimura 2-parameter) distance																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	<i>S. albaduncus</i> NRRL B-3605 ^T	-																	
2	<i>S. albaduncus</i> NRRL b-3605g ^T	0.000																	
3	VV/E1	0.028	0.028																
4	VV/R4	0.028	0.028	0.000															
5	<i>S. umbrinus</i> NRRL B-2572 ^T	0.094	0.094	0.092	0.092														
6	VV/R5	0.096	0.096	0.091	0.091	0.040													
7	<i>S. phaeochromogenes</i> NRRL B-1248 ^T	0.094	0.094	0.091	0.091	0.034	0.013												
8	<i>S. phaeochromogenes</i> subs. <i>phaeochromogenes</i> NRRL B-3010 ^T	0.094	0.094	0.091	0.091	0.034	0.013	0.000											
9	<i>S. helveticus</i> NRRL B-12365 ^T	0.124	0.124	0.130	0.130	0.121	0.124	0.126	0.126										
10	VV/E2	0.119	0.119	0.126	0.126	0.118	0.122	0.122	0.122	0.043									
11	<i>S. albidochromogenes</i> NRRL B-24308 ^T	0.113	0.113	0.117	0.117	0.110	0.112	0.113	0.113	0.045	0.057								
12	<i>S. flavidovirens</i> subs. <i>flavidovirens</i> NRRL B-2708 ^T	0.113	0.113	0.113	0.113	0.106	0.113	0.111	0.111	0.067	0.055	0.048							
13	<i>S. flavidovirens</i> DSM 40150 ^T	0.113	0.113	0.113	0.113	0.106	0.115	0.112	0.112	0.067	0.055	0.050	0.001						
14	<i>S. peucetius</i> subs. <i>peucetius</i> NRRL B-3826 ^T	0.104	0.104	0.105	0.105	0.111	0.113	0.108	0.108	0.131	0.128	0.131	0.124	0.124					
15	VV/E5	0.106	0.106	0.107	0.107	0.112	0.114	0.110	0.110	0.132	0.130	0.132	0.126	0.126	0.002				
16	<i>S. peucetius</i> CGMCC 4.1799 ^T	0.122	0.122	0.105	0.105	0.111	0.113	0.111	0.111	0.131	0.128	0.131	0.124	0.124	0.000	0.002			
17	VV/R1	0.219	0.219	0.187	0.187	0.201	0.200	0.196	0.196	0.218	0.212	0.214	0.210	0.210	0.108	0.108	0.108		
18	<i>S. xantholiticus</i> NRRL B-12153 ^T	0.106	0.106	0.108	0.108	0.113	0.114	0.111	0.111	0.130	0.125	0.128	0.124	0.124	0.012	0.014	0.112	0.112	-

^aOur isolates appear in bold

Fig. I-3 Macroscopic differences between *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 strains when they are growing in different culture media. According to MLSA analysis they could be the same species, although they show different morphological characteristics and sporulation patterns when grow in the same culture media. Note, like VV/E1 only sporulate in Czapek's medium, whereas VV/R4 sporulate in Czapek, ISP1, ISP2, ISP3, ISP7, MBA, MEY and TBO media.

VV/R1 and VV/E5 were placed in a well-delineated subclade including *Streptomyces peucetius* and *Streptomyces xantholiticus* strains. Both isolates shared a MLSA distance of 0.108, which indicates that they are different species. On the one hand, VV/E5 was classified as *Streptomyces peucetius* since the MLSA distance was 0.002 (smaller than 0.007). On the other hand, VV/R1 matched between *S. peucetius* (MLSA distance of 0.108) and *S. xantholiticus* (0.112 MLSA distance) suggesting that it could be a novel *Streptomyces* species.

VV/E2 was located in a subclade including *Streptomyces albidochromogenes*, *Streptomyces flavidovirens* and *Streptomyces helveticus*. However, the distances with these strains were always higher than 0.007, indicating that isolate VV/E2 could also be a novel species. Finally, VV/R5 was closely related to members of a subclade including *Streptomyces phaeochromogenes* and *Streptomyces umbrinus*, although the MLSA distance with regard to both species (0.013 and 0.040 respectively) indicated that this isolate belongs to a different species.

Growth and sporulation media for the other rhizosphere (VV/R1 and VV/R5) and endophytic (VV/E2 and VV/E5) strains appear is shown in Fig. SI-3.

I.4.1.3. Amplification of the *groEL2* gene

Since according to the 16S rRNA sequences, VV/E1 and VV/R4 seemed to be the same species, we carried out the sequence analysis of an additional *groEL2* gene (Duchêne *et al.*, 1994, Kim *et al.*, 2008). We also included in this analysis VV/E5 and VV/R1 strains (Fig. I-4).

Fig. I-4 Neighbour-Joining tree showing phylogenetic relationships based on *groEL2* gene sequences of actinobacterial strains with antifungal activity which belong to the same group according to 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are given in the nodes. Bar, 0.5% difference in nucleotide sequence.

Due to the lack of sequences for this gene in the databases, we made a phylogenetic tree comparing only the four strains in order to determine if, according to their *groEL2* sequences they belong to the same species.

According to this analysis, VV/E5 and VV/R1 were the same species, while VV/E1 and VV/R4 would belong to different species. Surprisingly, these results were just the opposite of those obtained for the MLSA analysis. However, as there

is few information of *groEL2* gene for *Streptomyces* species in the databases, we cannot ensure these classification of *Streptomyces* strains based on this gene.

I.4.2. Detection and selection of actinobacteria with a putative effect as biocontrol agents against phytopathogenic fungi involved in GTD.

Since one of the main goals of this work was to evaluate the putative application of selected actinobacteria to control fungal pathogens involved in GTD and YGD the antifungal activity of isolated actinobacteria was screened *in vitro* by bioassay.

All the strains were tested against *D. seriata* and *Da. macrodidyma* fungal pathogens. These fungi were selected to perform a bioassay due to their high growth rate. Later, the five endophytic and rhizosphere strains that showed the higher inhibition index values (I index) were also tested for their antifungal effect against other two fungi involved in YGD: *P. minimum* and *Ph. chlamydospora*. Data (Table I-3) are only shown for those strains that exhibited antifungal effect against any of the tested fungal trunk pathogens.

Chapter I

Table I-3. Antifungal activity of isolated actinobacteria. All the strains were tested against *D. seriata* and *Da. macrodidyma*. Those strains that showed the higher inhibition index values (I index) were also tested against *Ph. chlamydospora* and *P. minimum*. Only those strains that exhibited antifungal effect against at least one of the tested fungi are shown.

Isolate	Inhibition index (%)		Detection of antifungal activity	
	<i>D. seria</i>	<i>Da. macrodidyma</i>	<i>Ph. chlamydospora</i>	<i>P. minimum</i>
VV/E1	53.47	33.91	+	+
VV/E2	61.17	59.41	+	+
VV/E3	31.97	35.87	-	-
VV/E4	46.95	46.62	-	+
VV/E5	52.50	47.15	+	+
VV/E6	49.57	33.86	NP	NP
VV/E7	36.81	-	NP	NP
VV/E8	33.42	-	NP	NP
VV/E9	-	40.24	NP	NP
VV/E10	29.97	-	NP	NP
VV/E11	-	40.24	NP	NP
VV/R1	48.75	55.06	+	+
VV/R2	45.52	53.20	-	+
VV/R3	42.58	49.13	-	-
VV/R4	51.69	50.77	+	+
VV/R5	55.60	51.43	+	+
VV/R6	47.84	44.57	NP	NP
VV/R7	51.83	41.22	NP	NP
VV/R8	51.04	44.40	NP	NP
VV/R9	54.14	21.55	NP	NP
VV/R10	45.65	48.44	NP	NP
VV/R11	45.52	35.23	NP	NP
VV/R12	44.61	49.07	NP	NP
VV/R13	52.03	46.41	NP	NP
VV/R14	56.95	43.42	NP	NP
VV/R15	49.47	48.67	NP	NP
VV/R16	45.65	-	NP	NP
VV/R17	40.48	-	NP	NP
VV/R18	62.27	-	NP	NP
VV/R19	48.66	-	NP	NP
VV/R20	44.67	-	NP	NP
VV/R21	40.61	-	NP	NP
VV/R22	55.21	-	NP	NP
VV/R23	48.27	47.02	NP	NP
VV/R24	40.71	-	NP	NP
VV/R25	80.50	-	NP	NP
VV/R26	62.87	-	NP	NP
VV/R27	90.81	-	NP	NP
VV/R28	43.59	-	NP	NP
VV/R29	44.95	-	NP	NP

Inhibition index values (%) shown are the average of three independent experiments.

(-)-Species that not exhibited antifungal activity against any of the pathogens tested.

(+) antifungal activity.

NP means that data was not performed for this strains.

A bioassay-based *in vitro* evaluation showed that 15.5% (9 out of 58) and 13.8% (8 out of 58) of the endophytic isolates exhibited antifungal activity against *D. seriata* and *Da. macrodidyma* respectively. With regards to the rhizosphere strains, the 30.8% (29 out of 94) and 17.0% (16 out of 94) of the isolates exhibited antifungal activity against both pathogens. Based on these results, **three rhizosphere** (VV/R1, VV/R4 and VV/R5) **and three endophytic strains** (VV/E1, VV/E2 and VV/E5) **showing antifungal activity against all the pathogens analysed were selected for testing in field assays by their application on grafts.**

I.4.3. Field assays with the most promising actinobacteria

The 6 isolates that exhibited antifungal effect against all tested phytopathogenic fungi, were used for performing field assays in three different growing seasons. Two different analysis were performed. On the one hand, growth and mortality rates were checked and, on the other hand, pathogenic fungi were isolated at the end of the growing season.

I.4.3.1. Growth and mortality rates

I.4.3.1.1. Plant growth

Plant growth data were tested for univariate normality using the Shapiro-Wilk test. They were subjected to univariate analysis of variance using the general linear means procedure to determine if there were significant differences between treated and untreated plants. According to the analysis performed, the application of actinobacteria to grafted plants did not have a significant effect, either positive or negative, on the growth of the surviving plants, as determined by the measurement of total plant height [$F(6.413) = 0.37$, $p = 0.90$] (Fig. I-5A).

I.4.3.1.2. Total length of the 7th internode

Total length of the 7th internode was also analysed for univariate normality using the Shapiro-Wilk test and then subjected to univariate analysis of variance using the general means procedure. Results determined that the application of actinobacteria to grafted plants neither had a significant effect on the elongation of the seventh internode [$F(6.413) = 0.12$, $p = 0.29$] (Fig I-5B).

Fig. I-5 Total length (A) and elongation of the 7th internode (B) in grafted plants treated with different endophytic and rhizosphere actinobacteria compared to untreated control plants. Each bar corresponds to the arithmetic mean of the values determined for the total number of surviving plants in the three tested seasons and their corresponding standard errors. Bars marked with the same letter are not significantly different ($p > 0.05$).

I.4.3.1.3. Mortality rate

The average mortality rate of untreated (not inoculated, negative control) plants in the 3-year field study was 28.4% (64 dead plants out of a total of 225 plants). Significant differences in mortality rates were observed for two of the treatments, according to the chi-square tests. The mortality rates of plants inoculated with VV/R1 (19.1% [43 dead plants out of 225 plants]) [$\chi^2(1) = 5.41$, $p = 0.05$] or VV/R4 (17.8% [40 dead plants out of 225 plants]) [$\chi^2(1) = 7.20$, $p = 0.01$] strains were significantly lower. Average mortality rates appear in Fig. I-6.

According to these data, the odds of plant survival were 1.68 times higher on

VV/R1 treatment than on untreated control plants, whereas the probability of plant survival were 1.84 times higher on VV/R4 treatment than on untreated control plants.

Fig. I-6. Average mortality rates (%) and their corresponding standard error values of grafted grapevines plants observed in the experimental nurseries after treatment with different endophytic and rhizosphere strains compared to untreated control plants. Bars marked with the same letter are not significantly different ($p \geq 0.05$).

a reduction in the number of fungal pathogens involved in young grapevine decline comparing with the number of fungal pathogens isolated from control plants (Fig. I-7). Raw data of this study are shown in Table SI-4.

Fig. I-7 Relative isolation frequency (%) of fungal pathogens involved in young grapevine decline from grafted plants treated with different endophytic and rhizosphere actinobacteria regarding untreated plants (Control). A value of 100 was assigned to the total number of fungal pathogens of a particular species (*P. chlamydospora*, *P. minimum*, or *Dactylonectria sp.-Ilyonectria sp.* analysed, in untreated plants (control)). The relative frequency for each type of pathogen analysed in the plants treated with the different actinobacteria was calculated as a percentage by comparison against the value of 100% assigned to the control plants. Data shown corresponds to the average of 3 batches of 25 plants in 3 experimental nurseries belonging to three growing seasons.

I.4.3.1.4. Isolation of pathogenic fungi

Once the plants had completed their vegetative growth cycle and were in dormant state (around two hundred days after planting), vines were carefully removed from the soil to obtain the root system as intact as possible. The incidence of fungal trunk pathogens causing YGD (*Dactylonectria sp.-Ilyonectria sp.* group, *Ph. chlamydospora* and *P. minimum*), which are able to infect the plant through the root system, was determined by collecting wood samples from the root insertion point.

All the selected actinobacteria led to

The impact of the different actinobacteria strains on the number of fungal trunk pathogens involved in YGD was analysed using nested generalised linear models (Poisson distribution, with intercept versus intercept plus treatment as dependent variables). Moreover, the existence of significant differences between the number of fungal pathogens isolated from plants treated with the different actinobacteria, and the number of pathogens isolated from control plants was determined using chi-

square tests. Thus, in accordance with the nested generalized linear models, significant associations between the nature of the treatment and the number of fungal pathogens isolated were found in the *Dactylonectria* sp.-*Ilyonectria* sp. group and for *Ph. chlamydospora* ($p < 0.05$ in the chi-square tests conducted to compare the nested models) for all the actinobacteria assayed. On the other hand, no significant association ($p > 0.05$ in the chi-square tests) between the nature of the treatment and the number of fungal pathogens isolated was detected for *P. minimum*, probably due to the low number of individuals of this species isolated.

After that, chi-square and Fisher's tests were performed to establish the existence of significant differences between treatments. The results are shown as heatmaps of independence assumptions between the type of actinobacterial treatment and the number of fungal pathogens isolated (Fig. I-8).

Surprisingly, despite the data shown by nested generalized linear models, significant differences ($p < 0.05$) between the negative control and the three treatments (VV/E1, VV/R1, and VV/R4) were found with respect to *P. minimum* (Fig. I-7). However, these significant associations were very close to the edge of the significance threshold (0.05). It may be that the nested model tests are more conservative than individual tests performed between treatments.

When focusing on the highly significant association ($p < 0.01$ and $p < 0.001$) between the nature of the treatment and the number of isolated pathogens within the *Dactylonectria* sp.-*Ilyonectria* sp. group, the following results should be highlighted: control and VV/E1 [χ^2 (1) = 46.4, $p < 0.001$], control and VV/R4 [χ^2 (1) = 12.0, $p = 0.001$], control and VV/R1 [χ^2 (1) = 7.15, $p = 0.01$], and control and VV/R5 [χ^2 (1) = 5.91, $p < 0.01$] (Fig. I-8). In the case of *Ph.*

Fig. I-8. Analysis of significant differences between the number of fungal pathogens isolated from plants treated with the different actinobacteria versus the number of pathogens isolated from control plants. The analysis is shown as heatmaps of independence assumptions between the type of actinobacterial treatment and the number of fungal pathogens isolated. Colour key corresponds to the significance of chi-square or Fisher's test results. Data relative to *Dactylonectria* sp.-*Ilyonectria* sp. group (A). Data relative to *P. minimum* (B). Data relative to *Ph. chlamydospora* (C). Data shown correspond to 3 batches of 25 surviving plants in three experimental nurseries corresponding to three different growing seasons.

chlamydospora, highly significant associations detected were: control and VV/R4 [χ^2 (1) = 15.8, $p < 0.001$], control and VV/E1 [χ^2 (1) = 10.8, $p < 0.01$], control and VV/E2 [χ^2 (1) = 10.8, $p < 0.01$], control and VV/R1 [χ^2 (1) = 10.8, $p < 0.01$], and control and VV/R5 [χ^2 (1) = 9.43, $p < 0.01$] (Fig. I-8).

I.5. Discussion

It is well established that grapevine plants are mainly infected by fungal pathogens causing GTD through pruning wounds or the root system, with a reduced number of fungal strains that can use both paths of entry. Many studies have tested wound protection by the use of chemical antifungals (Rolshausen *et al.*, 2010; Díaz and Latorre, 2013; Sosnowski *et al.*, 2013), natural antifungal compounds (Cobos *et al.*, 2015) or BCAs (McMahan *et al.*, 2001; Schmidt *et al.*, 2008). **Given the many years that a plant remains in the ground, and the consequent extent and depth of the root development and soil penetration, it is clear that the protection of grapevines from fungi that penetrate through the root system is a hard issue.** Current studies have not gone much beyond the application of BCAs to the root system, mainly different strains of *Trichoderma* sp. (Fourie and Halleen, 2004; Marco and Osti, 2007; Halleen *et al.*, 2010; Kotze *et al.*, 2011; Mutawila *et al.*, 2011).

Actinobacteria play an important role in soil microbial communities representing around 10% of the total soil microbiome (Janssen, 2006). Notable among this microbiome is the genus *Streptomyces* due to its ability to produce a wide range of secondary metabolites, including antifungal compounds (Doumbou *et al.*, 2001; Schrey and Tarkka, 2008). **Several studies indicate that actinobacteria can enter root tissues and establish an endophytic lifestyle with plants** (Doumbou *et al.*, 2001; Schrey and Tarkka, 2008; Li *et al.*, 2012; Schrey *et al.*, 2012). As reported in other plants, **the grapevine root system contains many different endophytic actinobacteria, where *Streptomyces* and *Micromonospora* were the most frequently isolated genera**, representing up to 70.7% of the total isolates. Similarly, when the population of endophytic actinobacteria in *Artemisia annua* was analysed, *Streptomyces* and *Micromonospora* represented the 57.0% of the total isolates identified (Li *et al.*, 2012). Other minority genera like *Nonomuraea* and *Kribbella* have been found in both grapevine plants and *A. annua*, while *Corynebacterium* and *Saccharopolyspora* genera have only been isolated from *V. vinifera*. These data confirm that actinobacteria are frequent endophytes in the root system of both plants.

Streptomyces species identification is a great challenge, further complicated by factors such as the fact that there are a great number of species, many of which are poorly defined. The insufficient resolution of 16S rRNA as a phylogenetic marker, difficulties in

effectively addressing phenotypic differences, and the absence of rapid molecular identification methods applicable to a high number of species also hinder the accurate identification of species of *Streptomyces* (Rong and Huang, 2012; Labeda *et al.*, 2017). Since 16S rRNA sequencing does not provide sufficient resolution for species-level identification of streptomycetes, an MLSA study of the six isolates used in field trials was performed by partially sequencing five housekeeping genes. MLSA has proven to be an efficient molecular tool for the improved taxonomic resolution of *Streptomycetaceae* family (Labeda *et al.*, 2017), as well as for the right identification of *Streptomyces* species from the *S. albidoflavus* (Rong *et al.*, 2009), *S. griseus* (Guo *et al.*, 2008), and *S. hygroscopicus* (Rong and Huang, 2012) clades. These clades include some of the more widely studied species in the genera, many of which produce antibiotics and other industrially and agronomically important secondary metabolites (Rong and Huang, 2012). **In our case, the MLSA allowed us to identify the isolate VV/E5 as belonging to the *S. peucetius* species. Unfortunately, this study did not lead to an accurate identification of the rest of the strains at the species level, probably due to a poor characterization of those closely related phylogenetic strains.** This setback makes it difficult to establish whether these strains are unique to the root system of grapevines or to assess their ability to produce known antifungal compounds. We cannot even rule out that some of them are new species.

The case of *Streptomyces* sp. VV/E1 and VV/R4 is particularly intriguing, since the MLSA study indicated that the two isolates belong to the same species. In fact, these strains share 99.5% homology at the 16S rRNA level. However, they exhibit quite different macroscopic traits when growing on various culture media (Fig. I-3). Remember that *Streptomyces* sp. VV/E1 was isolated as an endophytic strain, whereas *Streptomyces* sp. VV/R4 was isolated from the rhizosphere environment. We can speculate that the observed macroscopic differences could be a putative adaptation to two different lifestyles. However, currently, we do not know for certain whether a particular strain present in the rhizosphere might also develop an endophytic lifestyle.

Recently, there have been several attempts to characterise the endophytic bacterial microbiota of grapevine plants. Andreolli and colleagues (2016) analysed the diversity of bacterial endophytes in 3- and 15-year-old plants. In 3-year-old plants, *Actinobacteria* was the second dominant class (26%), and the genera detected were *Nocardioides*, *Curtobacterium*, *Microbacterium*, *Brachybacterium*, *Kocuria*, and *Micrococcus* (Andreolli *et al.*, 2016). None of these genera were detected in our study, and surprisingly, Andreolli and colleagues did not detect any *Streptomyces* sp. strains. In 15-year-old plants, the population of endophytic actinobacteria was reduced to 5%, with only 2 strains detected, belonging to the *Microbacterium* and *Curtobacterium* genera. Also recently, Rezgui and colleagues analysed the population of endophytic bacteria that inhabit the wood tissues from grapevines of Tunisian vineyards (Rezgui *et al.*, 2016). *Actinobacteria* were hardly detected, and *Curtobacterium* was the only genus found. These differences

can be attributed to many different factors. First, in both studies, endophytes were isolated from vine stems and mature arms, which are aerial habitats far from the ground. In fact, it has been reported that the population of actinobacteria decreases along the plant axis from 20% in the root, to 5% in the graft union, and just 2% in the cane samples (Faist *et al.*, 2016). Second, the observed differences may be attributed to some extent to the different isolation culture media used, and even to the different molecular techniques used in the different works (Faist *et al.*, 2016; Rezgui *et al.*, 2016).

Lately, different studies have focused on the characterisation of the grapevine microbiome (Zarraonaindia *et al.*, 2015; Samad *et al.*, 2017) and vineyard soils (Burns *et al.*, 2015; Vega-Avila *et al.*, 2015), since *Actinobacteria* strains are abundant microorganisms in vineyard environments. For instance, in vineyards of Argentina they represented the second most abundant microorganisms after *Proteobacteria*, with averages of 9.72% of the total microorganisms detected (Vega-Avila *et al.*, 2015). Likewise, *Actinobacteria* have also been reported to be present with different relative abundance in vineyards in California (20.48%) (Burns *et al.*, 2015), New York (5.1%) (Zarraonaindia *et al.*, 2015) and Australia (around 44% in the rhizosphere and around 36% in roots) (Samad *et al.*, 2017). Unfortunately, most of these studies dealing with soil and root microbiomes did not delve deeply into the characterization of the actinobacteria detected beyond the taxonomic class level. Future studies, focussed more specifically on the actinobacteria population detected on the root system of grapevine plants at the genus or even species level, should shed light on many issues that remain unresolved.

Actinobacteria were also easily isolated from young grapevine plant rhizospheres. A screening based on the antifungal activity against fungi causing GTD and YGD resulted in the identification of a total of 29 isolates, all belonging to the genus *Streptomyces*. Furthermore, Loqman and colleagues had isolated strains of *Streptomyces* with antifungal activity against *Botrytis cinerea* from the rhizosphere of grapevine in Morocco (Loqman *et al.*, 2009). **These data indicate that, both rhizosphere and the inner of the grapevine root system, are highly interesting sources for actinobacteria, which may be potential BCAs for the treatment of different vineyard pathologies.**

Black-foot and Petri disease, recognised as prominent causes of YGD, together with the failure of planting material, are some of the most worrisome problems affecting the vine industry since the 1990s (Gramaje and Armengol, 2011). Many factors are involved in this decline. Among all the diseases, infections in the root system of grafted plants in nurseries, caused by fungal trunk pathogens, are the most relevant (Gramaje and Armengol, 2011). The main fungal trunk pathologies associated with YGD are Petri disease, primarily produced by *Ph. chlamydospora* and different *Phaeoacremonium* species, and black-foot disease, mainly produced by species of *Campylocarpon*, *Dactylonectria*, and *Ilyonectria*. These pathogens can infect a significant number of all

the grafted plants produced every year in nurseries worldwide. They are frequently found infecting the rootstock of mother vines (Fourie and Halleen, 2004; Gramaje and Armengol, 2011), but they can also infect grafts once they are planted in an open-root field nursery. This step seems to be particularly critical, since it is possible that soil pathogens can infect grafts through the incipient grapevine root system, necessary for plant survival and development in soil. Hence, when Halleen and colleagues (2003) analysed the presence of fungal pathogens involved in YGD in nurseries during the different steps of the propagation process, they found that less than 1% of the plants were infected with *Cylindrocarpon* spp. before planting in the nursery, whereas 50% or more were infected at the end of the season (Halleen *et al.*, 2003). This result is highly significant, since it demonstrates that **most of the grafted plants in the nurseries can be infected through the roots once they are planted in an open-root field nursery. Our study shows that the application of selected actinobacteria according to their *in vitro* antifungal properties against fungal pathogens involved in YGD, is highly effective in controlling the infection at this step.**

Application of all the selected actinobacteria resulted in a clear reduction in the isolation of fungal pathogens. Furthermore, the application of actinobacterial strains VV/E1, VV/R1, and VV/R4 led to a statistically significant decrease ($p < 0.05$) in the presence of three types of pathogenic fungi analysed, *Dactylonectria* sp.-*Ilyonectria* group, *Ph. chlamydospora*, and *P. minimum*. **None of the actinobacteria had a significant effect, either positive or negative, on the growth of surviving plants.** However, whereas the average of the mortality rate in untreated plants (negative control) in the 3-field study was 28.4%, the mortality rate was significantly reduced when the actinobacteria VV/R1 and VV/R4 were applied to the grafts.

Graft failure is a serious problem for nurseries since, on average, 30 to 60% of the grafts taken to the field resulted in failure. Many different factors can contribute to graft failure, including abiotic causes like improperly healed rootstock, disbudding sites and graft unions, or improper storage and management of the plant material used to obtain the grafts (Gramaje and Armengol, 2011). However, despite the large number of studies showing the presence of fungal pathogens in nursery plants at the end of the propagation process, to date, no clear data are available that correlate the presence of pathogens with mortality levels. In fact, after analysing the presence of fungal pathogens in plants with failed graft unions, (Rumbou and Rumbos, 2001) concluded that those pathogens were not the cause of YGD. **Our data suggest a putative relationship between the number of fungal pathogens and the mortality rate, since the application of actinobacterial strains VV/R1 and VV/R4 led to both a significant reduction in the number of fungal pathogens detected and a significant reduction in the mortality rate.** Interestingly, the dead plants at the end of the field trials showed a very small, almost nonexistent root system. We can speculate that the early infection with fungi causing YGD may interfere with the development of a potent root system, resulting in a premature graft death.

Clearly, graft failure is a complex problem with many different factors, but in view of these results, it seems that the presence of fungal pathogens could significantly contribute to this problem.

Finally, in recent years, it has become clear that endophytic organism, particularly actinobacteria, can be a significant reservoir of genetic diversity as well as an important source for the discovery of novel bioactive secondary metabolites (Joseph *et al.*, 2012), which can help control many different crop pathologies. **This work represents one of the first reports on the characterisation of the endophytic actinobacterial population in the root system of young grapevines at the levels of genus and species.** Several of the isolates tested had a beneficial impact when applied to grafts in nurseries, resulting in a significant decrease in both mortality and infection rates by fungal pathogens involved in YGD. Therefore, the development of technologies that are able to limit the infection of grapevines through the root system is of great interest in the wine industry, and the application of these selected actinobacteria might help to diminish this problem. **This novel, green, and promising methodology could be easily integrated into the industrial propagation of grapevines until the final aim to reduce the rate of fungal infections through the root system.**

I.6. References

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Figure SI-1 Neighbor-Joining tree showing phylogenetic relationships based on 16S rRNA gene sequences of culturable endophytic (VV/E1 to VV/E58) and rhizosphere actinobacterial (VV/R1 to VV/R29) strains with antifungal activity isolated from the root system of grafted grapevines. Isolates characterised in this work are in bold. Strains tested in field assays in experimental nurseries are highlighted in bold, underlined and shaded. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are given in the nodes. Bar, 1% difference in nucleotide sequence. Accession numbers are indicated between parentheses.

A

B

C

D

E

G

Fig. SI-2 A-I. MLSA analysis. *Streptomyces* phylogenetic tree inferred from concatenated partial sequences of the housekeeping genes (atp D, gyr B, rec A, rpo B and trp B) of endophytic (VV/E1, VV/E2, VV/E5) and rhizosphere (VV/R1, VV/R4, VV/R5) isolates with type strains obtained from the ARS Microbial Genomic Sequence Database server (<http://199.133.98.43>). The tree was constructed using the Maximum-Likelihood based on Kimura two-parameters model (nucleotide-based analysis). The trees were subjected to 1000 bootstrap replications and values (expressed as percentages) greater than 50% are given in the nodes. Subclades containing the isolated strains (bold highlighted) and related type strains are shown shaded. Bar scale reflects number of substitutions per site.

Fig SI-3. Macroscopic and sporulation differences for *Streptomyces* sp. VV/E2, VV/E5, VV/R1 and VV/R5 strains when they grow in different culture media. *Streptomyces* sp. VV/E5 does not sporulate in any of this media, so other culture media were tested. This strain sporulate in MEA medium.

Table SI-1 Composition of culture media used for testing sporulation in *Streptomyces* strains.

Media	Composition
2xTY (Sambrook <i>et al.</i> , 1989)	16.0 g/L tryptone 10.0 g/L yeast extract 5.0 g/L NaCl 20.0 g/L agar (pH 6.5)
Czapek agar (Czapek F, 1902)	30.0 g/L sucrose 4.0 g/L NaNO ₃ 1.0 g/L K ₂ HPO ₄ 1.0 g/L MgSO ₄ ·7H ₂ O 0.01 g/L FeSO ₄ ·7H ₂ O 20.0 g/L agar (pH 6.5)
ISP1 (Tryptone-yeast extract broth) (Shirling and Gottlieb, 1966)	5.0 g/L pancreatic digest of casein 3.0 g/L yeast extract 20.0 g/L agar (pH 7.2 ± 0.2)
ISP2 (Yeast extract-malt extract) (Shirling and Gottlieb, 1966)	4.0 g/L yeast extract 10.0 g/L malt extract 4.0 g/L dextrose 20.0 g/L agar (pH 7.2 ± 0.2)
ISP3 (Oatmeal agar)** (Shirling and Gottlieb, 1966)	20.0 g/L oatmeal 18.0 g/L agar 10.0 mL/L trace solution* (pH 7.2 ± 0.2)
ISP4 (Inorganic salts-starch agar) (Shirling and Gottlieb, 1966)	10.0 g/L soluble starch 1.0 g/L Na ₂ HPO ₄ 1.0 g/L MgSO ₄ ·7H ₂ O 1.0 g/L NaCl 2.0 g/L (NH ₄) ₂ SO ₄ 2.0 g/L CaCO ₃ 20.0 g/L agar 10.0 mL/L trace solution* (pH 7.2 ± 0.2)
ISP5 (Glycerol-asparagine agar) (Shirling and Gottlieb, 1966)	1.0 g/L L-asparagine (anhydrous base) 10.0 g/L glycerol 1.0 g/L K ₂ HPO ₄ (anhydrous basis) 10.0 mL/L trace solution* 20.0 g/L agar (pH 7.0 - 7.4)

* **Trace solution:** consists of a small amounts of different salts to improve the composition of several ISP media and, therefore, increase the actinobacteria growth. The composition of this solution is: 1.0 g/L FeSO₄·7H₂O; 0.1 g/L MnCl₂·4H₂O; 0.1 g/L ZnSO₄·7H₂O. It is sterilized on autoclave for 15 min / 121°C and added to the corresponding media when use.

** In order to prepare ISP3 medium, 20.0 g of oatmeal were boiled in 1 L of dH₂O for 20 min and filter through cotton cloth. After all, it was necessary to add dH₂O to restore the volume to 1 L. Then pH was adjusted to 7.2 with 1 N NaOH.

Chapter I

Table SI-1 (Continued)

Media	Composition
ISP6 (Peptone-iron agar) (Shirling and Gottlieb, 1966)	15.0 g/L peptone 5.0 g/L protease peptone 0.5 g/L ferric ammonium citrate 1.0 g/L sodium glycerophosphate 0.08 g/L sodium thiosulphate 15.0 g/L agar (pH 6.7 ± 0.2)
ISP7 (Tyrosine agar) (Shirling and Gottlieb, 1966)	15.0 g/L glycerol 0.5 g/L L-tyrosine 1.0 g/L L-asparagine 0.5 g/L K ₂ HPO ₄ (anhydrous basis) 0.5 g/L MgSO ₄ ·7H ₂ O 0.5 g/L NaCl 0.01 g/L FeSO ₄ ·7H ₂ O 10.0 mL/L trace solution* 20.0 g/L agar (pH 7.2 - 7.4)
MBA (Modified Bennet's Agar) (Jones, 1949)	1.0 g/L yeast extract 1.0 g/L beef extract 2.0 g/L N-Z-amine-A 10.0 g/L glycerol 20.0 g/L agar (pH 7.3)
MEA (Malt extract agar) (Commercial media purchased from Merck)	30.0 g/L malt extract 5.0 g/L microbiological peptone 15.0 g/L agar
MEY (Malt-yeast extract) (Kieser <i>et al.</i> , 2000)	10.0 g/L maltose 4.0 g/L yeast extract 0.01 g/L CoCl ₂ 20.0 g/L agar (pH 7.0)
TBO (Tomato paste-oatmeal agar) (Higgins <i>et al.</i> , 1974)	20.0 g/L tomato paste 20.0 g/L oat flakes 25.0 g/L agar (pH 6.5)

Among all the tested media, the selected ones for the sporulation of the different actinobacterias were the following: Czapek agar was used for the sporulation of *Streptomyces* sp. VV/E1, MEA for *Streptomyces* sp. VV/E5 and TBO for *Streptomyces* sp. VV/R1, VV/R4 and VV/E2. *Streptomyces* sp. VV/R5 did not sporulate in any of the tested media.

*Trace solution: consists of a small amounts of different salts to improve the composition of several ISP media and, therefore, increase the actinobacteria growth. The composition of this solution is: 1.0 g/L FeSO₄·7H₂O; 0.1 g/L MnCl₂·4H₂O; 0.1 g/L ZnSO₄·7H₂O. It is sterilized on autoclave for 15 min / 121°C and added to the corresponding media when use.

Table SI-2. Primers used for MLSA analysis. Five pair of primers were used for the amplification of five housekeeping genes (*atpD*, *gyrB*, *recA*, *rpoB* and *trpB*) of *Streptomyces* strains (VV/E1, VV/E2, VV/E5, VV/R1, VV/R4 and VV/R5).

Gene	Primer	Sequence (5'-3')	Amplified size (bp)	T_m (°C)	Reference
<i>atpD</i>	atpDPF atpDPR	GTCGGCGACTTCACCAAGGGCAAGGTGTTCAACACC GTGAACTGCTTGGCGACGTGGGTGTTCTGGGACAGGAA	998	63	Guo <i>et al.</i> , 2008
<i>gyrB</i>	gyrBPFA gyrBPRA	CTCGAGGGTCTGGACGCGGTCCGCAAGCGACCCGGTATGTA GAAGGTCTTCACCTCGGTGTTGCCCAGCTTCGTCTT	1060	62	Rong <i>et al.</i> , 2009
<i>recA</i>	recAPF recAPR	CCGCRCTCGCACAGATTGAACGSCAATTC GCSAGGTCGGGGTTGTCCTTSAGGAAGTTGCG	913	60	Guo <i>et al.</i> , 2008
<i>rpoB</i>	rpoBPF rpoBPR	GAGCGCATGACCACCCAGGACGTCGAGGC CCTCGTAGTTGTGACCCTCCACGGCATGA	994	65	Guo <i>et al.</i> , 2008
<i>trpB</i>	trpBPF trpBPR	GCGCGAGGACCTGAACCACACCGGCTCACACAAGATCAACA TCGATGGCCGGGATGATGCCCTCGGTGCGCGACAGCAGGC	822	66	Guo <i>et al.</i> , 2008

Table SI-3 GenBank accession numbers of DNA sequences from the endophytic and rhizosphere *Streptomyces* sp. strains obtained in the MSLA analysis.

Isolate	GenBank accession no.				
	<i>atpD</i>	<i>gyrB</i>	<i>recA</i>	<i>rpoB</i>	<i>trpB</i>
VV/E1	MF437320	MF437326	MF437332	MF437338	MF437344
VV/E2	MF437321	MF437327	MF437333	MF437339	MF437345
VV/E5	MF437322	MF437328	MF437334	MF437340	MF437346
VV/R1	MF437323	MF437329	MF437335	MF437341	MF437347
VV/R4	MF437324	MF437330	MF437336	MF437342	MF437348
VV/R5	MF437325	MF437331	MF437337	MF437343	MF437349

Table SI-4 Number of fungal pathogen isolated from grafted plants in experimental nurseries during three growing seasons after treating them with different endophytic and rhizosphere isolates, comparing to untreated plants (Control).

Treatment	<i>Dactylonectria– Ilyonectria</i> sp.	<i>P. minimum</i>	<i>Ph. chlamydospora</i>	Total fungal pathogens isolated
Control (untreated plants)	54	18	64	136
VV/E1	6	2	24	32
VV/E2	26	6	20	52
VV/E5	24	6	30	60
VV/R1	18	4	26	48
VV/R4	12	2	14	28
VV/R5	18	4	22	44

Data shown correspond to the analysis of 3 batches of 25 plants in 3 experimental nurseries during three different growing seasons.

CHAPTER II: DEVELOPING TOOLS FOR THE EVALUATION OF INOCULATION METHODS OF BIOCONTROL *STREPTOMYCES* SP. STRAINS INTO GRAPEVINE PLANTS

Chapter corresponding to the publication: **González-García S.**, Álvarez-Pérez J.M., Sáez de Miera L.E., Cobos R., Ibáñez A., Díez-Galán A., Garzón-Jimeno E., Coque J.J.R. 2019. Developing tools for evaluating inoculation methods of biocontrol *Streptomyces* sp. strains into grapevine plants. PLOS ONE 14, e0211225. <https://doi.org/10.1371/journal.pone.0211225>

II.1. Abstract

The endophytic *Streptomyces* sp. VV/E1, and rhizosphere *Streptomyces* sp. VV/R4 strains, isolated from grapevine plants were shown to reduce the infection rate of fungal pathogens involved in young grapevine decline. These actinobacteria were implanted in young grafted grapevines.

For this purpose, an unequivocal detection method for both actinobacteria was developed. We cloned fragments from randomly amplified polymorphic DNA (RAPD), and developed two stably diagnostic sequence-characterised amplified region (SCAR) markers of 182 and 160 bp for the VV/E1 and VV/R4 strains, respectively. The SCAR markers were not found in another 50 actinobacterial strains isolated from grapevine plants. Quantitative real-time PCR protocols based on the amplification of these SCAR markers were used for the detection and quantification of both strains in plant material. Two different inoculation methods were evaluated: perforation of the rootstock followed by injection of the microorganisms, or soaking the root system in a bacterial suspension. Both methods were combined with a booster treatment consisting of the direct addition of a bacterial suspension to the soil near the root system. Analysis of uprooted plants showed that those inoculated by injection exhibited the highest rate of colonization, with evidence of bacterial movement through both the upper and lower zones of the rootstock. Immersion method was also effective indicating that the actinobacteria likely come into the plant through the root system. Moreover, this was the preferably inoculation method since its reliable application in the industrial process. In contrast, direct addition of either strains to the soil did not lead to reliable colonisation.

Analysis of *Streptomyces* sp. VV/E1 along the grapevine plant led to the conclusion that this endophytic strain could move through the plant and reach the second petiole, but it could not be detected on the second leaf. Moreover, microscopy analysis showed the presence of *Streptomyces* sp. VV/E1 and VV/R4 strains in both the xylem vessels and on the root surface, respectively.

This study has developed molecular tools for evaluating different methods of grapevine plants inoculation with selected *Streptomyces* sp. strains which protect them from fungal infection that enter through their root system. **This research is of great applied interest since these tools could easily be established in nurseries to produce grafted grapevine plants that provide protection against fungal pathogens.** Finally, this methodology might also be applied to other vascular plants for their colonisation with beneficial biological control agents.

II.2. Introduction

Young grapevine decline (YGD) is a major threat to the wine and grape industry, especially since the 1990s when it entered a period of rapid expansion (Gramaje and Armengol, 2011; Álvarez-Pérez *et al.*, 2017). Many factors are involved in YGD, although it is accepted that fungal pathogens responsible for grapevine trunk diseases (GTDs) are one of the major causes of this syndrome (Gramaje and Armengol, 2011; Álvarez-Pérez *et al.*, 2017). Most of these pathogens can penetrate the plant through the root system (Gramaje and Armengol, 2011; Fourie and Halleen, 2004; Bertsch *et al.*, 2013), and therefore, the presence of endogenous pathogens in grafted plants (planting material) is also highly frequent. Accordingly, **there has been a growing demand among grape growers for methods to control fungi causing GTDs that infect the plant through the root system, and to reduce the infection rate of planting material produced in nurseries.**

As it was seen in chapter I, **several actinobacteria strains isolated and characterised from the rhizosphere or the inner tissues of the root system of young grapevine plants, exhibited antifungal activity against fungi that cause YGD** (Álvarez-Pérez *et al.*, 2017). Field trials carried out in a nursery led to the identification of one endophytic strain, *Streptomyces* sp. VV/E1, and two rhizosphere isolates, *Streptomyces* sp. VV/R1 and *Streptomyces* sp. VV/R4, which significantly reduced the infection rate produced by the fungal pathogens *Dactylonectria* sp., *Ilyonectria* sp., *Ph. chlamydospora* and *P. minimum*, involved in YGD (Álvarez-Pérez *et al.*, 2017).

In the current study, **SCAR (sequence characterized amplified region) markers and qPCR protocols for the *Streptomyces* sp. VV/E1, and *Streptomyces* sp. VV/R4 strains were developed to specifically detect and quantify their presence in plant material.** These molecular tools allowed us to analyse the effectiveness of different inoculation methods of young grapevine plants with these selected strains, as well as to determine the presence of these strains inside the root system of the inoculated plants.

II.3. Material and Methods

II.3.1. Microbial strains, culture media and growth conditions

Streptomyces strains VV/E1 and VV/R4 were isolated from the root system (endophytic) and the rhizosphere of young grapevine plants respectively (Álvarez-Pérez *et al.*, 2017). Both strains were grown in tryptic soy broth (TSB) medium (Sigma-Aldrich) at 220 rpm / 3 days / 30°C.

II.3.2. Isolation of genomic DNA

Genomic DNA of both strains was isolated from liquid cultures grown in TSB at 220 rpm / 3 days / 30°C following the protocol of Hopwood *et al.*, (1985) (See section I.3.2.1).

II.3.3. Sequence Characterized Amplified Region (SCAR) markers development

II.3.3.1. Random Amplified Polimorphic DNA (RAPD) amplification

A thirteen random primers set 10-mer long each, were used for developing RAPD-PCR protocols in order to identify RAPD polymorphisms of ten different actinobacteria (VV/E1-VV/E5 and VV/R1-VV/R5) (Álvarez-Pérez *et al.*, 2017) according to their electrophoretic band patterns. Primers used in this assay are shown in Table SII-1.

Amplification reactions were developed in 1x paq5000 buffer with 625 nM primer, 0.125 nM dNTP mix, 1U Paq5000 DNA polymerase and 30 ng template DNA. ddH₂O was used to bring the reaction volume to 20 µL. PCR amplification was carried out in 0.2 mL sterile tubes using a Mastercycler Gradient (Eppendorf) thermocycler. PCR conditions for primers R1, R3, OPA2, OPA9 and OPA10 were: 94°C for 3 min; 40 cycles of 94°C for 60 s, 40°C for 60 s, 72°C for 90 s; and a final extension of 72°C for 10 min. PCR conditions for the rest of the primers were: 94°C for 3 min; 45 cycles of 94°C for 30 s, 36°C for 30 s, 72°C for 60 s; and a final extension of 72°C for 10 min. Tubes were kept at 4°C until the reactions were analysed in 1.5% (w/v) agarose gels and photographed using VWR GenoSmart equipment (VWR).

II.3.3.2. Cloning and sequencing of RAPD fragments

Unique bands for each strain were excised from the agarose gel and purified by freeze-squeeze using a modified protocol of Tautz and Renz (1983), where glass wood was replaced by hydrophilic cotton due to its high toxicity. Freeze-squeeze protocol consisted of freeze the excised band from agarose gel O/N / -80°C, centrifuge at 11,000 rpm / 7 min / RT and a phenol-CIA extraction. Nucleic acid precipitation was carried out by adding 0.1 volumes of 3 M sodium acetate pH 5.2 and 3 volumes of absolute ethanol pre-cooled at -20°C. Tubes were carefully mixed by inversion and incubate for 2 h / -20°C before centrifuge at 14,000 rpm / 30 min / 4°C. Precipitated DNA was washed with 70% ethanol, dried on a vacuum system and re-suspended in

15.0 μL of ddH₂O. DNA concentration was measured on NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific).

Purified DNA fragments were ligated into pBluescript II KS (+) plasmid (Agilent Technologies) using T4 DNA ligase (Fermentas). Ligation reaction was performed in a reaction mixture containing 1x T4 ligase buffer (Fermentas), 5 ng/ μL pBluescript KS II (+) plasmid digested with EcoRV restriction enzyme (Fermentas), 200 ng/ μL purified DNA, 1/10 volume PEG4000 (Fermentas), 1U T4 DNA ligase (Fermentas). ddH₂O was used for bring the reaction volume to 10 μL .

Ligation reaction was transformed in *E. coli* DH5 α competent cells (Thermo Fisher Scientific) by mixing 100 μL of competent cells with the ligation reaction. The mix was then incubated for 30 min on ice before carrying out a heat shock for 45 s / 42°C in a water bath without shaking and later cooled on ice for 2 min. 250 μL of pre-warmed (37°C) SOC medium^{II.1} (Hanahan, 1983) were added to the mix and incubated at 225 rpm / 1 h / 37°C.

The transformation mix was spread on LB agar^{II.2} plates (Miller, 1972) supplemented with 50 $\mu\text{g}/\text{mL}$ ampicillin (Sigma), 50 $\mu\text{g}/\text{mL}$ X-Gal (Thermo Fisher Scientific) and 50 $\mu\text{g}/\text{mL}$ IPTG (Fermentas) and incubated O/N / 37°C. Recombinant clones were selected by a classical blue-white screening. White colonies were checked for the presence of the correct insert using a double digestion with EcoRI and SalI restriction enzymes (EURx), which cut immediately outside of the inserted fragment. The DNA from positive clones was then sequenced using universal primers T7 (5'-TAATACGACTCACTATAGGG-3') and M13 reverse (5'-CCTTTGTCGATACTGGTAC-3') (Yang *et al.*, 2013). Sequence data were analysed using BLASTx (basic local alignment search tools programme (<https://blast.ncbi.nlm.nih.gov/>)).

II.3.3.3. Amplification of Sequence-Characterized Amplified Region (SCAR) markers

The nucleotide sequence of each cloned RAPD fragments was used to design pairs of SCAR primers targeting either poorly conserved regions or intergenic regions. The pair of primers SCAR.E1_fw (5'-GGTTCACGGTATCTGTTTACTCACC-3') and SCAR.E1_rv (5'-GTTGGTTGAACCCTTCTTCCG-3') were used for the amplification of *Streptomyces* sp. VV/E1 isolate, while primers SCAR.R4_fw (5'-CCGAAGGGCTC TCTGAGTTGC-3') and SCAR.R4_rv (5'-CTTCGCCGCTGAGGCACG-3') were used for the specific amplification of *Streptomyces* sp. VV/R4 strain.

^{II.1} **SOC medium:** 20.0 g/L tryptone; 5.0 g/L yeast extract; 10.0 mM NaCl; 2.5 mM KCl; 10.0 mM MgCl₂, 10.0 mM MgSO₄; 20.0 mM glucose; pH 6.7 - 7.0

^{II.2} **LB (Luria-Bertani) agar:** 10.0 g/ tryptone; 5.0 g/L yeast extract; 10.0 g/L NaCl; 20 g/L agar; pH 7.5

SCAR amplification from genomic DNA samples was performed in a reaction containing 1x paq5000 buffer, 0.5 μ M each of the primers, 0.1 mM dNTP mix, 1U paq5000 DNA polymerase, and 30 ng genomic DNA. ddH₂O was used to bring the reaction volume to 25 μ L. PCR conditions were: 95°C for 2 min; 30 cycles of 95°C for 30 s, melting temperature (T_m) for 30 s, 72°C for 40 s; with a final extension of 72°C for 7 min. The T_m for SCAR.E1_fw/SCAR.E1_rv primers was 60°C, and 63°C for the SCAR.R4_fw/SCAR.R4_rv primers. The annealing temperature was selected performing a gradient PCR with temperatures ranged from 60 to 65°C for *Streptomyces* sp. VV/E1 and between 56 and 63°C for *Streptomyces* sp. VV/R4. Tubes were kept at 4°C until analysis on 1.5% (w/v) agarose gels. In order to check the specificity of the 2 sets of primers and the SCAR marker developed, genomic DNA from 50 actinobacterial strains isolated from the rhizosphere and the root systems of young grapevine plants (Álvarez-Pérez *et al.*, 2017) was also used as template in a conventional PCR under the same conditions.

II.3.4. Verification of the number of copies of the SCAR sequences into the *Streptomyces* sp. genomes by DNA hybridization

II.3.4.1. Probe labelling

Labelling reaction was performed following the protocol of Barreiro (2004). For this, 1.0 μ g DNA was denaturalized by heating for 10 min / 95°C. Then, it was quickly cooled on ice to avoid the DNA re-naturalization. Probe labelling was carried out with the hapten digoxigenin (Roche). The reaction mix was performed using 1x DIG-DNA labelling mix (Roche), 1x hexanucleotides mix (Roche), 1x reaction buffer for Klenow fragment (Fermentas), 1U Klenow polymerase (Fermentas) and 30 ng DNA in a final volume of 20 μ L. Samples were incubated O/N / 37°C. Then, labelling reaction was stopped by adding 0.25 mM EDTA pH 8.0 and kept protected from light at -20°C until use.

To check the label process, seven 5-fold serial dilutions were performed as follows: 1 μ L of each dilution was applied over the nylon membrane (HybondTM-N+ membrane optimised for nucleic acid transfer (GE Healthcare)) and DNA was fixed into the membrane by the application of UV light using a UV-Stratalinker (Stratagene).

Membrane verification was performed following a modified protocol of Godio Fernández (2007). In short, first the membrane was equilibrated with 30 mL buffer I^{II.3} for 1 min, under constant agitation, and blocked using 50 mL buffer II^{II.4} (30 min) under constant agitation. Then, membrane was placed in a new hybridization bag containing 15 mL of antibody solution^{II.5} and

^{II.3} **Buffer I:** 100 mM maleic acid; 150 mM NaCl, pH 7.5

^{II.4} **Buffer II:** buffer I supplemented with 1% blocking reagent (Roche)

^{II.5} **Antibody solution:** buffer II supplemented with a 1:15,000 diluted solution of Anti-Digoxigenin antibodies (Anti-Digoxigenin-AP Fab fragments) (Roche)

incubated for 30 min under constant agitation. After, the membrane was washed twice with 30 mL of buffer I for 15 min under constant agitation, and with 40 mL of buffer III^{II.6} for 2 min under the same conditions. Finally, the membrane was placed into a new hybridization bag and 10 mL of detection solution^{II.7} was added and incubated for 20 - 30 min under constant agitation (protected from light), before stopping the detection reaction by washing the membrane with dH₂O. Dark spots appeared on the membrane with lower intensity in serial dilutions.

II.3.4.2. DNA transfer to a membrane

The transfer of the digested DNA to a membrane is known as Southern blot (Southern, 1975). To carry out this technique, total genomic DNA was digested with several restriction enzymes [ApaI (Fermentas), BamHI (Fermentas), BstXI (Fermentas), PvuII (Fermentas), SalI (Fermentas), SmaI (Fermentas) and XhoI (EURx)], which did not cut inside the selected SCAR fragments. To determine the restriction enzymes that should be used, Restriction Mapper tool was used (<http://www.restrictionmapper.org/>).

The digested DNA was subjected to electrophoresis in a 0.8% (w/v) agarose gel, visualised by staining in ethidium bromide and photographed with a ruler in order to be able to measure the distance between the wells and the different bands (which will allow us to determine the size of the band that hybridize with the probe). Then, a nylon membrane (HybondTM-N+ membrane optimised for nucleic acid transfer (GE Healthcare) was cut 1 cm bigger (in each slide) than the gel and wet with transfer solution (20x SSC solution)^{II.8}. Next, the membrane was placed into the transfer system according to the manufacturer's instructions, the positions of the wells were marked into the membrane, and the vacuum system was connected. The gel was covered with different solutions in succession (all the solutions were added when needed to avoid that the gel dried up). First solution was depurination solution^{II.9} for 15 -20 min. Then, denaturation solution^{II.10} for 15 min - 20 min. This solution was replaced by neutralizing solution^{II.11} and incubate for 20 - 25 min. Finally, neutralizing solution was replaced by transfer solution (20x SSC) and incubated for 1 h. After the transfer process, the membrane was recovered and the DNA was fixed into the membrane by the application of three pulses of 120,000 μ J of UV light using a UV-Stratalinker.

In order to check that the transfer process worked, the agarose gel was recovered and visualised by staining again with ethidium bromide. No bands should appear on the gel.

^{II.6} **Buffer III:** 0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl; 500 mM MgCl₂·6H₂O

^{II.7} **Detection solution:** buffer III supplemented with 350 μ g/mL NTB (Roche) and 175 μ g/mL BCIP (Roche)

^{II.8} **20x SSC solution:** 3 M NaCl; 0.3 M sodium citrate; pH 7.0

^{II.9} **Depurination solution:** 0.25 M HCl

^{II.10} **Denaturation solution:** 1.5 M NaCl; 0.5 N NaOH

^{II.11} **Neutralizing solution:** 1.5 M NaCl; 0.05 M Tris-HCl, pH 7.2; 1 mM EDTA

II.3.4.3. DNA hybridization and detection

For the hybridization process, the membrane was wet with 20x SSC solution, placed into a hybridization bag containing 25 mL of hybridization solution^{II.12} and incubated in a water bath for 1 h / 42°C under constant agitation. Meanwhile, labelled probe was boiled for 10 min / 95°C in order to denaturalize the DNA and subsequently cooled on ice. Next, hybridization solution was replaced by 40 mL of the same solution supplemented with the denaturalized labelled probe and incubated at least for 8 h under the same conditions.

Once hybridization process was performed, the membrane was washed twice with 15 mL of wash buffer I^{II.13} for 15 min under constant agitation, and then washed twice with 15 mL wash buffer II^{II.14} for 25 min under the same conditions. Next, the same protocol detailed for the probe labelling (see section II.3.4.1) was followed (Godio Fernández, 2007). After the detection reaction, if the DNA sequence had a single copy in the genome, a single band for each restriction enzyme should be detected.

II.3.5. Quantitative real-time PCR (qPCR) assays

A quantitative SYBR Green real-time PCR (qPCR) technique (Bustin *et al.*, 2009) was carried out based on the developed SCAR primers. The reaction mixture contained 1x SYBR Green master mix (Takara), 0.2 µM of each primer and 5 µL bacterial DNA isolated from axenic cultures of VV/E1 and VV/R4 strains as templates. Nuclease-free water was used to bring the reaction volume to 20 µL. VV/E1 bacterial DNA, ranging in amounts from 5.65x10⁶ fg to 5.65x10² fg per reaction, and 5.2x10⁶ fg to 5.2x10² fg per reaction for VV/R4 strain, were used. Amplification was performed in qPCR 96-well plates (Agilent Technologies), and run in the Mx3005P qPCR System (Agilent Technologies). Thermal conditions were: 95°C for 2 min, 40 cycles of 95°C for 20 s, elongation at T_m for 20 s, followed by 1 cycle of 95°C for 1 min, 55°C for 30 s and 95°C for 30 s. T_m was the same as for the conventional PCR (section II.3.3.3). All analysis were performed as three independent experiments with three replicates for each dilution.

II.3.5.1. Sensitivity of actinobacterial detection in grapevine wood by qPCR

Detection and quantification of actinobacteria in grapevine plants was performed by quantitative real-time PCR (qPCR) as described above. Sensitivity was determined by calculating standard curves using 10-fold serial dilutions of genomic DNA: 5.65x10⁶ fg to 5.65x10² fg were used for *Streptomyces* sp. VV/E1, while 5.2x10⁶ fg to 5.2x10² fg were used for *Streptomyces* sp. VV/R4. Nuclease free water was used as non-template control (NTC).

^{II.12} **Hybridization solution:** 5x SSC; 1% blocking reagent (Roche); 30% formamide; 0.1% lauroylsarcosine; 0.02% SDS

^{II.13} **Wash buffer I:** 2x SSC; 0.1% SDS

^{II.14} **Wash buffer II:** 0.1x SSC; 0.1% SDS

II.3.5.2. Limit of quantification

The limit of quantification (LOQ) was estimated according to the coefficient of variation (*CV*) [$CV = 100 \times (SD/\text{mean value})$] of five replicates of genomic DNA from both *Streptomyces* strains (same concentrations of genomic DNA as for the construction of standard curves in section II.3.5.1). The smallest DNA concentration with a *CV* below 35% was assumed to be the LOQ (Forootan *et al.*, 2017).

II.3.5.3. Interference of genomic DNA from grapevine plants

To ascertain whether the designed sets of primers for the amplification of SCAR markers were reliable for the detection of *Streptomyces* strains without the interference of grapevine genomic DNA, qPCR assays were carried out in the presence of grapevine genomic DNA. Each PCR mixture contained 5.2 ng of *Streptomyces* sp. VV/E1 genomic DNA or 5.7 ng of *Streptomyces* sp. VV/R4 genomic DNA, 10-fold serially diluted up to 10^{-5} . The same reactions were supplemented with 5.2 and 5.7 ng of grapevine genomic DNA. The final ratio of actinobacteria-to-grapevine genomic DNA ranged from 1:1 to 1:100,000. The experiments were conducted twice with three replicates each.

II.3.5.4. Calculation of the correlation between quantity of genomic DNA from *Streptomyces* sp. VV/E1 and VV/R4 strains and number of cells

Once DNA from both strains was quantified by qPCR, the amount of detected DNA was correlated with cell number as follows. Both SCAR markers were amplified by conventional PCR, purified by NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and quantified on NanoDrop.

Since the size and nucleotide composition of each amplicon are known, the number of copies of each DNA template (SCAR copy numbers) were easily estimated using an on-line calculator (<http://cels.uri.edu/gsc/cndna.html>). PCR products were quantified and serially 10-fold diluted to be used as templates to generate standard curves, one for each SCAR marker (Fig. SII-1A). Genomic DNA quantified on NanoDrop was also 10-fold serially diluted and the copy number of each SCAR was estimated using the previous standard curves (Fig. SII-1B). Therefore, the number of cells (cells/mg grapevine wood) could be directly estimated through an additional correlation between the quantity of genomic DNA (previously estimated by qPCR method) and the corresponding number of copies of each amplicon (Fig. SII-1C). qPCR conditions were the same as described above.

II.3.6. Inoculation of grapevine plants with *Streptomyces* strains

II.3.6.1. Plants for testing the colonization of the root system by *Streptomyces* strains using different inoculation methods

Sixty 1-year-old *Vitis vinifera* (cv. Tempranillo) plants grafted on Richter 110 (110R) rootstock were supplied by Viveros Villanueva Vides S.L. (Larraga, Spain). Their root systems were uniformly trimmed to a length of 10 cm, inoculated with *Streptomyces* sp. VV/E1 or *Streptomyces* sp. VV/R4 strains (Álvarez-Pérez *et al.* 2017), and planted in plastic pots containing 50 L of Compo Sana 20-20-30 commercial substrate mixture (Compo Sana), which is suitable for grapevines. Potted vines were grown outdoors under shade-cloth and regularly irrigated with tap water. Both inoculation and experimental potted vineyards were performed in the facilities of the Instituto de Investigación de la Viña y el Vino (42°35'01.0"N 5°35'20.2"W).

For each *Streptomyces* sp. strain, 6 different batches were processed as follows: batch 1 consisted of non-inoculated plants (control); batch 2 corresponded to plants inoculated by immersion of the root system for 24 hours in a bacterial suspension (5×10^5 cells/mL); batch 3 was identical to batch 2, but an additional booster treatment was applied 30 days after planting. The booster treatment was added to the soil into a 15 cm hole, made using a hand drill in parallel to the rootstock. In this way the bacteria (10^7 cells; 20 mL of 5×10^5 cells/mL) were applied directly to each pot in the proximity of the root system. Batch 4 plants were inoculated by direct injection of bacteria into the rootstock (50 μ L of a bacterial culture containing 5×10^5 cells) 3 cm above the root insertion point (RI) via a pipette placed in a drill hole (5 mm deep) (Fig II-1A). Batch 5 was like batch 4 plus the additional booster treatment (in a similar way to treatment 3). Batch 6 plants were an additional control, where plants were not inoculated by either method, although the same booster treatment was applied 30 days after planting in order to observe the effect of direct actinobacteria addition to the soil. Plants were uprooted and analysed 180 days after being planted in pots.

Fig. II-1 Inoculation of grapevine plants in the rootstock by injection. Four 1.0 cm cylinders (Z1/RI, Z2, Z3 and Z4) were analysed once the plants were uprooted **(A)**. Zones analysed in plants inoculated by immersion in a cell suspension, defined as root insertion point (RI) and root system (RS) **(B)**. Note that Z1/RI and RI zones in plants inoculated by injection and immersion respectively are equivalent for analytical purposes.

II.3.6.2. Plants for the analysis of the movement of *Streptomyces* sp. VV/E1 through grapevine plants

Three 1-year-old *Vitis vinifera* (cv. Tempranillo) plants grafted on Richter 110 (110R) rootstock were supplied by Viveros Villanueva Vides S.L. (Larraga, Spain). As in the previous section, their root systems were uniformly trimmed to a length of 10 cm, inoculated by immersion of the root system for 24 hours in a bacterial suspension (5×10^5 cells/mL) with *Streptomyces* sp. VV/E1 (Álvarez-Pérez *et al.*, 2017), and planted in plastic pots containing 50 L of Compo Sana 20-20-30 commercial substrate mixture (Compo Sana). Potted vines were grown outdoors under shade-cloth and regularly irrigated with tap water.

Two different batches were processed. Batch 1 consisted of non-inoculated plants (control) and batch 2 corresponded to plants inoculated with the actinobacteria. In each plant, 6 different zones were analysed in order to detect the movement of the actinobacteria inside the plant. Plants were uprooted and analysed 90 days after being planted in pots.

II.3.7. Analysis of the experimental vines planted in pots: detection and quantification of *Streptomyces* sp. VV/E1 and VV/R4 strains in wood samples.

II.3.7.1. Analysis of the colonization of *Streptomyces* strains using different inoculation methods

Once uprooted, samples from all the plants were taken from the root system (RS), and the root insertion point (RI) (Fig II-1B) for analysis. Plants from treatment groups 3 and 4 (inoculated by injection) were subjected to a more detailed analysis by zones (Fig II-1A). Zone Z1 was located 2-3 cm below the inoculation point; zone Z2 was 0-1 cm below the inoculation point, while zones Z3 and Z4 were 0-1 cm and 2-3 cm above the inoculation point, respectively. These 1 cm wood fragments were surface-sterilized (Li *et al.*, 2012), cut into small pieces using a sterile scalpel, frozen in liquid N₂ and manually-ground up with a mortar into a very fine powder. DNA was extracted from 50 mg of dry weight material using NucleoSpin® Plant II Extraction kit (Macherey-Nagel). DNA concentration was estimated on NanoDrop and stored at -20°C until needed. The *Streptomyces* sp. VV/E1 and VV/R4 strains inoculated were detected and quantified by amplification of the developed SCAR markers by qPCR, as indicated above.

II.3.7.2. Analysis of the movement of *Streptomyces* sp. VV/E1 through grapevine plants

Once uprooted, samples from all the plants of the different batches were taken from different zones of the plant for analysis. The first zone (P1) corresponded to 3 cm of the root system. The second zone (P2) consisted of a sample of 2 cm from the root insertion point. The third zone (P3) corresponded to a sample taken from the middle of the rootstock. The fourth zone (P4) consisted of a 2 cm sample in the graft. The fifth zone (P5) corresponded to the second petiole. Finally, the

Fig. II-2. Image of the analysed zones (P1, P2, P3, P4, P5 and P6) of grapevine plants inoculated by immersion in order to detect the bacteria movement through the plant.

block out-of-focus light in image formation (Pawley, 2006). It was used for the detection of *Streptomyces* strains transformed with pGM1190-mCherry-GusA plasmid (See section II.3.9.1.1), in grapevine plants inoculated by immersion for 24 h in a bacterial suspension of *Streptomyces* sp. VV/E1 or *Streptomyces* sp. VV/R4. Control plants were immersed for 24 h in TSB broth.

Samples were processed according to the protocol provided by the Department of Cell Biology of the University of León (Anuncibay-Soto *et al.*, 2018). In brief, roots were cut into small pieces of 1 cm long and fixed to preserve their morphological structure in 4% (w/v) paraformaldehyde for 24 h / 4°C. Then, samples were washed twice in 150 mM phosphate-buffered saline solution (PBS) (Sigma) for 30 min and stored at 4°C until use.

Fixed samples were cut into 50 µm sections using a microtome (Micron HM450), fixed on a slide with 3% (v/v) DABCO (Sigma) and visualised in a Zeiss LSM800 confocal fluorescent microscope under fluorescent light (excitation 560 nm / emission 640 nm). Images were acquired using Zeiss Airyscan technology and a Plant-Apochromat 63x/1.40 Oil DIC M27 in a Zeiss LSM 800 confocal microscope.

sixth zone (P6) was the second leaf (Fig. II-2). These plant material were surface-sterilised and DNA was extracted following the same protocol as in the previous section.

II.3.8. Data analysis

Statistical analyses were performed with R software (version 3.3.1; <https://www.r-project.org/>). Analysis of variance was explored with linear models and level of significance between treatment groups was determined by an LSD-test (least significant difference) from the Agricolae R package (version 1.2-8, 2017) (De Mendiburu, 2009).

II.3.9. Actinobacteria detection by microscopy techniques

II.3.9.1. Fluorescent microscopy

Confocal microscopy increases the optical resolution and contrast using a spatial pinhole to

II.3.9.1.1. Transformation of *Streptomyces* strains with pGM1190-mCherry-GusA plasmid and intergenic conjugation in *Streptomyces* sp. strains.

pGM1190-mCherry-GusA plasmid (provided by Professor Dr. Tilmann Weber; Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark), is based on a single temperature sensitive vector pGM1190, in which a *Streptomyces* codon optimised *mCherry* and *GusA* gene have been cloned, under the control of an *ermE** promoter, ended with a *t0* terminator. The *mCherry* gene codifies for a red fluorescent protein derived from *Dicosoma* sp. (Shaner *et al.*, 2004).

The introduction of fluorescent plasmid pGM1190-mCherry-GusA in *Streptomyces* strains VV/E1 and VV/R4 was carried out by intergenic conjugation using as donor strain *E. coli* ET12567/pUZ8002, following the protocol of Tong *et al.* (2018). Briefly, 50 µL *E. coli* ET12567/pUZ8002 competent cells were transformed with 10 µL of plasmid DNA (section II.3.3.2). All the colonies were collected in a tube containing 5 mL LB supplemented with 50 µg/mL apramycin (Sigma), 25 µg/mL chloramphenicol (Intron Biotechnology) and 25 µg/mL kanamycin (Thermo Fisher Scientific) and incubated at 225 rpm / O/N / 37°C. 200 µL of this culture were inoculated in a flask containing 100 mL LB supplemented with the same antibiotics and incubated under the same conditions. The O/N culture was centrifuged at 8,000 rpm / 10 min / RT and donor cells were washed twice with 50 mL LB without antibiotics and re-suspended in 0.5 - 2.0 mL of LB broth.

For the intergenic conjugation, 100 µL of the donor cells (*E. coli* ET12567/pUZ8002) were mixed with 20 µL of *Streptomyces* sp. strains spores, plated on Cullum agar^{II.15} plates (Hobbs *et al.*, 1989) and incubated for 16-20 h / 30°C. After incubation, plates were overlaid with 1 mL sterile water supplemented with 25 µL of 25 mg/mL nalidixic acid (Sigma) and 25 µL of 50 mg/mL apramycin and incubated for 5-6 days / 30°C (or until single colonies appear). Single colonies were picked onto TSB agar plates with 50 µg/mL apramycin and 25 µg/mL nalidixic acid and incubated 2-5 days / 30°C until growth. Transformed colonies were checked by fluorescent microscopy (Nikon Eclipse E400 fitted with a Digital Sight DS-L1 camera) under fluorescent light (excitation 510 - 560 nm / emission 590 nm).

^{II.15} **Cullum agar:** 20.0 g/L mannitol; 20.0 g/L soy flour; 20.0 mM MgCl₂; 20.0 mM CaCl₂; 20.0 g/L agar. This media is prepared in tap water.

II.3.9.2. Scanning electron microscopy (SEM)

Scanning Electron Microscope (SEM) provides images of surfaces with high three-dimensional plasticity (Nguyen and Harbison, 2017). SEM was used to determine the presence of the biocontrol agents (BCAs) after inoculate them in young grapevine plants to elucidate their possible location and/or movement inside the plant. Samples used for this assay consisted of young grapevine plants corresponding to five different treatments: control (non-treated) plants, plants inoculated by immersion for 24 h in an actinobacterial suspension of *Streptomyces* sp. VV/E1, plants inoculated by immersion for 24 h in an actinobacterial suspension of *Streptomyces* sp. VV/E1 transformed with the fluorescent plasmid pGM1190-mCherry-GusA, plants inoculated by immersion for 24 h in an actinobacterial suspension of *Streptomyces* sp. VV/R4, and plants inoculated by immersion for 24 h in an actinobacterial suspension of *Streptomyces* sp. VV/R4 transformed with the fluorescent plasmid pGM1190-mCherry-GusA.

Sample preparation was carried out according to the protocol provided by the Microscopy service of the University of León. In brief, roots were cut into small pieces of 1 cm long and fixed to preserve their morphological structure in 2.5% (v/v) glutaraldehyde in PBS (Sigma) for 24 h / 4°C. Then, samples were washed three times in PBS for 30 min each washing step. Next, samples were post-fixed in 2% osmium tetroxide (TAAB Laboratories) for 2 h / RT protected from light, and washed three times in PBS for 30 min each washing step. Post-fished samples were dehydrated in solutions with increasing ethanol concentration (30, 50, 70, 90, three times in 96% (v/v) and three times in absolute ethanol) and dried by the critical point method, using a critical point dryer CPD 030. The critical point is defined by the pressure and temperature values of any element or compound, from which the density of the gas is the same as that of the liquid; in other words, the liquid phase is changed to the gas phase without visible limits and without distortion forces. This requires exchanging absolute ethanol for a liquid in which the critical point is easily reached: CO₂ (31°C and 73.8 bar).

Samples were mounted on a metal support and created a conductive surface by coating them with gold before visualising in a scanning electron microscope JEOL JSM-6480LV.

II.4. Results

II.4.1. Identification and characterization of differential RAPD amplification fragments of *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4

Different electrophoretic band patterns of 5 *Streptomyces* sp. rhizosphere strains (VV/R1 to VV/R5) and 5 endophytic actinobacterial strains (VV/E1 to VV/E5) (Álvarez-Pérez *et al.*, 2017) were obtained by the amplification of random polymorphic DNA sequences (RAPD) using up to thirteen different primers (Fig. SII-2).

A 916 pb specific band was solely amplified from *Streptomyces* sp. VV/E1 using P4 primer. In a similar way, a 1,862 bp specific band was only amplified from *Streptomyces* sp. VV/R4 using the OPA2 primer (Fig. II-3).

Fig. II-3 RAPD profiles generated by P4 primer **(A)** and OPA2 primer **(B)** for rhizosphere (VV/R1 to VV/R5) and endophytic (VV/E1 to VV/E5) *Streptomyces* sp. strains isolated from the root system of young grapevine plants. MW: GeneRuler 1kb DNA Ladder Plus (Thermo Fisher Scientific). Specific bands amplified from isolates VV/E1 and VV/R4 that were selected for SCAR markers design are indicated by arrows. NC: negative control.

Both DNA bands were excised and purified from agarose gels, cloned into pBluescript II KS (+) vector, transformed into *E. coli* DH5 α competent cells and sequenced. Sequences were compared with those already present in GenBank using the online tool BLASTx.

The *Streptomyces* sp. VV/E1 916 bp band (GenBank accession number MH048872) contained two partial open reading frames (ORF) (Fig. II-4A). The first one, extending from positions 1 to 401, exhibited a 61% amino acid identity with a TetR/AcrR family transcriptional regulator from *Streptomyces ipomoeae* 91-03 (accession number EKX60661.1), and lower amino acid identities with several other proteins from different actinobacteria strains, also identified as transcriptional regulators belonging to that family. The second ORF, almost complete, stretched from positions 597 to 916 and might

encoded a protein that showed a 94% amino acid identity with an hypothetical protein from *S. ipomoeae* (accession number WP_048822038.1), and lower amino acid identities with many other hypothetical proteins from several *Streptomyces* species.

Fig. II-4 Representative map of the sequenced RAPD amplification fragments for *Streptomyces* sp. VV/E1 (**A**) and *Streptomyces* sp. VV/R4 (**B**). Incomplete ORFs are shown in grey arrows whereas black arrows belong to complete ORFs. The relative positions of the amplified SCAR markers developed are indicated by a black line.

The *Streptomyces* sp. VV/R4 1,862 bp band (GenBank accession number MH048873) contained three putative ORFs (Fig. II-4B). The first incomplete ORF, stretching from positions 1 to 509, encoded a protein showing a very high amino acid identity (92%) with a hypothetical protein from *Streptomyces acidiscabies*

(accession number WP_075662455.1). The second ORF was complete, and it extended from positions 860 to 1306, encoding a protein with high similarity (87% amino acid identity) to a very short patch repair endonuclease from *Streptomyces* sp. strain (accession number WP_004939603.1). The third ORF, was also truncated, stretching from positions 1331 to 1862. It encoded a protein that exhibited 69% amino acid identity to a DNA cytosine methyltransferase from *Streptomyces* sp. (accession number WP_053914715.1).

II.4.2. Conversion of a RAPD marker into a SCAR marker

In order to improve reproducibility when identifying our strains, the nucleotide sequences of each RAPD fragment were used to design a pair of SCAR primers, targeting mainly poorly conserved intergenic regions. A pair of primers, SCAR.E1_fw (positions 415 to 439) and SCAR.E1_rv (positions 576 to 596) (Fig. SII-3A), were designed for *Streptomyces* sp. VV/E1 strain. Both primers were located in the intergenic region between the two ORFs detected. The SCAR.E1_fw and SCAR.E1_rv primers had 48.0% and 52.4% GC content, respectively. This pair of primers amplified from genomic DNA of VV/E1 strain a unique 182 bp PCR fragment when annealing temperatures ranged from 60 to 65°C, indicating a high specificity.

In a similar way, the pair of primers SCAR.R4_fw (positions 466 to 486), and SCAR.R4_rv (positions 608 to 625) (Fig. SII-3B) were designed to specifically amplify from VV/R4 genomic DNA a specific PCR band of 160 bp when annealing temperatures ranged from 57 to 63°C. SCAR.R4_fw primer was designed with 61.9% GC content and it annealed near the 3'-end of ORF1. The SCAR.R4_rv primer had a 72.2% GC content and was located in the ORF1-ORF2 intergenic region.

Both pairs of primers were designed to amplify a band with a single-copy in the genome. This fact was checked in a Southern blot assay (Fig. II-5).

To check the specificity of the designed primers, genomic DNA of 50 actinobacteria corresponding to 29 rhizosphere

Streptomyces sp. strains (VV/R1-VV/R29) and 21 endophytic actinobacteria strains (including 15 *Streptomyces* sp. isolates, 2 *Saccharopolyspora* sp. strains and 3 *Micromonospora* sp. isolates) (Álvarez-Pérez *et al.*, 2017) were amplified by conventional PCR. Results pointed out that PCR products were only amplified from VV/E1 and VV/R4 strains with the expected sizes of 182 bp and 160 bp respectively, with no other amplification in any tested strains (Fig. SII-4).

II.4.3. Development of qPCR methods for the detection of *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 in vegetable samples

Despite the fact that SCAR markers allowed to detect a specific strain, conventional PCR did not give an accurate quantification of the detected DNA. Accordingly, once the specific SCAR markers had been raised for both strains, a rapid, specific and sensitive method for their detection was developed in a SYBR Green qPCR system using the same primers tested before for conventional PCR. The sensitivity of the system was evaluated for *Streptomyces* sp. VV/E1 and VV/R4 strains using genomic DNA.

Assays for *Streptomyces* sp. VV/E1 were carried out using as template 10-fold serial dilutions of genomic DNA from 5.65×10^6 fg to 5.65×10^2 fg. For the DNA amplification curve, the cycle threshold (C_t) values were converted into amount of DNA using the linear regression equation $y = -3.567x + 39.116$, where x is the logarithm of the DNA quantity (fg) and y is C_t value. The regression coefficient obtained was close to 1 ($R^2 = 0.998$), and the amplification efficiency was 90.7% (Fig. II-6).

Fig. II-5. Southern blot assay of *Streptomyces* sp. VV/E1 (A) and *Streptomyces* sp. VV/R4 (B) to determine the number of copies of the DNA regions containing the SCAR marker region in the genome of each strain. The labelled probe (SCAR marker of each strain) hybridized in a single DNA fragment for each restriction endonuclease used, indicating that the SCAR marker was in a single copy in the genome of the corresponding strain.

Fig. II-6 Representative amplification curves obtained by plotting the mean *Ct* values with respect to the logarithm of genomic DNA quantity from *Streptomyces* sp. VV/E1 (black line) and *Streptomyces* sp. VV/R4 (grey line). Standard curves were generated with 10-fold serial dilutions of genomic DNA concentrations from 5.65×10^6 to 56.5 fg (VV/E1 strain) or 5.2×10^6 fg to 52.0 fg (VV/R4 strain) by SYBR green qPCR reactions.

In a similar way, assays for *Streptomyces* sp. VV/R4 strain were performed with 10-fold serial dilutions of genomic DNA from 5.20×10^6 fg to 5.20×10^2 fg. The results allowed us to define the equation $y = -3.356x + 39.033$ ($R^2 = 0.996$). The amplification efficiency was 98.6% (Fig. II-6).

The LOQ was 50.47 fg for *Streptomyces* sp. VV/E1 and 72.65 fg for *Streptomyces* sp. VV/R4 (Fig. II-7).

Fig. II-7 Limit of quantification (LOQ) from *Streptomyces* sp. VV/E1 and VV/R4 strains. Coefficient of variation [$CV = 100 \times (SD/\text{mean value})$] of the back-calculated amounts of genomic DNA from *Streptomyces* sp. VV/E1 (**A**) and *Streptomyces* sp. VV/R4 (**B**) by qPCR assays (5 replicates). Horizontal dashed lines indicate $CV = 35\%$ and vertical dashed lines indicate the lowest quantity of DNA with a CV below 35% (obtained by interpolation). Grey symbols indicate the presence of negative ("non-detected") replicates among samples.

In order to check that host (*Vitis vinifera*) DNA did not interfere with the quantification of the DNA of *Streptomyces* sp. strains over a wide range, additional standard curves for both strains were generated under the same conditions, but including grapevine genomic DNA extracted from wood (ranged from 1:1 to 1:100,000) of a non-inoculated grapevine plant (Fig. II-8).

Fig. II-8 Standard curves generated by SYBR Green qPCR using the primer set that amplified the SCAR markers developed. Curves generated with 10-fold serial dilution of genomic DNA from VV/E1 (**A**) and VV/R4 (**B**) strains are shown in black lines. Grey lines correspond to curves generated on the presence of grapevine genomic DNA. The final ratio of actinobacteria-to-grapevine DNA ranged from 1:1 to 1:100.000.

The linear regression equations obtained were very similar for each strain in both conditions, with high regression coefficients ($R^2 = 0.995$ for *Streptomyces* sp. VV/E1 and $R^2 = 0.999$ in the case of *Streptomyces* sp. VV/R4). Furthermore, the corresponding curve slopes did not show significant differences, according to a Student's t-test statistical analysis ($p \leq 0.05$), which means that the presence of genomic DNA from grapevine wood did not have a significant impact on the results.

II.4.4. Detection and quantification of *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 strains in wood samples of inoculated grapevine plants

The development of this fast, reliable, accurate and specific qPCR method allow us to analyse the colonization efficiency of the root system of young grapevines by both strains depending on two different inoculation methods, immersion and injection (see section II.3.6; Fig. II-1).

II.4.4.1. Definition of the lineal models of analysis

Lineal models were defined with the DNA quantification raw data (fg of DNA from both *Streptomyces* sp. strains detected in analysed samples), for every treatment (batch), and area analysed, in order to explain the variability obtained (dependent variable).

Three independent variables (treated as factors) were considered. The first variable was related to the *method of inoculation*, which included control treatment (non-inoculated plants, batches 1 and 6), inoculation by immersion (batches 2 and 3), and

inoculation by injection (batches 4 and 5). The second factor was related to *application of a booster treatment* in batches 2, 3 and 6. The third factor was the *plant area analysed*. In the case of the control and immersion treatments, the root insertion point (RI) and root system (RS) were analysed (Fig. II-1B), whereas in treated plants inoculated by injection four different zones were analysed (Z1/RI, Z2, Z3 and Z4) (Fig. II-1A).

Z1 zone analysed in injected plants was equivalent to RI area in control and immersion-treated grapevine plants.

II.4.4.2. Analysis of inoculation by immersion

After defining the lineal model, data analysis clearly indicated that the booster dose applied to the corresponding batches (3, 5 and 6) was not efficient at promoting bacterial colonization of the root system, with p -values of 0.9190 for *Streptomyces* sp. VV/E1 and 0.5520 for *Streptomyces* sp. VV/R4. Accordingly, the second factor (application of a booster treatment on soil) was not taken into account in the final model. Therefore, batches with the same inoculation method, with and without booster treatment were combined. Thus, further analysis was simplified by reducing treatments just to two: control samples (batches 1 and 6), and inoculation by immersion (batches 2 and 3), resulting in a larger sample size for each treatment and providing an increase in the robustness of the statistical analysis.

Genomic DNA quantities for both VV/E1 and VV/R4 strains were measured in both, the RI (Fig. II-9A) and the RS (Fig. II-9B). Data analysis showed the existence of significant differences ($p < 0.001$)

Fig. II-9 Box plot showing the DNA levels from *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 strains in plants subjected to inoculation by immersion and compared to control plants in the root insertion point (RI) (**A**) and the root system (**B**). Black rhombuses show the average values for each treatment. Asterisks indicate significant differences according to the LSD test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Circles correspond to data outliers (falling outside the Q1-Q3 range).

in $\log_{10}$ DNA values for both RI and RS with respect to control samples. Mean values detected in RI were higher for the VV/R4 strain (631.5 fg DNA) compared to the VV/E1 strain (310.9 fg DNA). The same trend was observed in the RS area: 734.6 fg of DNA detected for the VV/R4 strain and 331.9 fg of DNA for the VV/E1 strain. The correlation between the amount of DNA and the corresponding number of cells is shown in Table SII-2.

II.4.4.3. Analysis of inoculation by injection

Four different zones (Z1/RI to Z4) were analysed along the grapevine stem (Fig. II-1A). As in the previous treatment, a simplified model was used, since boosted treatment was not taken into account according to p -values obtained (0.6122 for VV/E1 and 0.2106 for VV/R4, respectively).

Data analysis was carried out firstly, by the comparison between injected and control plants for Z1/RI, (equivalent to RI area). The $\log_{10}$ DNA amount detected showed significant differences for both strains with p -values of 7.096×10^{-6} and 2.996×10^{-2} for VV/E1 and VV/R4, respectively in the Z1/RI area, compared to the negative control (untreated plants). In this case, the mean DNA value detected was clearly higher for the VV/E1 strain (321.3 fg), as compared to 83.5 fg for the VV/R4 strain (Fig. II-10).

Fig. II-10 Box plot showing the levels of DNA from *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 strains detected in the root insertion point (RI) zone in plants inoculated by injection compared to the DNA amount detected in control (untreated) plants. Note that in this figure DNA amount are shown in pg. Black rhombuses correspond to the average values. Asterisks indicate significant differences according to the LSD test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Circles correspond to data outliers (falling outside the Q1-Q3 range).

Secondly, an analysis of plant material around the injection point (Z1 to Z4 zones) was carried out (Fig. II-11). For the VV/E1 strain, significant differences were observed with respect to the distance from the inoculation point according to the LSD-test ($p = 2.575 \times 10^{-12}$). The amount of DNA detected in the distal areas Z1/RI (0.32 pg) and Z4 (0.33 pg) was low, whereas in the closer areas Z2 (105.7 pg) and Z3 (62.3 pg) the amount of DNA was significantly higher when compared to distal areas. Regarding the VV/R4 strain, there

Fig. II-11 Box plot corresponding to the DNA amount of *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 strains detected in the different zones (Z1 to Z4) analysed in the rootstock of plants inoculated by the injection method, with regard to the DNA amount detected in control (untreated) plants. Note that in this figure, the DNA amounts are shown in pg. Black rhombuses correspond to the averages values. Asterisks indicate significant differences according to the LSD test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Circles correspond to data outliers (falling outside the Q1-Q3 range).

were significant differences between Z1/RI (0.08 pg) and Z2-Z3-Z4 (values of 4.67, 3.34, and 0.64 pg, respectively) area samples ($p = 1.821 \times 10^{-3}$).

The correlation between the amount of DNA and the corresponding number of copies is shown in Table SII-2.

II.4.4.4. Analysis of the colonization of root insertion point (RI or Z1 zone)

As previously mentioned, Z1/RI and RI were the only common area to be analysed in plants inoculated by either immersion or injection. Consequently, **it was of great interest to analyse the rate of bacterial colonization of this area in plants subjected to both treatments**. As in previous cases, the full lineal model was also discarded since no influence of the booster treatment in soil was determined (p -values of 0.601 and 0.205 for VV/E1 and VV/R4, respectively). The differences in log₁₀ DNA amounts detected were significant ($p = 7.598 \times 10^{-8}$ for VV/E1, and $p = 2.691 \times 10^{-4}$ for VV/R4) compared to control plants. However, no significant differences between the two inoculation procedures could be detected. Indeed, VV/E1 strain mean values for both treatments were quite similar (310.9 and 331.3 fg DNA for immersion and injection, respectively). Nevertheless, a remarkable difference was noticed for the VV/R4

Fig. II-12 Box plot showing DNA amount in the root insertion point (RI or Z1) of *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 strains in plants inoculated by immersion or injection methods comparing to control (untreated) plants. Black rhombuses correspond to the average value for each treatment. Asterisks indicate significant differences according to the LSD test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Circles correspond to data outliers (falling outside the Q1-Q3 range).

strain, where higher DNA amounts were detected in plants inoculated by immersion (mean value of 631.5 fg of DNA) than in injected plants (83.48 fg of DNA) (Fig. II-12).

The correlation between DNA amount and the corresponding number of cells is shown in Table SII-2.

II.4.5. Movement of the *Streptomyces* sp. VV/E1 through the grapevine plant

Once analysed the colonization efficiency of the root system of young grapevines, the next step consisted of the analysis of the VV/E1 strain capability to colonise and move along the plant, after its inoculation through the root system. This strain was selected since it should be more adapted to an endophytic lifestyle as it had been isolated like an endophytic strain.

II.4.5.1. Definition of the lineal models of analysis

Lineal models were defined with the DNA quantification raw data (fg of DNA from *Streptomyces* sp. VV/E1 detected in analysed samples), for every treatment (batch), and area analysed, with the final objective of explaining the variability obtained (dependent variable). Two independent variables (treated as factors) were considered. The first factor was the *treatment*, which included control plants (non-inoculated) and plants inoculated by immersion with the bacteria. The second factor was the *plant area analysed*. In both cases, the areas analysed (Fig. II-2) were the root system (P1), the root insertion point (P2), the middle of the rootstock (P3), the graft (P4), the second petiole (P5) and the second leaf (P6).

II.4.5.2. Analysis of VV/E1 strain movement inside the plant

Values of genomic DNA quantities for VV/E1 were measured at the 6 different zones (Fig. II-13). Data analysis showed the existence of significant differences ($p < 0.001$) in the DNA values for treated plants with regard to control untreated plants.

Moreover, when comparing the interaction between zones and treatment, there were significant differences ($p = 0.02379$). The LSD test showed the existence of significant differences in both P1 (539.45 fg DNA), P2 (85.39 fg DNA), P3 (205.50 fg DNA), and P5 (179.6 fg DNA) zones, with respect to control samples (9.33, 2.49, 39.17 and 15.23 fg DNA respectively), while no differences would be detected on the graft (P4) and leaf (P6) regions.

Fig. II-13 Box plot showing level of DNA from *Streptomyces* sp. VV/E1 detected in different zones of grapevine plants inoculated by immersion with regard to the DNA amount detected in control (untreated) plants. Black rhombuses correspond to the average values. Asterisks indicate significant differences according to the LSD test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Plots marked with different letter are significantly different ($p < 0.05$). Circles correspond to data outliers (falling outside the Q1-Q3 range).

II.4.6. Analysis of colonization of *Streptomyces* sp. strains in grapevine roots by microscopy techniques

The presence of the *Streptomyces* sp. strains in the root system of grapevine plants inoculated by immersion method was visually checked using microscopic techniques. Treated and untreated (control) plants were visualised under fluorescent light in a confocal microscopy, using *Streptomyces* strains (VV/E1 and VV/R4) transformed with pGM1190-mCherry-GusA plasmid. Nevertheless, due to the very high inherent red fluorescence of the plant tissues, identification of transformed actinobacteria was not possible (Fig. II-14).

On the other hand, plants inoculated with *Streptomyces* sp. VV/E1, *Streptomyces* sp. VV/R4 strains and untreated (control) plants were analysed

by SEM. In this case, the presence of both strains could be observed, not only in the surface of the roots but also inside the xylem vessels (Fig. II-15).

Fig. II-14 Pure cultures of *Streptomyces* sp. VV/E1 (A) and *Streptomyces* sp. VV/R4 (B) transformed with pGM1190-mCherry-GusA fluorescent plasmid and *Streptomyces* sp. VV/E1 wild-type strain (C) visualised under fluorescent microscopy. Vine root section visualise under confocal fluorescent microscopy (D).

Fig. II-15 Scanning electron microscopy of *Streptomyces* sp. VV/E1 and VV/R4 showing colonization on the roots of grapevines. Root inner (A) and root surface (B) of untreated plants. Root inner of grapevines inoculated with *Streptomyces* sp. VV/E1 and (C) root surface of plants inoculated with *Streptomyces* sp. VV/R4 (D). Arrows indicate the presence of *Streptomyces* strain.

II.5. Discussion

Streptomyces strains are well known as secondary metabolite producers (Robertsen *et al.*, 2018) and for their ability to control plant diseases (Doubou *et al.*, 2001; Schrey and Tarkka, 2008; Schrey *et al.*, 2012; Barka *et al.*, 2016; Chater, 2016; van der Meij *et al.*, 2017; Vurukonda *et al.*, 2018). Plants, including grapevines, are a common host for numerous endophytic and rhizosphere strains of this genus. Moreover, some of these strains are effective against the infection of fungi responsible for YGD, emerging as putative biocontrol agents (Álvarez-Pérez *et al.*, 2017).

Nevertheless, several factors make their application difficult. Firstly, the lack of methods that allow to detect and quantify these strains in plant material. And secondly, the absence of effective and easy methods to their introduction into the plant to allow their colonization.

One purpose of this study was to develop simple molecular markers able to detect both strains in plant material. RAPDs are easy to develop as molecular markers, but their application is unreliable due to the lack of reproducibility. A better option to solve this problem is to convert RAPD amplicons into SCAR markers (Kiran *et al.*, 2010; Bhagyawant, 2016). In the current study, the specific SCAR markers developed for

Streptomyces sp. VV/E1 and VV/R4 were raised against intergenic regions located in the previously sequenced RAPDs. Since intergenic regions are poorly conserved between different species, or even strains, **a SCAR marker developed from an intergenic region would likely be highly specific from a particular strain or species.** In fact, the specificity of both SCAR markers was very high, considering that they did not allow amplification or cross-reactions with, genomic DNA from 50 other actinobacteria (mainly belonging to the genus *Streptomyces*) that had been isolated as endophytic and rhizosphere strains from the root system of young grapevine plants (Álvarez-Pérez *et al.*, 2017). However, we cannot totally rule out the existence of any taxon that could allow DNA amplification, for one or both developed markers, although the robustness of the qPCR technology would allow us to discriminate that it was a false negative. On the one hand, there would be differences in the dissociation curve obtained in the qPCR assay and, on the other, there could be differences in the size of the amplified fragment.

Although the use of SCAR markers allowed the detection of both strains from a low amount of DNA (30 ng genomic DNA), they did not allow an accurate quantification of the DNA detected. Accordingly, another goal of this work was the **development of a specific and sensitive qPCR assay for the detection and quantification of *Streptomyces* sp. VV/E1 and VV/R4 in plant material.** Given their high specificity, the same pairs of primers used to amplify SCAR markers were used as specific primers for the development of qPCR protocols. The *Ct* values were linearly correlated with the concentration of target DNA for both strains, signifying that this methodology was suitable for qualitative and quantitative assays. The LOQ was quite low for both strains, which indicates a good sensitivity of the developed methods. However, there are hardly any studies on the quantification of *Streptomyces* strains by qPCR that allow us to compare the LOQ values obtained (Auffret *et al.*, 2011). Since one of the objectives of this work was to quantify the presence of both strains in grapevine plants, we had to rule out the possible interference of grapevine genomic DNA in the qPCR assays. We tested broad concentration ratios (1:1 to 1:100,000) of both *Streptomyces* sp. strain to grapevine genomic DNA in qPCR experiments. The corresponding curve slopes did not show significant differences, which meant that the presence of grapevine genomic DNA did not interfere with the detection and quantification of the bacterial strains.

With the development of fast, reliable, accurate and specific **qPCR methods** for the detection of both strains in plant material, it was possible **to analyse the colonization efficiency in the root systems of young grapevines.** After comparing the two methods of inoculation with the corresponding plant areas analysed, several conclusions would be drawn. First, that both strains could be detected inside the root system of non-inoculated (control) plants, although at a very low rate if we compared with the DNA amount detected in inoculated plants. This is a logic result for *Streptomyces* sp. VV/E1 since it was originally isolated as an endophytic strain. In contrast, *Streptomyces* sp. VV/R4 was isolated as a rhizosphere strain. Consequently, the obtained results suggest that the line

that separates the definition of a particular strain as endophytic or rhizosphere is very diffuse. Rhizosphere-established streptomycetes are well known to have different effects on plants (Schrey and Tarkka, 2008; Schrey *et al.*, 2012; van der Meij *et al.*, 2017; Vurukonda *et al.*, 2018). Unfortunately, general knowledge about the impact of endophytic *Streptomyces* sp. strains on plant development is poorly understood, although it is clear that they can enhance the growth of some plants and help in the biocontrol of phytopathogens (Vurukonda *et al.*, 2018).

Since both strains were detected 180 days after their application and once completed a full growth cycle, it could be concluded that **immersion is an effective method for the actinobacterial colonization of grapevine plants**. Both strains were detected in both the root system and the root insertion point. This indicates that the actinobacteria probably enter the root system through injuries produced when the roots were trimmed before the bacterial application, but it cannot be discarded that the bacteria might enter through other microscopic injuries in the plant. Furthermore, their detection in the root insertion point suggested an upward movement of the bacteria. This movement might be carried out through the xylem vessels, since microscopy studies shown that *Streptomyces* sp. VV/E1 was present in the xylem vessels of inoculated plants. Moreover, the behaviour of both strains was similar, while it is true that VV/R4 was detected in a higher level than VV/E1 at both root system and root insertion point.

On the other hand, **injection of a bacterial suspension directly into the rootstock was also effective at inducing the colonization of grapevine plants**. The amount of genomic DNA quantified in the root insertion point was considerably higher than in control (untreated) plants. Contrary to what happened on plants inoculated by immersion, VV/E1 strain was detected in higher levels than VV/R4. The analysis of the different Z1/RI, Z2, Z3, and Z4 data samples around the injection point indicated that, from this point, the bacteria could move, at least a short distance (2-3 cm), up and down the inoculation point, as we can conclude by their levels in Z1/R1 and Z4 zones. The comparison of bacterial levels at the root insertion point suggested that for the VV/E1 strain, **both methods exhibited a similar efficiency at colonizing the root system, whereas immersion yielded a higher colonization rate than injection for VV/R4 strain**.

The possibility that the bacteria might reach upper sections in the plant was also evaluated, although only for the endophytic VV/E1 strain. The analysis of the bacteria movement through the grapevine plant confirmed the efficient colonization of the plant, while showing upward movement of the strain, reaching the second petiole but not the leaf. However, no significant differences between inoculated and control plants were detected on the graft area, although mean values indicated that the DNA amount detected in control samples was rather lower compared to treated plants.

We also analysed if the addition of a booster treatment in the proximity of the root system could reinforce the colonisation rate of both strains, previously inoculated by immersion or injection. Achieved results showed that this method was clearly inefficient, not allowing an improvement of colonisation levels in any strain.

Streptomyces strains have been associated with the suppression of plant diseases (Ryan et al., 2004; Hiltunen et al., 2009; Toumatia et al., 2016; Law et al., 2017). However, their application in soil as biocontrol agents remains unreliable (Kinkel *et al.*, 2012). This failure to colonise the plant from the ground could be due to multiple factors among which is the competition with other microbial species existing in the root environment. Taking into account these data, the potential for endophytic *Streptomyces* to control plant disease is becoming a focus of research. However, these studies are hampered by a poor understanding of the relationships between rhizosphere and endophytic populations, and how a *Streptomyces* strain becomes adapted to an endophytic way of life. It may happen that the protected endophytic habitat minimises the challenge of achieving successful colonisation, which has proven to be difficult in competitive soil communities (Kinkel et al., 2012; Schlatter and Kinkel, 2015).

Finally, we should emphasise that **these results show that *Streptomyces* sp. strains isolated from grapevine root environment as putative biocontrol agents to fight fungal pathogens causing YGD, can be introduced into young grapevine plants by both immersion or injection, where they can establish and colonise, not only the interior of the root system, but also upper regions on the plant.** Undeniably, immersion method should be the ideal option due to their easier application, not only in nurseries by the introduction of an additional inoculation step in the production of grafted plants, but also in cellars and vineyards, simply by placing the plants into a bacterial suspension before planting. **Therefore, the application of these biocontrol agents (BCA) prior to vineyard establishment is a promising tool for diminishing the incidence of YGD in new plantations all over the world.** These inoculation methods could be also potentially used for applying useful bacterial BCAs to other vascular plants of great agronomical and economical interest like fruit trees (citrus, almonds or olive among others) affected by trunk diseases.

II.6. References

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Supplemental material

Fig.SII-1 Correlation between quantity of genomic DNA and number of cells. Standard curves obtained by plotting the mean Ct values with respect to the logarithm of DNA copy number from purified and quantified PCR products from *Streptomyces* sp. VV/E1 (black line) and *Streptomyces* sp. VV/R4 (grey line) (A). Standard curves obtained by plotting the mean Ct values with respect to the logarithm of genomic DNA quantity (10-fold serial dilutions) from *Streptomyces* sp. VV/E1 (black line) and *Streptomyces* sp. VV/R4 (grey line) (B). Correlation (logarithm scales) between genomic DNA quantity and the corresponding number of copies of each amplicon from *Streptomyces* sp. VV/E1 (black line) and *Streptomyces* sp. VV/R4 (grey line) (C).

Fig. SII-2. RAPD profiles generated by the amplification of rhizosphere (VVR1 to VVR5) and endophytic (VVE1 to VVE5) actinobacteria strains isolated from the root system of young grapevine plants using different primers: OPA2 (A), OPA9 (B), OPA10 (C), P1 (D), P2 (E), P3 (F), P4 (G), P5 (H), R1 (I), R3 (J), SS1 (K), SS2 (L) and SS3 (M). MW: Generuler 1 kb DNA Ladder Plus (Thermo Fisher Scientific). NC is the negative control.

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WE1  AAGAGCCCGTACTCGATGGCAATGACGTCGAGCGGCTCGAAGTACGCGCGGATCATCGCGGCAGTCAGT
      20      40      60
WE1  GCGACGTGCTCGTCGCACATCTTCGTGGCGGCTAGACGTAGCGGACCAGTTTCATCAACCGGATCATCG
      80     100    120
WE1  GGTGATAGGTGCCGTCCTCCGAACCTTGCACGATCGGATCGATGACCGGTGCCAACACACGGGCGTGC
     140    160    180    200
WE1  GCCGCCCTCTAGAACGTCGTAATTCAACCGGAAGTGGTTAGACAGTGTGGTGAGGCCGACGCCTGCGGCT
     220    240    260
WE1  TCGGCGATCTGCTCGCGGGTGTAAATCACCCCGTTTCTGGTCGCTGTACAGGTCGATCGTGGACTGAAAG
     280    300    320    340
WE1  ATCCACTCCCGTGTTCGTTTGTGCTTGGTCTCTGCTGCCCGCTGCTGCGGGGTCTATCTCTCCGTCACG
     360    380    400
      SCAR.E1_fw
      420
WE1  GGTTCACGGTATCTGTTTACTCACCGACCAACAAGAAGGGAGGCACTTGCTGATCGGGGTGCTTGACG
     460    480
WE1  CTGAAGTGGACTATCTCCACTCTTGAGCTCTTCGACCTATCCGCTGCGGCCGGCTCGCTTGAACGCTG
     500    520    540
      SCAR.E1_rv
      580
WE1  CGCTCTACAGAGCTGCCAGGCACGGAAGAAGGGTTCAACCAACATGGGAAGCGAACTGACGCTGGGTG
     560    600    620
WE1  GCTTCGGTCTGTCAGCCGGAACGACGAGGACGACTACACAGGACCAACGGGCGCTCGTCCAGCGTGCCG
     640    660    680
WE1  AGCACGACCGCGACGCCCTTGCTGTTTCGAGGCGAAGAAGCAGGAGTTACCGCAGCATTACGACTGCGAA
     700    720    740
WE1  TGACGGAGGGCGCCATGCACGACGTCGCTGATGTCATGAAC TTGGCCCGTCAACTGGGAGGGGACGACC
     760    780    800    820
WE1  CCTACTTGCTTCGCGAACTTGGAAGTTGCAGCAGGAGTTCACAGGCAGACAGCACACGACATCCGCA
     840    860    880
WE1  GCTTCGGCAACGGGCTCTT
     900

```

Fig. SII-3A Sequence of the 916 bp DNA RAPD fragment amplified from *Streptomyces* sp. VV/E1 using the P4 primer. Sequence and position of the designed primers (SCAR.E1_fw and SCAR.E1_rv) are marked with light blue arrows.

Fig. SII-3B Sequence of the 1,862bp DNA RAPD fragment amplified from *Streptomyces* sp. VV/R4 using the OPA2 primer. Sequence and position of the designed primers (SCAR.R4_fw and SCAR.R4_rv) are marked with light blue arrows.

A **B**

Fig. SII-4 Absence of amplification from genomic DNA of 50 actinobacterial strains using SCAR primers SCAR.E1_fw/SCAR.E1_rv (**A**) and SCAR.R4_fw/SCAR.R4_rv (**B**). Specific amplification bands were only detected for strains *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4. MW: GeneRuler 1 kb DNA Ladder Plus (Thermo Fisher Scientific). NC: negative control.

Table SII-1. Primers used for RAPD amplification of different *Streptomyces* species in order to design specific SCAR markers for *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4

Primer	Sequence (5'-3')	T_m (°C)	Reference
OPA2	TGCCGAGCTG	40	Gharaibeh <i>et al.</i> , 2003
OPA9	GGGTAACGCC	40	Gharaibeh <i>et al.</i> , 2003
OPA10	GTGATCGCAG	40	Gharaibeh <i>et al.</i> , 2003
P1	GGTGCGGGAA	36	Nomura <i>et al.</i> , 2006
P2	GTTTCGCTCC	36	Nomura <i>et al.</i> , 2006
P3	GTAGACCCGT	36	Nomura <i>et al.</i> , 2006
P4	AAGAGCCCGT	36	Nomura <i>et al.</i> , 2006
P5	AACGCGCAAC	36	Nomura <i>et al.</i> , 2006
R1	CACGCCCTTC	40	Corte <i>et al.</i> , 2005
R1	ATGCAGCCAC	40	Corte <i>et al.</i> , 2005
SS1	GTCAACGCGG	36	González-García <i>et al.</i> , 2019
SS2	AACGGCTCGC	36	González-García <i>et al.</i> , 2019
SS3	GAGATCGCGC	36	González-García <i>et al.</i> , 2019

Table SII-2 Correlation between mean values of DNA amounts quantified by qPCR from *Streptomyces* sp. VV/E1 and VV/R4 strains and cell numbers.

Strain	Treatment	Zone or sample analysed *	<i>Streptomyces</i> sp. DNA (fg)	<i>Streptomyces</i> sp. cells/mg wood
VV/E1	<i>Control</i>	RI	4.07	1.62
	<i>Immersion</i>		310.9	26.5
	<i>Injection</i>		321.3	31.2
VV/R4	<i>Control</i>		5.91	1.76
	<i>Immersion</i>		631.5	20.9
	<i>Injection</i>		83.5	6.01
VV/E1	<i>Control</i>	RS	4.7	2.35
	<i>Immersion</i>		331.9	49.4
VV/R4	<i>Control</i>		1.96	1.22
	<i>Immersion</i>		734.6	67.5
VV/E1	<i>Injection</i>	Z1/RI	321.3	31.2
		Z2	105719	6344
		Z3	60261	3735
		Z4	327.6	20.4
VV/R4	<i>Injection</i>	Z1/RI	83.5	6.01
		Z2	4672	170.8
		Z3	3338	120.6
		Z4	642.5	20.9
VV/E1	<i>Control</i>	Z1/RI	4.07	1.62
	<i>Immersion</i>		310.9	26.5
	<i>Injection</i>		321.3	31.2
VV/R4	<i>Control</i>		5.91	1.76
	<i>Immersion</i>		631.5	20.9
	<i>Injection</i>		83.5	6.01

* Root insertion point (RI), Root System (RS), Zone 1 (Z1), Zone 2 (Z2), Zone 3 (Z3), Zone 4 (Z4) analysed in the rootstock as shown in Fig. II-1. Note that Z1 is equivalent to RI area for comparison purposes in plants subjected to inoculation by injection.

CHAPTER III: ANALYSIS OF ANTIFUNGAL PROPERTIES OF *STREPTOMYCES* SP. VV/E1 AND VV/R4 STRAINS

III.1. Abstract

Genome sequencing together with the development of computational tools, combined with molecular techniques have allowed the analysis and characterization of a vast number of microorganisms and their secondary metabolites. **Actinobacteria are one of the most important sources of natural bioactive products with relevant application in industries, including agriculture.**

The endophytic actinobacteria *Streptomyces* sp. VV/E1 and the rhizosphere strain *Streptomyces* sp. VV/R4 isolated from grapevine, have been reported as biocontrol agents against grapevine trunk diseases due to their ability to reduce the infection rates produced by fungal pathogens involved in young grapevine decline. Therefore, they could represent a promising eco-friendly tool for controlling trunk pathogens in nurseries and open fields. The analysis of their genomes could reveal the production of secondary metabolites related to the synthesis of antifungals, involved in their biocontrol activity.

Thus, **the aim of this study was to carry out a bioinformatics analysis of both genomes in order to identify clusters or molecules that could be involved in the biocontrol process.** Genome analysis showed that both strains have many common clusters, involved in the synthesis of the same secondary metabolites, some of them like antimycin or candicidin, described as antifungals.

III.2. Introduction

Streptomycetes are filamentous gram-positive bacteria recognised for their capabilities to produce secondary metabolites (Zhou *et al.*, 2012; Robertsen *et al.*, 2018; Tsois *et al.*, 2018), with great potential in pharmaceutical, agricultural and nutritional applications (Weber *et al.*, 2015a), like antibiotics, antifungals or antitumors (Zhou *et al.*, 2012; Stulberg *et al.*, 2016;). From the roughly 18,000 bioactive bacterial compounds known, more than half have been described to be produced by the genus *Streptomyces* (Weber *et al.*, 2015b). **Genome sequence analysis from multiple actinomycetes indicates that each bacterium can potentially produce approximately 10-fold more secondary metabolites than those that can be detected** (Weber *et al.*, 2015b). For this reason, **actinomycetes continue to be promising sources of novel bioactive compounds** (Weber *et al.*, 2015b).

In the early 2000s, when the complete genome of the model organism *Streptomyces coelicolor* (Bentley *et al.*, 2002) and the industrial strain *Streptomyces avermitilis* (Ikeda *et al.*, 2003) were published, **silent or “cryptic” biosynthetic gene clusters (BGCs) were discovered** (Robertsen *et al.*, 2018). **This revealed a hitherto untapped potential of microorganisms for the production of new compounds, which has stimulated new**

efforts to understand the complex regulation between primary and secondary metabolism (Robertsen *et al.*, 2018). This new trend had been accompanied by the development of novel computational resources (genome and compound mining tools), the generation of a range of high-quality omics data, the establishment of molecular tools, and other strains engineering strategies (Robertsen *et al.*, 2018).

Availability of genome data has spurred advances in relevant technologies covering computational resources (Robertsen *et al.*, 2018). Thus, over the last years, **genome mining of Biosynthetic Gene Clusters (BGCs) have become a key methodology to identify bioactive molecules** (Blin *et al.*, 2013). Several computational tools have been developed in this field, including antibiotics and Secondary Metabolites Analysis Shell (antiSMASH) (Medema *et al.*, 2011; Blin *et al.*, 2013; Weber *et al.*, 2015a; Blin *et al.*, 2017). This tool has served as a comprehensive web server for the genomic identification and analysis of BGCs of any type, and therefore, facilitating rapid genome mining of a wide range of bacterial strains (Weber *et al.*, 2015a). In addition to polyketides and non-ribosomal peptides, which are two of the most important and diverse classes of secondary metabolites (González *et al.*, 2005; Johnston *et al.*, 2015; Nimaichand *et al.*, 2015), other kind of clusters can be identified, such as terpenes, lantipeptides, bacteriocins, aminoglycosides/aminocyclitols, β -lactams, aminocumarins, indoles, butyrolactones, ectoines, siderophores, phosphoglycolipids, melanins, oligosaccharide antibiotics, phenazines, thiopeptides, homoserine lactones, phosphonates, furans and a generic class of clusters encoding unusual secondary metabolite biosynthetic genes (Blin *et al.*, 2013).

The genes encoding the biosynthesis pathways that are responsible for the production of secondary metabolites are usually clustered together on the chromosome (Weber *et al.*, 2015a), encoded by large secondary metabolism BGCs, such as polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) (Jackson *et al.*, 2018). Actually, actinobacteria synthesise functionally diverse classes of bioactive compounds, often through NRPS and PKS pathways (Dinesh *et al.*, 2017). Some of this secondary metabolites have been reported as antifungal agents (Busch and Hertweck, 2009; Arasu *et al.*, 2013; Arnison *et al.*, 2013; Liu *et al.*, 2015). Thus, **screening for genes associated with secondary metabolism is a helpful tool in evaluating the biosynthetic potential of the actinobacteria** (Nimaichand *et al.*, 2015).

Understanding bacterial metabolic regulation help to improve any genetic engineering approach (Tsolis *et al.*, 2018). Moreover, the availability of closely related genomes make it possible to assess the mechanisms underlying genome plasticity and systems adaptation (Zhou *et al.*, 2012). In terms of annotation and functional analysis of metagenomes, a common tool is RAST server (Meyer *et al.*, 2008; Overbeek *et al.*, 2014), which is included into the SEED database (Overbeek *et al.*, 2014). This tool allows a functional classification in subsystems, that is, a set of “functional roles” that together implement a specific process or structural complex (Overbeek *et al.*, 2005).

The aim of this study consists of the genome analysis and understanding of two *Streptomyces* strains with biocontrol activity to control fungal trunk pathogens involved in GTD and YGD, in order to identify the putative secondary metabolites responsible for their biocontrol effect.

III.3. Material and Methods

III.3.1. Strains used and genome sequencing

Streptomyces sp. VV/E1 and *Streptomyces* sp. VV/R4, isolated from the inner tissues and the rhizosphere of young grapevines plants (section I.3.1.2) (Álvarez-Pérez *et al.*, 2017) were used to carry out this work. They were grown in TSB (Sigma-Aldrich) at 220 rpm / 48 h / 30°C. Genomic DNA was extracted following the protocol detailed by Hopwood *et al.* (1985) (see section I.3.2.1).

Streptomyces sp. VV/E1 was sequenced using PacBio technology. DNA from this strain was send to Macrogen for sequencing, assembling and annotating. *Streptomyces* sp. VV/R4 was sequenced and assembled in a collaboration with Evogene (Rehovot, Israel).

III.3.2. Bioinformatics tools

Functional analysis of both genomes were carried out using RAST (Rapid Annotation using Subsystem Technology) server (<http://rast.theseed.org/FIG/rast.cgi>) (Aziz *et al.*, 2008; Overbeek *et al.*, 2014). For the analysis of secondary metabolites antiSMASH (Medema *et al.*, 2011; Blin *et al.*, 2013; Weber *et al.*, 2015) was run on the complete genome sequence of both *Streptomyces* strains.

III.3.3. Amplification of biosynthetic genes involved in secondary metabolism

To amplify three different genes involved in secondary metabolism, three sets of degenerate PCR primers were used: K1F (5'-TSAAGTCSAACATCGGBCA-3') and M6R (5'-CGCAGGTTSCSGTA CCAGTA-3') targeting PKS-I sequences (Ayuso-Sacido and Genilloud, 2004); KS α F (5'-TSGCSTGCTTCGAYGCSATC-3') and KS α R (5'-TGGAANCCGCC GAABCCGCT-3') targeting a portion of the KS α from PKS type II genes (Janso and Carter, 2010); while A3F (5'-GCSTACSYSATSTACACSTCSGG-3') and A7R (5'-SASGTCVCC SGTSCGGTAS-3') targeted NRPS sequences (Ayuso-Sacido and Genilloud, 2004). Amplification reactions were developed in 1x paq5000 buffer (Agilent Technologies) with 500 nM of each primer (IDT), 0.1 mM dNTP mix (EURx), 1U paq5000 DNA polymerase (Agilent Technologies) and 15 ng template DNA. ddH₂O was used to bring the reaction volume to 25 μ L.

PCR amplification was carried out in 0.2 mL sterile tubes using a Mastercycler Gradient Thermocycler. PCR conditions were: 96°C for 5 min; 30 cycles of 96°C for 1 min, T_m for 1 min, 72°C for 90 s; and a final extension of 72°C for 10 min. T_m was 57°C for PKS-I and NRPS

amplifications, while 58°C was the T_m for PKS-II amplification. Tubes were kept at 4°C until the reactions were analysed in 1% (w/v) agarose gels in 1x TAE buffer containing 0.15 µg/mL ethidium bromide (Sigma-Aldrich) and photographed using VWR GenoSmart equipment (VWR).

III.4. Results

III.4.1. Genome analysis of *Streptomyces* sp. VV/E1 and VV/R4 strains

Streptomyces sp. VV/E1 and *Streptomyces* sp. VV/R4 had been selected, after field assays, for their putative effect as BCAs against grapevine trunk pathogens. Sequence data of both genomes were analysed with different bioinformatics tools in order to characterise them.

III.4.1.1. Functional analysis

The functional analysis of both genomes was carried out with RAST server (Meyer *et al.*, 2008).

The genome size of *Streptomyces* sp. VV/E1 strain was 8,857,163 bp with a GC content of 71.4%. Moreover, it contained 89 RNAs and 7,866 coding genes. Subsystem distribution of this strain is shown in Fig. III-1A. Functional classification by subsystem database revealed the predominance of amino acids and derivatives subsystem (14.19%), followed by fatty acids, lipids and isoprenoids (12.45%), cofactors, vitamins and prosthetic group (12.32%), energy and precursor metabolites generation (11.64%) and protein synthesis (6.29%) subsystems.

In a similar way *Streptomyces* sp. VV/R4 genome was analysed. The size of this strain was 8,559,629 bp with a GC content of 71.5%. It contained 83 RNAs and 7,509 coding genes. Subsystem distribution (Fig III-1B) showed the predominance of amino acids and derivatives subsystem (14.07%), followed by cofactors, vitamins and prosthetic group (12.74%), fatty acids, lipids and isoprenoids (12.11%), energy and precursor metabolites generation (12.05%) and protein synthesis (6.25%) subsystems.

When **comparing the genomes of the two strains** using The SEED Viewer (version 2.0) (Fig. III-2), a high similarity could be observed. In fact, for most of the analysed proteins, **aminoacid identity was higher than 95%**. In addition, the distribution of subsystems (Fig. III-1) was also very similar in both strains.

Fig. III-1 Distribution of functional categories for subsystem database in *Streptomyces* sp. VV/E1 (**A**) and *Streptomyces* sp. VV/R4 (**B**) strains. Each slice represent the percentage of genes that match into a particular category.

Fig. III-2 Comparison of the genomes of *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 using The Seed Viewer tool. The colour code indicates the percent protein sequence identity.

III.4.1.2. Identification, annotation and analysis of secondary metabolite clusters using antiSMASH

AntiSMASH software was run for *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 in order to identify bioactive compounds that could be related to their antifungal activity.

On the one hand, the antiSMASH run for *Streptomyces* sp. VV/E1 identified 30 gene clusters, belonging to 19 secondary metabolite cluster types. Terpene-like compounds were the most abundant, representing the 20% (6 out of 30 BGCs) followed by

siderophore, which represented the 10% (3 out of 30 BGCs) (Table III-1A).

On the other hand, the antiSMASH run for *Streptomyces* sp. VV/R4 detected 36 gene clusters belonging to 19 secondary metabolite cluster types. Terpene and polyketide synthases type I were the most abundant, representing the 19.44% each one (7 out of 36 BGCs), followed by siderophores which represented the 8.33% (3 out of 36) (Table III-1B). The comparison between the relative abundance of the different classes of BGCs in both strains is shown in Fig. III-3.

Table III-1A AntiSMASH for the identification of secondary metabolites in *Streptomyces* sp. VV/E1. Putative antifungal compounds are marked with an asterisk (*) and highlighted in blue colour

Cluster	Type	Most similar known cluster	Genes show similarity (%)
Cluster 1	Butyrolactone	-	
Cluster 2	Siderophore	Desferrioxamine B biosynthetic gene cluster	100
Cluster 3	Melanin	Melanin biosynthetic gene cluster	100
Cluster 4	Nrps	SCO-2138 biosynthetic gene cluster	64
Cluster 5	Ladderane-Arylpolyene-Nrps	WS9326 biosynthetic gene cluster	95
Cluster 6	Ectoine	Ectoine biosynthetic gene cluster	100
Cluster 7	Nrps	Antimycin biosynthetic gene cluster*	100
Cluster 8	T2pks	Spore pigment biosynthetic gene cluster	83
Cluster 9	Terpene	Carotenoid biosynthetic gene cluster	54
Cluster 10	T1pks-Nrps	Albachelin biosynthetic gene cluster	80
Cluster 11	T1pks-Butyrlactone	Streptazone E biosynthetic gene cluster	75
Cluster 12	Siderophore	Grincamycin biosynthetic gene cluster	8
Cluster 13	Terpene	-	-
Cluster 14	Bacteriocin	-	-
Cluster 15	Other ks	-	-
Cluster 16	Indole	7-prenylisatin biosynthetic gene cluster	60
Cluster 17	Transatpks-Nucleoside_Nrps	Herboxidiene biosynthetic gene cluster	9
Cluster 18	Siderophore	-	-
Cluster 19	Terpene	Albaflavenone biosynthetic gene cluster	100
Cluster 20	Lantipeptide	-	-
Cluster 21	Linaridin	Legonaridin biosynthetic gene cluster	33
Cluster 22	T1pks	Zorbamycin biosynthetic gene cluster	8
Cluster 23	Terpene	Fluostatin biosynthetic gene cluster	4
Cluster 24	T1pks	Candididin biosynthetic gene cluster*	95
Cluster 25	Bacteriocin	Infomatipeptin biosynthetic gene cluster	57
Cluster 26	Terpene	-	-
Cluster 27	Transatpks-Ectoine-Lantipeptide-T1pks-Nrps	Daptomycin biosynthetic gene cluster	18
Cluster 28	Terpene	Hopene biosynthetic gene cluster	92
Cluster 29	Lantipeptide	SAL-2242 biosynthetic gene cluster	80
Cluster 30	Lasso peptide	SRO15-2005 biosynthetic gene cluster	40

Chapter III

Table III-1B AntiSMASH for the identification of secondary metabolites in *Streptomyces* sp. VV/R4. Putative antifungal compounds are marked with an asterisk (*) and highlighted in blue colour

Cluster	Type	Most similar known cluster	Genes show similarity (%)
Cluster 1	Terpene	Albaflavenone biosynthetic gene cluster	100
Cluster 2	Lantipeptide	-	-
Cluster 3	Butyrolactone	-	-
Cluster 4	Other ks	-	-
Cluster 5	Bacteriocin	-	-
Cluster 6	Terpene	-	-
Cluster 7	Siderophore	Grincamycin biosynthetic gene cluster	8
Cluster 8	Bacteriocin	Infomatipeptin biosynthetic gene cluster	57
Cluster 9	Terpene	-	-
Cluster 10	Transatpks-Ectoine-Lantipeptide-T1pks-Nrps	Daptomycin biosynthetic gene cluster	15
Cluster 11	T2pks	Spore pigment biosynthetic gene cluster	83
Cluster 12	T1pks-Nrps	Antimycin biosynthetic gene cluster*	100
Cluster 13	Nrps	SCO-2138 biosynthetic gene cluster	64
Cluster 14	Lasso peptide	-	-
Cluster 15	Siderophore	-	-
Cluster 16	Transatpks-Nucleoside-Nrps	Herboxidiene biosynthetic gene cluster	9
Cluster 17	Indole	7-prenylisatin biosynthetic gene cluster	60
Cluster 18	Terpene	Fluostatin biosynthetic gene cluster	4
Cluster 19	T1pks	Zorbamycin biosynthetic gene cluster	8
Cluster 20	Linaridin	Legonaridin biosynthetic gene cluster	33
Cluster 21	Terpene	Carotenoid biosynthetic gene cluster	54
Cluster 22	T1pks-Nrps	Albachelin biosynthetic gene cluster	80
Cluster 23	Nrps-Arylpolyene-Ladderane	WS9326 biosynthetic gene cluster	95
Cluster 24	Terpene	Borrelidin biosynthetic gene cluster*	6
Cluster 25	T1pks	Divergolide biosynthetic gene cluster	31
Cluster 26	T1pks	Nystatine-like <i>Pseudocardia</i> polyene biosynthetic gene cluster*	30
Cluster 27	Ectoine	Ectoine biosynthetic gene cluster	100
Cluster 28	Lantipeptide	SAL-2242 biosynthetic gene cluster	100
Cluster 29	Melanin	Melanin biosynthetic gene cluster	100
Cluster 30	T1pks	Candididin biosynthetic gene cluster*	90
Cluster 31	Siderophore	Desferrioxamine B biosynthetic gene cluster	100
Cluster 32	T1pks-Butyrolactone	Streptazone E biosynthetic gene cluster	58
Cluster 33	T1pks	Hygrocin biosynthetic gene cluster	16
Cluster 34	Terpene	Hopene biosynthetic gene cluster	61
Cluster 35	T1pks	-	-
Cluster 36	T1pks	-	-

Fig. III-3. Relative abundance of the different biosynthetic gene clusters in *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4.

Polyketides and non-ribosomal peptides are two of the most important and diverse classes of secondary metabolites. In *Streptomyces* sp. VV/E1, PKSs and NRPSs represented the 36.67% of the detected clusters (11 out of 30 BGCs), either as single classes (6 out of 30), as hybrids PKSs/NRPSs (1 out of 30) or associated with other secondary metabolite clusters (4 out of 30). In *Streptomyces* sp. VV/E4, PKSs and NRPSs represented the 44.44% (16 out of 36 BGCs), either as single classes (10 out of 36), as hybrids PKSs/NRPSs (2 out of 36), or linked to other secondary metabolite clusters (4 out of 36).

When comparing the antiSMASH of both strains, most of the BGCs were common, regulating the synthesis of the same secondary metabolites such as daptomycin, 7-prenylisatin, streptazone derivatives or hopene type terpenoids.

III.4.2. Detection of PKS-I, PKS-II and NRPS genes by PCR in the isolated strains

A PCR approach to screen the isolates for genes associated with secondary metabolite production was applied to evaluate the biosynthetic potential of the two selected actinobacteria (*Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4). Moreover, this assay was also performed for all the endophytic and rhizosphere actinobacteria isolated (Álvarez-Pérez *et al.*, 2017) in order to compare the presence of this genes with the effect showed in field assays (Table III-2).

Table III-2A Screening for genes associated with secondary metabolism through a PCR approach.

Isolate	PKS-I	PKS-II	NRPS	<i>Da. macrodidyma</i>	<i>D. seriata</i>
VV/E1	+	+	-	+	+
VV/E2	+	+	+	+	+
VV/E3	-	-	+	+	+
VV/E4	+	+	-	+	+
VV/E5	+	+	+	+	+
VV/E6	+	+	+	+	+
VV/E7	+	-	+	+	-
VV/E8	-	-	-	+	-
VV/E9	+	+	+	-	+
VV/E10	+	-	-	+	-
VV/E11	-	-	-	-	+
VV/E12	+	+	+	-	-
VV/E13	+	-	+	-	-
VV/E14	+	+	+	-	-
VV/E15	+	+	+	-	-
VV/E16	+	+	+	-	-
VV/E17	+	+	+	-	-
VV/E18	+	+	+	-	-
VV/E19	+	+	+	-	-
VV/E20	+	+	+	-	-
VV/E21	-	+	+	-	-
VV/E22	+	+	+	-	-
VV/E23	+	+	+	-	-
VV/E24	+	+	+	-	-
VV/E25	+	-	+	-	-
VV/E26	+	+	+	-	-
VV/E27	+	+	+	-	-
VV/E28	+	-	-	-	-
VV/E29	+	+	+	-	-
VV/E30	+	-	+	-	-
VV/E31	+	-	+	-	-
VV/E32	-	-	-	-	-
VV/E33	-	-	-	-	-
VV/E34	-	-	+	-	-
VV/E35	-	-	-	-	-
VV/E36	+	-	+	-	-
VV/E37	+	-	+	-	-
VV/E38	+	-	+	-	-
VV/E39	+	+	+	-	-
VV/E40	+	-	-	-	-
VV/E41	-	-	-	-	-
VV/E42	-	-	-	-	-
VV/E43	+	+	+	-	-
VV/E44	+	+	+	-	-
VV/E45	+	+	+	-	-
VV/E46	+	-	+	-	-
VV/E47	-	-	+	-	-
VV/E48	+	-	+	-	-
VV/E49	+	-	+	-	-
VV/E50	-	-	-	-	-
VV/E51	-	-	-	-	-
VV/E52	+	-	+	-	-
VV/E53	-	-	-	-	-
VV/E54	-	-	+	-	-
VV/E55	-	-	+	-	-
VV/E56	+	-	+	-	-
VV/E57	-	+	+	-	-
VV/E58	-	-	+	-	-

(+) indicates the presence of PKS-I, PKS-II and NRPS genes and antifungal activity in culturable endophytic actinobacteria isolated from the root system of grafted grapevines.

(-) lack of this genes/activity.

Table III-2B Screening for genes associated with secondary metabolism through a PCR approach.

Isolate	PKS-I	PKS-II	NRPS	<i>Da. macrodidyma</i>	<i>D. seriata</i>
VV/R1	+	+	-	+	+
VV/R2	+	+	+	+	+
VV/R3	+	-	+	+	+
VV/R4	+	+	+	+	+
VV/R5	+	+	+	+	+
VV/R6	-	+	+	+	+
VV/R7	-	+	-	+	+
VV/R8	+	+	-	+	+
VV/R9	-	+	+	+	+
VV/R10	+	+	+	+	+
VV/R11	+	+	+	+	+
VV/R12	+	-	+	+	+
VV/R13	-	+	+	+	+
VV/R14	+	+	+	+	+
VV/R15	+	-	-	+	+
VV/R16	-	-	+	+	-
VV/R17	+	+	+	+	-
VV/R18	+	+	+	+	-
VV/R19	-	-	+	+	-
VV/R20	-	+	+	+	-
VV/R21	+	+	+	+	-
VV/R22	+	+	+	+	-
VV/R23	-	+	-	+	+
VV/R24	-	+	-	+	-
VV/R25	+	+	+	+	-
VV/R26	+	+	+	+	-
VV/R27	+	+	+	+	-
VV/R28	+	+	+	+	-
VV/R29	-	+	+	+	-

(+) indicates the presence of PKS-I, PKS-II and NRPS genes and antifungal activity in culturable rhizosphere actinobacteria isolated from the rhizosphere of grafted grapevines.

(-) lack of this genes/activity.

Most of the strains showed amplification at least for one of the three genes. However **no clear relationship between the presence of these genes and the existence of antifungal activity against fungal trunk pathogens could be established.** On the one hand, some strains like VV/E15 and VV/E16 (among many others) had PKS-I, PKS-II and NRPS genes, but they did not exhibit antifungal activity against the tested pathogens. On the other hand, other strains like VV/E8 were negative for the amplification of these genes, but showed antifungal activity against *Da. macrodidyma*. Thus, different combinations could be detected, which did not allow to get any clear conclusion.

III.5. Discussion

Biocontrol bacteria strains and their secondary metabolites can reduce pathogen diseases, making them an important tool for the future control of trunk diseases in agriculture (Compant *et al.*, 2013). Thus, some compounds have been related to the increase of plant resistance against fungal pathogens (Río *et al.*, 2004). Phenolic compounds including tannins, phenolic acids, flavonoids and stilbenes are involved in the vine defence mechanism, increasing plant resistance (Río *et al.*, 2004). Other phenolic compounds like p-coumaric, caffeic acid or catechin inhibit the enzyme activity involved in ligning degradation after infection with fungi which cause Petri disease, suggesting that phenolics have a direct impact on fungal growth and sporulation (Rusjan *et al.*, 2017). Actinobacteria have also been reported to produce compounds with antifungal activity. In this sense, *Streptomyces* sp. AP-123 has been characterised for the production of polyketide like extracellular products effective against pathogenic bacteria and filamentous fungal pathogens (Arasu *et al.*, 2013). Polyene macrolides, produced by *Streptomyces* strains and other soil bacteria, are also antifungal agents due to their affinity for ergosterol, the predominant sterol in fungal membranes (Caffrey *et al.*, 2008).

The genus *Streptomyces* is widely recognised to **produce secondary metabolites with relevant bioactive activities** (Stulberg *et al.*, 2016; Robertsen *et al.*, 2018) **including antifungals** (Rolshausen *et al.*, 2010; Díaz and Latorre, 2013; Sosnowski *et al.*, 2013). In fact, in recent years, it has become clear that **actinobacteria** can be an **important reservoir of genetic diversity**, as well as an **important source for the discovery of bioactive secondary metabolites which can help control many crop pathologies** (Joseph *et al.*, 2012; Álvarez-Pérez *et al.*, 2017).

Genome and BGC analysis of both *Streptomyces* sp. genomes led to the identification of several bioactive molecules with a putative role in the biocontrol effect. Within the several secondary metabolites already described, PKS and NRPS are two of the most important and diverse classes of secondary metabolites (Johnston *et al.*, 2015; Nimaichand *et al.*, 2015). The genome of *Streptomyces* sp. VV/E1 and VV/R4 contains a high percentage (around 35 - 45% depending on the strain) of these two types of secondary metabolites. Moreover, when comparing the antiSMASH of both strains, they had most of the BGCs in common, which regulates the synthesis of the same secondary metabolites with antibiotic activity. For instance, daptomycin is a 13-aminoacid cyclic lipopeptide produced by a NRPS with antimicrobial activity against a wide spectrum of Gram-positive pathogens (Streit *et al.*, 2004; Miao *et al.*, 2005). Moreover, 7-prenylisatin has been reported as a isatin-type antibiotic produced by *Streptomyces* with an indole prenyltransferase that catalyses the conversion of tryptophan into 7-prenylisatin, a compound with antimicrobial activity against *Bacillus subtilis* (Wu *et al.*, 2015). Streptazone derivatives are piperidine alkaloids isolated from several *Streptomyces* species that display cytotoxicity and antibiotic activities (Ohno *et al.*, 2015).

Hopene-type terpenoids (such as sonhafouonic acid), have also been reported to have a moderate antifungal and cytotoxic activity (Kongue *et al.*, 2013).

Other **secondary metabolites, shared by both actinobacteria, have been specifically described as antifungal compounds.** In this way, **antimycin** exhibits a myriad of biological properties including antifungal activity (Hosotani *et al.*, 2005), owing to its ability to inhibit mitochondrial electron transport chains (Liu *et al.*, 2016; McLean *et al.*, 2016). **Candicidin**, a polyene macrolide, is also an important antifungal agent (van Keulen and Dyson, 2014; Zhang *et al.*, 2015), whose activity results from the specific binding to ergosterol, a major component of the fungal membrane, causing the formation of channels that lead to the leakage of cellular materials (Zhang *et al.*, 2015). BGCs for both antifungal compounds could be detected in both genomes. On the other hand, two BGCs that control the synthesis of secondary metabolites with antifungal activity were only detected in *Streptomyces* sp. VV/R4 genome. These antifungal compounds were borrelidin (Gao *et al.*, 2012; Fang *et al.*, 2015) and a nystatin-like polyene (Odds *et al.*, 2003).

In addition, in iron deficient conditions, some bacteria, including actinomycetes (Anwar *et al.*, 2016), secrete siderophores, a small molecular weight compounds with a high-affinity of iron chelating (Kodani *et al.*, 2015). Siderophore producing actinomycetes have been described to have antagonistic activity against plant pathogens (Tamreihao *et al.*, 2016). This antagonism is due to the fact that siderophores produce iron complexes in the rhizosphere making iron unavailable to the phytopathogen (Aznar and Dellagi, 2015; Anwar *et al.*, 2016).

In summary, **the genome of both strains contains BGCs related to the synthesis of antifungal compounds.** Their putative involvement in the biocontrol effect against grapevine trunk pathogens will be analysed in chapter IV.

III.6. References

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CHAPTER IV: GENOME EDITING IN *STREPTOMYCES* SP. VV/E1 BY CRISPR

CRISPR protocols and techniques used in this chapter were learnt during a 3-month PhD stay en the Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark (DTU), under the supervision of Professor Dr. Tilmann Weber. CRISPR plasmids (pUSER2-Cas9 and pUSER2-Cas9/ScaLigD) were kindly provided by Professor Dr. Tilmann Weber and his team group to perform this study.

Streptomyces griseus IMRU 3570 was kindly provide by Prof. Dr. Jose Antonio Gil (University of León, León, Spain)

HPLC-MS analysis was performed in collaboration with Biomar Microbial Technologies (León, Spain).

IV.1. Abstract

The genus *Streptomyces* has a very high potential to synthesise secondary metabolites, with diverse application in agriculture. In spite of their genetic potential, most of the genes responsible for the production of this bioactive compounds remain silent under standard laboratory conditions. Moreover, genetic manipulation in actinomycetes is much more difficult than in other model microorganisms.

Advances in the field of Bioinformatics have revealed a rich reservoir of natural products in *Streptomyces* genomes. Genetic manipulation based on clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) protein systems has considerably facilitate high-efficiency genome editing in *Streptomyces* strains. This tool has been used in *Streptomyces* sp. VV/E1 to knock-down genes related to the synthesis of secondary metabolites described as antifungals like antimycin, candicidin and also the *msiK* gene which is essential for chitinase production. Mutant strains for these genes were analysed by *in vitro* bioassay to check their putative involvement in the observed antifungal activity against grapevine trunk pathogens.

The production of secondary metabolites was also analysed by HPLC-MS in order to check their role on the biocontrol activity showed by *Streptomyces* sp. VV/E1 against fungal trunk pathogens.

Finally, some hormones like indole-3-acetic acid (IAA) provide several benefits to host plants including growth promotion. The production of this phytohormone was also analysed in order to complete the characterization of *Streptomyces* sp. VV/E1.

Despite the fact that this streptomycete shows a biocontrol effect against fungi that cause grapevine trunk diseases (GTD) and young grapevine decline (YGD), the identification of the secondary metabolites or enzymes involved in this control is a hard issue, and it remains unknown under *in vitro* conditions.

IV.2. Introduction

Actinobacteria are Gram-positive soil bacteria characterised by a GC rich genomic DNA (Barka *et al.*, 2016) and a complex secondary metabolism which undergoes a complicated regulation (Huang *et al.*, 2015). They produce many compounds with widely diverse structures and biological activities, including antifungals (Huang *et al.*, 2015; Low *et al.*, 2018). More than 10,000 bioactive bacterial compounds have been described to be produced by the genus *Streptomyces* (Weber *et al.*, 2015). **These secondary metabolites are not essential for bacterial growth, but instead play an important ecological role in mediating bacteria-host interactions** (Cimermancic *et al.*, 2014; Stulberg *et al.*, 2016).

Despite the genetic potential to synthesise multiple complex metabolites, only a fraction of metabolites encoded by biosynthetic gene clusters (BGCs) are detected in routine fermentations (Robertsen, 2017; Zhang *et al.*, 2017). Instead, **most of the BGC responsible for the production of these secondary metabolites are still silent under standard laboratory culture conditions** (Robertsen, 2017; Zhang *et al.*, 2017).

Actinobacteria, particularly those of genus *Streptomyces*, remain as invaluable hosts for the discovery and engineering of natural products and their biosynthetic pathways (Tong *et al.*, 2015; Cobb *et al.*, 2015). However, **in comparison with other model organisms, such as *Saccharomyces cerevisiae* or *Escherichia coli*, few genetic manipulation tools have been developed in this genus**, which hampers the functional genome research of this industrially important bacteria (Tong *et al.*, 2015; Tao *et al.*, 2018; Zhao *et al.*, 2018). This difficulty in their genetic manipulation is due, in part, to the diversity and extremely high GC content of their genomes, which is a bottleneck for systematic metabolic engineering (Tong *et al.*, 2015).

In the past decades, typically, for gene disruption in *Streptomyces*, single crossover integration of a suicide plasmid could be employed, resulting in the disruption of the gene of interest with a selectable marker (Cobb *et al.*, 2015). However, the restricted number of selectable markers limited the reusability of this approach. Further, disruption via single-crossover could revert in the absence of selective pressure, resulting in undesired restoration of the wild-type allele. Inclusion of flanking recombinase target sites and expression of the corresponding recombinase could enable recycling of markers and improved mutation stability, but this required additional steps and left a scar sequence at the target site. Alternatively, clean genomic deletions could be made via double-crossover integration. However, this multistep process was often labour and time intensive, if even possible (Cobb *et al.*, 2015).

Recently, the great advances in next-generation sequencing technologies and computational resources have rapidly expanded the genomic information of numerous

organisms and have revealed a rich reservoir of natural product gene clusters from microbial genomes, especially from *Streptomyces* (Wang *et al.*, 2016; Tao *et al.*, 2018; Tong *et al.*, 2018). As average, these microorganisms are supposed to possess 20 - 50 BGCs in a single genome, number that greatly exceed the already identified compounds (Robertsen, 2017; Tao *et al.*, 2018). Nevertheless, most BGCs are silent or poorly expressed in native host under conventional laboratory culture conditions (Robertsen, 2017; Zhang *et al.*, 2017; Tao *et al.*, 2018). To activate these cryptic BGCs, high-efficient approaches for genome editing and BGC engineering have gained widespread attention (Tao *et al.*, 2018). **Application of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) system, especially the CRISPR-Cas9 system, has greatly facilitated high-efficiency genome editing** (Cobb *et al.*, 2015; Tong *et al.*, 2015; Wang *et al.*, 2016; Tao *et al.*, 2018). It consists of an adaptive immune system distributed among most archaea and many bacteria (Makarova *et al.*, 2015), that has been extensively developed as a powerful technology for genome editing in both eukaryotes and prokaryotes (Zhao *et al.*, 2018). Since CRISPR/Cas system exhibits higher specificity and accuracy on sequence targeting, it has become excellent choice for precision genome editing (Tao *et al.*, 2018). This tool allows to delete gene(s) or gene cluster(s), implement precise gene replacement, and reversibly control gene expression in actinomycetes (Tong *et al.*, 2015).

This tool has widely used mainly in *Streptomyces coelicolor* A3(2) to knock-down the actinorhodin (ACT) (Huang *et al.*, 2015; Robertsen, 2017; Tong *et al.*, 2015), calcium-dependent antibiotic (CDA) (Zhao *et al.*, 2018) or undecylprodigiosin (RED) production (Huang *et al.*, 2015; Robertsen, 2017), but has been also used for the deletion of the same BGC (ACT and RED) in *Streptomyces lividans* (Zhang *et al.*, 2017). **In this study, different genes and BGCs have been knocked-down using this tool in *Streptomyces* sp. VV/E1 in order to detect those secondary metabolites involved in the biocontrol process against fungal trunk pathogens.**

Moreover, actinobacteria are also a promising source of a wide range of **extracellular enzymes** (Ranjani *et al.*, 2016; Dinesh *et al.*, 2017). In this sense, chitinases play a crucial role in maintaining soil ecology, fertility and health and stimulate plant growth (Jog *et al.*, 2012). Furthermore, in nature, this enzyme is involved in the degradation of chitin biopolymer, which is associated with fungal cell walls (Lacombe-Harvey *et al.*, 2018).

Another family of enzymes of great interest are cyclodipeptide synthases (CDPSs), which use two aminoacids, activated as aminoacyl-tRNAs, as substrates to synthesise cyclodipeptides (Gondry *et al.*, 2018). These cyclodipeptides, also named 2,5-diketopiperazines (2,5-DKPs) (Gondry *et al.*, 2018), can be produced by many *Streptomyces* strains (Wattana-Amorn *et al.*, 2016). They are the **precursors of many**

secondary metabolites with diverse and noteworthy biological activities (Moutiez *et al.*, 2014; Gondry *et al.*, 2018), including antifungals (Wang *et al.*, 2017; Liu *et al.*, 2018)

However, **actinobacteria**, and especially the genus *Streptomyces*, are not only known for its ability to produce secondary metabolites or enzymes, but they **have also been described as promising biocontrol agents to control plant diseases** (Doumbou *et al.*, 2001; Schrey and Tarkka, 2008; Schrey *et al.*, 2012; Barka *et al.*, 2016; Chater, 2016; Ranjani *et al.*, 2016). Actinobacteria associated with plants provide several benefits to host plants, including growth promotion (Gopalakrishnan *et al.*, 2013; Passari *et al.*, 2015). Therefore, microbiologists have widely studied the production of phytohormones by microorganisms to understand their effect on plant growth (Goswami *et al.*, 2015). In this sense, soil microorganisms, including *Streptomyces* species, are able to produce phytohormones, including auxins, (Glick, 2014; Goswami *et al.*, 2015), whose biosynthesis involve tryptophan as precursor (Goswami *et al.*, 2015). One of this important phytohormones is the indole-3-acetic acid (IAA) auxin.

In summary, *Actinobacteria* strains with biocontrol activity and their secondary metabolites can reduce pathogen diseases, either directly or indirectly, affecting pathogen mode of action (Compant *et al.*, 2013). Therefore, the **development of biocontrol strains has become an important tool for the future control of trunk diseases in agriculture** (Compant *et al.*, 2013). Several studies have focused on the use of beneficial microorganisms as biocontrol agents to combat fungal trunk pathogens (Di Marco *et al.*, 2004; Loqman *et al.*, 2009; Kotze *et al.*, 2011; Couillerot *et al.*, 2014; Andreolli *et al.*, 2016; Álvarez-Pérez *et al.*, 2017). **This study aims to focus on the identification of secondary metabolites or enzymes involved in the biocontrol activity of *Streptomyces* sp. VV/E1 to control fungal trunk pathogens which cause GTD and YGD.**

IV.3. Material and Methods

IV.3.1. Bacterial strains

E. coli **DH5 α** (Hanahan, 1983) was used for routinely transformation assays due to the high transformation efficiency of this competent cells (up to 5×10^8 cells / mg of plasmid DNA) and the lowest recombination rate. The genotype of this strain is: F- $\Phi 80lacZ\Delta M15 \Delta(lacZYA-argF)$ U169 *recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ -*.

E. coli **ET12567/pUZ8002** (Bierman *et al.*, 1992). It was used as donor strain during the intergenic conjugation process from *E. coli* to *Streptomyces* sp. VV/E1. This strain is resistant to chloramphenicol. Furthermore, it contains the pUZ8002 plasmid that carries the resistant gene for kanamycin and those genes which allow the conjugation (*tra* genes). The genotype of this strain is: *dam13::Tn9(ChlR) dcm-6 hsdM, hsdR recF143 zjj-201::Tn10 galK2 galT22 ara14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtII glnV44. pUZ8002(KanR)*.

One Shot® *ccdB* Survival™ 2 T1R chemically competent cells (Thermo Fisher Scientific). Competent cells used for the propagation of pUSER plasmids harbouring the *ccdB* and chloramphenicol resistant (*CmR*) gene cassette. The genotype of this strain is: F-*mcrA $\Delta(mrr-hsdRMS-mcrBC)$ $\Phi 80lacZ\Delta M15 \Delta lacX74 recA1 ara\Delta 139 \Delta(ara-leu)7697 galU galK rpsL$ (StrR) *endA1 nupG fhuA:IS2*.*

Streptomyces griseus **IMRU 3570** (Campello and Gil, 2002). *Streptomyces* strain used as positive control for candididin production.

IV.3.2. Plasmids

pUSER2-Cas9 and pUSER2-Cas9/ScaLigD (Robertsen, 2017) (Fig. IV-1). Both plasmids contain as a USER (uracil-specific excision reagent) cassette the *ccdB* gen (a suicide gen in *E. coli* DH5 α) and the *CmR* gene flanked by a restriction and a nicking enzyme. Both plasmids are based on pCRISPR-Cas9 and pCRISPR-Cas9/ScaLigD plasmids (Tong *et al.*, 2018). The nuclease Cas9 is under control of the thiostrepton inducible *tipA* promoter, which requires the presence of the thiostrepton responsive activator TipA. The ScaLigD expression cassette is composed of a *Streptomyces carneus LigD* gene controlled by *ermE** promoter, downstream flanked by a t0 terminator. This cassette can reconstitute the non-homologous end joining (NHEJ) pathway of actinomycetes without a complete NHEJ pathway because of lacking the ligase activity (Tong *et al.*, 2015).

Both plasmids were used to do genome editing of *Streptomyces* sp. VV/E1. On the one hand, pUSER-Cas9 allows to make a random size deletion, flanked by the double stranded break (DSB) site. It can also be used to knockout genes with a homologous recombination template. On the other hand, pUSER-Cas9-ScaLigD permits doing precise genome editing since it allows to knockout genes with an homologous recombination template due to the expression of the ligase D (*LigD*), a core component of the NHEJ pathway (Tong *et al.*, 2015).

Fig. V-1 Maps of pUSER2-Cas9 and pUSER2-Cas9/ScaLigD plasmid with the corresponding primers for clone verification.

IV.3.3. Knock-down of biosynthetic gene clusters by CRISPR

IV.3.3.1. *In silico* plasmid construction

Two different BGCs (cluster 7-NRPS and cluster 24-T1-PKS), and the *msiK* gene which, a priori, could be related to the biocontrol effect of *Streptomyces* sp. VV/E1, were selected to knock down by CRISPR techniques.

On the one hand, once selected the genes to knock down inside the BGCs it was necessary to design a single guide RNA (sgRNA), which interacts with Cas9 to form a complex, using CRISPy web (<https://crispy.secondarymetabolites.org/#/input>). This tool scans the full genome looking for sgRNAs (Blin *et al.*, 2016). In the case of the *msiK* gene, the sgRNA was manually designed based on its nucleotide sequence using the CLC-Main Workbench 8.

IV.3.3.2. Primers design

Depending on the plasmid used, different primers were needed in order to amplify the different constructions required.

For pUSER2-Cas9 it was necessary to amplify the 20 bp-sgRNA, the *ermE** promoter, and two sequences of 1 kb size homologous to both sides of the selected genes (hereinafter left and right arms). For pUSER2-Cas9/ScaLigD it was only necessary to amplify the 20 bp-sgRNA. Primers designed was performed with the online tool AMUSER 1.0 (<http://www.cbs.dtu.dk/services/AMUSER/>), an *in silico* tool for designing oligonucleotides for the USER assembly (Genée *et al.*, 2015) (Table SIV-1).

IV.3.3.3. Inactivation of selected genes and biosynthetic gene clusters.

IV.3.3.3.1. Amplification of the different constructions

For the amplification of the sgRNA and *ermE** promoter the template was a sgRNA purchased from IDT, while for left and right arms was genomic DNA of *Streptomyces* sp. VV/E1. The different constructions were amplified by PCR in a reaction containing 1x Phusion buffer (Phusion HF buffer for sgRNA and *ermE** and Phusion GC buffer for left/right arms amplification), 0.2 mM dNTP mix, 0.2 μ M each primer, 0.5 mM MgCl₂, 1U Phusion U Hot Start DNA polymerase (Thermo Fisher Scientific), and 20 ng DNA. For the amplification of left/right arms 1 M betaine was used to increase the primer specificity and improve the PCR reaction. ddH₂O was used to bring the reaction volume to 50 μ L.

PCR conditions were: 98°C for 30 s; 30 cycles of 98°C for 30 s, melting temperature (T_m) (up to 72°C, calculated by JusBio calculator (<http://www.justbio.com/hosted-tools.html>)) for 30 s, 72°C for 30 s/kb; with a final extension of 72°C for 5 min. Amplified fragments were run by electrophoresis in a 1% (w/v) agarose gel in 1x TAE running buffer, excised from the gel, purified with the commercial kit GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) and measured on NanoDrop (Tong *et al.*, 2018).

IV.3.3.3.2. USER cloning

USER cloning was performed in order to introduce the corresponding construction into pUSER plasmids. USER cloning represents an alternative to conventional ligation-based cloning that allows simultaneous assembly of multiple PCR products into USER-compatible vectors (Tong *et al.*, 2018). To calculate the molar ratios for the USER cloning, a ligation calculator (http://www.insilico.uni-duesseldorf.de/Lig_Input.html) was used. pUSER2-Cas9:left/right arms:sgRNA/*ermE** should be mixed in a 1:3:6 ratio, whereas pUSER2-Cas9/ScaLigD:sgRNA should be mixed in a 1:6 ratio. USER mix contains 0.5x CutSmart buffer, 1U USER enzyme (Biolabs) and the corresponding concentrations of linearized vector (digested with AsiSI restriction enzyme and Nb.BsmI nicking enzyme (New England Biolabs)), and DNA fragments in order to maintain the mix ratio. ddH₂O was used to bring the reaction mixture to 10 µL. Mix was incubated at 37°C for 15 min, followed by incubation at T_m for 15 min (the ligation temperature depends on the primers melting temperature) and 10°C for 10 min (Tong *et al.*, 2018).

IV.3.3.3.3. Transformation in *E. coli* DH5α

USER mix reaction was directly transformed in *E. coli* DH5α competent cells using a modified protocol based on that described by Tong *et al.* (2018). In brief, 90 µL of competent cells were transformed with 10 µL ligation mixture, incubated on ice for 30 min, heated shock at 42°C for 45 s, incubated again on ice for 2 min and cells were recovered in 250 µL SOC medium^{IV.1} (Hanahan, 1983) at 200 rpm / 1 h / 37°C. Next, 100 and 250 µL were plated on a pre-warmed (37°C) selective LB^{IV.2} agar plate (Miller, 1972) supplemented with 50 µg/mL apramycin (Sigma) and incubated 16 h / 37°C (Tong *et al.*, 2018).

IV.3.3.3.4. Clone verification

For clone verification and sequencing, new primers were designed to amplify the entire USER cassette (Table SIV-2). Single colonies from the transformation were used as template for PCR. Amplification reactions were performed containing 1x Dream Taq buffer, 0.2 mM dNTP mix, 0.5 µM each primer (t0_check_FW/Gene_midR_RV for pUSER2-Cas9 cloning and pGM1190_USERseq_FW/pGM1190_USERseq_RV for pUSER2_Cas9/ScaLigD cloning), 1U Dream Taq Polymerase (Thermo Fisher Scientific) and 2 µL of the colony (suspended in 20 µL ddH₂O). ddH₂O was used to bring the reaction volume to 20 µL. In both cases, a positive control (1 µL of the linearized plasmid) and a negative control were included (Tong *et al.*, 2018).

^{IV.1} **SOC medium:** 20.0 g/L tryptone; 5.0 g/L yeast extract; 10.0 mM NaCl; 2.5 mM KCl; 10.0 mM MgCl₂; 10.0 mM MgSO₄; 20.0 mM glucose; pH 6.7 - 7.0

^{IV.2} **LB (Luria-Bertani) agar:** 10.0 g/ tryptone; 5.0 g/L yeast extract; 10.0 g/L NaCl; 20.0 g/L agar; pH 7.5

PCR conditions were 95°C for 3 min; 40 cycles of 95°C for 30 s, 64°C for 30 s, 72°C for 90 s, and a final extension of 72°C for 5 min. Then, PCR amplifications were run by electrophoresis in a 1% (w/v) agarose gel in 1x TAE running buffer and photographed. Successful integration of the USER cassette should result in a PCR product of 1.7 kb for pUSER2-Cas9 and 1.1 kb for pUSER2-Cas9/ScaLigD constructions (Robertsen, 2017).

Colonies with a correct size of PCR product were subjected to Sanger sequencing (Eurofins and MacroGen), and analysed with CLC-Main Workbench 8 in order to check that they did not contain any point mutation.

IV.3.3.3.5. Transformation on ET12567/pUZ8002 competent cells

One clone per gene with the correct sequence was subjected to transformation in ET12567/pUZ8002 competent cells in order to perform the intergenic conjugation in *Streptomyces* sp. VV/E1. This transformation was performed in the same conditions as *E. coli* DH5 α transformation (section IV.3.3.3.3), using 2 - 5 μ L of the plasmid and all the reaction was spread in a single plate of LB agar supplemented with 50 μ g/mL apramycin. Next, all the colonies were collected in 5 mL LB medium supplemented with 50 μ g/mL apramycin, 25 μ g/mL chloramphenicol (Intron Biotechnology) and 25 μ g/mL kanamycin (Thermo Fisher Scientific) and incubated at 225 rpm / O/N / 37°C. In order to preserve those clones with the correct sequence, O/N cultures were mixed with glycerol in a final concentration of 25% (w/v) and store at -20°C. On the other hand, 100 μ L of the liquid culture were inoculated in 100 mL LB supplemented with the same antibiotics and incubated at 225 rpm / O/N / 37°C. Then, the culture was washed twice with 50 mL LB liquid medium without antibiotics supplementation and donor cells were re-suspended in 0.5-2.0 mL LB, depending on the pellet size (Tong *et al.*, 2018).

IV.3.3.3.6. Intergenic conjugation in *Streptomyces* sp. VV/E1

For the intergenic conjugation, 100 μ L ET12567/pUZ8002 donor cells were mixed with 20 μ L *Streptomyces* sp. VV/E1 spores and plated the mix onto Cullum agar^{IV.3} plates, incubated for 16 - 20 h / 30°C. Next day, plates were overlaid with 1 mL of sterilized ddH₂O containing 1 mg nalidixic acid (Sigma) and 1 mg apramycin, and incubated for 3-5 days / 30°C until single colonies grew. Then, 10 - 20 exconjugants were grown onto fresh TSB (Sigma-Aldrich) agar plates supplemented with 50 μ g/mL apramycin, 25 μ g/mL nalidixic acid and 1 μ g/mL thiostrepton (Sigma) for 5 - 7 days (Tong *et al.*, 2018).

^{IV.3} **Cullum agar:** 20.0 g/L mannitol; 20.0 g/L soy flour; 20.0 mM MgCl₂; 20.0 mM CaCl₂; 20.0 g/L agar. This media is prepared in tap water.

IV.3.3.3.7. Exconjugant verification

In order to check the exconjugant strains, new primers were designed for the amplification of an approximately 500 bp fragment around the DSB site of mutation (Table SIV-3). DSB produce mutations, which could be either insertion/deletion or substitution of one or more nucleotides. Thus, differences with regard to the wild-type strain will be looked for, which will mean that the gene is not expressed.

Exconjugants from TSB agar plates supplemented with apramycin, nalidixic acid and thiostrepton were resuspended in 20 μ L ddH₂O and 5 μ L were spreaded onto fresh TSB agar plates supplemented with 50 μ g/mL apramycin, 25 μ g/mL nalidixic acid. Excojugant verification was performed by PCR using the Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific). Amplification reaction contained 1x Phusion GC buffer, 0.2 mM dNTP mix, 0.5 μ M of designed primers, 1 M betaine, 1U Phusion Hot Start II DNA polymerase, 2 μ L of the colony resuspended in ddH₂O (heated 10 min / 90°C in a heating block). ddH₂O was used to bring the reaction volume to 20 μ L.

PCR conditions were: 98°C for 30 s; 35 cycles of 98°C for 10 s, T_m (up to 72°C, calculated by JusBio calculator) for 30 s, 72°C for 10 s (1 kb/15-30 s); and a final extension of 72°C for 5 min. Tubes were kept at 4°C. Amplified fragments were run by electrophoresis in a 1% (w/v) agarose gel in 1x TAE running buffer, excised from the gel, purified with the commercial kit NucleoSpin Gel and PCR (MachereyNagel) and measured on NanoDrop (Tong *et al.*, 2018). Colonies with the correct size of PCR product were subjected to Sanger sequencing, and sequences were analysed with CLC-Main Workbench 8.

IV.3.3.3.8. Verification of antifungal activity in mutant strains

Those exconjugants with any point mutation were checked in a bioassay to test if the corresponding gene, or BGC, could be involved in the antifungal activity. For the bioassay, the corresponding colony was grown in 50 mL TSB liquid culture supplemented with 25 μ g/mL nalidixic acid and incubated at 220 rpm / 2 - 5 days / 30°C. This culture was used for both prepare some glycerol stocks (preserve the mutant strains) and re-inoculate onto fresh TSB supplemented with the antibiotic in the same conditions (perform the bioassay).

For the bioassay, 10 µL of both the mutant and the wild-type strain cultures were spotted on TSB and ISP5^{IV.4} agar plates at 1 cm from the edge of a plate (the mutant in one side and the wild-type in the opposite) that contained an agar plug of the fungi (*Dactylonectria macrodidyma* CBS 120170 or *Diplodia seriata* CBS 112555) in the middle of the plate. Antifungal activity was detected as an inhibitory growth zone around the wild-type/mutant colony, where the fungi cannot grow. The lack of the antifungal activity regarding the wild-type strain, that is, the absence of this inhibitory zone around the mutant colony, would mean that the knocked out gene or BGC would be involved in the biocontrol process.

In the case of the *msiK* gene, responsible for chitinase production, wild-type and mutants were also grown in colloidal chitin agar media^{IV.5} (Passari *et al.*, 2015) to check their chitinase activity.

IV.3.4. Determination of actinobacterial growth: methylene blue method

Actinobacteria growth was determined by methylene blue method, using a modified protocol of Fischer and Sawers (2013). A pre-culture was started in 100 mL of ISP2^{IV.6} and inoculated with 1×10^7 spores of the strain. Liquid culture was incubated at 220 rpm / 24 h / 30°C. Then 10 mL of the pre-culture were used to start a new 100 mL culture (in triplicate) in the same media. They were incubated at 220 rpm / 30°C, and 500 µL samples were taken every 12 h during 3 days and every 24 h for 7 days more. Next, 100 µL of the culture were centrifuged at 10,000 rpm / 6 min / RT. After discarding the supernatant, 100 µL of methylene blue solution^{IV.7} were added to re-suspend the pellet. The mix was incubated at 850 rpm / 10 min / 80°C and cooled to 25°C before centrifuge at 10,000 rpm / 2 min / RT. Finally, the absorbance of 1 mL of a 1:200 dilution, in ddH₂O, of the supernatant was measured (660 nm) in a spectrophotometer Libra S70 (Biochrom). The same dilution of methylene blue was used as blank.

^{IV.4} **ISP5 (glycerol-asparagine agar):** 1.0 g/L L-asparagine (anhydrous basis); 10.0 g/L glycerol; 1.0 g/L K₂HPO₄ (anhydrous basis); 10.0 mL/L trace solution*; 20.0 g/L agar

* **Trace solution:** consists of a small amounts of different salts to improve the composition of several ISP media and, therefore, increase the actinobacteria growth. The composition of this solution is: 1.0 g/L FeSO₄·7H₂O; 0.1 g/L MnCl₂·4H₂O; 0.1 g/L ZnSO₄·7H₂O. It is sterilized on autoclave for 15 min / 121°C and added to the corresponding media when use

^{IV.5} **Colloidal chitin agar media:** 1.5% colloidal chitin**; 0.1% (NH₄)₂SO₄; 0.136% KH₂PO₄; 0.03% MgSO₄·7H₂O; 0.05% yeast extract; 2.0% agar

** **Colloidal chitin:** add 5 g of chitin slowly to 60 mL 37% (w/v) HCl, incubate under vigorous stirring 1 h / RT and filter through glass wool. Next, add 200 mL of 50% ethanol to the filtrate under vigorous stirring. Transfer the precipitate to a glass funnel with filter paper (80 g) and wash with ddH₂O until the colloidal chitin became neutral (pH 7.0). Store the colloidal chitin retained on the filter paper at 4°C in the dark, until further usage

^{IV.6} **ISP2 (yeast extract-malt extract):** 4.0 g/L yeast extract; 10.0 g/L malt extract; 4.0 g/L dextrose; pH 7.2 ± 0.2

^{IV.7} **Methylene blue solution:** 125 g of methylene blue (Fisher Scientific) dissolved in 260 mL 20% ethanol (v/v)

IV.3.5. Determination of chitinase activity

Chitinase activity was checked by spreading the *Streptomyces* strain in agar plates containing colloidal chitin agar media (Souza *et al.*, 2009; Passari *et al.*, 2015). This experiment was carried out in triplicate. Agar plates were incubated for 7 days / 30°C. Chitinase activity was detected by the formation of a clear halo zone around the colony, as a result of chitin degradation.

IV.3.6. Determination of indole-3-acetic acid production

The evaluation of the ability of *Streptomyces* strains VV/E1 to produce IAA was analysed following a modified protocol of Gopalakrishnan *et al.* (2013). Ten 7.0 mm agar plugs of the actinobacteria grown in TSB agar plates were inoculated in 100 mL ISP2 and incubated at 220 rpm / 24 h / 30°C. After, 10 mL of this culture were inoculated in 100 mL ISP2 supplemented with 0.2% L-tryptophan and incubate at 220 rpm / 10 days / 30°C. This assay was performed in triplicate. In order to determine the IAA production, 2.0 mL of the cell culture were taken every 12 hours for the first 3 days and then every 24 h for 7 days more and centrifuged at 13,000 rpm / 15 min / RT. Supernatant was collected and freeze at -20°C. For the analysis, 1.0 mL of the supernatant was mixed with 2.0 mL Salkowsky reagent^{IV.8} and incubated for 30 min / 28°C. Absorbance was measured in a spectrophotometer at 530 nm. IAA production was quantified according to a standard curve constructed using serial dilutions of 1 mg/mL IAA standard (Acros Organics) in acetone. IAA concentrations used for the standard curve were 100, 50, 20, 10, 5 and 0 µg/mL.

IV.3.7. HPLC-MS analysis

Streptomyces sp. VV/E1 was analysed for the production of secondary metabolites by HPLC-MS techniques in collaboration with Biomar Microbial Technologies. This strain was grown in ATCC 172^{IV.9} agar plates for 5 days. Then, 10 agar plugs (7.0 mm diameter) of VV/E1 strain were inoculated in 100 mL MIT medium (composition preserved by the company). The fermentation process was performed in 5 different media supplied and preserved by the company (F5-5, FA-5, MN-2, PAT and SAF), and the two media where the antifungal activity had been first detected in our laboratory: TSB and PDB (Conda Laboratories). Liquid extractions were performed in acetone:methanol:H₂O (1:1:0.2).

^{IV.8} **Salkowsky reagent:** 1 mL of 0.5 M FeCl₃ dissolved in 50 mL of 35% HClO₄

^{IV.9} **ATCC 172:** 10.0 g/L glucose; 20.0 g/L soluble starch; 5.0 g/L yeast extract; 5.0 g/L tryptone; 1.0 g/L CaCO₃; 15.0 g/L agar

The corresponding extracts were analysed using Liquid Chromatography-Mass Spectrometry (LC-MS) with two different detectors: a diode array detector (DAD) in which the UV absorption of the compounds present in the extract was detected, and a mass spectrometry detector (electrospray ionization (ESI) in positive mode) to detect the compounds. Retention time (rt), UV spectrum and mass spectrum were compared with the company database that includes more than 1,000 compounds to identify the metabolites present in the extract.

IV.3.7.1. Evaluation of the antifungal activity of maculosin and L-tryptophan

Eight different concentrations (250, 200, 150, 100, 75, 50, 25 and 10 µg/mL) of pure maculosin (Quimigen S. L.) and L-tryptophan (Acros Organics) were used to perform an *in vitro* bioassay against two fungi involved in GTD (*Dactylonectria macrodidyma* CBS 120170 and *Diplodia seriata* CBS 112555) on PDB-1% (w/v) agar plates. A 7.0 mm agar plug containing the fungi was placed in the middle of the plate and incubated at 25°C / 24 h. Then, 100 µL of each concentration of either maculosin or L-tryptophan were placed in a well into the agar plate at 1 cm from the edge of the plate (4 wells were uniformly distributed into the plate). Plates of *D. seriata* were incubated at 25°C / 10 days while plates of *Da. macrodidyma* were incubated at 25°C / 20 days. This assay was performed in duplicate for each fungi.

IV.3.8. Candicidin extraction

Candicidin extraction was performed with n-butanol as described Campello and Gil (2002). For this, *Streptomyces griseus* IMRU 3570 was grown at 220 rpm / 24 h / 30°C in YED^{IV.10}. Then, 100 mL of SPG^{IV.11} were inoculated with 10 mL of the YED culture and incubated at 220 rpm / 48 h / 30°C. Then, 50 mL of the culture were centrifuged at 10,000 rpm / 15 min / RT and the supernatant was recovered. Candicidin was extracted from the supernatant using the same volume of n-butanol. The upper phase, containing the candicidin was kept at 4°C for further usage. Candicidin was spectrophotometrically determined taking into account the highest adsorption peak at 380 nm (Fig. IV-2).

Fig. IV-2. Characteristic candicidin spectrum measured between 320 nm and 420 nm. It can be observed a highest adsorption peak at 380 nm and another two peaks at 360 nm and around 405 nm. Candicidin was extracted from *Streptomyces griseus* IMRU3570 after 48 h growing in SPG medium.

IV.10 YED: 10.0 g/L glucose; 10.0 g/L yeast extract.

IV.11 SPG 25.0 g/L soy peptone; 60.0 g/L glucose; 0.143 g/L zinc sulphate.

IV.4. Results

IV.4.1. Genome editing by CRISPR

Two BGCs (cluster 7 and cluster 24) detected by antiSMASH (Table III-1A) are involved in the synthesis of two compounds described as antifungals: candicidin and antimycin. To determine the putative role of this BGCs in the biocontrol effect against fungal trunk pathogens related to GTD and YGD, they were knocked-down using CRISPR techniques.

IV.4.1.1. Knock-down of antimycin biosynthetic gene cluster

Cluster 7 is an NRPS cluster type with a 100% similarity to the antimycin BGC deposited in databases. In order to inhibit the production of antimycin secondary metabolite, two different genes were chosen to knock-down this BGC: the condensation (C) domain of a dimodular NRPS (*dhbF_6*), and the ketosynthase (KS) domain of a phthiocerol synthesis type I PKS (*ppsE_1*) (Fig. IV-3).

The inactivation of genes involved in the biosynthesis of this secondary metabolite was carried out using plasmids pUSER2-Cas9 and pUSER2-Cas9/ScaLigD (section IV.3.2). The resulted constructs were verified by PCR and sequenced before go ahead with the intergenic conjugation in *Streptomyces* sp. VV/E1. After conjugation, resulted exconjugants were verified by sequencing, and those which presented any point mutation (mutants for the antimycin BGC) were selected. Only one mutant was obtained using the vector pUSER2-Cas9-ScaLigD: *Streptomyces* sp. VV/E1 Δ ant.

The antifungal activity of *Streptomyces* sp. VV/E1 Δ ant mutant was checked *in vitro* against *Da. macrodidyma* and *D. seriata* (Fig. IV-4A), and compared to the wild-type *Streptomyces* sp. VV/E1. Bioassay results showed that the lack of expression for the knocked-down gene did not correspond to the absence of antifungal activity, since the mutant strain exhibited the same antifungal activity as the wild-type. In order to check the antifungal activity of the pure compound, a bioassay was performed using different concentrations of antimycin (Sigma) against the same fungi (Fig. IV-4B). The analysis indicated that both were not sensitive to antimycin in the range 25 - 100 μ g/mL, and therefore, we can conclude that antimycin was not responsible for the antifungal effect against the tested fungi, at least, under *in vitro* conditions.

Fig. IV-4 *In vitro* assay for the evaluation of the antifungal activity of antimycin mutant (Δ ant) against *D. seriata* compared to the wild-type (WT) (A) and different concentrations of pure antimycin against *Da. macrodidyma* (B).

A **B**

ORF	Start	End	Strand	Sequence	PAM	0 bp mismatches	1 bp mismatches	2 bp mismatches
79	102	-1	GCCGTCGAGCAGGATGTGAT	GGG	0	0	31
192	215	-1	GATGCCACGCGGACCAGCGA	GGG	0	3	83

Fig. IV-3 Schematic representation of the antimycin biosynthetic gene cluster (**A**). Prediction of 20 bp protospacer sequences (CRISPy web; <https://crispy.secondarymetabolites.org/#/input>) specific for *dhbF-6* and *ppsE_1*, two genes that are part of the antimycin biosynthetic gene cluster (**B**).

IV.4.1.2. Knock-down of candicidin biosynthetic gene cluster

In a similar way cluster 24 was analysed. This cluster encoded a type I PKS with a 95% similarity to the candicidin BGC deposited in the databases.

For the knock-down of candicidin BGC, two different 20 bp protospacers were selected in the *fscRI* regulator (Fig. IV-5).

The inactivation of this regulator was also performed using both pUSER plasmids (section IV.3.2). The resulted constructs were verified by PCR and sequenced, prior to intergenic conjugation in *Streptomyces* sp. VV/E1 strain. After conjugation, resulted exconjugants were verified by sequencing. The only mutant obtained using pUSER2-Cas9/ScaLigD (*Streptomyces* sp. VV/E1 Δ can) was selected to perform an *in vitro* bioassay against the two pathogenic fungi (Fig.

Fig. IV-6 *In vitro* assay for the evaluation of the antifungal activity of candicidin mutant (Δ can) compared to the wild-type (WT) against fungal pathogens *Dactylonectria macrodidyma* and *Diplodia seriata*.

IV-6). The inactivation of this gene neither produce an absence of antifungal activity. Therefore, a priori, this secondary metabolite was not responsible for the biocontrol effect. The impossibility to find commercial candicidin made it impossible to perform a bioassay with the pure compound. However, this polyene compound was extracted from *Streptomyces griseus* IMRU 3570 according to Campelo and Gil (2002) and checked spectrophotometrically (the three characteristic peaks of this compound could be observed in Fig. IV-2). The extracted candicidin was used to perform an *in vitro* bioassay against *D. seriata*. Results showed that different concentrations of this compound inhibited the growth of *D. seriata* (Fig. IV-7). The same bioassay was performed using the same dilutions of n-butanol obtaining as a result that the n-butanol did not interfere in the antifungal effect.

Fig. IV-7 *In vitro* assay for the evaluation of the antifungal activity of different concentrations of candicidin extracted from *S. griseus* IMRU 3570 with n-butanol against *D. seriata*.

Fig. IV-5 Schematic representation of the candidin biosynthetic gene cluster (**A**). Prediction of 20 bp protospacer sequences (CRISPy web; <https://crispy.secondarymetabolites.org/#/input>) specific for the *fscRI* regulator, which coordinately controls the biosynthesis of both candidin and antimycin (**B**).

IV.4.2. HPLC analysis

Since those BGCs edited by CRISPR were not involved in the biocontrol activity showed by *Streptomyces* sp. VV/E1 against fungal trunk pathogens, this strain was analysed by HPLC-MS with the objective of identifying the production of secondary metabolites that could be related to the antifungal effect.

Fermentation was performed in 7 different culture media (section IV.3.7). Among all the tested media, TSB was the most suitable for further analysis according to the results obtained from a bioassay performed against *Da. macrodidyma* and *D. seriata*. After 5 days growing at 220 rpm / 30°C, the corresponding extract was analysed by HPLC-MS using DAD and a mass spectrometry detector.

Two different peaks were identified. On the one hand, a peak with a rt of 0.518 min, with a molecular ion corresponding to the protonated molecule $[M+H]^+ = 205$ (rt = 0.563 min) and a characteristic UV spectrum that allowed us to determine that this molecule was tryptophan (Fig. IV-8). Another peak with a rt of 0.618, a molecular ion corresponding to the protonated molecule $[M+H] = 261$ (rt = 0.662 min) and a characteristic UV spectrum led to the identification of this molecule as cyclo(L-Propyl-L-Tyrosil), also known as maculosin (Fig. IV-9). The chemical structures of both compounds are shown in the figures (Fig. IV-8 and IV-9).

The analysis of the antifungal activity of both compounds in a bioassay against both fungi indicated that these compounds were not involved in the biocontrol effect of *Streptomyces* sp. VV/E1, unless under *in vitro* conditions (Fig. SIV-1).

Fig. IV-8 HPLC analysis of *Streptomyces* sp. VV/E1 extract compared to tryptophan spectrum using diode array detector (DAD) to establish the UV absorption spectrum of the compounds (**A**), mass spectrometry to identify the ionization of the compounds (**B**), and characteristic UV absorption spectra of both the extract and the identified compound (**C**). Chemical structure for this compound (**D**).

A

B

C

D

Fig. IV-9 HPLC analysis of *Streptomyces* sp. VV/E1 extract compared to Cyclo(L-Prolyl-L-Tyrosyl) spectrum using diode array detector (DAD) to establish the UV absorption spectra of the compounds (A), mass spectrometry to identify the ionization of the compounds (B), and characteristic UV absorption spectra of both the extract and the identified compound (C). Chemical structure for this compound (D).

IV.4.3. Analysis of chitinase involvement in biocontrol activity

The analysis of chitinase production in colloidal chitin agar plates, through the formation of a clear halo zone around the colony showed that *Streptomyces* sp. VV/E1 was positive for enzyme production with clearing zones of 4.67 ± 0.58 mm (Fig. IV-10).

To check whether this enzyme could be involved in the biocontrol process, its production was truncated by CRISPR techniques.

IV.4.3.1. Knock-down chitinase production

For the knock-down of chitinase production, two different 20 bp protospacers were selected in the *msiK* gene. The inactivation of the *msiK* gene, essential for the induction of chitinase production (Saito *et al.*, 2008), was carried out using pUSER2-Cas9/ScaLigD. Resulted constructs were verified by PCR and sequenced prior to intergenic conjugation in *Streptomyces* sp. VV/E1 strain. After conjugation, resulted exconjugants were verified by Sanger sequencing.

Three mutants were obtained (*Streptomyces* sp. VV/E1 Δ chi1, *Streptomyces* sp. Δ chi2 and *Streptomyces* sp. Δ chi3). The lack of chitinase production was checked in colloidal chitin agar plates. Those exconjugants which no longer hydrolysed chitin (Fig. IV-11B), or even with a reduced growth rate in a colloidal chitin medium, were selected to perform a bioassay against the two pathogenic fungi (Fig. IV-11A). The lack of expression of chitinases was not related to the lack of antifungal activity. Therefore, this enzyme seemed not to be responsible for the biocontrol effect, unless under *in vitro* conditions.

IV.4.4. Production of indole-3-acetic acid

For the characterization of *Streptomyces* sp. VV/E1, the production of the auxin hormone indole-3-acetic acid (IAA) was also analysed (Fig. IV-12). The spectrophotometric analysis determined that this strains was able to produce IAA by tryptophan metabolism,

Fig. IV-10 Chitinase production by *Streptomyces* sp. VV/E1 in colloidal chitin agar medium after incubating 7 days / 30 °C. Enzyme production was visualised as a clear halo around the colony where chitin had been degraded as result of the extracellular enzyme activity.

Fig. IV-11 *In vitro* assay for the evaluation of the antifungal activity of chitinase mutants (Δ chi) compared to the wild-type (WT) against fungal pathogen *Diplodia seriata* grown in ISP5 media (A). The different strains were grown in colloidal chitin agar plates to detect chitin degradation. Note that only the wild-type strain had chitinase activity (white arrow) (B).

with a maximum production of 33.95 ± 3.05 $\mu\text{g/mL}$ after 10 days growing in liquid medium supplemented with 0.2% of L-tryptophan.

Fig. IV-12 Standard curve to quantify the IAA ($\mu\text{g/mL}$) production by the *Streptomyces* sp. VV/E1 strain, generated with serial dilutions of IAA from 0 to 100 $\mu\text{g/mL}$ (A). Representation of the growth (black line) and the IAA production (grey line) of *Streptomyces* sp. VV/E1 (B). Bars indicate the standard deviation.

IV.5. Discussion

Recent genome sequencing of actinomycetes has shown that their genome contains multiple BGCs predicted to encode the biosynthesis of poliketides or non-ribosomal peptides by PKSs and NRPs, respectively (Kodani *et al.*, 2015; Evans, 2016). Both types of metabolites represent **larges families of natural products biologically important, applied in pharmaceutical, agrochemical and veterinary fields** (Zhang *et al.*, 2014). Due to the **development of bioinformatics tools** together with the existence of numerous genetic data, the **prediction of natural compounds from biosynthetic genes** by genome-mining has been possible (Kodani *et al.*, 2015).

The use of antiSMASH for predicting BGCs in *Streptomyces* sp. VV/E1 led to the selection of two BGCs responsible for the putative production of antimycin and candicidin secondary metabolites, to knock-down using CRISPR techniques. Their selection was based on the fact that both compounds had been previously described as antifungal agents (Hosotani *et al.*, 2005; Qi *et al.*, 2015; Liu *et al.*, 2016; McLean *et al.*, 2016).

Antimycins are a large class of natural products, widely produced in *Streptomyces* species, with a myriad of biological properties (Liu *et al.*, 2016; McLean *et al.*, 2016). They are encoded by a hybrid NRPS/PKS pathway (McLean *et al.*, 2016). Among these bioactive activities, antifungal, insecticidal and nematocidal effects are included, owing to their ability to inhibit mitochondrial cytochrome c reductase (McLean *et al.*, 2016). For instance, *Streptomyces* sp. AZ-AR-262 antimycin was active against yeasts (*Candida*

albicans and *Saccharomyces cerevisiae*) and filamentous fungi strains, such as several *Aspergillus* sp. *Fusarium oxysporum*, *Rhizoctonia solani*, *Botrytis fabae* and *Penicillium chrysogenum* (Atta and Ahmad, 2009).

Candicidin, a polyene compound synthesised by a type I PKS, also presents antifungal activity as a result of their specific binding to ergosterol, a major component of fungal membranes, causing the formation of channels that lead to monovalent iron leakage (van Keulen and Dyson, 2014; Zhang *et al.*, 2015; Ngo *et al.*, 2016;). In this sense, mutants of *Streptomyces* sp. FR-008 for candicidin production did not show antifungal effect against *Rhodotorula rubra* compared to the wild-type strain (Zhang *et al.*, 2015). Furthermore, candicidin cluster-situated regulator *fscRI* had been described to coordinately controls the biosynthesis of both candicidin and antimycin metabolites (McLean *et al.*, 2016). Recently the *fscRI* gene has been knocked-down by CRISPR-Cas9 editing and the resulting mutants tested against *Candida albicans*, getting as a result that the mutant strains no longer inhibited the growth of the unicellular fungi (McLean *et al.*, 2016). Unfortunately, **both the VV/E1 mutant strain for antimycin, and the mutant for the *fscRI* regulator that controls the biosynthesis of both candicidin and antimycin production, showed antifungal effect against grapevine trunk pathogens *Da. macrodidyma* and *D. seriata*, indicating that the biocontrol activity detected was not exerted by those compounds.**

An enriched extract from culture supernatants of the candicidin producer *Streptomyces griseus* IMRU 3570 showed a clear antifungal activity under *in vitro* conditions against *D. seriata*. In parallel, both the wild-type (*Streptomyces* sp. VV/E1) and the mutant for candicidin production (*Streptomyces* sp. VV/E1 Δ can) exhibited the same antifungal activity against this pathogen. No differences in the *in vitro* bioassays indicate that the antifungal activity would be exerted by other(s) bioactive compound(s). However, it cannot be discarded a putative role of candicidin in the observed effect, maybe due to a low rate production of the wild-type strain. That is, **the antifungal effect in *Streptomyces* sp VV/E1 could not be only attributable to candicidin, so the most important antifungal compound(s) remain(s) unidentified.**

Not only computational resources have contributed to the understanding of the complex regulatory networks used by streptomycetes to balance primary and secondary metabolism (Robertsen *et al.*, 2018). The identification of metabolic profiles can also be analysed by other traditional methods such as HPLC techniques (Frisvad *et al.*, 2008; Djinni *et al.*, 2014). Through the use of this technique, **maculosin** was discovered to be produced by *Streptomyces* sp. VV/E1. This cyclic dipeptide composed of L-proline-L-tyrosine (Wattana-Amorn *et al.*, 2016), synthesised by CDPSs (González *et al.*, 2017) has also been **reported to have a very strong antifungal activity against some pathogenic fungi**, and therefore, this compound may have a practical application in agriculture as

safe fungicide (Managamuri *et al.*, 2017). **Despite this, maculosin had no effect against *Da. macrodidyma* and *D. seriata*, unless under *in vitro* conditions.**

The antagonistic activity of *Streptomyces* strains against phytopathogens is due to, not only the production of antifungal compounds, but also to **the production of extracellular enzymes such as chitinases** (Loqman *et al.*, 2009; Jog *et al.*, 2012; Tamreihao *et al.*, 2016), able to break down chitin (Schrey and Tarkka, 2008), and therefore degrade fungal cell walls (Passari *et al.*, 2015). Although *Streptomyces* sp. VV/E1 strain exhibited a degradation halo, its chitinase production was low when comparing to other actinomycetes reported in the literature. Thus, *Micobacterium* sp. BPSAC21 exhibited a colloidal chitin degradation zone of 17 mm (Passari *et al.*, 2015), while *Streptomyces corchorusii* UCR3-16 produced a clear zone of 14 mm (Tamreihao *et al.*, 2016), compared to the 4.5 mm showed by VV/E1 strain. Regarding its antifungal activity, chitinases from *Bacillus subtilis* MBCU4 had been reported to present an inhibitory effect against five fungal phytopatogens (*Macrophomia phaseolina*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotium rofsi* and *Sclerotinia sclerotiorum*) (Pandya *et al.*, 2014). The *msiK* gene has been described to encoded the ATP-hydrolysing component of N,N'-diacetylchitobiose ABC transporters, essential for the induction of chitinase production in *Streptomyces coelicolor* A3(2) (Saito *et al.*, 2008). Furthermore, disruption of this gene severely affected the bacterial ability to utilise maltose, cellobiose, starch, cellulose, chitin and chitosan (Saito *et al.*, 2008). **Unfortunately, the deletion of the *msiK* gene by CRISPR-Cas9 editing showed that mutant strains inhibited the growth of fungal trunk pathogens, and therefore we can conclude that chitinases were not involved in the biocontrol process.**

Actinobacteria associated with plants also provide several benefits to host plants, including promoting their growth (Gopalakrishnan *et al.*, 2013; Passari *et al.*, 2015). Microbiologist have widely studied the production of phytohormones by microorganisms so as to understand their effect on plant growth (Goswami *et al.*, 2015). Soil microorganisms are able to produce phytohormones like auxins, gibberellins, cytokinins, ethylene and abscisic acid (Glick, 2014; Goswami *et al.*, 2015). One of this important phytohormones is the IAA auxin, produced from tryptophan (Goswami *et al.*, 2015). It has been reported that 80% of the microorganisms isolated from the rhizosphere of crops are able to synthesise and release auxins as secondary metabolites which promote root elongation and plant growth (Gopalakrishnan *et al.*, 2013). In addition to stimulate plant growth, it has been suggested that IAA also acts on a common regulatory cascade for sporulation and secondary metabolite production in streptomycetes (Matsukawa *et al.*, 2007; Jog *et al.*, 2012). The IAA production of the VV/E1 strain tested in this study (34 µg/mL; section IV.4.4) was comparable to other actinomycetes reported in the literature. For instance, *Streptomyces* sp. BPSAC34 produced 32.0 µg/mL IAA, *S. rochei* IDWR19 production was 17.8 µg/mL (Jog *et al.*, 2012) and *S. albidoflavus* observed production

was 7.4 µg/mL (Narayana *et al.*, 2009). Nimnoi and col. isolated 10 endophytic actinobacteria that produced IAA in a range between 9.85 and 15.14 µg/mL (Nimnoi *et al.*, 2010), Khamna and col. isolated 36 actinomycetes whose IAA production varied between 5.47 and 143.95 µg/mL (Khamna *et al.*, 2009), and Sreevidya and colleagues isolated 10 *Streptomyces* strains which produced from 3.6 to 14.6 µg/mL IAA (Sreevidya *et al.*, 2016).

Taking everything into account, ***Streptomyces* sp. VV/E1 has been described as BCA against the main fungi related to GTD and YGD (Álvarez-Pérez *et al.*, 2017).** However, **attempts to identified secondary metabolites or other type of factors (as enzymes) involved in the biocontrol process** against these fungi were unsuccessful, indicating that their identification is a hard issue. Likely, **the antifungal effect might be due to the interaction of several secondary metabolites and enzymes under *in vivo* conditions, or alternatively it could be due to some antifungal compounds not identified yet.**

IV.6. References

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Supplemental material

Fig. SIV-1 Absence of antifungal activity of Cyclo(L-Prolyl-L-Tyrosyl) (maculosin) **(A)** and L-tryptophan **(B)** against *D. seriata* and *Da. macrodidyma* using different concentrations of the pure compound.

Table SIV-1A Primers designed to amplify constructions using pUSER2-Cas9/ScaLigD plasmids

Primer	Sequence (5'-3')	Region	T_m (°C)	Fragment size (bp)
ANT_C_sgRNA_FW	CGTGCGAUGCCGTCGAGCAGGATGTGATGTTTTAGAG	sgRNA	67	400
ANT_KS_sgRNA_FW	CGTGCGAUGATGCCACGCGGACCAGCGAGTTTTAG	sgRNA	69	400
CAN1_sgRNA_FW	CGTGCGAUAAACACGGTGGTCATTTCAGTGGTTTTAGAG	sgRNA	64	400
CAN2_sgRNA_FW	CGTGCGAUGTGCGTCCACTGCGTGCGAAGTTTTAG	sgRNA	67	400
CHI1_sgRNA_FW	CGTGCGAUGGGTAGACCCGGGTCGCCTTGT	sgRNA	67	400
CHI2_sgRNA_FW	CGTGCGAUTGCCGCAGCCGGACGGGC	sgRNA	66	400
sgRNA_LigD_RV	CACGCGAUCCTCAGGTGACCTCAGAACTCCATCTG	sgRNA	67	400

Table SIV-1B Primers designed to amplify constructions for knocking-down candidin BGC with pUSER2-Cas9 plasmid.

Primer	Sequence (5'-3')	Region	T_m (°C)	Fragment size (bp)
CAN_1_sgRNA_FW	CGTGCGAUAAACACGGTGGTCATTTCAGTGGTTTTAGAG	sgRNA	64	500
CAN_2_sgRNA_RV	CGTGCGAUGTGCGTCCACTGCGTGCGAAGTTTTAG		67	
CAN_sgRNA_RV	ACCGCTCCUGTCCTCAGGTGACCTCAGAACTCCATC		67	
CAN_Left_FW	AGGAGCGGUCACCGTCCGCCACGCCCCG	Left arm	76	1,000
CAN_Left_RV	ACCGCGCCCGGUCCGGGCCGCCCCG		75	
CAN_ermE_FW	ACCGGGCGCGGUCGATCTTGACGGCTGGCGAGAGG	ermE promotor	67	114
CAN_ermE_RV	ATCCCCGUCGATCCTACCAACCGGCACGATTG		65	
CAN_Right_FW	ACGGGGAUGCGCTTGATCCCACTGAATCGCCG	Right arm	70	1,000
CAN_Right_RV	CACGCGAUTGGGCCATCGGGGTACACCGC		67	

Table SIV-1C Primers designed to amplify constructions for knocking-down antimycin BGC using pUSER2-Cas9 plasmid.

Primer	Sequence (5'-3')	Region	T_m (°C)	Fragment size (bp)
ANT_C_sgRNA_FW	CGTGCGAUGCCGTCGAGCAGGATGTGATGTTTTAGAG	sgRNA	67	500
ANT_C_sgRNA_RV	ACCTCAGGUGACCTCAGAACTCCATCTGGATTTGTTTCAG		66	
ANT_C_Left_FW	ACCTGAGGUCGCCGAGGAGGCGCAGCGCGTAC	Left arm	72	1,000
ANT_C_Left_RV	ACCGCGGGGUCGTGAGGTCCGCGCGGATCCA		73	
ANT_C_ermE_FW	ACCCGCGGGUCGATCTTGACGGCTGGCGAGAG	<i>ermE</i> promotor	65	114
ANT_C_ermE_RV	ACCGGCACGAUTGTGCCCACAACAGCATCGCGG		65	
ANT_C_Right_FW	ATCGTGCCGGUTGGTAGGATCGACGGCACCACCGAGCCGCAGGACG	Right arm	69	1,000
ANT_C_Right_RV	CACGCGAUGGCCACCTCGATGGGGTCGCC		69	
ANT_KS_sgRNA_FW	CGTGCGAUGATGCCACGCGGACCAGCGAGTTTTAG	sgRNA	69	500
ANT_KS_sgRNA_RV	AGCCTCAGGUGACCTCAGAACTCCATCTGGATTTGTTTCAGAACG		69	
ANT_KS_Left_FW	ACCTGAGGCUGGCGGCCAAGGTGGACGGAGCG	Left arm	72	1,000
ANT_KS_Left_RV	ACCGCGGCCUCGGCCCTCTCGTGCCGT		72	
ANT_KS_ermE_FW	AGGCCGCGGUCGATCTTGACGGCTGGCGAGAG	<i>ermE</i> promotor	65	114
ANT_KS_ermE_RV	ACGCGGCCGCCGUCGATCCTACCAACCGGCACGATTG		65	
ANT_KS_Right_FW	ACGGCGGCCGCGUCCCGCGAGGAGG	Right arm	68	1,000
ANT_KS_Right_RV	CACGCGAUCGGCCCAGCGCCTCCGCG		68	

Table SIV-2 Primers used for clone verification.

Primer	Sequence (5'-3')	Use	Vector construction	Reference
t0_check_FW	GGCGGCAACGCAGCG	Amplification/sequencing	pUSER2-Cas9	H. Robertsen personal communication
pGM1190_USER_seq_FW	GTTCGAGGTCATGTCCGAGGAG	Amplification/sequencing	Both	Robertsen (2017)
pGM1190_USER_seq_RV	CCGTCTGACGCCCCGATCACG	Amplification/sequencing	pUSER2-Cas9/ScaligD	Robertsen (2017)
sgRNA_seq_FW	CGCTGAGGGACCGCCATG	Sequencing	pUSER2-Cas9	H. Robertsen personal communication
USER_seq_pGM1190_RV	GCGTACCGCTTCGGGCCC	Sequencing	pUSER2-Cas9	Tong <i>et al.</i> (2018)
ANT_C_midL_FW	CCGTGATGTGCCGTAGAACCG	Sequencing	pUSER2-Cas9	This study
ANT_C_midL_RV	CGGTTCTACGGCACATCACGG	Sequencing	pUSER2-Cas9	This study
ANT_C_midR_FW	CTACGAACTGGGCCTCACCG	Sequencing	pUSER2-Cas9	This study
ANT_C_midR_RV	CGGTGAGGCCAGTTCGTAG	Amplification/sequencing	pUSER2-Cas9	This study
ANT_KS_midL_FW	CACGTGGGTGCTGCGCTCG	Sequencing	pUSER2-Cas9	This study
ANT_KS_midL_RV	CGAGCGCAGCACCCACGTG	Sequencing	pUSER2-Cas9	This study
ANT_KS_midR_FW	CTTCCGCGTGACCGTCG	Sequencing	pUSER2-Cas9	This study
ANT_KS_midR_RV	CGACGGTGACGCGGAAG	Amplification/sequencing	pUSER2-Cas9	This study
CAN_midL_FW	CGGGCCTGGACGTCACCAG	Sequencing	pUSER2-Cas9	This study
CAN_midL_RV	CTGGTGACGTCCAGGCCCCG	Sequencing	pUSER2-Cas9	This study
CAN_midR_FW	CTTCTCCACGGCCCGGGAG	Sequencing	pUSER2-Cas9	This study
CAN_midR_RV	CTCCCGGGCCGTGGAGAAG	Amplification/sequencing	pUSER2-Cas9	This study

Table SIV-3. Designed primers for checking exconjugants by PCR.

Primer	Sequence (5'-3')	<i>T_m</i> (°C)	Fragment size (bp)
ANT_C_Check_exc_FW	GTGCGACCTCCCCTTCCACC	63	1,096
ANT_C_Check_exc_RV	GAACTCGCCCCGGTACTGC	62	
ANT_KS_Check_exc_FW	GAACTTCTTCGACCTGGGCGG	62	1,112
ANT_KS_Check_exc_RV	CGTCCTCCAGCCGCTTCAG	62	
CAN_Check_exc_FW	CGGTTCGGCACCTTGAGCTTAC	62	1,140
CAN_Check_exc_RV	GGCCGTGGAGAAGCACCTC	62	
CHI_Check_exc_FW	GTCCAGGTACTCGGTGAGGTC	63	639
CHI_Check_exc_RV	CGAAACAGCACGAGCACGGC	62

GENERAL DISCUSSION

Grapevines are severely affected by GTD worldwide (Morales-Cruz *et al.*, 2017; Gramaje *et al.*, 2018). These pathologies are caused by pathogenic fungi resulting in the vine deterioration, or even the early plant death (Hofstetter *et al.*, 2012; Stempien *et al.*, 2018). Black-foot and Petri disease, recognised as prominent causes of YGD, together with the failure of planting material, are some of the most worrisome problems affecting the vine industry since the 1990s (Gramaje and Armengol, 2011). The occurrence of these diseases has increased in the last decades, leading to massive economic losses estimated to exceed one billion dollars per year (Hofstetter *et al.*, 2012; Stempien *et al.*, 2018). Since full effective plant protection strategies are not available to date, GTD are considered as a major threat to the wine industry, and therefore a true challenge to winegrowers (Álvarez-Pérez *et al.*, 2017; Stempien *et al.*, 2018).

Grapevine protection against GTD is a hard issue given the many years that a plant remains in the ground and, furthermore, their management depends on both, the disease/pathogen, and the geographical area, which make almost impossible their complete eradication. **Control programmes are essential for the proactive management of GTD**, and consequently, **to reduce fungal infections in grapevine nurseries** (Halleen and Fourie, 2016; Gramaje *et al.*, 2018). Several strategies, including chemical, biological and physical treatments are being investigated, with special interest on eco-friendly alternatives. As a result, **beneficial microorganisms inhabiting plant rhizosphere and/or endosphere are becoming promising tools** on this matter (Compant *et al.*, 2013; Álvarez-Pérez *et al.*, 2017).

Actinobacteria as biocontrol agents

Actinobacteria, specially the genus *Streptomyces*, are widely distributed in soils playing an important role in soil microbiota (Janssen, 2006). They represent a high proportion of rhizosphere microbial communities (Loqman *et al.*, 2009; Barka *et al.*, 2016), although they are also colonisers of plant inner tissues, being found in nearly every plant worldwide, not only as mycorrhizal, but also as endophytic associated strains (Li *et al.*, 2012; Santoyo *et al.*, 2016). Its natural presence in vineyards (Loqman *et al.*, 2009; Álvarez-Pérez *et al.*, 2017), in conjunction with their production of a vast range of bioactive products (Couillerot *et al.*, 2013; Palazzotto and Weber, 2018), make these Gram-positive bacteria good candidates to control plant diseases (Couillerot *et al.*, 2014; Andreolli *et al.*, 2016; Ranjani *et al.*, 2016; Álvarez-Pérez *et al.*, 2017). Moreover, since many of them produce spores, that help in their dissemination and confer resistance against adverse conditions, they can be a promising tool for the **development of novel biofertilizers and long-life biocontrol agents** (Tamreihao *et al.*, 2016).

In this work, a screening based on the antifungal activity against fungi that cause GTD and YGD led to the identification of many isolates, where the vast majority belonged to

the *Streptomyces* genus. These data revealed that **both the rhizosphere and the root system of grapevines are an interesting source for actinobacteria, which might be potential BCAs** for the treatment of different vineyard pathologies.

Considering that most of the grafted plants in nurseries can be infected through the root system once they are planted in an open-field nursery (Halleen *et al.*, 2003), the **application of selected actinobacteria** has been demonstrated to be highly effective in controlling the infection at this step, since their application **resulted in a clear reduction of fungal pathogens isolated**. In fact, the application of three actinobacterial strains (VV/E1, VV/R1 and VV/R4) led to a statistically significant decrease ($p < 0.05$) in the presence of the three types of fungal pathogens analysed (*Dactylonectria* sp. – *Ilyonectria* sp. group, *Ph. chlamydospora* and *P. minimum*), with no significant effect, either positive or negative, on the growth of surviving plants.

The development of technologies that are able to limit the infection of grapevines through the root system is of great interest in the wine industry and, given that several of the tested isolates had a beneficial impact when applied to grafts in nurseries, the implementation of these selected actinobacteria might help to diminish this problem. **This eco-friendly methodology could be easily integrated into the industrial propagation of grapevines with the final aim of reducing the rate of fungal infections through the root system.**

Colonization of grapevine plants

Since selected *Streptomyces* strains (VV/E1 and VV/R4) were effective in controlling YGD, it was necessary to analyse the most effective protocol to apply them in nurseries or vineyards. Several factors, including the lack of effective and easy methods to introduce them into the root system to allow colonization, make their application difficult. In these dissertation, two different inoculation methods were analysed: direct immersion of the root system in a bacterial suspension, and injection of a bacterial suspension into the rootstock. Both methods displayed a similar effectiveness, ensuring the plant colonization after 6 months in pots.

The development of SCAR markers allowed the detection of both strains in plant material with a high specificity and reproducibility, since they were designed against poorly conserved DNA regions between species, or even strains. By their use, grapevine colonization of both strains could be easily detected by qPCR methodologies. Moreover, the movement inside the plant was analysed, using the same molecular techniques, for the endophytic strain VV/E1. After 3 months in pots, the actinobacteria could move upwards through the rootstock and reach the second petiole.

These results showed that *Streptomyces* strains isolated from grapevine root environment as putative BCAs against fungal pathogens can be easily introduced into

young grapevine plants. **The immersion of the root system** is the ideal option due to its simplicity to be **implemented in the industrial process of vines production in nurseries**. The actinobacteria can be added to the rooting hormone solution avoiding additional steps, which would slow down the production process. Moreover, contrary to the injection of the bacterial suspension into the rootstock, this methodology does not require additional handling of the plants avoiding possible situations of plant stress. Therefore, **their application prior to vineyard establishment is a promising tool to decrease the incidence of YGD in new plantations worldwide.**

***Streptomyces* sp. as secondary metabolite producers**

The recent sequencing of actinomycetes genomes has revealed that they contain many BGCs predicted to encode large families of natural products with relevant bioactive activities (Zhang *et al.*, 2014; Kodani *et al.*, 2015; Stulberg *et al.*, 2016), including antifungals (Rolshausen *et al.*, 2010; Díaz and Latorre, 2013; Sosnowski *et al.*, 2013). As a matter of fact, it has become clear that actinobacteria can be an important reservoir of genetic diversity, as well as an essential source of natural products, biologically important, that can help control plant pathologies (Joseph *et al.*, 2012; Álvarez-Pérez *et al.*, 2017). However, the secondary metabolites and other related compounds involved in the biocontrol effect against grapevine trunk pathogens remain unknown.

The putative role of several secondary metabolites, enzymes and other protective compounds in *Streptomyces* sp. VV/E1 have been analysed without success. Genome analysis identified several BGCs encoding bioactive molecules that could be related to the biocontrol effect. Two of them should be highlighted, since they regulate the synthesis of two compounds described as antifungals: antimycin and candicidin (Hosotani *et al.*, 2005; van Keulen and Dyson, 2014; Zhang *et al.*, 2015). Mutant strains for these biosynthetic gene clusters were obtained by CRISPR techniques. Unfortunately, the analysis of mutant strains for both BGCs, showed that they were not responsible for the biocontrol effect against fungal trunk pathogens *Dactylonectria macrodidyma* and *Diplodia seriata*, at least under *in vitro* conditions. The role of maculosin, described as antifungal in the literature (Managamuri *et al.*, 2017) was also tested against the same phytopathogenic fungi, showing no effect. Moreover, chitinases (extracellular enzymes able to degrade fungal cell walls) (Passari *et al.*, 2015) were also found not to be involved in the biocontrol effect.

As a general conclusion, several *Streptomyces* species have been described as BCAs against the main fungi involved in YGD (Álvarez-Pérez *et al.*, 2017). However, in-depth analysis of one of the most promising strains (VV/E1) led to the conclusion that the **identification of the secondary metabolites related to the biocontrol activity is a hard issue**, and, the mechanism of action to control the growth of fungal pathogens remains

misunderstood. Likely, the **antifungal effect might be due to the interaction of several secondary metabolites and enzymes under *in vivo* conditions**, some of which may be currently unknown. To clarify this issue, future research would be necessary.

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General discussion

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FUTURE PERSPECTIVES

Future perspectives

Most of the biosynthetic gene clusters are expressed at very low rates under laboratory conditions (Rutledge and Challis, 2015; Weber *et al.*, 2015). The expression of these cryptic secondary metabolite biosynthetic genes may result in the production of novel secondary metabolites (Ochi, 2017). In order to activate or increase the production of “cryptic” secondary metabolites that would allow to identify those compounds related to the antifungal activity showed by *Streptomyces* sp. VV/E1 against grapevine trunk pathogens, two approaches should be considered: regulatory gene activation and use of elicitors.

- **Regulatory gene activation.** PAS-LuxR regulators are highly conserved proteins devoted to the control of antifungal production by binding to operators located in given promoters of polyene biosynthetic genes (Vicente *et al.*, 2015). This fact make them an interesting tool for the activation of biosynthetic gene transcription, and therefore, increase the production of polyene macrolides (Santos-Aberturas *et al.*, 2011).

Thus, the introduction of the gene *pimM* and its promoter in *Streptomyces* sp. VV/E1 could increase the production of antifungal polyenes involved in the biocontrol effect, and thereby their corresponding identification by HPLC-MS techniques.

- **Elicitors.** These signalling compounds have been described to stimulate the activation or synthesis of other compounds (Tanaka *et al.*, 2010; Robertsen *et al.*, 2018). In particular, the addition of low concentrations of rare earth elements, such as scandium or lanthanum, to a culture has been found to enhance antibiotic production, ranging from 2.5 to 12 fold (Tanaka *et al.*, 2010).

Thus, the addition of some elicitors to a pure culture of *Streptomyces* sp. VV/E1 and the subsequent analysis by HPLC-MS could allow the detection of novel antifungal compounds.

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Future perspectives

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CONCLUSIONS **(CONCLUSIONES)**

Conclusions

In view of the results obtained in this dissertation, it can be concluded that:

1. The rhizosphere and inner tissues of the grapevine root system are highly interesting sources for the isolation of actinobacteria selected as biocontrol agents (BCAs) against fungal pathogens causing grapevine trunk diseases (GTD) and young grapevine decline (YGD).
2. The strains *Streptomyces* sp. VV/E1 and VV/R4 are highly effective to reduce the infection rate of fungal pathogens involved in young grapevine decline through the root system.
3. Both strains can be easily introduced into the plant by immersion of the root system in a bacterial suspension or by the direct injection to the rootstock.
4. Both strains are able to efficacy colonise the root system, and at least in the case of *Streptomyces* sp. VV/E1 strain, can move from the root system up to the second petiole, colonising inner tissues of the plant.
5. The genomes of both strains contain several clusters encoding compounds described as bioactive secondary metabolites.
6. The secondary metabolites antimycin, candicidin or maculosin, described as antifungals, are not responsible for the biocontrol effect observed in *Streptomyces* sp. VV/E1 strain against grapevine trunk pathogens.
7. The production of extracellular chitinases is not responsible for the biocontrol effect observed in VV/E1 strain.
8. The biocontrol effect of *Streptomyces* sp. VV/E1 strain remains unknown suggesting that is mediated by secondary metabolites, enzymes or other mechanisms not identified yet.

Conclusiones

En relación con los resultados obtenidos se pueden destacar las siguientes conclusiones:

1. La rizosfera y el interior del sistema radicular de las plantas de vid constituyen una importante fuente de aislamiento de actinobacterias como agentes de biocontrol frente a los hongos patógenos causantes de las enfermedades de madera de vid y el decaimiento de las plantas jóvenes de vid.
2. Las cepas *Streptomyces* sp. VV/E1 y VV/R4 son altamente efectivas reduciendo la tasa de infección de hongos patógenos involucrados en el decaimiento de plantas jóvenes de vid a través del sistema radicular.
3. Ambas cepas pueden ser fácilmente introducidas en plantas de vid mediante inmersión del sistema radicular en una suspensión de bacterias o por inyección en el portainjertos.
4. Ambas cepas son capaces de colonizar de manera eficiente el sistema radicular, y al menos en el caso de la cepa *Streptomyces* sp. VV/E1, de moverse desde el sistema radicular hasta el segundo peciolo, colonizando tejidos internos de la planta.
5. Los genomas de ambas cepas contienen varias agrupaciones génicas que codifican para compuestos descritos como metabolitos secundarios bioactivos.
6. Los metabolitos secundarios antimicina, candicina o maculosina, descritos como antifúngicos, no son responsables del efecto de biocontrol observado en la cepa de *Streptomyces* sp. VV/E1 frente a patógenos de madera de vid.
7. La producción de quitinasas extracelulares no es responsable del efecto de biocontrol observado en la cepa VV/E1.
8. El efecto de biocontrol de la cepa de *Streptomyces* sp. VV/E1 sigue siendo desconocido, lo que sugiere que está mediado por metabolitos secundarios, enzimas u otros mecanismos aún no identificados.

APPENDIX

1. Abbreviations

antiSMASH: antibiotic Secondary Metabolites Analysis Shell	MLSA: multilocus sequence analysis
ACT: actinorhodin	MS: mass spectrometry
BCA: biocontrol agent	NC: negative control
BCG: biosynthetic gene cluster	NHEJ: non-homologous end joining
BLAST: basic local alignment search tools	NRPS: non-ribosomal peptide synthetases
bp: base pair	NTC: non-template control
Cas: CRISPR-associated	O/N: over night
CDA: calcium dependent antibiotic	OIV: International Organisation of Vine and Wine
CDPS: cyclodipeptide synthase	ORF: open reading frame
CmR: chloramphenicol resistance marker	PAM: protospacer associated motif
CRISPR: clustered regularly interspaced short palindromic repeats	PBS: phosphate-buffered saline
<i>Ct</i> : cycle threshold	PCR: polymerase chain reaction
<i>CV</i> : coefficient of variation	PKS: polyketide synthase
DABCO: 1,4-Diazabicyclo[2.2.2]octane	qPCR: quantitative real-time PCR
DAD: diode array detector	RAPD: random amplified polymorphic DNA
dH ₂ O: distilled water	RED: undecylprodigiosin
ddH ₂ O: double-distilled water	RI: root insertion point
DSB: double stranded break	rpm: revolutions per minute
EDTA: ethylenediaminetetraacetic acid	RS: root system
GTD: grapevine trunk diseases	rt: retention time
HPLC: high-performance liquid chromatography	RT: room temperature
IAA: indole-3 actic acid	SCAR: sequence characterized amplified region
IPTG: isopropyl-β-D-thio-galactopyranoside	SD: standard deviation
ITS: internal transcribed spacers	SEM: scanning electron microscopy
J: Joules	sgRNA: single guide RNA
kb: kilobase	<i>T_m</i> : melting temperature
KS: ketosynthase	U: unit of enzymatic activity
LC-MS: liquid chromatography - mass spectrometry	USER: uracil-specific excision reagent
<i>LigD</i> : ligase D	UV: ultraviolet
LOQ: limit of quantification	v/v: volume/volume
LSD: least significant difference	w/v: weight/volume
MhL: millions of hectolitres	X-Gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
	YGD: young grapevine decline

2. Units

Type	Unit
Concentration	g/L (grams/litre)
	mL/L (millilitre/litre)
	µg/mL (micrograms/millilitre)
	ng/µL (nanograms/microlitre)
Length	cm (centimetre)
	mm (millimetre)
	nm (nanometre)
	µm (micrometre)
Mass	g (grams)
	mg (milligrams)
	µg (micrograms)
	ng (nanograms)
	pg (picograms)
Concentration	fg (femtograms)
	M (molar)
	mM (millimolar)
	µM (micromolar)
	nM (nanomolar)
Power	W (watt)
Pressure	bar
Temperature	°C (degree centigrade)
Time	h (hour)
	min (minute)
	s (second)
Volume	L (litre)
	mL (millilitre)
	µL (microlitre)

3. Equipment

The equipment used during this dissertation was the following:

- Autoclave (JP Selecta, Barcelona, Spain)
- Centrifuges:
 - Sorvall legend Micro17 centrifuge (Thermo Scientific, Waltham, Massachusetts, USA)
 - High speed Brushless centrifuge MPW-350R (MPW Med. Instruments, Warsaw, Poland)
 - Heraeus Multifuge X3 FR centrifuge (Thermo Fisher Scientific)
- Critical point dryer CPD 030 (BALTEC Inc., Los Angeles, California, USA)
- Electrophoresis equipment
 - Electrophoresis chamber Horizon 11.14 (Biometra, Gottingen, Germany) / Mini-Sub® Cell GT Systems (BIO RAD, California, USA)

- Eletrophoresis power supply unit: PowerPac 300 (BIO RAD)
- UV transilluminator: Safe Imager™ 2.0 (Invitrogen, California, USA)
- Visualized and photographed equipment: VWR GenoSmart equipment (VWR, Barcelona, Spain).
- FastPrep-24 homogenizer (MP Biomedicals, Frederick, Maryland, USA)
- Flow bench Telstar AV-100 (Telstar, Tarrasa, Spain)
- Horizontal shaker: Certomat® BS-1 (Sartorius Stedim Biotech, Gotinga, Germany)
- Micropipettes (Gilson, Middleton, Wisconsin, USA)
- Microscope and stereomicroscope:
 - Confocal microscope: Zeiss LSM800 with AiryScan (Zeiss, Oberkochen, Germany).
 - Fluorescent microscope: Nikon Eclipse E400 (Nikon, Minato, Tokyo, Japan) fitted with a Digital Sight DS-L1 camera (Nikon)
 - Optical microscope: Olympus CX41 (Olympus, Shinjuku, Tokyo, Japan)
 - Scanning electron Microscope: JEOL JSM-6480LV (JEOL Ltd., Akishima, Tokio, Japan).
 - Stereomicroscope: Nikon SMZ1500 (Nikon).
- Microtome: Micron HM450 (Thermo Fisher Scientific)
- Mixer: Heidolph polymax 1040 (Heidolph, Schwabach, Germany)
- Multichannel pipettes (VWR)
- NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).
- pH-meter: pH-meter pH526 (WTW, New York, New York, USA)
- Purify water system types I (capsule filter 0.2 µm) and II (analytical grade water) (Wasserlab, Barbataín, Navarra, Spain).
- qPCR system: Mx3005P QPCR system (Agilent technologies, Saint Claire, California, USA)
- Spectrophotometer: Spectrophotometer libra S70 (Biochrom, Holliston, Massachusetts, USA)
- Speed-vac: centrivap vacuum concentrator (Labconco, Kansas City, Missouri, USA)
- Thermocycler: Mastercycler gradient (Eppendorf) / C1000™ Thermal Cycler (BIO RAD, California, USA)
- Thermomixer comfort (Eppendorf, Hamburg, Germany)
- Ultrasound bath: Ultrasons (JP Selecta)
- UV-Stratalinker 2400 (Stratagene, San Diego, California, USA)
- Vacuum system CVC 3000, (VWR, Barcelona, Spain)
- Water bath: Unitronic-320 (JP Selecta)

4. Software

The software used during this dissertation was the following:

- CLC Main Workbench 8 (Qiagen, Hilden, Germany)
- CorelDRAW X8 (Corel Corporation, Ottawa, Canada)
- DNASTAR-SeqBuilder Pro, software for Sequence Editing and Annotation (Madison, Wisconsin, USA)

Appendix

- EzTaxon-e database: Kim O.S., Cho, Y.J., Lee K., Yoon S.H., Kim M., Na H., Park S.C., Jeon Y.S., Lee J.H., Yi H., Won S., Chun J. (2012). "Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species". *International Journal of Systematic and Evolutionary Microbiology* 62(3): 716–21.
- MEGA6: Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.
- MxPro QPCR software (Agilent Technologies, Saint Claire, California, USA)
- R software (3.3.1): R Foundation for Statistical Computing (Vienna, Austria) (<https://www.r-project.org/>).
- RAST (Rapid Annotation using Subsystem Technology) (DuPage Country, Illinois, USA) (<http://rast.theseed.org/FIG/rast.cgi>).
- SeqBuilder Pro (Software for Sequence Editing and Annotation, Automated Virtual Cloning and Primer Design) (DNASTAR, Madison, Wisconsin, USA)
- SigmaPlot12 (Systat Software, Inc., San Jose, California, USA)
- SnapGene Viewer (GSL Biotech LLC, Chicago, Illinois, USA) (<http://www.snapgene.com/>).

5. Suppliers

The main suppliers of reagents, enzymes and kits used during this dissertation was the following:

- Acros Organics (Waltham, Massachusetts, USA)
- Agilent Technologies (Santa Clara, California, USA)
- Compo Sana (Barcelona, Spain)
- Conda Laboratories (Torrejón de Ardoz, Madrid, Spain)
- Eurofins (Luxembourg, Luxembourg)
- EURx (Gdansk, Poland)
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PUBLICATIONS

Use of Endophytic and Rhizosphere Actinobacteria from Grapevine Plants To Reduce Nursery Fungal Graft Infections That Lead to Young Grapevine Decline

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ABSTRACT Endophytic and rhizosphere actinobacteria isolated from the root system of 1-year-old grafted *Vitis vinifera* plants were evaluated for their activities against fungi that cause grapevine trunk diseases. A total of 58 endophytic and 94 rhizosphere isolates were tested. Based on an *in vitro* bioassay, 15.5% of the endophytic isolates and 30.8% of the rhizosphere isolates exhibited antifungal activity against the fungal pathogen *Diplodia seriata*, whereas 13.8% of the endophytic isolates and 16.0% of the rhizosphere isolates showed antifungal activity against *Dactylonectria macrodidyma* (formerly *Ilyonectria macrodidyma*). The strains which showed the greatest *in vitro* efficacy against both pathogens were further analyzed for their ability to inhibit the growth of *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (formerly *Phaeoacremonium aleophilum*). Based on their antifungal activity, three rhizosphere isolates and three endophytic isolates were applied on grafts in an open-root field nursery in a 3-year trial. The field trial led to the identification of one endophytic strain, *Streptomyces* sp. VV/E1, and two rhizosphere isolates, *Streptomyces* sp. VV/R1 and *Streptomyces* sp. VV/R4, which significantly reduced the infection rates produced by the fungal pathogens *Dactylonectria* sp., *Ilyonectria* sp., *P. chlamydospora*, and *P. minimum*, all of which cause young grapevine decline. The VV/R1 and VV/R4 isolates also significantly reduced the mortality level of grafted plants in the nursery. This study shows that certain actinobacteria could represent a promising new tool for controlling fungal trunk pathogens that infect grapevine plants through the root system in nurseries.

IMPORTANCE Grapevine trunk diseases are a major threat to the wine and grape industry worldwide. They cause a significant reduction in yields as well as in grape quality, and they can even cause plant death. Trunk diseases are caused by fungal pathogens that enter through pruning wounds and/or the root system. Although different strategies have recently been developed to protect pruning wounds using antifungal compounds (natural or synthetic) or biocontrol agents, no tools are yet available for controlling soil pathogens that infect plants through their root system. This study shows that different actinobacterial isolates, when applied to grafts in a nursery, can significantly reduce the infection rate caused by fungal pathogens that enter through the root system. This is a new, promising, and green alternative for preventing the decline of young grapevines in nurseries and vineyards.

KEYWORDS young grapevine decline, trunk diseases, actinobacteria, rhizosphere, endophytes

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Grapevines are one of the most important economic crops worldwide. Grapevine trunk diseases (GTD) are a major threat for the wine sector, causing serious economic losses to the wine industry (1, 2). GTD include different fungal diseases, the most relevant of which are *Botryosphaeria* dieback, esca, *Eutypa* dieback, Petri disease, and blackfoot. The incidence of GTD has increased over the last decades, primarily due to the lack of effective strategies for fighting these diseases (3, 4). Adult plant infections in mature vineyards can occur through the root system. This is the primary mode of colonization for pathogens belonging to the *Dactylonectria* or *Ilyonectria* genus (5), which are responsible for blackfoot disease. Other pathogens, such as species of the *Botryosphaeriaceae* family and *Eutypa lata*, mainly infect the plant through pruning wounds produced at the end of the growing season each year (6–8). Finally, other pathogens, like *Phaeoacremonium minimum* (formerly *Phaeoacremonium aleophilum*) or *Phaeomoniella chlamydospora*, can use both pathways to penetrate the plant, and these two microorganisms are the primary causal agents of Petri disease (9, 10). Young grapevine plants can be infected in the field by the same mechanisms described for adult plants. It is well known that planting material (grafted plants) produced in nurseries is frequently infected with fungal pathogens, especially those involved in blackfoot and Petri diseases. In fact, it has been widely reported that grafts can be infected at different stages of the propagation process that takes place in the nurseries (10, 11). GTD, particularly blackfoot disease and Petri disease, are also recognized as prevalent causes of young grapevine decline (YGD). Decline symptoms in young vineyards have dramatically increased all over the world since the early 1990s, and these plants are primarily infected through their root system (10).

Accordingly, in recent years, there have been many different studies aimed at controlling or diminishing fungal pathogen infection rates (6, 12–23). Most studies have focused on protecting pruning wounds by applying natural antifungal compounds (12) or different chemical fungicides. Benomyl and flusilazole are the most effective at controlling *Eutypa lata* infections (13, 14), thiophanate-methyl is used for treating both Petri disease and several *Botryosphaeriaceae* species (6) infections, and benzimidazole works effectively in protecting pruning wounds infected by *Diplodia seriata*, *P. chlamydospora*, or *Inocutis* sp. (15). The efficacy of several biological control agents (BCAs), including natural epiphytes and colonizers of pruning wounds (16–18), has been tested. The application of several strains of *Trichoderma* (19–22) has also been attempted.

Unfortunately, there is currently no treatment available to protect grafts in nurseries or grapevines in vineyards from phytopathogenic fungi that can infect plants through their root system. Nevertheless, some studies have been focused on the application of different *Trichoderma* strains to soils or the grapevine root system to prevent infection by fungi that cause GTD. Thus, in a nursery trial, Fourie and Hallen (23) reported that the application of *Trichoderma* strains had a growth-stimulating effect and reduced the infection rates of *Cylindrocarpon* sp., *Phaeoacremonium* sp., and *P. chlamydospora*. More recently, a study involving the application of *Trichoderma harzianum* at different vine growth stages in a nursery indicated that application during the rooting stage was the most effective in protecting against *P. chlamydospora* infections. Unfortunately, an increase in vine mortality was observed at the end of the growing season (19).

Actinobacteria, particularly streptomycetes, are a complex group of Gram-positive bacteria making up around 10% of the total soil microbiome (24). Although they are known as soil and rhizosphere bacteria, several reports indicate that they are also more intimately associated with plants, either as endophytic strains (25–27) or mycorrhiza-associated strains (28, 29). They are well known as important secondary metabolite producers, excreting antibiotic and antifungal compounds, and for their ability to control plant diseases (29–31).

Recently, bacterial communities associated with *Vitis vinifera* rhizospheres and their variations, including factors like geographical features (32) and vineyard management, have been characterized (33, 34). Although some reports indicate that grapevine rhizosphere-associated bacteria could have some beneficial effects on the plants, particularly in protecting them from reactive oxygen species (35), no special attention

has been focused on actinobacteria and streptomycetes as a potential group from which to isolate strains to control phytopathogenic fungi that cause GTD. Loqman and colleagues (36) isolated 142 actinobacterial strains from rhizospheres of *V. vinifera* plants cultivated in Moroccan soils, and some of these isolates were effective against different fungal pathogens not involved in GTD. This study further characterizes the endophytic and rhizosphere microbiota associated with the grapevine root system, particularly as a potential source of strains of interest in controlling the incidence of GTD produced by fungi that penetrate plants through their root system and which are therefore involved in YGD. The best-performing isolates were tested in a 3-year field trial with grafted vines in a commercial nursery in Spain between 2013 and 2015.

RESULTS

Isolation and identification of culturable endophytic actinobacteria associated with the *Vitis vinifera* root system and their antifungal activity. A total of 58 endophytic strains were obtained from four different young *V. vinifera* plants using various culture media (Table 1). 16S rRNA gene sequencing of all the strains revealed their genus levels. *Streptomyces* was the most abundant genus (26 out of 58 strains), representing 44.8% of the total isolates. *Micromonospora* (15 out of 58) and *Saccharopolyspora* strains (10 out of 58) represented 25.9% and 17.2% of the total isolates, respectively (see Fig. S1 in the supplemental material). Microorganisms from other taxa were also isolated in smaller numbers, including the rare actinomycete taxa *Nonomuraea* (37), *Kribbella*, *Rathayibacter*, and *Corynebacterium* (4 out of 58 strains [6.9%]). These strains, 1 out of 58 in each case, represented 1.7% of the total isolates. Since the primary goal of this work was to evaluate the putative application of selected actinobacteria (able to infect plants through their root system in order to control fungal pathogens involved in GTD and YGD), we screened all the endophytic strains for antifungal activity. A bioassay-based *in vitro* evaluation showed that 15.5% (9 out of 58) and 13.8% (8 out of 58) of the endophytic isolates, respectively, exhibited antifungal activity against *D. seriata* and *Dactylonectria macrodidyma* pathogens (Table 1). These pathogens were initially selected for their high growth rate. The five strains showing higher inhibition (I) index values were also tested for their antifungal activities against *P. chlamydospora* and *P. minimum*. Three endophytic isolates, *Streptomyces* sp. VV/E1, *Streptomyces* sp. VV/E2, and *Streptomyces* sp. VV/E5, showed antifungal activities against all the pathogens tested. These were selected for field assays.

Antifungal activities of rhizosphere actinobacteria. A total of 94 rhizosphere strains were isolated. Bioassay-based *in vitro* screening showed that 30.8% (29 out of 94) and 16.0% (15 out of 94) of the isolates exhibited antifungal activity against *D. seriata* and *D. macrodidyma* pathogens, respectively (Table 2). Only those isolates exhibiting antifungal activity were identified by 16S rRNA sequencing, and all turned out to be members of the *Streptomyces* genus (Fig. S1). The five strains which showed the highest I index against *D. macrodidyma* were also tested for their antifungal activities against *P. chlamydospora* and *P. minimum*. Three rhizosphere isolates, *Streptomyces* sp. VV/R1, *Streptomyces* sp. VV/R4, and *Streptomyces* sp. VV/R5, showed antifungal activity against all the pathogens tested and accordingly were selected for field assays.

Multilocus sequence analysis of actinobacterial strains selected for field assays. As previously indicated, preliminary characterization of the selected endophytic and rhizosphere actinobacteria based on 16S rRNA sequencing led to their identification as species of the *Streptomyces* genus. To further characterize and more precisely identify them, we performed a multilocus sequence analysis (MLSA) study of five housekeeping genes (*atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*).

MLSA (Fig. S2) confirmed that isolates VV/E1 and VV/R4 are the same species (sharing an MLSA evolutionary distance of 0.000; Table 3), although they exhibit macroscopic differences when grown in different culture media (Fig. S3). Both strains are closely related to *Streptomyces albaduncus* (showing an MLSA distance of 0.028 with this species; Table 3). Isolates VV/R1 and VV/E5 were located in a well-delineated

TABLE 1 Antifungal activity and culture media for the isolation of culturable endophytic actinobacteria isolated from the root system of grafted grapevines

Isolate	I index ^a		Detection of antifungal activity ^b		Isolation medium	16S rRNA sequence accession no.
	<i>D. seriata</i>	<i>D. macrodidyma</i>	<i>P. chlamydospora</i>	<i>P. minimum</i>		
<i>Streptomyces</i> sp. VV/E1	53.47	33.91	+	+	SC	KY978646
<i>Streptomyces</i> sp. VV/E2	61.17	59.41	+	+	ISP2	KY978647
<i>Saccharopolyspora</i> sp. VV/E3	31.97	35.87	—	—	SC	KY978648
<i>Streptomyces</i> sp. VV/E4	46.95	46.62	—	+	SC	KY978649
<i>Streptomyces</i> sp. VV/E5	52.50	47.15	+	+	SC	KY978650
<i>Streptomyces</i> sp. VV/E6	49.57	33.86	ND	ND	DPA	KY978651
<i>Saccharopolyspora pathumthaniensis</i> VV/E7	36.81	—	ND	ND	ISP2	KY978652
<i>Streptomyces</i> sp. VV/E8	33.42	—	ND	ND	DPA	KY978653
<i>Streptomyces</i> sp. VV/E9	—	40.24	ND	ND	DPA	KY978654
<i>Saccharopolyspora</i> sp. VV/E10	29.97	—	ND	ND	ISP2	KY978655
<i>Streptomyces</i> sp. VV/E11	—	40.24	ND	ND	SAA	KY978656
<i>Streptomyces atrovirens</i> VV/E12	—	—	ND	ND	ISP2	KY978657
<i>Streptomyces</i> sp. VV/E13	—	—	ND	ND	ISP2	KY978658
<i>Streptomyces</i> sp. VV/E14	—	—	ND	ND	SAA	KY978659
<i>Streptomyces</i> sp. VV/E15	—	—	ND	ND	SC	KY978660
<i>Streptomyces</i> sp. VV/E16	—	—	ND	ND	SC	KY978661
<i>Streptomyces</i> sp. VV/E17	—	—	ND	ND	ISP2	KY978662
<i>Streptomyces</i> sp. VV/E18	—	—	ND	ND	SAA	KY978663
<i>Micromonospora</i> sp. VV/E19	—	—	ND	ND	ISP2	KY978664
<i>Micromonospora</i> sp. VV/E20	—	—	ND	ND	ISP2	KY978665
<i>Micromonospora</i> sp. VV/E21	—	—	ND	ND	SC	KY978666
<i>Streptomyces</i> sp. VV/E22	—	—	ND	ND	SC	KY978667
<i>Streptomyces</i> sp. VV/E23	—	—	ND	ND	DPA	KY978668
<i>Streptomyces</i> sp. VV/E24	—	—	ND	ND	SC	KY978669
<i>Streptomyces</i> sp. VV/E25	—	—	ND	ND	SC	KY978670
<i>Streptomyces</i> sp. VV/E26	—	—	ND	ND	DPA	KY978671
<i>Micromonospora palomenae</i> VV/E27	—	—	ND	ND	ISP2	KY978672
<i>S. pathumthaniensis</i> VV/E28	—	—	ND	ND	SC	KY978673
<i>Streptomyces</i> sp. VV/E29	—	—	ND	ND	ISP2	KY978674
<i>Streptomyces</i> sp. VV/E30	—	—	ND	ND	SC	KY978675
<i>S. pathumthaniensis</i> VV/E31	—	—	ND	ND	ISP2	KY978676
<i>Streptomyces</i> sp. VV/32	—	—	ND	ND	SC	KY978677
<i>S. pathumthaniensis</i> VV/E33	—	—	ND	ND	ISP2	KY978678
<i>Rathayibacter caricis</i> VV/E34	—	—	ND	ND	ISP2	KY978679
<i>Nonomuraea kuesteri</i> VV/E35	—	—	ND	ND	ISP2	KY978680
<i>S. pathumthaniensis</i> VV/E36	—	—	ND	ND	SC	KY978681
<i>N. kuesteri</i> VV/E37	—	—	ND	ND	SC	KY978682
<i>S. pathumthaniensis</i> VV/E38	—	—	ND	ND	ISP2	KY978683
<i>Micromonospora</i> sp. VV/E39	—	—	ND	ND	ISP2	KY978684
<i>Micromonospora</i> sp. VV/E40	—	—	ND	ND	SC	KY978685
<i>Micromonospora</i> sp. VV/E41	—	—	ND	ND	ISP2	KY978686
<i>Micromonospora</i> sp. VV/E42	—	—	ND	ND	ISP2	KY978687
<i>Micromonospora</i> sp. VV/E43	—	—	ND	ND	ISP2	KY978688
<i>Streptomyces</i> sp. VV/E44	—	—	ND	ND	SAA	KY978689
<i>Micromonospora</i> sp. VV/E45	—	—	ND	ND	SC	KY978690
<i>Micromonospora</i> sp. VV/E46	—	—	ND	ND	ISP2	KY978691
<i>Micromonospora</i> sp. VV/E47	—	—	ND	ND	ISP2	KY978692
<i>Micromonospora</i> sp. VV/E48	—	—	ND	ND	SC	KY978693
<i>S. pathumthaniensis</i> VV/E49	—	—	ND	ND	SC	KY978694
<i>Micromonospora</i> sp. VV/E50	—	—	ND	ND	ISP2	KY978695
<i>Nonomuraea</i> sp. VV/E51	—	—	ND	ND	SC	KY978696
<i>S. pathumthaniensis</i> VV/E52	—	—	ND	ND	ISP2	KY978697
<i>Streptomyces</i> sp. VV/E53	—	—	ND	ND	DPA	KY978698
<i>Nonomuraea</i> sp. VV/E54	—	—	ND	ND	ISP2	KY978699
<i>Kribbella</i> sp. VV/E55	—	—	ND	ND	SAA	KY978700
<i>Corynebacterium</i> sp. VV/E56	—	—	ND	ND	ISP2	KY978701
<i>Micromonospora</i> sp. VV/E57	—	—	ND	ND	ISP2	KY978702
<i>Streptomyces</i> sp. VV/E58	—	—	ND	ND	SAA	KY978703

^aInhibition index (I index) values shown are the average of three independent experiments. —, species not showing antifungal activity against *D. seriata* or *D. macrodidyma*.

^bND, not determined.

TABLE 2 Antifungal activity and culture media for the isolation of culturable rhizospheric actinobacteria isolated from the root system of grafted grapevines

<i>Streptomyces</i> isolate	I index ^a		Detection of antifungal activity ^b		Isolation medium	16S rRNA sequence accession no.
	<i>D. seriata</i>	<i>D. macrodidyma</i>	<i>P. chlamydospora</i>	<i>P. minimum</i>		
VV/R1	48.75	55.06	+	+	ISP2	KY978704
VV/R2	45.52	53.20	—	+	SC	KY978705
VV/R3	42.58	49.13	—	—	ISP2	KY978706
VV/R4	51.69	50.77	+	+	SC	KY978707
VV/R5	55.60	51.43	+	+	SC	KY978708
VV/R6	47.84	44.57	ND	ND	SC	KY978709
VV/R7	51.83	41.22	ND	ND	DPA	KY978710
VV/R8	51.04	44.40	ND	ND	SAA	KY978711
VV/R9	54.14	21.55	ND	ND	ISP2	KY978712
VV/R10	45.65	48.44	ND	ND	SC	KY978713
VV/R11	45.52	35.23	ND	ND	SC	KY978714
VV/R12	44.61	49.07	ND	ND	SC	KY978715
VV/R13	52.03	46.41	ND	ND	ISP2	KY978716
VV/R14	56.95	43.42	ND	ND	SC	KY978717
VV/R15	49.47	48.67	ND	ND	ISP2	KY978718
VV/R16	45.65	—	ND	ND	DPA	KY978719
VV/R17	40.48	—	ND	ND	SC	KY978720
VV/R18	62.27	—	ND	ND	SC	KY978721
VV/R19	48.66	—	ND	ND	SC	KY978722
VV/R20	44.67	—	ND	ND	SAA	KY978723
VV/R21	40.61	—	ND	ND	SAA	KY978724
VV/R22	55.21	—	ND	ND	ISP2	KY978725
VV/R23	48.27	47.02	ND	ND	SC	KY978726
VV/R24	40.71	—	ND	ND	ISP2	KY978727
VV/R25	80.50	—	ND	ND	ISP2	KY978728
VV/R26	62.87	—	ND	ND	DPA	KY978729
VV/R27	90.81	—	ND	ND	SC	KY978730
VV/R28	43.59	—	ND	ND	SC	KY978731
VV/R29	44.95	—	ND	ND	ISP2	KY978732

^aInhibition index (I index) values shown are the average of three independent experiments. —, species not showing antifungal activity against *D. seriata* or *D. macrodidyma*.

^bND, not determined.

subclade including *Streptomyces peucetius* and *Streptomyces xantholiticus* strains (Fig. S2). Both isolates share an MLSA distance of 0.108, indicating they are different species. Isolate VV/E5 was identified as *S. peucetius*, as it had MLSA distances smaller than 0.007 (Table 3) with the *S. peucetius* species. Strain VV/R1 matched between *S. peucetius* (0.108 MLSA distance) and *S. xantholiticus* (0.112 MLSA distance) (Fig. S2), suggesting that it could be a novel *Streptomyces* species. Isolate VV/E2 was located in a subclade including *Streptomyces albidochromogenes*, *Streptomyces flavidovirens*, and *Streptomyces helveticus* (Fig. S2). MLSA distances with these strains were always higher than 0.007, indicating that isolate VV/E2 could be a novel species. Isolate VV/R5 is closely related to members of a subclade including *Streptomyces phaeochromogenes* and *Streptomyces umbrinus* (Fig. S2), although its MLSA distance with respect to both species clearly indicated that this isolate belongs to a different species.

Field assays. (i) Growth data and mortality rate. The application of actinobacteria to grafted plants did not have a significant effect, either positive or negative, on the growth of the surviving plants, as determined by measurement of total plant height [$F(6.413) = 0.37, P = 0.90$] (Fig. 1A) or elongation of the seventh internode [$F(6.413) = 0.12, P = 0.29$] (Fig. 1B).

The average mortality rate of noninoculated untreated (negative control) plants in the 3-year field study was 28.4% (64 dead plants out of a total of 225 plants) (Fig. 2). Significant differences in mortality rates were observed for two of the treatments, according to the chi-square tests. The mortality rates of plants inoculated with VV/R1 (19.1% [43 dead plants out of 225 plants]) [$\chi^2(1) = 5.41, P < 0.05$] or VV/R4 (17.8% [40 dead plants out of 225 plants]) [$\chi^2(1) = 7.20, P < 0.01$] strains were significantly lower (Fig. 2). Thus, we could conclude that the odds of plant survival were 1.68 times higher

TABLE 3 MLSA distances for strains phylogenetically near to our isolates

		MLSA (Kimura 2-parameter) distance																	
No.	Strain ^a	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	<i>S. albaduncus</i> NRRL B-3605 ^T																		
2	<i>S. albaduncus</i> NRRL b-3605g ^T	0.000																	
3	VV/E1	0.028	0.028																
4	VV/R4	0.028	0.028	0.000															
5	<i>S. umbrinus</i> NRRL B-2572 ^T	0.094	0.094	0.092	0.092														
6	VV/R5	0.096	0.096	0.091	0.091	0.040													
7	<i>S. phaeochromogenes</i> NRRL B-1248 ^T	0.094	0.094	0.091	0.091	0.034	0.013												
8	<i>S. phaeochromogenes</i> subsp. <i>phaeochromogenes</i> NRRL B-3010 ^T	0.094	0.094	0.091	0.091	0.034	0.013	0.000											
9	<i>S. helvaticus</i> NRRL B-12365 ^T	0.124	0.124	0.130	0.130	0.121	0.124	0.126	0.126										
10	VV/E2	0.119	0.119	0.126	0.126	0.118	0.122	0.122	0.122	0.043									
11	<i>S. albidochromogenes</i> NRRL B-24308 ^T	0.113	0.113	0.117	0.117	0.110	0.112	0.113	0.113	0.045	0.057								
12	<i>S. flavidovirens</i> subsp. <i>flavidovirens</i> NRRL B-2708 ^T	0.113	0.113	0.113	0.113	0.106	0.113	0.111	0.111	0.067	0.055	0.048							
13	<i>S. flavidovirens</i> DSM 40150 ^T	0.113	0.113	0.113	0.113	0.106	0.115	0.112	0.112	0.067	0.055	0.050	0.001						
14	<i>S. peucetius</i> subsp. <i>peucetius</i> NRRL B-3826 ^T	0.104	0.104	0.105	0.105	0.111	0.113	0.108	0.108	0.131	0.128	0.131	0.124	0.124					
15	VV/E5	0.106	0.106	0.107	0.107	0.112	0.114	0.110	0.110	0.132	0.130	0.132	0.126	0.126	0.002				
16	<i>S. peucetius</i> CGMCC 4.1799 ^T	0.122	0.122	0.105	0.105	0.111	0.113	0.111	0.111	0.131	0.128	0.131	0.124	0.124	0.000	0.002			
17	VV/R1	0.219	0.219	0.187	0.187	0.201	0.200	0.196	0.196	0.218	0.212	0.214	0.210	0.210	0.108	0.108	0.108		
18	<i>S. xantholiticus</i> NRRL B-12153 ^T	0.106	0.106	0.108	0.108	0.113	0.114	0.111	0.111	0.130	0.125	0.128	0.124	0.124	0.012	0.014	0.112	0.112	

^aOur isolates are in bold.

on VV/R1 treatment than on untreated control plants, whereas the odds of plant survival were 1.84 times higher on VV/R4 treatment than on untreated control plants.

(ii) Isolation of pathogenic fungi. Two hundred days after planting (once the plants had completed their vegetative growth cycle and were in a dormant state), vines were carefully removed from the soil to obtain a root system that was as intact as possible. The incidence of fungal pathogens causing YGD (*P. chlamydospora*, *P. minimum*, and *Dactylonectria* sp.-*Ilyonectria* sp. group) and able to infect the plants through their root system was determined by collecting samples of wood taken from the root insertion point. All the selected actinobacteria led to a reduction in the number of fungal pathogens involved in YGD (Fig. 3) compared to the number of fungal pathogens isolated from control plants (raw data corresponding to this study are shown in Table S1).

In order to analyze the impact of every actinobacterial treatment on the number of each particular type of fungal pathogen involved in YGD, we used nested generalized linear models (Poisson distribution, with intercept versus intercept plus treatment as dependent variables into the model). The existence of significant differences between the number of fungal pathogens isolated from plants treated with the different actinobacteria and the number of pathogens isolated from control plants was determined using chi-square tests. Thus, in accordance with the nested generalized linear models, significant associations between the nature of the treatment and the number of fungal pathogens isolated were found in the *Dactylonectria* sp.-*Ilyonectria* sp. group and for *P. chlamydospora* ($P < 0.05$ in the chi-square tests conducted to compare the nested models) for all the actinobacteria assayed. On the other hand, no significant association ($P > 0.05$ in the chi-square tests) between the nature of the treatment and the number of fungal pathogens isolated was detected for *P. minimum*, probably due to the low number of individuals of this species isolated.

After that, chi-square and Fisher's tests were performed to establish the existence of significant differences between treatments. The results are shown as heatmaps of independence assumptions between the type of actinobacterial treatment and the number of fungal pathogens isolated (Fig. 4). Surprisingly, despite the data shown by nested generalized linear models, significant differences ($P < 0.05$) between the negative control and the three treatments (VV/E1, VV/R1, and VV/R4) were found with respect to *P. minimum* (Fig. 3). However, these significant associations were very close

FIG 1 Plant growth in the experimental nurseries (2013, 2014, and 2015 growing seasons) as determined by total height (A) or length of the 7th internode (B) in grafted plants treated with different endophytic (VV/E1, VV/E2, or VV/E5) or rhizosphere (VV/R1, VV/R4, or VV/R5) actinobacteria compared to untreated control plants. Each bar corresponds to the arithmetic mean of the values determined for the total number of surviving plants in the three seasons tested and their corresponding standard error (SE). Bars marked with the same letter are not significantly different ($P \geq 0.05$).

to the edge of the significance threshold (0.05). It may be that the nested model tests are more conservative than individual tests performed between treatments.

In the cases related to the *Dactylonectria* sp.-*Ilyonectria* sp. group isolation, and solely focusing on those highly significant associations between the nature of the treatment and the number of fungal pathogens isolated ($P < 0.01$ and < 0.001), the following results should be highlighted: control and VV/E1 [χ^2 (1) = 46.4, $P < 0.001$], control and VV/R4 [χ^2 (1) = 12.0, $P < 0.001$], control and VV/R1 [χ^2 (1) = 7.15, $P < 0.01$], and control and VV/R5 [χ^2 (1) = 5.91, $P < 0.01$] (Fig. 4). If we focus on *P. chlamydospora*, the following highly significant associations were detected: control and VV/R4 [χ^2 (1) = 15.8, $P < 0.001$], control and VV/E1 [χ^2 (1) = 10.8, $P < 0.01$], control and VV/E2 [χ^2 (1) = 10.8, $P < 0.01$], control and VV/R1 [χ^2 (1) = 10.8, $P < 0.01$], and control and VV/R5 [χ^2 (1) = 9.43, $P < 0.01$] (Fig. 4).

(iii) Rootstock colonization by selected actinobacteria. The ability of the selected actinobacteria to colonize rootstocks at the root insertion point was analyzed 10 and 200 days after their application. As shown in Table 4, 10 days after application, all the actinobacteria were successfully recovered from rootstocks at percentages ranging between 11.43% (*Streptomyces* sp. VV/R5) and 36.43% (*Streptomyces* sp. VV/E1), indicating that the application method was effective enough to allow actinobacterial

FIG 2 Average mortality rates (%) and their corresponding SE values of grafted grapevine plants observed in the experimental nurseries (2013, 2014, and 2015 growing seasons) after treatment with different endophytic (VV/E1, VV/E2, or VV/E5) or rhizosphere (VV/R1, VV/R4, or VV/R5) strains compared to untreated control plants. Bars marked with the same letter are not significantly different ($P \geq 0.05$).

penetration into the rootstock. The differences were significant in all cases with respect to the isolation values for the same strain from control plants.

At the end of the trials (200 days after planting), only four strains could be reisolated from the plants, and these were always at lower levels than those detected 10 days after application. Significant differences compared to the control were observed for *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/E2 strains. These data showed that with the passage of time, the degree of colonization tends to decrease.

DISCUSSION

It is well established that grapevine plants are mainly infected by fungal pathogens causing GTD through pruning wounds or the root system, with a reduced number of

FIG 3 Relative isolation frequency (%) of fungal pathogens involved in young grapevine decline from grafted plants treated with different endophytic and rhizosphere actinobacteria with respect to untreated plants. A value of 100 was arbitrarily assigned to the total number of fungal pathogens of a particular species (*P. chlamydospora* or *P. minimum*) or *Dactylonectria* sp.-*Ilyonectria* sp. group analyzed once the plants had been removed from the field. The relative frequency for each type of pathogen analyzed in the plants treated with the different actinobacteria was calculated as a percentage (%) compared against the value of 100 assigned to the control plants. Data shown correspond to the average of 3 batches of 25 plants in 3 experimental nurseries (2013, 2014, and 2015 growing seasons).

FIG 4 Analysis of significant differences between the number of fungal pathogens isolated from plants treated with the different actinobacteria versus the number of pathogens isolated from control plants. The analysis is shown as heatmaps of independence assumptions between the type of actinobacterial treatment and the number of fungal pathogens isolated. Color key corresponds to the significance of chi-square or Fisher's test results (according to expected cell values). (I) Data relative to *Dactylonectria* sp.-*Ilyonectria* sp. group; (II) data relative of *P. minimum*; (III) data relative to *P. chlamydospora*. Data shown correspond to 3 batches of 25 surviving plants in 3 experimental nurseries (2013, 2014, and 2015 growing seasons).

fungal strains that can use both routes of entry. There have been many different studies to test pruning wound protection with chemical antifungals (6, 13–15), natural anti-fungal compounds (12), or BCAs (16–18). Given the many years that a plant remains in the ground and the consequent extent and depth of root development and soil penetration, it is clear that the protection of grapevines from fungi that penetrate

TABLE 4 Rootstock colonization rates by selected actinobacteria at 10 and 200 days after their application

Actinobacterium isolated by batch of plants	% of isolated actinobacteria after ^a :	
	10 days	200 days
Control plants		
VV/E1	6.43 (9)	4.29 (6)
VV/E2	1.43 (2)	0.71 (1)
VV/E5	4.29 (6)	2.14 (3)
VV/R1	3.57 (5)	ND (0)
VV/R4	2.86 (4)	3.57 (5)
VV/R5	ND (0)	0.71 (1)
Treated plants		
VV/E1	36.43 (51)	13.57 (19)
VV/E2	27.14 (38)	7.86 (11)
VV/E5	16.43 (23)	4.29 (6)
VV/R1	30.00 (42)	ND (0)
VV/R4	20.71 (29)	7.14 (10)
VV/R5	11.43 (16)	ND (0)

^aND, not detected. The percentage of each particular strain was calculated as the number of clones growing from wood chips and identified by sequencing of 16S rRNA against a number of 140 chips tested under each condition (7 chips isolated from two stubs per 10 plants analyzed). Values are the average of two independent experiments. Raw data (number of each particular actinobacterium isolated from 140 chips) are indicated in parentheses.

through the root system is much more complicated. Current studies have not gone much beyond the application of BCAs to the root system, mainly different strains of *Trichoderma* sp. (19, 23). Thus, it seems evident that development of technologies that can limit the infection of vine plants through their root systems is of great interest for the grape and wine industry. Thus, we decided to investigate the putative application of certain actinobacteria that might help mitigate this problem.

Actinobacteria are an integral part of soil microbial communities, making up around 10% of the total soil microbiome (24), and they stand out, particularly streptomycetes, for their ability to produce a wide range of secondary metabolites, including antifungal compounds (29–31). Several studies indicate that actinobacteria can enter root tissues and establish an endophytic lifestyle with plants (26–30). As reported in other plants, the grapevine root system contains many different endophytic actinobacteria. *Streptomyces* and *Micromonospora* were the most frequently isolated genera, representing up to 70.7% of the total isolates. Similarly, when the population of endophytic actinobacteria in *Artemisia annua* was analyzed, *Streptomyces* and *Micromonospora* represented 57.0% of the total identified isolates (26). Other minority genera found in grapevine plants, like *Nonomuraea* and *Kribbella*, were also detected in *A. annua* (26). However, *Corynebacterium* and *Saccharopolyspora*, genera isolated from *V. vinifera*, were not detected in *A. annua* (26). These data confirm that actinobacteria are frequent endophytes in the root systems of both plants.

Streptomyces species identification is a great challenge, further complicated by factors, such as the fact that there are a great number of species, many of which are poorly defined. The insufficient resolution of 16S rRNA as a phylogenetic marker, difficulties in effectively addressing phenotypic differences, and the absence of rapid molecular identification methods applicable to a high number of species also hinder the accurate identification of species of *Streptomyces* (38, 39). Since 16S rRNA sequencing does not provide sufficient resolution for species-level identification of streptomycetes, we performed an MLSA study of the six isolates used in field trials by partially sequencing five housekeeping genes. MLSA has proven to be an efficient molecular tool for the improved taxonomic resolution of *Streptomycetaceae* family (39), as well as for the right identification of *Streptomyces* species for the *S. albidoflavus* (40), *S. griseus* (41), and *S. hygroscopicus* (38) clades. These clades include some of the more widely studied species in the genera, many of which produce antibiotics and other industrially and agronomically important secondary metabolites (38). In our case, the MLSA al-

lowed us to identify the isolate VV/E5 as belonging to the *S. peucetius* species. Unfortunately, this study did not lead to an accurate identification of the rest of the strains at the species level, probably due to a poor characterization of those closely related phylogenetic strains. This setback makes it difficult to establish whether these strains are unique to the root system of grapevines or to assess their ability to produce known antifungal compounds. We cannot even rule out that some of them are new species. The case of *Streptomyces* sp. VV/E1 and VV/R4 is particularly intriguing, since the MLSA study indicated that the two isolates belong to the same species. In fact, these strains share 99.5% homology at the 16S rRNA level. However, they exhibit quite different macroscopic traits when growing on various culture media, as shown in Fig. S3. Remember that *Streptomyces* sp. VV/E1 was isolated as an endophytic strain, whereas *Streptomyces* sp. VV/R4 was isolated from the rhizosphere environment. We can speculate that the observed macroscopic differences could be a putative adaptation to two different lifestyles. However, currently, we do not know for certain whether a particular strain present in the rhizosphere might also develop an endophytic lifestyle. Works under way for the development of molecular markers for the specific detection of these strains, as well as genome sequencing of the VV/E1 strain, should enable us in the future to address many of these issues that remain unanswered for now.

Recently, there have been several attempts to characterize the endophytic bacterial microbiota of grapevine plants. Andreolli et al. (42) analyzed the diversity of bacterial endophytes in 3- and 15-year-old plants. In 3-year-old plants, *Actinobacteria* was the second dominant class (26%), and the genera detected were *Nocardioides*, *Curtobacterium*, *Microbacterium*, *Brachy bacterium*, *Kocuria*, and *Micrococcus*. None of these genera were detected in our study, and surprisingly, Andreolli and colleagues did not detect any *Streptomyces* sp. strains. In 15-year-old plants, the population of endophytic actinobacteria was reduced to 5%, with only 2 strains detected, belonging to the *Microbacterium* and *Curtobacterium* genera. Also recently, Rezgui et al. (43) analyzed the population of endophytic bacteria that inhabit the wood tissues from grapevines of Tunisian vineyards. Actinobacteria were hardly detected, and *Curtobacterium* was the only genus found. These differences can be attributed to many different factors. First, in both studies, endophytes were isolated from vine stems and mature arms, which are aerial habitats far from the ground. In fact, it has been reported that the population of actinobacteria decreases along the plant axis from 20% in the root, to 5% in the graft union, and just 2% in the cane samples (44). Second, the observed differences may be attributed to some extent to the different isolation culture media used, and even to the different molecular techniques used in the two works (43, 44).

Recently, different studies have focused on the characterization of the grapevine microbiome (45, 46) and vineyard soils (32, 34). After *Proteobacteria*, *Actinobacteria* are the second most abundant microorganisms in vineyard soils, representing averages of 9.72% and 20.48% of the total microorganisms detected in vineyards of Argentina (34) and California (32), respectively. In New York vineyards, *Actinobacteria* were present at 5.1% in grapevine roots, whereas their relative abundance was lower in all above-ground samples (grapes, leaves and flowers) tested (45). *Actinobacteria* are also a prominent group detected in both rhizosphere (around 44%), and roots (around 36%) of plants from Austrian vineyards (46). Unfortunately, most of these studies dealing with soil and root microbiomes do not delve deeply into characterization of the actinobacteria detected beyond the taxonomic class level. Future studies, focused more specifically on the population of actinobacteria detected in the root system of grapevine plants at the genus or even species level, should shed light on many issues that remain unresolved.

Actinobacteria were also easily isolated from young grapevine plant rhizospheres, and a screening based on antifungal activity against fungi causing GTD and YGD resulted in the identification of a total of 29 isolates, all belonging to the genus *Streptomyces*. Furthermore, Loqman and colleagues (36) have isolated strains of *Streptomyces* with antifungal activity against *Botrytis cinerea* from a grapevine rhizosphere in Morocco. Together, these data indicate that both the rhizosphere and the interior of the

grapevine root system are highly interesting sources for actinobacteria which may be potential BCAs for the treatment of different vineyard pathologies.

Blackfoot and Petri diseases, recognized as prominent causes of YGD, along with failure of planting material, are some of the most worrisome problems affecting the wine grape industry since the 1990s (10). Evaluations of declining young vineyards has revealed that many factors are involved. Among all the diseases, the grafted plant root system infections by fungal trunk pathogens in nurseries are the most relevant (10). The main fungal trunk pathologies associated with YGD are Petri disease, primarily produced by *P. chlamydospora* and different *Phaeoacremonium* species, and blackfoot disease, mainly produced by species of *Campylocarpon*, *Dactylonectria*, and *Ilyonectria*. These pathogens can infect a significant portion of all the grafted plants produced every year in nurseries all around the world. They are frequently found infecting the rootstock mother vines (10, 23). They can also infect grafts once they are planted in an open-root field nursery.

This step seems to be particularly critical, since it is supposed that the pathogens present in the soil can infect grafts through the incipient root system that the vines must produce to survive and develop in soil. Thus, when Halleen and colleagues analyzed the presence of fungal pathogens involved in YGD in nurseries during the different steps of the propagation process, they found that less than 1% of the plants were infected with *Cylindrocarpon* spp. before planting in the nursery, whereas 50% or more of the plants were infected at the end of the season (47). This result is highly significant, since it demonstrates that most of the grafted plants in the nurseries can be infected through the roots once they are planted in an open-root field nursery. Our study shows that the application of actinobacteria, selected for their *in vitro* antifungal properties against fungal pathogens which cause YGD, is highly effective in controlling the infection at this step. Application of all the selected actinobacteria resulted in a clear reduction in the isolation of fungal pathogens involved in YGD from surviving plants. Furthermore, the application of actinobacterial strains VV/E1, VV/R1, and VV/R4 led to a statistically significant decrease ($P < 0.05$) in the presence of three types of pathogenic fungi analyzed, *Dactylonectria* sp.-*Ilyonectria* group, *P. chlamydospora*, and *P. minimum*. None of the actinobacteria had a significant effect, either positive or negative, on the growth of the surviving plants. However, whereas the average mortality rate of untreated (negative control) plants in the 3-year field study was 28.4%, the mortality rate was significantly reduced when the actinobacteria VV/R1 and VV/R4 were applied to the grafts. Graft failure is a serious problem for nurseries since, on average, it results in the failure of 30 to 60% of the grafts taken to the field. Many different factors can contribute to graft failure, including abiotic causes, such as improperly healed rootstock, disbudding sites and graft unions, or improper storage and management of the plant material used for obtaining grafts (10).

However, despite the large number of studies showing the presence of fungal pathogens in nursery plants at the end of the propagation process, to date, no clear data are available that correlate the presence of pathogens with mortality levels. In fact, after analyzing the presence of fungal pathogens in plants with failed graft unions, Rumbos and Rumbou concluded that those pathogens were not the cause of YGD (48). Our data suggest a putative relationship between the number of fungal pathogens and the mortality rate, since the application of actinobacterial strains VV/R1 and VV/R4 led to both a significant reduction in the number of fungal pathogens detected and a significant reduction in the mortality rate. Interestingly, the dead plants at the end of the field trials showed a very small, almost nonexistent root system. We can speculate that the early infection with fungi causing YGD may interfere with the development of a potent root system, resulting in a premature graft death. Clearly, graft failure is a complex problem with many different factors, but in view of these results, it seems that the presence of fungal pathogens could significantly contribute to this problem.

Finally, in recent years, it has become clear that endophytic microorganisms, particularly actinobacteria, can be a significant reservoir of genetic diversity and an important source for the discovery of novel bioactive secondary metabolites (27) that

can help control many different crop pathologies. This work represents one of the first reports on the characterization of the endophytic actinobacterial population in the root system of young grapevines at the levels of genus and species. Several of the isolates tested had a beneficial impact when applied to grafts in nurseries, resulting in a significant decrease in both the mortality and infection rates by fungal pathogens involved in YGD. Therefore, the application of endophytic and/or rhizosphere actinobacteria to grafts in nurseries is a novel, green, and promising technology that can reduce the incidence of grapevine plant fungal infections through the root system.

MATERIALS AND METHODS

Isolation of culturable endophytic and rhizosphere actinobacteria from the grapevine root system. A total of four 7-month-old *Vitis vinifera* cv. Tempranillo plants grafted on Richter 110 (110R) rootstock were collected from an open-root field nursery (Viveros Villanueva Vides S.L.) located in Larraga (Spain) at 350 m above sea level (a.s.l.) (01°48'45.8"W and 42°32'12.9"N). Actinobacteria associated with the root system were isolated from root-adjacent soil (rhizosphere) and from interior root tissue (endophytic strains). Roots and soil were placed in sterile containers to be taken to the lab. Two different approaches were used to isolate the culturable actinobacterial population associated with the root system. Rhizosphere strains were isolated from 1 g of root-adjacent soil samples obtained from every plant collected. Soil samples were suspended in a final volume of 10 ml of sterile water and homogenized using a vortex (1 min). Tenfold serial dilutions were made in sterile water, and 0.1-ml aliquots of each dilution were inoculated on starch-casein (SC) (49), ducitol-proline agar (DPA) (26), International *Streptomyces* Project 2 (ISP2) (50), and sodium succinate-asparagine agar (SAA) media (26), supplemented with cycloheximide (150 $\mu\text{g} \cdot \text{ml}^{-1}$) and nalidixic acid (25 $\mu\text{g} \cdot \text{ml}^{-1}$) (Sigma-Aldrich, Madrid, Spain) in order to prevent fungal and Gram-negative bacterial growth, respectively. Plates were incubated at 28°C for 3 to 7 days.

Endophytic strains were isolated from root tissue samples. Two different sections of root tissue were used: 2-cm stubs corresponding to the root insertion point, and root fragments corresponding to the 6-cm apical ends. Wood from both samples was mixed, and microorganisms were extracted as indicated below. Roots were washed in sterile phosphate-buffered saline (PBS) and sonicated (160 W; 2 min) to dislodge soil and organic matter from the sample surface. After drying at room temperature (RT), roots were cut into 2.0-cm-long fragments. Root fragments were surface-sterilized by immersing in 20 ml of Tween 20 (0.1%) (Sigma-Aldrich) for 30 s, followed by sodium hypochlorite (1%) for 6 min and then $\text{Na}_2\text{S}_2\text{O}_3$ (2.5%) (Panreac, Barcelona, Spain) for 10 min to remove the residual chlorine. After that, samples were washed three times with sterile water. Next, the root fragments were submerged in 70% (vol/vol, in sterile water) ethanol for 6 min, followed by three washes with sterile water and air-dried (in petri plates) in a laminar flow hood (Telstar AV-100; Telstar, Terrassa, Spain). To confirm the effectiveness of the surface disinfection process, 0.2 ml of liquid from the final washing step was spread onto ISP2 medium and incubated at 28°C. Two different methods were used to isolate endophytic actinobacteria from the disinfected root fragments. The first method involved the disruption of 5 g of root material using a mortar and pestle. A volume of 20 ml of 0.9% (wt/vol) NaCl was added to the samples, and they were incubated at 28°C with strong agitation (220 rpm) to favor microorganism extraction. The second method entailed disruption of the samples (0.1 g) in 2-ml tubes containing glass beads (0.5 mm diameter) with a FastPrep-24 homogenizer (MP Biomedicals, Santa Ana, CA) at 4,000 oscillations per min for 8 s, for a total of 10 times. Samples from both methods were diluted 10-fold, and 0.1-ml aliquots were plated on SC, DPA, ISP2, and SAA media. Plates were incubated at 28°C for 3 to 7 days.

Preliminary identification of actinobacteria by 16S rRNA sequencing. Different isolates were selected according to their morphological and cultural characteristics, including colony properties, presence/absence of aerial mycelia, spore mass color, distinctive reverse colony color, and production of diffusible pigments. Isolates were routinely cultivated and maintained on ISP2 medium at 4°C. Spore-producing isolates were maintained as spore suspensions at -20°C in glycerol (40%). Endophytic and rhizosphere strains exhibiting antifungal activity were identified by 16S rRNA sequencing. Briefly, genomic DNA extraction was performed as described by Hopwood et al. (51). 16S rRNA genes were amplified using the oligonucleotides 27F and 1492R (52). Isolates were identified by comparing them to corresponding sequences of the type strain found on the EzTaxon-e database (53) (<http://www.ezbiocloud.net/eztaxon/identify>). Sequence alignment and generation of phylogenetic trees were performed using the MEGA 6.0 software (<http://www.megasoftware.net/>) using a neighbor-joining (NJ) algorithm. Evolutionary distance was calculated using the Kimura two-parameter (K2P) model for nucleotide sequences (54).

Identification of the actinobacteria selected by field assays using multilocus sequence analysis. Multilocus sequence analysis (MLSA) was carried out using five housekeeping genes, namely, *atpD* (ATP synthase F1, beta subunit), *gyrB* (DNA gyrase B subunit), *recA* (recombinase A), *rpoB* (RNA polymerase, beta subunit), and *trpB* (tryptophan synthase, beta subunit) (38). PCR amplification of housekeeping genes was carried out using the primers and under the amplification conditions previously described by Guo et al. (41) and Rong et al. (40). The GenBank accession numbers of DNA sequences from the housekeeping genes are listed in Table 5. Phylogenetic trees were constructed from a concatenation of the five housekeeping genes. All the sequences were concatenated by joining them head to tail. DNA sequences were manually trimmed at the same position before being aligned using MEGA 6.0 software with sequences from type strains obtained from the ARS Microbial Genomic Sequence Database server

TABLE 5 GenBank accession numbers of DNA sequences from the endophytic and rhizosphere *Streptomyces* sp. strains isolated from the root system of grapevine plants used in the MLSA

Genus/species	Isolate	GenBank accession no.				
		<i>atpD</i>	<i>gyrB</i>	<i>recA</i>	<i>rpoB</i>	<i>trpB</i>
<i>Streptomyces</i> sp.	VV/E1	MF437320	MF437326	MF437332	MF437338	MF437344
<i>Streptomyces</i> sp.	VV/E2	MF437321	MF437327	MF437333	MF437339	MF437345
<i>Streptomyces peucetius</i>	VV/E5	MF437322	MF437328	MF437334	MF437340	MF437346
<i>Streptomyces</i> sp.	VV/R1	MF437323	MF437329	MF437335	MF437341	MF437347
<i>Streptomyces</i> sp.	VV/R4	MF437324	MF437330	MF437336	MF437342	MF437348
<i>Streptomyces</i> sp.	VV/R5	MF437325	MF437331	MF437337	MF437343	MF437349

(<http://199.133.98.43>). Phylogenetic trees were constructed using the maximum likelihood method with the Kimura two-parameter model (54). MLSA evolutionary distances were calculated using MEGA 6.0 by calculating the K2P distance. Strain pairs having ≤ 0.007 MLSA evolutionary distance were considered conspecific based on the guideline empirically determined by Rong and Huang (38).

Evaluation of antifungal activity. All isolates were tested for antifungal activity using an *in vitro* antifungal assay, as described by Lamsal et al. (55), with minor modifications. Briefly, isolates were inoculated on potato dextrose agar (PDA) plates (Sigma-Aldrich) in a 1.0-cm² area (4 isolates per plate inoculated at 1 cm from the edge of the plates). An agar plug (0.7 mm) containing *D. seriata* CBS 112555 or *Ilyonectria macrodidyma* CBS 120170 (now reclassified as *Dactylonectria macrodidyma*) was placed in the middle of the plates. The plates were incubated up to 12 days at 25°C to detect growth inhibition areas. Growth was quantified by calculating the inhibition index (I index) according to the following formula: I index (%) = $[1 - (Ra - R)/(Rc - R)] \times 100$, where Ra is the radius of the fungal colony opposite the bacterial colony, Rc is the maximum radius of the fungal colony (farther away from the bacterial effect), and R is the radius of the agar plug containing the fungi (3.5 mm). This assay was performed in triplicate for each actinobacterium tested (Fig. S4A).

Strains showing antifungal activity against both pathogens were also screened for their ability to inhibit the growth of *P. chlamydospora* and *P. minimum* in PDA, Czapek Dox (Sigma-Aldrich), or ISP5 (50) agar plates as follows (Fig. S4B): fungal pathogens were inoculated on an agar plate forming a circle 2 cm from the periphery of the petri dish. Actinobacterial strains were inoculated in 2.25-cm² patches located 5 mm from the edge of the fungal strain. Plates were incubated at 25°C for up to 20 days to detect the effect of growth inhibition.

Field evaluation in nurseries. Field assays were conducted in three experimental open-root field nurseries of grafted *Vitis vinifera* cv. Tempranillo plants that were grafted on Richter 110 (110R) rootstock in 2013, 2014, and 2015. Every experimental nursery consisted of 525 plants. Grafts were subdivided into 7 batches of 75 units (3 replicas of 25 units per batch), each of which was treated with one of the 6 selected actinobacteria, 3 endophytic (VV/E1, VV/E2, and VV/E5) and 3 rhizosphere (VV/R1, VV/R4, and VV/R5) isolates. A negative-control batch (grafts not treated with actinobacteria) was also included in the study. The biomass production of each strain was obtained from 200-ml liquid cultures of tryptic soy broth (TSB) medium (Sigma-Aldrich) incubated at 220 rpm and 28°C for 3 days in 500-ml Erlenmeyer flasks that were inoculated with 8×10^8 spores. Cells were centrifuged (20 min, 12,000 rpm), washed with saline solution (0.9% [wt/vol]), and resuspended in a final volume of 50.0 ml of saline solution. Actinobacterial isolates were applied to a batch of 75 grafts by partially immersing the grafts (up to a depth of 10 cm) in a rooting hormone solution (indole-3-butyric acid; 0.2 mg · ml⁻¹) containing the selected actinobacteria (10^7 CFU · ml⁻¹) for 24 h at RT. Whole sets of plants were planted (during the month of May in 2013, 2014, and 2015) in an open-root field nursery. After 60 days in the field (month of July), several parameters were measured: mortality rate, total plant height, and elongation of the 7th internode in surviving plants. Two hundred days after planting (month of December), once the plants had completed their cycle of vegetative growth and were in a dormant state, they were carefully removed from the soil to obtain a root system as intact as possible to be analyzed for the presence of fungal pathogens as described below. A total of 75 live plants per batch (corresponding to three replicates of 25 plants) were analyzed.

Analysis of rootstock colonization by selected actinobacteria. In order to check rootstock colonization by the selected actinobacteria, we analyzed the graft interiors. A total of 10 grafts inoculated with each actinobacteria were analyzed 10 days and 200 days (at the time of uprooting the plants) after their application. The wood was surface-sterilized by immersion in 70% ethanol (1 min), 4% sodium hypochlorite (2 min), and then again in 70% ethanol (1 min). Two 1-cm-long stubs (isolated from 2 and 5 cm from the lower end of the rootstock) were split longitudinally, and samples were taken from 7 tissue sections (wood chips of 3 by 2 by 2 mm obtained using a sterile scalpel). Wood samples were plated on ISP2 agar medium containing cycloheximide (200 μ g · ml⁻¹) (Sigma-Aldrich) to prevent fungal growth and nalidixic acid (25 μ g · ml⁻¹) to inhibit the growth of Gram-negative bacteria. Plates were incubated at 28°C, and actinobacteria growing from the chips were identified by amplification and sequencing of 16S rRNA genes, as described above.

Fungal isolation from wood and pathogen identification. Plants were removed from the soil and analyzed for the presence of pathogenic fungi. A 1-cm stub was cut 1 cm above the root insertion point. The wood was surface-sterilized as described above, and inner tissue sections were obtained as previously indicated and placed on PDA plates supplemented with chloramphenicol (150 μ g · ml⁻¹) to

prevent bacterial growth. Plates were incubated at 25°C for up to 4 weeks, subculturing where necessary. Pathogenic fungi were identified by their cultural-morphological traits as *P. chlamydospora*, *P. minimum*, *Dactylonectria* sp., and *Ilyonectria* species. Subsequently, their identities were confirmed by sequencing their internal transcribed spacer 1 (ITS1)–5.8S–ITS2 regions using ITS1 and ITS4 primers under the PCR conditions described by White et al. (56). Other isolates were identified by sequencing the same region. DNA from wood samples was isolated using the REExtract-N-Amp kit (XNAP) (Sigma-Aldrich).

Statistical data analysis. Plant growth data were tested for univariate normality using the Shapiro-Wilk test. They were subjected to univariate analysis of variance using the general linear means procedure to determine if there were significant differences between treated and untreated plants. Previously, total height and length of the 7th internode data were tested for univariate normality using the Shapiro-Wilk test and then subjected to univariate analysis of variance using the general linear means procedure. In the case of grafted grapevine mortality data, we used a chi-square to test the null hypothesis of no differences in mortality rates against a two-tailed alternative hypothesis that the difference in mortality rates was not zero. When the expected frequencies were less than 5, Fisher's exact test was used. The chances of plant survival versus untreated control plants were calculated as the ratio between the odds of vine survival between a particular treatment and the control (the odds ratio for a particular treatment is the quotient between the number of live and dead plants). To research the treatment efficacy on the number of fungal pathogens isolated, we used the Poisson distribution (comparing the results achieved with nested models) to construct generalized linear models. The null hypothesis of the independence assumption between the frequency of fungal pathogen isolation and treatments was checked using either the chi-square or Fisher's exact test, depending on the nature of the data. Statistical analyses were performed using R Core Team (3.3.0) software (<http://www.R-project.org/>).

Accession number(s). The accession numbers for the 16S RNA sequences in this article are presented in Tables 1, 2, and 5.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01564-17>.

SUPPLEMENTAL FILE 1, PDF file, 4.7 MB.

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RESEARCH ARTICLE

Developing tools for evaluating inoculation methods of biocontrol *Streptomyces* sp. strains into grapevine plants

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Abstract

The endophytic *Streptomyces* sp. VV/E1, and rhizosphere *Streptomyces* sp. VV/R4 strains, isolated from grapevine plants were shown in a previous work to reduce the infection rate of fungal pathogens involved in young grapevine decline. In this study we cloned fragments from randomly amplified polymorphic DNA (RAPD), and developed two stably diagnostic sequence-characterized amplified region (SCAR) markers of 182 and 160 bp for the VV/E1 and VV/R4 strains, respectively. The SCAR markers were not found in another 50 actinobacterial strains isolated from grapevine plants. Quantitative real-time PCR protocols based on the amplification of these SCAR markers were used for the detection and quantification of both strains in plant material. These strains were applied on young potted plants using two methods: perforation of the rootstock followed by injection of the microorganisms or soaking the root system in a bacterial suspension. Both methods were combined with a booster treatment by direct addition of a bacterial suspension to the soil near the root system. Analysis of uprooted plants showed that those inoculated by injection exhibited the highest rate of colonization. In contrast, direct addition of either strain to the soil did not lead to reliable colonization. This study has developed molecular tools for analyzing different methods for inoculating grapevine plants with selected *Streptomyces* sp. strains which protect them from fungal infections that enter through their root system. These tools are of great applied interest since they could easily be established in nurseries to produce grafted grapevine plants that are protected against fungal pathogens. Finally, this methodology might also be applied to other vascular plants for their colonization with beneficial biological control agents.

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Introduction

Young grapevine decline (YGD) is a major threat to the wine and grape industry, especially since the 1990s when it entered a period of rapid expansion. Many factors are involved in YGD, although it is accepted that the fungal pathogens responsible for grapevine trunk diseases (GTDs) are one of the major culprits [1]. The main GTDs associated with YGD are Petri disease, primarily caused by *Phaeoconiella chlamydospora* and several species of the genus *Phaeoacremonium* [1], and black-foot disease, caused by different species belonging to the genera *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria* and *Ilyonectria* [2, 3]. Most of these pathogens can penetrate the plant through the root system [1, 4, 5], and therefore, the presence of endogenous pathogens in grafted plants (planting material) produced in nurseries is also highly frequent. This infection is due either to prior infection of the parent plants from which the rootstocks are obtained, or contamination occurring during the propagation process [1]. Accordingly, there has been a growing demand among winemakers for methods to control fungi causing GTDs that infect the plant through the root system, and for reducing the infection rate of planting material produced in nurseries. Unfortunately, there is not much information available about this subject. Some nursery trials have shown that application of different *Trichoderma* strains to soils or the grapevine root system had a growth-stimulating effect and reduced the infection rate of *Cylindrocarpon* sp., *Phaeoacremonium* sp., and *P. chlamydospora* [5, 6]. Another study involving the application of *Trichoderma harzianum* at different vine-growth stages in a nursery indicated that its application during the rooting stage showed some efficacy in protecting against *P. chlamydospora* infections. However, an increase in vine mortality was observed at the end of the growing season [7].

Recently, several actinobacterial strains isolated and characterized from the rhizosphere, or the inner tissues of the root system of young grapevine plants, exhibited antifungal activity against fungi that cause YGD [8]. Field trials carried out in a nursery led to the identification of one endophytic strain, *Streptomyces* sp. VV/E1, and two rhizosphere isolates, *Streptomyces* sp. VV/R1 and *Streptomyces* sp. VV/R4, which significantly reduced the infection rate produced by the fungal pathogens *Dactylonectria* sp., *Ilyonectria* sp., *P. chlamydospora* and *Phaeoacremonium minimum* involved in YGD [8]. In the current study, SCAR (sequence characterized amplified region) markers and qPCR protocols for the *Streptomyces* sp. VV/E1, and *Streptomyces* sp. VV/R4 strains were developed to specifically detect and quantify their presence in plant material. These molecular tools allowed us to analyse the effectiveness of different inoculation methods of young grapevine plants with these selected strains, as well as to determine the presence of these strains inside the root system of the inoculated plants.

Materials and methods

Microbial strains, culture media and growth conditions

Streptomyces strains VV/E1 and VV/R4 were isolated from the root system (endophytic) and the rhizosphere of young grapevines, respectively [8]. Both strains were grown in tryptic soy broth (TSB) medium (Sigma-Aldrich, St. Louis, Missouri, USA) at 220 rpm and 30°C for 3 days.

Isolation of genomic DNA

Genomic DNA of both strains was isolated from liquid cultures grown in TSB medium (Sigma-Aldrich) as described by Hopwood *et al.* [9]. DNA was checked by electrophoresis in a 0.8% (w/v) agarose gel. DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stored at -20°C.

Amplification of random polymorphic DNA sequences (RAPD)

Thirteen random primers, 10-mer each, were used for developing RAPD-PCR protocols in order to identify the differential polymorphism of 10 different actinobacteria (VV/E1-VV/E5 and VV/R1-VV/R5) [8] according to their electrophoretic band patterns. Primers used were: OPA2 (5′-TGCCGAGCTG-3′), OPA9 (5′-GGGTAACGCC-3′), OPA10 (5′-GTGATCGCAG-3′) [10], P1 (5′-GGTGC GGGA-3′), P2 (5′-GTTTCGCTCC), P3 (5′-GTAGACCCGT-3′), P4 (5′-AAGAGCCCGT-3′), P5 (5′-AACGCGCAAC-3′) [11], R1 (5′-CACGCCCTTC-3′), R3 (5′-ATGCAGCCAC-3′) [12], SS1 (5′-GTCAACGCGG-3′), SS2 (5′-AACGGCTCGC-3′) and SS3 (5′-GAGATCGCGC-3′). Amplification reactions were developed in 1X paq5000 buffer (Agilent Technologies, Santa Clara, California, USA) with 625 nM primer (IDT, Leuven, Belgium), 0.125 nM dNTP mix (EURx, Gdansk, Poland), 1 U Paq5000 DNA polymerase (Agilent Technologies) and 30 ng template DNA. Nuclease-free water was used to bring the total reaction volume to 20 µL. PCR amplification was carried out in 0.2 mL sterile tubes using a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). PCR conditions for primers R1, R3, OPA2, OPA9 and OPA10 were: 94°C for 3 min; 40 cycles of 94°C for 60 s, 40°C for 60 s, 72°C for 90 s; and a final extension of 72°C for 10 min. PCR conditions for the rest of the primers were: 94°C for 3 min; 45 cycles of 94°C for 30 s, 36°C for 30 s, 72°C for 60 s; and a final extension of 72°C for 10 min. Tubes were kept at 4°C until the reactions were analysed in 1.5% agarose gels in 1X TAE buffer containing 0.15 µg/mL ethidium bromide (Sigma-Aldrich) and photographed using VWR GenoSmart equipment (VWR, Barcelona, Spain).

Cloning and sequencing of DNA fragments

Two distinctive bands of 916 (VV/E1 strain) and 1862 bp (VV/R4 strain) were excised from the gel and the DNA was recovered and purified using freeze-squeeze [13]. Purified DNA was ligated into pBluescript (II) KS (+) (Agilent Technologies) using T4 DNA ligase (Fermentas, Glen Burnie, Maryland, USA) and transformed into *E. coli* DH5α competent cells (Thermo Fisher Scientific). Recombinant clones were selected on LB agar plates (Sigma-Aldrich) containing 50 µg/mL ampicillin, 50 µg/mL X-Gal, and 50 µg/mL IPTG. Blue-white screening was used and white colonies were checked for the presence of the correct insert using a double digestion with *EcoRI*-*SalI* restriction enzymes (EURx). The DNA was then sequenced using universal primers T7 (5′-TAATACGACTCACTATAGGG-3′) and M13 reverse (5′-CCTTTGTCGATACTGGTAC-3′) [14]. Sequence data were analysed using BLASTx (Basic Local Alignment Search Tool program) (<https://blast.ncbi.nlm.nih.gov/>).

Amplification of SCAR markers

The nucleotide sequence of each of the cloned RAPD fragments was used to design pairs of SCAR primers targeting poorly conserved regions or intergenic regions. The paired primers, SCAR.E1_fw (5′-GGTTCACGGTATCTGTTTACTCACC-3′) and SCAR.E1_rv (5′-GTTGGTTGAACCTTCTTCCG-3′) were used for amplification of a VV/E1 SCAR marker, while primers SCAR.R4_fw (5′-CCGAAGGGCTCTCTGAGTTGC-3′) and SCAR.R4_rv (5′-CTTCGCCGCTGAGGCACG-3′) were used for the VV/R4 strain. SCAR amplification from genomic DNA samples was performed in a reaction containing 1X paq5000 buffer (Agilent Technologies), 0.5 µM each of the primers (IDT), 0.1 mM dNTP mix (EURx), 1 U Paq5000 DNA polymerase (Agilent Technologies), and 30 ng genomic DNA. Nuclease-free water was used to bring the reaction volume to 25 µL. PCR conditions were as follows: 95°C for 2 min; 30 cycles of 95°C for 30 s, melting temperature (*T_m*) for 30 s, 72°C for 40 s; with a final extension of 72°C for 7 min. The *T_m* for SCAR.E1_fw/SCAR.E1_rv primers was 60°C, and 63°C for the

SCAR.R4_fw/SCAR.R4_rv primers. Tubes were kept at 4°C until analysis on 1.5% (w/v) agarose gels (as described above). In order to check specificity of the 2 sets of primers and the SCAR marker developed, genomic DNA from 50 actinobacterial strains isolated from rhizospheres and the root systems of young grapevine plants [8] were used as templates in a conventional PCR under the same conditions.

Quantitative SYBR Green real-time PCR (qPCR) assays

The qPCR technique [15] was developed based on the above mentioned SCAR primers. The reaction mixture contained 1X SYBR Green master mix (Takara, Shiga, Japan), 0.2 µM of each primer and 5 µL bacterial DNA isolated from axenic cultures of VV/E1 and VV/R4 strains as templates. Nuclease-free water was used to bring the reaction volume to 20 µL. VV/E1 bacterial DNA, ranging in amounts from 5.65×10^6 fg to 5.65×10^2 fg per reaction, and 5.2×10^6 fg to 5.2×10^2 fg per reaction for VV/R4 strain, were used. Amplification was performed in qPCR 96-well plates (Agilent Technologies), and run in the Mx3005P qPCR System (Agilent Technologies). Thermal conditions were: 95°C for 2 min, 40 cycles of 95°C for 20 s, elongation at T_m for 20 s, followed by 1 cycle of 95°C for 1 min, 55°C for 30 s and 95°C for 30 s. T_m was the same as for the conventional PCR. All analyses were performed as three independent experiments with three replicates for each dilution.

Sensitivity of actinobacterial detection in grapevine wood

It was determined by calculating qPCR standard curves using 10-fold serial dilutions of genomic DNA (5.65×10^6 fg to 5.65×10^2 fg DNA were used for *Streptomyces* sp. VV/E1, whereas 5.2×10^6 fg to 5.2×10^2 fg were used for *Streptomyces* sp. VV/R4). Nuclease-free water was used as non-template control (NTC). The limit of quantification (LOQ) was estimated according to the coefficient of variation [$CV = 100 \times (SD/mean\ value)$] of back-calculated concentrations (5 replicates) of genomic DNA from both *Streptomyces* strains: a CV below 35% was assumed to be the LOQ (S2 Fig) [16]. To ascertain whether the primer sets designed for the amplification of SCAR markers were reliable for the *Streptomyces* sp. PCR detection in grapevine wood, without the interference of any grapevine genomic DNA, quantitative real-time assays were carried out in the presence of grapevine genomic DNA. Each PCR mixture contained 5.2 ng of *Streptomyces* sp. VV/E1 genomic DNA, or 5.7 ng of *Streptomyces* sp. VV/R4 genomic DNA, serially diluted tenfold up to 10^{-5} . The same reactions were supplemented with 5.2 and 5.7 ng grapevine genomic DNA. The final ratio of actinobacteria-to-grapevine genomic DNA ranged from 1:1 to 1:100,000. The experiments were conducted twice with three replicates each.

Calculation of the correlation between quantity of genomic DNA from *Streptomyces* sp. VV/E1 and VV/R4 strains and number of cells

Both SCAR markers were amplified by conventional PCR, purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and quantified on NanoDrop. Since the size and nucleotide composition of each amplicon are known, the number of copies of each DNA template (SCAR copy numbers) were easily estimated using an online calculator (<http://cels.uri.edu/gsc/cndna.html>). Thus, PCR products were serially diluted 10-fold and used as templates to generate qPCR standard curves, one for each SCAR marker (S3A Fig). Genomic DNA quantified on NanoDrop were also serially diluted 10-fold and the copy number of each SCAR was estimated using the previous standard curves (S3B Fig). Thus, the number of cells (cells/mg of grapevine wood) could be directly estimated through the additional correlation between

genomic DNA quantity and the corresponding number of copies of each amplicon (S3C Fig). qPCR conditions were the same as described above.

Inoculation of grapevine plants with *Streptomyces* strains

Sixty 1-year-old *Vitis vinifera* (cv. Tempranillo) plants grafted on Richter 110 (110R) rootstock were supplied by Viveros Villanueva Vides S.L. (Larraga, Spain). Their root systems were uniformly trimmed to a length of 10 cm, and immediately inoculated with *Streptomyces* sp. VV/E1 or *Streptomyces* sp. VV/R4 strains [8], and planted in plastic pots containing 50 L of Compo Sana 20-20-30 commercial substrate mixture, which is suitable for grapevines (Compo Sana, Barcelona, Spain). Potted vines were grown outdoors under shade-cloth and regularly irrigated with tap water. Experimental potted vineyards were developed in the facilities of the Instituto de Investigación de la Viña y el Vino (Universidad de León, Spain; 42°35'01.0"N 5°35'20.2"W).

For each *Streptomyces* sp. strain, 6 different batches were processed as follows: batch 1 consisted of non-inoculated plants (control); batch 2, plants inoculated by immersion of the root system for 24 hours in a bacterial suspension (5×10^5 cells/mL); batch 3 was identical to batch 2, but an additional booster treatment was applied 30 days after planting. The booster treatment was added to the soil into a 15 cm hole made using a hand drill in parallel to the rootstock. In this way the bacteria (10^7 cells; 20 mL of 5×10^5 cells/mL) were applied directly to each pot in the proximity of the root system. Batch 4 plants were inoculated by direct injection of bacteria into the rootstock, (50 μ L of a bacterial culture containing 5×10^5 cells) 3 cm above the root insertion point (RI) via a pipette placed in a drilled hole (5 mm deep) (Fig 1A). Batch 5 was like batch 4, but with an additional booster treatment applied (similar to treatment 3). Batch 6 plants were an additional control. They were not inoculated by either method, although the same booster treatment was applied 30 days after planting in order to observe the effect of direct actinobacteria addition to the soil. Plants were uprooted and analysed 180 days after potting.

Analysis of the experimental grapevines planted in pots: Detection and quantification of *Streptomyces* sp. VV/E1 and VV/R4 strains in wood samples

Once uprooted, samples from all the plants were taken from the root system (RS), and the root insertion point (RI) (Fig 1B). Plants from treatment groups 3 and 4 (inoculated by injection)

Fig 1. Scheme of inoculation of grapevine plants and areas analysed. Image of (A) the inoculation point in the rootstock of plants treated by injection, as well as the four 1.0 cm cylinders (Z1/RI, Z2, Z3 and Z4) analysed once the plants were uprooted, and (B) the zones defined as root insertion point (RI) and root system (RS) also analysed in plants inoculated by immersion in a cell suspension. Note that Z1/RI and RI zones in plants inoculated by injection or immersion are equivalent for analytical purposes.

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were subjected to a more detailed analysis by zones (Fig 1A). Zone Z1 was located 2–3 cm below the inoculation point; zone Z2 was 0–1 cm below the inoculation point, while zones Z3 and Z4 were 0–1 cm and 2–3 cm above the inoculation site, respectively. 1 cm wood fragments were surface-sterilized [17], cut into small pieces using a sterile scalpel, frozen in liquid N₂ and manually-ground up with a mortar into a very fine powder. DNA was extracted from 50 mg of dry weight material using a Nucleo Spin Plant II Extraction kit (Macherey-Nagel). DNA concentration was estimated using a NanoDrop and stored at -20°C. *Streptomyces* sp. VV/E1 and VV/R4 strains were detected and quantified by qPCR amplification of the developed SCAR markers, as indicated above.

Data analysis. Statistical analyses were performed with R software (ver. 3.3.1; <https://www.r-project.org/>). Analysis of variance was explored with linear models and level of significance between treatment groups was determined by an LSD-test (least significant difference) from the Agricolae R package (ver. 1.2–8, 2017) [18].

Results

Identification and characterization of differential RAPD amplification fragments of *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4

Different electrophoretic band patterns of 5 *Streptomyces* sp. rhizosphere strains (VV/R1 to VV/R5), and 5 actinobacterial endophytic strains (VV/E1 to VV/E5) [8] were obtained by amplification of RAPD sequences using up to 13 different primers (S1 Fig). A 916 bp band was uniquely amplified from *Streptomyces* sp. VV/E1 strain using the P4 primer. Similarly, a 1862 bp band was amplified from only *Streptomyces* sp. VV/R4 using the OPA2 primer (Fig 2). Both DNA bands were purified from agarose gels, cloned, sequenced and compared with those already present in GenBank database.

The *Streptomyces* sp. VV/E1 916 bp band (GenBank accession number MH048872) contained two partial open reading frames (ORF) (Fig 3A). The first one, extending from positions 1 to 401, exhibited a 61% amino acid identity with a TetR/AcrR family transcriptional regulator from *Streptomyces ipomoeae* 91–03 (accession number EKK60661.1), and lower amino acid identities with several other proteins from different actinobacterial strains also identified as transcriptional regulators belonging to that family. The second ORF, almost complete, stretched from positions 597 to 916 and might encode a protein that showed a 94% amino acid identity with a hypothetical protein from *S. ipomoeae* (accession number WP_048822038.1), and lower amino acid identities with many other hypothetical proteins from several *Streptomyces* species.

The *Streptomyces* sp. VV/R4 1862 bp band (GenBank accession number MH048873) contained 3 putative ORFs (Fig 3B). The first incomplete ORF, stretching from positions 1 to 509, encoded a protein with a very high amino acid identity (92%) to a hypothetical protein from *Streptomyces acidiscabies* (accession number WP_075662455.1). The second ORF was complete, and extended from positions 860 to 1306, encoding a protein with high similarity (87% amino acid identity) to a very short patch repair endonuclease from a *Streptomyces* sp. strain (accession number WP_004939603.1). The third ORF was also truncated, stretching from positions 1331 to 1862. It encoded a protein that exhibited 69% amino acid identity to a DNA cytosine methyltransferase from *Streptomyces* sp. (accession number WP_053914715.1).

Conversion of a RAPD marker into a SCAR marker

The nucleotide sequences of each of the cloned RAPD fragments were used to design pairs of SCAR primers targeting poorly conserved intergenic regions. After several attempts, a pair of

Fig 2. RAPD profiles. RAPD profiles generated by (A) P4 primer and (B) OPA2 primer for rhizosphere (VV/R1 to VV/R5) and endophytic (VV/E1 to VV/E5) *Streptomyces* sp. strains isolated from the root system of young grapevine plants. MW: GeneRuler 1kb DNA Ladder Plus (Thermo Fisher Scientific). Specific bands amplified from isolates VV/E1 and VV/R4 that were selected for SCAR markers design are indicated by arrows. NC (negative control).

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primers, SCAR.E1_fw (positions 415 to 439; Fig 3A) and SCAR.E1_rv (positions 576 to 596; Fig 3A), were selected for *Streptomyces* sp. VV/E1 strain. Both primers were in the intergenic region located between the two ORFs detected. The SCAR.E1_fw and SCAR.E1_rv primers were designed with a 48.0% and 52.4% GC content, respectively, and they amplified a unique

Fig 3. Representative maps of the characterized RAPD fragments. (A) *Streptomyces* sp. VV/E1 and (B) *Streptomyces* sp. VV/R4. Incomplete ORFs are indicated by grey arrows, whereas black arrows correspond to complete ORFs. The relative positions of the amplified SCAR markers developed are indicated by a black line. GeneBank accession numbers: MH048872 (*Streptomyces* sp. VV/E1) and MH048873 (*Streptomyces* sp. VV/R4).

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182 bp PCR fragment from genomic DNA of the VV/E1 strain when annealing temperatures ranged from 60 to 65°C, indicating a high specificity of the amplification reaction.

Similarly, SCAR.R4_fw (positions 466 to 486; Fig 3B), and SCAR.R4_rv (positions 608 to 625; Fig 3B), were designed to amplify a specific 160 bp PCR fragment from VV/R4 strain genomic DNA when annealing temperatures ranged from 57 to 63°C. The SCAR.R4_fw primer had a 61.9% GC content and annealed near the 3'-end of ORF1. The SCAR.R4_rv primer had a 72.2% GC content and was located in the ORF1-ORF2 intergenic region (Fig 3B).

To test the specificity of the designed SCAR primer pairs, they were used to check if they amplified the genomic DNA of a total of 29 rhizosphere *Streptomyces* sp. strains (VV/R1 to VV/R29), and 21 endophytic actinobacterial strains, including 15 *Streptomyces* sp. isolates, 2 *Saccharopolyspora* sp. strains, and 3 *Micromonospora* sp. isolates [8] (S4 Fig) by conventional PCR. Only PCR products with the expected sizes were amplified from strains VV/E1 (182 bp) and VV/R4 (160 bp) (S4 Fig), with no other amplification in the other strains tested.

Development of qPCR methods for the detection of *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 in vegetal samples

Once specific SCAR markers had been raised for both strains, rapid, sensitive, and specific methods for their detection were developed using quantitative real-time qPCR in plant material. The same primers used earlier were tested in a SYBR green qPCR system. The sensitivity of this system was evaluated for *Streptomyces* sp. VV/E1 and VV/R4 strains using purified DNA isolated from liquid culture mycelia.

Assays for *Streptomyces* sp. VV/E1 were performed using 10-fold serial dilutions of genomic DNA as templates. For the DNA amplification curve, the cycle threshold (C_t) values were converted into amount of DNA using the linear regression equation $y = -3.567x + 39.116$, where x is the logarithm of the DNA quantity (fg) and y is the C_t value. There was a high regression coefficient close to 1 ($R^2 = 0.998$) and the amplification efficiency (E) was 90.7% (Fig 4). Similarly, assays for *Streptomyces* sp. VV/R4 strain were also carried out with 10-fold serial dilutions of genomic DNA as templates. The results allowed us to define the equation $y = -3.356x + 39.033$ ($R^2 = 0.996$). The amplification efficiency (E) was 98.6% (Fig 4).

The LOQ was estimated to be 50.47 fg for VV/E1 and 72.65 fg for VV/R4 strains (S2 Fig).

To check that host (*Vitis vinifera*) DNA did not interfere with quantification of *Streptomyces* sp. strain DNA over a wide range, additional standard curves for both strains were

Fig 4. qPCR standard curves. Representative amplification curves obtained by plotting the mean *Ct* values with respect to the logarithm of genomic DNA quantity from *Streptomyces* sp. VV/E1 (black line) and *Streptomyces* sp. VV/R4 (grey line). Standard curves were generated with 10-fold serial dilutions of genomic DNA concentrations from 5.65×10^6 fg to 56.5 fg (VV/E1 strain) or 5.2×10^6 fg to 52.0 fg (VV/R4 strain) by SYBR green qPCR reactions.

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generated under the same conditions, but including grapevine genomic DNA extracted from wood (range from 1:1 to 1:100,000) of a non-inoculated grapevine plant. The linear regression equations obtained (S5 Fig) were very similar for each strain under both conditions. Furthermore, the corresponding slopes (S5 Fig) did not show significant differences, according to a Student's t-test statistical analysis ($p \leq 0.05$), which means that the presence of genomic DNA from grapevine wood did not have a significant impact on the results.

Detection and quantification of *Streptomyces* sp. VV/E1 and VV/R4 strains in wood samples of inoculated grapevine plants

The plant inoculation efficacy of the different methods assayed was analysed 180 days after application, once the plants were uprooted. *Streptomyces* sp. VV/E1 and VV/R4 strains were quantified in plant material isolated from different zones (see Fig 1) according the inoculation method used in the batches analysed.

(i) Definition of the linear analysis models. Linear models were defined with the DNA quantification raw data (fg of DNA from both *Streptomyces* sp. strains detected in analysed samples), for every treatment (batch), and area analysed in order to explain the variability obtained (dependent variable). Three independent variables (treated as factors) were considered, as described in the materials and methods section. One variable is related to the inoculation methodology: we had control treatments (uninoculated plants, batches 1 and 6), inoculation by immersion (batches 2 and 3), and inoculation by injection (batches 4 and 5)

treatments. The second factor was related to application of a booster treatment in batches 2, 3 and 6. The third factor was the plant area analysed. In the case of the control and immersion treatments, the RI and RS samples were analysed (Fig 1B). In grapevine plants inoculated by injection, four different zones were analysed (Z1/RI, Z2, Z3 and Z4; Fig 1A); Z1 was equivalent to the RI area in the control and immersion-treated grapevine plants.

(ii). Analysis of the inoculation efficiency by immersion. Once the linear model had been defined by considering the three independent variables treated as qualitative factors, the data analysis clearly indicated that the booster dose applied in batches 3, 5, and 6 was not efficient at promoting bacterial colonization of the root system (p values of 0.9190 and 0.5520 for VV/E1 and VV/R4, respectively). Accordingly, the second factor (application of a booster treatment on soil) was not taken into account in the final model. Therefore, batches with the same inoculation method, with and without booster treatment, were combined. Thus, further analysis was simplified by reducing treatments to just two: control samples (batches 1 and 6), and inoculation by immersion (batches 2 and 3), resulting in a larger sample size for each treatment and providing an increase in the robustness of the statistical analysis. Genomic DNA quantities for both VV/E1 and VV/R4 strains were measured at RI (Fig 5A) and RS (Fig 5B) in the samples. Data analysis showed the existence of significant differences ($p < 0.001$) in $\log_{10}$ DNA values for both RI and RS areas with respect to controls (batches 1 and 6). Mean values detected in RI were higher for the VV/R4 strain (631.45 fg DNA), compared to the VV/E1 strain (310.87 fg DNA). A similar trend was observed in the RS area: 734.62 fg of DNA detected for the VV/R4 strain and 331.87 fg of DNA for the VV/E1 strain. To check the correlation between the amount of DNA detected, and the corresponding number of cells, S1 Table should be consulted.

(iii). Analysis of the injection inoculation efficiency. Four different zones (Z1/RI to Z4) were analysed along the grapevine stem (Fig 1A). As in the previous treatment, a simplified model (according to p values of 0.6122 and 0.2106 for VV/E1 and VV/R4, respectively), in which the booster treatment was not taken into account, was used. Data analysis was carried out firstly by comparing injected and control plants at the RI point (or Z1/RI area in Fig 1A) (Fig 6). The $\log_{10}$ DNA amounts detected showed significant differences (p values of 7.096×10^{-6} and 2.996×10^{-2} for VV/E1 and VV/R4, respectively in the Z1/RI area) compared to the negative control (untreated plants). In this case, the mean DNA value, 321.3 fg, detected was clearly higher for the VV/E1 strain, as compared to 83.5 fg for the VV/R4 strain.

Secondly, we analysed the presence of DNA from *Streptomyces* sp. strains in the Z1/RI to Z4 zones (Fig 1A) around the injection point (Fig 7). For the VV/E1 strain, significant differences were observed with respect to the distance from the inoculation point according to the LSD-test ($p = 2.575 \times 10^{-12}$). The amount of DNA detected in the distal areas Z1/RI (0.32 pg) and Z4 (0.33 pg) was low, whereas in the closer areas, Z2 (105.7 pg) and Z3, (62.3 pg) the amount of DNA was significantly higher when compared to Z1/RI and Z4 samples. As for the VV/R4 strain, there were significant differences ($p = 1.821 \times 10^{-3}$) between the Z1/RI (0.08 pg) and Z2-Z3-Z4 (values of 4.67, 3.34, and 0.64 pg, respectively) areas.

(iv) Comparison of the colonization rate of RI in plants inoculated by immersion or injection. As we previously indicated, Z1/RI and RI were the only common areas to be analysed in plants inoculated by either immersion or injection (Fig 1). Consequently, it was of great interest to analyse the rate of bacterial colonization of this area in plants inoculated by both treatments. As in prior analysis, the full linear model was discarded, since no influence of the booster treatment in soil (p values of 0.601 and 0.205 for VV/E1 and VV/R4, respectively) was observed. The differences in $\log_{10}$ DNA amounts detected were significant ($p = 7.598 \times 10^{-8}$ for VV/E1, and $p = 2.691 \times 10^{-4}$ for VV/R4) with respect to control plants (Fig 8). However, no significant differences between the two inoculation procedures could be detected. Indeed, VV/E1 strain mean values for both treatments were quite similar (310.87 and 331.33

Fig 5. Quantification in RI and RS DNA samples from the *Streptomyces* sp. strains inoculated by immersion. Box plot showing the DNA levels from *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 strains in plants subjected to inoculation by immersion as compared to control plants in the (A) RI, and (B) RS areas. Black rhombuses show the average values (back transformed) for each treatment. Asterisks indicate existence of significant differences according the LSD-test performed: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Circles correspond to data outliers (falling outside the Q1-Q3 range).

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fg DNA for immersion and injection, respectively). A remarkable difference was noticed for the VV/R4 strain, since higher DNA amounts were detected in plants inoculated using the immersion procedure (mean value of 631.45 fg of DNA) than in injected plants (83.48 fg of DNA) (Fig 8).

Fig 6. Quantification at RI of DNA from *Streptomyces* sp. strains inoculated by injection. Box plot showing the levels of DNA from *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 strains detected at RI in plants inoculated by injection, compared to the amount of DNA detected in control (untreated) plants. Black rhombuses correspond to average values (back transformed). Asterisks indicate significant differences according the LSD test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Circles correspond to outliers of the data (data falling outside the Q1-Q3 range).

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Fig 7. Quantification of *Streptomyces* sp. strain DNA at different zones of the rootstock inoculated by injection. Box plot corresponding to the DNA amounts of *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 strains detected in the different zones (Z1/R1 to Z4) analysed in the rootstock of plants inoculated by injection. Note that in this figure, DNA amounts are shown in pg. Black rhombuses correspond to average values (back transformed). Asterisks indicate significant differences according to the LSD test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Circles correspond to data outliers (outside the Q1-Q3 range).

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Discussion

Bacteria belonging to the *Streptomyces* genus are well known as important secondary metabolite producers, excreting both antibacterial and antifungal compounds, and for their ability to

Fig 8. Comparison of the effectiveness of inoculation by immersion vs. injection at RI. Box plot showing DNA amounts at RI of *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/R4 strains in plants inoculated by immersion or injection as compared to control (untreated) plants. Black rhombuses correspond to average values (back transformed) for each treatment. Asterisks indicate significant differences according to the LSD test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Circles correspond to data outliers (outside the Q1-Q3 range).

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control plant diseases [19–23]. The recent finding that the grapevine plant root system is home to numerous endophytic and rhizosphere *Streptomyces* species, and that some of these strains are effective at reducing the infection rate by fungi causing young grapevine decline (YGD) [8] in grafted grapevine plants, has suggested a putative use for these strains as biocontrol agents in vine nurseries [8].

However, this application was hampered by several factors including the absence of methods to specifically detect and quantify these strains in plant material, and effective, easy methods for introducing them into the grapevine plant root system to allow their colonization.

One of the objectives of the current study was to develop a simple molecular marker to detect *Streptomyces* sp. VV/E1 and VV/R4 strains in plant material. RAPDs are easy to develop as molecular markers, but lack of reproducibility blocks their reliable use. To improve their reproducibility, we converted RAPD amplicons into SCAR markers [24, 25] for VV/E1 and VV/R4 strains, raised against intergenic regions located inside the previously sequenced RAPDs. Since intergenic regions are poorly conserved between different species, or even strains, a SCAR marker developed from an intergenic region would likely be highly specific for a particular strain or species. In fact, the specificity of both SCAR markers was very high, since they did not allow amplification of, or cross-react with, genomic DNA from 50 other species of actinobacteria (mainly belonging to *Streptomyces* genus) that had been isolated as endophytic or rhizosphere strains from the root system of young grapevine plants [8]. However, we cannot totally rule out the existence of any taxon that could allow DNA amplification, for one, or both of the developed markers, although the robustness of the qPCR technology would allow us to discriminate that it was a false positive. On the one hand, there would be differences in the dissociation curve obtained in the qPCR assay, and on the other, there could even be differences in the size of the amplified fragment.

Although the use of SCAR markers allowed the detection of both strains from a low amount of only 30 ng genomic DNA, they did not allow an accurate quantification of the DNA detected. Accordingly, another goal of the current study was the development of a specific, sensitive qPCR assay for the detection and quantification of both strains in plant material. Given their high specificity, the same pair of primers used to amplify SCAR markers was used as specific primers for development of a qPCR protocol. The *Ct* values were linearly correlated with the concentration of target DNA for both strains, signifying that this methodology was suitable for qualitative and quantitative assays. The LOQ for both strains were quite low, indicating a good sensitivity of the methods. Unfortunately, there are hardly any studies on the quantification of *Streptomyces* strains by qPCR that allow comparison of the LOQ values obtained [26]. Since one of the objectives of this work was to quantify the presence of both strains in grapevine plants, we had to rule out the possible interference of grapevine genomic DNA in the qPCR assays. We tested broad concentration ratios (1:1 to 1:100,000) of DNA from both *Streptomyces* sp. strains against grapevine genomic DNA in qPCR experiments. The corresponding curve slopes did not show significant differences, which meant that the presence of grapevine genomic DNA did not interfere with the detection and quantification of the bacterial strains.

With the development of fast, reliable, accurate and specific qPCR methods for the detection of both strains in plant material, it was possible to analyse the colonization efficiency in the root system of young grapevine plants using two different inoculation methods: immersion and injection. Data analysis led us to several conclusions. First, that both strains could be detected inside the root system of non-inoculated (control) plants, although at a very low rate if we compared with the DNA amounts detected in inoculated plants. This result seems obvious for the VV/E1 strain since it was isolated as an endophytic strain. However, the VV/R4 strain was isolated as a rhizosphere strain. This data suggests that the line that separates the

definition of a particular strain as endophytic or rhizosphere is very diffuse. Rhizosphere-established streptomycetes are well known to have different effects on plants [20–23]. Unfortunately, general knowledge about the impact of endophytic *Streptomyces* sp. strains on plant development is poorly understood, although it is clear that they can enhance the growth of some plants and help in the biocontrol of phytopathogens [22].

Since both strains were detected 180 days after their application and once the potted plants had completed a full growth cycle, it is clear that immersion of the root system in a bacterial suspension is a method that allows effective colonization. Both strains were detected at the RS as well as at the RI points. This indicates that the bacteria probably enter the root system through injuries produced when the roots were trimmed, before the bacterial application, although we cannot discard that the bacteria might enter through other microscopic injuries in the plants. RI detection also suggested an upward movement of the bacteria. Perhaps the bacteria move upward through the xylem vessels. Further experiments, including microscopy studies, could shed some light. The possibility that the bacteria might reach upper sections in the plant was not investigated. Current experiments should clarify this point. The behaviour of both strains was similar, although the VV/R4 strain was detected at higher levels than the VV/E1 strain at both RS and RI.

Injection of a bacterial suspension directly into the rootstock at a point 3 cm above the RI area was also effective at inducing colonization of the root system. The amount of genomic DNA detected at RI was clearly higher than that obtained for control (non-injected) plants. Nevertheless, the VV/E1 strain levels were higher than those detected for the VV/R4 strain (just the opposite result observed in the case of application by immersion). Taken together, immersion and injection data suggest that we cannot conclude that either of the strains is more adapted to an endophytic lifestyle. Analyses of the different Z1/RI, Z2, Z3, and Z4 samples around the injection point suggest that, from this point, the bacteria can move at least a short distance (2–3 cm) up and down the plants. Whether the bacteria could have travelled longer distances was not evaluated. The comparison of bacterial levels at the RI (Fig 8) suggest that for the VV/E1 strain, both methods exhibited a similar efficiency at colonizing the root system. However, immersion of the VV/R4 strain yielded a higher colonization rate than injection. We also analysed if the addition of a booster treatment, with direct addition of a bacterial suspension in the proximity of the root system, could reinforce the colonization rate of both strains previously inoculated by immersion or injection. According to our data, there was no improvement of colonization levels by either strain.

Since high densities of antagonistic *Streptomyces* are associated with plant disease suppression in some soils, some efforts have been made to apply the bacteria onto seeds (mainly by soaking the seeds in a suspension containing the biocontrol agent), or into soil at the highest possible density using different methods like mixing it with soil or into sowing furrows, or simply spreading it on the field using dripping systems [19, 22]. However, despite some success with this approach [27–30], the application of *Streptomyces* to soils as a biocontrol agent remains unreliable [31]. This failure to colonize the plant from the ground could be due to multiple factors among which is the competition with other microbial species existing in the root environment. Since the plants in this study were grown in a vegetal substrate, not in classical soil, it is possible that this is not the most appropriate substrate to allow for proper survival of the *Streptomyces* species. Therefore, we cannot rule out that the results obtained would have been different had we used a more traditional soil in the pots. However, our data indicate that *Streptomyces* sp. strains selected as biocontrol agents can be easily introduced into the root system of woody plants by simply soaking their trimmed root system in a bacterial suspension, or alternatively by direct injection into the vegetal tissues, thus providing a new methodology of inoculation. Taking into account these data, the potential

for using endophytic *Streptomyces* to control plant disease is becoming a focus of research. Nonetheless, these studies are hampered by a poor understanding of the relationships between rhizosphere and endophytic populations, and how a *Streptomyces* strain becomes adapted to an endophytic way of life. It may be that the protected endophytic habitat minimizes the challenge of achieving successful colonization, which has proven to be difficult in competitive soil communities [31, 32].

Finally, we should emphasize that the current work shows that *Streptomyces* sp. strains isolated from grapevine root environment as putative biocontrol agents to fight fungal pathogens causing YGD, can be easily introduced into young grapevine plants by both immersion or injection, where they can establish and colonize the interior of the root system. Obviously, due to its ease of application, immersion should be the preferred option. This inoculation method could easily be applied in both nurseries, by the introduction of an additional inoculation step in the production of grafted plants, before young grapevine plants go on the market, as well as in cellars and vineyards, simply by placing the plants into a bacterial suspension prior to planting. Therefore, these biocontrol agents are a promising tool for diminishing the incidence of YGD in new plantations all over the world. These inoculation methods could also be potentially used for applying useful bacterial biocontrol strains to other vascular plants of great agro-nomical and economical interest like fruit trees (citrus, almonds or olive trees among others) affected by trunk diseases.

Supporting information

S1 Fig. RAPD amplification profiles. RAPD profiles generated by the amplification of rhizosphere (VV/R1 to VV/R5) and endophytic (VV/E1 to VV/E5) actinobacteria strains isolated from the root system of young grapevine plants using different primers: (A) OPA2, (B) OPA9, (C) OPA10, (D) P1, (E) P2, (F) P3, (G) P4, (H) P5, (I) R1, (J) R3, (K) SS1, (L) SS2 and (M) SS3. Specific bands selected for SCAR markers design are indicated by arrows. MW: Generuler 1 kb DNA Ladder Plus (Thermo Fisher Scientific). NC is the negative control.
(PDF)

S2 Fig. Limit of quantification (LOQ) from *Streptomyces* sp. VV/E1 and VV/R4 strains. Coefficient of variation [$CV = 100 \times (SD/mean \text{ value})$] of the back-calculated amounts of genomic DNA from (A) *Streptomyces* sp. VV/E1 and (B) *Streptomyces* sp. VV/R4 by qPCR assays (5 replicates). Horizontal dashed lines correspond to $CV = 35\%$ and vertical dashed lines indicate the lowest quantity of DNA with a CV below 35% (obtained by interpolation). Grey symbols indicate the presence of negative (“non-detected”) replicates among samples.
(PDF)

S3 Fig. Correlation between quantity of genomic DNA and number of cells. Standard curves obtained by plotting (A) the mean C_t values with respect to the logarithm of DNA copy number from purified and quantified PCR products. Standard curves obtained by plotting (B) the mean C_t values with respect to the logarithm of genomic DNA quantity (10-fold serial dilutions). (C) Correlation (logarithm scales) between genomic DNA quantity and the corresponding number of copies of each amplicon. *Streptomyces* sp. VV/E1 (black line) and *Streptomyces* sp. VV/R4 (grey line).
(PDF)

S4 Fig. Specificity of SCAR primers. Absence of amplification from the genomic DNA of 50 actinobacterial endophytic and rhizosphere strains isolated from grapevine plants of the selected SCAR markers using SCAR primers (A) SCAR.E1_fw/SCAR.E1_rv and (B) SCAR.R4_fw/SCAR.R4_rv. Specific amplification bands were only detected for strains VV/E1 and

VV/R4. MW: GeneRuler 1kb DNA Ladder Plus (Thermo Fisher Scientific). NC (negative control).

(PDF)

S5 Fig. qPCR standard curves supplemented with grapevine genomic DNA. Standard curves obtained by SYBR Green qPCR using the primer sets that amplified the SCAR markers and generated with 10-fold serial dilutions of genomic DNA from (A) VV/E1 and (B) VV/R4 strains are shown in black lines. Grey lines correspond to the curves generated in the presence of grapevine genomic DNA. The final ratio of actinobacteria-to-grapevine DNA ranged from 1:1 to 1:100,000.

(PDF)

S1 Table. Correlation between mean values of DNA amounts quantified by qPCR from *Streptomyces* sp. VV/E1 and VV/R4 strains and cell numbers.

(DOCX)

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