INFERENCES FROM INVENTORIES OF MICROBES IN ECOLOGICAL VINEYARD SETTINGS

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Abstract

Malfunctioning soils are specifically problematic in organically cultivated cropping systems, where plants are relying on well functioning soil processes. The here presented experiments aim at describing the effect of degraded soil conditions and compost, green manure or dry mulching based organic amendments on microbial communities associated with the rhizosphere and roots of grapevines. They were performed within the frame of project CORE Organic Plus ReSolVe. Several relationships based on Dice cluster analyses of DGGE gel profiles suggest that fungal and bacterial communities from degraded and non-degraded areas differ. Results also suggest that composted organic amendments and green manure can support the development or enhancement of root associated communities that are different from those in degraded areas. However, diversity indexes did not show that bacterial or fungal communities differed in species number or evenness when degraded and non-degraded plots were compared. With culture dependent methods, a high diversity of *Streptomyces* spp., best known for their potential to produce antibiotics and increasingly depicted as beneficial plant associated bacteria, were isolated from degraded and non-degraded areas.

Keywords: *Microbial community structure, DGGE, Fungi, Bacteria, Actinobacteria, Streptomyces*

Introduction

Soil environments accommodate diverse ecological niches for microorganisms and are considered hotspots for microbial diversity (Roesch et al., 2007). Bacteria and fungi provide many essential services in soils as decomposers of dead biomass, drivers of nutrient cycles, nutrient solubilizers, plant growth promoting rhizosphere colonizers, and root associated endo- and ectosymbionts (Aislabie and Deslippe, 2013). Moreover, microorganisms accommodate an essential position in food webs because numerous components of the mesofauna feed either directly or indirectly on bacterial and/or fungal biomass (Grandy et al., 2016). Diverse abiotic properties effect or determine soil functionality, plant and mesofauna community structures, and organic matter contents. All these biotic and abiotic factors affect microbial community compositions either directly or indirectly. Accordingly it is to be expected that factors modifying soil characteristics and functionality, including

DOI: 10.6092/issn.2281-4485/7921

agricultural or horticultural management systems influence soil microorganisms, soil microbial processes and microbial community compositions (Tian et al., 2017). ReSolVe, a CORE Organic Plus Project, focused on areas in vineyards showing soil malfunctioning caused by excessive soil erosion or improper earthworks before plantation and targeted thus an extreme form of unsuitable horticultural land management. Degraded areas were characterized by badly performing grapevines and showed little development of natural vegetation cover. In general, causes of soil malfunctioning were related to a reduced soil water availability and capacity, lowered organic carbon and total nitrogen content, reduced cation exchange capacity, and higher concentrations of carbonates (Costantini et al., 2018). Research activities aimed at comparing degraded (DEG) and non-degraded (ND) areas within the same vineyards and re-storing soil functionality in degraded areas by increasing soil organic matter. This paper describes grapevine root-and rhizosphere associated microbial communities from degraded and non-degraded areas of vineyards and tests whether soil restoration strategies have an effect on these communities.

Materials and methods

Study area

Experimental vineyards were the (i) Fontodi farm in Chianti (Firenze, Italy) (It-FON) cultivating Sangiovese, (ii) Château Maison Blanche farm in Montagne Saint-Émillion (France) (Fr-MB) cultivating Cabernet Franc, and (iii) Brajniki farm with vineyards in Bonini and Prade (Slovenia) (Si-VL, Si-VS) cultivating Refošk (Table 1 in D'Avino et al., this issue). Degraded and non-degraded areas within vineyards were determined according to personal communications with farmers and soil profile analyses in 2015. Degraded areas were subdivided into 4 plots in spring 2015, of which one was used as control plot (CONTR) and the other three were managed with restoration practices that focused on increasing soil organic matter by means of (i) seeded cover crops used as dry mulch (DM), (ii) seeded cover crops incorporated into top soil layer as green manure (GM), or (iii) organically produced composted organic amendments incorporated into the top soil layer (COMP) (D'Avino et al., this issue). Different combinations of winter legume and cereal species were used for DM and GM in each country; composted organic amendments were either based on animal manure only or a combination of pruning residues and animal manure (Table 2 in D'Avino et al., this issue). The experimental design included a further control plot (ND) that showed non-degraded soil characteristics. Cover crops were seeded and compost was disseminated in November 2015, while cover crops were mowed (for GM, DM) and incorporated into the top soil layer in May/June 2016 (GM). For further information regarding the set-up of plots, see D'Avino et al. (this issue).

Root and rhizosphere soil sampling and processing

Soil profile exposed roots were collected in April 2015 from vineyards in Italy, France, and Slovenia from a depth typically of 10-30 cm. In May 2017, roots from

10-30 cm depth were retrieved from shovel-dug holes. In both years, roots from a distance up to 50 cm from grapevine trunks were considered. Terminal roots of ca. 1 mm or less were cut in up to 2 cm long pieces and collected in 50 ml Falcon tubes. Rhizosphere soil was collected by washing roots with sterile distilled water (SDW) through centrifugation at 2200 g for 5 min (Biofuge Stratos, Heraeus instruments). Remaining roots were processed by following the principles described by Collado et al. (2007). Included sample pulverization was achieved with an Ultra-Turrax T25 (IKA Labs) after root pieces were washed 5 times with SDW, once in 75% ethanol for 1 min, once in a 1% NaClO solution (kemika 14552, Zagreb) for 3 min, once in 75% ethanol for 30 sec., and 3 times again with SDW. Pieces of 100-250 µm were eventually retrieved by using Retsch sieves.

Culture independent microbial community fingerprinting

DNA extractions were based on 100 mg pulverized root material and performed with the NucleoSpin Plant II kit (Macherey-Nagel) by selecting the CTAB based lysis buffer or 100-250 mg rhizosphere soil and performed with the PowerSoil® DNA Isolation Kit (MO BIO Laboratories). Cell lyses were done with a Qiagen Retsch Tissue Lyser. DNA extracted from rhizosphere soil and roots was used for the specific amplification of the V6-V8 region of bacterial 16S rDNA by using primers GC968f and 1401r following procedures described in Castaldini et al. (2005). Extracts were also used in a nested PCR system with primers targeting the internal transcribed spacer region 1 of the fungal ribosomal DNA gene cluster using primers EF4 and ITS4 and GCITS1f and ITS2 according to Anderson et al. (2003). For the nested PCR system, the Kapa 2G Robust HS PCR Kit (Kapabiosystems, Sigma Aldrich) with amplifications at a temperature of 55°C was used. Mixed amplification products from three individual PCR reactions per DNA extract were analyzed by using DGGE approaches on 6% acrylamide/bis gels with a 50 to 60% denaturing gradient for bacterial amplicons (Castaldini et al., 2005) and on 8% acrylamide/bis gels with a 10 to 50% denaturing gradient for fungal amplicons. Calculated indexes (Shannon, Pielou Evenness) were used to describe observed diversity of taxa and similarities of community fingerprints were assessed by Dice cluster analyses of DGGE gel profiles (Castaldini et al., 2005).

Culture dependent microbial community analysis

Aliquots of suspended pulverized root material from Si-VS and Si-VL were diluted to a concentration of 10-20 pieces per 10 µl. The concentration was adjusted through counting the number of pieces microscopically in aliquots of 10 µl pipetted on glass slides. Aliquots of 60 µl were pipetted on agar media in 9 cm Petri-Dishes. Rhizosphere soil slurry was diluted in serial dilutions and 60 µl of the dilution at 10^{-4} were pipetted on agar medium. Inocula were disseminated with drigalski spatel and incubated at room temperature. Bacteria were enriched on soil extract agar (DSMZ 12 medium) and 1 g nutrient broth (Biolife) per liter agar (Difco) medium. Growth of fungi was suppressed by adding 75 mg cycloheximide per liter agar medium. Twenty to 30 colony forming units were pure-cultured per sample, and re-grown on yeast (2 g, Biolife) malt (10 g, Oxoid) extract agar for DNA

DOI: 10.6092/issn.2281-4485/7921

extraction (UltraClean Microbial DNA Isolation Kit, MO BIO Laboratories. Strains selected for DNA extraction / PCR covered the range of macroscopical colony characteristics such as colony and aerial mass pigmentation characteristic for actinobacteria or *Streptomyces* spp. (Taddei et al., 2006). Partial 16S rDNA was amplified and sequenced with primers 27F and 1492R (Lane 1991) for identifying pure cultured strains. PCR was performed with the Kapa 2G Robust HS PCR Kit using an annealing temperature at 60°C. Retrieved sequences of selected actinobacteria were compared in Blast searches with databased sequences available in Genbank (Benson et al., 2010) and their similarity with taxonomic reference material was assessed in a neighbor joining tree analysis that adopted the Jukes-Cantor substitution model, uniform rates among sites, pairwise deletion of gaps/missing data and 1000 bootstrap repetitions to test node support (Tamura et al., 2013).

Results and discussion

Culture independent microbial community fingerprinting

No clear differences in overall diversity indices were observed for rhizosphere or root associating fungi and bacteria in DEG and ND areas in 2015 samples (Figure 1).

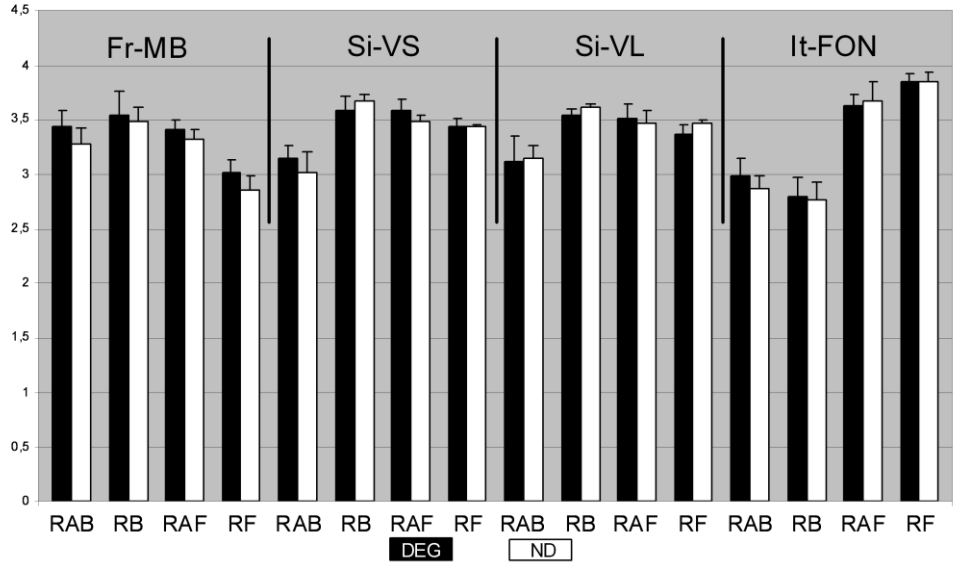

Figure 1. *Shannon diversity indices describing species richness of root-associated bacteria/fungi (RAB/RAF) or rhizosphere bacteria/fungi (RB/RF) from degraded (DEG, black bars) and non-degraded (ND, white bars) areas in vineyards in France (Fr-MB), Slovenia (Si-VS/VL), and Italy (IT-Fon). Error indicators, Std.*

Pielou indices (not shown) suggested high species evenness in the studied DEG and ND areas. The indices therefore did not support the hypothesis that the level of

soil degradation has an influence on the overall diversity / numbers of bacterial and fungal species in the studied root-influenced habitats. Similar results were encountered in our 2017 inventories that also tested the impact of treatments with cover crops (DM, GM) and compost (COMP). Our findings are in accordance with the results retrieved by Ondreičková et al. (2018) for bacterial communities investigated with molecular methods targeting differing soil management strategies in Slovakia and by Costantini et al. (2015) targeting a vineyard in Tuscany.

Dice cluster analyses of DGGE gel profiles allow the detection of qualitative differences between microbial communities (here, communities from ecological niches in degraded versus non-degraded areas). They may also provide ideas of how the communities have changed over time or as a consequence of strategies used to re-install soil functionality including GM, DM, and COMP treatments. Analyses were performed on root and rhizosphere associated fungi and bacteria from 2 experimental plots in France and Italy and 2 vineyards in Slovenia.

Dice cluster analyses performed on samples from Slovenia suggest that microbial communities retrieved from DEG areas differed from those retrieved from ND areas for both vineyards (Figure 2, root associated bacteria in VS and VL (A), rhizosphere bacteria in VS (B), rhizosphere fungi in VL (D)). An extreme degradation, caused by stoniness, was encountered in the Bonini site (VS) (Priori et al., 2018).

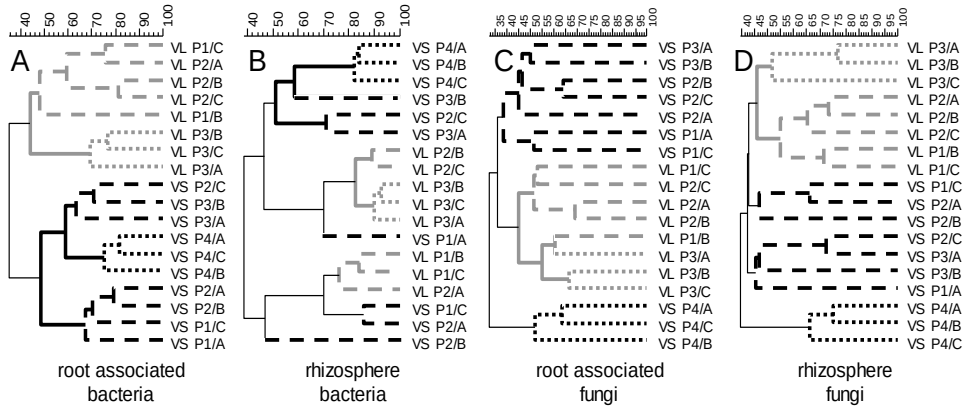

Figure 2. *Dice cluster analyses of DGGE gel profiles from bacterial (A, B) and fungal (C, D) communities obtained from roots (A, C) or rhizosphere soil (B, D) in Slovenian vineyards (2015). Black terminal lineages represent vineyard Bonini (VS); grey lineages, vineyard Prade (VL). P1-4 represent different soil excavation holes used to sample roots; letters A-C, sample replications. Dashed terminal lines represent samples from degraded areas (P1, 2 (VL) and P1-3 (VS)); square-dotted terminal lines, non-degraded areas (P3 for VL; P4 for VS).*

This extreme degeneration may explain why root and rhizosphere associated fungi from the ND plot differed more markedly from those of DEG plots with respect to the communities from the Prade vineyard VL (Figure 2, rhizosphere and root

DOI: 10.6092/issn.2281-4485/7921

associated fungi in VS/VL (C and D)).The specific situation encountered in the Bonini vineyard (VS) did not allow growth of green cover plants seeded for implementing DM and GM based restoration strategies and only little natural vegetation spontaneously grew up. By contrast, composted organic amendments had an immediate and strong effect on the spontaneously developing natural vegetation. The Dice cluster analyses indicated that the treatment with mature compost or the spontaneously developing natural vegetation stimulated by compost had a strong effect at least on the root associated fungal community as compost supports the development or enhancement of a root associated fungal community that is different from that in CONTR and ND plots (Figure 3, A for VS).

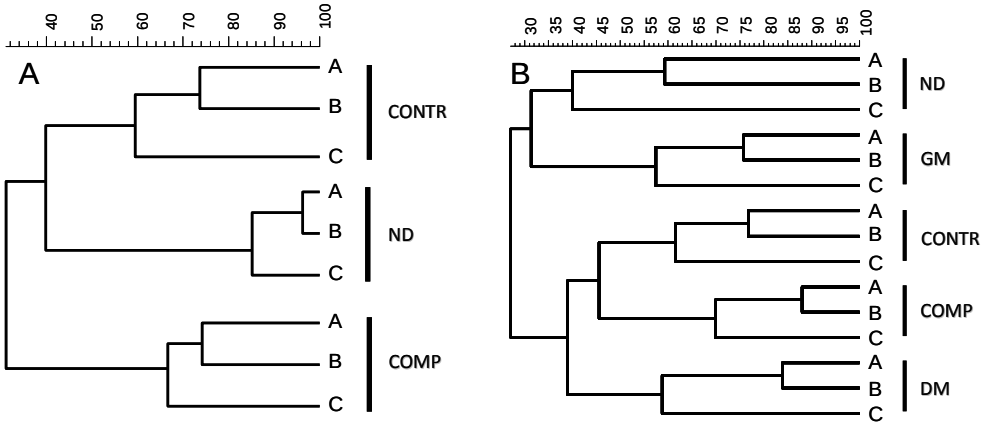

Figure 3. *Dice cluster analyses of DGGE gel profiles from fungal communities obtained from roots in vineyards VS (A) and VL (B) (Slovenia) (2017). Denotations right of bars represent plots treated with compost (COMP), green manure (GM), dry mulch (DM), or non-degraded (ND) and degraded (CONTR). Letters A-C represent sample replications.*

In the Prade vineyard (VL), strong development of cover crops seeded for implementing DM and GM based restoration strategies and also a strong development of spontaneously developing natural vegetation was observed on the compost treated plot. The cluster analyses indicated differentiation of the root associated fungal communities in the differently treated degraded plots, none of them similar to the DEG area as in 2015 (Figure 2, C). In addition, more similar communities were encountered from the GM treated plot and the ND site in 2017 (Figure 3, B); so, at least for root associated fungi, the treatment with green manure shaped a community structure that was more similar to that from the ND plots. Fungal rhizospheric communities from degraded (DEG) and non-degraded (ND) areas did not exhibit a great degree of similarity across the three repetitions in the Italian FON vineyard (Figure 4, A) in 2015, perhaps because of unidentified, however, non-homogeneous factors in the three experimental blocks. A similar situation was observed in samples from the French vineyard MB, i.e., samples neither from DEG or ND areas from different experimental blocks clustered

together (not shown). As in the Bonini vineyard in Slovenia, the specific situation encountered at FON did not allow the development of green cover plants seeded for implementing DM and GM based restoration strategies. Therefore, only compost treated plots were taken into account. Other as in the Slovenian site, fungal communities from compost treated plots clustered together with those from the CONTR plots and differed from those of the ND controls (Figure 4, B), suggesting that compost treatments were not able to reshape the rhizospheric fungal community structures in a "not degraded similar" way. Interestingly, communities from the Fontodi block 1 separated from those representing the same treatments in the other 2 blocks (Figure 4, B), supporting the hypothesis that these blocks were non-homogeneous. Relative similar bacterial rhizosphere communities were observed in the French vineyard MB in 2017 in ND plots and in plots treated with COMP, DM and GM, while the communities from the CONTR plots differed (Figure 4, C). The result may provide an indication for a positive effect of the treatments, especially green manure, on community profiles because they are more similar to those from the ND controls.

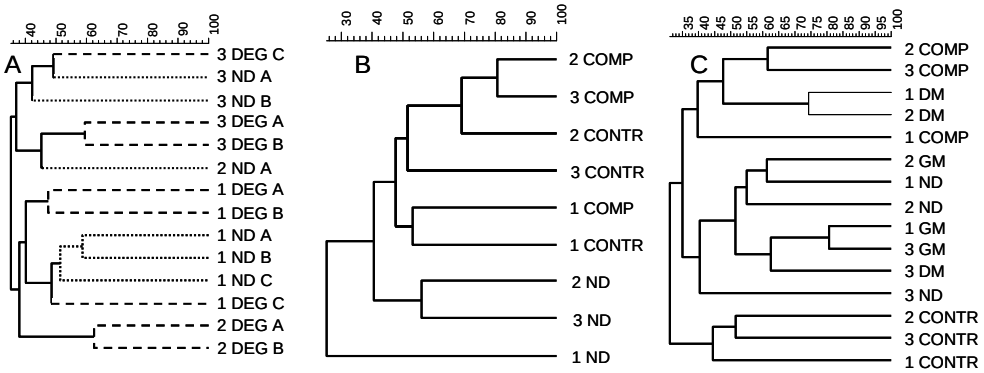

Figure 4. *Dice cluster analyses of DGGE gel profiles from fungal communities obtained from roots in Italian vineyard FON in 2015 (A) and 2017 (B) and rhizosphere bacteria in French vineyard MB in 2017 (C). Degraded areas are identified by dashed terminal lines and DEG in A or CONTR in B and C; non-degraded areas (ND) by square-dotted terminal lines in A. DM (dry mulch), GM (green manure) and COMP (composted organic amendments) denote soil restoration strategies. Numbers in the lineage names represent experimental blocks 1-3; letters A-C, sample replications.*

Culture dependent microbial community analysis

Actinobacterial species of genus *Streptomyces* are known for their potential to produce natural compounds that can have antibiotic, thus potentially biopesticidal effects, or plant growth promoting and other plant beneficial effects (reviewed in Barka et al., 2016). Life cycle characteristics render *Streptomyces* important versatile species. They can dominate under nutrient-rich and limited conditions during their mycelial vegetative stage and produce resource protecting antibiotics and dormant spores when nutrients are depleting. Our results in 2015 confirmed

DOI: 10.6092/issn.2281-4485/7921

that they are common rhizosphere colonizers as they were isolated from roots in degraded (VLP1, VLP2; VSP1-3) and non-degraded plots (VLP3, VSP4) (Figure 5); high numbers of isolates were also obtained from any tested plot in 2017 (details not shown).

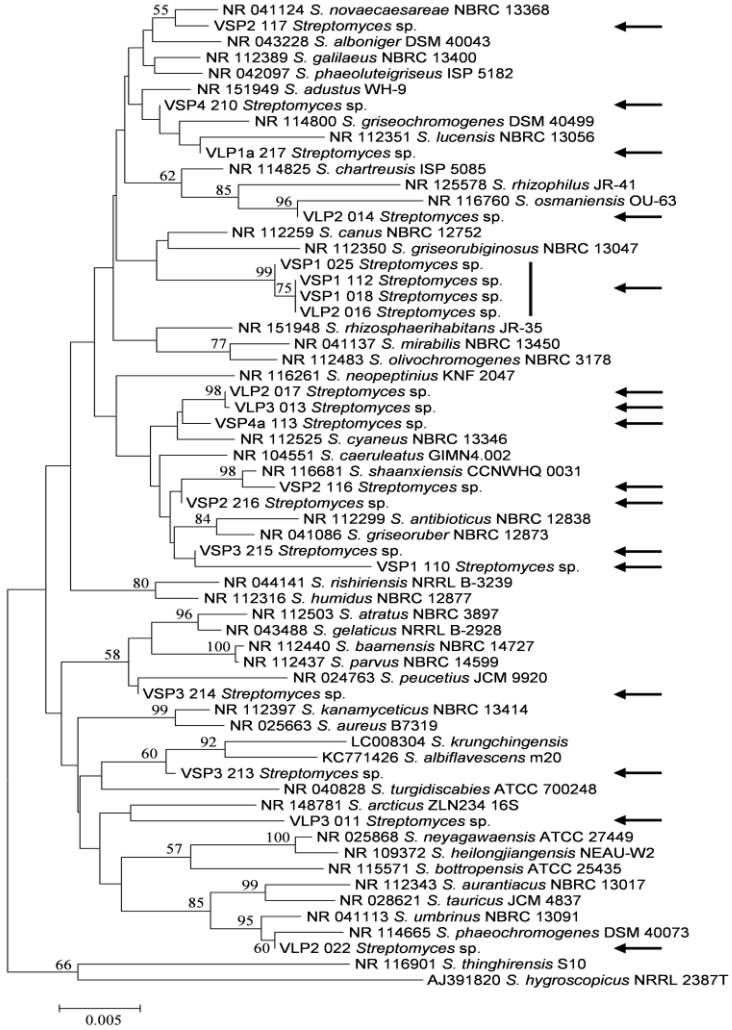

Figure 5. *Neighbor joining tree comparing strains identified as* Streptomyces *sp. and isolated from the rhizosphere of grapevines in Slovenian vineyards VL and VS (arrow right of tree) with aligned 16S rDNA sequences of taxonomic reference strains downloaded from Genbank. Numbers near nodes describe bootstrap proportions. Scale, expected number of substitutions per site.*

With more than 500 described species, *Streptomyces* is one of the largest genera of the Actinobacteria (Viaene et al., 2016). None of the here identified *Streptomyces* strains could be identified with high confidence, i.e., 100% sequence identity,

although several clustered together with either *S. novaecaesareae*, *S. griseochromogenes*/*S. lucensis*, *S. osmaniensis*, *S. cyaneus*, *S. shaanxiensis*, *S. albiflavescens*/*S. krungchingensis* or, respectively, *S. phaeochromogenes*. One group of strains isolated from the vineyards VL and VS formed a statistically supported unique lineage (Figure 5, vertical bar at right side of tree). None of the isolated strains were closely related to *S. thinghirensis*, originally described from grapevine rhizosphere (Loqman et al., 2009). The results add therefore new members to the group of *Streptomyces* taxa known to associate plants or, especially, grapevine roots. Selected *Streptomyces* strains can suppress root pathogenic fungi causing young grapevine decline (Álvarez-Pérez et al., 2017).

Acknowledgments

This contribution was carried out in the framework of the EU project RESOLVE (Restoring optimal Soil functionality in degraded areas within organic vineyards), supported by transnational funding bodies, being partners of the FP7 ERA-net project, CORE Organic Plus, and the cofound from the European Commission. Authors express their gratitude to the vineyard owners that hosted the field trials. Authors thank all the people who helped during field-work and laboratory analysis. We thank Brice Giffard (INRA, Université de Bordeaux, Bordeaux Sciences Agro) for critically reading a previous version of this text.

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