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Diversity of bacterial endophytes in 3 and 15 year-old grapevines of *Vitis vinifera* cv. Corvina and their potential for plant growth promotion and phytopathogen control

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ABSTRACT

This study represents the first investigation on ecology of endophytic bacteria isolated from 3 and 15 year-old vine stems of *Vitis vinifera* cv. Corvina. The analysis was performed by means of culture-dependent techniques. The obtained results showed that new grapevine endophytic genera are being discovered. Moreover, *Bacilli* and *Actinobacteria* are frequently isolated from 3 year-old plants, whereas Alpha- and Gamma- *Proteobacteria* classes are more prevalent in the 15 year-old plants. Shannon–Wiener (*H*) index and analysis of rarefaction curves revealed greater genus richness in young grapevine plants. Furthermore, results evidenced an increase of genotypic group number within specific genera (e.g., *Rhizobium* and *Pantoea*).

Among isolated strains from 3 and 15 year-old stems, respectively, 34 and 39% produce siderophores; 22 and 15% secrete ammonia; 22 and 21% produce indole-3-acetic acid; 8.7 and 41% solubilize phosphate. Besides, two strains isolated from 15 year-old grapevines showed 1-aminocyclopropane-1-carboxylate deaminase activity. Antifungal activity analysis evidenced that two *Bacillus* strains possess growth antagonistic effect toward all the tested fungal strains. Therefore, the present study extends our knowledge of the diversity of the endophytic bacteria by providing new insights into the complexity of the grapevine microbiome.

1. Introduction

In both natural and anthropic ecosystems, plants interact with a wide range of microorganisms including bacteria. These latter can have pathogenic, beneficial or neutral relationships with their hosts. Recently Hardoim et al. (2015) described endophytes as "all microorganisms which for all or part of their lifetime colonize internal plant tissues". Endophytic bacteria have been isolated from different plant tissues, such as roots, stems, leaves, flowers, and seeds (Rosenblueth and Martínez-Romero, 2006; Compant et al., 2011). Endophytes can colonize plants by entering plant cuttings or other vegetative plant parts and often seeds (Ait Barka et al., 2002). Moreover, even rhizospheric bacteria can penetrate, colonize and endure within root tissues from which they can then move to other plant anatomical compartments, assuming an endophytic behavior (Bohm et al., 2007; Compant et al., 2010). Albeit most of them show undetectable impact on the host plants (Kado, 1992), it is widely demonstrated that endophytic bacteria can produce beneficial effects on their hosts (Taechowisan and Lumyong, 2003; Andreolli et al., 2013; Naveed et al., 2014). For instance, endophytes can enhance the nutrient assimilation through solubilization of mineral phosphate, production of siderophores or ammonia release (Loaces et al., 2011; Natul et al., 2013). Additionally, they have been shown to possess growth-promoting traits related to growth regulatory mechanisms such as ethylene-1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity and synthesis of indole-3-acetic acid (IAA) or other auxins (Taghavi et al., 2009). Moreover, endophytic bacteria and their secondary metabolites can directly or indirectly contrast and reduce incidence of infectious plant diseases by means of antibiosis, competition for ecologic niches or nutrients, and stimulation of plant defense mechanisms (Cabanás et al., 2014; Melnick et al., 2011).

Grapevine is the most cultivated fruit plant in the world as well as the first economically important tree crop whose berries are

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mainly processed in wine making. It covers worldwide more than 7.5 million of hectare, with 27 million ton of wine produced by year (FAOSTAT, 2011). Corvina grapevine is an indigenous variety that represents the key constituent of Valpolicella wines such as Valpolicella Classico, Amarone, Ripasso, and Recioto. The importance of this grapevine variety is clearly underlined by transcriptomic, proteomic and metabolomic studies available in the literature (Di Carli et al., 2011; Toffali et al., 2011; Venturini et al., 2013).

Although several investigations have already revealed many important aspects of grapevine biology, physiology and genetics (Dai et al., 2011; Guerra and Steenwerth, 2012), little information exists about the actual occurrence and role of bacterial endophytes in this plant. Descriptions of bacterial endophytic cenoses have been carried out either by means of culture-independent techniques (Bulgari et al., 2009; West et al., 2010) or by culture-dependent methods (Bell et al., 1995; Compant et al., 2011; Campisano et al., 2015) The results so far achieved describe marked differences among endophytic bacterial populations from wild and cultivated plants (Campisano et al., 2015), from different organs (Compant et al., 2011), from healthy and diseased plants (Bulgari et al., 2009, 2011), or from vines that have undergone different pest management strategies (Campisano et al., 2014). On the other hand, information is lacking about the dynamics of culturable endophytic populations throughout different grapevine ages.

The ultimate objective of this study is to assess the composition of the endophytic bacterial community in young (3 year-old) and old stems (15 year-old) of Corvina grapevine. Stems obtained from 3 and 15 year-old plants were considered for the isolation and identification of endophytic bacteria to be then characterized for their plant growth-promoting capabilities and antagonistic potential against fungal phytopathogens. The present study regarding the succession dynamics of endophytes in grapevine of different ages represents an absolute novelty in this field of investigation.

2. Materials and methods

2.1. Arrangement of vine stems and isolation of bacterial endophytes

Stem portions of Corvina grapevine (*Vitis vinifera* cv. Corvina, clone 48) were collected at the experimental vineyard of the "Centro Sperimentale Vitivinicolo della Provincia di Verona" (Verona Provincial District's Experimental Station for Viticulture and Oenology) (Italy) in October 2013. Five 3 year-old (3Y) and five 15 year-old (15Y) plants without any eye-visible injury were randomly chosen in order to aseptically cut the stems. Both 3Y and 15Y plants were cultivated in the same vineyard (where a homogenous climate was assumed), and exposed to the same phytosanitary and fertilization management. Stem diameters ranged between 1.5–2.5 cm and 5.0–6.5 cm from 3Y and 15Y, respectively. Isolation of endophytic bacteria from the stems was performed in quadruplicate. Stems were vigorously washed in distillated water for 5 min and their surface was then treated for 10 min with a 1% (w/v) active chloride (as sodium hypochlorite [NaOCl]) solution added with a drop of Tween 80 every 100 ml of solution. Stem portions were rinsed three times in sterile distilled water and 3Y trunk segments were longitudinally opened by means of a sterile blade of a chisel. Thus, their inner sections were scraped until 50 mg sawdust aliquots were finally collected in sterile 2-ml Eppendorf tubes. On the other hand, core samples from 15Y stem portions were obtained by using a sterile increment borer, equipped with a threaded auger and an extractor tray. Increment cores were cut and scraped with a sterilize chisel, and 50 mg of sawdust was collected in a sterile 2-ml Eppendorf tubes. Thereafter, 450 μl of physiological solution (0.9% [w/v] NaCl) was added to each tube and thus placed for 1 h on an orbital shaker (250 rpm). Serial dilutions were made and 100 μl of samples were plated on R_2A-agar (yeast extract 0.5 g l^{-1}, peptone 0.5 g l^{-1}, casein acid hydrolysate 0.5 g l^{-1}, glucose 0.5 g l^{-1}, starch 0.5 g l^{-1}, sodium pyruvate 0.3 g l^{-1}, K_2HPO_4 0.3 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 0.05 g l^{-1}, agar 15.0 g l^{-1}), Trypticase Soy Agar (pancreatic digest of casein 15 g l^{-1}, peptic digest of soybean meal 5 g l^{-1}, NaCl 5 g l^{-1}) and Yeast Mannitol Agar (K_2HPO_4 0.5 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 0.1 g l^{-1}, NaCl 0.1 g l^{-1}, yeast extract 0.4 g l^{-1}, mannitol 10 g l^{-1} and agar 15.0 g l^{-1}). Both chisel and increment borer were sterilized before each sampling procedure by relying on the same protocol used for trunk portions. A 100 μl samples of water derived from the third rinsing of stems, chisel and increment borer were plated on above-mentioned media to verify that sterilization had effectively occurred. All plates were incubated for 5 days at 27 °C. Colonies grown on those Petri dishes inoculated with core specimen suspensions of different grapevine stem portions were picked up from the plates and purified by repeated streaking until axenic bacterial cultures were obtained. Totally, more than 150 isolates were eventually obtained. Bacterial cultures were cultivated also in R_2A, TSB and YMB liquid media of the same compositions described above except for the agar not supplied.

2.2. BOX-PCR and amplification of 16S rRNA gene

Total DNA from bacterial colony was obtained as described by Andreolli et al. (2011). BOX-PCR reactions were performed in a 25 μl total volume containing 2 μM of the primer AR1 (CTACGGCAAGGCGACGCTGACG) (Van Belkum et al., 1996), 0.4 mM of dNTPs, 1 U of GoTaq™ DNA polymerase (Promega Italia Srl, Milan, Italy) and 5 μl of 5× PCR buffer. PCR conditions were as follows: 95 °C for 5 min, then 30 cycles of 95 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min, with a final extension step at 72 °C for 5 min. PCR products were run on a 1.5% agarose gel in Tris–borate–EDTA buffer for 2 h at 100 V. The gels were thus stained by using Eurosafe (EuroClone SpA, MI, Italy) following the instruction's manual. The banding patterns data were evaluated both by visual inspection and through software package UVIbandmap (UVItec Ltd., Cambridge, UK) in comparison with standard 100 bp and 1 Kb molecular markers (EuroClone SpA).

The gene encoding for 16S rRNA gene (1500 bp) was amplified using FD1 and rp2 primers (Weisburg et al., 1991). PCR reaction was carried out in 25 μl of total volume containing 0.8 μM of each primer, 0.4 mM of dNTPs, 1 U of GoTaqTM DNA polymerase (Promega Italia Srl) and 5 μl of 5× PCR buffer. PCR conditions were as follows: 95 °C for 5 min, then 30 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, with a final extension step at 72 °C for 5 min.

2.3. Cloning, sequencing and phylogenetic analysis

16S rRNA gene PCR products were transformed in *Escherichia coli* Xl1-blue by using the Promega pGEM-T vector system, according to the manufacturer's instructions, and the corresponding nucleotide sequences were obtained from Primm Srl (Milan, Italy). Bacterial sequences were searched for homology by relying on the EzTaxon-E database (Chun et al., 2007). The sequences were aligned by means of multiple alignment program CLUSTAL_X 1.83 (Thompson et al., 1997). A phylogenetic tree based on the neighbor-joining method using the MEGA version 5.0 software package was thus constructed (Kumar et al., 2008). Bootstrap analysis was performed on the basis of 1000 bootstrap replications.

Isolated strains with less than 98% *16S rRNA* gene similarity with type strain identified through EzTaxon-E were further analyzed by means of the BLASTN database (Altschul et al., 1997).