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ISOLATION AND CHARACTERISATION OF ENDOPHYTES FROM *VITIS VINIFERA*

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ABSTRACT

Endophytes are microorganisms that colonize the plant's internal tissues and can have beneficial growth promoting effects for the host plant. Endophytic bacteria and microscopic fungi have also been isolated from grapevine (*Vitis vinifera*), however, the complete structure as well as the role of endophytes in grapevine have not been thoroughly studied.

The aim of this work was

- To isolate endophytes from leaves and green shoots from 4 grapevine varieties.
- To identify the isolated bacteria by MADI-TOF mass spectrometry.
- To test the isolated bacteria for plant growth promoting properties (such as production of antioxidants and siderophores, or minerals phosphate solubilization and atmospheric nitrogen fixation).

The isolated microbes have proved to have beneficial effects on the plant such as growth promotion or resistance to biotic and abiotic stress.

Key words: Endophyte, bacteria, symbiosis, *Vitis vinifera*.

RESUMEN

Los endófitos son microorganismos que colonizan los tejidos internos de la planta y pueden tener efectos beneficiosos promoviendo el crecimiento de la planta huésped. Se han aislado bacterias endófitas y hongos microscópicos de la vid (*V. vinifera*), sin embargo, la estructura completa, así como el papel de los endófitos en la vid no se han estudiado a fondo.

El objetivo de este trabajo fue:

- Aislar endófitos de hojas y brotes verdes de 4 variedades de vid.
- La identificación de bacterias aisladas se realizó mediante espectrometría de masas MALDI-TOF.
- Los microorganismos aislados fueron evaluados para determinar las propiedades de la planta huésped (como la producción de antioxidantes y sideróforos, o la solubilización de fosfatos minerales y la fijación atmosférica de nitrógeno).

Los microorganismos aislados demostraron tener efectos beneficiosos sobre la planta tales como la promoción del crecimiento o la resistencia al estrés biótico y abiótico.

Palabras clave: Endófito, bacteria, simbiosis, *Vitis vinifera.*

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1. INTRODUCTION

Endophytes can be defined as those endosymbiont microorganisms that colonize the internal tissue of the plant, at least during part of its life cycle, showing no external sign of infection or negative effect on their host.

Over the last years, endophytes have been acquiring such an important role, becoming the spotlight of numerous researches. The feature which makes them valuable is their capacity to develop highlighted effects in plants, such as accelerating seedling emergence, promoting plant establishment under adverse conditions such as drought or salinity, enhancing plant growth and preventing disease development, through the synthesis of secondary metabolites as well as their phytoremediation potential. Bacterial endophytes colonize an ecological niche similar to that of phytopathogens, which makes them suitable as biocontrol agents. In fact, it was demonstrated that endophytic microorganisms are able to control plant pathogens, insects and nematodes. In exchange the plant supplies the endophyte with nutrients and protection, acting as a host.

Endophytes are associated with plants in various forms, including bacteria (actinomycetes or mycoplasma) or fungi that colonize the inner plant tissues. More than 200 genera from 16 phyla of bacterial species have been reported to be associated with endophytes and among them, most of the species belong to the phyla Actinobacteria, Proteobacteria, and Firmicutes (Günter Brader et al., 2014).

Endophytes may be transmitted either vertically (directly from parent to offspring) directly within the seeds or horizontally (among individuals) through asexual conidia or sexual spores letting them to spread around plants community.

During this study, endophytes were isolated from leaves and green shoots from 4 grapevine varieties. The importance to study *Vitis vinifera* endophytes comes from the overuse of fungicides and fertilizers, which results in

environmental pollution, the emergence of resistant microbial pathogens as well as undesirable effects on non-target organisms, including humans. Some fungicides even have phytotoxic effects, although little is known about the practical impact of this phenomenon (Dias 2012). These concerns have increased the demand for alternative crop protection products, including biopesticides and biofertilizers, with a natural origin and that are safe for both humans and the environment (Yoon et al. 2013). Other researchers have considered the possibility of managing natural microbial endophytes as biological control agents to confer or induce resistance against phytopathogens in crops such as grapevine (Andreolli M. et al. 2017).

The potential of endophytes to positively influence the growth of grapevine is also of great importance and more investigation is clearly needed for a better understanding of the interactions between endophytes and grapevine.

An important growth promoting property is the ability to utilize 1-aminocyclopropane-1-carboxylate (ACC, the precursor of ethylene). Ethylene synthesis in plants is a result of the plant's response to stressful conditions (such as the presence of metals, drought or humidity, ultraviolet light, and mechanical wounding, etc. (Abeles et al. 1992). One model that describes the ethylene synthesis in plants says that the ethylene is synthesized in two peaks (Glick et al. 2007). The first smaller ethylene peak occurs after the consumption of an existing ACC pool in stressed plant tissues. The second larger peak occurs after an additional ACC synthesis in response to stress and is often involved in initiating processes for various plant damages as a result of stress. Any significant reduction in ACC levels might simultaneously decrease the damage to the plant, such assenescence, chlorosis and leaf abscission, that occurs as a consequence of stress (Robison et al. 2001).

A very important mechanism to promote plant growth involves the production of siderophores by endophytes (Ghavami et al. 2017). Most of the iron in soil is bound in insoluble complexes and is therefore unavailable to the plant or other organisms. Siderophores are secondary metabolites produced by both microorganisms and plants, which help the organism to satisfy its iron requirement by causing solubilisation and chelation of iron from organic or inorganic complexes present in soil (Singh et al. 2017).

Industrial and agricultural waste is one of the main source of metals accumulation which could be toxic in excessive amounts. These minerals promote the generation of reactive oxygen species (ROS) which can cause the oxidation of proteins, lipids, alter the integrity of the proteins, membranes, photosynthesis, growth and induce cell death. Among the biochemical defense mechanisms that are carried out in response to oxidative damage is the production of antioxidants by endophytes. In this study, an antioxidant assay with 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), organic chemical compound composed for stable free-radical molecules, was conducted (Janet María et al.2012).

Nitrogen is the most limiting nutritional factor for the growth of plants since plants are unable to reduce atmospheric nitrogen and it must be first reduced to ammonia. Some endophytes are capable of atmospheric N_2 fixation and subsequent conversion to nitrogen compounds that can be easily utilized by plants (Hongrittipun et al., 2014).

The second most important nutrient after nitrogen is phosphorus, which can be found in soil bound in mineral salts or incorporated into organic compounds. The low availability of phosphorus in soils was recognized as a major growth limiting factor for plants (Daniels et al., 2009). Secretion of organic acids by endophytes is an important mechanism to dissolve insoluble phosphorus in soil and make it thus more available for plants (Illmer et al., 1995).

In this work, the identification of isolated bacteria was performed by MADI-TOF mass spectrometry and the host plant properties which have been tested with the isolated microorganisms were the production of antioxidants and siderophores, minerals phosphate solubilization, ACC deaminase activity, antioxidant production and atmospheric nitrogen fixation.

2. MATERIALS AND METHODS

2.1. Isolation and identification

The endophytes were isolated from shoots and leaves of four different grapevine varieties (Muller Thurgau, Rheinriesling, Pinot gris and Pinot noir) grown in Prague, Czech republic. The grapevine variety and the abbreviations used in this work are mentioned in Table 1.

Table 1. Grapevine varieties and their abbreviations used in this work

Abbreviation	Grapevine variety
MT	Muller Thurgau
RM	Pinot noir
RŠ	Pinot gris
RR	Rheinriesling

Pieces of the size of 5 cm were cut off the plant with sterile scissors and transferred into a sterile 10mM solution of $MgSO_4$, cooled by crushed ice. The plant material was then within 24 hours sterilized in a solution consisting a disinfectant containing sodium hypochlorite, distilled water (in a ratio of 1:7) with an addition of a detergent. After 7 minutes of shaking (150 rpm), the plant material was then transferred into a 70 % ethanol solution for another 3 minutes. Afterwards it was washed four times with sterile distilled water. The sterile material (2 g) was homogenized (Polytron, 1 min, max. Speed) in 20 ml sterile 10 mM phosphate buffer. The mixture was filtered through a 100 µm porosity filter and a dilution of 1:100, 1:1000 and 1:10 000 was prepared from which 100 µl was applied to plates with TSA media with added fungal growth inhibitor (cycloheximidine 100 mg/l). Petri dishes were incubated at 28°C for 3-5 days. After the visual characterization, the colonies were inoculated on Petri dishes with PCA. After 24 hours of cultivation on PCA, the individual colonies were transferred to a specific position on a metal plate for MALDI TOF MS identification. The sample was applied in three parallels, then covered with 1 µl of matrix and allowed to crystallize at room temperature. The identification took place on MALDI TOF MS (Bruker Daltonics, DE) with MALDI BioTyper database (Brucker Daltonics, DE).

2.2. Determination of siderophore production

This assay is performed by cultivation of the bacteria on CAS agar for 48 hours at 28°C. Chromium Azurol S (CAS) and hexadecyltrimethylammonium (HDTMA) bind ferrous ion contained in the medium to produce a blue agar color. If the iron is removed from the complex by siderophores, the blue color of the agar changes to the yellow/orange.

CAS agar: 60.5 mg of CAS was dissolved in 50 ml of deionized water and mixed with 10 ml of solution containing ferrous ion (2.7 mg of $FeCl_3.6H_2O$ dissolved in 10 mM HCl). While stirring, 72.9 mg of hexadecyltrimethylammonium (HDTMA) dissolved in 40 ml of distilled water were added to this solution. The resulting blue solution was autoclaved (0.12 MPa, 121 ° C, 30 min). A second solution was prepared by dissolving 15 g of bacteriological agar and 22 g of LB medium in 900 ml of distilled water.30.24 g of PIPES (piperazine-N, N-bis- (2-ethanesulfonic acid)) was added. The pH of the mixture was adjusted to 6.8 with a solution of 4.16 M NaOH. The resulting solution was autoclaved (0.12MPa, 121°C, 30 min). Both solutions were mixed after sterilization and poured into sterile Petri dishes.

2.3. Determination of ACC deaminase activity

In this assay, the reaction of ninhydrin with the amino group in 1-aminocyclopropane-1-carboxylic acid (ACC) is used. When culturing bacteria with ACC deaminase activity in the presence of ACC, degradation occurs, which can be measured after the reaction with ninhydrin, after which the absorbance at the wavelength of 570 nm decreases.

One colony of endophytic bacteria grown on TSA was inoculated into 5 ml of liquid LB medium and cultured for 24 hours at 28°C with constant shaking. Subsequently, 2 ml of culture were collected and centrifuged (5 min, 8000 x g). The pellet was washed twice with 1 ml of DF medium and suspended in 2 ml of DF-ACC medium and incubated for 24 hours at 28 ° C with constant shaking. At the same time, the DF-ACC medium alone (negative control) was incubated. 1 ml of each culture was centrifuged (5 min, 8000 x g). 100 μl of each supernatant was withdrawn and 1 ml of DF medium was added. 60 μl of the diluted supernatant was pipetted in 3 parallels into a PCR strip and 120 μl of ninhydrin reagent was added. The strips were incubated in boiling water

for 30 minutes. From each tube, 100 μl of the sample was pipetted into a microtiter plate and the absorbance was measured at 570 nm.
DF minimal salt medium: KH_2PO_4 (2 gl^{-1}), Na_2HPO_4 (6 gl^{-1}), $MgSO_4$. $7H_2O$ (0.2 gl^{-1}), D-glucose (2 gl^{-1}), gluconic acid (2 gl^{-1}), citric acid (2 gl^{-1}), $(NH_4)_2SO_4$ (2 gl^{-1}).
Trace element solutions: H_3BO_3 (10 μgl^{-1}), $MnSO_4$. H_2O (11.19 μgl^{-1}), $FeSO_4$. 7H2O (1 mgl^{-1}), $ZnSO_4$. $7H_2O$ (124.6 μgl^{-1}), $CuSO_4$. $5H_2O$ (78.22 μgl^{-1}), MoO_3 (10 μgl^{-1})
The base medium was sterilized in an autoclave (0.12MPa, 121°C, 30 min). Trace element solutions were sterilized by filtration using 0.22 μm membrane filters (Millipore®). To 994 ml of sterile DF medium, 1 ml of the each of the trace element solution was added.
DF-ACC media: ACC was dissolved in distilled water and sterilized by filtration with a 0.22 μm membrane filter (Millipore®). To 50 ml of DF medium without $(NH_4)_2SO_4$, 300 μl of a 0.5 M solution of ACC was added.

2.4. Determination of phosphate solubility

This assay is performed by culturing microorganisms on Pikovskaya agar, which is opaque because of the insoluble $Ca_3(PO_4)_2$, for 48 hours at 28°C. Phosphate solubilization is manifested by halo/clear zones around the microbial colonies.
Pikovskaya agar: D- glucose (10 gl^{-1}), agar (23 gl^{-1}), $Ca_3(PO_4)_2$ (5 gl^{-1}), $(NH_4)_2SO_4$ (0.5 gl^{-1}), KCl (0.2 gl^{-1}), yeast extract (0.5 gl^{-1})
Trace element solutions: $MgSO_4$. $7H_2O$ (0.1 gl^{-1}), $FeSO_4$. $7H_2O$ (1 mgl^{-1}), $MnSO_4$. $2H_2O$ (1 mgl^{-1})
The base medium was sterilized in an autoclave (0.12MPa, 121°C, 30min). Trace element solutions were sterilized by filtration using 0.22 μm membrane filters (Millipore®).To 997 ml of sterile base medium, 1 ml of each of the trace element solution was added and the medium was poured into sterile Petri dishes.

2.5. Determination of nitrogenase activity

Nitrogenase activity is an indicator of the bacteria's ability to fix atmospheric nitrogen. The principle of the determination is the ability of bacteria with nitrogenase activity to grow in medium with a minimum amount of nitrogen. Endophytic bacteria were inoculated into 5 ml of liquid LB medium and incubated for 24 hours at 28°C, 150 rpm. 100 μl of the bacterial culture was inoculated into 5 ml of a liquid medium for nitrogenase activity testing. Cultivation was carried out for 48 hours at 28°C with constant shaking. Subsequently, the optical density of the individual samples was measured at a wavelength of 550 nm. As a negative control, the medium for testing nitrogenase activity itself was used.

Liquid medium for nitrogenase activity testing: Yeast extract (0.2 gl^{-1}), K_2HPO_4 (0.5 gl^{-1}), $MgSO_4$. $7H_2O$ (0.2 gl^{-1}), NaCl (0.1 gl^{-1}), $CaCO_3$ (0.5 gl^{-1}), manitol (10 gl^{-1}), sodium glutamate (0.32 gl^{-1}), sodium succinate (1.6 gl^{-1}) The medium was sterilized in an autoclave (0.12MPa, 121°C, 30 min).

2.6. Determination of antioxidant production

5 ml of liquid LB medium was inoculated with endophytic bacteria. Cultivation was carried out for 7 days at 28 ° C with constant shaking. The cultured colonies of endophyte bacteria were then centrifuged (5 min, 5000 g) and 1 ml of the supernatant was analysed. The presence of antioxidants was expressed as ascorbic acid equivalents. 25 mg of ascorbic acid was dissolved in 50 ml of distilled water to give a stock solution of 500 mgl^{-1}. A calibration series was prepared from this stock solution at the concentrations: 2.5; 5; 7.5; 10; 12.5 and 15 mgl^{-1}. Subsequently, a solution of 52 mg/l of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was prepared. Measurement was carried out in a microtiter plate, 200 μl of DPPH was added to 100 μl of the tested sample, incubated for 15 minutes in the dark, and the absorbance was measured at 517 nm.

3. RESULTS AND DISCUSSION

85 bacteria strains were isolated from 4 different grapevine varieties, from leaves as well as green shoots (Table 2) and numbered from 1 to 85: The isolated bacteria proved to be different not only in different varieties but also in different parts of the same plant.

Table 2. Number of isolates from different parts and different varieties of *Vitis vinifera*

	Muller	Rheinriesling	Pinot noir	Pinot gris
Leaves	10	11	12	10
Shoots	12	7	3	20

From these 85, 34 bacterial strains were successfully identified with MALDI TOF MS analysis (Table 3):

The higher the score number is, the more likely that the bacteria was identified correctly: those bacteria with a MALDI score between 2.300 and 3.000 are described as highly probable species identification; those with 2.000 and 2.299 are recognized as secure genus identification and probable species identification; between 1.700 and 1.999 a probable genus identification is obtained; finally, between 0 and 1.699, not reliable identification is performed. As we can observe in the Table 3, almost all of them are secure genus identification and probable identification, so we can state that it is quite reliable.

Furthermore, some specific bacteria have been found in different varieties of *Vitis vinifera* and from different parts of the plant as well (L corresponds to "Leaves" and S to "Shoots").

Table 3. Identified endophytic bacteria isolated from four different grapevine (MT- Muller, RM- Pinot noir, RŠ- Pinot gris, RR- Rheinriesling) varieties and two different plant parts (L- leaves, S- shoots) and their MALDI score

Isolate number	Identification	Score
MT L 5	*Pseudomonas syringae*	2,103
MT L 6	*Bacillus subtilis*	2,098
MT L 8	*Bacillus subtilis*	1,905
MT L 9	*Bacillus mojavensis*	2,025
MT S 15	*Pantoea agglomerans*	2,417
MT S 20	*Staphylococcus saprophyticus*	2,051
MT S 22	*Kocuria rhizophila*	1,812
RM L 24	*Rhodococcus fascians*	1,998
RM L 25	*Pantoea sp.*	2,334
RM L 27	*Pantoea sp.*	2,113
RM L 28	*Pseudomonas caricapapaya*	2,022
RM L 29	*Pseudomonas congelans*	2,104
RM L 31	*Rhodococcus fascians*	1,775
RM L 33	*Pseudomonas congelans*	2,123
RŠ L 38	*Pseudomonas graminis*	2,269
RŠ L 39	*Pseudomonas graminis*	2,291
RŠ L 42	*Staphylococcus hominis*	2,077
RŠ L 43	*Pantoea sp.*	2,446
RŠ L 44	*Staphylococcus saprophyticus*	2,012
RŠ L 47	*Pseudomonas graminis*	2,248
RŠ S 49	*Staphylococcus saprophyticus*	2,106
RŠ S 50	*Pantoea agglomerans*	2,400
RŠ S 58	*Bacillus subtilis*	1,758
RŠ S 59	*Pantoea agglomerans*	2,428
RŠ S 67	*Bacillus subtilis*	1,950
RR L 68	*Pantoea agglomerans*	2,320
RR L 69	*Micrococcus luteus*	2,028
RR L 71	*Pantoea agglomerans*	2,517
RR L 73	*Micrococcus luteus*	2,044

“Table 3 continues in next page”

RR L 74	*Pantoea agglomerans*	2,391
RR L 75	*Kocuria rosea*	2,004
RR L 78	*Kocuria rosea*	2,448
RR S 81	*Curtobacterium flaccumfaciens*	2,066
RR S 82	*Pantoea sp.*	2,375

SIDEROPHORE PRODUCTION:

From 85 bacteria, including those which were identified and those classified as unknown, 69 were siderophore producers. Thus, it could be said that a broad number of bacteria are siderophores producers, since 81% of the microorganisms analysed where positive in siderophore tests (Fig. 1).

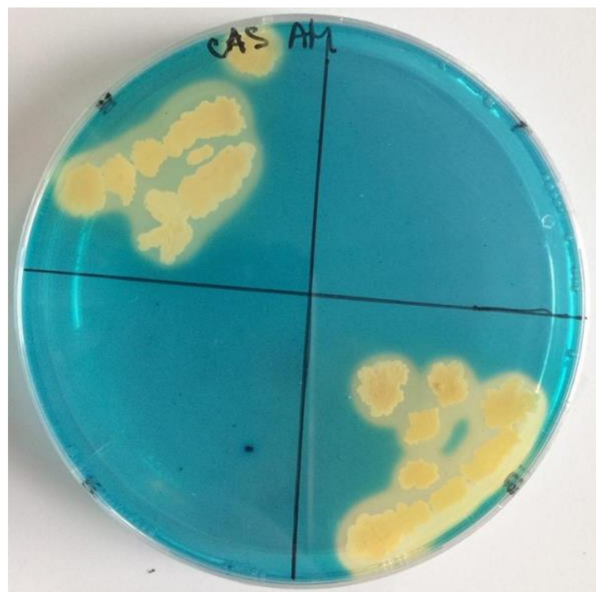

Fig. 1 Petri dish with CAS agar and endophytic bacteria producing siderophores

At the same time, 28 of them were successfully identified by MALDI-TOF MS (Table 4) while the remaining 41 siderophore producers were not among the unidentified endophytic bacteria (Table 5).

Table 4. Siderophore production by endophytic bacteria identified by MALDI-TOF MS (+++: strong activity, ++: mediocre activity, +: weak activity)

Degree of production	Identified bacterial strains
+++	*Bacillus subtilis 6, Bacillus subtilis* 8*, Bacillus subtilis* 58, *Bacillus mojavensis* 9, *Pseudomonas graminis* 39*, Micrococcus luteus* 73*, Kocuria rosea* 78
++	*Pseudomonas congelans* 33*, Staphylococcus saprophyticus* 44, *Pantoea agglomerans* 68*, Pantoea agglomerans* 59
+	*Pantoea agglomerans* 71*, Staphylococcus saprophyticus* 49, *Kocuria rhizophila* 22*, Pantoea sp.* 82, *Pseudomonas caricapapaya* 28*, Pseudomonas congelans* 29*, Rhodococcus fascians* 31*, Pseudomonas graminis* 47*, Kocuria rosea* 75*, Curtobacterium flaccumfaciens* 81

As we can see in (Table 4), there are several identical bacteria species whose production degree can be different due to differences between the strains. E.g, two strains of *Pseudomonas graminis* are in "+++" group as well as in "+" group.

Table 5. Siderophore production by endophytic bacteria not identified by MALDI-TOF MS (+++: strong activity, ++: mediocre activity, +: weak activity)

Degree of production	Unidentified bacterial strains
+++	4,72,77,85
++	10,34,45,62,63,66
+	2,11,12,13,14,16,17,18,19,21,23,30,35,36,37,40,46,51,53,54,55,56,60,61,64,65,70,76,79,80,83,84

It could be interesting the study of specific strains producers of high amount of siderophores because, as it can be observed in (Table 4), it does not only depend on the species but also on the strain. Each culture with its respective

strain was numbered allowing us to focus on the strains with higher yields. In the literature, similar results were found. Those strains with the highest halo-diameter were selected to quantify the rate of siderophore production using CAS-liquid assay as well. The strains with more yield according to the production of siderophores such us hydroximates or carboxylates were sequenced through 16S ribosomal ribonucleic acid (rRNA) sequence analysis. Andreolli et al. (2016) investigated both the ecology and the growth-promoting traits of endophytic bacteria isolated from 3-year-old and 15-year-old *V. vinifera* cv. Corvina stems. In their study, 36 % of the siderophores were positive producers and the production was mainly attributed by genus *Rizhobium*.

PRODUCTION OF ANTIOXIDANTS

All 85 bacterial strains were antioxidants producers. (Table 6) show those identified and unidentified bacteria ordered in 3 groups according to their antioxidant production. Each group is organised starting from the strain with most production to the least efficient one.

Both plant parts (leaves and shoots) were exposed to sunlight, producing reactive oxygen species (ROS) by excitation radiation. Therefore, it is understandable that all isolated bacteria produce antioxidants as an answer to such stressful conditions (Gill and Tuteja, 2010). It is questionable whether the production of antioxidants alone by endophyte is sufficient to protect the plants against oxidative stress. However, a study by White and Torres (2010) suggests that a major benefit for plants might be the formation of a small amount of ROS by an endophyte microorganisms, thereby adapting the plant cells to oxidative stress.

Table 6. Antioxidant production by endophytic bacteria in [mg·l^{-1}].

Antioxidant concentrations [mg·l^{-1}]	Identified bacterial strains	Unidentified bacterial strains
19,1 – 25,2	*Curtobacterium flaccumfaciens 81, Staphylococcus saprophyticus 49*	80, 83, 48
13,1 – 19,0	*Bacillus subtilis 58, Pseudomonas graminis 47 , Pseudomonas graminis 38 Pantoea* sp. 82, *Rhodococcus fascians 31, Pseudomonas caripapaya 28, Bacillus subtilis 67, Staphylococcus hominis 42, Pseudomonas graminis 39, Pantoea agglomerans 59, Pseudomonas congelans 33, Pantoea agglomerans 50, Pantoea agglomerans 74, Staphylococcus saprophyticus 44, Micrococcus luteus 69, Pantoea agglomerans 71,*	76, 85, 35,61, 53, 56, 65, 57, 52, 40, 41, 70, 34, 54, 45, 55, 66, 51, 37, 80, 49, 87, 63, 79
7,0 – 13,0	*Micrococcus luteus* 73*, Pantoea agglomerans* 68*, Bacillus subtilis* 8*, Kocuria rhizophila* 22*, Pantoea sp.* 25*, Staphylococcus saprophyticus* 20*, Bacillus mojavensis* 9*, Bacillus subtilis* 6*, Pantoea agglomerans* 15, *Pantoea sp.* 27*, Pseudomonas syringae* 5*, Rhodococcus fascians* 24*, Pseudomonas congelans* 29	62, 4, 14, 18, 2, 10, 12, 30, 3, 23, 7, 26, 17, 19, 21, 11, 13, 1, 16

NITROGENASE ACTIVITY:

All 85 isolated bacteria had nitrogenase activity. The results are mentioned in Table 7 and Table 8 ordered by degree of production for identified and unidentified bacterial strains respectively.

Table 7. Nitrogenase activity in identified endophytic bacteria (+++: strong activity, ++: mediocre activity, +: weak activity)

Degree of production	Identified bacterial strains
+++	*Bacillus subtilis 6, Bacillus subtilis 8, Bacillus mojavensis 9, Kocuria rhizophila 22, Rhodococcus fascians 31, Pseudomonas congelans 33, Staphylococcus saprophyticus 49, Bacillus subtilis 58, Bacillus subtilis 67, Micrococcus luteus 69, Pantoea agglomerans 74, Curtobacterium flaccumfaciens 81.*
++	*Pseudomonas syringae 5, Pantoea agglomerans 15, Staphylococcus saprophyticus 20, Rhodococcus fascians 24, Pantoea sp. 25, Pantoea sp. 26, Pseudomonas caricapapaya 28, Pseudomonas congelans 29, Pantoea sp. 43, Pantoea aglomerans 59, Pantoea aglomerans 71 Kocuria rosea 75, Kocuria rosea 78.*
+	*Pseudomonas graminis 38, Pseudomonas graminis 39, Staphylococcus hominis 42, Staphylococcus saprophyticus 44, Pseudomona graminis 47, Pantoea agglomerans 50, Pantoea agglomerans 68, Micrococcus luteus 73, Pantoea sp 82.*

Table 8. Nitrogenase activiy in unidentified endophytic bacteria (+++: strong activity, ++: mediocre activity, +: weak activity)

Degree of production	Unidentified bacterial strains
+++	7, 10, 11, 13, 23, 26, 51, 53, 57, 60, 61, 66, 70, 72, 79, 85
++	1,2,3, 12, 16, 17, 18, 41, 46, 52, 54, 56, 64, 77, 80, 84
+	4, 14, 19, 21, 30, 32, 34, 35, 36, 37, 40, 45, 48, 55, 62, 63, 65, 76, 83

It can be observed some identified species which could be fully studied such us *Bacillus subtilis* or *Kocuria rhizophila*. Moreover, it would be interesting to sequence those unidentified strains with strong activity.

In Andreolli et al. (2016) assays, 19% bacterial strains secreted ammonia from dinitrongen gas (N2). Although the presence of nitrogenase was not directly tested in our experiments, Dadarwal et al.(1981) proved in their work that all bacteria able to grow on the media for nitrogenase activity testing proved to have this ability.

PHOSPHATE SOLUBILITY:

From 85 bacteria, 37 were able to solubilize insoluble phosphate and 18 of them were identified bacteria by MALDI TOF MS. The strains were tested on Pikovskaya agar (Fig. 2). Andreolli et al. (2016) isolated endophytic bacteria from grapevine stems and proved that 25 % of the isolated bacteria were able to solubilize phosphate. However, there was a big difference in the number of bacteria able to solubilize phosphate, which were isolated from young, 3 year old, plants (8.7 %) in comparison to those isolated from older, 15 years old, plants (41 %). In our case, the bacteria were isolated from 26 years old grapevines, therefore, the number of bacteria able to solubilize phosphate (43.5 %) was more similar to Andreolli et al. results from older plants. In addition, about half of all the bacterial species that were able to solubilize phosphate represented the genus *Pantoea*. In our case, genus *Bacillus*, *Pseudomoas* and *Pantoea* among others represent this group.

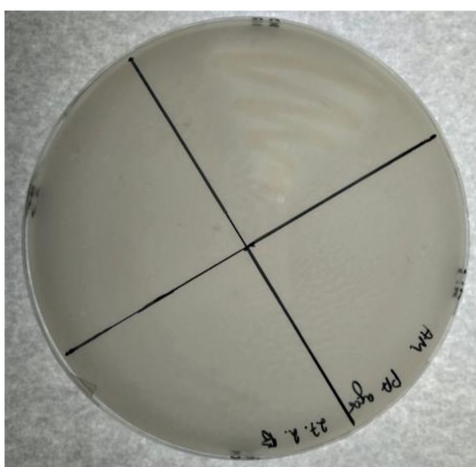

Fig. 2 Petri dish with Pikovskaya agar and endophytic bacteria able to solubilize $Ca_3(PO_4)_2$

Table 9. Identified bacteria capable to solubilize phosphate (+++: strong activity, ++: mediocre activity, +: weak activity)

Degree of production	Identified bacterial strains
+++	*Pseudomonas congelans 33, Kocuria rosea 78*
++	*Staphylococcus saprophyticus 20, Pseudomonas caricapapaya 28, Pseudomonas graminis 39, Staphylococcus saprophyticus 49, Micrococcus luteus 73*
+	*Bacillus subtilis 6, Bacillus subtilis 8, Bacillus mojavensis 9, Rhodococcus fascians 31, Pantoea agglomerans 50, Bacillus subtilis 58, Pantoea agglomerans 59, Bacillus subtilis 67, Pantoea agglomerans 68, Micrococcus luteus 69, Curtobacterium flaccumfaciens 81*

Phosphorus is considered the most important key element in the nutrition of plants after nitrogen. An adequate supply of phosphorus during early phases of plant development is important for laying down the primordia of plant reproductive parts. It plays significant role in increasing root ramification and strength thereby imparting vitality and disease resistance capacity to plant. It also helps in seed formation and in early maturation of crops. Its presence in soil is abundant, however the availability of soluble phosphate is limited. Therefore, P is usually added to soil as chemical P fertilizer, nevertheless synthesis of chemical P fertilizer is highly energy intensive processes, and has long term impacts on the environment. The isolation and development of efficient strains which are able to solubilize the phosphate present in soil would be interesting. According to these results, further studies with *Pseudomonas* sp. could be carried out and genetic modification may be realized to improve strains capacity.

Table 10. Unidentified bacteria with the ability to solubilize phosphate (+++: strong activity, ++: mediocre activity, +: weak activity

Degree of production	Unidentified bacterial strains
+++	-
++	14,16,17,18,19,62,63
+	2, 10, 30, 34, 45, 53, 56, 57, 60, 66, 72, 80

ACC DEAMINASE ACTIVITY

A small percentage of bacteria had ACC deaminase activity. From 85 bacteria, 21 of them had this property.

Table 11. Identified and unidentified strains with ACC deaminase activity

Bacteria with ACC deaminase activity	
Identity	**Strain**
Identified	*Bacillus subtilis 6, Bacillus subtilis 8, Kocuria rhizophila 22, Rhodococcus fascians 31, Pseudomonas graminis 39, Staphylococcus hominis 42, Pantoea sp. 43, Staphylococcus saprophyticus 44.*
Unidentified	1,2,3,10, 12, 13, 14, 18, 19, 35, 36, 40, 41.

Plant growth-promoting bacteria that contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase facilitate plant growth and development by decreasing plant ethylene levels, especially following a variety of environmental stresses. Those bacteria that possess the enzyme ACC deaminase can degrade ACC and utilize it as a carbon source. Under such conditions, re-uptake by the roots is prevented and the level of ACC in the roots is reduced. As a consequence, ethylene production by the roots is lowered, relieving inhibition of root growth.

Andreolli et al. (2016) study demonstrated that only 1% bacteria strains had ACC deaminase activity which according to our results it is smaller since we obtained a percentage around 25%. Nevertheless, comparing with the rest of endophyte traits, ACC deaminase activity was the one trait with the lowest frequency among the strains.

4. CONCLUSION

This study has demonstrated the potential and the wide range of endophytes which are present in *Vitis vinifera* varieties. Many of the isolated bacteria showed one or more plant growth promoting features. However, further studies are needed to find out those strains which better plant growth promoting features. This could be accomplished through the study and genetic modification of those interesting strains which are already identified as well as sequencing the strains that seem to be interesting based on the obtained results.

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6. ACADEMIC HONESTY DECLARATION

I, the undersigned, declare that the attached assignment/project is wholly my own work, and that no part of it has been:

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