

Unveiling of beneficial bacteria in Douro Vineyards

Pesquisa de bactérias benéficas nas Vinhas do Douro

El Hadi Erbiai^{1,*}, Fernando Alves² & Guilhermina Marques^{1,3}

¹ Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Department of Agronomy, University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal

² Symington Family Estates, Vinhas SA, Travessa Barão de Forrester 86, 4431-901 Vila Nova de Gaia, Portugal

³ Institute for Innovation, Capacity Building and Sustainability of Agri-Food Production (Inov4Agro), UTAD, 5000-801 Vila Real, Portugal

(*E-mail: el.hadi.erbiai@gmail.com)

<https://doi.org/10.19084/rca.38525>

Recebido/received: 2024.08.31

Aceite/accepted: 2024.10.25

ABSTRACT

Microbial biodiversity in soil vineyards is a key determinant of grapevine (*Vitis vinifera* L.) growth and wine quality. Vineyards are subject to many pests and fungal pathogens responsible for significant losses in the wine sector. Recently, beneficial bacteria have received increasing attention as a promising sustainable alternative to chemical fertilizers and pesticides. This work aimed to isolate and characterize bacteria, with biofertilizer and biopesticide potential, from vineyards in the Douro Wine Region. The isolates were tested for their antagonistic activity against 4 grapevine phytopathogenic fungi. Thirty antagonistic isolates were identified by 16S rDNA sequences and subjected to the test of their plant growth-promoting properties. Identification results revealed the presence of 7 bacteria genera, mainly *Bacillus* species. Strains of *Burkholderia* sp. (Gs3) and *Streptomyces* sp. showed the highest antifungal effect. Concerning biofertilizer potential, all isolates produced IAA (values between 1 and 53 µg/mL). The strain Gs3 was the highest producer of IAA and the most powerful phosphate solubilizer. For siderophores, *Bacillus* spp. were the main producing strains. Therefore, it can be suggested that these bacteria can be an alternative to agrochemicals. However, further studies are required to evaluate the properties of these bacteria in field conditions, separately and/or in combination, aiming to develop commercial products.

Keywords: Phytopathogenic fungi, biocontrol, biofertilizer, grapevine, beneficial bacteria.

RESUMO

A biodiversidade microbiana no solo das vinhas é determinante do crescimento da videira (*Vitis vinifera* L.) e da qualidade do vinho. As videiras estão sujeitas a diversas pragas e doenças responsáveis por perdas significativas no sector vitivinícola. Recentemente, as bactérias têm recebido uma atenção crescente como alternativa sustentável e promissora aos fertilizantes e pesticidas químicos. Este trabalho teve como objetivo isolar e caracterizar bactérias, com potencial biofertilizante e biopesticida, provenientes de vinhas da região do Douro. Os isolados foram testados para as atividades antagonistas contra 4 fungos fitopatogénicos da videira. Trinta isolados antagonistas foram identificados por análise das sequências da região 16S do rDNA e submetidos à avaliação das suas propriedades promotoras do crescimento de plantas. Os resultados revelaram a presença de 7 géneros de bactérias, predominando as espécies de *Bacillus*. Estirpes de *Burkholderia* sp. (Gs3) e *Streptomyces* sp. apresentaram o maior efeito antifúngico. Quanto ao potencial biofertilizante, todos os isolados produziram IAA. A estirpe Gs3 foi a maior produtora de IAA e o mais eficiente solubilizador de fosfato. As espécies do género *Bacillus* destacaram-se na produção de sideróforos. Assim, pode concluir-se que estas bactérias podem ser uma alternativa aos agroquímicos. No entanto, são necessários mais estudos para avaliar as suas propriedades em condições de campo, isoladamente e/ou em combinação, visando o desenvolvimento de produtos comerciais.

Palavras-chave: fungos fitopatogénicos, biocontrolo, biofertilizante, videira, bactérias benéficas

INTRODUCTION

The grapevine (*Vitis vinifera* L.) is one of the most economically important fruit crops worldwide (Grassi and De Lorenzis, 2021). In Portugal, the viticultural sector is central to the national economy, ranking third among the nation's largest crop production (Costa *et al.*, 2015). However, the grapevine is often affected by many fungal and viral diseases that are responsible for significant losses in the wine sector, and managing plant diseases has become a challenge for plant pathologists in more sustainable agriculture (Akhtar and Siddiqui, 2010). Using chemical pesticides and fertilizers causes hazardous effects on human health and the environment. Therefore, it is necessary to manage plant diseases and enhance crop production using eco-friendly methods and sources. Recently, beneficial microorganisms, including fungi and bacteria, have received increasing attention as a promising sustainable alternative to chemical fertilizers and pesticides (Wang *et al.*, 2022). Plant growth-promoting bacteria (PGPB) play a vital role in managing plant diseases and directly and indirectly affect plant growth by producing several phytohormones, enzymes, siderophores, antibiotics, and solubilizing minerals (Akhtar and Siddiqui, 2010). The microbial diversity found in soil vineyards is

a key determinant of grapevine growth and wine quality. Herein, this work aimed to isolate and characterize bacteria, with biofertilizer and biopesticide potential, from vineyards' soils located in the Douro Wine Region. The isolated bacteria were tested for their antagonistic effects (biopesticide) against four phytopathogenic fungi including *Fusarium oxysporum*, *F. solani*, *Botrytis cinerea*, and *Alternaria alternata*. The antagonistic isolates were identified by 16S rDNA gene sequences and subjected to evaluating their plant growth-promoting properties (biofertilizer), including the ability to produce the indole-3-acetic acid (IAA), siderophores and solubilize phosphates.

MATERIAL AND METHODS

Isolation of Bacteria and Evaluation of Antagonistic Activity

The bacteria were isolated from three different samples, including leaves, berries, and soils of several grapevine varieties located in the Quinta do Ataíde (Symington), Vila Flor, Trás-os-Montes (Figure 1). The isolation was done by inoculating suspension solutions containing samples with autoclaved distilled water on Petri dishes with two

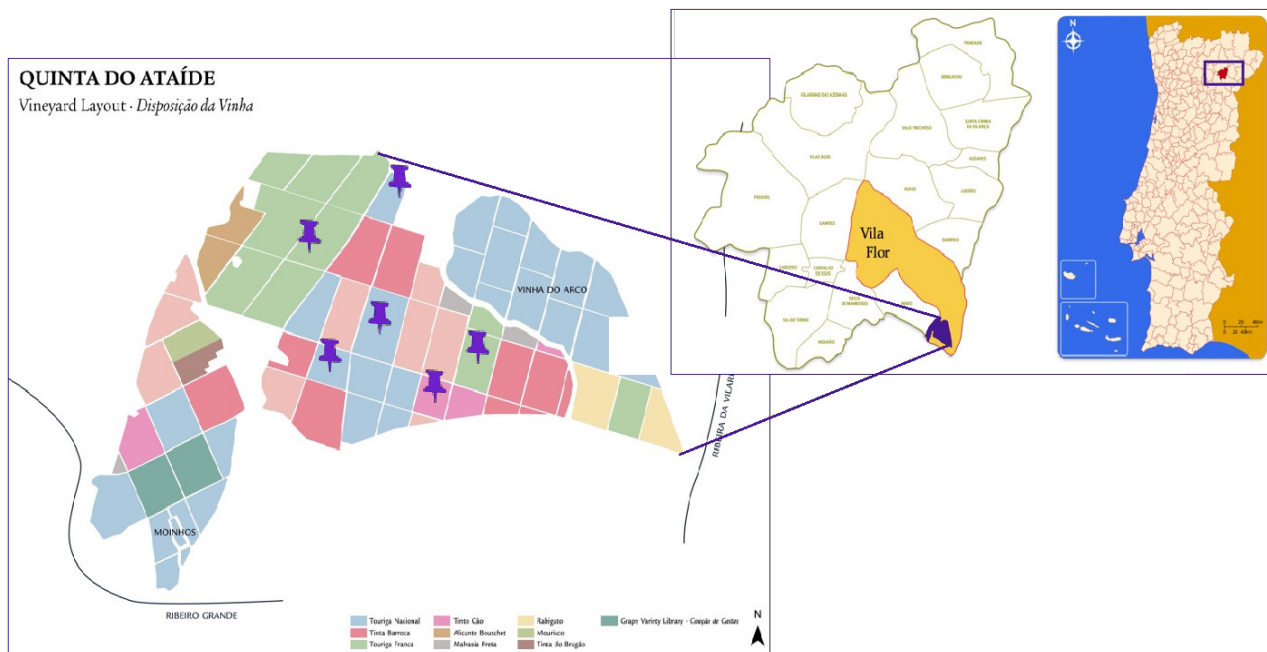


Figure 1 - Geographical location of the study area: "Quinta do Ataíde", Vila Flor, Trás-os-Montes.

culture media Trypticase Soy Agar (TSA) and Potato Dextrose Agar (PDA), separately. The plates were incubated at 25-28°C. Then, the bacteria isolates were repeatedly subcultured onto fresh agar plates until getting a pure culture.

The purified bacteria isolates were used to evaluate their antagonistic effects (biopesticide) against four well-known selected phytopathogenic fungi namely *Fusarium oxysporum*, *F. solani*, *Botrytis cinerea*, and *Alternaria alternata* using the dual-culture method and PDA medium. The Petri dishes containing fungi and bacteria isolates were incubated at 28-30°C for 7 days, and then the results were presented as distance (mm) between bacteria and pathogen edges.

Molecular Identification of Antagonistic Bacteria

The bacteria that showed the highest antagonistic activities against at least two pathogens were subjected to molecular identification. DNA extraction was done using the CTAB method, and 16S rDNA amplification was carried out with the primers 27F and 1942R (Laranjeira *et al.*, 2021). The PCR products were sequenced by StabVida (Lisbon, Portugal) and compared with the GenBank nucleotide data bank from the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). Identification was performed by phylogenetic analysis using MEGA X software (Kumar *et al.*, 2018).

Determination of the Plant Growth-Promotion Properties

Determination of the plant growth promotion properties (biofertilizer potential) was performed by evaluating the abilities of antagonistic bacteria to produce the indole-3-acetic acid (IAA), siderophores, and solubilize phosphates using the same procedures, equipment, and conditions used previously by Laranjeira *et al.* (2021).

The bacteria's ability to produce IAA was estimated by colorimetric assay using the Salkowski reagent method (Patten and Glick, 2002). The IAA concentration was calculated using the calibration curve of pure IAA used as a standard.

The ability of bacteria to produce siderophore was tested qualitatively by the Chrome Azurol S assay (CAS) (Alexander and Zuberer, 1991). After incubation at 30°C for 8 days, positive siderophore production was indicated by a colour change of the CAS medium from blue to orange.

For phosphate solubilizing ability, the antagonistic bacteria were evaluated for their ability to dissolve phosphate using the National Botanical Research Institute's Phosphate (NBRIP) solid and liquid medium. For solid medium, tricalcium (TCP, $\text{Ca}_3(\text{PO}_4)_2$) and aluminum (AlPO_4) phosphates were used as single phosphorus sources, and the bacteria P-solubilizing activity was detected by the formation of a distinguished clear halo around the colonies (Mehta and Nautiyal, 2001; Laranjeira *et al.*, 2021). Concerning the liquid medium, TCP was used as the only phosphate source to quantify P-solubilization by measuring the level of free phosphate, and pH values, and calculating the efficiency of P-solubilization (Suleimanova *et al.*, 2023).

RESULTS AND DISCUSSION

Antagonistic bacteria and their molecular identification

More than 120 bacteria were isolated from vineyard samples, and most of them were isolated from berries and soils. These isolates were evaluated for their antagonistic effect against four well-known phytopathogenic fungi: *F. oxysporum*, *F. solani*, *B. cinerea*, and *A. alternata* (Figure 2). Thirty among them were chosen to present their results in this work, as they exhibited high antagonistic activity. These antagonistic isolates were identified through molecular techniques, and their plant growth-promoting properties were tested.

The molecular identification using 16S rDNA sequence revealed the presence of 7 bacteria genera, namely *Bacillus*, *Streptomyces*, *Burkholderia*, *Calidifontibacillus*, *Paenibacillus*, *Priestia*, and *Alcaligenes*, whereas, most identified species belong to *Bacillus*, particularly *B. subtilis* and *B. amyloliquefaciens* species.

The isolates Gs3 (*Burkholderia* sp.), 42s5 (*Streptomyces* sp.), Qs6 (*Alcaligenes* sp.), Cb5 (*Paenibacillus* sp.),

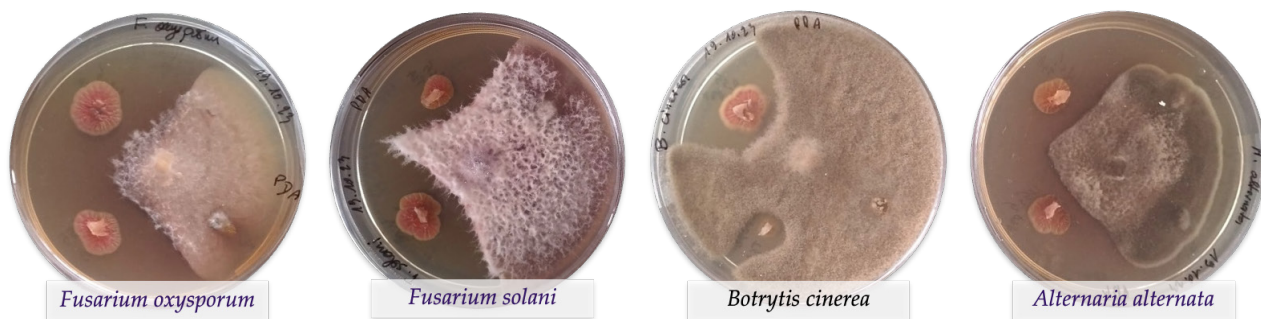


Figure 2 - Example of bacteria isolates showing strong antagonistic effects against the four phytopathogenic fungi.

and Nb4 (*Bacillus* sp.) showed the highest antifungal effect. Generally, *F. oxysporum* was the more bacteria resistant phytopathogenic fungi, while *A. alternata* was the most sensitive. The strong antagonistic activity of isolated bacteria may be due to these bacteria being able to synthesize biologically active compounds with high antifungal properties (Ranjbariyan *et al.*, 2011). Overall, these findings showed that vineyards are an important source of beneficial bacteria able to eco-friendly manage several plant diseases.

Plant Growth-Promotion Properties of Antagonistic Bacteria

According to, the obtained data on the production ability of indole-3-acetic acid (IAA), all 30 antagonistic bacteria showed the ability to produce IAA with values ranging between 1.5 and 53 $\mu\text{g/mL}$. The isolate Gs3 (*Burkholderia* sp.) was the one with the highest capability to produce IAA, followed by Cs4 (*Priestia* sp.) and Nb1 (*Bacillus* sp.).

The qualitative determination of siderophore production by isolated bacteria was done by CAS assay, and results demonstrated that all bacteria were able to produce siderophores by developing a yellow halo around inoculated isolates. The most efficient isolates were Nb4 (*Bacillus* sp.), 42s3 (*Bacillus* sp.), and Qs6 (*Alcaligenes* sp.). Generally, the *Bacillus* species showed better results than other isolates.

The phosphate solubilization ability of the studied bacteria was evaluated qualitatively on solid medium and quantitatively in liquid medium to confirm their capacity to solubilize phosphate. Concerning the solid medium, the results were highly variable among the isolates, where some bacteria showed well-developed halo zones on plates containing TCP, particularly *Burkholderia* sp. (Gs3), while the other bacteria only changed the colour of the medium. None of the bacteria studied formed halos on plates containing AlPO_4 . Based on qualitative results, the TCP was chosen as a source of phosphate to quantify the P-solubilisation using NBRIP liquid medium. The quantification results showed that all antagonistic bacteria had the capacity for solubilizing the TCP with concentrations varying between 85 and 800 $\mu\text{g/mL}$, whereas Gs3 (*Burkholderia* sp.) was the highest-produced isolate followed by the isolates 42s5 (*Streptomyces* sp.) and Cb5 (*Paenibacillus* sp.) (Figure 3). The phosphate solubilization was accompanied by an acidification of the medium, which is likely due to the important production of organic acids.

Generally, the antagonistic bacteria in the current work showed high capacities of producing phytohormone (IAA) and organic compounds (including siderophores, organic acids, etc.) and solubilization of inorganic phosphates, which positively influence plant growth and development and play an important role in the suppression of plant pathogens.

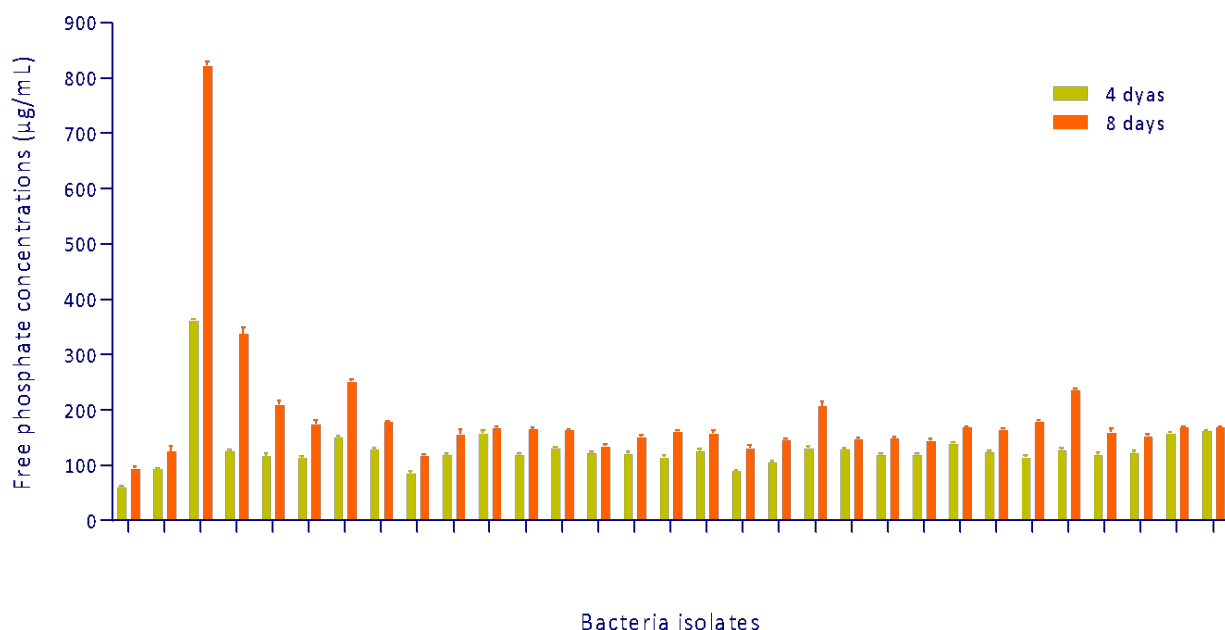


Figure 3 - Inorganic phosphate solubilization ability of the 30 bacteria isolates in NBRIP liquid medium with TCP (tricalcium phosphate) as phosphate sources, after 4 and 8 days of incubation.

CONCLUSION

In summary, the results demonstrated that vineyards soils in the Douro Wine Region (Portugal) are a rich source of beneficial bacteria, which showed interesting results as biofertilizers and biopesticides. These beneficial bacteria can be suggested as a sustainable alternative to agrochemicals. However, further studies are required to evaluate their properties in field conditions, separately and/or in combination, aiming to develop commercial products.

ACKNOWLEDGMENTS

This work was supported by the project “Vine&Wine Portugal- PRR004 – B6.1 – BioGrape-Sustain: Use of grapevine and microbiome diversity for sustainable production” funded by *Plano de Recuperação e Resiliência* (PRR) and by European Funds NextGeneration EU, through “Agendas Mobilizadoras para a Reindustrialização.” This work was also supported by National Funds by the Portuguese Foundation for Science and Technology (FCT) under the projects UIDB/04033/2020 (CITAB) and LA/P/0126/2020 (Inov4Agro). EHE acknowledges PRR and European Funds NextGeneration EU for their fellowship grant (BIPD/UTAD/3/2023).

REFERENCES

- Akhtar, M.S. and Siddiqui, Z.A. (2010) - Role of Plant Growth Promoting Rhizobacteria in Biocontrol of Plant Diseases and Sustainable Agriculture. In: Maheshwari, D.K. (Ed.) - *Plant Growth and Health Promoting Bacteria*, vol.18, p. 157–195. Microbiology Monographs. Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-13612-2_7
- Alexander, D.B. and Zuberer, D.A. (1991) - Use of Chrome Azurol S Reagents to Evaluate Siderophore Production by Rhizosphere Bacteria. *Biology and Fertility of Soils*, vol. 12, n. 1, p. 39–45. <https://doi.org/10.1007/BF00369386>
- Costa, R.; Fraga, H.; Malheiro, A.C. and Santos, J.A. (2015) - Application of Crop Modelling to Portuguese Viticulture: Implementation and Added-Values for Strategic Planning. *Ciência e Técnica Vitivinícola*, vol. 30, n. 1, p. 29–42. <https://doi.org/10.1051/ctv/20153001029>
- Grassi, F. and De Lorenzis, G. (2021) - Back to the Origins: Background and Perspectives of Grapevine Domestication. *International Journal of Molecular Sciences*, vol. 22, n. 9, art. 4518. <https://doi.org/10.3390/ijms22094518>
- Kumar, S.; Stecher, G.; Li, M.; Knyaz, C. and Tamura, K. (2018) - MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution*, vol. 35, n. 6, p. 1547-1549. <https://doi.org/10.1093/molbev/msy096>
- Laranjeira, S.; Fernandes-Silva, A.; Reis, S.; Torcato, C.; Raimundo, F.; Ferreira, L.; Carnide, V. and Marques, G. (2021) - Inoculation of Plant Growth Promoting Bacteria and Arbuscular Mycorrhizal Fungi Improve Chickpea Performance under Water Deficit Conditions. *Applied Soil Ecology*, vol. 164, art. 103927. <https://doi.org/10.1016/j.apsoil.2021.103927>
- Mehta, S. and Nautiyal, C.S. (2001) - An Efficient Method for Qualitative Screening of Phosphate-Solubilizing Bacteria. *Current Microbiology*, vol. 43, n. 1, p. 51–56. <https://doi.org/10.1007/s002840010259>
- Patten, C.L. and Glick, B.R. (2002) - Role of *Pseudomonas putida* Indoleacetic Acid in Development of the Host Plant Root System. *Applied and Environmental Microbiology*, vol. 68, n. 8, p. 3795–3801. <https://doi.org/10.1128/AEM.68.8.3795-3801.2002>
- Ranjbariyan, A.R.; Shams-Ghahfarokhi, M.; Kalantari, S. and Razzaghi-Abyaneh, M. (2011) - Molecular Identification of Antagonistic Bacteria from Tehran Soils and Evaluation of Their Inhibitory Activities toward Pathogenic Fungi. *Iranian Journal of Microbiology*, vol. 3, n. 3, p. 140–146.
- Suleimanova, A.; Bulmakova, D.; Sokolnikova, L.; Egorova, E.; Itkina, D.; Kuzminova, O.; Gizatullina, A. and Sharipova, M. (2023) - Phosphate Solubilization and Plant Growth Promotion by *Pantoea Brenneri* Soil Isolates. *Microorganisms*, vol. 11, n. 5, art. 1136. <https://doi.org/10.3390/microorganisms11051136>
- Wang, G.; Ren, Y., Bai, X.; Su, Y. and Han, J. (2022) - Contributions of Beneficial Microorganisms in Soil Remediation and Quality Improvement of Medicinal Plants. *Plants*, vol. 11, n. 23, art. 3200. <https://doi.org/10.3390/plants11233200>