SCAP
SOCIEDADE DE CIÊNCIAS AGRÁRIAS DE PORTUGAL

Diversity of Entomopathogenic Fungi in vineyard soils in Portugal

Diversidade de fungos entomopatogénicos em solos de vinhas em Portugal

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https://doi.org/10.19084/rca.38903

Received/recebido: 2024.08.31
Accepted/aceite: 2024.10.25

A B S T R A C T

Entomopathogenic fungi (EPF) are important parasitic microorganisms of soil-dwelling insect pests, playing an important role in the dynamics of insect populations in agricultural soils. The increased global demand for sustainable grapevine cultivation and pest control has highlighted the need for a better understanding of these naturally occurring antagonists. To improve the efficacy of the EPF as microbial insecticides it is necessary to understand their habitat selection. Therefore, this study aimed to isolate and identify EPF from several vineyard soils. Soil samples were collected to obtain the isolates using *Tenebrio molitor* as bait. Based on ITS sequence analysis and phylogeny, most fungal isolates were identified as *Metarhizium robertsii* followed by *Clonostachys rosea*, *Beauveria bassiana*, *Purpureocillium lilacinum*, *Blackwellomyces pseudomilitaris*, *Flavocillium bifurcatum*, *Lecanicillium dimorphum, Sesquicillium rossmaniae and Trichoderma gamsii*. This work will provide insights into the biodiversity of entomopathogenic fungi, which can be exploited for the biological control of vineyard pests.

Keywords: Soil microorganisms, biological control, functional diversity, microbial insecticides

R E S U M O

Os fungos entomopatogénicos (EPF) são importantes microrganismos parasitas de insetos que habitam o solo, desempenhando um papel importante na dinâmica das populações de algumas pragas em solos agrícolas. A crescente procura global por métodos mais sustentáveis de proteção da vinha contra pragas, reforça a importância de um melhor conhecimento destes antagonistas naturais nestes agroecossistemas, em particular no estudo do seu habitat natural, tendo em vista a melhoria da sua eficácia como insecticidas microbianos. Assim, este estudo teve como objetivo isolar e identificar EPF associados a diversas vinhas. Os isolados foram obtidos a partir de amostras de solo utilizando *Tenebrio molitor* como armadilha. Com base na sequência ITS e na análise filogenética, a maioria dos isolados fúngicos foram identificados como *Metarhizium robertsii* seguido por *Clonostachys rosea*, *Beauveria bassiana*, *Purpureocillium lilacinum*, *Blackwellomyces pseudomilitaris*, *Flavocillium bifurcatum*, *Lecanicillium dimorphum*, *Sesquicillium rossmaniae* e *Trichoderma gamsii*. Este trabalho contribui para o conhecimento da biodiversidade de fungos entomopatogénicos, que podem ser explorados para o controlo biológico de pragas na vinha.

Palavras-chave: Microrganismos do solo, controlo biológico, diversidade funcional, insecticidas microbianos

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is a woody, perennial plant of high economic importance worldwide (Bettenfeld *et al*., 2022). Vineyards are one of the most emblematic icons of Mediterranean landscapes, renowned for their cultural, environmental and identity value with a particular socioeconomic significance in Portugal (Silva *et al*., 2018). These agroecosystems face threats from various insect pests, including the European Grapevine Moth (*Lobesia botrana*). This pest can reduce yields by up to 50% at the time of harvest in countries such as Portugal (Carlos *et al*., 2013). Therefore, developing effective strategies for managing vineyard pests is crucial from an economic perspective.

The chemical control measures employed against the pest were found to be ineffective. In addition, the frequent use of pesticides causes problems such as the development of resistance in the pest population (Fofana *et al*., 2023), the reduction of the populations of natural enemies and environmental pollution. With increased awareness of the impact of certain chemical insecticides in the environment, biological methods to control crop pests such as biopesticides based on entomopathogenic fungi (EPF) have been receiving greater attention as alternatives to chemical pesticides (Jaronski, 2010). EPF are important pathogens that infect arthropod hosts, playing a crucial role in regulating insect populations and facilitating biotransformation in natural ecosystems. Unlike bacterial and viral pathogens, which infect insects through the mouthparts and gut, EPF invades their hosts through the cuticle. As indicators of soil health and contributors to sustainable agroecosystem management, EPF have been recognized for their importance in environment-friendly agriculture (Lacey *et al*., 2015). Additionally, EPF interacts with plants as growth promoters, beneficial rhizosphere colonizers, and biofertilizers, further highlighting their significance in maintaining ecological balance and promoting sustainable agricultural practices (Lacey *et al*., 2015). Therefore, to improve the efficacy of the EPF as microbial insecticides this study was aimed to study the biodiversity of EPF in Portuguese vineyards which can be exploited for the biological control of vineyard pests.

MATERIAL AND METHODS

A total of fifty rhizospheric soil samples were analyzed for the isolation of EPF, collected during in the late summer season, from vineyards of Pegões and Palmela (Setúbal District, Portugal). The soil samples were processed in two ways: treated soils were dried at 35°C for 24 hours in glass Petri plates, while non-treated soils were left at room temperature. For insect baiting, four late instar *Tenebrio molitor* larvae were placed on the soil surface and plates were kept in the growth chamber (Panasonic MLR-352H-PE) at a temperature of 22°C with relative humidity of 85%, in the dark for the total incubation period of two weeks (Sharma *et al*., 2018).

Figure 1 - Process of isolation and purification of entomopathogenic fungi.

The isolation of EPFs was carried out by recuperation, sterilization (by 1% sodium hypochlorite solution) and cultivation of infected larvae, showing fungal growth, on Rose Bengal Chloramphenicol Agar medium. Then, subsequent subcultures were done on Potato Dextrose Agar (PDA) until obtaining purified isolates (Figure 1).

For molecular identification of the isolates, DNA was extracted from fungal mycelium by the CTAB method as described in Sharma *et al.* (2018). The fungal internal transcribed spacer (ITS) region was amplified using the forward primer ITS1 (5'CTTGGTCATTTAGAGGAAGTAA-3') and reverse primer ITS4 (5'TCCTCCGCTTATTGATATGC-3') (Gardes and Burns, 1993). The PCR amplifications were carried out using a T100 Thermal Cycler (Bio-Rad). The thermal profile for both primer sets included pre-PCR denaturation at 95°C for 5 minutes followed by 35 cycles of denaturing at 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 2 minutes, and a final extension at 72°C for 7 minutes. The amplified PCR products were electrophoresed on 1.5% agarose gel in Tris borate EDTA buffer (TBE) (0.5X) for 25 minutes under an electrical voltage of 90V and visualized in Gel Doc™ EZ Imaging System (Bio-Rad). Successfully amplified PCR products were sequenced at STABvida (Lisbon, Portugal). The fungal strains were identified by searching the NCBI nucleotide database using the Basic local alignment search tool (BLASTn). Sequences were aligned using MEGA X software and a maximum-likelihood (ML) phylogenetic tree was constructed. The robustness of ML tree topology was confirmed via using 1000 bootstrap replications.

RESULTS AND DISCUSSION

Molecular characterization has revealed nine EPF species. The occurrence of EPF species was significantly higher in Pegões (125%) compared to Palmela (70%). Also, EPF species were more abundant in the treated soil samples (soil dried at 35°C for 24 hours) compared to the non-treated ones, in both regions (Figure 2). In Pegões, the most prevalent fungal species were *Metarhizium robertsii* (59%), *Clonostachys rosea* (30%), *Beauveria bassiana* (16%), *Purpureocillium lilacinum* (12%), *Blackwellomyces pseudomilitaris* (4%), *Flavocillium bifurcatum*

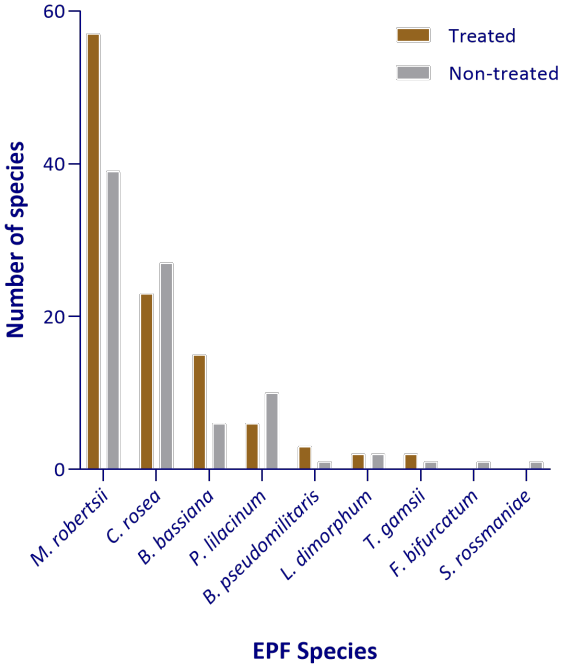

Figure 2 - EPF isolated from treated soil (samples dried at 35°C for 24 hours) and non-treated soils.

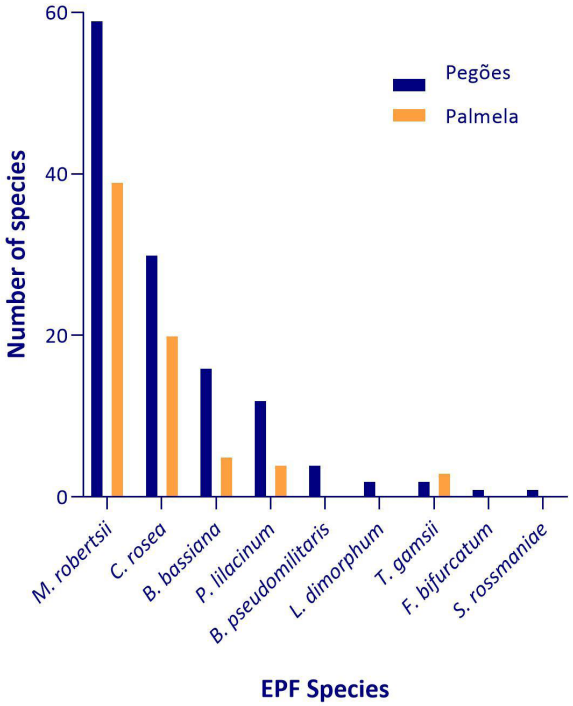

Figure 3 - Distribution of EPF isolates from different sampling sites.

(2%), *Lecanicillium dimorphum* (2%) and *Sesquicillium rossmaniae* (1%). In contrast, a lower diversity of EPF was found in Palmela, with *M. robertsii* (39%), *C. rosea* (20%), and *B. bassiana* (5%) being the most prominent (Figure 3). *M. robertsii and B. bassiana* were the most abundant isolated EPF species from treated soils, while *C. rosea* and *P. lilacinum* were more abundant in non-treated soils. Two species *Flavocillium bifurcatum* and *Sesquicillium rossmaniae* were isolated only once in the non-treated soil from Pegões (Figure 3). Cultivated soils are likely to harbor a diverse range of fungal species due to agricultural practices, e.g ploughing, which create niche opportunities for EPF and foster an increase in fungal diversity (Sharma *et al*., 2021).

CONCLUSION

This work is a contribution for expanding the knowledge on the natural abundance and diversity of EPFs in Portuguese vineyards. These isolates are valuable resources for to test their efficiency in biological control of some important pests in these agroecosystems.

ACKNOWLEDGEMENTS

This work was supported by the project "Vine&Wine Portugal- PRR004 – B6.1 – BioGrapeSustain: 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 the Portuguese Foundation for Science and Technology (FCT) through the project "DrosuGreen: Controlling the quarantine pest *Drosophila suzukii* through epidemiological studies and new Green biocontrol techniques"-PTDC/ASP-PLA/4477/2020, and the projects UIDB/04033/2020 (CITAB) and LA/P/0126/2020 (Inov4Agro).

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