**Grapevine-associated microorganisms as biocontrol agents against the proliferation of pathogenic fungi**

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This PhD project aim at selecting potential biocontrol agents (BCAs) able to prevent, or limit, the infection of grapevine and grape (table and wine) by pathogenic fungi, reducing the use of chemical fungicides. The first performed activities of the project are described. Firstly, the evaluation of endophytic community in *Vitis vinifera* and *V. sylvestris* through set up of protocols for the cultural isolation and biomolecular identification. Secondly, the investigation of BCA-plant interaction through assessment of plant-BCA assays in grapevine cell cultures.

**Microorganismi associati alla vite come agenti di biocontrollo contro la proliferazione di funghi patogeni**

Il seguente progetto di dottorato mira a selezionare dei potenziali agenti di biocontrollo (BCAs) in grado di prevenire, o limitare, l’infezione di vite e uva (da tavola e vino) da parte di funghi patogeni, in modo da ridurre l’utilizzo dei fungicidi chimici. In seguito, sono descritte le prime attività del progetto svolte. In primo luogo, la valutazione della comunità endofita in piante di *V. vinifera* e *V. sylvestris* tramite messa a punto di protocolli per l’isolamento culturale e identificazione biomolecolare. Successivamente, l’indagine delle interazioni BCA-pianta tramite utilizzo di colture cellulari di vite.

**Key words**: biocontrol agents, endophytes, grape cell culture.

# **1. Introduction**

In accordance with the PhD thesis project previously described (Pizzi, 2022), this poster reports the main results of the activities concerning:

**WP1.** Evaluation of endophytic community in *V. vinifera* and *V. sylvestris*:

*T1.1* Set up of protocols for the cultural isolation of endophytes (M1.1, Sterilization step optimized)

*T1.2* Isolation of culturable microorganisms from grape flowers, leaves and fruits (M1.2, Isolation of at least 10 culturable species)

**WP3.** Investigation of BCA-plant interaction

*T3.1* Set up of protocols for BCA-plant interaction (M3.1 Identification of the best cultivar for grapevine cell cultures)

Nowadays, the following activities are ongoing:

*T2.1* Screening of potential biocontrol capability (M2.1 Selection of at least 1 potential BCA against *Botrytis cinerea*)

*T2.3* Determination of the mechanisms of action of BCAs (M2.3 Report on the main mode of action of at least 1 potential BCA against *B. cinerea*)

*T3.2* Assessment of plant-BCA assays in grapevine cell cultures evaluating the interaction in presence/absence with *B. cinerea* (M3.2 Inoculum and cultural conditions for the preparation of plant-pathogen-BCA validated)

# **2. Materials and Methods**

*T1.1* Grapevines located in four locations of the norther Italy were sampled in six vineyards chosen for their different conduction. The sampling was carried out during different phenological stages from April until July 2022. To check the efficacy of surface sterilization for endophytes isolation, a challenge test was performed through contamination with *Saccaromyces cerevisiae EC1118 UMY341* and two protocolsof sterilization were evaluated.

*T1.2* Endophytic populations were isolated from distinct parts of the plants (i.e., shoots, leaves and berries) and each sample was sterilized with ethanol and sodium hypochlorite, with different pre-treatments depending on the analyzed part of the plant. The shoots were cut in sections and placed with the vascular vessels facing the medium, the leaves were shattered with Bead beater (Biomedicals), and the berries were homogenized with NaCl 0.8%. All plates were incubated at 26°C for one week. Then, all isolates were stored at -80°C in glycerol 20% (v/v). Species identification was performed using the sequencing of taxonomically relevant regions within the ribosomal DNA of bacteria (16S rDNA) and fungi (ITS sequences).

*T3.1* For the callus preparation, leaves were sterilized, put on solid Murashige and Skoog (MS) medium supplemented with hormones and maintained at 26°C in darkness for two weeks until callus cells appear. To obtain liquid culture, 2 g of fresh callus was inoculated in the same medium without agar in the same condition of callus.

The following activities are on-going:

*T2.2* Evaluation of the efficacy of BCAs in controlling the *B. cinerea* infection by culture inhibition tests (dual-culture plate and double Petri dish)

*T2.3* Assessment of the biocontrol mechanisms of action of BCAs by VOCs analysis. VOC production will be measured in solid and liquid co-culture with plant/BCA/pathogen contact using SPME coupled with GC/MS analysis.

*T3.2* Microscopy observation of the plant/BCA/pathogen interaction in cell cultures and detection of resveratrol production (HPLC) and/or the expression of specific target genes (RT-PCR).

# **3. Results and Discussion**

To evaluate the efficacy of the sterilization step on the samples collected for the endophytes isolation different protocols were applied. Challenge tests, using a contamination with *S. cerevisiae* [$10^{7}UFC$/mL], demonstrated that to rinse the sample in 90% ethanol and then in 2.5% sodium hypochlorite with further three washing steps in sterile water, each for 3 min, allowed the sterilization of the material. In accordance with Campisano *et al.* (2014), the same concentration of ethanol and sodium hypochlorite in contact with shoot samples was proved efficient to remove the presence of superficial epiphytes.

A total of 220 endophytes were isolated from grapes, consisting of 72 fungi and 148 bacteria. Specifically, 69 endophytes were isolated from shoots, 120 from leaves and 31 from berries. The results obtained from the species identification are summarised in Table 1.

***Table 1*** Species identification by sequencing of ribosomal DNA of bacteria (16S rDNA) and fungi (ITS sequences)

|  |  |
| --- | --- |
| Bacteria species identification | Fungal species identification |
| *Bacillus cereus*  | *Massilia sp.* | *Alternaria alstroemeriae* | *Elsinoe sp.*  |
| *Bacillus megaterium*  | *Microbacterium sp.* | *Alternaria alternata* | *Filobasidium wieringae* |
| *Bacillus velezensis*  | *Mycobacteroides abscessus*  | *Alternaria infectoria* | *Paraconiothyrium brasiliense* |
| *Brevibacillus sp.*  | *Okibacterium fritillariae*  | *Aureobasidium pullulans* | *Plenodomus enteroleucus* |
| *Burkholderia sp.*  | *Pantoea agglomerans*  | *Chaetomium globosum* | *Talaromyces amestolkiae* |
| *Curtobacterium sp.* | *Paracoccus yeei*  | *Ciboria rufofusca* |  |
| *Deinococcus citri* | *Pseudomonas coleopterorum* | *Cladosporium sp.* |  |
| *Dermacoccus nishinomiyaensis* | *Ralstonia pickettii* | *Cryptovalsa ampelina* |  |
| *Dermacoccus sp.*  | *Sphingomonas echinoides* | *Cytospora cedri* |  |
| *Enterococcus faecium* | *Staphylococcus warneri*  | *Diatrype stigma*  |  |
| *Kocuria sp.*  | *Stenotrophomonas sp.*  | *Didymella pinodella*  |  |
| *Leifsonia sp.* |  | *Diplodia seriata* |  |

The bacterial endophyte population usually present in grape is variable because it can depend on different conditions (i.e., environmental, vineyard conduction, grape cultivar). As shown in several studies, the most isolated bacteria belong to the genus *Ralstonia sp., Burkholderia sp., Pseudomonas sp., Agrobacterium sp., Bacillus sp.,* and *Curtobacterium sp.* Similarly, fungal endophytes have been shown to belong to the genus *Alternaria sp., Didymella sp.,* and *Cladosporium sp.* (Campisano *et al.,* 2014, Pancher *et al.,* 2012, Aleynova *et al.,* 2021).

Regarding the ongoing activities, three BCA candidates were chosen to evaluate the activity against two strains of *B. cinerea.* Preliminary results obtained from dual-culture plate and double Petri dish have shown that BCAs are able to reduce the mycelium growth both by direct contact (production of metabolite/proteins/enzyme) and production of VOCs. GC/MS analysis have shown that in a liquid co-culture system, where all components are in direct contact, volatile compounds such as acids, alcohols, and a low quantity of terpenes are released (e.g., benzyl alcohol, butanoic acid, longifolene). Moreover, the microscopy observation has shown that, when placed in co-culture, fungi and BCAs interact directly with plant cells. Finally, a reduction in the expression of genes belonging to the phenylpropanoid pathway in a co-culture system compared with callus/*B. cinerea* control culture has been shown which suggests that BCAs may have inhibitory properties against fungal activity.

# **4. References**

Aleynova OA, Suprun AR, Nityagovsky NN, Dubrovina AS, Kiselev KV (2021) The influence of the grapevine bacterial and fungal endophytes on biomass accumulation and stilbene production by the in vitro cultivated cells of *Vitis amurensis Rupr*, *Plants (Basel)*. **10**(7):1276.

Campisano A, Antonielli L, Pancher M, Yousaf S, Pindo M, Pertot I (2014) Bacterial endophytic communities in the grapevine depend on pest management, *PLoS One*. **9**(11), e112763.

Pancher M, Ceol M, Corneo PE, Longa CM, Yousaf S, Pertot I, Campisano A (2012) Fungal endophytic communities in grapevines (*Vitis vinifera L.*) respond to crop management, *Appl Environ Microbiol*. **78**(12): 4308-4317.