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BIOCONTROL BACTERIA AGAINST BOTRYTIS GRAY MOLD AND OTHER STRAWBERRY FUNGAL PATHOGENS

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Abstract
Strawberries are listed among the functional foods. Beside their appreciated taste, they are rich in antioxidant compounds. Due to their sugars, fleshy pulp and thin skin, as well as dwarf habitus, they are exposed to various plant diseases, especially fungi. Botrytis gray mold is the most destroying pathogen, but there are other fungi that can cause losses in the field or after harvest. The aim of this study is to select some bacterial strains that could be easily formulated as biocontrol agents against various molds. These bacteria were isolated from winery semi-composted marc and analyzed through specific microbiologic methods. Based on their in vitro antagonistic activity, two bacterial isolates were selected. These strains revealed up to 84.2% inhibition of Botrytis cinerea growth, and high antifungal activity against various other fungi, such as Fusarium oxysporum, Macrophomina phaseolina and Sclerotinia sclerotiorum. Qualitative enzymatic tests, as well as microscopic analysis of the microbial interactions revealed fungal degradative enzymes produced by the bacterial strains. As the selected strains were determined to be spore producing bacteria, long term preservation is an advantage for their formulation as plant protection inoculants. Moreover, these bacteria revealed no phytotoxic activity against test plants.

Keywords: Bacillus, biocontrol bacteria, gray mold, strawberries.

1. INTRODUCTION

Strawberries are listed among the functional foods (Basu et al., 2014). Beside their appreciated taste, they are rich in antioxidant compounds (Azzini et al., 2010). However, due to their sugars, fleshy receptacle pulp and thin skin, as well as dwarf habitus, they are exposed to various plant diseases, especially fungi (Garrido et al., 2011).

Botrytis gray mold is the strawberries most common pathogen (Petrasch et al., 2019). It can cause flowers or fruits rot, and can be installed also after harvest. Beside *Botrytis cinerea*, there are other fungi that can cause losses in the field or after harvest (Garrido et al., 2011).

Although the preventing phytosanitary measures are the healthiest way of production, sometime, they are not enough, and should be sustained through various treatments. Nowadays consumers are oriented towards organic products or, at least obtained with less toxic interferences (Cierniak-Emerych et al., 2018). Among the biological methods for plant protection, the microbial resources are an environmentally friendly approach, some microbial strains being able to promote or stimulate plant growth (Bhargava et al., 2017).

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The aim of this study is to select some bacterial strains that could be easily formulated as biocontrol agents against various molds that can infect strawberry plants.

2. MATERIALS AND METHODS

Bacterial isolation

A winery semi-composted marc was used as microbial resource. Serial dilutions were prepared in sterile distilled water, for bacterial isolation. The first dilution resulted after one hour of continuous orbital shaking at 120 rpm, while the others were made by vortexing one minute. From the third to the fifth dilutions, 100 µl were plated on Luria Bertani (LB) agar and incubated at 28ºC. Cultivable microorganisms obtained on the LB medium were quantified and visually analyzed. The most abundant bacterial colonies, showing similar morphology, were selected for microbial isolation. New isolates were purified using the streak plate method on LB agar.

Bacterial characterization

New isolated bacterial strains were microbiological characterized to reveal their colony morphology, size, shape, margins and elevation, biomass consistency and color on LB agar. Gram reaction was also analyzed. Some qualitative tests were performed (table 1) by inoculating the isolates in spots on solid media.

Table 1. Qualitative assays

No.	Purpose	Culture media	Growth conditions	References
1	Phosphate solubilization	Modified Pikovskaya agar supplemented with bromothymol blue (PKV blue)	Seven days incubation at 28ºC	Boiu-Sicuia and Cornea, 2020
2	Phytate solubilization	Phytate salt agar medium (PSM)	Three days incubation at 28ºC	Dobre et al., 2016
3	Zinc solubilization	Bunt & Rovira (B&R) solid medium supplemented with zinc oxide (ZnO)	Seven days incubation at 28ºC	Abaid-Ullah et al., 2015
4	Arginine decarboxylase activity & potential polyamine synthesis	Arginine solid medium with phenol red	Overnight incubation at 28ºC	Sicuia et al., 2015

Microbial antagonism

Antifungal potential was tested using the modified double culture technique, on potato dextrose agar medium (PDA), in 9 cm diameter in Petri plates. The tests were performed against *Botrytis cinerea* mold and other three phytopathogens that can cause strawberry plant diseases, *Fusarium oxysporum* responsible of plant wilt, *Macrophomina phaseolina* (sin. *Sclerotium bataticola*) that can cause charcoal crown rot, and *Sclerotinia sclerotiorum* that can produce white rot. The fungal pathogens were inoculated in the center of the plate, as mycelial plugs of 6 mm in diameter. Each trial the inoculum was provided from fresh cultures on PDA. Tested bacterial antagonists were inoculated equidistant, in spots, at 2 cm away from the fungi. Control plates, inoculated only with the fungi, were also prepared each trial. Tests were performed in two trials of three replicates each. Plates were incubated at 27ºC for 10 days and analyzed periodically, after 3, 5, 7 and 10 days of incubation, and that stored at room temperature for three weeks.

Antifungal efficacy was evaluated according to Lahlali and Hirji (2010), using the following formula: **E(%) = (Rc-Ri)/Rc × 100**, where *E(%)* is the antifungal efficacy, *Rc* represents the radius

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of fungal growth in control plates, and *Ri* represents the radius of fungal growth influenced by the bacterial strain.

3. RESULTS AND DISCUSSIONS

Bacterial load

The analyzed winery semi-composted marc revealed a microbial load of $8.1\times10^6 \pm 7.45$ culturable bacteria. From the predominant colonies, with differentiated morphology, 6 isolates were selected and purified. These bacteria were named MTC 2.1 to 2.6.

Bacterial strains characterization

The bacterial strains isolated from the winery semi-composted marc were characterized for their colony and cell morphology (table 2), as well as for some beneficial traits than can be useful in plant growth stimulation, such as phosphorus solubilization from inorganic and organic sources, increased zinc bioavailability and arginine decarboxylation.

Table 2. Microbiologic characteristics of the isolated strains

Bacterial isolate	Colony morphology on LB agar	Cell characteristics
MTC 2.1	Small, flat colonies, rough, opaque and cream in color, irregular shaped, with undulate edge.	G+, rod
MTC 2.2	Medium size colonies of 3 mm in diameter, raised, rough, opaque and cream in color, irregular shaped, with undulate edge.	G+, rod
MTC 2.3	Extended filamentous growth, flat, opaque, whitish-cream in color.	G+, rod
MTC 2.4	Colonies of 3 to 5 mm in diameter, opaque, cream color, rhizoid with lobate edge, and umbonate elevation	G+, rod
MTC 2.5	Large colonies of 5 mm in diameter, raised, rough, opaque, whitish-cream in color, rhizoid shaped, with lobate edge	G+, rod
MTC 2.6	Small, semi-translucid colonies, with irregular edge and yellowish color.	G-

Tricalcium phosphate solubilization was attributed to the production of organic acids. This fact was highlighted by color change of PKV blue medium, from blue (pH 7.5) to green (pH 6.8) by the MTC 2.2 and MTC 2.3 strains, then to yellow (pH 5.5-6.0) by the other 4 isolates. The most intense activity of inorganic phosphorus solubilization was observed in MTC 2.4 and MTC 2.5 strains which produced 4 mm yellow halos around the colonies, due to organic acids production (figure 1).

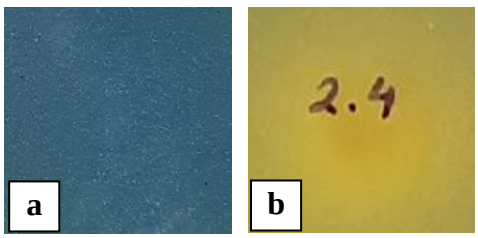

Figure 1. Tricalcium phosphate solubilization through bacterial organic acid production.
a. Uninoculated PKV blue medium, **b.** MTC2.4 strain modifying the pH and the color of the medium after the first 3 days of incubation

Organic phosphorus was also solubilized by tested bacterial strains. The MTC 2.4 and MTC 2.5 strains had the higher solubilizing activity of the calcium phytate, showing 4 mm clear halos around

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their colonies (figure 2). However, these strains were not able to grow on B&R medium supplemented with ZnO. The only strains revealing ZnO solubilization were MTC 2.2 and MTC 2.3 (table 3).

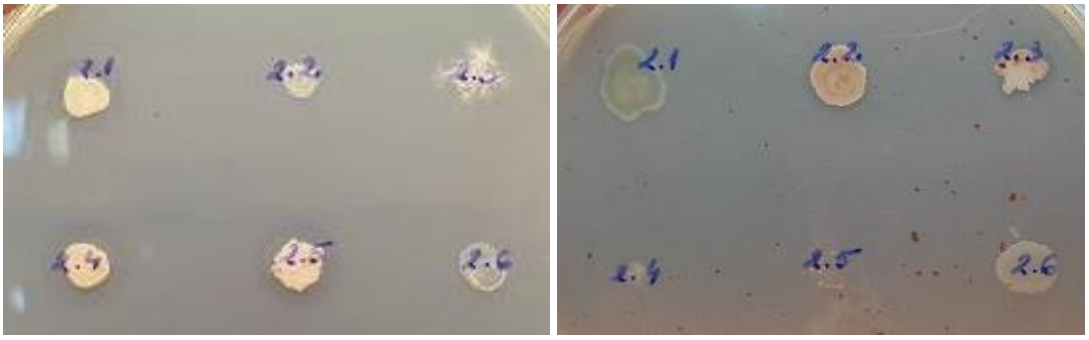

Figure 2. Phytate and ZnO solubilisation revealed by incubating the bacterial strains on PSM (left) and B&R+ZnO media (right) The clear halos arround bacterial colonies are indicating the solubilizing strains

Table 3. Solubilizing ability of the bacterial strains

Bacterial isolate	Phosphate solubilization after 7 days	Phytate solubilization after 3 days	ZnO solubilization after 7 days
MTC 2.1	+ 2mm yellow footprint and hallo	3mm halo	no halo
MTC 2.2	± green footprint and halo	no halo	1mm halo
MTC 2.3	± green footprint and halo	0.5mm halo	1mm halo
MTC 2.4	++ 4mm yellow hallo and footprint	4mm halo	no growth
MTC 2.5	++ 4mm yellow hallo and footprint	4mm halo	no growth
MTC 2.6	+ yellow footprint and green halo	0.5mm halo	no halo

Arginine decarboxylase production was revealed by all tested strains. This enzyme is the first step in polyamine pathway (Fortes et al., 2011). While the biosynthesis of the polyamine compounds plays important role in bacterial cell growth and proliferation (Suzuki and Kurihara, 2015), when these compounds are delivered to plants, they act as systemic protectants against environmental stress, beside their involvement in embryo development and plant flowering. Moreover, they can delay the senescence process, prolonging vegetation in harsh environmental conditions (Chen et al., 2019).

Antifungal activity

The newly isolated bacterial strains were analyzed *in vitro* for their antifungal potential. The antagonistic efficacy was evaluated after one week of co-cultivation with the tested phytopathogens, *Botrytis cinerea*, *Fusarium oxysporum*, *Macrophomina phaseolina*, and *Sclerotinia sclerotiorum*. The clear inhibition zones were also biometric evaluated.

Among tested bacteria, only MTC 2.4 and MTC 2.5 isolates revealed antifungal potential (table 4) and clear inhibition zones (figure 3). Their antagonistic activity was maintained during the incubation and storage time.

Although *Bacillus* spp. bacteria are mentioned to have biocontrol activity also *in vivo* trials (Albayrak, 2019), they are less used in commercial strawberry protection (Pertot et al., 2017). *Bacillus* sp. are not the only bacteria to be used in biocontrol, however they are preferred due to

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their spore forming ability (Kefi et al., 2015), adaptability to various and unstable conditions (Valdivia-Anistro et al., 2018), as well as their safeness for untargeted hosts and human health (Koutsoumanis et al., 2020; Spears et al., 2021).

Table 4. Antifungal activity

Bacterial isolate	*Botrytis cinerea*		*Fusarium oxysporum*		*Macrophomina phaseolina*		*Sclerotinia sclerotiorum*	
	E (%)	C.Z. (mm)	E (%)	C.Z. (mm)	E (%)	C.Z. (mm)	E (%)	C.Z. (mm)
MTC 2.1	5.26±0.16	0	2.63±0.18	0	5.12±0.78	0	5.00±0.53	0
MTC 2.2	7.89±0.95	0	2.63±0.13	0	5.12±0.82	0	N.A.	
MTC 2.3	21.05±0.26	0	2.63±0.21	0	2.56±0.19	0	5.00±0.55	0
MTC 2.4	84.21±1.73	5	73.68±1.23	6	84.61±2.13	5	87.50±0.27	8
MTC 2.5	81.57±2.71	2	73.68±1.69	2	84.61±2.53	2	82.50±1.34	6
MTC 2.6	2.63±0.23	0	5.26±0.28	0	2.56±0.53	0	N.A.	

Legend: E(%) is the antifungal efficacy percentage; C.Z. is the clear zone, were the media in not colonized by the microorganisms, due the antimicrobial compounds released in the substrate by the antagonistic strain; N.A. means not available.

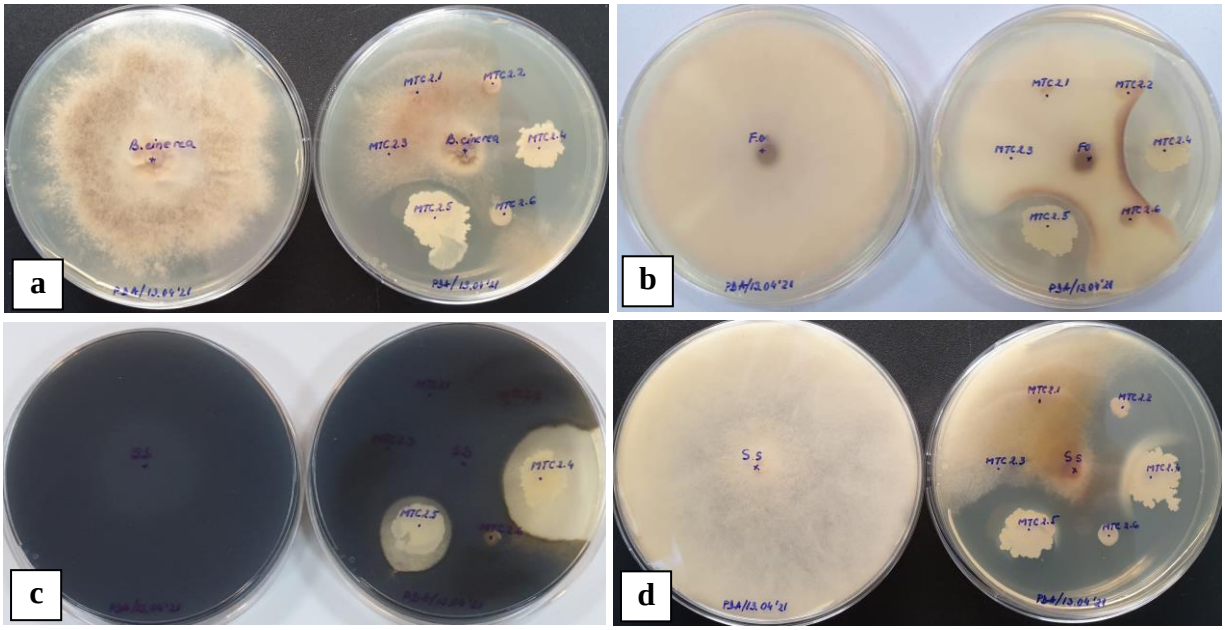

Figure 4. Antifungal activity of tested bacterial strains against various pathogens, after 10 days of incubation a. Botrytis cinerea, b. Fusarium oxysporum, c. Macrophomina phaseolina, and d. Sclerotinia sclerotiorum. Control plates are in the left part, while test plates at the right.

MTC 2.4 and MTC 2.5 proved a broad-spectrum antifungal activity, *in vitro*, and biocontrol potential against these strawberry phytopathogens. Due to the wider clear zone around the MTC 2.4 isolate, when tested against the phytopathogenic fungi, it is believed that this bacterial strain can release a wider amount and variety of antimicrobial compounds, compared to MTC 2.5 strain. However, these isolates expressed a synergic effect when tested together towards *B. cinerea* and *S. sclerotiorum*.

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Microscopic analysis of direct interaction between the newly isolated antifungal bacteria and studied fungal phytopathogens revealed extreme mycelia alterations and cell disruptions (Figure 5).

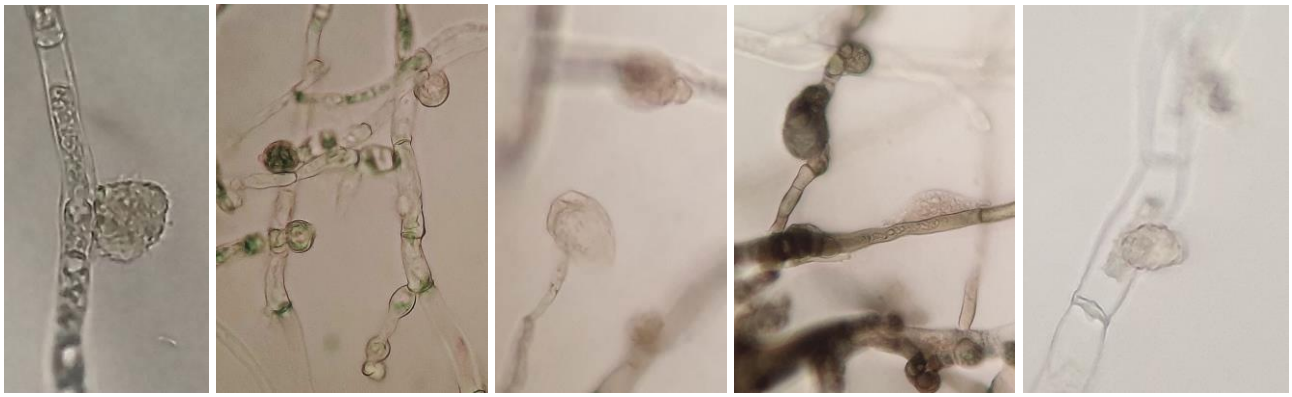

Figure 5. Mycelia alterations and cell disruptions caused by biocontrol bacteria to various fungal pathogens a. Botrytis cinerea, b. Fusarium oxysporum, c. & d. Macrophomina phaseolina, and e. Sclerotinia sclerotiorum.

The biocontrol bacteria induced cell lysis in *Botrytis cinerea*, *Macrophomina phaseolina*, and *Sclerotinia sclerotiorum* during microbial interaction. There could be seen fungal cell perforation and cytoplasm leakage, only were the mycelia grew in the proximity of the antagonistic bacteria. To *Macrophomina phaseolina* swelling were seen before cells' damage (Figure 5 c, d). Disruptions were noticed also in *Fusarium oxysporum* interaction with the studied biocontrol bacteria. Where the mycelia suffered severe cells' swelling (Figure 5b). Similar aspects were previously described in bacteria-fungal antagonistic interactions by our research group (Boiu-Sicuia et al., 2017, 2021) and other scientists (Dijksterhuis et al., 1999; Bapat and Shah, 2000).

4. CONCLUSIONS

Winery semi-composted marc is a source of plant beneficial microorganisms with plat growth promoting traits and antifungal potential, able to inhibit various phytopathogenic molds and rots. The microbial load in the winery semi-composted marc revealed 10^6 cfu/g. Within the isolated strains plant beneficial bacteria having nutrient solubilizing activity were detected. Bacterial strains MTC2.4 and MTC2.5 were able to solubilize mineral and organic phosphorus, and to inhibit the growth of *Botrytis cinerea*, *Fusarium oxysporum*, *Macrophomina phaseolina* and *Sclerotinia sclerotiorum* pathogens.

5. ACKNOWLEDGEMENTS

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