Biocontrol perspectives of *Aspergillus carbonarius*, *Botrytis cinerea* and *Pencillium expansum* on grapes using epiphytic bacteria isolated from Romanian vineyards

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Abstract
A total of 85 microbial strains (70 fungal strains and 15 bacterial strains) isolated from natural epiphytic flora of grapes from three Romanian vineyards were assessed in a preliminary screening for their antagonistic properties against *Aspergillus carbonarius*, *Botrytis cinerea*, *Penicillium expansum* and *Fusarium oxysporum*. Two bacterial isolates (FRI11V and FRI19V) were selected for their inhibition on the mycelia growth and antisporulation activity on these filamentous fungi. Furthermore, these bacterial isolates were assessed in vivo on wounded grape berries Fetească Regală to reduce the decay incidence of grapes caused by *A. carbonarius*, *B. cinerea* and *P. expansum*. The high inhibition of *A. carbonarius* and *B. cinerea* which ranged between 75 and 80% was observed in all samples treated with selected bacterial isolates. However, no inhibition effect was observed in the case of *P. expansum*.

Keywords: antagonistic bacteria, biocontrol, alteration molds, grapes.

1. Introduction
The grape microflora is complex and includes bacteria, yeasts and filamentous fungi. In the last years, the research has focused on filamentous fungi as potential spoilage agents which are responsible for significant economic losses in vineyards worldwide. Among spoilage microflora, *Aspergillus carbonarius* (the causal agent of black mold), *Botrytis cinerea* (the causal agent of gray mold) and *Penicillium expansum* (the causal agent of blue mold) were frequently detected on grapes which affects their characteristics and can greatly influence the hygienic or sensory quality of wine and derived products (ROUSSEAUX & al., [1]; DIGUTA & al., [2]; SAGE & al., [3], SERRA & al., [4]). Thus, the monitoring of this spoilage fungi is essential in the vineyard, in order to prevent/combat the contaminated grapes. Systematic application of synthetic fungicides such as fenhexamid, iprodione, cyprodinil, boscalid, pyrimethanil has been generally used in different phenological growth stages of the grapevine for the management of fungal decay incidence (DIGUTA & al., [5]; VALERO & al., [6]; DE COSTA AND BEZERRA, [7]; GABRIOLLOTTO & al., [8]). However, the excessive use of synthetic fungicides can have severely negative effects on epiphytic microbial communities, chemical residues on the treated grapes and environment (GABRIOLLOTTO & al., [8]; BELLÍ & al., [9]; CADEZ & al., [10], DEBODE & al., [11]; MILANOVIĆ & al., [12]). Several studies
demonstrated that the fungal pathogens developing resistance to the most common fungicides (ERRAMPALLI & al., [13]; DE MICCOLIS ANGELINI & al., [14]). Moreover, in view of the growing public concerns over environmental and health hazards, a strict legislation about fungicides’ use has been established in the European Union. The use of antagonistic microorganisms has emerged as a potential alternative to synthetic fungicides and offers economically viable and environmentally friendly for sustainable management of decay diseases caused by A. carbonarius, B. cinerea and P. expansum. The main mechanisms of action of the antagonistic microorganisms are: (i) competition for space and nutrients, (ii) production of bioactive molecules, such as antibiotics and cell-wall degrading enzymes, (iii) induction of defense-related responses in grapevine (GABRIOLLOTTO & al., [8]; MAGNIN-ROBERT & al., [15]; TROTEL-AZIZ & al., [16]; SHARMA & al., [17]). Bacteria, yeasts and yeast-like fungus are the main antagonistic microorganisms isolated and used as microbial biocontrol agents (BCAs) in pre and/or post-harvest of fruits. Numerous studies have been focused to isolate and identify the antagonistic yeasts and yeast-like fungus: Aureobasidium pullulans (SCHENA & al., [18]; DIMAKOPOULOU & al., [19]; RASPOR & al., [20]; DE CURTIS & al. [21]), Pichia guilliermondii (RASPOR & al., [20]; CHALUTZ & al., [22]), Metschnikowia fructicola (KARABULUT & al., [23]), Metschnikowia pulcherrima (RASPOR & al., [20]; DE CURTIS & al., [21], [24]; BLEVE & al., [25]; CSUTAK & al., [26]), Saccharomyces cerevisiae (RASPOR & al., [20]; NALLY & al., [27]), Cryptococcus laurentii (LIU & al., [28]; YU & al., [30]) Issatchenkia terricola, (BLEVE & al., [25]) Candida incommunis (BLEVE & al., [25]), Kluyveromyces thermotolerans (BLEVE & al., [25]; PONSONE & al., [31]).

Several bacterial biocontrol agents have isolated from vineyards: Acinetobacter lwoffii (MAGNIN-ROBERT & al., [15]; TROTEL-AZIZ & al., [16]), Pseudomonas fluorescens (MAGNIN-ROBERT & al., [15]; TROTEL-AZIZ & al., [16]), Pantoea agglomerans (MAGNIN-ROBERT & al., [15]; TROTEL-AZIZ & al., [16]), Bacillus subtilis (TROTEL-AZIZ & al., [16]).

However, only several commercial biocontrol products are available: Serenade ® (Bacillus subtilis), Pantovital™ (Bacillus amyloliquefaciens), Shemer™ (Metschnikowia fructicola), Candifruit™ (Candida sake) and Boni-Protec™ (the yeast-like fungus, Aureobasidiun pullulans).

The objectives of this work were: (i) to assess in vitro antagonistic microorganisms isolated from the surface of grapes collected from Romanian vineyards for their efficiency for the control of Aspergillus carbonarius, Botrytis cinerea, Penicillium expansum and Fusarium oxysporum, and (ii) to investigate in vivo the most effective antagonistic isolates on detached grape berries.

2. Materials and methods

Antagonistic microorganisms

70 fungal strains and 15 bacterial strains were isolated from different wine grapes from three Romanian vineyards (Valea Călugărească, Pietroasa, Minis-Măderat), during 2014 and used in this study. The bacterial strains were maintained and tested on Nutrient Agar (Merck, Germany) at 37°C, using 24 h old inoculums. Fungal strains were maintained and tested on Yeast Extract Glucose Agar (Merck, Germany) at 28°C, using 48h old inoculums. The number of cells was determined with the aid of a haemocytometer and adjusted to 10⁸ cells/mL with sterile distilled water.
**Fungal pathogens**

*Aspergillus carbonarius* (1 isolate), *Botrytis cinerea* (2 isolates), *Penicillium expansum* (4 isolates) and *Fusarium oxysporum* (1 isolate) were used in this study. The pathogen molds were isolated from Romanian vineyards and maintained on Potato-Dextrose Agar (PDA) (Meconti, Poland) medium and stored at 4°C. The pathogen inoculum consisted of spores and/or mycelium were released from 7-days-old cultures at 25°C by flooding sterile distilled water containing 0.2% (v/v) Tween 80. The number of spores was determined with the aid of a haemocytometer and adjusted to $10^5$ spores/mL with sterile distilled water. Fungal pathogens were identified by molecular method based on PCR-RFLP of the 5.8S-ITS region (DIGUTA & al., [2]).

**Dual cultural screening of antagonistic microorganisms**

All microbial isolates were tested by *in vitro* preliminary screening for their antagonistic activity against alteration molds. For this purpose, a loop of microbial cells from 24-48 h cultures was streaked in line at the distance of 2.5 cm from the centre of the PDA plate (9 cm diameter). After 24h, a fungal pathogen disk (5 mm diameter) was placed in the centre of each plate. The plates were incubated at 28°C for 7 days. The growth and spore production inhibition was observed. Results are the mean of three separate experiments with three repetitions for each sample.

**In vivo biological control A. carbonarius B. cinerea and P. expansum on grapes**

The most promising 2 bacterial isolates (FRVII11 and FRVII19) were assessed *in vivo* for biocontrol activity in a detached berry. Fresh, visually healthy grapes Fetească Regală (3-4 for each replicate) were disinfected with 1% (v/v) sodium hypochlorite for 5 min and then three times with sterile distilled water. Finally, grape berries were kept in 0.2% Carboxymethyl cellulose (CMC) for 2 min. Then, the grape berries were placed in covered sterile plastic boxes to maintain high humidity conditions by addition of 5 mL sterile water in each box. The grapes were wounded (approximately 3 mm wide and 3 mm deep) using a sterile needle. The bacterial suspension ($10^8$CFU/mL) was sprayed on each grape. After 2 h of incubation at room temperature, 10 μL of pathogen spore suspension of alteration molds ($10^5$ spores/mL) were pipetted into the wounds. The plastic boxes were incubated at different temperatures 15°C for 14 days, 28°C and 37°C for 5 days. Control negative was treated with the same volume of sterile distilled water and control positive was treated with spore suspension of alteration molds, under the same conditions. The results obtained are the mean of three independent experiments. After incubation period, wounds were examined and the percentage of fungal inhibition was determined as follows: Inhibition (%) = 100-(Number of decayed wounds/Number of total wounds×100). A reduction in disease incidence ranged 50%-70%, compared to the control treatment was adopted as criteria of selection of the best biocontrol bacteria.

3. Results and discussions

70 fungal strains and 15 bacterial strains isolated from different wine grapes from three Romanian vineyards (Valea Călugărească, Pietroasa, Miniş-Măderat) were used in this study. Antifungal activity was observed by inhibition mycelia growth and spore production in the zone surrounding the bacterial isolates (Fig.1).
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Aspergillus carbonarius

In our study, three of the 15 tested bacterial isolates showed antagonistic properties against *Aspergillus carbonarius* isolate (Fig. 1) under in vitro conditions. No inhibition effect of any other fungal isolates was observed in the case of *A. carbonarius* (data not shown). Two bacterial isolates (FRI11V and FRI19V) were assessed in vivo and was observed the reduction of infections of detached wounded berries by *A. carbonarius* with 75% compared to fungal growth on control berries (Fig. 2). The high biocontrol activity was observed at temperatures 28°C - 37°C. Others studies have been focused to isolate the antagonistic yeasts and yeast-like fungus. It is well known the potential antifungal activity *Aureobasidium* sp. and *Metschnikowia* sp. to control pre and/or post-harvest diseases when applied as biological control agents. DIMAKOPOULOU & al. [19] isolated an efficient *Aureobasidium pullulans* strain (Y-1) to control sour rot, *A. carbonarius* infection and the consequent OTA contamination in must. DE CURTIS & al. [24] showed that two yeasts species (*A. pullulans* and *Metschnikowia pulcherrima*) are capable of reducing infection by *Aspergillus carbonarius* and ochratoxin A production in healthy wine grape bunches (cv. ‘Montepulciano’). PONSENE & al. [31] isolated antagonistic yeasts from the epiphytic flora associated with grape berries (cv. Negroamaro) which reduced the *A. carbonarius* and *A. niger* colonization on grape berry. In particular, *Issatchenkia orientalis* isolates expressed high levels of antagonistic activity. Other antagonistic yeast, *Candida sake* presented inhibitory activity against *A. carbonarius* on grapes (22.8% disease incidence) (NALLY & al., [27]).

**Figure 1** Biocontrol activity *in vitro* of a three bacterial isolates towards alteration molds.
1- bacterial isolate FRI11V; 2- bacterial isolate FRI19V; 3- bacterial isolate FRCI25
A – *Aspergillus carbonarius*; B- *Botrytis cinerea*; C– *Penicillium expansum*; D – *Fusarium oxysporum*

**Figure 2** *In vivo* biological control *Aspergillus carbonarius* (A) and *Botrytis cinerea* (B) on detached grapes berries at 28°C
1- bacterial isolate FRI11V; 2- bacterial isolate FRI19V; C- control
Botrytis cinerea

Two bacterial isolates (FRI11V and FRVI19V) presented inhibitory activity against Botrytis cinerea isolates (Fig. 1) under in vitro conditions. Only, one fungal isolate showed lower antifungal activity (data not shown). The both bacterial isolates inhibited infections of detached wounded berries by B. cinerea (Fig. 2) with 80% compared to fungal growth on control berries. The high biocontrol activity was observed at temperatures of 28°C - 37°C. In the literature, several bacterial strains (Acinetobacter lwoffii, Bacillus subtilis, Pseudomonas fluorescens, Pantoaea agglomerans) exhibited biological control of Botrytis cinerea infections (MAGNIN-ROBERT & al., [15]; TROTEL-AZIZ & al., [16]). Numerous others studies have been focused to isolate and identify the antagonistic yeasts and yeast-like fungus. Metschnikowia species (M. fructicola and M. pulcherrima) have been assessed as biocontrol agents against Botrytis cinerea and others fungal pathogens (DE CURTIS & al., [21], [24]; RASPOR & al., [20]; CHALUTZ & al., [22]; KARABULUT & al., [23] BLEVE & al., [25]; CSUTAK & al., [26]). Different Aureobasidium pullulans strains showed high antifungal efficacy against B. cinerea and Monilinia laxa on single-wounded berries of sweet cherries and table grapes (SCHENA & al., [18]).

Penicillium expansum

The same bacterial isolates showed largest inhibition halos against P. expansum isolates under in vitro conditions (Fig.1). Two fungal isolates showed lower antifungal activity (data not shown). However, none bacterial isolate was able to reduce fungal growth of P. expansum on detached berries. In the literature, several antagonistic yeasts have been reported to be an effective as biocontrol agents against P. expansum and/or others fungal pathogens in fruits: Rahnella aquatilis (CALVO& al., [32]); Cryptococcus laurentii (LI and TIAN, [29]; YU & al., [30]), M. pulcherrima (JANISIEWICZ & al., [33]).

Fusarium oxysporum

Two bacterial isolates (FRI11V and FRVI19V) presented inhibitory activity against Fusarium oxysporum isolate. However, Fusarium sp. has been occasionally detected in epiphytic microflora of grapes (DIGUTA & al., [2]; Sage & al., [3]; Serra & al., [4]). In the literature, two yeasts isolates, Saccharomyces kluveri and Torulaspora delbrueckii from different viticultural environments significantly reduced F. oxysporum growth; the least isolate showed a higher antagonistic activity NALLY & al., [27].

4. Conclusions

The results reported in the present study showed that the microflora associated with grape berries in Romanian vineyards is an important source of biocontrol agents against spoilage microflora. Thus, our results show that two bacterial isolates (FRI11V and FRVI19V) exhibited the highest antifungal activity against four phytopathogenic fungi under in vitro conditions. Antagonistic activity was observed by inhibition mycelia growth and spore production in the zone surrounding of the bacterial isolates. The both bacterial isolates controlled A. carbonarius and B. cinerea infections on detached wounded berries. This work represents an initial step for further researches to identify antagonistic bacteria, to investigate the mode of action of the selected antagonistic bacteria, to enhance the biocontrol activity by combination with other antimicrobial compounds and to evaluate their effect directly on grape in different development stages; which could lead at an effective and affordable approach in the management of the spoilage microflora of the grapes.
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References
Aspergillus carbonarius (sour rot) incidence in wine-producing vineyards in Greece, Biological Control, 46, 158–165, (2008).