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BACTERIAL EXTRACTS AS NATURAL PREPARATIONS TO CONTROL FUNGAL PLANT DISEASES

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Abstract

Microorganisms represent a rich source of biologically active substances including antifungal compounds that can potentially be used as alternative methods to control fungal plant diseases. In the present study, a number of bacterial strains producing and releasing antifungal compounds were identified from natural forest soils and utilized to obtain antifungal preparations. Selected bacteria were associated to 4 different 16S genotypes clustering within the genus *Pseudomonas spp*. Alcoholic extracts from these bacteria proved a significant antifungal activity by inhibiting the mycelial growth of typical postharvest pathogens including *Botrytis cinerea, Penicillium digitatum, Penicillium italicum, Penicillium expansum, Monilinia fructicola,* and *Colletrotrichum acutatum*. The same extracts were particularly effective in reducing the incidence of rots on different artificially inoculated fruits such as citrus, apricots, sweet cherries, table grape, and tomatoes. On tangerines, the selected extracts also showed a curative activity since treatments made 24 h after the inoculations of *P. digitatum* reduced rots, suggesting the possible control of already established infections. In addition to the direct antifungal activity, the induction of resistance in treated host tissues was also demonstrated. In fact, on apricots inoculated with *M. fructicola* and on table grape and tomatoes inoculated with *B. cinerea* a significant reduction of rots was achieved without a direct contact between extracts and pathogens. Furthermore, these extracts proved effective under semi-commercial conditions by reducing natural rots on olive drupes naturally infected by *Colletotrichum* spp. and on sweet cherries subjected to cold storage and shelf-life. In field conditions, the most effective extract significantly reduced the incidence and severity of grape powdery mildew. According to metabolomics analyses, 521 target features were detected within the extracts of 3 selected bacteria. Although most of them were not annotated, some common *Pseudomonas* membrane structural lipids as well as natural products such as phenazine and orfamide analogs, known to have some antimicrobial and antifungal activity, were identified. Additional investigations are needed to correlate the composition of extracts with their biological activity, to identify putative active ingredients and ascertain their safety for humans' health and the environment. However, currently available data shows important features including: i) a high efficacy under different conditions and against a broad range of diseases, ii) complex mechanisms of action that include a direct antimicrobial activity and the induction of resistance in treated host tissues, and iii) the possible control of already established infection (curative action). Furthermore, selected bacteria can be easily grown with simple and cheap culturing media and their extracts are active at a very low concentration, suggesting

the possible development cost effective formulations, able to compete with traditional chemical compounds.

Keywords: *bacterial extract; Pseudomonas spp; soil; bioactive metabolites; biological control; plant pathogens*

Riassunto

I microrganismi rappresentano una ricca fonte di sostanze biologicamente attive tra cui composti con azione antifungina che possono essere potenzialmente utilizzati in strategie di lotta alternative contro le malattie fungine delle piante. Nel presente studio, numerosi ceppi batterici che producono e rilasciano nell'ambiente composti antifungini sono stati isolati da terreni forestali naturali ed è stato utilizzato per ottenere preparati antifungini. I batteri selezionati sono stati associati a 4 diversi genotipi del gene 16S tutti rientranti nel genere *Pseudomonas spp*. Gli estratti alcolici di questi batteri hanno dimostrato una significativa attività antifungina inibendo la crescita miceliare di alcuni tra i più importanti patogeni del post-raccolta tra cui *Botrytis cinerea, Penicillium digitatum, Penicillium italicum, Penicillium expansum, Monilinia fructicola,* e *Colletrotrichum acutatum.* Gli stessi estratti sono risultati particolarmente efficaci nel ridurre l'incidenza dei marciumi su diversi frutti inoculati artificialmente come agrumi, albicocche, ciliegie, uva da tavola e pomodori. Sui mandarini, alcuni estratti hanno mostrato anche un'attività curativa poiché hanno significativamente ridotto le infezioni di *P. digitatum* anche quando somministrati 24 ore dopo l'inoculazione del patogeno. Oltre all'attività antifungina diretta, è stata dimostrata la capacità di indurre resistenza nei tessuti dell'ospite. Infatti, su albicocche inoculate con *M. fructicola* e su uva da tavola e pomodori inoculati con *B. cinerea* i marciumi sono stati significativamente ridotti anche in assenza di un contatto diretto tra estratti e patogeni. Inoltre, questi estratti si sono dimostrati efficaci in condizioni semi-commerciali, riducendo i marciumi su olive naturalmente infettate da *Colletotrichum* spp. e su ciliegie sottoposte a frigoconservazione e shelf-life. In prove sperimentali di pieno campo, l'estratto più efficace ha inoltre significativamente ridotto l'incidenza e la gravità dell'oidio dell'uva da vino. Le analisi metabolomiche condotte sugli estratti di tre batteri hanno permesso il rilevamento di 521 *"target features"*. Sebbene la maggior parte di esse non siano state annotate è stato possibile identificare alcuni lipidi strutturali di membrana tipici di *Pseudomonas* e prodotti naturali come gli analoghi della fenazina e dell'orfamide, noti per avere una certa attività antimicrobica e antifungina. Ulteriori indagini sono necessarie per correlare la composizione degli estratti con la loro attività biologica, per identificare i principi attivi e per verificare che non rappresentino un pericolo per la salute umana e per l'ambiente. Tuttavia, i dati attualmente disponibili mostrano importanti caratteristiche tra cui: 1) un'elevata efficacia in diverse condizioni e contro un'ampia gamma di malattie, 2) complessi meccanismi d'azione che comprendono attività antimicrobica diretta e induzione di resistenza nei tessuti dell'ospite, e 3) la possibilità di controllare le malattie anche in una fase avanzata dell'infezione (azione curativa). Inoltre, i batteri selezionati possono essere facilmente coltivati con mezzi di coltura semplici

ed economici ed i loro estratti sono efficaci ad una concentrazione molto bassa, suggerendo il possibile sviluppo di formulazioni economiche, in grado di competere con i composti chimici tradizionali.

Parole chiave: *estratti batterici; Pseudomonas spp; suolo; metaboliti bioattivi; lotta biologica; patogeni delle piante*

1. Introduction

Fruits and vegetable are an important part of the human diet since they are a source of vitamins and minerals and contain important compounds such as antioxidants. The importance for human health of a diet rich in these products is increasing their consumption, as well as the request for high quality and safe products free of pesticide residues and toxins. Fruit and vegetables are characterized by a high perishability at every stage of production, especially in the post-harvest and storage phases when they are subject to multiple biotic and abiotic stresses. It has been estimated that about 20–25% of the harvested fruits and vegetables are lost during post-harvest handling (El-Ghaouth et al., 2004; Korsten, 2006; Zhu, 2006; Singh and Sharma, 2007). This is particularly relevant in developing countries due to inadequate storage and transportation. Different methods used for controlling phytopathogens have been proposed and, among them, chemical control based on synthetic products has represented the mainstay of crop protection for immediate effectiveness and ease of application. The use of synthetic chemicals as fungicides is also a primary method of control of post-harvest fungal decay of fruit. However, with the latest national and international legislative restrictions and the rise of consumer awareness in human and environmental health, the development of safe and eco-friendly alternative control means to control plant diseases has become an imperative need (Aktar et al., 2009; Wisniewski et al., 2016; Belgacem et al., 2021). The consumer's trend appears to be shifting towards reduced use of fungicides on produce and hence, there is strong consumers desire to seek safer and more ecofriendly alternative control means (Feliziani and Romanazzi, 2013, Mari et al., 2014). Among different alternative approaches, particular interest has been shown in the use of the microbial antagonists like fungi and bacteria (Eckert and Ogawa, 1988; Wilson, 1994; Korsten, 2006). In recent years, various biocontrol agents (BCA) have been developed and commercialized to be used against phytopathogenic fungi (El-Baky and Amara, 2021)

1.1. *Mechanisms of action*

The mechanisms of action of bacteria and fungi as BCAs have been extensively investigated in the last 30 years however, currently available information are still incomplete mainly because of difficulties associated with the study of complex interactions between a host, a pathogen, and an antagonist (Fig. 1) (Droby et al., 2009; Bahadou, S.A, et al 2018; Thokchom, E, et al 2017). Notably space and nutrient competition appears to play a major role in the biocontrol activity of many postharvest

antagonists, but it's rare that a single mechanism of action might be involved in suppressing a disease (Janisiewicz et al., 2000). A biocontrol agent is generally equipped with several features that working in concert, can be crucial for controlling disease development. For example, nutrient competition may be related to the ability of biocontrol agents to adhere to specific sites of host and pathogen tissues (Wisniewski et al., 1991, 2007). Furthermore, microorganisms can induce resistance in host tissue, can produce antimicrobial substances (water soluble or volatile) and eventually can also produce specific active metabolites upon the interaction with fruit/plant tissues (Droby et al., 2002; Schotsmans et al., 2008).

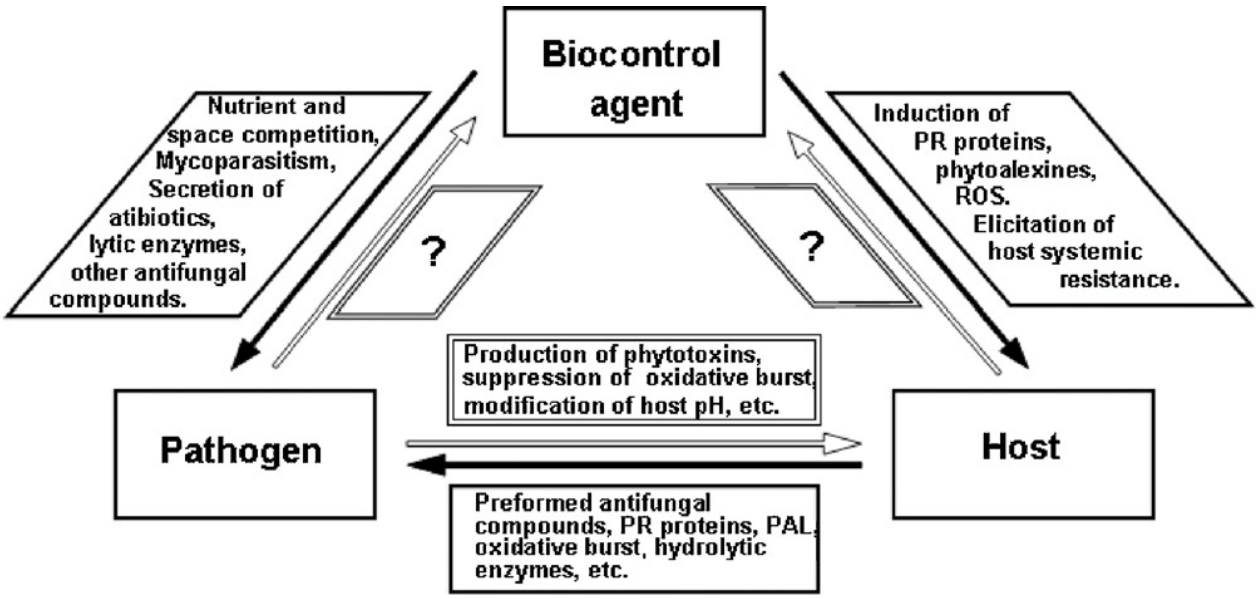

Fig. 1: *Diagram of possible interactions between host, pathogen and antagonist. Question marks indicate interactions that have not been studied (Droby et al., 2009).*

1.1.1. Competition for space and nutrients

Competition for space and nutritive sources is one of the main antagonistic mechanisms exerted by biocontrol bacteria and yeasts against phytopathogens (Di Francesco et al, 2016). The capacity of bacteria to colonize damaged fruit depends on the higher assimilation of the necessary sources for survival and multiplication, limiting nutrients disposition for the phytopathogen and reducing spore germination and capacity to invade host tissues (Hernandez-Montiel et al, 2018). Different studies have demonstrated that antagonistic microorganisms limit phytopathogen fungi subtracting different carbon

sources. For instance, the bacteria *Pseudomonas putida* inhibits the spore germination of *Penicillium digitatum* reducing the nutrient availability (Yu et al, 2015). Another essential element for microorganism growth is iron (Terpilowska et al, 2019). BCAs can produce small, high-affinity iron-chelating compounds called siderophores that can capture iron molecules (Golonka, et al, 2019). Consequently, siderophores inhibit phytopathogens by limiting spore germination and mycelial growth (Cordova-Albores et al., 2016). Diverse types of siderophores produced by antagonistic bacteria have been identified and reported. Their production confers competitive advantages to the bacteria used as BCA by excluding other microorganisms including phytopathogens (Drehe et al., 2018; Carmona-Hernandez et al., 2019).

1.1.2. Parasitism

Parasitism takes place when the antagonist feeds on the phytopathogen, producing partial or total lysis of its structures. Bacteria have been reported to feed on the cell walls of fungi, mainly on chitin, glucans, and proteins (Spadaro et al, 2016). Bacteria decompose the fungal cell wall by lithic enzymes, mainly β-1,3-glucanase, chitinase, and protease (Safdarpour et al., 2019). During the last years, different research studies have been performed on hydrolytic enzymes produced by bacteria (Bahadou et al., 2018). The genera *Bacillus* and *Pseudomonas* are considered as some of the most efficient antagonists in phytopathogen control due to the direct action of chitinases (Yu et al., 2008). Chitinases hydrolyze chitin, the non-branched homopolymeric N-Acetyl glucosamine in a 1,4 link by exo-chitinase or N-Acetyl-b-glucosaminidase, which sequentially segments the NAG extreme residues and endo-chitinase, which activates links in aleatory sites along the polymer chain (Stoykov et al., 2015; Carmona-Hernandez et al., 2019).

1.1.3. Induction of resistance

The activation of resistance responses, associated with the use of alternative control means, is considered the preferred approach in integrated control strategies against postharvest diseases (Ippolito et al., 2000; Droby et al., 2002). During ripening and senescence of fruit and other vegetative plant parts after harvest, disease resistance generally declines leading to increased susceptibility to pathogen attack. Hence, the use of resistance inducers may be useful to restore an acceptable level of resistance. In this regard, a variety of physical and biological elicitors are capable of inducing resistance responses, but the nature of responses to different elicitors is largely unknown (Wilson, 1994). The use of the microbial

antagonists as inducers of resistance is a developing and very promising field of research in plant protection (Ippolito et al., 2000; Romanazzi et al., 2016). Induction of disease resistance responses was reported, for example, in pineapple, avocado, and citrus fruits. Microbial antagonists can induce disease resistance in the harvested commodities by the production of antifungal compounds (Yak-oby et al., 2001), or by accumulation of phytoalexins, like scoparone and scopoletin in citrus fruit (Rodov et al., 1994). The production of such antifungal compounds by microbial antagonists in the host cells premises to induce defense mechanisms and provide phytopathogen control on the harvested commodities.

1.1.4. Production of antibiotics

Antibiosis is one of the most important mechanisms of action of BCA. Antibiosis can be performed through the production of nonvolatile high-molecular-weight metabolites or by low-molecular-weight volatile metabolites. Latter compounds are called volatile organic compounds (VOCs) and can have a direct effect on the growth of phytopathogen. These compounds can travel great distances in structurally heterogeneous environments, as well as in solid, liquid, or gas compounds, which is a great advantage for BCA. Example of antibiotics produced by microorganisms are pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG) and phenazines produced by *Pseudomonas spp.* These compounds can play an important role in biocontrol (Neidig et al. 2011; Nandi et al. 2015; Mishra and Arora 2018). Pyrrolnitrin is a phenylpyrrole with strong antifungal activity by inhibiting the fungal respiratory chains (Kirner et al. 1998). DAPG is toxic to a wide range of pathogenic fungi (Bangera and Thomashow 1999). Pyoluteorin is toxic against oomycetes, while phenazines are low-molecular-weight metabolites with a broad-spectrum antimicrobial activity. Most studies have described that the antimicrobial effects of phenazines are mainly attributed to their ability to generate reactive oxygen species (ROS) (Laursen and Nielsen, 2004, Mavrodi et al., 2006). Specifically, phenazine-1-carboxylic acid (PCA) and pyocyanin (PYO) were found to be very effective in the treatment of fungal diseases. Strain B2017 of *Pseudomonas putida* proved to be an effective BCA against *Fusarium oxysporum f.sp. radicis-lycopersici, Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Oliver et al. 2019). The analysis of secondary metabolites highlighted that *P. putida* does not produce toxic metabolites/antibiotics for mammalians (Mudgal et al. 2013; OECD 2018).

1.1.5. Microorganisms as a source of active metabolites

Although antibiosis is considered one of the most important mechanisms of action of BCAs the use of microbial bioactive compounds (MBCs) as natural antifungal molecules is largely uninvestigated. However, in the last few years the interest toward compounds derived from the metabolism of microorganisms is rapidly increasing (Alfonzo et al., 2021). In fact, the great genetic diversity of microorganisms represents an incredible source of natural antimicrobials and may be the key to control phytopathogens in a more sustainable and environmentally friendly way (Bastida et al., 2021). In particular, soil is one of the environments with the highest microbial diversity. Interestingly, various antimicrobials also induce systemic resistance in host tissues greatly increasing their efficacy. Among the most studied microorganisms filamentous actinobacteria stands out as the largest group of metabolite producers, followed by the genera *Pseudomonas* and *Bacillus* (Navarro et al., 2019).

1.2. *Aim of the thesis*

Aim of the present study was to evaluate the potential use of microbial bioactive compounds (MBCs) as alternative, environmentally compatible bio-pesticides for plant disease management. A selective method to identify microorganisms (mainly bacteria) producing antifungal compounds was developed and evaluated. Selected bacteria were identified and their extracts were extensively evaluated to determine *in vitro* and *in vivo* efficacy again major fungal plant pathogens. Furthermore, the efficacy of extracts in controlling plant diseases was evaluated both in experimental conditions as well as semi-commercial trials. Finally, specific metabolomics analyses were performed to evaluate the composition of the most effective extracts.

2. Materials and Methods

2.1. Inoculum of fungal pathogens

Fungal pathogens utilized in the present study comprised an isolate of *Penicillium digitatum* from oranges, *Penicillium expansum* from apples, *Botrytis cinerea* from table grape berries, *Colletotrichum acutatum sensu stricto* and *Alternaria alternata* from olive drupes and *Monilinia fructicola* from apricots. All isolates came from the Fungal Culture Collection of the Department of Agriculture, University Mediterranea of Reggio Calabria, and were stored on Potato Dextrose Agar (PDA) (Oxoid, UK) at 4°C. To facilitate the production of conidia, all fungi were grown in PDA plates at approximately 20°C for 7-10 days and receive indirect sunlight during the daytime. Conidia were collected with a sterile spatula, suspended in sterile distilled water, filtered through a double layer of sterile gauze and vortexed for 1 min to ensure uniform mixing. The concentration of conidia was evaluated using a Thoma cell counting chamber (Marienfeld, Germany) and properly diluted to have stock solutions of 10^6 conidia/ml.

2.2. Source of bacteria producing antifungal compounds

Soil samples were collected in five different forests or woodlands located in Calabria, Southern Italy (Table 1). In all localities, the litter was removed and the upper layer of true soil commonly containing the highest population of microorganisms was harvested using a sterile gardening shovel and kept in sterile plastic bags. In each locality, 10 subsamples of approximately 100 g were collected within a radius of 50 m, pooled and accurately mixed to have a soil sample of 1 kg per locality.

2.3. Selection of bacteria producing antifungal compounds

Soils were preliminary sieved with a 2 mm mesh to eliminate residues of leaves, roots, small stones and other debris. Per each soil, three subsamples of 10 g were suspended in 100 ml of water in Erlenmeyer flasks, and vigorously shaken, using magnetic stirrers. Per each subsample one ml of soil suspension was collected while keeping soils agitated and serially diluted (1:10; 1:100; 1:1000) with sterile distilled water.

One hundred µl of each diluted soil suspension were mixed with and equal volume of a spore suspension of *Penicillium digitatum* (1 x 10^6 CFU/ml), poured into Petri dishes (Ø 90 mm) containing PDA and uniformly distributed using a sterile cell spreader. Petri dishes (5 per each dilution) were incubated at 25 ± 2°C.

From 4 to 7 days after inoculation plates were periodically observed to identify bacteria producing inhibition halos around their colonies. They appeared as small spots of agar without mycelium of *P. digitatum* with a bacterial colony in the middle. Altogether, 125 bacterial colonies showing clear inhibition halos were collected using a sterile loop and subjected to streaking on nutrient broth agar (NBA) (Oxoid, UK) in order to have bacterial isolates from single cell (Table 1).

A preliminary gross screening of these bacteria was performed by accurately observing morphological features of colonies (shape, color and size) after 3-7 days of incubations at 24°C. Colonies showing identical morphological characteristics and obtained from the same soil sample were assumed to be the same strain and, consequently, a single representative isolate was kept.

According to this preliminary screening 22 bacteria were selected and kept on NBA at 5°C for short term storage and in nutrient broth (NB) (Oxoid, UK) containing 15% glycerol at - 80°C for long term storage.

Table 1. *Source of soils utilized to isolate bacteria, number of isolates collected from each soil, and representative isolates (22) selected according to morphological features.*

Locality	**Prevalent plant species**	**GPS coordinates**	**Collected bacteria (N.)**	**Selected bacteria isolates**
Reggio Calabria	*Pinus pinea*	38°07'10"N/15°40'05"E	17	B2, B3, B9, B17, B18
		38°07'12"N/15°40'01"E	23	B5, B15, B10
		38°07'16.3"N/15°40'07.1"E	18	B13, B16, B19, B20
Serra S. Bruno (VV)	*Abies alba*	38°33'42.4"N/16°18'54.2"E	20	B1, B11, B12
Stalettì (CZ)	*Quercus ilex*	38°45'26.7"N/16°33'16.4"N	15	B4
Arena (VV)	*Fagus sylvatica*	38°33'43.1"N/16°12'37.4"E	19	B6, B7, B8, B14, B22
Bova (RC)	*Pinus nigra laricio*	38°02'15.9"N/15°57'14.1"E	13	B21
Tototal			**125**	**22**

2.4. In vitro antifungal and antioomycete activity of bacteria

A dual culture assay method was utilized to estimate the capability of producing antimicrobial substances by the preliminary 22 selected bacterial strains. Tests were performed as described by Ibrahim *et al.* (2017), with two fungal (*Botrytis cinerea* and *Alternaria alternata*) and an oomycete (*Phytophtora palmivora*) pathogens. A 0.4 cm PDA agar plug, taken from 5 days fungal/oomycete cultures was placed on a side of a 90 mm Petri dish containing PDA. Bacteria isolates were inoculated in a single point in the opposite side of the dish using a needle. Three plates were inoculated per each BCA/pathogen combination. All plates were incubated at 22 ± 2°C in the dark and the radial growth of pathogens was measured after 5 days. Controls plates were only inoculated with the pathogens.

The inhibition activity was evaluated by measuring the radius of the pathogen colonies without (R1) and with the bacterial colonies (R2). The two readings were transformed into percentage inhibition of radial growth (PIRG) using the formula developed by Skidmore and Dickinson (1976)

$$\text{PIRG (\%)} = \frac{(R1-R2)}{R1} \text{ x100}$$

2.5. Screening of bacteria on artificially inoculated fruit

The preliminary 22 selected bacteria strains were tested on artificially inoculated fruit. Tests were performed on tangerines, cv. Avana inoculated with *P. digitatum*, tomatoes, cv Datterino inoculated with *A. alternata* and *B. cinerea*, apples, cv Golden Delicious inoculated with *P. expansum*, table grape berries, cv Italia inoculated with *B. cinerea* and olives drupes, cv Ottobratica inoculated with *C. acutatum*. All fruits were selected for uniformity in size and color. Fruits were surface sterilized by immersion in a 2% sodium hypochlorite solution for 2 min, washed with tap water, air-dried and fixed on polypropylene pads using a double-sided tape (Li Destri Nicosia et al., 2016). Fruits were kept 1–2 cm apart to avoid nesting and wounded to a uniform and standard depth of 3 mm using a nail with diameter of 1 mm. Wounds were inoculated by applying 10 µl of bacterial suspensions or water (control) and inoculated 2h later with 10 µl of fungal conidial suspension. A pomegranate peel extract (PGE) at the concentration 6 mg/L was also included in all trials as addition control (Li Destri et al., 2016; Belgacem et al., 2021)

The inoculum of bacteria was prepared by growing each isolate in 5 ml of Luria – Bertani broth (LB) (Difco, USA) in 25 ml Erlenmeyer flasks on a rotary shaker at 100 rpm for 48 h at 25°C. Cells were

harvested by centrifugation (1100 x g for 15 min), rinsed twice with water, and resuspended in 5 ml of distilled water. All bacterial suspensions were diluted 10 times and utilized to inoculate fruits. The pathogen inoculum was prepared as described above (section 2.1) and diluted with distilled water to have $5x10^4$ (*P. digitatum*) or 10^5 (*P. expansum, A. alternata, C. acutatum* and *B. cinerea*) conidia/ml. Mock wounds treated with sterile water and inoculated with the pathogens served as controls.

In all trials, treated fruits were maintained at room temperature (22±2°C) in plastic boxes containing wet paper to ensure high relative humidity (RH). Decays were evaluated daily starting from 4 days post inoculation by recording the percentage of infected wounds. The incidence of rots was utilized to evaluate the percentage of inhibition (INHIB)

$$\text{INHIB (\%)} = \frac{(IRc-IRx)}{IRc} \text{ x100}$$

Where IRc and IRx indicate the incidence of the rot on control fruit and on fruit treated with each specific bacterium, respectively.

2.6. Molecular characterization of selected microorganisms

Eight bacteria (B1, B2, B5, B6, B7, B8, B9 and B10) selected according to results of preliminary *in vitro* and *in vivo* screenings were molecularly characterized by sequencing the 16S ribosomal RNA and by Box-PCR. To extract nucleic acids, bacterial cells were collected from single colonies using a sterile inoculating loop and suspended in 300 µl of sterile distilled water in 1.5 ml Eppendorf tubes. After brief vortexing, bacterial suspensions were mechanically lysed in a Ribolyzer for 30 s at 6.5 m/s. Eppendorf were centrifuged for 3 min at 4000 rpm and the supernatant (approximately 200 µl) was transferred in clean tubes and used for PCR amplification.

Box-PCR reactions were performed using the BOX-A1R primer (Smith et al., 2001; Versalovic et al., 1994) following the official EPPO protocol (PM 7/100(1), 2010). Amplification products were analysed by electrophoresis (5 hours at 110V) in 1.5% agarose gels containing SYBR Safe™ DNA gel stain (Invitrogen) in TBE buffer and visualized with UV light.

The 16S region was amplified with primers 27F-1492R (Heuer et al., 1997). Amplifications were performed in a total volume of 30 µL containing 1 µL of template DNA, 10 µM of each primer and 6 µL of Taq&Go polymerase (MP Biomedicals). PCR amplification conditions consisted of: 1 cycle of 94°C

for 4 min; 25 cycles of 98°C for 30 s, 50°C for 30s, 72 °C for 90s; and a final cycle of 72°C for 5 min. PCR bands were purified with the MinElute PCR Purification Kit (Quiagen Ltd.West Sussex, UK) to remove excess primers and nucleotides. Sequencing was carried out with the same primers utilized for the amplification in a dye-terminator cycle-sequencing reaction (FS sequencing kit, Applied Biosystems,Warrington, UK) and run on an ABI373 automated sequencer (Applied Biosystems). The CHROMASPRO v. 1.5 software (http://www.technelysium.com.au/) was used to evaluate reliability of sequences and to create consensus sequences.

Reliable DNA sequences were deposited in GenBank with accession numbers from OK135771 to OK135778. Sequences were aligned using the software MUSCLE and compared by constructing a phylogenetic tree using the Maximum Likelihood method and the Tamura-Nei models as implemented in MEGA X (Kumar et al., 2018). Bootstrap values were obtained from 500 repetitions. Furthermore, sequences were compared with GenBank available sequences according to nucleotide BLASTN analyses (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Following BLAST analyses the Multiple Sequence Alignment Viewer (MSA Viewer) tool was utilized to compare our sequences with other sequences deposited in GenBank.

2.7. Alcoholic extraction of compounds with antifungal activity

Extractions were performed from the 8 selected bacteria (B1, B2, B5, B6, B7, B8, B9 and B10) with water/ethanol solution (80%). To perform extractions, bacteria were grown on NA and cells were collected with a sterile loop (Ext.1). Two additional extraction methods (Ext.2 and Ext.3) were only tested for bacteria B1, B5 and B9. These methods provided the growth of bacteria on NB and the extraction from bacterial cells after harvesting by centrifugation (Ext.2) or from both bacteria and culturing medium (Ext.3). All extracts were dried, suspended in absolute (100%) ethanol to have stock solutions containing 3 g/L of dried extract, and stored at -20°C until use. Details about extraction methods are not reported because extracts are currently under patenting.

2.8. Minimal Inhibitory Concentration (MIC) of alcoholic extracts

The Minimal Inhibitory Concentration (MIC) of alcoholic extracts was determined for the eight selected isolates (B1, B2, B5, B6, B7, B8, B9 and B10) using extracts obtained with the first method (Ext.1). Furthermore, the MIC of isolates B1, B5 and B9 was also tested for extracts obtained with the other two methods (Ext.2 and Ext.3) in order to compare extraction methods.

In all trials, 7 sterile absorbent paper pads with a diameter of 3 mm (Whatman Schleicher & Schuell) were placed inside a Petri dish containing PDA after uniform distribution of 100 µl of spore suspension of *P. digitatum* (10^5 conidia/ml). The extract was diluted just before its use with sterile distilled water to have final concentrations of 1, 3, 10, 30, 100 and 300 mg/L. Ten µl of each dilution were added on each pad. Ten µl of a water solution containing 100 ml/L of ethanol were added to control pads since this was the concentration of ethanol in most concentrated extract (300 mg/L). All plates (3 per each concentration and extract) were wrapped in parafilm and incubated at 25°C. The size of the eventual inhibition halo was recorded after 3 days.

2.9. Evaluation of bacteria extracts on artificially inoculated fruit (preventive activity)

Tests were performed with extracts obtained with the first method (Ext.1) from the eight selected isolates (B1, B2, B5, B6, B7, B8, B9 and B10). Tangerines, cv. Avana, inoculated with *P. digitatum*, tomatoes, cv Datterino, inoculated with *B. cinerea* and apricots, cv Tsunami, inoculated with *M. fructicola* were used as host/pathogen combinations. Fruit were selected to be uniform, surface sterilized, fixed on polypropylene pads and wounded as described in section 2.5. Each wound received 10 µl of bacterial extract diluted with sterile distilled water just before use to have final concentrations of 1.5, 3, 6, 12, 24 and 36 mg/L. Wounds treated with 10 µl of a water solution containing 12 ml/L of ethanol were used as a control since this was the concentration of ethanol in most concentrated extracts (36 mg/L). Once dry (approximately 2 h) wounds were inoculated with 10 µl of a spore suspension containing $5x10^4$ (*P. digitatum*) or $5x10^5$ (*B. cinerea* and *M. fructicola*) conidia/ml. In all trials, treated fruits were maintained at room temperature (22±2°C) in plastic boxes containing wet paper to ensure high relative humidity (RH).

2.10. Curative activity of bacterial extracts on artificially inoculated fruit

Curative trials were performed with extracts obtained with the first method (Ext.1) from isolates B1, B5 and B9. Three different concentrations of the extracts were used (1.5, 12, and 36 mg/L) on tangerines, cv. Avana inoculated with *P. digitatum*. Experiments were conducted as described in section 2.9, but the extracts were applied 24 hour after the inoculation of the pathogen. This time was considered enough for the germination of the conidia of *P. digitatum* and the starting of the infection process.

2.11. Induction of resistance of bacterial extracts on artificially inoculated fruit

Trials were performed with extracts obtained with the first method (Ext.1) on apricots (cv Tsunami) inoculated with *M. fructicola* and on table grape (cv Italia) and tomatoes (cv Datterino) inoculated with *B. cinerea*. Fruits were selected to be uniform, surface sterilized, fixed on polypropylene pads and wounded as described in section 2.5. However, unlike previous trials, the extracts and the pathogens were applied in two distinct wounds made in the equatorial zone at a distance of approximately 2 cm. The distance between wounds ensured the absence of a physical contact and excluded a direct antimicrobial action of the extracts. A first series of tests was conducted with extracts from isolates B1, B2, B5, B6, B7, B8, B9 and B10 on apricots and tomatoes. In these tests, the pathogens were inoculated approximately 1 h after the application of the extract. A second series of tests were performed with the most effective extracts (B1, B5 and B9) and provided the inoculation of the pathogen 1, 6 and 24 h after the extracts. In all experiments, fruit wounds received 10 µl of extracts at a concentration of 1.5, 12 and 36 mg/L. Similarly, the inoculation of the pathogens was made by applying 10 µl of a suspension containing $5x10^4$ (*P. digitatum*) or $5x10^5$ (*B. cinerea* and *M. fructicola*) conidia/ml. In all trials wounds treated with 10 µl of a water solution containing 12 ml/L of ethanol were used as a control since this was the concentration of ethanol in most concentrated extracts (36 mg/L).

2.12. Comparison of the efficacy of extracts obtained with different methods

Trials were conducted with extracts of isolates B1, B5, and B9 obtained with the three different extraction methods (Ext.1, Ext.2, and Ext.3). Extracts were used at a concentration of 1.5, 12 and 36 mg/L. Experiments were conducted as described in section 2.9 on fruit of tangerines, cv. Avana, inoculated with *P. digitatum*.

2.13. Evaluation of extracts against natural rots

2.13.1. Trials on olives

Tests were conducted with organic olives of cv Ottobratica from an orchard located in the Gioia Tauro plain where the disease is endemic (Graniti, 2008). Olive drupes were accurately selected to be uniform in size and ripeness and to avoid fruits with apparent lesions and/or symptoms of rot. Selected drupes were immersed for 2 min in water solutions containing extract of bacteria B1, B5 and B9 at a concentration of 12 mg/L. Fruits dipped in tap water and untreated fruits were used as controls. Each treatment was performed with 3 kg of olives (approximately 1500 drupes). Treated olives were

maintained on blotting paper at room temperature for approximately 2 h. Once dry, olives from each treatment were divided into equal part. One part did not receive any additional treatment while the second part was artificially inoculated by nebulization of a conidia suspension (10^5 conidia/mL) of *C. acutatum* s.s. After inoculation, olives were dried for approximately 2 h at room temperature. All olives were transferred in plastic boxes and incubated at (22±2°C). Boxes contained wet paper to ensure high relative humidity (RH) while avoiding the presence of free water.

The incidence of the disease was assessed daily starting from 7-day post inoculation (dpi). Furthermore, an empirical scale was used to evaluate the severity of the disease: 0 (absence of rots) and 1, 2, 3 and 4 for 1-25, 26-50, 51-75, and more than 76% of olive surface with symptoms of rots. This empirical scale made it possible to calculate the McKinney index (McKinney, 1923), expressing the weighted average of the disease severity as actual percentage in terms of the maximum disease severity:

$$\text{McKinney index} = \frac{\Sigma d \cdot f}{Tn \cdot D} \text{ x100}$$

where *d* is the degree of disease severity and I is the frequency, Tn is the total number of samples examined (healthy and diseased) and D is the maximum degree of symptom intensity of the scale.

2.13.2. Trials on sweet cherries

Trials were conducted on sweet cherries of both cvs. Ferrovia and Giorgia, trying to simulate as much as possible commercial practical conditions. Cherries were harvested in a commercial orchard located Apulia, Southern Italy (GPS coordinates 40°51'42.1"N 17°17'15.3"E) and accurately selected to be uniform in size and ripeness. All cherries with apparent lesions and/or rot symptoms were discarded. Selected fruit were dipped for 2 min in an extract solution of strain B9 at different concentrations (6, 12 and 24 mg/L), dried at room temperature for 2 h on blotting paper, placed in plastic trays (approximately 50 fruits per tray), covered with plastic sheet, and stored at 2±1°C. Fruit dipped in tap water were used as controls. Incidence and severity of rots was evaluated after 14 days of cold storage, using the same empirical scale described for trials on olives to evaluate the McKinney index (see paragraph 2.13.1). For cherries of cv Giorgia the disease was also evaluated after 1, 2 and 3 days of shelf-life at room temperature. This evaluation was not possible for cherries of cv Ferrovia because rapid nesting diffusion of secondary rots, mainly caused by *Rhizopus stolonifer*.

2.13.3. Field trials on wine grapes

Field trials were conducted to control the powdery mildew in a grapes vine orchard, cv Gaglioppo, located in Cirò, Crotone, Southern Italy (GPS coordinates 39°.3609321" N, 17°.0686961"E). The vineyard was 10 years old and was cultivated under an organic farming system. Trials were conducted according to a completely randomized block design with three block corresponding to uniform grape rows. Each block comprised 4 experimental units of at least 8 grape plants that received:

- The extract from strain B9 at the concentration of 24 mg/L
- A pomegranate peel extract (PGE) at the concentration of 6 mg/L (Pangallo et al., 2021; Belgacem et al., 2021)
- Chemicals commonly utilized in the control schedule of the farm to protect grapes (Chemical control)
- No treatment (Control).

All treatments were made following the normal chemical schedule utilized in the farm. In the year of experimentation, the incidence of powdery mildew was quite low and, consequently the schedule provided only two sprays with formulates containing copper (Coprantol Hi Bio 2.0, Syngenta) and sulfur (Tjovit Jet, Syngenta) on June 6 and July 5, 2021. Biological and chemical formulates were applied with a commercial backpack atomizer (Volpi V. black comfort 16, AgriEuro, Italy) using approximately 5 liters of solution to enable the uniform wetting of the vegetation without water dropping.

The incidence and severity of the disease was evaluated on July 3 and July 29 on leaves and bunches. Leaves were divided in mature and young to differentiate those already present at the time of the treatment from those that developed later (did not directly receive the treatment). For each treatment, a total of 72 bunches and 90 young and mature leaves were evaluated. The following empirical scale was utilized to quantify the severity of the disease: 0 = no damage; and 1, 2, 3, and 4 for 1-20, 21-40, 41-60 and more than 60% of infected leaf surface or grape berries. This empirical scale made it possible to calculate the McKinney index (See section 2.13.1). Finally, to evaluate the effect of different treatments on the content of sugar, the total soluble solids (°Brix) were evaluated with a refractometer on August 31, 2021. To this aim a total of 144 berries (48 per block) were collected per each treatment and crushed to get the juice.

2.14. Metabolomics characterization of Pseudomonas *active extracts*

To investigate the chemical composition of extracts, a metabolomics-based approach was applied. Metabolomics is the large-scale study of small molecules (metabolites) and their association within a biological system (Peng et al., 2015). Since we did not have any information about the composition of extracts, an untargeted approach was used to look at differences between metabolomes and attempt to discover molecules (Peng et al., 2015; Luzzatto Knaan et al., 2017). The used strategy significantly differ from the targeted ones which is based on hypothesis driven approaches to search for specific molecules. Mass spectrometry (MS) analyses were conducted according to Luzzatto Knaan et al. (2015). It is the main analytical tool with complementary and orthogonal techniques providing investigators with molecular formulas, isotopic profiles and fragmentation data (tandem MS) that are especially useful for structure elucidation, as well as spatial and temporal distribution of the chemical composition within a sample, without the need for labels or probes.

Analyses were performed on extracts obtained from the three best performing isolates (B1, B5 and B9) obtained with the three tested extraction methods (Ext.1, Ext.2, nad Ext.3).

2.14.1. Liquid Chromatography Mass Spectrometry

Liquid chromatography was carried out on a UPLC ultimate 3000 Dionex system with a C-18 column (Phenomenex 1.7 µm C18 50 × 2.1 mm) in the following conditions: A- ACN (Acetonitrile): 0.1% FA (Formic Acid); B- H20: 0.1% FA. Flow: 0.5 ml/min; 0 min: 90% B, 0.5 min: 90% B, 3 min: 50% B, 8 min: 1% B, 11 min: 1% B, 11.5 min: 90% B, 12.5 min: 90% B.

Mass spectrometry measurements were carried out on a Bruker Maxis impact QTQF system in an ESI positive mode. The method used was tune positive MSMS with MS conditions as follows: Ionization mode: ESI positive; Capillary: 4000V; Corona: 4000nA; Nebulizer: 2Bar; Dry Gas: 5 l/min; Dry temp: 200; Vaporizer Temp: 450; Active: 5; Exclude after: 4; Release after: 0.5 min; Absolute Threshold: 213 cts; Relative Threshold: 0%; Spectra rate: 1 Hz; Precursor Ion list: exclude. For MS2 Auto MS\MS transitions were used.

2.14.2. Data processing and GNPS workflow

LC-MS raw data files were converted to mzXML format by Compass DataAnalysis 4.2 software (Bruker Daltonics). Feature detection was performed by GNPS molecular networking based on MS/MS spectra.

A molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). The precursor ion mass tolerance was set to 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da. A network was then created where edges were filtered to have a cosine score above 0.6 and more than 4 matched peaks. The spectra in the network were then searched against GNPS' spectral libraries followed by additional steps for class annotations by the MolNetEhnacer workflow (Wang et al., 2016; Ernst et al., 2019). Networks with integrated data and annotations visualized by the open source software platform Cytoscape (Otasek et al., 2019).

2.15. Statistical analysis

In vivo experiments were made using three repetitions. In trials with artificially inoculated fruits, each repetition consisted of 10 (olives, tomatoes and table grape) and 5 (tangerines, apricots and apples) fruit kept in separate plastic boxes. In semi-commercials trials, each repetition consisted of 150 olives and approximately 300 fruits of sweet cherries. In vitro experiments were made using three repetitions consisting of 3 PDA plates. The data were submitted to the analysis of variance (ANOVA) and means were compared using the Tukey test ($p < 0.05$) to determine the significance of the treatments. The data were analyzed using IBM SPSS Statistic v. 20 software.

3. Results

3.1. Selection of bacteria producing antifungal compounds

The protocol utilized to select soil bacteria producing antifungal compounds proved very effective enabling the identification on many different bacteria producing clear inhibition halos without mycelium of *P. digitatum* around their colonies. A total of 125 colonies were selected and transferred in PDA plates to be morphologically characterized. A preliminary screening enabled the selection of 22 colonies representative of different soils and/or showing different morphological features (Table 1).

3.2. In vitro antimicrobial activity of bacteria

Dual culture assays were carried out to preliminary screen select bacteria and identify those producing more antimicrobial substances (Fig. 2).

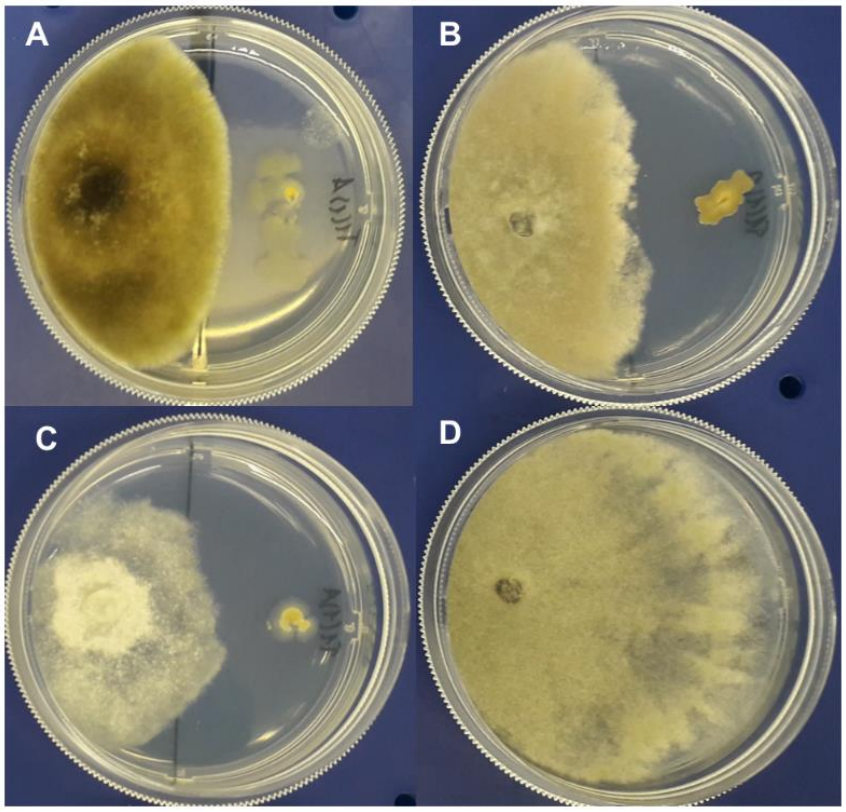

Fig. 2: *Representative Petri dishes utilized for dual culture assays containing colonies of* A. alternata *(A),* B. cinerea *(B) and* P. palmivora *(C) on the left side and the bacterium isolate B9 on the right side. The last Petri dish (D) contains a representative colony of* B. cinerea *without any bacterial isolate.*

Most of the tested bacteria determined a significant inhibition of the mycelial growth with an overall average PIRG of 26,60 (Table 2). Similar average levels of inhibitions were recorded against the two fungi *A. alternata* and *B. cinerea* (PIRGs of 27.70 and 26.65, respectively), and the oomycete *P. palmivora* (average PIRG of 25.80). However, single bacterial strains performed differently against different pathogens (Table 2). For instance, assuming empirically 40 as high PIRG value, the threshold was exceeded by 11 strains for *B. cinerea* (B2, B3, B5, B9, B10, B13, B14, B17, B18, B19, B21), two strains for *A. alternata* (B13, B21), and 7 strain for *P. palmivora* (B2, B3, B5, B9, B17, B22). None of the tested bacteria showed a PIRG higher than 40 for all tested pathogens although as average PIRG, the threshold of 40 was exceeded by 6 isolates (B3, B5, B9, B13, B17, B21).

3.3. Preventive effect of bacterial isolates on artificially inoculated fruit

Preliminary selected bacterial strains were also tested on artificially inoculated fruit to evaluate their biocontrol activity with different host/pathogen combinations. On average, INHIB value determined by all bacteria varied from a minimum 7.8% in the case of tomatoes inoculated with *A. alternata* and a maximum of 34.5% for olives inoculated with *C. acutatum s.s.* (Table 2). However, bacteria showed a much diversified level of activity, which also great varied according to hosts and pathogens (Table 2). Considering all host/pathogen combination isolate B9 was the most effective isolate with an average inhibition of rots of 80.6% followed by isolates B5, B10, B1, B8, B2, B6, and B7 with an inhibition of rots of 74.2, 36.7, 35.0, 34.5, 30.4, 21.7, and 20.6%, respectively. Isolate B9 proved very effective in most host pathogen/combinations since it reduced the incidence of rots by 86.7% on grapes and tomatoes inoculated with *B. cinerea* and by 100.0, 96.7, and 96.7% on apples inoculated with *P. expansum*, olives inoculated with *C. acutatum s.s.* and tangerines inoculated with *P. digitatum*, respectively. A lower level of efficacy was only recorded on tomatoes inoculated with *A. alternata* (27% of reduction). Excepted than on this last host/pathogen combination, no significant differences were observed between isolate B9 and PGE.

***Table 2**: Percentage of inhibition of radial growth (PIRG) evaluated according to Skidmore and Dickinson (1976) for 22 selected bacterial isolates using* Botrytis cinerea, Alternaria alternata *and* Phytophthora palmivora *as representative plant pathogens. Per each pathogen, different letters indicate significantly different values according to Tukey's test (P≤0.05)*.*

Strains	*B. cinerea*		*A. alternata*		*P. palmivora*		Average	
	PIRG (%)	P*	PIRG (%)	P*	PIRG (%)	P*	PIRG (%)	P*
B1	6.74	h	27.45	efg	25.13	e	19.78	c
B2	45.86	bc	29.63	cde	51.12	a	42.20	a
B3	45.47	bc	32.03	cd	46.04	a	41.18	a
B4	35.73	d	32.90	c	35.96	cd	34.86	b
B5	46.09	bc	32.01	cd	44.22	a	40.78	a
B6	2.12	i	24.62	efg	10.60	f	12.49	c
B7	7.32	gh	23.97	g	8.25	fgh	13.18	c
B8	7.51	gh	24.62	fg	15.80	f	15.98	c
B9	51.45	a	37.69	efg	43.52	cd	44.22	a
B10	50.10	ab	27.02	efg	34.99	cd	37.37	ab
B11	0.19	i	12.85	h	5.63	gh	6.22	d
B12	0.19	i	28.54	def	11.22	fg	13.32	c
B13	46.27	bc	41.46	b	34.04	d	40.59	ab
B14	45.09	c	32.03	cd	39.83	bc	38.98	ab
B15	0.00	i	13.07	h	5.15	hi	6.07	d
B16	12.91	f	2.36	i	3.19	hi	6.15	d
B17	51.64	a	32.46	cd	44.88	ab	42.99	a
B18	51.64	a	27.02	efg	36.93	cd	38.53	ab
B19	51.64	a	28.54	def	24.09	e	34.76	ab
B20	11.66	fg	11.33	h	0.00	i	7.66	cd
B21	44.15	c	52.92	a	31.63	d	42.90	a
B22	16.23	f	32.46	cd	44.88	ab	31.19	b
Average	**27.70**	**-**	**26.65**	**-**	**25.80**	**-**	**26.60**	**-**

Table 3: *Percentage of inhibition of rots on fruits treated with 22 selected bacterial isolates and inoculated with* Botrytis cinerea *on table grape and tomatoes,* Alternaria alternata *on tomatoes,* Penicillium expansum *on apples,* Colletotrichum acutatum s.s. *on olives, and* Penicillium digitatum *on citrus. Per each pathogen, different letters indicate significantly different values according to Tukey's test (P≤0.05)*.*

Strains	*B.cinerea table grapes*		*B.cinerea tomatoes*		*A.alternata tomatoes*		*P.expansum* apples		*C.acutatum* olives		*P.digitatum* tangerines		Average	
	INHIB	P*	INHIB	P*	INHIB	P*	INHIB	P*	INHIB	P*	INHIB	P*	INHIB	P*
B1	50.00	cd	13.30	bc	40.00	b	70.00	b	10.00	cd	26.67	cdef	35.00	c
B2	36.67	f	20.00	bc	33.30	b	35.50	c	16.67	cd	40.00	bcd	30.36	c
B3	40.00	f	20.00	bc	0.00	d	22.40	cd	16.67	cd	20.00	defg	19.84	c
B4	20.00	ef	6.70	bc	6.70	cd	0.00	f	23.33	cd	10.00	efg	11.12	d
B5	73.33	bc	71.70	a	20.00	bcd	93.40	a	96.67	a	90.00	a	74.18	b
B6	10.00	f	20.00	bc	6.70	cd	20.00	cde	67.00	ab	0.00	g	20.62	cd
B7	6.67	f	33.30	b	0.00	d	0.00	f	83.33	a	6.67	fg	21.66	cdé
B8	60.00	cd	26.70	bc	20.00	bcd	0.00	f	86.67	a	3.33	fg	34.45	c
B9	86.67	ab	86.70	a	27.00	bc	100.00	a	96.67	a	96.67	a	80.62	b
B10	100.00	a	0.00	c	6.70	cd	0.00	f	86.67	a	26.67	cdef	36.67	c
B11	0.00	f	0.00	c	0.00	d	0.00	f	93.33	a	0.00	g	15.56	cd
B12	0.00	f	0.00	c	0.00	d	0.00	f	0.00	d	50.00	bc	8.33	d
B13	0.00	f	0.00	c	0.00	d	0.00	f	0.00	d	0.00	g	0.00	e
B14	20.00	ef	10.00	bc	5.00	cd	10.00	def	20.00	cd	20.00	defg	14.17	cd
B15	0.00	f	0.00	c	0.00	d	0.00	f	25.70	cd	25.70	cdefg	8.57	d
B16	15.70	ef	6.70	bc	3.50	cd	2.30	ef	0.00	d	0.00	g	4.70	d
B17	0.00	f	0.00	c	0.00	d	0.00	f	0.00	d	0.00	g	0.00	e
B18	0.00	f	5.00	c	0.00	d	0.00	f	0.00	d	55.00	b	10.00	cd
B19	0.00	f	0.00	c	0.00	d	0.00	f	0.00	d	0.00	g	0.00	e
B20	0.00	f	0.00	c	0.00	d	0.00	f	0.00	d	0.00	g	0.00	e
B21	10.70	f	2.50	c	2.00	cd	6.70	def	35.40	bc	35.40	bcde	15.45	cd
B22	0.00	f	0.00	c	0.00	d	0.00	f	0.00	d	0.00	g	0.00	e
PGE6	100.00	a	93.30	a	86.70	a	100.00	a	100.00	a	100.00	a	96.67	a
Average**	24.08		14.67		7.76		16.38		34.46		23.01		20.06	

*The average was calculated excluding PGE

3.4. Molecular characterization

Selected bacteria were associated to 4 different 16S genotypes. Identical sequences were identified for isolates B2, B5, B9 and B10 and for isolates B7 and B8, while unique sequences were associated to B1 and B6 (Fig. 3). These clustering of isolates was confirmed by Box-PCR since isolates B2, B5, B9 and B10 as well as isolates B7 and B8 showed identical banding patterns (Fig. 4)

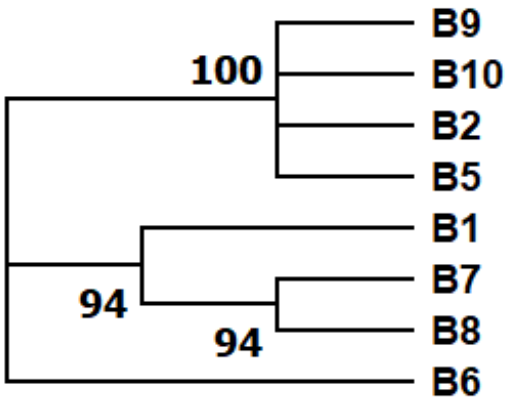

Fig. 3: *Phylogenetic tree based on 16S sequences of selected bacterial strains as inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Analyses were conducted in MEGA X (Kumar et al., 2018)*

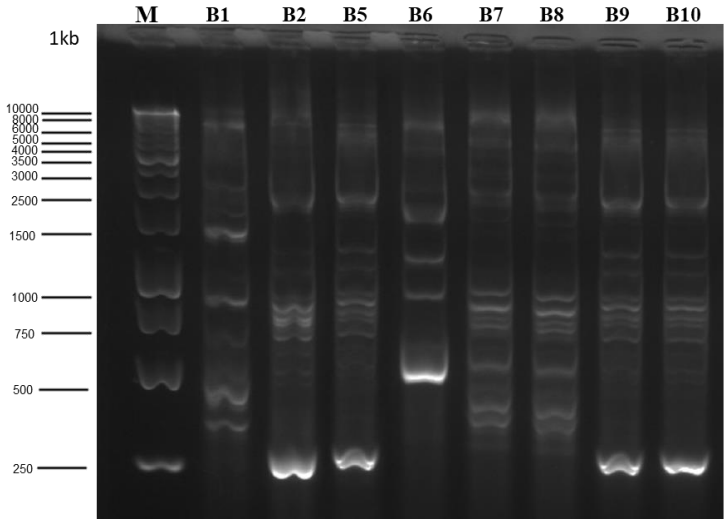

Fig. 4: *BOX-PCR profile of 9 selected bacteria strains. Lane M denotes 1 kb DNA ladders.*

According to BLASTN analyses and the following comparison of closely related sequences all isolates clustered within the genus *Pseudomonas*. A single nucleotide differentiated isolates B7 and B8 from that of an uncultured *Pseudomonas* sp. clone (JF500997), while at least 2 bases differentiated this isolate from other closely related sequences mainly deposited as *Pseudomonas* sp., and *P. stutzeri*. Isolates B2, B5, B9, and B10 shared an identical 16S sequence with a strain (O9-10) deposited as *P. putida* (KT215479) and were differentiated by one (MW037647) or two (MW037676) bases from strains generically deposited as "Bacterium". At least 4 differences were found between our isolates and other GenBank items, including several strains deposited as *P. putida*. Isolate B1 was differentiated by at least two bases from GenBank sequences being the most closely related deposited as *Pseudomonas* sp. and *P. stutzeri.* Finally, isolate B6 showed a base difference as compared to an isolated deposited as *Pseudomonas* sp (JF327483) and two bases differences from several other sequences mainly deposited as *Pseudomonas* sp., *P. fragi*, and *P. psychrophila.*

3.5. Alcoholic extractions

The three tested methods utilized to obtain alcoholic extracts yielded a similar quantity of dry pellet (approximately 0.2 g) from bacteria collected on 20 plates (Ext.1) or grown in 20 ml (Ext.2 and Ext.3) of liquid medium. Furthermore, the same yield was confirmed in large-scale trials since 2 g of dry pellet were obtained from 200 ml of bacterial suspension. According to metabolomics and biological analyses extracts obtained with different methods had a significant different composition. In agreement with the different composition, MIC analyses and tests on artificially inoculated tangerines showed a slight higher activity for Ext.3 (see specific sections).

3.6. Minimal Inhibitory Concentration (MIC) of alcoholic extracts

Results of MIC analyses revealed an overall low antifungal activity since most extract did not show any inhibition halo around the absorbent paper pads. In fact, as shown in Fig. 5 the analysis of extracts obtained with the first method (Ext.1) determined a significant halo only for the extract of isolate B6 at the three highest concentrations (30, 100 and 300 mg/L). Furthermore, a very faint halo was observed for the extract from isolate B9 at the highest concentration (Fig. 5).

The comparison of extracts obtained with the three tested methods (Ext.1, Ext.2, and Ext.3) from the three isolate that overall provided the best results on artificially inoculated fruits (B1, B5, and B9) showed and important impact of the extraction method. In fact, if on the one hand Ext.2 confirmed results

obtained with Ext.1 (absence of inhibition halos), on the other hand Ext.3 showed a clear inhibition for the extract of isolate B9 at 100 and 300 mg/L (Fig. 6). A clear inhibition halo was also observed for isolate B1 and, with a lesser extent, for isolate B5 at 300 mg/L (Fig. 6).

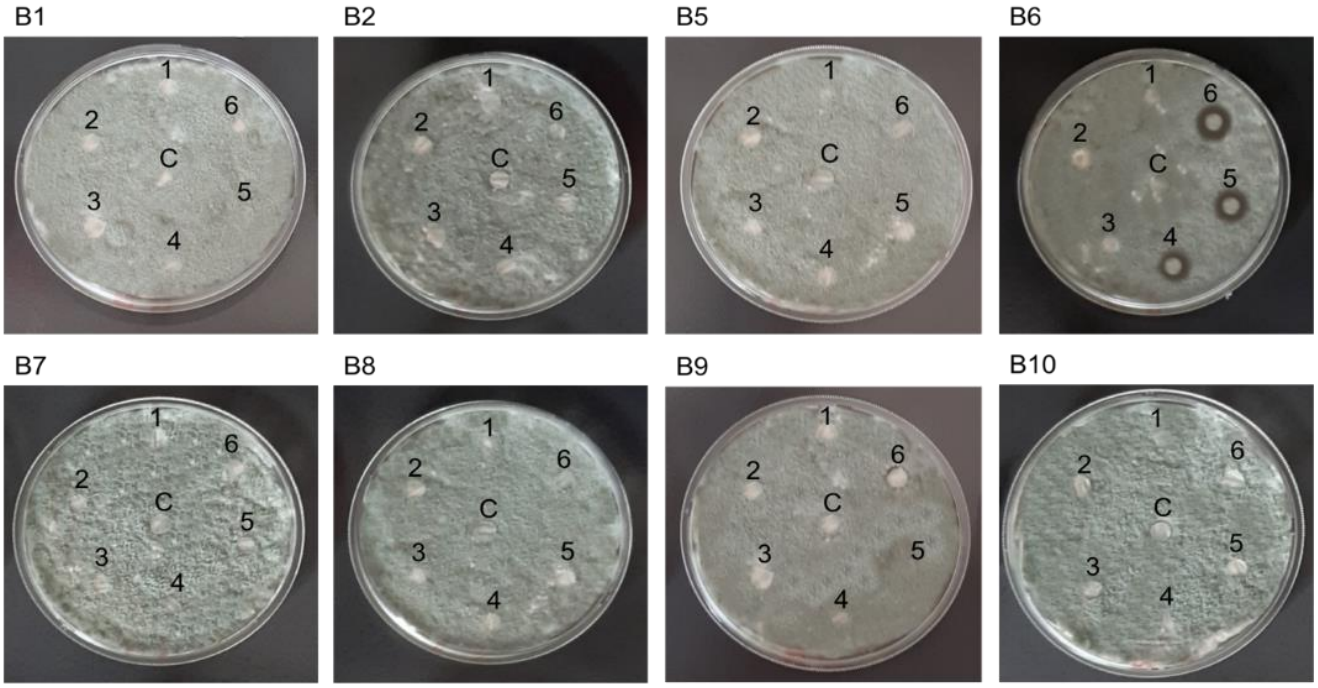

Fig. 5: *Minimal Inhibitory Concentration (MIC) of alcoholic extracts obtained with the first method (Ext.1) from selected isolates (B1, B2, B5, B6, B7, B8, B9 and B10). Tested concentrations (1, 3, 10, 30, 100 and 300 mg/L) are represented by number from 1 to 6, respectively. The control (C) contained ethanol at 100 ml/L as well as the most concentrated extract (300 mg/L).*

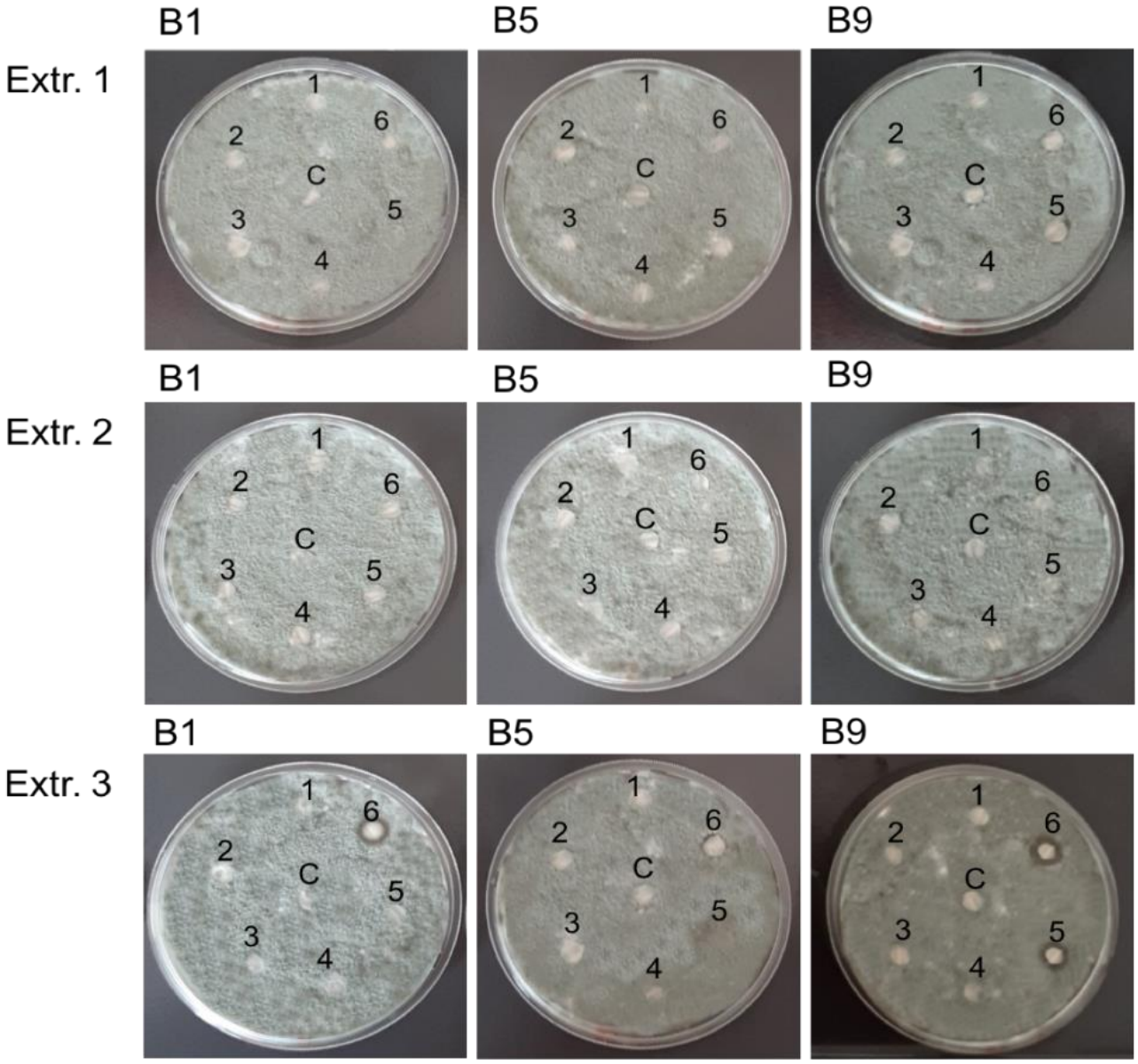

Fig. 6: *Minimal Inhibitory Concentration (MIC) of alcoholic extracts obtained with the three tested methods (Ext.1, Ext.2 and Ext.3) from three selected isolates (B1, B5, B9). Tested concentrations (1, 3, 10, 30, 100 and 300 µg/mL) are represented by number from 1 to 6, respectively. The control (C) contained ethanol at 100 ml/L as well as in the most concentrated extract (300 mg/L).*

3.7. Preventive effects of bacterial extracts on artificially inoculated fruit

Bacterial extracts proved a high efficacy in reducing the incidence of rots on artificially inoculated fruit when applied before the pathogen (preventive treatments). Considering all tested pathogen/combinations (*P. digitatum*/tangerines, *B. cinerea*/tomatoes, and *M. fructicola*/apricots) and concentrations (1.5, 12, and 36 mg/L), the average reduction of the incidence of rots varied between 51.8 and 95.2 (Table 4). Extracts proved slightly more effective on apricots inoculated with *M. fructicola*, followed by tomatoes inoculated with *B. cinerea*, and tangerines inoculated with *P. digitatum*. The concentration was always an important factor in determining the level of efficacy, although most extracts provided a significant reduction of rots on most host/pathogen combination, even with the lowest tested concentration (1.5 mg/L). The most effective extracts obtained from isolates B1, B5 and B9 provided an average reduction of 90.7, 92.7, and 95.2%, respectively. These extracts determined the complete inhibition of rots in most host/pathogen combinations and at all concentrations.

Table 4: *Preventive effect of bacterial extracts on the incidence of decay on artificially inoculated fruits. Tangerines, tomatoes and apricots were treated with bacterial extracts at three different concentrations (1.5, 12, or 36 mg/L) and then, approximately after two hours, with* P. digitatum, B. cinerea, *and* M. fructicola, *respectively. Mock fruit treated with a solution of EtOH (12 ml/l) and inoculated with the pathogens served as controls. Numbers indicate the percentage of reduction of infected wounds as compared to the control wounds. Per each host/pathogen combination and tested concentration different letters indicate significantly different values according to Tukey's test (P≤0.05).*

Extracts	*P. digitatum - Tangerines*						*B. cinerea - Tomatoes*						*M. fructicola - Apricots*						**Average**
	1,5 mg/L		12 mg/L		36 mg/L		1,5 mg/L		12 mg/L		36 mg/L		1,5 mg/L		12 mg/L		36 mg/L		
B1-E	100	a	100	a	100	a	70	a	73.3	a	86.7	a	86.6	b	100	a	100	a	**90.7**
B2-E	55.6	b	88.9	b	100	a	70	a	90	a	80	a	66.7	b	80	b	93.3	a	**80.5**
B5-E	77.8	b	100	a	100	a	70	a	93.3	a	93.3	a	100	a	100	a	100	a	**92.7**
B6-E	100	a	100	a	100	a	56.7	a	83.3	a	86.7	a	86.7	b	86.7	b	100	a	**88.9**
B7-E	0	d	0	d	0	c	60	a	80	a	86.7	a	80	b	80	b	80	b	**51.8**
B8-E	5.7	c	11.2	c	44.5	b	43.3	a	80	a	86.7	a	53	b	80	b	86.7	b	**54.5**
B9-E	100	a	100	a	100	a	60	a	96.7	a	100	a	100	a	100	a	100	a	**95.2**
B10-E	0	d	0	d	0	c	56.7	a	43.3	b	43.3	b	80	b	80	b	80	b	**42.6**
EtOH	0	d	0	d	0	c	0	b	0	c	0	c	0	c	0	c	0	c	**0.0**
Average	**54.9**		**62.5**		**68.1**		**60.8**		**79.9**		**82.9**		**81.6**		**88.3**		**92.5**		

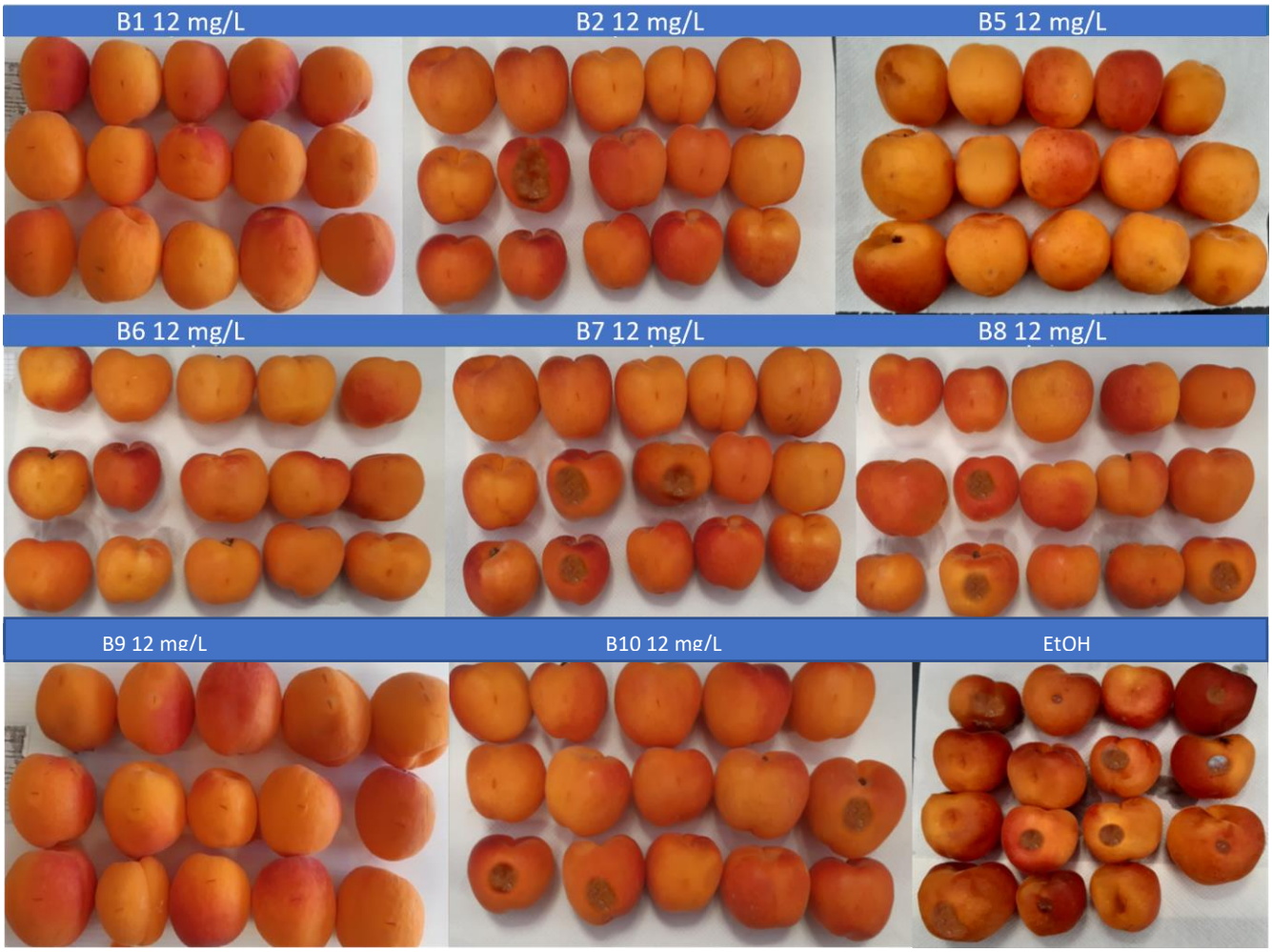

Fig. 7: *Representative photo of preventive trials conducted on apricots treated with extracts from isolates B1, B2, B5, B6, B7, B8, B9, and B10 and artificially inoculated with* Monilinia fructicola. *Mock fruit treated with a solution of EtOH (12 ml/L) and inoculated with the pathogens served as controls.*

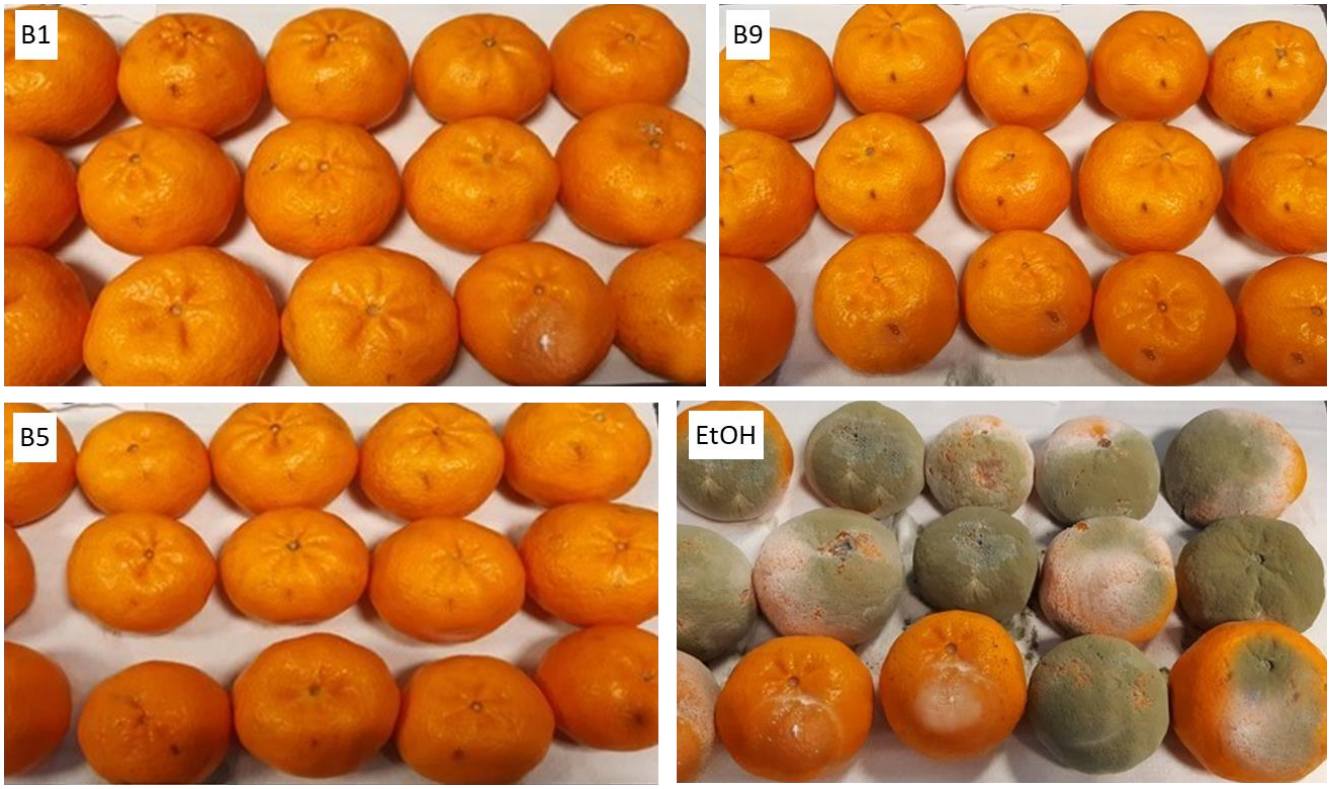

Fig. 8: *Representative photo of preventive trials conducted on tangerines artificially inoculated with* Penicillium digitatum *using extracts from isolates B1, B5, and B9. Mock fruit treated with a solution of EtOH (12 ml/l) and inoculated with the pathogens served as controls.*

3.8. Curative effects of bacterial extracts on artificially inoculated fruit

Extracts from bacteria B1, B5, and B9 significantly reduced the incidence of rots when applied 24 h after the inoculation of the pathogens (Fig. 9). On tangerines, the B1 at 1.5, 12 and 36 mg/L reduced *P. digitatum* rots by 60.0, 73.3, and 86.7%, respectively. Similarly, 1.5 and 12 mg/L of extracts were enough to reduce rots by 60.0% and 86.7% (B5) and by 53.4 and 93.3% (B9), respectively. A complete inhibition of rots (100% of inhibition) was achieved with extracts of isolates B5 and B9 at 36 mg/L (Fig. 9).

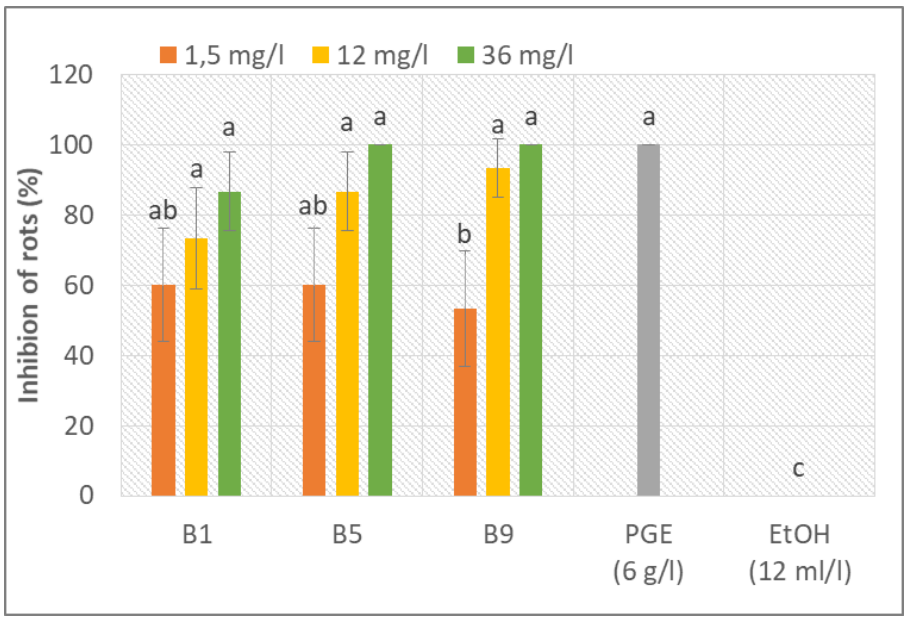

Fig. 9: *Incidence of decays on Tangerines artificially inoculated with* Penicillium digitatum *and treated 24 hour later with extracts from isolates B1, B5, and B9 at 1.5, 12, or 36 mg/L (curative activity). A 6 g/L pomegranate peel extract was included in the experiment as a reference treatment. Fruit inoculated with the pathogen and than treated with a solution of EtOH (12 mg/L) and served as controls. Bars indicate standard errors of the means. Per each tested concentration different letters indicate significantly different values according to Tukey's test (P≤0.05).*

3.9. Induction of resistance

Experiments, conducted in two independent series of trials, clearly showed a strong activation of resistance in treated host tissues since the incidence of rots was significantly reduced by applying extracts and pathogens in spatially separated wounds. Results of the first series of tests performed with extracts of isolates B1, B2, B5, B6, B7, B8, B9 and B10 and using apricots/*M. fructicola* and tomatoes/*B. cinerea* as pathosystems, are reported in Table 5. In these trials, all extracts applied approximately 1 h before the inoculation of the pathogens produced a significant reduction of rots as compared to the control (fruits treated with EtOH at 12 mg/L). Considering both, trials conducted on apricots and tomatoes, and all tested concentrations (1.5, 12 and 36 mg/L) the average reduction of rots ranged between 45.7% (extract of isolate B8) and 100%. In particular, extracts from isolates B1, B5 and B9 completely inhibited the development of rots at all tested concentrations (Table 5). The same level of protection was achieved with PGE at 6 g/L (Table 5).

Results of the second series of tests conducted with extracts from isolates B1, B5, and B9 and using table grapes/*B. cinerea* and tomatoes/*A. alternata* as pathosystems, are reported in Figure 10. In these trials, extracts were applied 1, 6 or 24 h before the inoculation of the pathogens. Extracts always determined a significant reduction of rots at all concentration (1.5, 12 and 36 mg/L), for both pathosystems (grapes/*B. cinerea* and tomatoes/*A. alternata*) and with all timings of application (1, 6, and 24 h). A single exception was represented by the extract of isolate B1 at the concentration of 1.5 mg/L when applied 24 h before the pathogen. On average higher levels of protection were achieved on grape against *B. cinerea* as compared to tomatoes inoculated with *A. alternata*.

Interestingly, the timing of pathogen inoculations proved to be an important factor in determining the level of induced resistance. In fact, the highest levels of protection with all pathosystems, extracts and concentrations were achieved when the pathogens were inoculated 1 h after the extracts. A lower reduction of rots was overall achieved inoculating the pathogens 6 h after extracts and, the level of protection further decreased after 24 h. For instance, considering cumulatively all extracts and concentrations rots caused by *B. cinerea* on grapes were reduced by 92.6, 61.5, and 60.7 when the pathogen was inoculated 1, 6 and 24 h after the extracts, respectively. Similarly, rots caused by *A. alternata* on tomatoes were reduced by 77.0, 65.2, and 53.3% inoculating the pathogen 1, 6 and 24 hs after the extracts, respectively. Finally, the overall observation of the data confirmed the importance of the concentration of extracts in determining the level of efficacy, although differences among different concentrations were not always present (Fig. 10). Considering all extracts, pathosystems and timings of applications the average percentage of rot reduction was 54.8, 71.1, and 79.2 for the tested concentrations of 1.5 mg/L, 12 mg/L, and 36 mg/L, respectively.

Table 5: *Percentage of inhibition of rots in apricots and tomatoes treated with bacterial extracts and then inoculated with M. fructicola or B. cinerea in spatially separated wounds. Bacterial extracts were applied at different concentrations (1.5, 12 or 36 mg/L) 1 h before the pathogens. Fruit treated with EtOH or PGE and inoculated with the pathogens served as controls. Per each pathosystem and tested concentration, different letters indicate significantly different values according to Tukey's test (P≤0.05).*

Extracts	*M. fructicola* - Apricot						*B. cinerea* - Tomato						**Average**
	1,5 mg/L		12 mg/L		36 mg/L		1,5 mg/L		12 mg/L		36 mg/L		
B1	100	a	100	a	100	a	100	a	100	a	100	a	**100**
B2	86.6	b	93.3	a	100	a	50.2	b	53.4	b	60	b	**73.9**
B5	100	a	100	a	100	a	100	a	100	a	100	a	**100**
B6	66.6	b	80	ab	86.6	a	33.4	c	53.4	b	86.6	ab	**67.8**
B7	86.6	b	100	a	100	a	33.4	c	40	c	60	b	**70,0**
B8	40	b	46	b	53.3	b	40	bc	45.2	bc	49	b	**45.7**
B9	100	a	100	a	100	a	100	a	100	a	100	a	**100**
B10	80	b	100	a	100	a	33.4	c	53.4	b	80	ab	**74.4**
PGE6	100	a	100	a	100	a	100	a	100	a	100	a	**100**
EtOH	0	c	0	c	0	c	0	d	0	d	0	c	**0.0**
Average	**76.0**		**82.0**		**93.3**		**59.0**		**64.5**		**73.5**		

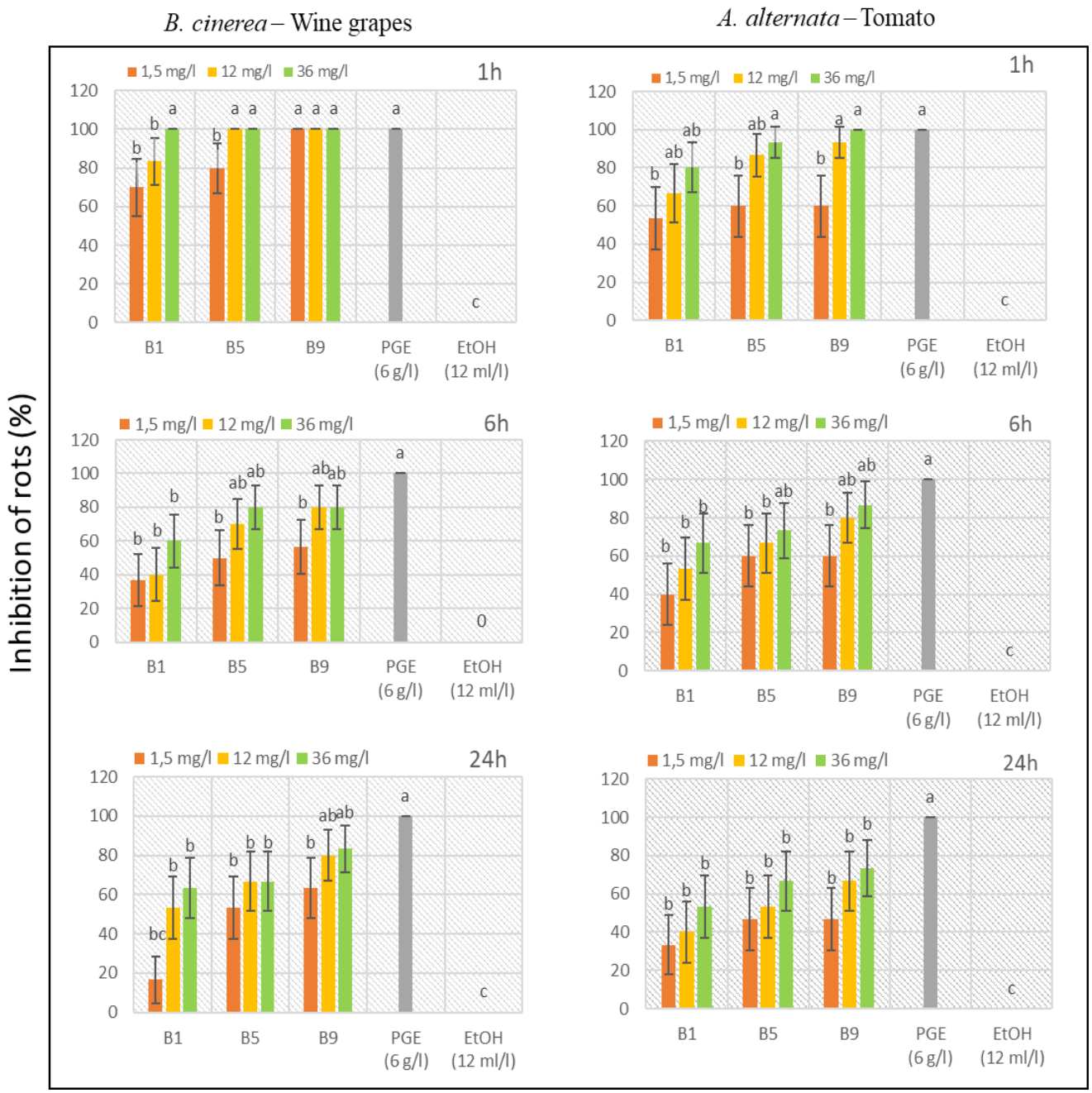

Fig. 10: *Percentage of inhibition of rots on table grapes and tomatoes treated with bacterial extracts and then inoculated with* B. cinerea *or* A. alternata *in spatially separated wounds. Bacterial extracts were applied at three different concentrations (1.5, 12 or 36 mg/L) 1, 6 or 24 hs before the inoculation of the pathogens. Fruit treated with EtOH or PGE and inoculated with the pathogens served as controls. Per each pathosystem and timing of inoculation, different letters indicate significantly different values according to Tukey's test (P≤0.05).*

Fig. 11: *Representative photo of trials conducted on table grapes to evaluate the induction of resistance. Grapes berries received 10 µl of EtOH at 12 mg/L (left) or B9 extract at 36 mg/L (right) and, approximately 1 hour later, were inoculated with a spore suspension of* Botrytis cinerea *in a spatially separated wound.*

3.10. Comparison of the efficacy of extracts obtained with different methods

The efficacy of extracts from isolates B1, B5 and B9, obtained with the three extraction methods was compared on tangerines inoculated with *P. digitatum*. For all tested isolates, no significant differences were observed (Fig. 12). In fact, extracts from isolate B9 completely inhibited the development of rots regardless of the extraction method, while extracts from B1 and B5 proved slightly more effective when obtained with the third method (Ext.3) but difference was not statistically significant (Fig. 12).

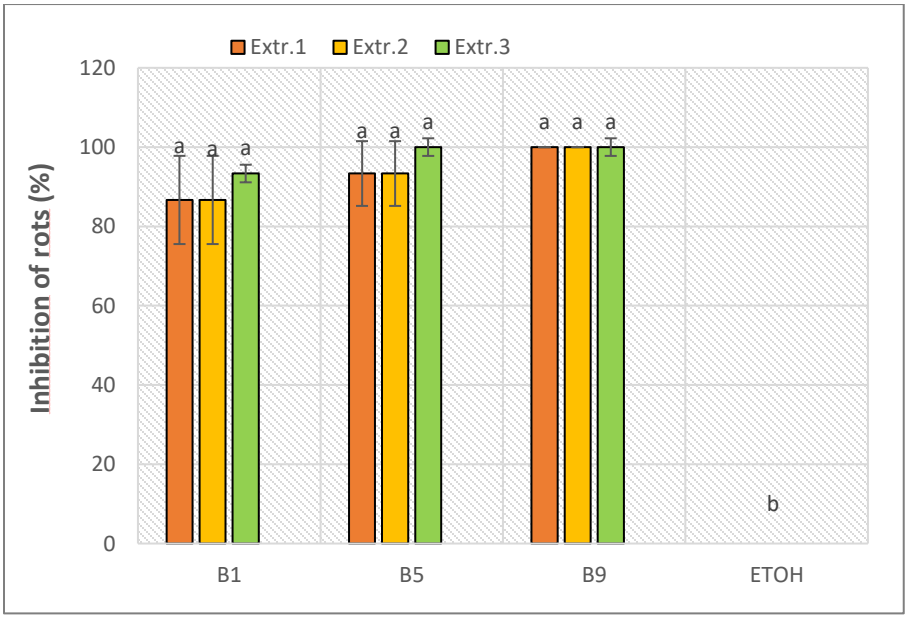

Fig. 12*: Comparison of the efficacy of extracts obtained with the three tested methods (Ext.1, Ext.2, and Ext.3) from isolates (B1, B5 and B9). Tests were conducted on tangerines inoculated with P. digitatum.*

Fruit treated with EtOH and inoculated with the pathogens served as controls. Bars indicate standard errors of the means. Per each bacterium, the same letter indicates the absence of significantly different values according to Tukey's test (P≤0.05).

3.11. Evaluation of extracts against natural rots

3.11.1. Trials on olives

A very high incidence of olive anthracnose was recorded on olives drupes collected in the Gioa Tauro plane. In fact, all untreated drupes as well as olives dipped in tap water showed typical symptoms of olive anthracnose after 7 days of incubation in moist chambers at 22±2°C. The same disease incidence and severity were recorded on olives inoculated by nebulization with a conidia suspension of *C. acutatum s.s.* Treatments with extracts from isolates B1, B5, and B9 at 12 mg/L proved very effective since they reduced the incidence and severity by 70.8, 84.7%, and 80.8% respectively (Fig. 13). No significant differences were recorded among extracts. Almost identical levels of protection were achieved with artificially inoculated olives. On the other hand, a significant higher efficacy was recorded for PGE at 6 g/L since rots were completely inhibited regardless of the artificial inoculation (Fig. 13).

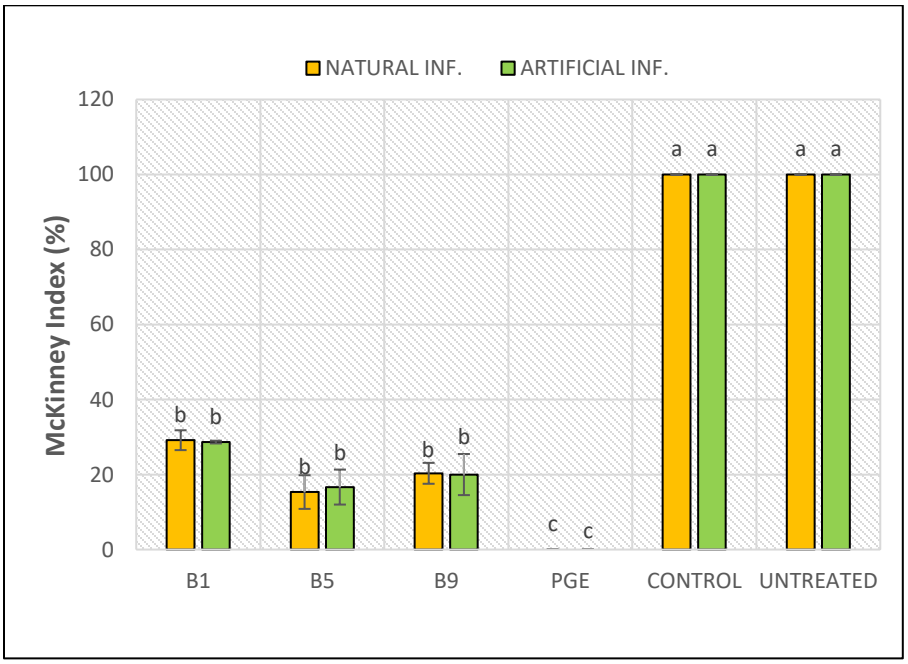

Fig. 13: *Incidence and gravity (McKinney index) of anthracnose on olives dipped in a solution of bacterial extracts (B1, B5, and B9) or PGE. Untreated olives and olives dipped in tap water were used as controls. Experiments were conducted with non-inoculated olives (left) and with olive inoculated by*

nebulization with a conidia suspension of C. acutatum s.s. (right). Bars indicate standard errors of the means. Different letters indicate significantly different values according to Tukey's test (P≤0.05).

3.11.2. Trials on sweet cherries

The dipping of sweet cherries in solutions of B9 extract at different concentrations significantly reduced the development of natural rot on both cultivar Ferrovia and Giorgia in conditions simulating those of commercial packinghouses (Figs. 14 and 15). On cv. Ferrovia, a high incidence of disease was recorded at the end of the cold storage with a disease index of 21.8% on control cherries dipped in tap water. On these cherries, the extract at 24, 12, and 6 mg/L significantly reduced natural rots by 89.0, 62.3, and 48%, respectively (Fig. 14). On cv Giorgia a much lower incidence of disease was recorded at the end of the cold storage but rots rapidly increased after 1, 2 and 3 days of shelf life. The extract at all tested concentration significantly reduced rots in all assessment times. On average considering all assessment times rots were reduced by 86.8, 62.0, and 35.0% with the extract at 24, 12, and 6 mg/L, respectively (Fig.15). On both cultivars PGE at 6 g/L proved a level of comparable to that of B9 extract at 24 g/L.

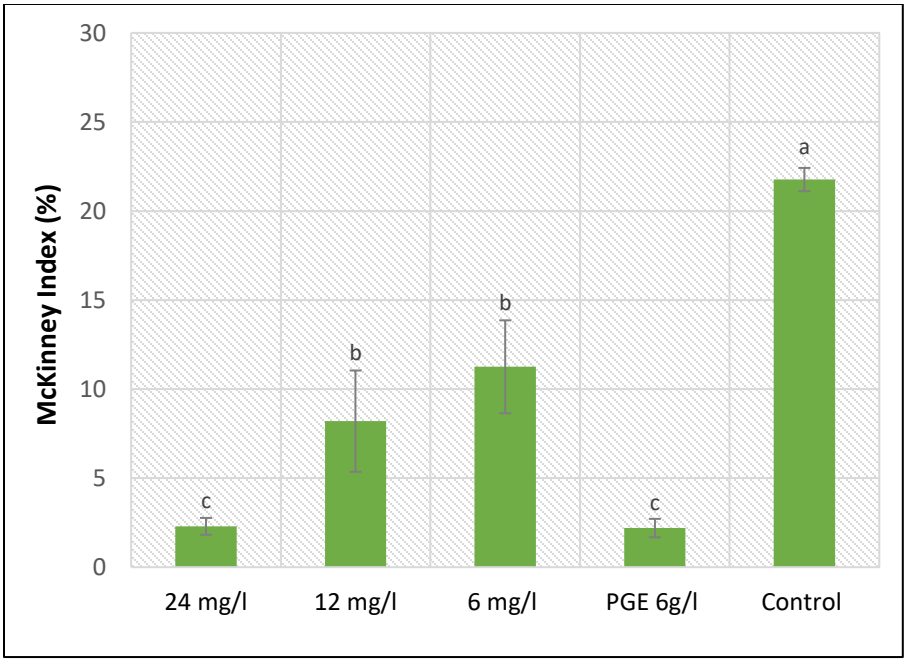

Fig. 14: *Incidence and gravity of natural rots (McKinney index) on sweet cherries, cv Ferrovia, dipped in a solution of B9 extract at 24, 12, and 6 mg/L. Rots were evaluated after 14 days of cold storage at 1°C. Sweet cherries dipped in a solution of PGE at 6 g/L or in tap water were used as controls. Bars*

indicate standard errors of the means. Different letters indicate significantly different values according to Tukey's test (P≤0.05).

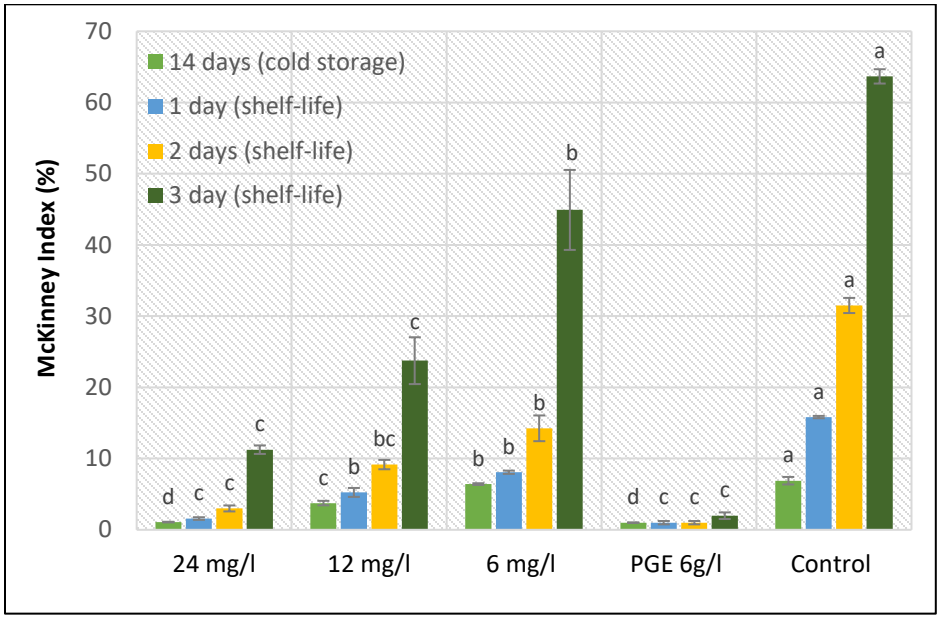

Fig. 15: *Incidence and gravity of natural rots (McKinney index) on sweet cherries, cv Giorgia, dipped in a solution of B9 extract at 24, 12, and 6 mg/L. Rots were evaluated after 14 days of cold storage at 1°C and then after 1, 2 and 3 days of shelf-life. Sweet cherries dipped in a solution of PGE at 6 g/L or in tap water were used as controls. Bars indicate standard errors of the means. Per each assessment time, different letters indicate significantly different values according to Tukey's test (P≤0.05).*

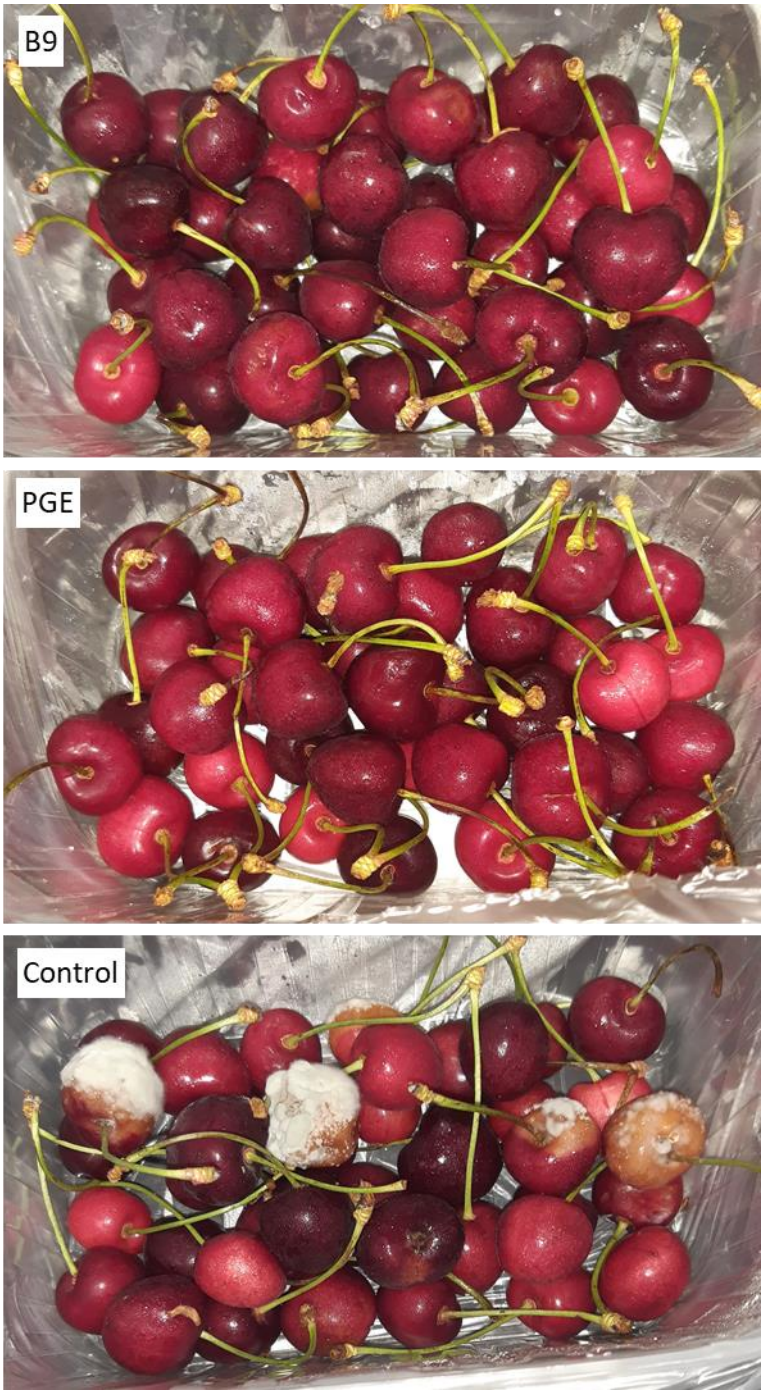

Fig. 16: *Representative photo of trials conducted with sweet cherries cv Ferrovia. Cherries were dipped in a solution of B9 extract at 24 mg/L, PGE at 6 g/L or tap water (control). The photo was made after 14 day of cold storage.*

3.11.3. Field trials on wine grapes

Field treatments with B9 extract at a concentration of 24 mg/L proved effective in controlling powdery mildew in practical conditions. Compared to the untreated grapes, a significant reduction of disease incidence and gravity was observed on both mature leaves and grape berries analyzed on July 3 and on July 29, 2021. A lower incidence and gravity of the disease was also observed on young leaves that did not directly receive the treatment, but differences were not statistically supported. No significant differences were observed as compared to PGE at 6 g/L and the chemical control on both leaves and berries and in both assessment times (Fig. 17). Similarly, the evaluation of total soluble solids (°Brix) showed a tendential higher content of sugar in grapes treated with B9 extract and PGE as well as in the chemical control but differences were not statistically significant (Fig. 18).

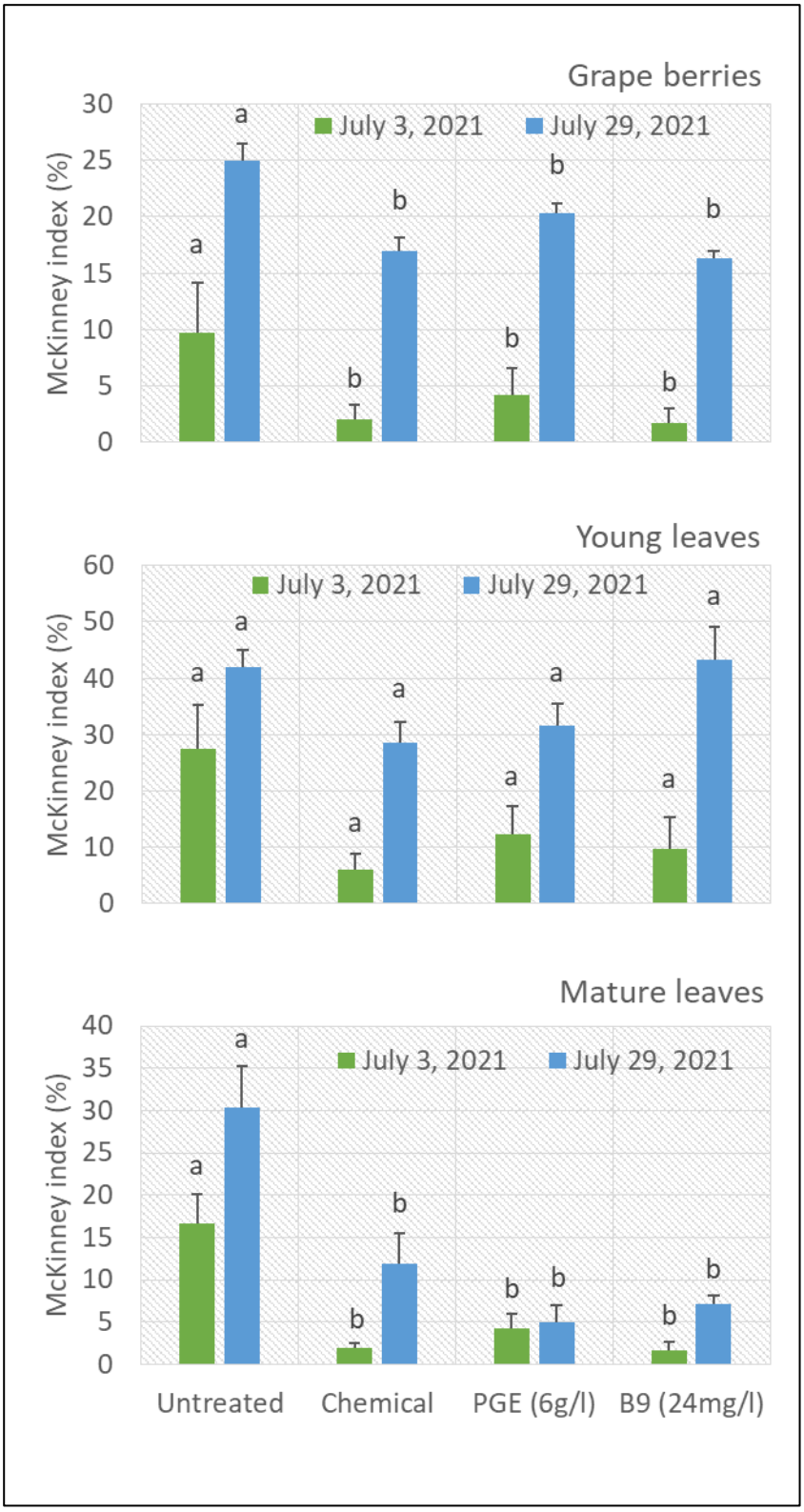

***Fig. 17**: Incidence and gravity of powdery mildew (McKinney index) on grape berries, young leaves, and mature leaves treated with B9 extract at 24 mg/L. Untreated plants, and plants sprayed with PGE at 6 g/L or with chemicals commonly utilized in control schedule of the farm were used as controls. Treatments were made on June 6 and July 5, 2021 while incidence and gravity of the disease was evaluated on July 3 and 29, 2021. Bars indicate standard errors of the means. For each assessment time columns with different letters are statistically different according to the Tukey test ($P \leq 0.05$).*

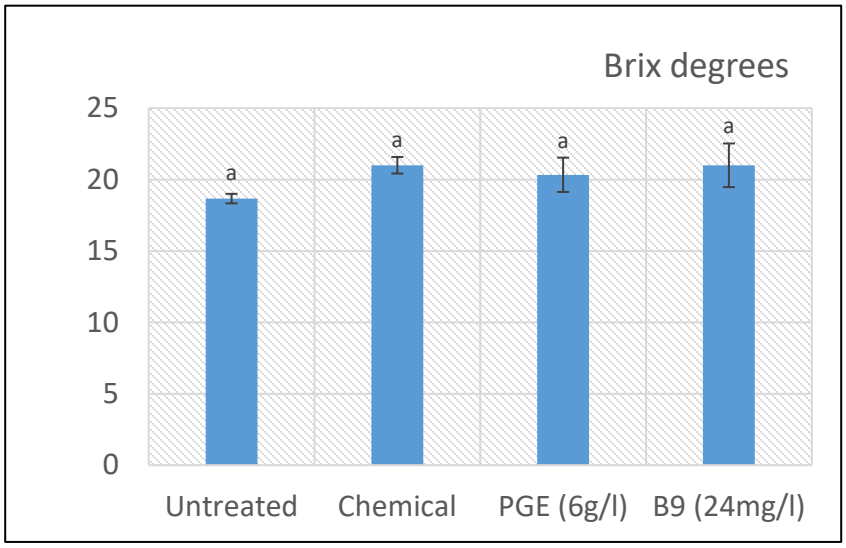

Fig. 18: *Total soluble solids (°Brix) in grape berries treated with B9 extract, or PGE as compared to grapes untreated or sprayed with chemicals. Bars indicate standard errors of the means. The same letter indicates the absence of significantly different values according to Tukey's test (P≤0.05).*

3.12. Metabolomics characterization

Differential untargeted metabolomics was used to tap on the differences between three microbial isolates (B1, B5, B9) and the three extraction methods (E1, E2, E3) demonstrating various antifungal efficiencies (refer to fig from activity). Extracts were analyzed by liquid chromatography- high resolution tandem mass spectrometry (LC-HRMS/MS). Feature detection and data annotation was performed via the Global Natural Products Social (GNPS) Molecular Networking interface (Wang et al., 2016; Ernst et al., 2019).

To estimate whether the difference in metabolome is mostly affected by the extraction method or the bacterial isolate, we refer to each sample detected features as a whole metabolome. Principal Coordinate Analysis (PCoA) demonstrated that the extraction method is a key factor. The metabolomes of the different bacteria were more similar (Fig 19a) than the various extraction methods of the same bacteria (Fig 19b). It should be noted that ethanol is the solvent used in all extractions. Changes are most likely affected by the extraction of biomass versus extraction with media. Both media components may alter the metabolomics profile as well as secreted compounds that may be lost or reduced by extraction of biomass alone.

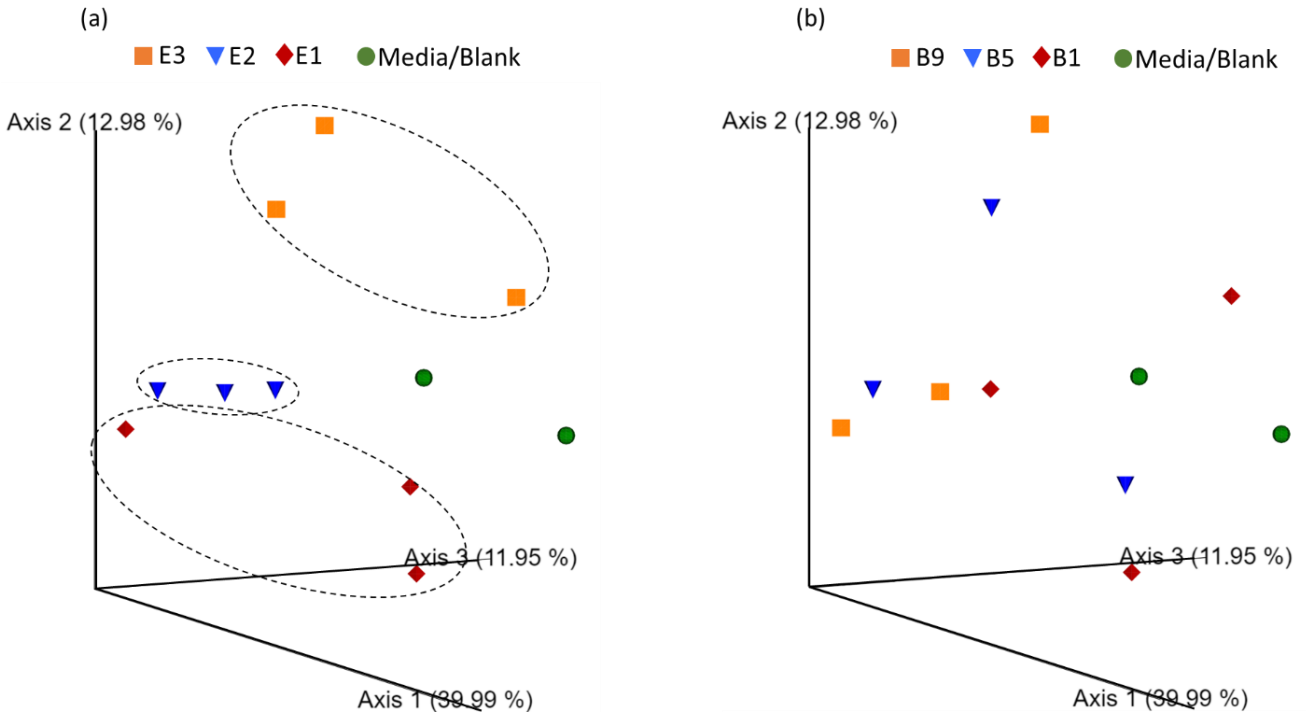

Fig. 19: *PCoA plots display the distance between metabolomes based on (a) extraction method (E1, E2, E3) and (b) bacteria (B1, B5, B9).*

A total of 889 features were detected in all samples. Background features were excluded, as well as those common with the control media, resulting in 521 target features. Applying tandem MS (MS/MS) provided the fragmentation pattern for detected molecules. By applying force (collision energy) molecules are fragmented based on the molecule chemical bonds. This enabled to get insight on the structural characteristics of compounds in the extracts and to putatively annotate them to chemical classes using available spectral libraries and databases. Ninety percent of the features had no spectral matches to the available resources. Yet, 10% were annotated and classified into 7 chemical superclasses (Fig 20a), with additional sub-classification as shown in figure 20b.

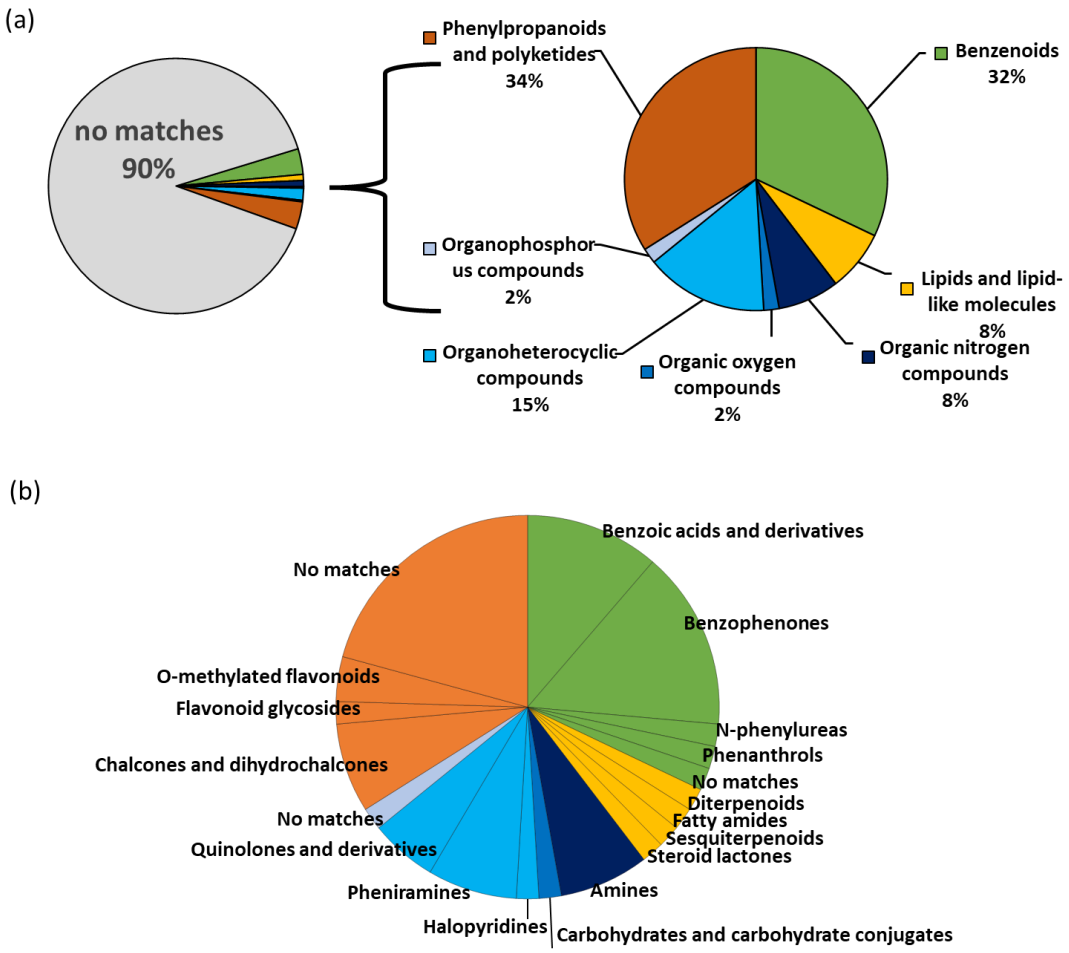

Fig. 20: *Pie charts representing the percentage of unique molecular features attributed to (a) superclass and (b) subclass.*

Because the fragmentation of molecules does not take place randomly and some chemical bonds are more prone for breaking, it is possible to classify compounds by patterns of fragments and neutral losses (Luzzatto Knaan et al., 2015). This information can also be used to organize the data based on structural similarity. Using the molecular networking workflow on GNPS, spectra are aligned and organized in a way that matching spectra are considered the same molecule (represented as a node) and similar spectra, clusters together into a molecular family (structurally related molecules) connected by edges. Numerous molecular families are creating a molecular network (Wang et al., 2016). Comparing the data to spectral libraries enabled the putative annotations of specific compounds as highlighted in Figure 21. Additional metadata could be added to highlight the origin and abundance of each feature.

Based on spectral alignment, some common Pseudomonas membrane structural lipids were annotaded as well as natural products such as phenazine and orfamide analogs, compounds are known to have some antimicrobial and antifungal activity.

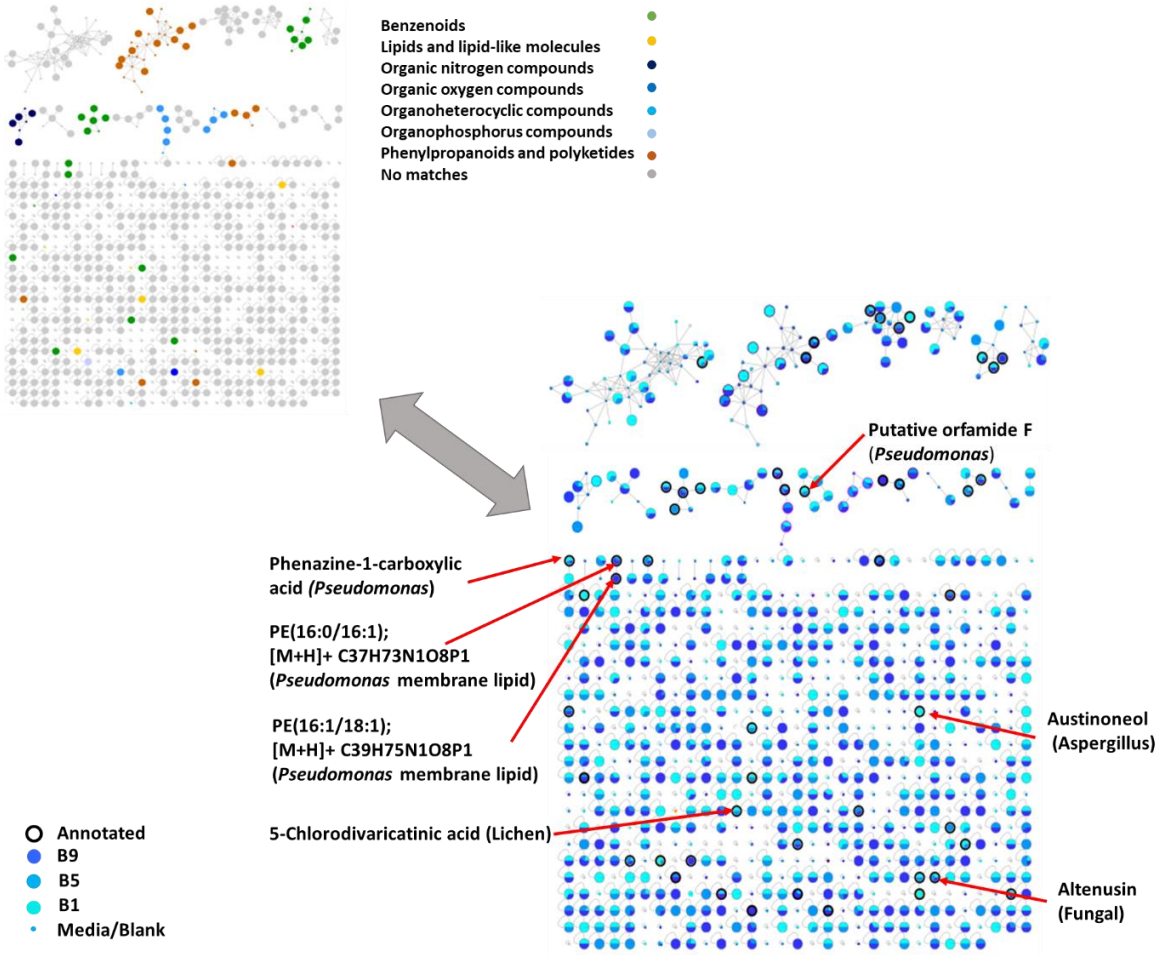

Fig. 21: *Molecular network of detected molecules, represented by superclass assessment and some annotations created by a match to the GNPS spectral library. Pie charts represent the abundance of a feature from a bacterial isolate origin.*

Extraction method 3 (E3) was found to be the most effective, as observed when the chromatograms of these samples from the three different bacterial isolates were compared (Fig 22).. Overall, chromatograms are very much alike, however differences in intensities of some selected peaks were detected, corresponding with the PCoA analysis (Fig 19a).

While the activity of the bacterial isolate B9 was higher than B1 and B5 in all the tested extractions, Extract B9E3 was the most active. In Figure 23, the chromatograms of the three B9 extract were compared highlighting the differences in detected peaks. Table 6 display the list of compounds that

were detected in B9 extracts only. These compounds can be further explored in future targeted studies to get a deeper understanding about the active compounds in B9 by activity guided isolation.

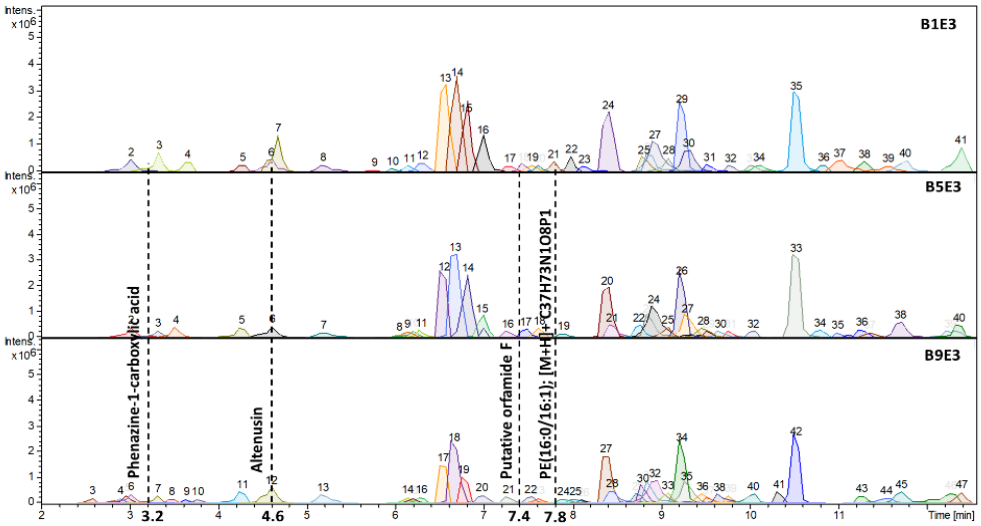

Fig. 22: *Extracted ion chromatograms of the E3 extraction from B1, B5 and B9 bacterial isolates. Dashed lines highlight related peaks for annotated features matched by mean retention time.*

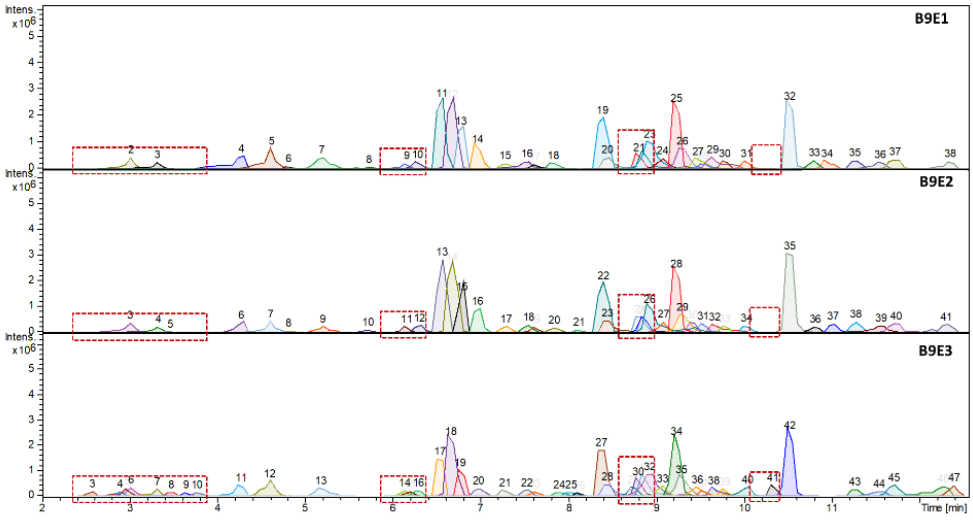

Fig. 23: *Extracted ion chromatograms of the B9 bacterial isolate extracted by E1, E2 and E3 extraction methods. Dashed rectangular highlight some differentiate peaks.*

Table 6: *The detected features from B9 bacterial cells extracted by different methods (E1, E2, E3).*

Bacteria	Extraction	Class	Subclass	Superclass	Ccluster index	Compound_Name	Parent Mass	RTMean
B9	E3	no matches	no matches	no matches	8577		318.11	0.40
B9	E2,E3	no matches	no matches	no matches	6252		282.68	0.51
B9	E2,E3	no matches	no matches	no matches	4476		229.17	2.03
B9	E1	no matches	no matches	no matches	8259		313.21	2.12
B9	E3	no matches	no matches	no matches	3987		211.50	2.20
B9	E3,E1	no matches	no matches	no matches	5713		263.69	2.21
B9	E3	no matches	no matches	no matches	3517		189.49	2.28
B9	E3,E1	no matches	no matches	no matches	7044		297.98	2.37
B9	E2,E3	no matches	no matches	no matches	6121		279.07	2.64
B9	E1	no matches	no matches	no matches	4381		227.49	2.81
B9	E2	no matches	no matches	no matches	4096		219.20	3.02
B9	E3	no matches	no matches	no matches	15314		552.34	3.19
B9	E2,E3	no matches	no matches	no matches	4706		237.21	4.15
B9	E3,E1	Organonitrogen compounds	Amines	Organic nitrogen compounds	9386		348.29	4.59
B9	E2,E1	no matches	no matches	no matches	4300		226.24	4.59
B9	E2,E1	no matches	no matches	no matches	11368		410.25	5.85
B9	E2,E3,E1	no matches	no matches	no matches	12091		419.25	5.92
B9	E2	no matches	no matches	no matches	6380		283.93	6.81
B9	E2,E3	Linear 1,3-diarylpropanoids	Chalcones and dihydrochalcones	Phenylpropanoids and polyketides	8226	MLS002695918-01!70356-09-1	312.86	6.88
B9	E2,E3,E1	no matches	no matches	no matches	17634		712.45	7.41
B9	E2,E3	no matches	no matches	no matches	6451		284.28	7.61
B9	E2,E3	Benzene and substituted derivatives	Benzophenones	Benzenoids	7710	Cryptotanshinone	299.04	7.62
B9	E3,E1	no matches	no matches	no matches	17650	PE(16:1/18:1);[M+H]+ C39H75N1O8P1	716.48	7.76
B9	E3,E1	no matches	no matches	no matches	17824		738.46	7.80
B9	E2,E1	no matches	no matches	no matches	7091		297.22	8.12
B9	E2,E3	no matches	no matches	no matches	17292		689.93	8.19
B9	E1	no matches	no matches	no matches	10412		379.27	8.27
B9	E1	no matches	no matches	no matches	14814		532.33	8.71
B9	E2,E3	no matches	no matches	no matches	18630		909.60	8.74
B9	E1	no matches	no matches	no matches	18492		865.57	8.77
B9	E2,E3	no matches	no matches	no matches	16741		669.69	8.81
B9	E2	no matches	no matches	no matches	2237		158.96	8.82
B9	E3,E1	no matches	no matches	no matches	17289		689.48	8.83
B9	E2,E1	no matches	no matches	no matches	18092		778.01	8.89
B9	E1	Flavonoids	Flavonoid glycosides	Phenylpropanoids and polyketides	13908	Massbank:PR301905 Gossypetin-8-C-glucoside	482.30	8.97
B9	E2,E3	no matches	no matches	no matches	13322		449.33	9.24
B9	E3,E1	no matches	no matches	no matches	13584		467.96	9.94
B9	E2,E3	no matches	no matches	no matches	15550		575.40	9.94
B9	E2,E1	no matches	no matches	no matches	17129		686.40	9.97
B9	E1	no matches	no matches	no matches	9493	1-[2-hydroxy-4-(3-hydroxy-5-methylphenoxy)-6-methylphenyl]-3-methylbutane-2,3-diol	354.39	10.06
B9	E2,E3,E1	no matches	no matches	no matches	15997		607.49	10.25
B9	E2,E3	no matches	no matches	no matches	18518		872.56	10.36
B9	E3,E1	no matches	no matches	no matches	10145		368.38	10.39
B9	E3,E1	no matches	no matches	no matches	9490		354.39	10.44
B9	E2,E3	no matches	no matches	no matches	9478		354.38	10.56
B9	E3,E1	no matches	no matches	no matches	15859		595.46	10.61
B9	E2,E1	no matches	no matches	no matches	18318		823.46	10.75
B9	E2,E3	no matches	no matches	no matches	18317		823.46	10.79
B9	E2,E1	no matches	no matches	no matches	18320		824.00	10.82
B9	E2,E3	no matches	no matches	no matches	9571		354.69	11.14
B9	E1	no matches	no matches	no matches	16943		685.40	11.21
B9	E2,E3	no matches	no matches	no matches	14859		538.11	11.44
B9	E3	no matches	no matches	no matches	16714		667.32	11.53
B9	E3,E1	no matches	no matches	no matches	18184		789.57	12.02

4. Discussion and conclusions

The main goal of the present study was the demonstration of the great potentiality of microbial bioactive compounds (MBCs) to develop alternative control methods against plant diseases. This goal was achieved through a number of consequential and strictly related experiments aimed at the selection of microorganisms, at the extraction and characterization of bioactive compounds and at their evaluation as preparations to control plant diseases. The first step was the development of a simple method to select microorganisms producing MBCs effective against fungi. This method proved appropriate since it enabled the identification of many soil-inhabiting bacteria producing a clear inhibition halo as a consequence of the synthesis and release of extracellular antifungal compounds in the culturing medium. These findings confirmed the great potential of natural soils as a reservoir of MBCs. In preliminary trials, other potential sources of MBC-producing microorganisms such as manure and "*ricotta forte*", a traditional whey cheese obtained through natural fermentation of fresh ricotta, were tested but did not yield a consistent number of microorganisms (data not shown). Our findings are not surprising since the rhizosphere is considered one of the most complex and rich microbiological niches (Lakshmanan et al., 2014; Bandyopadhyay et al., 2017; Thompson et al., 2017).

In our trials *P. digitatum* was utilized as representative plant pathogen to select MBC-producing microorganisms from soil but the same method is putatively implementable with any necrotrophic fungus. Regardless of the pathogen utilized for the selection, most bacteria proved to be effective against a much broader range of plant pathogens. In particular, *in vitro* dual culture tests showed a clear inhibition also against other two fungal species (*B. cinerea* and *A. alternata*) and an oomycete (*P. palmivora*). Furthermore, *in vivo* trials on artificially inoculated fruits highlighted an interesting activity of most selected bacteria in six different host/pathogen combinations (Table 3). These findings are important since indicate a broad range of activity of bacteria selected with *P. digitatum*. Importantly, as later discussed therein, the same conclusion arose from results of trials with extracts. However, an in-depth analysis of result shows that the same conclusion does not fit with all bacterial isolates. In fact, few isolates were very effective against some tested microorganisms (*in vitro* tests) and in some host/pathogen combinations (*in vivo* tests) but much less in other. These findings clearly indicate that pathogen and/or host can significantly influence the activity of bacteria and their MBCs. As a consequence, the selection MBC-microorganisms using propagules of the same pathogens to be controlled may be advisable in future investigations.

Eight bacterial strains (B1, B2, B5, B6, B7, B8, B9) were selected according to both *in vitro* dual culture tests and *in vivo* trials on artificially inoculated fruits. According to 16S gene sequences and Box-

PCR profiles, these strains were associated to four different genotypes. BLAST analyses enabled the accurate identification of the genus since all genotypes clearly clustered within the genus *Pseudomonas*. On the contrary the identification of the species was not possible because the rate of taxonomic errors in GenBank databases is low until the genus level (< 1%) but became very high at the species level (Leray et al., 2019). In agreement with this consideration the use of the MSA Viewer tool could enable the identification of several sequences very similar or even identical to those of our genotypes but deposited with different names. Interestingly, extracts from strains sharing the same 16S gene showed an overall different level of efficacy in controlling plant diseases. For instance, extracts of strain B9 were usually more effective than those of strain B5 that, in turn, were more effective than those of isolates B2 and B10. The existence of differences between strains B1, B5 and B9 was also confirmed by metabolomics analyses although the PCoA analysis did not enable a clear clustering of the extracts. The sequencing of the genome, currently in progress, should enable the identification of genes associated to the different biological activity and will also clarify if B5 and B9 are different strains of the same species or closely related species sharing the same 16S gene (Lalucat et al., 2020).

Three different extraction methods based on the use of ethanol as solvent were tested in the present study. The first two methods used bacterial cells collected with a spatula from agar plates or by centrifugations from liquid cultures. The third method extracted compounds from both cells and culturing liquid medium. The three methods yielded and similar quantity of lyophilized extract (approximately 0.2 g of dry material) and their extracts showed a similar efficacy in reducing the incidence of *P. digitatum* on artificially inoculated tangerines. However, MIC analyses showed a higher antifungal activity of extracts obtained with the third method. Furthermore, metabolomics analyses showed a clear clustering according to the extraction method and the presence of molecules specifically associated to each method. In particular, extracts obtained with the third method were clearly differentiated from the other two. This result is not surprising considering that the third method provided the extraction from both bacterial cells and culturing media and that microorganisms produce and secrete many primary and secondary metabolites to the surrounding environment during their growth (Pinu and Villas-Boas, 2017). As a consequence, although most preliminary trials were conducted using extracts obtained with the first method, above results and considerations prompted the use of the third extraction method for large scale trials. In our opinion, this method guarantees greater chances of success for future putative practical application also because of its simplicity considering that cultured bacteria can be subjected to extractions without any preliminary preparation.

MIC analyses highlighted an overall low *in vitro* antifungal activity of extracts since most of them did not show a clear inhibition halo, even when utilized at high concertation (300 mg/L). The same extracts proved much more effective at much lower concentrations (ranging from 1.5 and 36 mg/L) when applied on artificially inoculated fruits or even against natural infections. If on the one hand a dilution effect of the extracts applied on paper pad may have contributed in determining the low efficacy in MIC analyses on the other hand a key role of the plant response seem to be unquestionable to justify the much higher *in vivo* efficacy. In agreement with this hypothesis, extracts of isolate B6 provided the strongest inhibition halos in MIC tests but were not as effective as other extracts such as those of isolates B1, B5 and B9 in reducing rots on artificially inoculated fruits. In particular, a lower efficacy of B6-extracts was recorded in tests performed to evaluate the induction of resistance in treated host tissues by applying extracts and pathogens in spatially separated wounds. These results clearly shows that extracts of isolates B6 contains metabolites with a strong antifungal activity but less effective in activating resistance responses in host tissues. On the other hand, the most effective extracts obtained from isolates B1, B5, and B9 showed a very faint inhibition halo (only at the highest concentration and only when obtained with the third extraction methods) but determined a strong activation of resistance responses in treated fruits. The induction of resistance to control plant diseases has been largely investigated in the last two decades since it is considered one of the most promising alternative control strategies to reduce the use of convention chemical fungicides (Wilson et al., 1994; Romanazzi et al., 2016; Walters et al., 2007; Pangallo et al., 2017; Wang and Bi, 2021). In any case, a complex mechanism of action that include direct antimicrobial activity and induction of resistance in treated plant tissues may be important to increase reliability and spectrum of activity (Belgacem et al., 2021).

Interestingly, extracts also showed a relevant curative activity. The control of already established infections is important since most currently available alternative control means exert a preventive activity and are effective only when applied before the infection takes place (Belgacem et al., 2021). The control of already established infections may be strategic for postharvest diseases since it enables the reduction of latent infections, which play a fundamental role in the epidemiology of many fungal diseases (Wenneker and Thomma, 2020). The mechanisms by which extracts exerted their curative action is unknown. A direct antifungal activity against the colonizing fungi might be theoretically possible, but it seems unlikely considering the low activity of extracts determined in MIC analyses. Furthermore, extracts were used on fruits at a low concentration (1.5, 12 and 36 mg/L) and their penetration and diffusion within host tissue would determine a further decrease of concentration. More likely, the observed curative activity was a consequence of the rapid activation of resistance responses and the

consequent production of antifungal compounds as recently reported for fruits treated with a pomegranate peel extract (Pangallo et al., 2017; Belgacem et al., 2019).

In agreement with above considerations, tests conducted to evaluate the induction of resistance showed a very quick activation of responses since rots were strongly reduced on fruits treated with the extract and then inoculated with pathogens in spatially separated wounds just one hour later. Interestingly, the quick activation of the resistance response was followed by a quite quick reduction, since a lower level of protection was recoded when the pathogens were inoculated 6 h after the extracts and, this trend was confirmed after 24 h. The quick response is undoubtedly a positive feature of tested microbial extracts since the timing of defense responses is critical and reflects the difference between coping and succumbing to the challenge of necrotizing pathogens (Choudhary and Johri, 2007). On the other hand, the rapid decrease of the resistance response may indicate a short-term protection and the consequent need for repeated treatments. Future investigations are required to investigate these important aspects since they are essential to optimize application strategies.

Trials conducted on olives and sweet cherries simulating practical commercial conditions were important because confirmed the potential of tested microbial extracts as reliable alternative control means. In fact, several safe methods have been proposed in the last 20-30 years after provided promising results in experimental conditions, but then failed in practical commercial conditions. This is particularly true for products based on microbial antagonists, which are greatly influenced by external factors including environmental conditions and host species (Ippolito et al., 2005). In the present thesis, particularly relevant are also result of field trials conducted with extracts of isolate B9 to control grape powdery mildew. The extract significantly reduced the incidence and severity of the disease on grape berries and leaves, showing a level of efficacy comparable to that of chemicals commonly used under filed conditions and PGE, a well-known alternative control mean (Belgacem et al., 2021). The incidence of the disease was not significantly reduced on the new vegetation that did not directly receive the treatment. This result may be related to the above discussed low persistence of the induced resistance although specific investigations are needed to confirm this hypothesis. In any case, an equal disease index was recoded on plant sprayed with chemicals and PGE.

In conclusion, results of the present study showed a great potentiality of bacterial microbial extracts as alternative methods to control plant diseases also because no signs of phytotoxicity were recorded. The most effective extracts showed a broad spectrum of activity being effective in several different host/pathogen combination including disease caused by several necrotrophic fungi and, importantly, also hemibiotrophic (*Colletotrichum* spp. on olives) and biotrophic (*Oidium tuckeri* on

grape) pathogens. Interestingly, they proved a high level of efficacy comparable to that of a well-characterized plant extract (PGE) and, in the case of filed trial, to that of conventional chemicals. PGE as well as other pomegranate peel extracts have been proposed as reliable and versatile natural alternative control methods with high efficacy and a broad range of activity. Their use to obtain low-cost formulations able to compete with traditional chemical compounds is currently under investigation (Belgacem et al., 2020). To this regard, it is important to point out that bacterial extracts tested in the present study were utilized at a much lower concentration (between 1.5 and 36 mg/L) as compared to PGE (6 g/L). Since the concentration is a key factor directly affecting the efficacy of any control molecule or preparation, these data further highlight the potentiality of tested microbial extracts. In fact, it is also important to point out that in the present study a high quantity of lyophilized extracts (2 g of dry matter) were obtained from a low quantity bacterial suspension (200 ml). Therefore, few milliliters of bacterial cultures (200 ml) were enough to have more than 83 liters of working solution of extracts at 24 mg/L i.e. the concentration used in large-scale trials. On a practically point of view these considerations are very important in light of a possible development of cost-effective formulations. Furthermore, since the bacteria were obtained from natural soils it can be likely assumed they are very competitive microorganisms without particular nutritional requirements and easily culturable on simple and cheap substrates. For example, it would be interesting to evaluate their growth on nutritive media obtained with by-products of the food industry.

The main obstacle to the possible future commercialization of formulations having microbial extracts as active substances is, undoubtedly, the verification of their safety for the environment and for humans and, consequently, their acceptance by the public opinion. In the present study, metabolomic analyses highlighted an extremely complex composition and the presence among other of membrane structural lipids substances and natural products such as phenazine and orfamide analogs, which are known to have some antimicrobial and antifungal activity. Additional accurate investigations are needed to clarify the composition of extracts, identify active ingredients and exclude the presence of putative dangerous molecules. Encouraging aspects can be identified in the very low concentration at which extracts exert their activity, the mechanism of action mainly based on the induction of resistance and last, but not least, the natural origin of compounds that should guaranty a quick and complete degradation after their release in the environment.

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7. List of figures

Fig. 8: *Representative photo of preventive trials conducted on tangerines artificially inoculated with Penicillium digitatum using extracts from isolates B1, B5, and B9. Mock fruit treated with a solution of EtOH (12 ml/l) and inoculated with the pathogens served as controls.*

Fig. 9: *Incidence of decays on Tangerines artificially inoculated with Penicillium digitatum and treated 24 hour later with extracts from isolates B1, B5, and B9 at 1.5, 12, or 36 mg/L (curative activity). A 6 g/L pomegranate peel extract was included in the experiment as a reference treatment. Fruit inoculated with the pathogen and than treated with a solution of EtOH (12 mg/L) and served as controls. Bars indicate standard errors of the means. Per each tested concentration different letters indicate significantly different values according to Tukey's test (P≤0.05).*

Fig. 10: *Percentage of inhibition of rots in table grapes and tomatoes treated with bacterial extracts and then inoculated with B. cinerea or A. alternata in spatially separated wounds. Bacterial extracts were applied at three different concentrations (1.5, 12 or 36 mg/L) 1, 6 or 24 hs before the inoculation of the pathogens. Fruit treated with EtOH or PGE and inoculated with the pathogens served as controls. Per each pathosystem and timing of inoculation, different letters indicate significantly different values according to Tukey's test (P≤0.05).*

Fig. 11: *Representative photo of trials conducted on table grapes to evaluate the induction of resistance. Grapes berries received 10 µl of EtOH at 12 mg/L (left) or B9 extract at 36 mg/L (right) and, approximately 1 hour later, were inoculated with a spore suspension of Botrytis cinerea in a spatially separated wound.*

Fig. 12*: Comparison of the efficacy of extracts obtained with the three tested methods (Ext.1, Ext.2, and Ext.3) from isolates (B1, B5 and B9). Tests were conducted on tangerines inoculated with P. digitatum. Fruit treated with EtOH and inoculated with the pathogens served as controls. Bars indicate standard errors of the means. Per each bacterium, the same letter indicate the absence of significantly different values according to Tukey's test (P≤0.05).*

Fig. 13: *Incidence and gravity (McKinney index) of anthracnose on olives dipped in a solution of bacterial extracts (B1, B5, and B9) or PGE. Untreated olives and olives dipped in tap water were used as controls. Experiments were conducted with non-inoculated olives (left) and with olive inoculated by*

nebulization with a conidia suspension of C. acutatum s.s. (right). Bars indicate standard errors of the means. Different letters indicate significantly different values according to Tukey's test (P≤0.05).

Fig. 14: *Incidence and gravity of natural rots (McKinney index) on sweet cherries, cv Ferrovia, dipped in a solution of B9 extract at 24, 12, and 6 mg/L. Rots were evaluated after 14 days of cold storage at 1 °C. Sweet cherries dipped in a solution of PGE at 6 g/L or in tap water were used as controls. Bars indicate standard errors of the means. Different letters indicate significantly different values according to Tukey's test (P≤0.05).*

Fig. 15: *Incidence and gravity of natural rots (McKinney index) on sweet cherries, cv Giorgia, dipped in a solution of B9 extract at 24, 12, and 6 mg/L. Rots were evaluated after 14 days of cold storage at 1 °C and then after 1, 2 and 3 days of shelf-life. Sweet cherries dipped in a solution of PGE at 6 g/L or in tap water were used as controls. Bars indicate standard errors of the means. Per each assessment time, different letters indicate significantly different values according to Tukey's test (P≤0.05).*

Fig. 16: *Representative photo of trials conducted with sweet cherries cv Ferrovia. Cherries were dipped in a solution of B9 extract at 24 at 24 mg/L, PGE at 6 g/L or tap water (control). The photo was made after 14 day of cold storage*

Fig. 17*: Incidence and gravity of powdery mildew (McKinney index) on grape berries, young leaves, and mature leaves treated with B9 extract at 24 mg/L. Untreated plants, and plants sprayed with PGE at 6 g/L or with chemicals commonly utilized in control schedule of the farm were used as controls. Treatment were made on June 6 and July 5, 2021 while incidence and gravity of the disease was evaluated on July 3 and 29, 2021.* Bars indicate standard errors of the means. *For each assessment time columns with different letters are statistically different according to the Tukey test (P ≤ 0.05).*

Fig. 18: *Total soluble solids (°Brix) in grape berries treated with B9 extract, or PGE as compared to grapes untreated or sprayed with chemicals. Bars indicate standard errors of the means. The same letter indicate the absence of significantly different values according to Tukey's test (P≤0.05).*

Fig. 19: *PCoA plots display the distance between metabolomes based on (a) extraction method (E1, E2, E3) and (b) bacteria (B1, B5, B9).*

Fig. 20: *Pie charts representing the percentage of unique molecular features attributed to (a) superclass and (b) subclass.*

Fig. 21: *Molecular network of detected molecules, represented by superclass assessment and some annotations created by a match to the GNPS spectral library. Pie charts represent the abundance of a feature from a bacterial isolate origin.*

Fig. 22: *Extracted ion chromatograms of the E3 extraction from B1, B5 and B9 bacterial isolates. Dashed lines highlight related peaks for annotated features matched by mean retention time.*

Fig. 23: *Extracted ion chromatograms of the B9 bacterial isolate extracted by E1, E2 and E3 extraction methods. Dashed rectangular highlight some differentiate peaks.*

8. List of tables