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Heat-inactivated *Lysobacter capsici* AZ78 cells effectively control *Peronospora belbahrii* and *Plasmopara viticola* through direct and indirect mechanisms

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Abstract The bacterial strain *Lysobacter capsici* AZ78 is an effective biocontrol agent against several plant-pathogenic oomycetes. This study demonstrates that heat-inactivated *L. capsici* AZ78 cells retain strong protective activity against *Plasmopara viticola* and *Peronospora belbahrii*, comparable to that of viable *L. capsici* AZ78 cells, as well as a copper-based fungicide and a plant resistance inducer. Microscopic analyses showed that both heat-inactivated and viable *L. capsici* AZ78 cells were toxic to *P. viticola* sporangia. Moreover, microscopic analyses also revealed their ability to induce callose deposition and reactive oxygen species accumulation in basil and grapevine leaves, indicating the stimulation of plant resistance mechanisms. Chemical analyses identified dihydromaltophilin and maltophilin, two heat-stable polycyclic tetramate macrolactams, in the membranes of *L. capsici* AZ78 cells. Isolated dihydromaltophilin and maltophilin, individually or in combination, were highly effective against *P. viticola* and triggered callose deposition in grapevine leaf discs. These findings demonstrate that *L. capsici* AZ78 and its heat-stable polycyclic tetramate macrolactams act through a dual mechanism, direct antimicrobial activity and induction of plant defence responses. The use of heat-inactivated *L. capsici* AZ78 cells and their secondary metabolites offers a promising, environmentally friendly alternative to copper-based products for the sustainable management of downy mildews.

Keywords *Lysobacter* · Downy mildew · Biocontrol · Polycyclic Tetramate Macrolactams · Dihydromaltophilin · Plant resistance

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Introduction

In the field, crop plants have to face diverse biotic and abiotic stresses continuously. Among these, plant diseases caused by (micro)organisms represent a major factor contributing to global yield losses and pose

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a serious threat to agricultural production (Savary et al., 2019). To sustain and improve crop plant productivity, it is therefore crucial to develop strategies that effectively manage plant pathogenic (micro) organisms. Given the environmental and regulatory concerns associated with chemical inputs in agriculture, there is increasing interest in environmentally friendly and sustainable approaches for detecting and controlling plant pathogenic (micro)organisms (Helepciuc & Todor, 2022; Song et al., 2025).

One promising strategy involves the use of microbial biopesticides, plant protection products that employ microbial BioControl Agents (mBCAs) as active ingredients. These are effective in controlling plant pathogenic (micro)organisms, promote plant growth, and improve nutrient uptake (Puopolo, 2022; Ray et al., 2020). Interest in microbial biopesticides has grown rapidly, with 13 bacterial mBCAs already authorised for commercial use in the European Union (https://food.ec.europa.eu/plants/pesticides/eu-pesticides-database_en). The efficacy of mBCAs largely depends on their ability to produce a wide array of secondary metabolites, including lytic enzymes, siderophores, toxins, volatile organic compounds (VOCs), polyketides, and non-ribosomal peptides (Sarrocco, 2023). For example, *Bacillus subtilis* can suppress the growth of several plant-pathogenic (micro)organisms by producing secondary metabolites (Kiesewalter et al., 2021). Similarly, certain secondary metabolites produced by *Pseudomonas* spp. exhibit antagonistic activity against bacteria, fungi, and oomycetes (Raio & Puopolo, 2021). Furthermore, secondary metabolites extracted from *Trichoderma* spp. are often more effective as plant protection products than the organisms themselves (Vinale et al., 2009). Plants have also evolved mechanisms to recognise mBCAs and their bioactive compounds, thereby enhancing their capacity to resist pathogen attacks by triggering plant resistance mechanisms (Hönig et al., 2023). In particular, cyclic lipopeptides such as orfamide and surfactins, produced respectively by plant-beneficial *Pseudomonas* spp. and *Bacillus* spp., can be perceived by plants and stimulate defence mechanisms against plant-pathogenic (micro)organisms (Nimbeshaho et al., 2024; Omoboye et al., 2019).

Within this context, the genus *Lysobacter* (family Lysobacteraceae) has emerged as an important reservoir of mBCAs and secondary metabolites. They are well recognised for their ability to produce a broad spectrum of antimicrobial compounds, enabling them to prey on other microorganisms, particularly plant pathogenic (micro)organisms (Meers et al., 2018; Puopolo et al., 2018; Vlassi et al., 2020a). Among the most significant secondary metabolites are polycyclic tetramate macrolactams (PTMs), produced by many *Lysobacter* species (Li et al., 2021; Liu et al., 2022). One member of the PTM family is dihydromaltophilin (DMP), also known as the heat-stable antifungal factor (HSAF) due to its stability at high temperatures. Chemically, DMP is classified as a polycyclic macrolactam, a structural motif common in natural bioactive products that contain a tetramic acid, a class of compounds known for their antibiotic and anticancer properties (Schobert & Schlenk, 2008). DMP has a distinctive mode of action compared with previously known antifungal secondary metabolites. Indeed, it acts on multiple molecular pathways and interferes with a broad range of essential fungal cellular structures (Chen et al., 2024; Li et al., 2006, 2021).

DMP was first isolated from *Streptomyces* sp. (Graupner et al., 1997) and later identified in *L. enzymogenes* C3, where it demonstrated antagonistic activity against plant-pathogenic fungi (Li et al., 2006). It exerts its antifungal activity by targeting specific sphingolipids that are essential for polarised filament growth in fungal cells. Studies have demonstrated the negative impact of this molecule on various fungi, including *Alternaria alternata*, *Aspergillus nidulans*, and *Candida albicans* (Ding et al., 2016; He et al., 2018; Li et al., 2006). Notably, DMP was found to disrupt fungal growth via a reactive oxygen species (ROS)-dependent pathway in *C. albicans*, and its unique and selective mode of action makes it highly suitable for sustainable control of plant-pathogenic fungi. Specifically, DMP from *L. enzymogenes* C3 (previously identified as *Stenotrophomonas maltophilia* C3) inhibited the growth of plant-pathogenic fungi such as *Bipolaris sorokiniana* and *Uromyces appendiculatus* (Yuen et al., 2001; Zhang & Yuen, 1999), as well as oomycetes like *Pythium ultimum* (Kobayashi et al., 2005). In another study, DMP isolated from *L. enzymogenes* OH11 protected pear fruit against fungal decay caused by *Colletotrichum fruticola*. The protection was attributed to hyphal deformation and depolarisation, as well as inhibition of conidial germination (Li et al., 2021). Similarly, Liu et al. (2022) reported that DMP from *L. enzymogenes*

OH11 inhibited the growth of *Neurospora crassa* by activating fungal genes involved in cell wall formation. Additionally, crude DMP extracts from *L. enzymogenes* OH11 showed efficacy in managing Fusarium head blight (Zhao et al., 2019) and suppressing *Pythium graminicola* in maize (Ren et al., 2020). In the case of *Fusarium graminearum*, the causal agent of Fusarium head blight, DMP was recently shown to impact cell membrane and ergosterol biosynthesis by interacting with the oxysterol-binding protein FgORP1 (Chen et al., 2024).

Recent studies have further provided insight into the delivery mechanisms of DMP. DMP and other secondary metabolites are transported via outer-membrane vesicles (OMVs), which facilitate long-distance delivery to target plant pathogenic fungi (Yue et al., 2021). This vesicle-mediated mechanism has also been reported in *L. enzymogenes* C3, highlighting its relevance as a general strategy in *Lysobacter*–plant pathogen interactions. Specifically, DMP comes into contact with the outer membranes of *L. enzymogenes* C3 cells, from which it is released into the environment, where it is entrapped in OMVs until it establishes physical contact with plant-pathogenic fungi, thereby inhibiting their growth (Meers et al., 2018).

Among *Lysobacter* biocontrol agents, *L. capsici* AZ78 (AZ78) exhibits strong antagonistic activity against several economically important plant pathogens, including *Phytophthora infestans* and *Plasmopara viticola*, the causal agents of potato late blight and grapevine downy mildew, respectively (Puopolo et al., 2014a, 2014b). The biocontrol efficacy of AZ78 is associated with its production of multiple antagonistic factors, such as ammonia, antibiotics, lytic enzymes, and VOCs (Brescia et al., 2021; Puopolo et al., 2014a; Vlassi et al., 2020a, 2020b). Metabolomic analyses confirmed the presence of PTMs, notably DMP and its precursor maltophilin (MP), both of which exhibit potent activity against plant pathogens (Brescia et al., 2021). However, although these secondary metabolites have been identified in AZ78, their specific role in controlling plant-pathogenic oomycetes and the mechanisms by which they inhibit infection of crop plants remain largely unknown, prompting their reanalysis in this study.

To address this knowledge gap, we investigated whether the application of heat-inactivated AZ78 cells and the PTMs DMP and MP could be effective in controlling *P. viticola* and *Peronospora belbahrii*, the causal agent of basil downy mildew. Based on the results, we examined the mechanisms underlying the control of oomycetes using heat-inactivated AZ78 cells and assessed the role of PTMs in these processes. Overall, this study provides new insights into the multifaceted mechanisms of AZ78-mediated plant protection and highlights the potential of heat-inactivated AZ78 cells and their PTMs as promising components of next-generation microbial biopesticides.

Material and methods

Maintenance of bacterial strains

AZ78 and *Escherichia coli* DH5α (DH5α) were stored as 40% (v/v) glycerol stocks at − 80 °C. For routine culture, both bacterial strains were grown on Nutrient Agar (NA) in 90 mm Petri dishes and incubated for 48 h at 27 °C. To prepare bacterial cell suspensions, the Petri dishes were flooded with 5 mL of sterile distilled water, and the cells were gently scraped from the surface using sterile L-shaped spatulas. The resulting cell suspensions were homogenised in sterile distilled water. The absorbance at 600 nm was adjusted to 0.1, corresponding to approximately 1×10^8 colony-forming units (CFU)/mL, using a spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan) (Puopolo et al., 2014b). This bacterial cell concentration was used in all experiments unless otherwise specified.

Preparation of heat-inactivated bacterial cells

AZ78 and DH5α cell suspensions were transferred into sterile 15 mL centrifuge tubes and exposed to a thermal shock at 90 °C for 20 min using a thermomixer (T-shaker EMS100 Euro Clone, IT), followed by incubation at − 20 °C for five minutes (Brescia et al., 2021). After the thermal shock, cell viability was assessed by spot-inoculating 100 µL of each bacterial cell suspension onto NA. Once inoculated, Petri dishes were incubated at 27 °C. After 72 h of incubation, the occurrence of colonies was visually assessed. Five Petri dishes were prepared for each treatment.

Plants and respective plant pathogenic oomycetes

Sweet basil (*Ocimum basilicum* L.) plants were cultivated from seeds (cv. Italiano classico, Franchi Sementi, Italy) in a 1:1 (v/v) mix of peat and vermiculite in 0.5 L pots under controlled conditions (25 ± 1 °C, 70 ± 10% relative humidity (RH), and a 16/8 h day/night photoperiod) until they reached the 8–10-leaf stage. *P. belbahrii*, originally isolated from diseased basil plants in a local garden, was maintained by manually spraying the sporangial suspension onto both leaf surfaces until runoff occurred. Following inoculation, the plants were placed in a humid chamber (100% RH, 20 °C) to promote infection. After 20 h, basil plants were transferred to a growth chamber at 25 ± 1 °C, 70 ± 10% RH, and a 16/8-h day/night light regime to facilitate *P. belbahrii* colonisation. Six days post-inoculation (dpi), the plants were placed in the humid chamber at 20 ± 1 °C and 100% RH in darkness for 20 h to induce sporulation (Cohen & Rubin, 2015). To prepare *P. belbahrii* inoculum, diseased, chlorotic basil leaves bearing sporangia on the abaxial surface were harvested and rinsed with distilled water. The resulting sporangial suspension was filtered through cheesecloth to remove plant debris, and the sporangial concentration was adjusted to 1×10^5 sporangia/mL using a haemocytometer under a light microscope (Gilardi et al., 2013). This sporangial concentration was used in all experiments unless otherwise specified.

Vitis vinifera cv. Pinot Noir grapevine plants, grafted onto Kober 5BB rootstock, were grown in 2.5 L pots containing a 3:1 (v/v) mixture of peat and pumice in a greenhouse (20 ± 0.5 °C, 70 ± 10% RH) for 60 days, until the plants produced two shoots with at least nine leaves each. *P. viticola*, originally isolated from an untreated vineyard in San Michele all'Adige (Italy) in 2024, was maintained on healthy grapevine plants through weekly inoculations according to Puopolo et al. (2014b) under controlled conditions (20 ± 0.5 °C, 70 ± 10% RH). Briefly, grapevine plants showing oil-spot symptoms were kept overnight in the dark at 20 ± 0.5 °C and 100% RH. The inoculum was then prepared by rinsing sporulating lesions on the abaxial leaf surface with cold (4–5 °C) distilled water and adjusting the concentration to 2.5×10^5 sporangia/mL by counting under a light microscope using a haemocytometer. This sporangial concentration was used in all experiments unless otherwise specified.

Assessment of the efficacy of heat-inactivated and viable *L. capsici* AZ78 cells in controlling *P. viticola* on grapevine leaf discs

To prepare grapevine leaf discs, the third and fourth leaves from the top of healthy grapevine plants were collected and used to cut 12 mm discs using a cork borer. These discs were placed with the abaxial side facing upwards in 90 mm Petri dishes containing three layers of absorbent paper soaked in 8 mL of sterile distilled water to prevent the discs from drying out.

Once prepared, the grapevine leaf discs were sprayed with the following treatments: 1) sterile distilled water (untreated control); 2) copper-based fungicide Coprantol Hi Bio (copper hydroxide 20%, Syngenta; 2 g/L solution); 3) resistance inducer BION 50 WG (benzothiadiazole-7-carbothioic acid S-methyl ester 50%, Syngenta; 50 mg/L); 4) viable AZ78 cells; 5) heat-inactivated AZ78 cells; 6) viable DH5α cells; and 7) heat-inactivated DH5α cells. All treatments were applied manually using a nebuliser until complete runoff, followed by a five-minute drying period under a chemical fume hood. Petri dishes were then transferred to the greenhouse at 20 ± 0.5 °C.

After 24 h, the leaf discs were sprayed with *P. viticola* sporangial suspension (2 mL per Petri dish) and kept in darkness at 20 ± 0.5 °C and 80–99% RH overnight. Subsequently, the leaf discs were incubated for six days in a greenhouse at 25 ± 1 °C and 70–80% RH (Brescia et al., 2021). Disease severity (percentage of abaxial leaf area covered by sporulating lesions) was evaluated at seven dpi according to the EPPO standard scale (EPPO, 2004) and expressed as a percentage. Each treatment consisted of five Petri dishes containing five leaf discs, and the experiment was repeated.

Evaluation of the efficacy of heat-inactivated and viable *L. capsici* AZ78 cells in controlling *P. belbahrii* and *P. viticola in planta*

Regarding efficacy against *P. viticola*, viable and heat-inactivated AZ78 cells were applied to both leaf surfaces of grapevine plants grown in the greenhouse using a hand sprayer. The treatments

applied were: 1) sterile distilled water (untreated control); 2) copper-based fungicide (Coprantol Hi Bio, 2 g/L); 3) viable AZ78 cells; and 4) heat-inactivated AZ78 cells. Once treated, grapevine plants were maintained at 25 ± 0.5 °C and 60–80% RH with a 16/8 h day/night light regime.

After 24 h, *P. viticola* sporangial suspension was uniformly sprayed onto the abaxial surfaces of fully expanded grapevine leaves using a hand sprayer. Subsequently, the plants were incubated in the dark at 20 ± 0.5 °C and 80–99% RH for 24 h, followed by maintenance at 25 °C (60–80% RH) under a 16/8 h day/night regime. After six days, the plants underwent an overnight dark incubation at 20 ± 0.5 °C and 80–99% RH to induce sporulation (Puopolo et al., 2014b). Disease severity was evaluated at seven dpi using the EPPO standard scale (EPPO, 2004). Each treatment was applied to five plants (replicates), and the experiment was repeated three times.

For efficacy against *P. belbahrii*, viable and heat-inactivated AZ78 suspensions were applied to basil plants. The treatments were: 1) sterile distilled water (untreated control); 2) copper-based fungicide Cuprotax S.D.I. (copper sulfite 15.20%, Sipcam; 0.594 mL/L) (Gilardi et al., 2020); 3) viable AZ78 cells; and 4) heat-inactivated AZ78 cells. Treatments 3 and 4 were used at an absorbance of 0.4 at 600 nm, corresponding to 1×10^9 CFU/mL. The treatments were applied using a hand sprayer (5 mL per plant), and the plants were maintained under controlled greenhouse conditions at 25 ± 1 °C (60–80% RH) with a 16/8 h day/night light regime.

After 24 h, each plant was sprayed with *P. belbahrii* sporangial suspension (5 mL per plant) and transferred to a humid chamber set at 100% RH and 20 ± 0.5 °C for 20 h. Subsequently, the plants were moved to a greenhouse chamber at 25 ± 1 °C (60–80% RH) with a 16/8 h day/night regime. Six dpi, the plants were again placed overnight in a humid chamber at 100% RH (Cohen & Rubin, 2015). After seven days, disease severity was assessed on eight leaves per plant using a 0–100 scale, where 0 represented no symptoms and 100 indicated complete coverage of the leaf surface by sporulating lesions. Lower leaves were excluded from the evaluation (Elad et al., 2016). Each treatment was applied to five plants (replicates), and the experiment was repeated three times.

Determination of the ability of heat-inactivated and viable *L. capsici* AZ78 cells to stimulate the deposition of callose and accumulation of reactive oxygen species in basil and grapevine leaves

For visualising callose deposition and reactive oxygen species (ROS) production, basil and grapevine leaf discs were used. Grapevine leaf discs were prepared as described above. The experimental treatments included: 1) distilled water (untreated control); 2) BION 50 WG (50 mg/L) as a positive control for callose deposition; 3) laminarin at a concentration of 2 mL/L as a positive control for ROS production; 4) viable AZ78 cells; and 5) heat-inactivated AZ78 cells. All treatments were applied to grapevine leaf discs 24 h before inoculation with *P. viticola*.

In the case of basil, treatments 1–5 were applied to the plants one day before inoculation with the *P. belbahrii* sporangial suspension. Twenty-four hours after *P. belbahrii* inoculation, 12 mm leaf discs were carefully excised from each treated plant using a sterile cork borer. These leaf discs were cut immediately before staining to minimise potential wounding effects, thereby ensuring accurate and reliable subsequent analyses. In both cases, leaf discs were stained and observed at two dpi.

To assess callose deposition in the guard cells of basil and grapevine leaf discs, a fluorescence microscopy method was used, staining plant tissues with 0.05% aniline blue in 0.06 M K_2HPO_4 (pH 8) (Palmieri et al., 2012). For staining grapevine leaf discs, samples were placed in 50 mL sterile centrifuge tubes containing 1 M KOH and immersed in a thermal bath at 95 °C for 15 min. After decantation, the discs were rinsed three times for 15 s each with sterile distilled water to remove KOH residues. Subsequently, the leaf discs were submerged in 0.05% aniline blue solution for 15 min before being mounted on glass slides. The slides were covered with coverslips and sealed along the edges for long-term preservation. All slides were wrapped in aluminium foil until observation. Callose deposition was visualised as blue fluorescence in guard cells. For basil leaf discs, aniline blue staining followed the same steps, except that the boiling period in KOH was shortened to 11 min at 95 °C due to the delicate nature of the leaves.

To assess ROS production, 3,3′-diaminobenzidine (DAB) staining was used. Briefly, basil and grapevine leaf discs were incubated for five hours in 1 mg/

mL DAB staining solution in HCl-acidified water at pH 3.8, as described by Thordal-Christensen et al. (1997). Afterwards, basil and grapevine leaf discs were incubated for 15 min at 90 °C in a destaining solution containing ethanol, acetic acid, and glycerol (3:1:1) to remove chlorophyll, carotenoids, and other plant pigments (Daudi & O'Brien, 2012). Due to their tenderness, the basil leaf discs were incubated in the destaining solution for only 11 min.

Subsequently, microscopic observations were conducted to assess callose deposition and ROS accumulation using a Leica LMD7000 microscope (Germany). ROS were visualised under bright field as dark reddish-brown spots resulting from DAB absorption and polymerisation. For callose deposition, an A4 filter (BP 320–400 nm excitation, 400 nm dichroic mirror, BP 470 nm emission) was used for blue-fluorescence imaging. Five slides per treatment were prepared in both cases, and the experiments were repeated three times.

Quantification of callose deposition and ROS accumulation in basil and grapevine leaves

To determine callose deposition, the proportion of stomata exhibiting detectable aniline blue fluorescence was calculated. To do that, stained basil and grapevine leaf discs were examined under a Leica LMD7000 microscope (Germany), using the same magnification and exposure settings as reported above. For each treatment, five biological replicates (slides) were prepared, and the experiments were repeated three times. From each biological replicate, five randomly selected, non-overlapping fields of view were analysed. In each field, all clearly visible stomata were counted. Stomata were scored as callose-positive when a distinct blue, fluorescent ring or localised deposition was observed surrounding the guard cells. At least 100 stomata were counted per biological replicate. The callose deposition was calculated as:

$$Callose-positive\ stomata\ (\%) = (Number\ of\ stomata\ with\ callose/Total\ number\ of\ stomata\ observed) \times 100$$

For the ROS production, the digital images of the leaf discs obtained under a bright field microscope, as reported above, were quantified using ImageJ software (version 1.53, NIH, USA) according to Sekulska-Nalewajko et al. (2016) with some modifications. For each basil and grapevine leaf disc, the total leaf disc area was selected and measured using the selection tool. DAB-stained regions (dark reddish-brown spots) were then identified using image thresholding and quantified. The percentage of leaf disc area stained with DAB was then calculated using the formula:

$$ROS-positive\ leaf\ disc\ area\ (\%) = (DAB-stained\ leaf\ disc\ area/Total\ leaf\ disc\ area) \times 100$$

Five biological replicates (leaf discs) were analysed per treatment, and the experiment was repeated three times.

Determination of the toxicity ofheat-inactivated and viable *L. capsici* AZ78 cells against *P. viticola* sporangia

The toxicity of heat-inactivated and viable AZ78 cells towards *P. viticola* sporangia was evaluated according to Puopolo et al. (2014a), with slight modifications. Briefly, 50 µL of freshly prepared *P. viticola* sporangial suspension (2.5×10^5 sporangia/mL) was transferred into sterile 1.5 mL centrifuge tubes and kept on ice to prevent sporangial mortality. Subsequently, an equal volume of each treatment was added to the tube. The treatments consisted of 1) distilled water (untreated control); 2) copper-based fungicide (Coprantol Hi Bio, 2 g/L); 3) AZ78 viable cell suspension (1×10^9 CFU/mL); and 4) AZ78 heat-inactivated cell suspension (1×10^9 CFU/mL). After treatment, the tubes were transferred to an incubator at 17 °C for one hour.

After incubation, the mixtures were stained with 5,6-carboxyfluorescein diacetate (Sigma-Aldrich, 0.05%) in DMSO (Sigma-Aldrich, 0.01%) and propidium iodide (Sigma-Aldrich, 0.01%). The former dye conferred green fluorescence to viable sporangia, while the latter imparted red fluorescence to dead sporangia.

After staining, the mixtures were carefully spread onto glass slides and covered with coverslips.

The prepared slides were examined under a Leica LMD7000 microscope (Germany) using the B/G/R filter to count dead sporangia (excitation filter: BP 550–590 nm; dichroic mirror: 590 nm; suppression filter: BP 600–680 nm) and the H3 filter to count viable sporangia (BP 420–490 nm excitation; dichroic mirror: 510 nm; LP 515 nm emission) (Trouvelot et al., 2008). Low-intensity fluorescence was used to minimise dye degradation. The ratio of live to dead sporangia was then calculated for each treatment. Three slides per treatment were prepared, and 100 sporangia were randomly counted on each slide, with five replicates per treatment. The experiment was repeated three times.

Chemical analytical tools

Analytical and preparative thin-layer chromatography (TLC) was performed on silica gel plates (Kieselgel 60, F_{254}, 0.25 and 0.5 mm, respectively) or reverse-phase plates (Kieselgel 60 RP-18 F_{254}, 0.20 mm), and the compounds were visualised by exposure to UV light and/or iodine vapours and/or by sequential spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography (CC) was performed using silica gel (Merck, Kieselgel 60, 0.063–0.200 mm). Solid-phase extraction (SPE) was carried out using SUPELCO Supelclean LC-18 SPE 2 g/12 mL cartridges (Merck, Darmstadt, Germany) on a SUPELCO Visiprep™ SPE Vacuum Manifold system (Merck, Darmstadt, Germany). Unless specified otherwise, all reagents were purchased from Sigma-Merck (Milan, Italy).

High-performance liquid chromatography (HPLC) was performed using a Hitachi 1260 Infinity system equipped with a 5160 pump and a 5410 spectrophotometric detector (Merck, Darmstadt, Germany). The HPLC separations were conducted with a Purospher STAR RP-18 column (5 µm, 125 × 4.0 mm; Merck, Darmstadt, Germany). Electrospray ionisation mass spectrometry (ESIMS) was performed using an LC/MS TOF AGILENT 6230B system (Agilent Technologies, Milan, Italy).

Clog P values were calculated using ChemOffice v20.1 (PerkinElmer, Waltham, MA, USA) with the corresponding tool in ChemDraw Professional (Vraka et al., 2017; Zorrilla et al., 2024).

Extraction and purification of bioactive metabolites from *L. capsici* AZ78 cells

A volume of 50 µL of AZ78 cell suspension was evenly distributed on the surface of 90 mm Petri dishes containing 15 mL of solid Luria–Bertani agar (LBA) 1:10 medium using sterile L-shaped spatulas, and the dishes were incubated at 27 °C. After 72 h, bacterial cells were scraped from the surface of the growth medium using sterile spatulas and placed into sterile flasks containing 30 mL of an extraction solution consisting of methanol (MeOH), acetonitrile (ACN), and water (1.5:1.5:1, v/v/v) plus 0.1% formic acid (v/v). The extraction was not performed on heat-inactivated AZ78 cells, as thermal shock can cause artefactual leakage of intracellular contents (Russell, 2003), which could affect the extraction of bioactive metabolites associated with cell membranes.

The flasks were stirred for one hour at room temperature, and the extracts were then transferred into sterile 15 mL centrifuge tubes. Cell-free extracts were obtained by centrifugation at 35,000 × g for 30 min. The samples were frozen at − 80 °C and lyophilised using a freeze dryer (FreeZone 6 Plus, Labconco, Kansas City, MO, USA) for 94 h. The dried samples were reconstituted in 3 mL of extraction solution. Five hundred microlitres of the concentrated extracts were centrifuged at 30,000 × g for 20 min at − 4 °C. The supernatant was filtered through a Millex-GV PVDF syringe filter (0.22 µm, Merck, Darmstadt, Germany) and transferred to 2 mL HPLC vials, which were stored at − 80 °C until analysis (Brescia et al., 2021).

The AZ78 cell-free extract (800 mL) corresponding to 240 Petri dishes (3.6 L of LBA 1:10) was concentrated under vacuum to 400 mL, and ammonium sulphate ($(NH_4)_2SO_4$) was added to a final concentration of 0.5 mg/mL. The solution was stirred for one hour, left overnight at 4 °C, and then centrifuged at 3,600 rpm for 50 min. The supernatant was discarded, and the pellet was washed twice with 3 mL of methanol, then left for one hour at room temperature. The sample was centrifuged at 10,000 rpm for 30 min, and the supernatant was applied to a C18 SPE column. The SPE column was preconditioned with 6 mL of ACN and equilibrated with 12 mL of distilled

water. The purification yielded five fractions (F1–F5), obtained by stepwise elution with H_2O (6 mL), ACN/H_2O (3:7, v/v; 12 mL), ACN/H_2O (1:1, v/v; 12 mL), ACN/H_2O (7:3, v/v; 12 mL), and ACN (12 mL), respectively.

Subsequently, the organic solvents were removed under reduced pressure, and the remaining aqueous phases were lyophilised. The residue of F3 (8.1 mg), which exhibited high anti-oomycete activity against *Pythium ultimum*, was analysed by ^{1}H NMR and further purified by TLC using the mixture CH_2Cl_2:MeOH:conc. NH_4OH (60:36:4, v/v/v) as the eluent. The main fractions obtained, F3.1C (1.7 mg, Rf 0.4) and F3.2E (1.0 mg, Rf 0.6), were purified by HPLC using multiple injections on a reverse-phase column to obtain dihydromaltophilin and maltophilin.

The mobile phase used to elute the samples was a mixture of H_2O (0.1% formic acid) and ACN (0.1% formic acid) at a flow rate of 0.5 mL/min. The analysis was performed using a gradient starting from 60% ACN (0.1% formic acid), increased linearly to 80% over 20 min, and finally re-equilibrated to the initial conditions for 10 min. Detection was performed at 220 nm, and samples were injected using a 20 µL loop and monitored for 35 min.

Evaluation of the application of dihydromaltophilin and maltophilin for the control of *P. viticola* on grapevine leaf discs

To evaluate the potential of the isolated metabolites, namely dihydromaltophilin (DMP) and maltophilin (MP), they were individually tested on grapevine leaf discs against *P. viticola* at various concentrations. The treatments included: 1) distilled water (untreated control); 2) copper-based fungicide (Coprantol Hi Bio, 2 g/L); 3) DMP at 5 mg/L; 4) DMP at 2.5 mg/L; 5) DMP at 0.5 mg/L; 6) MP at 5 mg/L; 7) MP at 2.5 mg/L; and 8) MP at 0.5 mg/L. All treatments were mixed with equal volumes of *P. viticola* inoculum to reach the final concentrations and stored under cold conditions.

The treatments were applied by spotting five drops of 20 µL each onto each grapevine leaf disc. After application, the Petri dishes were wrapped in aluminium foil and incubated at 25 °C for 16 h. This was followed by a drying period of the Petri dishes under a chemical fume hood, after which they were transferred to the greenhouse at 25 °C (60–80% RH) with a 16/8 h day/night light regime. Disease severity was measured on the seventh day post-inoculation. Each treatment consisted of five Petri dishes containing three leaf discs, resulting in 15 replicates (15 spots), and the experiment was repeated.

Assessment of the plant protection efficacy, toxicity against *P. viticola* sporangia and induction of plant resistance mechanisms of a mixture of dihydromaltophilin and maltophilin

To evaluate the plant protection efficacy of a mixture of DMP and MP against *P. viticola* on grapevine leaf discs, the following treatments were tested: 1) distilled water (untreated control); 2) DMSO 0.1% (v/v); 3) DMSO 0.05% (v/v); 4) copper-based fungicide (Coprantol Hi Bio, 2 g/L); 5–8) DMP and MP mixture prepared at a 1:1 (v/v) ratio tested at total concentrations of 50, 5, 2.5, and 0.5 mg/L, corresponding to equal concentrations of DMP and MP in each mixture. The DMSO concentrations corresponded to those present in the DMP and MP mixtures at 50 mg/L and 2.5 mg/L, respectively. The treatments were applied as described above. Each treatment consisted of five Petri dishes containing three leaf discs, resulting in 15 replicates (15 spots), and the experiment was repeated.

The same DMP and MP mixtures, along with their respective controls, were also evaluated for their toxicity against *P. viticola* sporangia, following the procedure described above. Three slides per treatment were prepared, and 100 sporangia were randomly counted per slide (five replicates). The experiment was repeated three times.

DMP and MP mixtures at varying concentrations were also evaluated for their ability to stimulate callose deposition using the procedures previously described. The treatments were: 1) distilled water (untreated control); 2) DMSO 0.1%; and 3–6) leaf discs treated with DMP and MP mixture at 50 mg/L, 25 mg/L, 2.5 mg/L, and 0.5 mg/L, respectively. Each treatment consisted of five biological and three technical replicates, and all experiments were repeated three times.

Statistical analysis

Because the experimental factor was not significant, data from the independent experiments were pooled, treated as biological replicates, and analysed using one-way analysis of variance (ANOVA) in R v4.3.0 with RStudio v2024.12.1–563. Data were analysed

for normality using the Shapiro–Wilk test ($p > 0.05$) and for homogeneity of variances using Levene's test. Tukey's HSD post hoc test was performed using the 'agricolae' (v 1.3–7) and 'emmeans' (v1.10.0) package, with adjustments for multiple comparisons and a significance level of $\alpha = 0.05$.

Results

Application of heat-inactivated and viable *L. capsici* AZ78 cells effectively controlled *P. viticola* on grapevine leaf discs

No colony formation occurred on NA dishes spot-inoculated with AZ78 and DH5α cells after thermal shock, indicating that all treated bacterial cells died. The plant protection efficacy of heat-inactivated and viable AZ78 and DH5α cells was evaluated against *P. viticola*. Grapevine leaf discs treated with distilled water exhibited a disease severity of $90.00 \pm 2.33\%$. In contrast, the copper-based fungicide provided the highest level of protection, resulting in a disease severity of $0.24 \pm 0.16\%$ (Fig. 1). Similarly, the application of BION 50 WG significantly reduced disease severity ($2.56 \pm 0.62\%$) (Fig. 1). Application of heat-inactivated and viable DH5α cells resulted in only a slight but significant reduction in disease severity compared with the untreated control. Notably, the application of heat-inactivated and viable AZ78 cells significantly reduced *P. viticola* infections, performing better

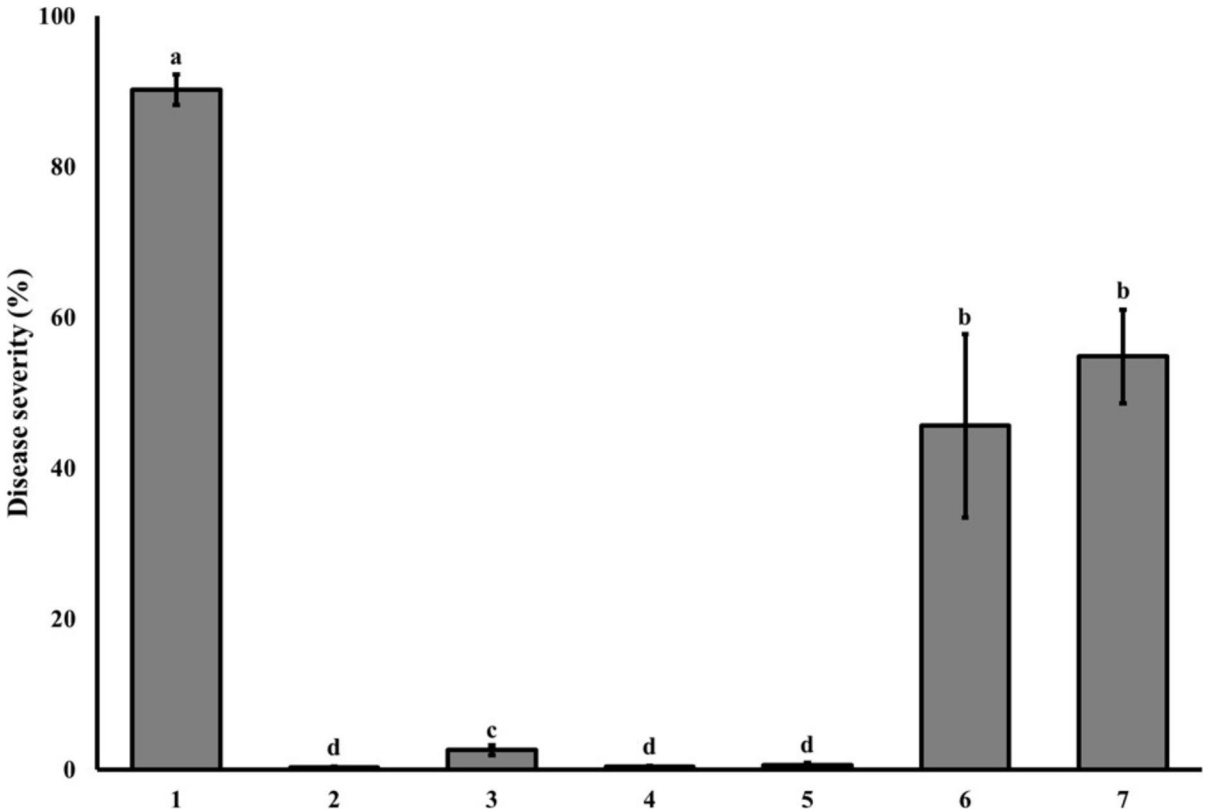

Fig. 1 Control of *Plasmopara viticola* on grapevine leaf discs through the preventive application of heat-inactivated and viable *Lysobacter capsici* AZ78 cells. The treatments included: **1)** distilled water (untreated control); **2)** copper-based fungicide [Coprantol Hi Bio (Copper hydroxide 20%, Syngenta; solution 2 g/L)]; **3)** plant resistance inducer [BION 50 WG (benzothiadiazole-7-carbothioic acid S-methyl ester 50%, Syngenta; 50 mg/L)]; **4)** viable *L. capsici* AZ78 cells (1×10^8 CFU/mL); **5)** heat-inactivated *L. capsici* AZ78 cells (1×10^8 CFU/mL); **6)** viable *Escherichia coli* DH5α cells (1×10^8 CFU/mL); and **7)** heat-inactivated *E. coli* DH5α cells (1×10^8 CFU/mL), applied 24 h before *P. viticola*. Disease severity is expressed as the mean percentage of symptomatic grapevine leaf disc area ± standard error of two replicated bioassays ($n = 2$). Columns bearing the same letters are not significantly different according to Tukey's HSD test ($\alpha = 0.05$)

than BION 50 WG and equivalent to the copper-based fungicide (Fig. 1).

Heat-inactivated and viable *L. capsici* AZ78 cells effectively suppressed downy mildew in grapevine and basil plants

In the next step, heat-inactivated and viable AZ78 cells were tested on whole grapevine and basil plants to evaluate their plant-protection efficacy under more complex conditions. For grapevine plants, the untreated control showed the highest disease severity (82.24 ± 2.18%), whereas the copper-based fungicide achieved the lowest (0.12 ± 0.12%; Fig. 2A). The application of heat-inactivated and viable AZ78 cells showed excellent efficacy, resulting in disease severities of 0.97 ± 0.23% and 0.34 ± 0.75%, respectively (Fig. 2A).

For basil plants, the untreated control exhibited a notably high disease severity (94.58 ± 0.75%). In contrast, the copper-based fungicide significantly reduced disease severity (1.46 ± 0.55%). Both viable and heat-inactivated AZ78 cells demonstrated strong plant-protection efficacy. Specifically, treatment with viable AZ78 cells (0.63 ± 0.36%) produced results comparable to those obtained with the copper-based fungicide. Heat-inactivated AZ78 cells reduced disease severity to 8.13 ± 2.01%, which was slightly higher than that achieved by the copper-based fungicide and viable AZ78 cells, but still significantly lower than the untreated control (Fig. 2B).

Application of heat-inactivated and viable *L. capsici* AZ78 cells stimulated callose deposition and reactive oxygen species accumulation in basil and grapevine leaf discs

In basil and grapevine leaf discs treated with distilled water, no callose deposition was observed before the inoculation of *P. belbahrii* and *P. viticola*, respectively (Fig. S1A, D), whereas the presence of zoospores was observed at one and two days post-inoculation (dpi; Fig. S1B, 2E, 3A, 3E). Finally, *P. belbahrii* and *P. viticola* sporulation with the formation of sporangiophores and sporangia was observed at seven dpi (Fig. S1C, F).

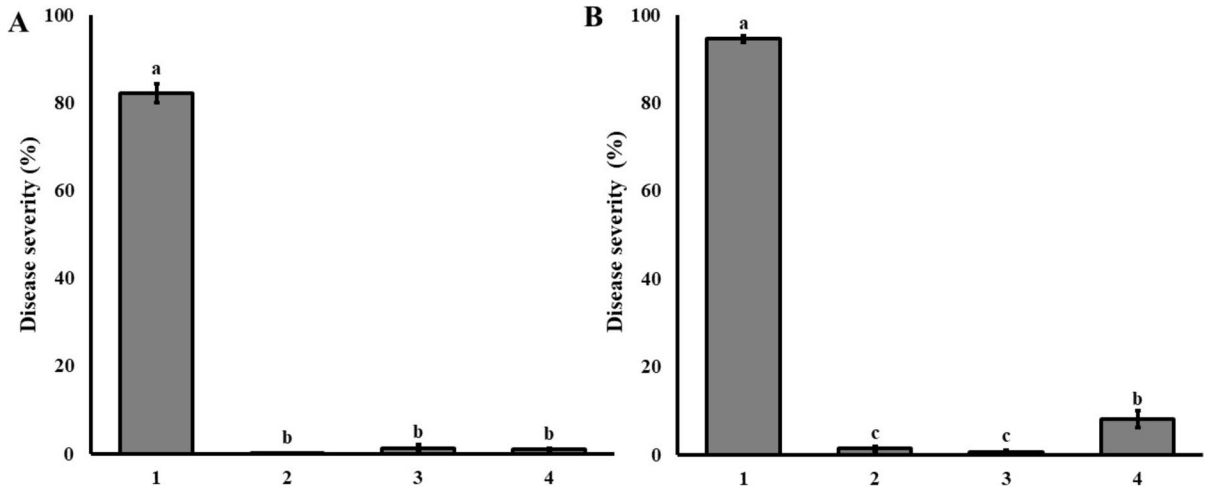

Fig. 2 Preventive application of heat-inactivated and viable *Lysobacter capsici* AZ78 cells effectively protects basil and grapevine plants against plant pathogenic oomycetes. Heat-inactivated and viable *L. capsici* AZ78 cell suspensions (1×10^8 CFU/mL) were applied to grapevine plants and treated with *Plasmopara viticola* after 24 h (**A**). Similarly,heat-inactivated and viable *L. capsici* AZ78 cell suspensions (1×10^9 CFU/mL) were applied to basil plants and inoculated with *Peronospora belbahrii* after 24 h (**B**). In both cases, treatments included: **1)** distilled water (untreated control); **2)** copper-based product, in particular Coprantol Hi Bio (copper hydroxide 20%, 2 g/L) for grapevine plants and Cuprotax S.D.I. (copper sulfite 15.20%, 0.594 mL/L) for basil plants; **3)** viable *L. capsici* AZ78 cells; and **4)** heat-inactivated *L. capsici* AZ78 cells. Disease severity is expressed as the mean percentage of symptomatic grapevine leaf disc area ± standard error of three replicated bioassays ($n = 3$). Columns bearing the same letters are not significantly different according to Tukey's HSD test ($\alpha = 0.05$)

In contrast, the application of the resistance inducer BION 50 WG resulted in pronounced callose deposition around stomata in both basil and grapevine leaf discs (Fig. 3B, F), consistent with its known resistance-inducing activity. Notably, the application of both heat-inactivated and viable AZ78 cells prevented the presence of *P. belbahrii* and *P. viticola* zoospores or mycelium, and, similar to BION 50 WG, promoted a marked callose deposition around stomata in basil and grapevine leaf discs (Fig. 3C, D, G, H).

Quantitative analysis confirmed these observations (Table S1). The application of distilled water did not increase the percentage of callose-positive stomata in basil and grapevine leaf discs. In contrast, the application of the resistance inducer BION 50 WG significantly increased in both basil ($48.19 \pm 3.10\%$) and grapevine ($54.91 \pm 3.03\%$) leaf discs (Table S1). Interestingly, the callose deposition around stomata was significantly enhanced by the application of heat-inactivated and viable AZ78 cells compared with the untreated controls in both grapevine and basil leaf discs. In particular, the effect of the application of heat-inactivated ($48.41 \pm 5.81\%$) and viable ($53.66 \pm 7.41\%$) AZ78 cells was not significantly different from the application of BION 50 WG in the case of grapevine leaf discs (Table S1). However, a significantly lower percentage of callose-positive stomata following the application of heat-inactivated ($39.69 \pm 4.00\%$) and viable ($40.67 \pm 2.32\%$) AZ78 cells compared to BION 50 was observed in the case of basil leaves.

Regarding ROS production, basil and grapevine leaf discs treated with distilled water showed minimal ROS accumulation (Fig. 4A, E). A moderate increase in ROS accumulation in basil and grapevine leaf discs was observed in the case of the application of laminarin, in agreement with its already known capacity to induce ROS accumulation in plant tissues (Fig. 4B, F). In contrast, treatment with both viable and heat-inactivated AZ78 cells resulted in a marked increase in ROS accumulation in basil and grapevine leaf discs at two dpi (Fig. 4C–H).

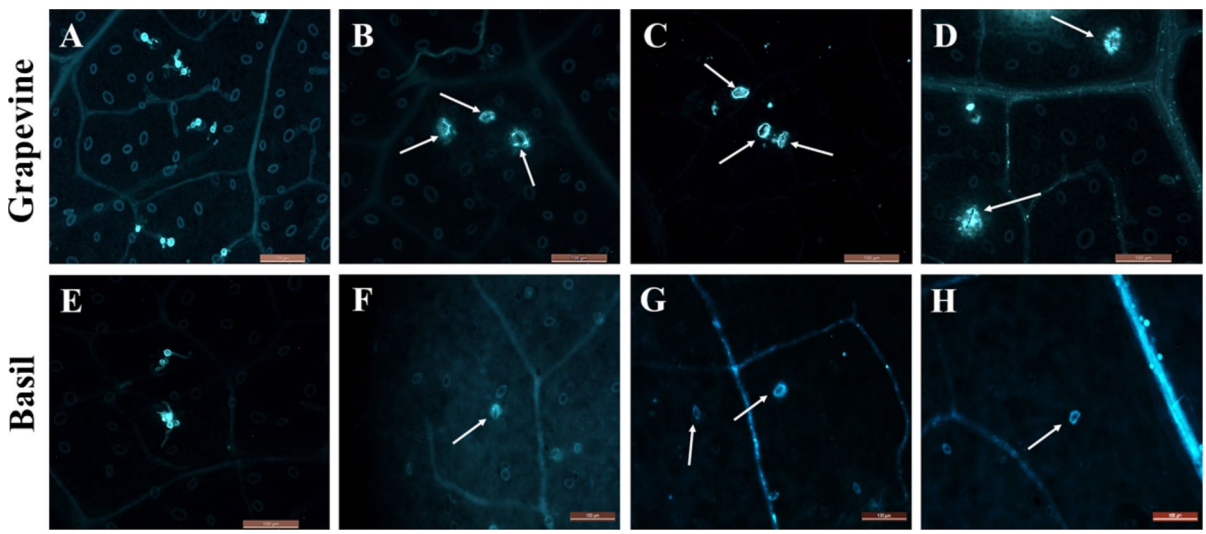

Fig. 3 Promotion of callose deposition in grapevine and basil leaf discs following the application of heat-inactivated and viable *Lysobacter capsici* AZ78 cells. Heat-inactivated and viable *L. capsici* AZ78 cell suspensions (1×10^8 CFU/mL) were applied to grapevine leaf discs. In contrast, basil plants were treated with heat-inactivated and viable *L. capsici* AZ78 cell suspensions at 1×10^9 CFU/mL. Grapevine leaf discs (**A**-**D**) and basil plants (**E**–**F**) were inoculated with *Plasmopara viticola* (2.5×10^5 sporangia/mL) and *Peronospora belbahrii* (1×10^5 sporangia/mL) 24 h after the application of different treatments, respectively. After 48 h, callose deposition was assessed in basil and grapevine leaf discs using aniline blue staining. Representative micrographs illustrate callose deposition in different treatments. The treatments included leaf discs treated with distilled water (untreated control) (**A**, **E**); leaf discs treated with BION 50 WG (benzothiadiazole-7-carbothioic acid S-methyl ester 50%, 50 mg/L) (**B**, **F**); leaf discs treated with viable *L. capsici* AZ78 cells (**C**, **G**) and heat-inactivated *L. capsici* AZ78 cells (**D**, **H**). Arrows indicate callose deposition around stomatal openings, visible as blue fluorescence. Scale bars: 100 µm

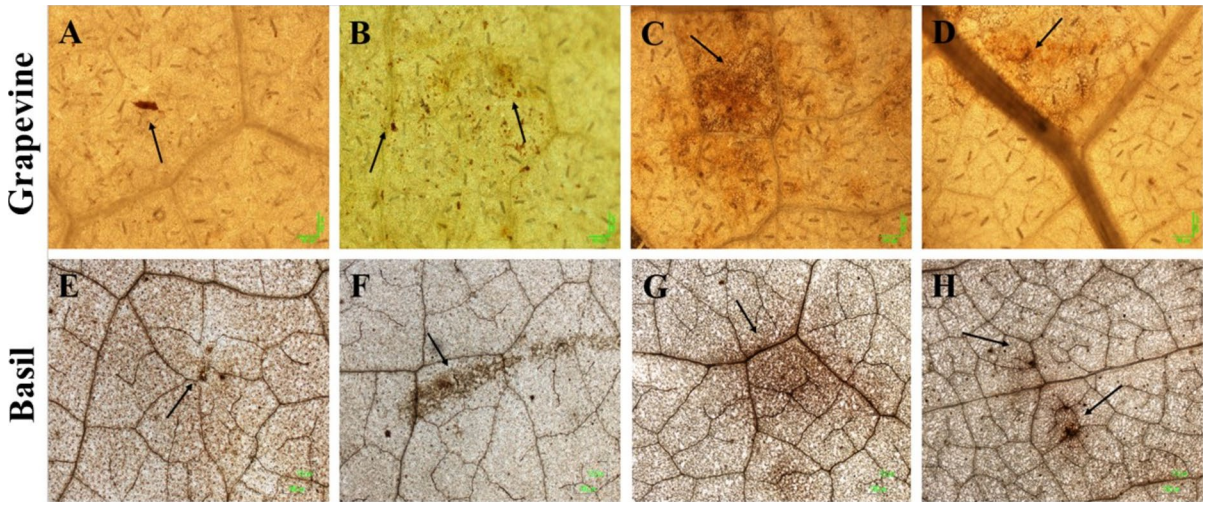

Fig. 4 Promotion of reactive oxygen species (ROS) accumulation in grapevine and basil leaf discs following the application of heat-inactivated and viable *Lysobacter capsici* AZ78 cells. Heat-inactivated and viable *L. capsici* AZ78 cell suspensions (1×10^8 CFU/mL) were applied to grapevine leaf discs. In contrast, basil plants were treated with heat-inactivated and viable *L. capsici* AZ78 cell suspension at 1×10^9 CFU/mL. Grapevine leaf discs (**A**–**D**) and basil plants (**E**–**H**) were inoculated with *Plasmopara viticola* (2.5×10^5 sporangia/mL) and *Peronospora belbahrii* (1×10^5 sporangia/mL), respectively, and assessed for ROS accumulation at 48 dpi using 3,3′-diaminobenzidine (DAB) staining solution. DAB-stained leaves revealed localised H_2O_2 accumulation. The treatments were as follows: leaf discs treated with distilled water (untreated controls) (**A**, **E**); leaf discs treated with laminarin (2 mL/L) (**B**, **F**); leaf discs treated with viable *L. capsici* AZ78 cells (**C**, **G**) and *L. capsici* AZ78 heat-inactivated cells (**D**, **H**). Arrows indicate ROS production, visible as brown precipitated spots. Scale bars: 100 µm

Quantitative analysis confirmed these observations (Table S2). The application of distilled water had no effect on the ROS accumulation of basil and grapevine leaf discs. In contrast, the application of laminarin promoted the ROS accumulation in both basil ($34.31\pm0.18\%$) and grapevine ($30.51\pm2.98\%$) leaf discs (Table S2). The percentage of DAB-stained leaf disc area was significantly higher than that of the untreated controls when both basil and grapevine leaf discs were treated with heat-inactivated and viable AZ78 cells (Table S2). Notably, the application of heat-inactivated ($41.65\pm4.70\%$) and viable ($44.76\pm0.95\%$) AZ78 cells resulted in a significantly higher percentage of DAB-stained grapevine leaf disc area than that with laminarin. Moreover, the application of heat-inactivated ($53.77\pm3.41\%$) and viable ($58.23\pm1.57\%$) AZ78 cells elicited the greatest ROS accumulation among treatments in basil leaf discs (Table S2).

Effect of heat-inactivated and viable *L. capsici* AZ78 on the viability of *P. viticola* sporangia

P. viticola sporangia treated with distilled water demonstrated $51.02\pm5.19\%$ dead sporangia. In contrast, treatments with heat-inactivated and viable AZ78 cells significantly reduced sporangial viability, with $81.05\pm1.50\%$ and $80.08\pm9.48\%$ dead sporangia, respectively (Fig. S2). Notably, the efficacy of viable AZ78 cells was higher than that of the copper-based fungicide, which caused $65.00\pm2.00\%$ sporangial devitalisation. Similarly, heat-inactivated AZ78 cells outperformed the untreated control, confirming their potential to reduce *P. viticola* sporangial viability and to suppress *P. viticola* infection (Fig. S2).

Identification of dihydromaltophilin and maltophilin in extracts from *L. capsici* AZ78 cells

As described above, the AZ78 cell-free extract was purified by precipitation with ammonium sulphate. The methanol-soluble compounds obtained were separated by C18 solid-phase extraction (SPE), yielding a fraction (F3) whose ^{1}H NMR spectrum (Fig. S3) displayed signals corresponding to olefinic, methine, and methylene protons, similar to those reported for tetramic acid-containing metabolites (Graupner et al., 1997). This fraction was further purified through two successive steps of TLC and HPLC, resulting in two pure compounds eluted at retention times (t_n) of 5.73 and 7.79 min (Fig. S4 and S5).

These were pooled, concentrated under reduced pressure, and lyophilised. Subsequently, the two compounds were identified as dihydromaltophilin (DMP), also known as HSAF (0.53 mg, t_n 5.73) (Fig. S6A), and maltophilin (MP; 0.83 mg, t_n 7.79 min) (Fig. S6B; Graupner et al., 1997).

Their ESI–MS spectra showed the dimer protonated adduct $[2\ M+H]^+$ and the protonated adduct $[M+H]^+$ ions at *m/z* 1025 and 513 for DMP (Fig. S7) and at *m/z* 1021 and 511 for MP (Fig. S8).

Impact of dihydromaltophilin and maltophilin application on *P. viticola* infection in grapevine leaf discs

The results demonstrated a strong protective effect of the two metabolites, DMP and MP, at varying concentrations on disease severity. In the control group, grapevine leaf discs exhibited a high disease severity ($90.74 \pm 6.23\%$; Fig. 5). Treatments with DMP at 5 mg/L and 2.5 mg/L, as well as MP at 5 mg/L, completely suppressed disease development, resulting in the absence of symptoms, comparable to those observed with the copper-based fungicide. However, when applied at the lowest concentration (0.5 mg/L), both DMP and MP were less effective, with disease severity remaining high ($77.41 \pm 7.71\%$ and $81.48 \pm 3.76\%$, respectively; Fig. 5). Additionally, MP applied at 2.5 mg/L exhibited a moderate reduction in disease severity ($64.52 \pm 3.96\%$).

Impact of a mixture of dihydromaltophilin and maltophilin on *P. viticola* infection in grapevine leaf discs

Grapevine leaf discs treated with distilled water were covered entirely with sporulating *P. viticola* mycelium, showing 100% disease severity. Treatments with DMSO at lower concentrations (0.1% and 0.05%) and with the DMP and MP mixture at 0.5 mg/L exhibited high levels of disease severity, not significantly different from the untreated control (Fig. 6). In contrast, the DMP and MP mixture resulted in complete disease suppression (0% severity), matching the efficacy of the copper-based fungicide when applied at concentrations of 50 mg/L, 5 mg/L, and 2.5 mg/L (Fig. 6).

Effect of dihydromaltophilin and maltophilin mixture on *P. viticola* sporangia

Fluorescence microscopy revealed $26.00 \pm 1.00\%$ dead sporangia in the untreated control (Fig. S9). Varying concentrations of DMSO did not show any significant effect on sporangial viability, with DMSO at 0.1% and 0.05% yielding $38.50 \pm 1.50\%$ and $45.00 \pm 3.00\%$ dead sporangia, respectively (Fig. S9).

Conversely, the application of the DMP and MP mixture was highly toxic to *P. viticola* sporangia at concentrations as low as 2.5 mg/L. Notably, the DMP and MP mixture at 50 mg/L resulted in a higher percentage of dead sporangia than the copper-based fungicide (Coprantol Hi Bio). Conversely, the DMP and MP mixture at 0.5 mg/L produced levels of sporangial devitalization comparable to those of the untreated control (Fig. S9).

Application of a ihydromaltophilin and maltophilin mixture stimulates callose deposition in grapevine leaf discs

Microscopic observations revealed that grapevine leaf discs treated with distilled water and 0.1% DMSO exhibited extensive *P. viticola* mycelial growth and no callose deposition (Fig. 7A-B). Notably, callose deposition was clearly observed in grapevine leaf discs treated with the DMP and MP mixture at concentrations of 50 mg/L, 5 mg/L, and 2.5 mg/L (Fig. 7C-E),

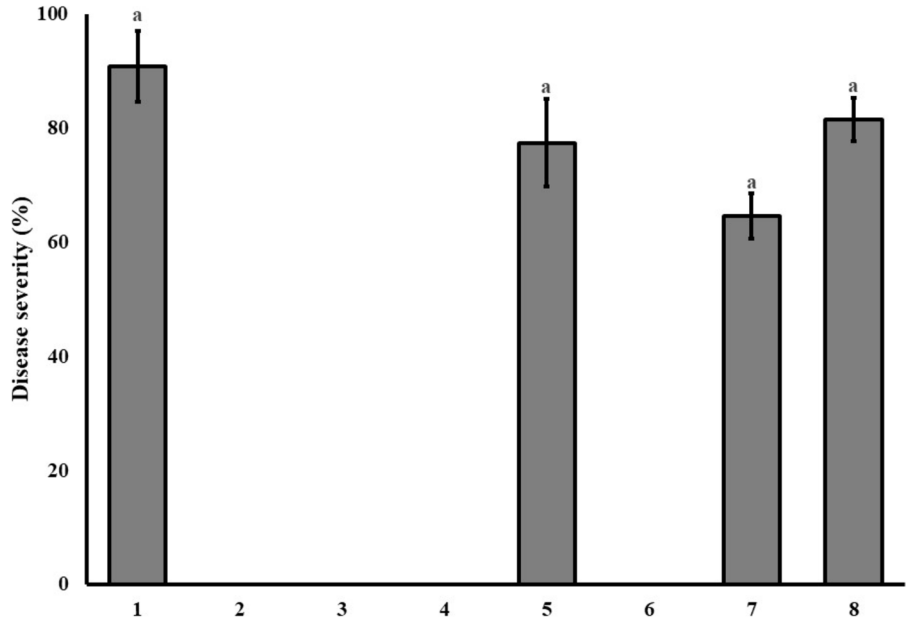

Fig. 5 Effective control of *Plasmopara viticola* infections on grapevine leaf discs through the preventive application of dihydromaltophilin and maltophilin. Grapevine leaf discs were subjected to various treatments, including: **1)** distilled water (untreated control); **2)** copper-based fungicide (Coprantol Hi Bio, copper hydroxide 20%, 2 g/L); DMP at **3)** 5 mg/L; **4)** 2.5 mg/L; **5)** 0.5 mg/L; and MP at **6)** at 5 mg/L;**7)** 2.5 mg/L; **8)** 0.5 mg/L. Disease severity is expressed as the mean percentage of symptomatic grapevine leaf disc area ± standard error of two replicated bioassays ($n = 2$). Columns bearing the same letters are not significantly different according to Tukey's HSD test ($\alpha = 0.05$)

whereas the lowest concentration tested (0.5 mg/L) did not induce callose deposition (Fig. 7F).

As reported above, treatment with distilled water did not increase the percentage of callose-positive stomata in grapevine leaf discs (0.94 ± 0.54%). However, this value was significantly higher than that observed in leaf discs treated with DMSO (0.1%), in which no callose-positive stomata were detected (Table S3). The DMP and MP mixture at various concentrations significantly induced callose deposition in grapevine leaf discs in a dose-dependent manner (Table S3). The highest response was observed at 50 mg/L (46.53 ± 2.35%), followed by 25 mg/L (18.19 ± 3.17%) and 2.5 mg/L (11.52 ± 0.81%). In contrast, the application of the DMP and MP mixture at 0.5 mg/L reached a percentage of callose-positive stomata (0.93 ± 0.53%) that was not significantly different from that observed on grapevine leaf discs treated with distilled water (untreated control).

Discussion

Biocontrol *Lysobacter* spp. strains are emerging as promising candidates for the development of new, eco-friendly products suitable for the management of plant-pathogenic (micro)organisms (Puopolo et al., 2018). In particular, *Lysobacter* spp. strains have shown remarkable efficacy in controlling plant diseases caused by pathogenic oomycetes (Lin et al., 2021), with AZ78 being one of the few examples of a biocontrol agent capable of controlling *P. viticola* in vineyards (Markellou et al., 2022). In this study, we further characterised the mechanisms employed by AZ78 to control plant-pathogenic oomycetes, focusing on *P. viticola* and *P. belbahrii* on grapevine and basil plants, respectively. We have previously demonstrated that AZ78 produces heat-stable polycyclic tetramate macrolactams (PTMs), including dihydromaltophilin (DMP), which retain antifungal activity after thermal treatment (Brescia et al., 2021; Li

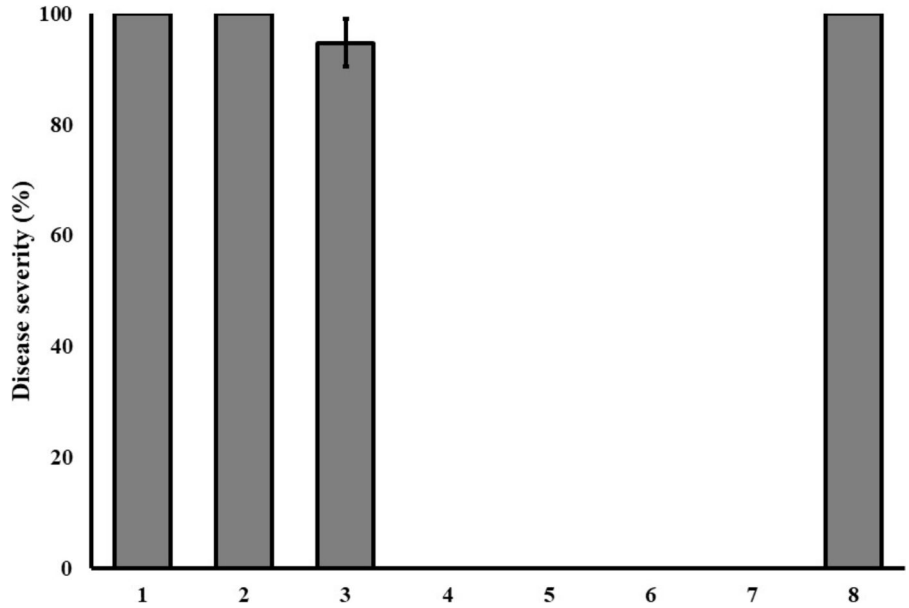

Fig. 6 Control of *Plasmopara viticola* infections on grapevine leaf discs through the preventive application of a mixture of dihydromaltophilin and maltophilin. Grapevine leaf discs were subjected to various treatments, including: **1)** distilled water (untreated control); **2)** DMSO 0.1% (*v/v*); **3)** DMSO 0.05% (*v/v*); **4)** copper-based fungicide (Coprantol Hi Bio, copper hydroxide 20%, 2 g/L). The dihydromaltophilin and maltophilin mixture was applied at the following concentrations: **5)** 50 mg/L, **6)** 5 mg/L, **7)** 2.5 mg/L, and **8)** 0.5 mg/L. Mixtures consisted of DMP and MP at a 1:1 (v/v) ratio; concentrations refer to the total mixture concentration. Disease severity is expressed as the mean percentage of symptomatic grapevine leaf disc area ± standard error of two replicated bioassays ($n = 2$)

et al., 2008; Zhang & Yuen, 2000). Moreover, DMP and analogues have been shown to associate with the outer membrane of *Lysobacter* cells (Yue et al., 2021), suggesting that a fraction of these secondary metabolites may remain cell-associated rather than being fully released into the surrounding environment. Based on these observations, we hypothesised that heat-inactivated AZ78 cells could retain antioomycete activity comparable to that of viable cells, owing to the persistence of membrane-associated, heat-stable PTMs.

To verify this hypothesis, we conducted efficacy trials on grapevine leaf discs, and the results clearly showed that heat-inactivated AZ78 cells provided significant plant protection efficacy, comparable to that of a copper-based fungicide and viable AZ78 cells. Similarly, heat-inactivated AZ78 cells drastically reduced infections caused by *P. viticola* on grapevine and *P. belbahrii* on basil under greenhouse conditions. Beyond the efficacy of heat-inactivated AZ78 cells, it is noteworthy that AZ78 is among the few bacterial biocontrol agents capable of efficiently controlling basil downy mildew, performing comparably to a copper-based fungicide. Until now, the development of resistant basil cultivars, the use of chemical active substances, physical control measures, and agronomic strategies have represented the main approaches for managing basil downy mildew (Ben-Naim et al., 2018; La Placa et al., 2025; López-López et al., 2014; Patel et al., 2021). Although several alternative products have been tested for controlling basil downy mildew in the USA, most have failed to control *P. belbahrii* effectively (Patel et al., 2021; Wyenandt et al., 2015). Thus, AZ78 could represent a valuable new resource for the development of a commercial microbial biopesticide targeting basil downy mildew, providing an environmentally sustainable alternative to chemical fungicides.

Given the efficacy of both heat-inactivated and viable AZ78 cells, we proceeded to characterise their mode of action. Firstly, we assessed the toxicity of AZ78 cells on *P. viticola* sporangia and found that sporangia treated with AZ78 displayed a significantly higher proportion of dead sporangia. Notably,

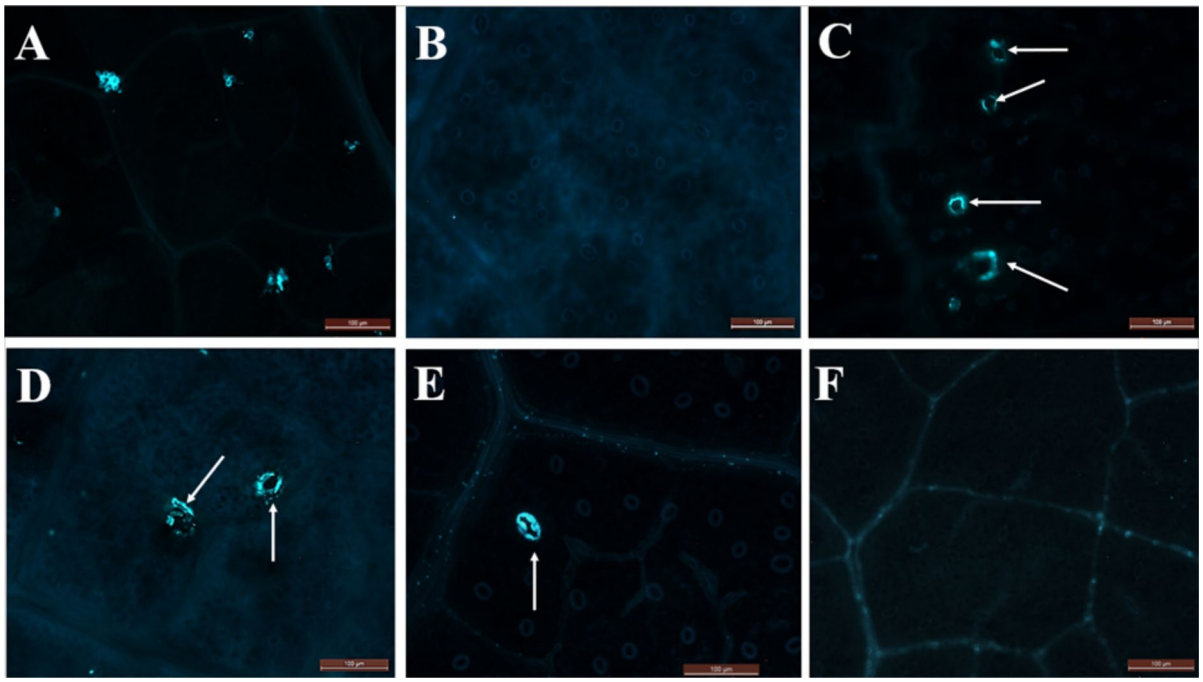

Fig. 7 Promotion of callose deposition in grapevine and basil leaf discs following the application of a dihydromaltophilin and maltophilin mixture. The treatments included: leaf discs treated with distilled water (untreated control) (**A**); leaf discs treated with DMSO 0.1% (**B**); and leaf discs treated with the dihydromaltophilin (DMP)-maltophilin (MP) mixture at 50 mg/L (**C**), 25 mg/L (**D**), 2.5 mg/mL (**E**), and 0.5 mg/L (**F**). Mixtures consisted of DMP and MP at a 1:1 (v/v) ratio; concentrations refer to the total mixture concentration. Grapevine leaf discs (**A**–**F**) were inoculated with *P. viticola* (2.5×10^5 sporangia/mL) 24 h after the application of the treatments. Arrows indicate callose deposition around stomatal openings, visible as blue fluorescence. Scale bars: 100 µm

viable AZ78 cells resulted in a greater proportion of dead sporangia than the copper-based fungicide. Previously, we showed that AZ78 can kill *P. viticola* sporangia through the production of the 2,5-diketopiperazine cyclo(L-Pro-L-Tyr); thus, it is plausible that AZ78 also produces this metabolite when applied to grapevine leaf discs (Puopolo et al., 2014a). However, as heat-inactivated AZ78 cells also showed strong efficacy, the toxicity could additionally be associated with the presence of heat-stable secondary metabolites.

Since heat-inactivated AZ78 cells are non-viable, their protective effect may also be mediated by the elicitation of plant innate immunity. Plant-beneficial bacteria can stimulate plant resistance mechanisms through recognition of conserved microbial-associated molecular patterns (MAMPs), including components of the cell envelope (e.g. lipopolysaccharides, peptidoglycans) and bioactive secondary metabolites. These components are perceived by plant pattern-recognition receptors, triggering defence signalling pathways involving salicylic acid, jasmonic acid, and ethylene (Boller & Felix, 2009; Pieterse et al., 2014). For example, the biocontrol bacterium *P. putida* WCSWCS358 stimulates systemic resistance in bean and tomato, an effect associated with its lipopolysaccharides, components of the bacterial cell envelope, and the production of the siderophore pseudobactin (Meziane et al., 2005). Production of the cyclic lipopeptides is the main molecular mechanism responsible for the elicitation by *B. velezensis* FZB42 of systemic resistance in rice plants against *Rhizoctonia solani* (Ali et al., 2025). Because members of the genus *Lysobacter* share Gram-negative cell envelope structures and produce a wide array of bioactive secondary metabolites, it is conceivable that stimulation of plant resistance mechanisms may contribute to the plant protection efficacy observed with heat-inactivated AZ78 cells.

Among resistance mechanisms, we focused our attention on callose deposition and ROS production, as they are two processes commonly associated with induced resistance triggered by plant-beneficial (micro)organisms and chemical resistance inducers (Mersha et al., 2012; Palmieri et al., 2013; Trouvelot et al., 2008; Van der Ent et al., 2008). ROS are typically produced in response to stress and play a pivotal role in activating plant-defence signalling pathways. The localised accumulation of ROS at plant pathogen entry sites leads to oxidative bursts that are toxic to invading (micro)organisms (Averyanov, 2009). For instance, ROS accumulation is associated with the hypersensitive response in resistant grapevine plants following *P. viticola* infection (Kortekamp & Zyprian, 2003). Additionally, callose, a β-(1,3)-D-glucan, reinforces plant cell walls and restricts plant pathogen spread (Wang et al., 2021). Its deposition around stomata is crucial for preventing *P. viticola* infection in resistant grapevine plants (Gindro et al., 2003).

In our study, substantial callose deposition was observed near stomata in both basil and grapevine leaf discs treated with heat-inactivated or viable AZ78 cells, comparable to the response induced by the resistance activator BION 50WG. The ability of AZ78 cells, whether heat-inactivated or viable, to stimulate callose deposition near the stomata in basil and grapevine leaves is essential for limiting infection by *P. viticola* and *P. belbahrii*, as both plant pathogens use stomata as their primary penetration sites (Fröbel & Zyprian, 2019; Koroch et al., 2013). Similarly, a marked deposition of ROS was detected in grapevine and basil leaf discs treated with both viable and heat-inactivated AZ78 cells, and this oxidative burst likely forms the basis of the host response to *P. viticola* and *P. belbahrii* infection. Alongside callose deposition, ROS accumulation may play a significant role in protecting basil and grapevine plants by triggering a hypersensitive response against these plant pathogenic oomycetes (Delledonne et al., 2001). Based on these results, it is conceivable that AZ78 contributes to plant protection not only through the release of secondary metabolites toxic to sporangia but also by activating host-defence mechanisms.

Plant-beneficial and pathogenic bacteria possess cell components and secreted proteins that can be recognised by plants, and this recognition forms the basis of pathogen- or microbe-associated molecular pattern (PAMP/MAMP)-triggered immunity (Meziane et al., 2005; Pruitt et al., 2015; Willmann et al., 2011; Yu et al., 2017). To exclude the possibility that heat shock could cause bacterial cells to release components and/or other compounds recognisable by plants, we also assessed the plant-protection efficacy of heat-inactivated *E. coli* DH5α, which has never been associated with resistance induction in plants. However, it is worth noting that another *E. coli* strain has been reported to promote resistance mechanisms in *Arabidopsis thaliana* (Seo & Matthews, 2012); thus, some *E. coli* strains may indeed possess elicitors that plants can recognise.

Neither heat-inactivated nor viable DH5α cells were effective in controlling *P. viticola*, confirming the inability of this strain to stimulate plant-resistance mechanisms. Based on these findings, it is plausible that AZ78 cells, unlike DH5α, contain metabolites and/or cell components capable of eliciting defence responses in grapevine, similarly to BION 50WG, which was used as a reference resistance inducer (Perazzolli et al., 2011).

Among *Lysobacter* spp. biocontrol agents, similar results were obtained with the application of heat-inactivated *L. enzymogenes* C3 cells, which were able to control *Bipolaris sorokiniana* in tall fescue and *Fusarium graminearum* on wheat (Jochum et al., 2006; Kilic-Ekici & Yuen, 2003). Thus, AZ78 and *L. enzymogenes* C3 may share heat-resistant elicitors that can stimulate defence mechanisms in plants. Notably, both AZ78 and *L. enzymogenes* C3 release PTMs such as DMP and maltophilin (MP), which remain stable even after exposure to high temperatures (Brescia et al., 2021; Yu et al., 2007).

The involvement of DMP (also known as Heat-Stable Antifungal Factor) in the biocontrol efficacy of *L. enzymogenes* C3 has been thoroughly investigated. This PTM displays potent toxicity against a wide range of fungi (e.g. *Fusarium graminearum*), nematodes (e.g. *Heterodera schachtii*), and oomycetes (e.g. *Pythium ultimum*) (Jochum et al., 2006; Kobayashi et al., 2005; Yuen et al., 2018). Regarding its mode of action, DMP produced by *L. enzymogenes* C3 was shown to severely impair the polarised growth of *Aspergillus nidulans* hyphae by influencing microfilament assembly at hyphal tips through interactions with the ceramide biosynthetic pathway (Li et al., 2006). Additionally, Ding et al. (2016) demonstrated that DMP application to *Candida albicans* induces

apoptosis in yeast cells by stimulating ROS production. Interestingly, Tomada et al. (2017) reported that genes involved in PTM biosynthesis were upregulated in AZ78 during its interaction with *Phytophthora infestans*. The plant pathogen itself responded by upregulating genes associated with apoptotic processes (Tomada et al., 2017).

Alongside the characterisation of the DMP mode of action, Meers et al. (2018) demonstrated that *L. enzymogenes* C3 releases DMP and related analogues into the environment via outer membrane vesicles (OMVs). Thus, it is conceivable that PTMs come into direct contact with the cell membranes of *Lysobacter* spp. producing strains. Based on this assumption, we isolated and characterised secondary metabolites associated with AZ78 cell membranes. Purification of the extracts through chromatographic techniques (SPE C18, TLC, and HPLC) led to the identification of two key metabolites: DMP and MP.

Once isolated, we tested DMP, MP, and their mixture at varying concentrations against *P. viticola* only, due to the limited amount of metabolites obtained. When applied individually at 2.5 mg/L, only DMP achieved complete control of *P. viticola* on grapevine leaf discs. The same level of efficacy was achieved with the mixture of DMP and MP at 2.5 mg/L. Overall, these results are consistent with those obtained by applying heat-inactivated and viable AZ78 cells to grapevine leaf discs and plants. Moreover, these findings align with those of Graupner et al. (1997), who isolated and tested DMP and MP from a *Streptomyces* sp. strain in Indiana (USA) against *P. viticola* on grapevine plants, demonstrating high efficacy for both PTMs.

To further characterise how these PTMs could control *P. viticola*, we applied a mixture of DMP and MP, as both metabolites were extracted from AZ78 cells. Similar to heat-inactivated and viable AZ78 cells, the DMP and MP mixture significantly reduced the viability of *P. viticola* sporangia. A clear dose-dependent effect was observed, with higher concentrations of the DMP and MP mixture exhibiting greater efficacy in devitalising *P. viticola* sporangia. Notably, at 50 mg/L, the DMP and MP mixture was as effective as the copper-based fungicide, and at 500 mg/L, its efficacy surpassed that of the fungicide. Importantly, the DMP and MP mixture remained toxic at 2.5 mg/L, indicating that these PTMs may serve as promising candidates for the development of new active substances for the sustainable control of grapevine downy mildew within integrated pest management programmes.

Along with its toxic activity against *P. viticola* sporangia, the DMP and MP mixture also stimulated callose deposition near stomatal guard cells in grapevine leaf discs, even at a concentration of 2.5 mg/L. To the best of our knowledge, this is the first report demonstrating that these PTMs can influence plant tissues by modulating callose deposition in grapevine. Recently, Lin et al. (2023) reported that a *L. enzymogenes* OH11 mutant deficient in DMP production was still able to induce the expression of defence genes involved in the salicylic acid and jasmonic acid signalling pathways in the *Phytophthora capsici–Nicotiana benthamiana* pathosystem. Based on these findings, we cannot exclude the possibility that AZ78 cells also contain other elicitors capable of stimulating resistance mechanisms in grapevine plants.

In future studies, it will be essential to investigate the presence of additional potential elicitors in AZ78, alongside experiments aimed at elucidating the molecular pathways activated in grapevine following the application of the DMP and MP mixture. Furthermore, identifying the specific grapevine receptors that recognise and bind these PTMs will be a crucial step towards understanding the molecular basis of AZ78-induced resistance and developing novel, targeted biocontrol strategies.

Conclusion

This study demonstrates that heat-inactivated AZ78 cells retain significant biocontrol activity against *P. viticola* and *P. belbahrii* on grapevine and basil plants, respectively. Their efficacy is comparable to that of a commercial copper-based fungicide and a resistance inducer. The results showed that heat-inactivated AZ78 cells were highly toxic to *P. viticola* sporangia, similarly to viable AZ78 cells. Moreover, both heat-inactivated and viable AZ78 cells induced callose deposition and ROS accumulation in basil and grapevine leaves, thereby inhibiting plant pathogen entry during the early stages of infection.

We attribute this activity to the deposition of thermostable polycyclic tetramate macrolactams (PTMs), specifically dihydromaltophilin (DMP) and

maltophilin (MP), in AZ78 cell membranes. Indeed, we isolated these PTMs from AZ78 cells and demonstrated their toxicity against *P. viticola* sporangia, as well as their ability to stimulate callose deposition in grapevine leaf discs, mirroring the effects of heat-inactivated and viable AZ78 cells. These complementary modes of action, combining direct antimicrobial activity with plant resistance induction, highlight the potential of AZ78 and its metabolites as eco-friendly biocontrol agents for the sustainable management of downy mildews.

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Data Availability The generated and analysed data during the current study are available from the corresponding author on reasonable request.

Declarations

Human and animal rights This article does not contain any studies with human participation or animals performed by any of the authors.

Conflicts of interest The authors declare that no known conflicts of interest exist.

References

Ali, Q., Khan, A. R., Yujie, W., Chenjie, Y., Zhao, Q., Ayaz, M., Raza, W., Gu, Q., Wu, H., Mundra, S., & Gao, X. (2025). Antimicrobial metabolites of *Bacillus velezensis* FZB42 reshape rice rhizosphere microbial community composition and induce host resistance against *Rhizoctonia solani*. *Current Plant Biology, 41*, 100440. https://doi.org/10.1016/j.cpb.2025.100440

Averyanov, A. (2009). Oxidative burst and plant disease resistance. *Frontiers in Bioscience-Elite, 1*, 142–152. https://doi.org/10.2741/E14

Ben-Naim, Y., Falach, L., & Cohen, Y. (2018). Transfer of downy mildew resistance from wild basil (*Ocimum americanum*) to sweet basil (*O. basilicum*). *Phytopathology, 108*(1), 114–123. https://doi.org/10.1094/PHYTO-06-17-0207-R

Boller, T., & Felix, G. (2009). A renaissance of elicitors: Perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology, 60*, 379–406. https://doi.org/10.1146/annurev.arplant.57.032905.105346

Brescia, F., Vlassi, A., Bejarano, A., Seidl, B., Marchetti-Deschmann, M., Schuhmacher, R., & Puopolo, G. (2021). Characterisation of the antibiotic profile of *Lysobacter capsici* AZ78, an effective biological control agent of plant pathogenic microorganisms. *Microorganisms, 9*, 1320. https://doi.org/10.3390/microorganisms9061320

Chen, W., Tang, B., Hou, R., Sun, W., Han, C., Guo, B., Zhao, Y., Li, C., Sheng, C., Zhao, Y., & Liu, F. (2024). The natural polycyclic tetramate macrolactam HSAF inhibit *Fusarium graminearum* through altering cell membrane integrity by targeting FgORP1. *International Journal of Biological Macromolecules, 261*(Pt 1), 129744. https://doi.org/10.1016/j.ijbiomac.2024.129744

Cohen, Y., & Rubin, A., E. (2015). Daytime solar heating controls downy mildew *Peronospora belbahrii* in sweet basil. *PLoS ONE, 10*, 1–25. https://doi.org/10.1371/journal.pone.0126103

Daudi, A., & O'Brien, J. A. (2012). Detection of hydrogen peroxide by DAB staining in *Arabidopsis* leaves. *Bio-Protocol Journal, 2*(18), e263.

Delledonne, M., Zeier, J., Marocco, A., & Lamb, C. (2001). Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proceedings of the National Academy of Sciences of the United States of America, 98*(23), 13454–13459. https://doi.org/10.1073/pnas.231178298

Ding, Y., Li, Z., Li, Y., Lu, C., Wang, H., Shen, Y., & Du, L. (2016). HSAF-induced antifungal effects in *Candida albicans* through ROS-mediated apoptosis. *RSC Advances, 6*, 30895–30904. https://doi.org/10.1039/c5ra26092b

Elad, Y., Omer, C., Nisan, Z., Harari, D., Goren, H., Adler, U., Silverman, D., & Biton, S. (2016). Passive heat treatment of sweet basil crops suppresses *Peronospora belbahrii* downy mildew. *Annals of Applied Biology, 168*, 373–389. https://doi.org/10.1111/aab.12269

EPPO. (2004). EPPO Standards PP1/31(3). *Efficacy Evaluation of Fungicides & Bactericides., 2*, 37–39.

Fröbel, S., & Zyprian, E. (2019). Colonization of different grapevine tissues by *Plasmopara viticola*-a histological study. *Frontiers in Plant Science, 10*, 951. https://doi.org/10.3389/fpls.2019.00951

Gilardi, G., Demarchi, S., Garibaldi, A., & Gullino, M. L. (2013). Management of downy mildew of sweet basil (*Ocimum basilicum*) caused by *Peronospora belbahrii* by means of resistance inducers, fungicides, biocontrol agents and natural products. *Phytoparasitica, 41*, 59–72. https://doi.org/10.1007/s12600-012-0264-y

Gilardi, G., Garibaldi, A., & Gullino, M. L. (2020). Integrated management of downy mildew of basil. *Crop Protection, 137*, 105202. https://doi.org/10.1016/j.cropro.2020.105202

Gindro, K., Pezet, R., & Viret, O. (2003). Histological study of the responses of two *Vitis vinifera* cultivars (resistant and susceptible) to *Plasmopara viticola* infections. *Plant Physiology and Biochemistry, 41*, 846–853. https://doi.org/10.1016/S0981-9428(03)00124-4

Graupner, P. R., Thornburgh, S., Mathieson, J. T., Chapin, E. L., Kemmitt, G. M., Brown, J. M., & Snipes, C. E. (1997). Dihydromaltophilin; a novel fungicidal tetramic acid containing metabolite from *Streptomyces* sp. *Journal of Antibiotics, 50*, 1014–1019. https://doi.org/10.7164/antibiotics.50.1014

He, F., Li, B., Ai, G., Kange, A. M., Zhao, Y., Zhang, X., Jia, Y., Dou, D., Liu, F., & Cao, H. (2018). Transcriptomics analysis of the Chinese pear pathotype of *Alternaria alternata* gives insights into novel mechanisms of HSAF antifungal activities. *International Journal of Molecular Sciences, 19*, 1–20. https://doi.org/10.3390/ijms19071841

Helepciuc, F. E., & Todor, A. (2022). EU microbial pest control: A revolution in waiting. *Pest Management Science, 78*(4), 1314–1325. https://doi.org/10.1002/ps.6721

Hönig, M., Roeber, V. M., Schmülling, T., & Cortleven, A. (2023). Chemical priming of plant defense responses to pathogen attacks. *Frontiers in Plant Science, 14*, 1146577. https://doi.org/10.3389/fpls.2023.1146577

Jochum, C. C., Osborne, L. E., & Yuen, G. Y. (2006). Fusarium head blight biological control with *Lysobacter enzymogenes*. *Biological Control, 39*, 336–344. https://doi.org/10.1016/j.biocontrol.2006.05.004

Kiesewalter, H. T., Lozano-Andrade, C. N., Wibowo, M., Strube, M. L., Maróti, G., Snyder, D., Jørgensen, T. S., Larsen, T. O., Cooper, V. S., Weber, T., & Kovács, Á. T. (2021). Genomic and chemical diversity of *Bacillus subtilis* secondary metabolites against plant pathogenic fungi. *mSystems, 6*(1), e00770-20. https://doi.org/10.1128/mSystems.00770-20

Kilic-Ekici, O., & Yuen, G. Y. (2003). Induced resistance as a mechanism of biological control by *Lysobacter enzymogenes* strain C3. *Phytopathology, 93*, 1103–1110. https://doi.org/10.1094/PHYTO.2003.93.9.1103

Kobayashi, D. Y., Reedy, R. M., Palumbo, J. D., Zhou, J. M., & Yuen, G. Y. (2005). A *clp* gene homologue belonging to the Crp gene family globally regulates lytic enzyme production, antimicrobial activity, and biological control activity expressed by *Lysobacter enzymogenes* strain C3. *Applied and Environmental Microbiology, 71*, 261–269. https://doi.org/10.1128/AEM.71.1.261-269.2005

Koroch, A. R., Villani, T. S., Pyne, R. M., & Simon, J. E. (2013). Rapid staining method to detect and identify downy mildew (*Peronospora belbahrii*) in basil. *Applications in Plant Sciences, 1*, 1300032. https://doi.org/10.3732/apps.1300032

Kortekamp, A., & Zyprian, E. (2003). Characterization of *Plasmopara*-resistance in grapevine using *in vitro* plants. *Journal of Plant Physiology, 160*(11), 1393–1400. https://doi.org/10.1078/0176-1617-01021

La Placa, L., Cornali, S., Bertinaria, F., Rossetti, A., Marchini, M., Reggiani, R., & Battilani, P. (2025). Cropping system support in downy mildew control in basil in organic farming: A two-year open field experiment. *Journal of Plant Pathology, 107*, 167–179. https://doi.org/10.1007/s42161-024-01651-x

Li, S., Du, L., Yuen, G., & Harris, S. D. (2006). Distinct ceramide synthases regulate polarized growth in the filamentous fungus *Aspergillus nidulans*. *Molecular Biology of the Cell, 17*, 1218–1227. https://doi.org/10.1091/mbc.e05-06-0533

Li, S., Jochum, C. C., Yu, F., Zaleta-Rivera, K., Du, L., Harris, S. D., & Yuen, G. Y. (2008). An antibiotic complex from *Lysobacter enzymogenes* strain C3: Antimicrobial activity and role in plant disease control. *Phytopathology, 98*, 695–701. https://doi.org/10.1094/PHYTO-98-6-0695

Li, C., Tang, B., Cao, S., Bao, Y., Sun, W., Zhao, Y., & Liu, F. (2021). Biocontrol ability and action mechanism of dihydromaltophilin against *Colletotrichum fructicola* causing anthracnose of pear fruit. *Pest Management Science, 77*, 1061–1069. https://doi.org/10.1002/ps.6122

Lin, L., Yang, Z., Tao, M., Shen, D., Cui, C., Wang, P., Wang, L., Jing, M., Qian, G., & Shao, X. (2023). *Lysobacter enzymogenes* prevents *Phytophthora* infection by inhibiting pathogen growth and eliciting plant immune responses. *Frontiers in Plant Science, 14*, 1–11. https://doi.org/10.3389/fpls.2023.1116147

Lin, L., Xu, K., Shen, , Chou, S. H., Gomelsky, M., & Qian, G. (2021). Antifungal weapons of *Lysobacter*, a mighty biocontrol agent. *Environmental Microbiology*, *23*, 5704–5715. https://doi.org/10.1111/1462-2920.15674

Liu, X., Jiang, X., Sun, H., Du, J., Luo, Y., Huang, J., & Qin, L. (2022). Evaluating the mode of antifungal action of Heat-Stable Antifungal Factor (HSAF) in *Neurospora crassa*. *Journal of Fungi, 8*, 252. https://doi.org/10.3390/jof8030252

López-López, A., Koller, M., Herb, C., & Schaerer, H. J. (2014). Influence of light management on the sporulation

of downy mildew on sweet basil. *Acta Horticulturae, 1041*, 213–219. https://doi.org/10.17660/ActaHortic.2014.1041.24

Markellou, E., Kapaxidi, E., Karamaouna, F., Samara, M., Kyriakopoulou, K., Anastasiadou, P., Vavoulidou, E., Meidanis, M., Machera, K., Mandoulaki, A., Margaritopoulou, T., Giovannini, O., Tomada, S., Pertot, I., & Puopolo, G. (2022). Evaluation of plant protection efficacy in field conditions and side effects of *Lysobacter capsici* AZ78, a biocontrol agent of *Plasmopara viticola*. *Biocontrol Science and Technology, 32*, 930–951. https://doi.org/10.1080/09583157.2022.2064431

Meers, P. R., Liu, C., Chen, R., Bartos, W., Davis, J., Dziedzic, N., Orciuolo, J., Kutyla, S., Pozo, M. J., Mithrananda, D., Panzera, D., & Wang, S. (2018). Vesicular delivery of the antifungal antibiotics of *Lysobacter enzymogenes* C3. *Applied and Environmental Microbiology, 84*, 1–16. https://doi.org/10.1128/AEM.01353-18

Mersha, Z., Zhang, S., & Raid, R. N. (2012). Evaluation of systemic acquired resistance inducers for control of downy mildew on basil. *Crop Protection, 40*, 83–90. https://doi.org/10.1016/j.cropro.2012.04.031

Meziane, H., Van Der Sluis, I., Van Loon, L. C., Höfte, M., & Bakker, P. A. (2005). Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. *Molecular Plant Pathology, 6*(2), 177–185. https://doi.org/10.1111/j.1364-3703.2005.00276.x

Nimbeshaho, F., Nihorimbere, G., Arias, A. A., Liénard, C., Steels, S., Nibasumba, A., Nihorimbere, V., Legrève, A., & Ongena, M. (2024). Unravelling the secondary metabolome and biocontrol potential of the recently described species *Bacillus nakamurai*. *Microbiological Research, 288*, 127841. https://doi.org/10.1016/j.micres.2024.127841

Omoboye, O. O., Oni, F. E., Batool, H., Yimer, H. Z., De Mot, R., & Höfte, M. (2019). *Pseudomonas* cyclic lipopeptides suppress the rice blast fungus *Magnaporthe oryzae* by induced resistance and direct antagonism. *Frontiers in Plant Science, 10*, 901. https://doi.org/10.3389/fpls.2019.00901

Palmieri, M. C., Perazzolli, M., Matafora, V., Moretto, M., Bachi, A., & Pertot, I. (2012). Proteomic analysis of grapevine resistance induced by *Trichoderma harzianum* T39 reveals specific defence pathways activated against downy mildew. *Journal of Experimental Botany, 63*, 6237–6251. https://doi.org/10.1093/jxb/ers279

Patel, J. S., Wyenandt, C. A., & McGrath, M. T. (2021). Effective downy mildew management in basil using resistant varieties, environment modifications, and fungicides. *Plant Health Progress, 22*(3), 226–234. https://doi.org/10.1094/PHP-02-21-0041-FI

Perazzolli, M., Roatti, B., Bozza, E., & Pertot, I. (2011). *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defense without costs for grapevine *Biological Control*, *58*, 74–82. https://doi.org/10.1016/j.biocontrol.2011.04.006

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C., & Bakker, P. A. (2014). Induced systemic resistance by beneficial microbes. *Annual Review of Phytopathology, 52*, 347–375. https://doi.org/10.1146/annurev-phyto-082712-102340

Pruitt, R. N., Schwessinger, B., Joe, A., Thomas, N., Liu, F., Albert, M., Robinson, M. R., Chan, L. J., Luu, D. D., Chen, H., Bahar, O., Daudi, A., De Vleesschauwer, D., Caddell, D., Zhang, W., Zhao, X., Li, X., Heazlewood, J. L., Ruan, D., … Ronald, P. C. (2015). The rice immune receptor XA21 recognizes a tyrosine-sulfated protein from a Gram-negative bacterium. *Science Advances, 1*(6), e1500245. https://doi.org/10.1126/sciadv.1500245

Puopolo, G., Cimmino, A., Palmieri, M. C., Giovannini, O., Evidente, A., & Pertot, I. (2014a). *Lysobacter capsici* AZ78 produces cyclo(l-Pro-l-Tyr), a 2,5-diketopiperazine with toxic activity against sporangia of *Phytophthora infestans* and *Plasmopara viticola*. *Journal of Applied Microbiology, 117*, 1168–1180. https://doi.org/10.1111/jam.12611

Puopolo, G., Giovannini, O., & Pertot, I. (2014b). *Lysobacter capsici* AZ78 can be combined with copper to effectively control *Plasmopara viticola* on grapevine. *Microbiological Research, 169*, 633–642. https://doi.org/10.1016/j.micres.2013.09.013

Puopolo, G., Tomada, S., & Pertot, I. (2018). The impact of the omics era on the knowledge and use of *Lysobacter* species to control phytopathogenic micro-organisms. *Journal of Applied Microbiology, 124*, 15–27. https://doi.org/10.1111/jam.13607

Puopolo, G. (2022) Microbial Biocontrol Agents Developing Effective Biopesticides. CABI. ISBN 9781789249200, ePub 9781789249187.

Raio, A., & Puopolo, G. (2021). *Pseudomonas chlororaphis* metabolites as biocontrol promoters of plant health and improved crop yield. *World Journal of Microbiology and Biotechnology, 37*(6), 99. https://doi.org/10.1007/s11274-021-03063-w

Ray, P., Lakshmanan, V., Labbé, J. L., & Craven, K. D. (2020). Microbe to microbiome: A paradigm shift in the application of microorganisms for sustainable agriculture. *Frontiers in Microbiology, 11*, 1–15. https://doi.org/10.3389/fmicb.2020.622926

Ren, X., Ren, S., Xu, G., Dou, W., Chou, S. H., Chen, Y., & Qian, G. (2020). Knockout of diguanylate cyclase genes in *Lysobacter enzymogenes* to improve production of antifungal factor and increase its application in seed coating. *Current Microbiology, 77*, 1006–1015. https://doi.org/10.1007/s00284-020-01902-x

Russell, A. D. (2003). Lethal effects of heat on bacterial physiology and structure. *Science Progress, 86*(1–2), 115–137. https://doi.org/10.3184/003685003783238699

Sarrocco, S. (2023). Biological disease control by beneficial (micro)organisms: Selected breakthroughs in the past 50 years. *Phytopathology, 113*(4), 732–740. https://doi.org/10.1094/PHYTO-11-22-0405-KD

Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., & Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution, 3*(3), 430–439. https://doi.org/10.1038/s41559-018-0793-y

Schobert, R., & Schlenk, A. (2008). Tetramic and tetronic acids: An update on new derivatives and biological aspects. *Bioorganic & Medicinal Chemistry, 16*, 4203–4221. https://doi.org/10.1016/j.bmc.2008.02.069

Sekulska-Nalewajko, J., Gocławski, J., & Chojak-koz, J. (2016). Automated image analysis for quantification of reactive oxygen species in plant leaves. *Methods, 109*, 114–122. https://doi.org/10.1016/j.ymeth.2016.05.018

Seo, S., & Matthews, K. R. (2012). Influence of the plant defense response to *Escherichia coli* O157: H7 cell surface structures on survival of that enteric pathogen on plant surfaces. *Applied and Environmental Microbiology, 78*(16), 5882–5889. https://doi.org/10.1128/AEM.01095-12

Song, H., Huang, X., Wang, Z., Hu, J., Zhang, H., & Yang, H. (2025). ADAM-DETR: An intelligent rice disease detection method based on adaptive multi-scale feature fusion. *Plant Methods, 21*, 108. https://doi.org/10.1186/s13007-025-01429-x

Thordal-Christensen, H., Zhang, Z., Wei, Y., & Collinge, D. B. (1997). Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *The Plant Journal, 11*, 1187–1194. https://doi.org/10.1046/j.1365-313X.1997.11061187.x

Tomada, S., Sonego, P., Moretto, M., Engelen, K., Pertot, I., Perazzolli, M., & Puopolo, G. (2017). Dual RNA-Seq of *Lysobacter capsici* AZ78 - *Phytophthora infestans* interaction shows the implementation of attack strategies by the bacterium and unsuccessful oomycete defense responses. *Environmental Microbiology, 19*(10), 4113–4125. https://doi.org/10.1111/1462-2920.13861

Trouvelot, S., Varnier, A.-L., Allègre, M., Mercier, L., Baillieul, F., Arnould, C., Gianinazzi-Pearson, V., Klarzynski, O., Joubert, J. M., Pugin, A., & Daire, X. (2008). A β-1,3 glucan sulfate induces resistance in grapevine against *Plasmopara viticola* through priming of defense responses, including HR-like cell death. *Molecular Plant-Microbe Interactions, 21*, 232–243. https://doi.org/10.1094/MPMI-21-2-0232

Van der Ent, S., Verhagen, B. W., Van Doorn, R., Bakker, D., Verlaan, M. G., Pel, M. J., Joosten, R. G., Proveniers, M. C., Van Loon, L. C., Ton, J., & Pieterse, C. M. (2008). MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in Arabidopsis. *Plant Physiology, 146*(3), 1293–1304. https://doi.org/10.1104/pp.107.113829

Vinale, F., Ghisalberti, E. L., Sivasithamparam, K., Marra, R., Ritieni, A., Ferracane, R., Woo, S., & Lorito, M. (2009). Factors affecting the production of *Trichoderma harzianum* secondary metabolites during the interaction with different plant pathogens. *Letters in Applied Microbiology, 48*, 705–711. https://doi.org/10.1111/j.1472-765X.2009.02599.x

Vlassi, A., Nesler, A., Parich, A., Puopolo, G., & Schuhmacher, R. (2020a). Volatile-mediated inhibitory activity of rhizobacteria as a result of multiple factors interaction: The case of *Lysobacter capsici* AZ78. *Microorganisms, 8*, 1–17. https://doi.org/10.3390/microorganisms8111761

Vlassi, A., Nesler, A., Perazzolli, M., Lazazzara, V., Büschl, C., Parich, A., Puopolo, G., & Schuhmacher, R. (2020b). Volatile organic compounds from *Lysobacter capsici* AZ78 as potential candidates for biological control of soilborne plant pathogens. *Frontiers in Microbiology, 11*, 1–11. https://doi.org/10.3389/fmicb.2020.01748

Vraka, C., Nics, L., Wagner, K. H., Hacker, M., Wadsak, W., & Mitterhauser, M. (2017). LogP, a yesterday's value? *Nuclear Medicine and Biology, 50*, 1–10. https://doi.org/10.1016/j.nucmedbio.2017.03.003

Wang, Y., Li, X., Fan, B., Zhu, C., & Chen, Z. (2021). Regulation and function of defense-related callose deposition in plants. *International Journal of Molecular Sciences, 22*, 1–15. https://doi.org/10.3390/ijms22052393

Willmann, R., Lajunen, H. M., Erbs, G., Newman, M. A., Kolb, D., Tsuda, K., Katagiri, F., Fliegmann, J., Bono, J. J., Cullimore, J. V., Jehle, A. K., Götz, F., Kulik, A., Molinaro, A., Lipka, V., Gust, A. A., & Nürnberger, T. (2011). Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. *Proceedings of the National Academy of Sciences of the United States of America, 108*(49), 19824–19829. https://doi.org/10.1073/pnas.1112862108

Wyenandt, C. A., Simon, J. E., Pyne, R. M., Homa, K., McGrath, M. T., Zhang, S., Raid, R. N., Ma, L. J., Wick, R., Guo, L., & Madeiras, A. (2015). Basil downy mildew (*Peronospora belbahrii*): Discoveries and challenges relative to its control. *Phytopathology, 105*(7), 885–894. https://doi.org/10.1094/PHYTO-02-15-0032-FI

Yu, F., Zaleta-Rivera, K., Zhu, X., Huffman, J., Millet, J. C., Harris, S. D., Yuen, G., Li, X. C., & Du, L. (2007). Structure and biosynthesis of heat-stable antifungal factor (HSAF), a broad-spectrum antimycotic with a novel mode of action. *Antimicrobial Agents and Chemotherapy, 51*, 64–72. https://doi.org/10.1128/AAC.00931-06

Yu, X., Feng, B., He, P., & Shan, L. (2017). From chaos to harmony: Responses and signaling upon microbial pattern recognition. *Annual Review of Phytopathology, 55*, 109–137. https://doi.org/10.1146/annurev-phyto-080516-035649

Yue, H., Jiang, J., Taylor, A. J., Leite, A. D. L., Dodds, E., & Du, L. (2021). Outer membrane vesicle-mediated codelivery of the antifungal HSAF metabolites and lytic polysaccharide monooxygenase in the predatory *Lysobacter enzymogenes*. *ACS Chemical Biology, 16*, 1079–1089. https://doi.org/10.1021/acschembio.1c00260

Yuen, G. Y., Steadman, J. R., Lindgren, D. T., Schaff, D., & Jochum, C. (2001). Bean rust biological control using bacterial agents. *Crop Protection, 20*, 395–402. https://doi.org/10.1016/S0261-2194(00)00154-X

Yuen, G. Y., Broderick, K. C., Jochum, C. C., Chen, C. J., & Caswell-Chen, E. P. (2018). Control of cyst nematodes by *Lysobacter enzymogenes* strain C3 and the role of the antibiotic HSAF in the biological control activity. *Biological Control, 117*, 158–163. https://doi.org/10.1016/j.biocontrol.2017.11.007

Zhang, Z., & Yuen, G. Y. (1999). Biological control of *Bipolaris sorokiniana* on tall fescue by *Stenotrophomonas maltophilia* strain C3. *Phytopathology, 89*, 817–822. https://doi.org/10.1094/PHYTO.1999.89.9.817

Zhang, Z., & Yuen, G. Y. (2000). The role of chitinase production by *Stenotrophomonas maltophilia* strain C3 in biological control of *Bipolaris sorokiniana*. *Phytopathology, 90*, 384–389. https://doi.org/10.1094/PHYTO.2000.90.4.384

Zhao, Y., Cheng, C., Jiang, T., Xu, H., Chen, Y., Ma, Z., Qian, G., & Liu, F. (2019). Control of wheat fusarium head blight by heat-stable antifungal factor (HSAF) from

Lysobacter enzymogenes. *Plant Disease, 103*, 1286–1292. https://doi.org/10.1094/PDIS-09-18-1517-RE

Zorrilla, J. G., Giovannini, O., Nadalini, S., Zanini, A., Russo, M. T., Masi, M., Puopolo, G., & Cimmino, A. (2024). Suppressive activity of *Glechoma hederacea* extracts against the phytopathogenic oomycete *Plasmopara viticola*, and first screening of the active metabolites. *Agriculture, 14*, 58. https://doi.org/10.3390/agriculture14010058