

ORIGINAL ARTICLE

Lysobacter capsici* AZ78 produces cyclo(L-Pro-L-Tyr), a 2,5-diketopiperazine with toxic activity against sporangia of *Phytophthora infestans* and *Plasmopara viticolaG. Puopolo¹, A. Cimmino², M.C. Palmieri¹, O. Giovannini¹, A. Evidente² and I. Pertot¹

1 Department of Sustainable Agro-Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach (FEM), S. Michele all'Adige (TN), Italy

2 Dipartimento di Scienze Chimiche, Università degli Studi di Napoli 'Federico II', Napoli, Italy

Keywords

anti-oomycete activity, cyclo(L-Pro-L-Tyr), *Lysobacter capsici*, *Phytophthora infestans*, *Plasmopara viticola*.

Correspondence

Gerardo Puopolo, Department of Sustainable Agro-Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach (FEM), 38010 S. Michele all'Adige (TN), Italy.

E-mail: gerardo.puopolo@fmach.it

2014/0840: received 23 April 2014, revised 10 July 2014 and accepted 24 July 2014

doi:10.1111/jam.12611

Abstract

Aims: To investigate low molecular weight compounds produced *in vitro* by *Lysobacter capsici* AZ78 and their toxic activity against sporangia of plant pathogenic oomycetes.

Methods and Results: Assays carried out *in vitro* showed that *L. capsici* AZ78 drastically inhibits the growth of plant pathogenic oomycetes. Accordingly, the preventive application of culture filtrates of *L. capsici* AZ78 on grapevine and tomato plants reduced the infections, respectively, caused by *Plasmopara* (*Pl.*) *viticola* and *Phytophthora infestans*. The subsequent chemical analysis of the culture filtrates of *L. capsici* AZ78 by spectroscopic (essentially 1D and 2D ¹H NMR and ¹³C NMR and ESI MS spectra) and optical methods led to the identification of the 2,5-diketopiperazine cyclo(L-Pro-L-Tyr) that inhibited the development of *P. infestans* sporangia *in vitro* and on tomato leaves. Furthermore, a genomic region with high sequence identity with genes coding for a hybrid polyketide synthase and nonribosomal peptide synthetase was detected in *L. capsici* AZ78.

Conclusions: *Lysobacter capsici* AZ78 produces cyclo(L-Pro-L-Tyr) *in vitro* that was effective in killing the sporangia of *P. infestans* and *Pl. viticola* *in vitro*. Moreover, this low molecular weight compound prevents the occurrence of late blight lesions when applied on tomato leaves.

Significance and Impact of the Study: The application of *L. capsici* AZ78 cells or its own culture filtrates effectively controls both *P. infestans* and *Pl. viticola*. Cyclo(L-Pro-L-Tyr) produced by *L. capsici* AZ78 is toxic against sporangia of both these oomycetes. These data enforce the potential in the use of *Lysobacter* members for the control of plant pathogenic oomycetes and provide the basis for the development of new low-impact fungicides based on cyclo(L-Pro-L-Tyr).

Introduction

A high number of destructive plant diseases are caused by plant pathogenic oomycetes. *Phytophthora infestans* Mont. (De Bary), the causal agent of late blight of potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*), was responsible for instance for the epidemics that led to the Irish potato famine (Fry 2008). Another example of

important plant diseases caused by oomycetes is downy mildew of grapevine (*Vitis vinifera*) caused by the obligate parasite *Plasmopara* (*Pl.*) *viticola* (Gessler *et al.* 2011). The control of both these plant pathogenic oomycetes relies mainly on intensive application of chemical fungicides and copper in conventional and organic agriculture, respectively (Fry and Goodwin 1997; Kato *et al.* 1997a; Gessler *et al.* 2011). Concerns about the negative

impact of chemical fungicides and copper on the environment and human health are however increasing, and more environmental-friendly solutions are being increasingly demanded. The use of micro-organisms as active ingredients in plant protection products represents a sustainable alternative to synthetic substances. Microbial strains should however be carefully characterized before being applied as a biopesticide; thus, determining the secondary metabolic profile is crucial to the development of plant protection products.

There are at present few bacterial strains capable of controlling *Pl. viticola* (Tilcher *et al.* 1994, 2002), while bacteria, mainly belonging to the genera *Bacillus* and *Pseudomonas* (*Ps.*), have so far given the most interesting and promising results in controlling *P. infestans* on tomato plants (Yan *et al.* 2002; Klopper *et al.* 2004; Tran *et al.* 2007; Halfeld-Vieira *et al.* 2008; Lamsal *et al.* 2013). Some of these strains indirectly reduce the attacks of *P. infestans* by triggering induced systemic resistance in tomato plants (Yan *et al.* 2002; Tran *et al.* 2007). Biological control bacteria may also be mixed to control tomato late blight, and the concurrent application of a rhizobacterial *Bacillus cereus* strain with epiphytic antagonists belonging to *Cellulomonas flavigena*, *Candida* sp. and *Cryptococcus* sp. produced a higher level of protection against *P. infestans* (Lourenço Júnior *et al.* 2006). Bacterial antagonists protect tomato plants also by acting directly on the zoospores and/or sporangia of *P. infestans*, as in the case of *Pseudomonas fluorescens* SS101 capable of killing *P. infestans* zoospores through biosynthesis of the cyclic lipopeptide massetolide A (Tran *et al.* 2007). Although several examples of biocontrol bacteria are reported in the literature, none of them has led to the development of a biofungicide that has been proved successful against these plant pathogenic oomycetes. There is therefore still a need to identify new bacterial strains to serve as active ingredients of low-impact biofungicides for use against *P. infestans* and *Pl. viticola* (Mizubuti *et al.* 2007; Gessler *et al.* 2011).

The bacterial genus *Lysobacter* (Christensen and Cook 1978) encompasses species that share several biological features that make them a suitable source of new biological control agents of plant diseases caused by plant pathogenic oomycetes (Hayward *et al.* 2010). For instance, *Lysobacter enzymogenes* strain 3.1T8 is active against different plant pathogenic oomycetes by producing lytic enzymes, biosurfactants and anti-oomycete compounds (Folman *et al.* 2001, 2003, 2004). The application of *Lysobacter* sp. SB-K88 effectively controls the damping-off of sugar beet caused by *Pythium* spp. in soil (Homma *et al.* 1993; Nakayama *et al.* 1999). Recently, we showed that the prophylactic application of *Lysobacter capsici* AZ78 to grapevine leaves results in the drastic reduction

of grapevine downy mildew caused by the plant pathogenic oomycete *Pl. viticola* (Puopolo *et al.* 2014). Furthermore, we also produced evidence that members of the *Lysobacter antibioticus*, *L. capsici*, *L. enzymogenes* and *Lysobacter gummosus* species are resistant to copper and this biological feature permits the combination of *L. capsici* AZ78 with a low dose of a copper-based fungicide for more effective control of grapevine downy mildew (Puopolo *et al.* 2014).

Building on our previous results, the current study aimed at investigating the secondary metabolites with anti-oomycete activity released *in vitro* by *L. capsici* AZ78. Our attention was focused on low molecular weight compounds synthesized by *L. capsici* AZ78, and we characterized in particular the direct activity of one of these against sporangia of *P. infestans* and *Pl. viticola*.

Materials and methods

Lysobacter capsici AZ78 and plant pathogenic oomycetes

Lysobacter capsici AZ78 was stored at length in glycerol 40% at -80°C and routinely grown on Luria-Bertani agar (LBA; Sigma-Aldrich, St. Louis, MO) in Petri dishes (90 mm diameter). To produce the culture filtrates, *L. capsici* AZ78 was grown in a modified LB medium [tryptone (Sigma-Aldrich) 10 g l^{-1} and yeast extract (Sigma-Aldrich) 5 g l^{-1}]. Sodium chloride was not employed in modified LB medium so as to avoid any phytotoxic effects on plants.

Isolates of the plant pathogenic oomycetes *Phytophthora cactorum*, *Phytophthora capsici*, *Phytophthora cinnamomi* and *Pythium* (*Py.*) *ultimum*, from our laboratory culture collection, were stored in sterile tubes containing potato dextrose agar (PDA; Oxoid, Cambridge, UK) and routinely grown on PDA plates at 22°C . *Phytophthora infestans* provided by M. Finckh and A. Butz (University of Kassel, Germany) was maintained on pea agar medium (PAM, 12.5% frozen pea and 1.2% agar in distilled water) at 17°C and stored at length in glycerol 20% at -80°C . The inoculum was prepared by collecting fresh sporangia from sporulating colonies developed after 21 days at 17°C on PAM (Sharma *et al.* 2010). Petri dishes were flooded with 5 ml of cold ($4-5^{\circ}\text{C}$) distilled water, and sporangia were scraped using sterile spatulas and transferred into sterile 50-ml tubes. *Plasmopara viticola* was isolated from an untreated vineyard in S. Michele all'Adige (Italy) in 2013 and maintained on grapevine plants (*V. vinifera* cv. Pinot Noir plants, grafted onto Kober 5BB) by subsequent weekly inoculations. To obtain sporangia, grapevine plants showing oil spot symptoms were kept overnight in the dark at $20-21^{\circ}\text{C}$ and 100% RH. The inoculum of *Pl. viticola* was prepared

by washing the freshly sporulating lesions on the abaxial leaf surface with cold (4–5°C) distilled water. Unless otherwise stated, the sporangial suspensions of *P. infestans* and *Pl. viticola* were then adjusted to a concentration of 2.5×10^5 sporangia ml⁻¹ by counting with a haemocytometer (Thoma chamber; HBG, Giessen-Lützelindem, Germany) under a light microscope and used in all the experiments.

Characterization of the activity spectrum of *Lysobacter capsici* AZ78 against plant pathogenic oomycetes *in vitro*

To define the activity spectrum of *L. capsici* AZ78 against plant pathogenic oomycetes, its ability to inhibit the mycelial growth of five oomycetes was evaluated in dual culture on PDA (*P. cactorum*, *P. capsici*, *P. cinnamomi* and *Py. ultimum*) and PAM (*P. infestans*) dishes. Briefly, 20 µl of a *L. capsici* AZ78 cell suspension (1×10^8 CFU ml⁻¹) was spotted on two opposite edges of dishes containing the above-mentioned growth media and kept at 27°C for 24 h. After this incubation period, plugs of mycelium (5 mm diameter) were cut from the edge of the young oomycete colonies and placed at the centre of the dishes containing *L. capsici* AZ78 macrocolonies. PDA and PAM dishes seeded only with mycelium plugs were used as controls. Inhibition of mycelial growth was evaluated by scoring the oomycete colony diameters after 7-day incubation at 20°C. Each treatment was carried out on three dishes (replicates), and experiments were repeated.

Assessment of the efficacy of culture filtrates of *Lysobacter capsici* AZ78 to control *Phytophthora infestans* and *Plasmopara viticola* *in vivo*

In each experiment, *L. capsici* AZ78 cell suspensions and its own supernatants were prepared according to the following procedure. Bottles containing 500 ml of modified LB medium were inoculated with 5 ml of *L. capsici* AZ78 cell suspension (1×10^8 CFU ml⁻¹) and incubated at 27°C for 72 h on a rotary shaker at 200 rev min⁻¹. At the end of this period, the content of the flasks was centrifuged at 4300 g for 20 min, and the supernatants were filter-sterilized (0.22 µm; Sigma-Aldrich) and collected in sterile 1-l bottles. Pelleted cells were resuspended in sterile distilled water to a final optical density at 600 nm of 0.1 corresponding to $\approx 1 \times 10^8$ CFU ml⁻¹.

The bioassays for the evaluation of the ability of *L. capsici* AZ78 and its own culture filtrates to control *P. infestans* and *Pl. viticola* were, respectively, carried out on tomato (*S. lycopersicum* cv. Tondo Rosso) and on grapevine plants. Two-year-old grapevine plants were

grown in a greenhouse under controlled conditions (20 ± 0.5°C; 70 ± 10% relative humidity, RH; 16-h photoperiod) in 2.5-l pots containing a mixture of peat and pumice (3 : 1) until the plants had produced two shoots with at least nine leaves each. Pregerminated tomato seeds were sown on sterile peat (autoclaved twice for 20 min at 120°C with an interval of 24 h) in sterile 50-ml pots maintained under greenhouse-controlled conditions (25 ± 1°C; 70 ± 10% RH; 16-h photoperiod). Grapevine and tomato plants were ready for experimentation approximately 2 months after planting.

Lysobacter capsici AZ78 cells and its own culture filtrates were applied on grapevine and tomato plants 24 h before inoculation with *Pl. viticola* and *P. infestans*, respectively. Treatments were applied on adaxial and abaxial leaf surfaces using a hand sprayer. Distilled water, modified LB medium and copper hydroxide (Kocide® 3000; Du Pont de Nemours, Wilmington, DE, 2.5 g l⁻¹) were used as controls and were applied 6 h before inoculation. Each grapevine and tomato plant was, respectively, sprayed with 40 and 20 ml of each treatment preparation.

Tomato plants were artificially inoculated by spraying the entire plant with *P. infestans* sporangial suspensions. The plants were kept in the dark at 100% RH and 18°C for 24 h and then transferred in a growth cabinet at 18°C at 80% RH with a 16/8-h day/night light regime. *Plasmopara viticola* inoculum was sprayed onto the abaxial surface of each fully expanded leaf using a hand sprayer. Once inoculated, grapevine plants were incubated at 20 ± 0.5°C (80–99% RH) in the dark for 24 h, then maintained at 25°C (60–80% RH) with a 16/8-h day/night light regime. Seven days after inoculation, the plants were incubated overnight in the dark at 20 ± 0.5°C and 80–99% RH to induce sporulation.

Each treatment was carried out on nine plants (replicates), and experiments were repeated. Seven days after pathogen inoculation, disease incidence (percentage of symptomatic leaves) and disease severity (percentage of leaf area showing symptoms) were visually assessed and expressed as percentages. The efficacy of treatments in reducing disease severity was calculated according to the following formula:

$$\text{Plant protection efficacy} = \left[\frac{(\text{disease severity in untreated plants} - \text{disease severity in treated plants})}{(\text{disease severity in untreated plants})} \right] \times 100.$$

The population of *L. capsici* AZ78 on grapevine and tomato leaves was assessed at the beginning and at the end of the greenhouse experiments using the dilution plating method according to Puopolo *et al.* (2014).

Extraction and purification of metabolites from *Lysobacter capsici* AZ78 culture filtrates

The culture supernatants of *L. capsici* AZ78 produced in modified LB medium as described above were filtered and lyophilized. Subsequently, culture filtrates (3.0 l) were dissolved in 10^{-1} of initial volume with distilled water and extracted with EtOAc (4×300 ml). The organic extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure to yield a brown solid residue (261.5 mg). The residue was then submitted to bioassay-guided fractionation through column chromatography (800×25 mm) on silica gel, eluted with the CHCl_3 -*i*-PrOH (9/1 v/v) until CHCl_3 -*i*-PrOH (7/3 v/v). One hundred and fifty tubes (10 ml) were checked on thin-layer chromatography (TLC) and combined. Four homogenous chromatographic fraction groups were evaluated for their toxicity against sporangia of *P. infestans* and *Pl. viticola*, as described below, at a final concentration of 1 mg ml^{-1} .

The residue (56.5 mg) of the second chromatographic fraction group was further purified by TLC on silica gel and eluted with the CHCl_3 -*i*-PrOH (8/2 v/v), yielding a homogeneous amorphous solid [R_f 0.69, eluent CHCl_3 -*i*-PrOH (8/2 v/v)].

Identification and characterization of *Lysobacter capsici* AZ78 low molecular weight secondary metabolites

Optical rotation was measured in MeOH on a polarimeter (Jasco, Easton, MD). IR spectra were recorded as deposit glass film on a Perkin-Elmer (Norwalk, CT) Spectrum. One FT-IR spectrometer and UV spectra were measured in MeCN on a Perkin-Elmer Lambda 23 UV/vis spectrophotometer. ^1H NMR and ^{13}C NMR spectra were recorded at 600 or 400 and 125 or 100 MHz, respectively, in CDCl_3 , on spectrometers (Bruker, Leipzig, Germany). The same solvent was used as internal standard. Carbon multiplicities were determined by distortionless enhancement by polarization transfer (DEPT) spectra (Berger and Braun 2004). DEPT, correlation spectroscopy (COSY-45), heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC) and nuclear overhauser enhancement spectroscopy (NOESY) experiments were performed using standard Bruker microprograms (Berger and Braun 2004). High-resolution (HR) and electrospray ionization mass spectrometry (ESIMS) spectra were recorded on Thermo LTQ Velos (Thermo Fisher Scientific, Bremen, Germany) and 6120 Quadrupole LC/MS (Agilent Technologies, Santa Clara, CA), respectively. Analytical and preparative TLC was performed on silica gel [Kieselgel 60, F_{254} , 0.25 and 0.5 mm (Merck, Darmstadt,

Germany)] dishes. The spots were visualized by exposure to UV light at 254 nm, iodine vapours, or by 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 was performed on a Kieselgel 60, 0.063–0.200 mm silica gel column (Merck).

Evaluation of the anti-oomycete activity against *Phytophthora infestans* and *Plasmopara viticola* sporangia of *Lysobacter capsici* AZ78 secondary metabolites

Fifty microlitres of each chromatographic fraction group was added to 50 μl of freshly prepared *P. infestans* or *Pl. viticola* sporangia (2×10^6 sporangia ml^{-1}) in sterile 1.5-ml tubes. The mixtures were then incubated for 60 min at 17°C and then stained for 5 min with a combination of 5,6-carboxyfluorescein diacetate (Sigma-Aldrich; 0.01%) in DMSO (Sigma-Aldrich; 0.01%) and propidium iodide (Sigma-Aldrich; $100 \mu\text{g ml}^{-1}$), protocol adapted from Sergeeva *et al.* (2002) and Trouvelot *et al.* (2008). DMSO (0.01%) and sterile distilled water were used as controls.

After the incubation period, the suspensions were transferred on glass slides (Fast-Read 102; Biosigma F, Cona, Italy) and observed under microscope Leica LMD7000 (Leica, Wetzlar, Germany) using the B/G/R filter (excitation filter: BP 550–590 nm; dichromatic mirror: 590 nm; suppression filter: BP 600–680 nm) to count dead sporangia (emitting propidium iodide-dependent red fluorescence) and H3 filter (excitation filter: BP 420–490 nm; dichromatic mirror: 510 nm; suppression filter: LP 515 nm) to count viable sporangia (emitting 5,6-carboxyfluorescein diacetate-dependent green fluorescence) (Trouvelot *et al.* 2008).

Each experiment involved three replicates (three 1.5-ml tubes), and a minimum of 100 sporangia was counted each time (five repetitions) according to Trouvelot *et al.* (2008). Three independent experiments were carried out.

Confirmation of anti-oomycete activity of cyclo(L-Pro-L-Tyr) on *Phytophthora infestans* sporangia *in vivo*

The anti-oomycete activity of cyclo(L-Pro-L-Tyr) was confirmed on *P. infestans* sporangia on detached tomato leaves according to Tran *et al.* (2007) with modifications. Purified cyclo(L-Pro-L-Tyr) isolated from *L. capsici* AZ78 culture filtrates was sprayed onto leaves collected from tomato plants (2 months old). The metabolite was sprayed at the following concentration: 10, 25, 50, 75 and $100 \mu\text{g ml}^{-1}$. Additional detached tomato leaves were treated with the

L. capsici AZ78 cell-free culture filtrates. Leaves were maintained at room temperature for 1 h under laminar flow to dry. Subsequently, leaves were transferred in 90-mm Petri dishes (one leaf each dish) on sterile wet filter paper, and the abaxial part was inoculated with three drops (10 μ l) of a *P. infestans* sporangia suspension (2×10^3 sporangia ml^{-1}) on each side of the leaf's midvein (six drops per leaf). Once inoculated, Petri dishes were allowed to dry under laminar flow for 20 min and then were placed in an incubator at 18°C. Tomato leaves treated with sterile distilled water, DMSO (0.01%) and copper hydroxide were used as controls. Four days after sporangial inoculation, disease incidence was assessed by scoring the occurrence of late blight lesions developed from the sporangial droplets. Each treatment was composed of five replicates (leaves), and the experiment was repeated.

At the end of the experiments, the occurrence of *L. capsici* AZ78 cells on detached tomato leaves was assessed using the dilution plating method as described above.

Determination of a gene coding for polyketide synthase in *Lysobacter capsici* AZ78

The genomic DNA of *L. capsici* AZ78 was extracted with a Genomic DNA isolation Kit (Qiagen, Holden, Germany) and used as the template in PCRs aimed at detecting the presence of gene coding for a polyketide synthase. The primer pair AZ78_pks1 F (5'-GGT TCC TCA AGC AGC TCA A-3')/AZ78_pks1 R (5'-GGA ACA GGT CGG TGT TGT A-3') was designed based on the nucleotide sequence from *L. enzymogenes* C3 coding for a hybrid polyketide synthase and nonribosomal peptide synthetase (GenBank Accession No. EF028635.2). In these PCRs, 2 μ l of genomic DNA was used as template in 25- μ l reaction including 1X Dream Taq Green PCR Mastermix (Thermo Fisher Scientific) and 0.2 $\mu\text{mol l}^{-1}$ of primer AZ78_pks1 F/AZ78_pks1R. These PCRs involved a first cycle at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 1 min with a final extension step at 72°C for 5 min.

PCR products were purified using Exo-Sap (Euroclone S.p.a., Italy) according to manufacturer's instructions. Purified DNA amplicons were sequenced using BigDye Terminator v 3.1 (Thermo Fisher Scientific), and the resulting nucleotide sequences were analysed using BLASTN to find homologies with DNA sequences already deposited in GenBank. The most similar nucleotide sequences were collected from GenBank and used for phylogenetic analyses. All the sequences were aligned with CLUSTAL X (Thompson et al. 1997), and the alignment profile was then used to establish the evolutionary distances by applying Kimura's two-parameter model (Kimura 1983) implemented in the MEGA3 program (Kumar et al. 2004).

The same software was used to construct the best phylogenetic tree with the neighbour-joining method (Saitou and Nei 1987). Bootstrap analysis with 1000 replicates was performed to assess confidence levels for the branches (Felsenstein 1985).

Statistical analyses

The data attained in all the experiments were subjected to a two-way ANOVA. If no significant differences were found, the data were pooled. In the case of data coming from dual-plate method, differences between mean values of oomycete colony diameters were statistically analysed by Student's *t*-test ($\alpha = 0.05$). In all the other cases, the data were arcsine-transformed prior to the two-way ANOVA and then analysed by ANOVA and mean comparisons were performed with a Tukey's test ($\alpha = 0.05$). The data of plant protection efficacy were also checked for normality (Kolmogorov–Smirnov's test) and homoscedasticity (Bartlett's test) before ANOVA. All the statistical analysis was carried out using STATISTICA software 7.0 (Statsoft, Tulsa, OK).

Results

Characterization of the activity spectrum of *Lysobacter capsici* AZ78 against plant pathogenic oomycetes *in vitro*

The ability of *L. capsici* AZ78 to inhibit mycelial growth of some plant pathogenic oomycetes *in vitro* was chosen as first step in the investigation on the mechanisms involved in the antagonism aptitude of this bacterial strain. *Lysobacter capsici* AZ78 was able to inhibit the mycelial growth of the five plant pathogenic oomycetes tested (Fig. 1), and particularly, *Phytophthora infestans* growth was drastically impaired in the presence of the bacterium ($\approx 73\%$ inhibition).

Assessment of the efficacy of culture filtrates of *Lysobacter capsici* AZ78 to control *Phytophthora infestans* and *Plasmopara viticola* *in vivo*

Based on the strong pathogen inhibition recorded in the dual-culture test, we hypothesized that *L. capsici* AZ78 releases secondary metabolites with anti-oomycete activity in the medium. To validate this hypothesis and identify the active molecules, we assessed the efficacy of the culture filtrates of *L. capsici* AZ78 for the control of two selected oomycetes *P. infestans* and *Pl. viticola* on tomato and grapevine plants, respectively. No statistical differences were recorded between the level of disease severity of the grapevine (28 ± 6 and 24 ± 3) and tomato (58 ± 12 and 46 ± 14) plants, respectively, treated with

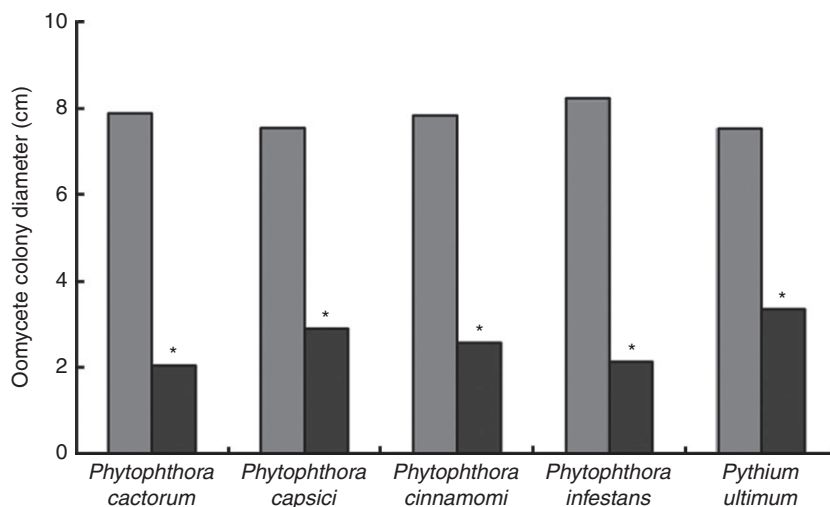


Figure 1 Mycelial growth of plant pathogenic oomycetes in dual culture by *Lysobacter capsici* AZ78. The black and grey histograms refer to the growth of the oomycetes in the presence of and in the absence of *L. capsici* AZ78. Oomycete colony diameters were scored after 7 days of incubation at 20°C. Three dishes were used for each treatment, and data originating from two independent experiments were pooled. *Values differ significantly according to Student's *t*-test ($\alpha = 0.05$).

distilled water and modified LB medium. The plant protection efficacy of *L. capsici* AZ78 cells on tomato leaves did not significantly differ from that achieved with copper hydroxide with values close to 100% (Fig. 2a). The application of *L. capsici* AZ78 culture filtrates resulted in a plant protection efficacy of 74% (Fig. 2a), higher than the values achieved using modified LB (15.1%, Fig. 2a). Similar results were obtained against *Pl. viticola* on grapevine. In this case, plant protection efficacy of *L. capsici* AZ78 culture filtrates was 66.2% and lower than that achieved on plants treated with *L. capsici* AZ78 cells (92.4%) and the copper hydroxide (90.7%) (Fig. 2b). Additionally, the application of *L. capsici* AZ78 culture filtrates resulted also in a reduction of the incidence of symptomatic leaves, but the values were not significantly different from plants treated with distilled water or modified LB medium (Table S1). On the other hand, application of *L. capsici* AZ78 cells in both the pathosystems determined a drastic reduction of disease incidence similar to that achieved with copper treatments (Table S1). *Lysobacter capsici* AZ78 cells were never isolated from leaves collected from plants treated with distilled water, modified LB medium, AZ78 culture filtrates and copper-based fungicide, either at the beginning or at the end of the greenhouse trials. *Lysobacter capsici* AZ78 was recovered only from plants treated with *L. capsici* AZ78 cells. At the beginning of the trial, the population recovered from tomato leaves was $6.56 \pm 0.16 \log_{10}$ CFU g^{-1} of tomato leaf, while at the end of trial, population reached $5.01 \pm 0.02 \log_{10}$ CFU g^{-1} of tomato leaf. A similar population size was recorded on grapevine leaves at the beginning and at the end of the trials (5.16 ± 0.12 and $5.18 \pm 0.14 \log_{10}$ CFU g^{-1} of grapevine leaf, respectively).

Lysobacter capsici AZ78 produces cyclo(L-Pro-L-Tyr) a 2,5-diketopiperazine toxic against *Phytophthora infestans* and *Plasmopara viticola* sporangia

The fact that *L. capsici* AZ78 culture filtrates reduced the disease in both pathosystems prompted us to investigate the low molecular weight secondary metabolites with anti-oomycete activity produced by *L. capsici* AZ78 during its growth in modified LB medium. For this purpose, the liquid culture filtrates of *L. capsici* AZ78 were exhaustively extracted with ethyl acetate and the organic extract, showing high toxic activity against *P. infestans* and *Pl. viticola* sporangia (data not shown), was fractionated by column chromatography in four fractions (named Fraction A, B, C and D) that were then assessed for their capacity to kill sporangia of both the plant pathogenic oomycetes.

After 60 min of incubation in different solutions, both water- and DMSO (0.01%)-treated sporangia of *P. infestans* and *Pl. viticola* (control samples) showed an 80% viability (Fig. S1a,b). The incubation in Fraction A and B caused, respectively, a decrease to 49 and 34% of sporangial viability in *P. infestans* (Fig. S1a) while a viability decrease to 19% was found in the case of *Pl. viticola* (Fig. S1b). On the other hand, Fraction C and D showed a limited negative effect on *Pl. viticola* sporangial viability and almost no effect on *P. infestans* (Fig. S1a,b).

The residue (56.5 mg) of Fraction B was further purified by TLC yielding a homogeneous amorphous solid (30.0 mg, corresponding to $10 \mu g \text{ ml}^{-1}$). The amorphous compound was identified as a cyclo(L-Pro-L-Tyr) (Fig. 3a), a (3*S*,8*aS*) pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro-3-[(4-hydroxyphenyl)methyl], by comparing its spectroscopic (IR, UV, ^1H ; Fig. 3b) and ^{13}C NMR

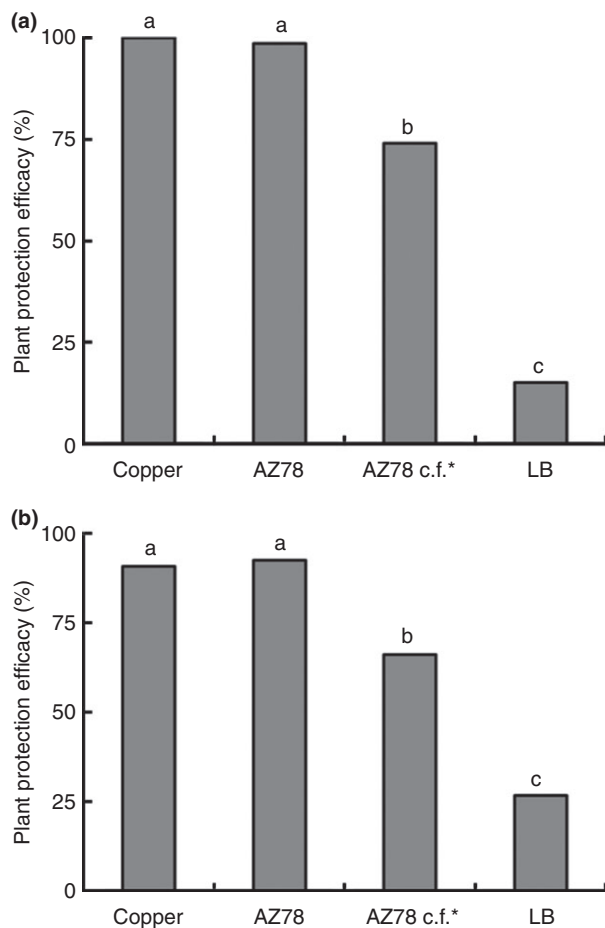


Figure 2 Plant protection efficacy against *Phytophthora infestans* (a) and *Plasmopara viticola* (b) achieved by the application of *Lysobacter capsici* AZ78 cells and its culture filtrates (c.f.*). Plant protection efficacy is expressed as (disease severity in untreated plants – disease severity in treated plants)/(disease severity in untreated plants) × 100. Each treatment was applied on five plants (replicates), experiments were repeated, and data were pooled. Histograms with the same letters are not significantly different according to Tukey's test ($\alpha = 0.05$).

(Fig. S2) and optical properties with those already reported in the literature (all the chemical characteristics are reported in Supporting information). The identification of cyclo(L-Pro-L-Tyr) was also confirmed by the couplings observed in the COSY and HSQC spectra (Figs S3 and S4) and by the correlation recorded in the NOESY spectrum (Fig. S5). Furthermore, the structure was supported by the several long-range coupling observed in the HMBC spectrum (Fig. S6) and by the data of its HRESI MS spectrum, which showed the dimeric sodiated $[2M+Na]^+$ and protonated $[2M+H]^+$ form, the sodium cluster $[M+Na]^+$ and the pseudomolecular ion $[M+H]^+$ at m/z 543·2217, 521·2397, 283·1051 and 261·1231, respectively (Fig. S7). The absolute configuration to cyclo

(L-Pro-L-Tyr) was assigned by comparing its specific optical rotatory power with that reported in the literature.

Confirmation of anti-oomycete activity of cyclo (L-Pro-L-Tyr) on *Phytophthora infestans* sporangia

The anti-oomycete activity cyclo(L-Pro-L-Tyr) was confirmed in a bioassay carried out on detached tomato leaves. *Lysobacter capsici* AZ78 culture filtrates reduced *P. infestans* disease incidence ($10.25 \pm 5.27\%$) similar to copper hydroxide ($7.00 \pm 4.99\%$, Fig. 4). A reduction in disease severity, although lower than *L. capsici* AZ78 culture filtrates, was observed with cyclo(L-Pro-L-Tyr) applied at concentrations ranging from 10 to $100 \mu\text{g ml}^{-1}$ (Fig. 4), and a clear dose effect of cyclo(L-Pro-L-Tyr) was recorded (Fig. S8). In more detail, the maximum level of protection was achieved by applying the cyclo(L-Pro-L-Tyr) at $100 \mu\text{g ml}^{-1}$ ($26.67 \pm 4.21\%$) while the lowest concentrated solution determined a reduced protective effect (66.67 ± 4.71) that, however, was statistically lower than the disease incidence observed on leaves treated with sterile distilled water and DMSO (0.01% , Fig. 4). At the end of the experiments, no *L. capsici* AZ78 cells were detected in any of the detached tomato leaves through dilution plating method.

Detection of a gene coding for polyketide synthase in *Lysobacter capsici* AZ78

A nucleotide region showing high sequence identity with polyketide synthase was detected in *L. capsici* AZ78. A DNA fragment of 1132 bp was amplified using the primer pair AZ78_PKS1 F/R specific for a nucleotide region coding for a hybrid polyketide synthase and nonribosomal peptide synthetase of *L. enzymogenes* C3. The resulting nucleotide sequence (GenBank accession no. KF841351) showed the highest sequence identity with the gene coding for polyketide synthase from *L. capsici* C3 (Fig. 5). The nucleotide region obtained from *L. capsici* AZ78 also shared high sequence identity with other bacterial strains such as *Salinispora arenicola* CNS-205 and others of different *Streptomyces* species (Fig. 5).

Discussion

Finding sustainable alternatives to chemical fungicides against destructive diseases caused by plant pathogenic oomycetes, such as late blight and grapevine downy mildew, is a priority for the future. Microbial biocontrol agents already offered a valid alternative in different pathosystems (Raaijmakers and Mazzola 2012), and *Lysobacter* genus could be a promising source for new active ingredients in biological plant protection products.

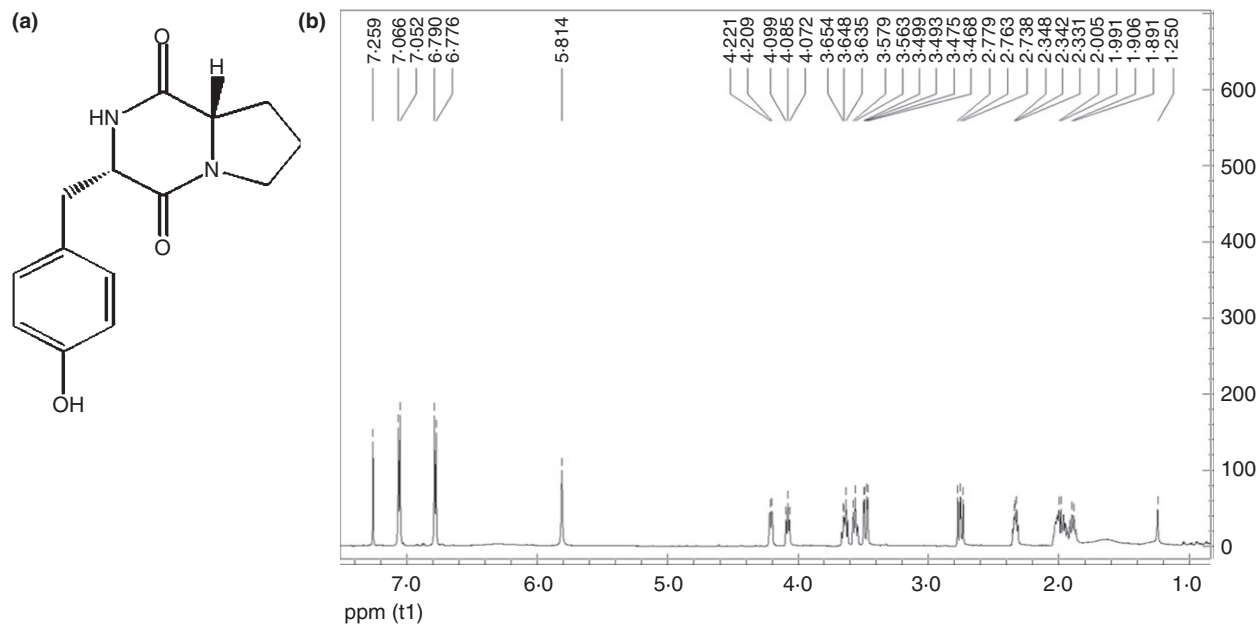


Figure 3 Identification of cyclo(L-Pro-L-Tyr) a 2,5-diketopiperazine produced by *Lysobacter capsici* AZ78. (a) Structural formula of cyclo(L-Pro-L-Tyr); (b) ¹H nuclear magnetic resonance (NMR) spectrum of cyclo(L-Pro-L-Tyr) was recorded at 600 MHz in monodeuterated chloroform (CDCl₃).

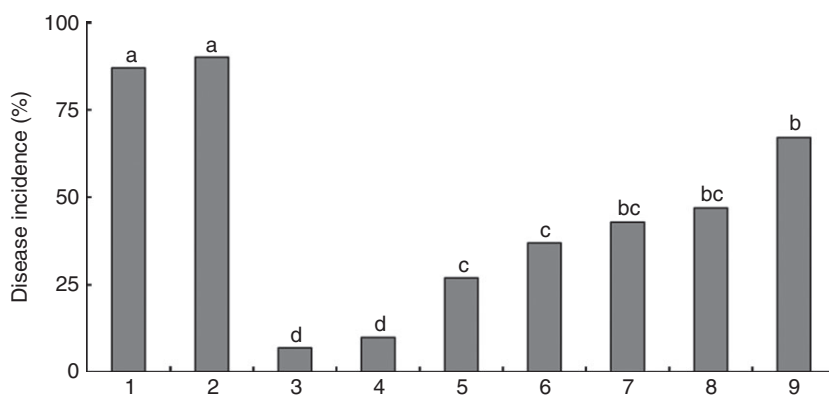


Figure 4 Capability of cyclo(L-Pro-L-Tyr) to protect detached tomato leaves against *Phytophthora infestans* attacks. The protective effect was evaluated by monitoring the disease incidence that is expressed as the percentage of late blight lesions developed from sporangial droplets. Five tomato leaves were used for each treatment, and experiments were repeated. Histograms with the same letters are not significantly different according to Tukey's test ($\alpha = 0.05$). (1) Sterile distilled water; (2) DMSO 0.01 %; (3) copper hydroxide; (5,6,7,8 and 9) cyclo(L-Pro-L-Tyr) at 100, 75, 50, 25 and 10 $\mu\text{g ml}^{-1}$.

We previously showed that *L. capsici* AZ78 effectively controls *Pl. viticola* in combination with a copper-based fungicide (Puopolo et al. 2014). Based on this evidence, we wanted to see whether its activity could be extended to other plant pathogenic oomycetes. We further investigated the low molecular weight secondary metabolites with anti-oomycete activity released *in vitro* by *L. capsici* AZ78.

Lysobacter capsici AZ78 has a direct effect on the growth of several oomycetes when tested in dual culture,

which leads to a hypothesis of the production of compounds toxic against plant pathogenic oomycetes. We subsequently carried out trials under controlled conditions where *L. capsici* AZ78 and its own culture filtrates were used for the biological control of *P. infestans* and *Pl. viticola*. We proved that the application of *L. capsici* AZ78 cells on tomato leaves strongly reduces tomato late blight, similar to application of the copper-based fungicide Kocide® 3000. To the best of our knowledge, this is the first time that a member of the genus

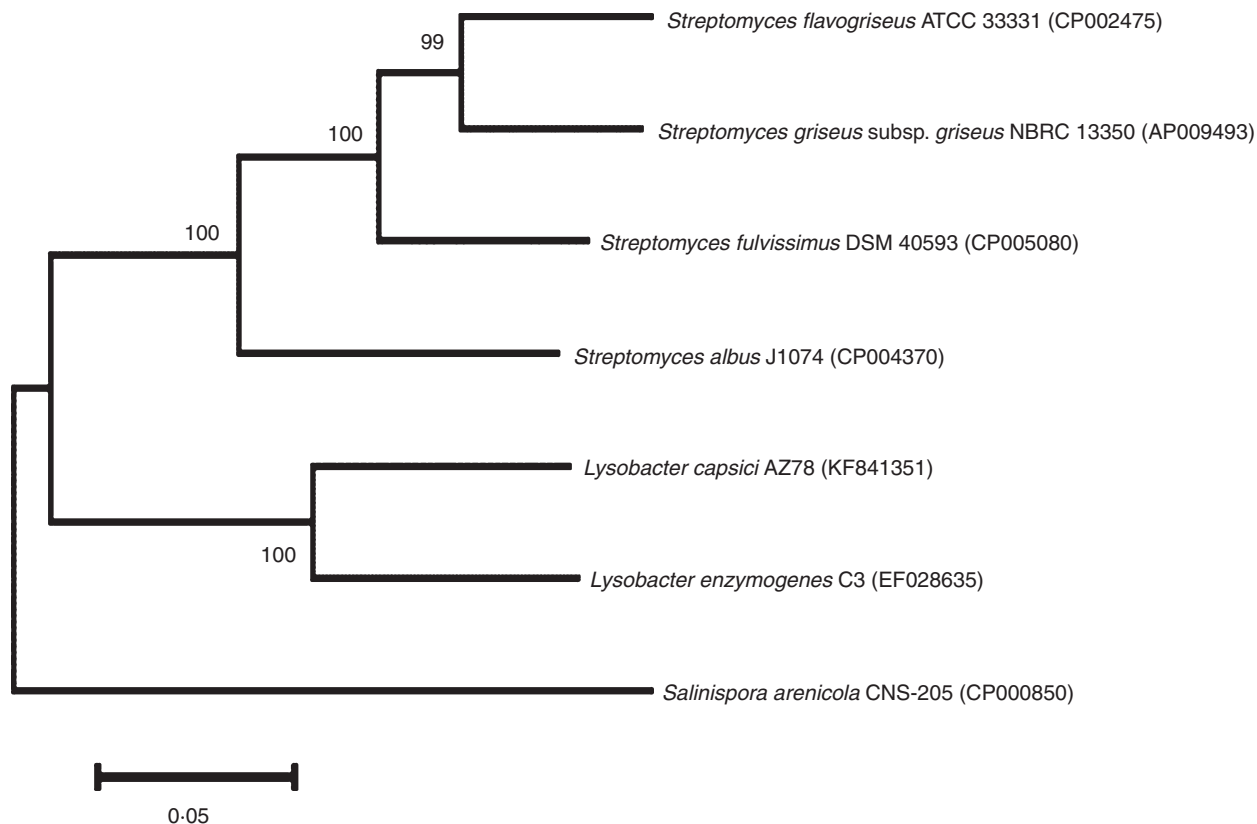


Figure 5 Analysis of sequence identity of a partial region of a gene coding for a hybrid polyketide synthase and nonribosomal peptide synthetase from *Lysobacter capsici* AZ78. The phylogenetic tree was obtained using the neighbour-joining method, and evolutionary distances were calculated using Kimura's two-parameter model (Kimura 1983). Bootstrap values (Felsenstein 1985) higher than 50 are shown at the branch points.

Lysobacter has been reported to control *P. infestans* on tomato plants. Previous studies have shown that other *Lysobacter* strains control soilborne plant pathogenic oomycetes such as *Aphanomyces cochlioides* and *Py. aphanidermatum* (Nakayama et al. 1999; Folman et al. 2001, 2003, 2004; Islam et al. 2005;). Recently, Ko et al. (2009) demonstrated that *L. antibioticus* HS124 effectively protects pepper plants against *P. capsici* attacks and linked this activity with the production of lytic enzymes (i.e. β -1,3-glucanase) and an antibiotic compound (4-hydroxyphenylacetic acid). The results obtained in our study thus further strengthen the potential of members of the bacterial genus *Lysobacter* for biological control of different plant pathogenic oomycetes.

Concurrently, results achieved with the application of *L. capsici* AZ78 cell-free culture filtrates on plants for the control of *P. infestans* and *Pl. viticola* clearly suggested that *L. capsici* AZ78 was producing secondary metabolites that are toxic against both the oomycetes tested. The evidence that a *Lysobacter* member produces secondary metabolites with anti-oomycete activity is also corroborated by previous studies carried out on *Lysobacter*

sp. SB-K88, *L. antibioticus* HS124 and *L. capsici* YC5194 (Homma et al. 1993; Nakayama et al. 1999; Islam et al. 2005; Park et al. 2008; Ko et al. 2009). In particular, *Lysobacter* sp. SB-K88 synthesizes Xanthobaccin A, B and C, which are highly effective *in vitro* against *Ap. cochlioides*, *Phytophthora vignae* f. sp. *adzukicola* and *Py. ultimum* (Nakayama et al. 1999) and in suppressing damping-off of sugar beet caused by *Pythium* spp. in soil (Homma et al. 1993; Nakayama et al. 1999).

These three macrocyclic lactams were isolated and identified after the screening of chromatographic fractions for their ability to inhibit the mycelial growth of *Py. ultimum* and *Rhizoctonia solani* (Nakayama et al. 1999). In our study, on the other hand, we focused our attention on low molecular weight compounds, and the chromatographic fraction groups were evaluated for their toxicity against sporangia of *P. infestans* and *Pl. viticola* as they represented the primary inoculum source for the trials carried out under controlled conditions. One of the four chromatographic fraction groups was found to be highly toxic against the sporangia of both the plant pathogenic oomycetes. The preliminary NMR investigation,

using both ^1H NMR and ^{13}C NMR spectra, showed that the toxic secondary metabolite was a cyclic dipeptide. On the basis of a comparison of its spectroscopic and optical properties with those already reported in the literature (Tatsuno *et al.* 1971; Stierle *et al.* 1988; Jayatilake *et al.* 1996; Cain *et al.* 2003), the secondary metabolite present in the chromatographic Fraction B was subsequently identified as the already-known cyclo(L-Pro-L-Tyr), a cyclic dipeptide that belongs to the group of the proline derivatives of 2,5-diketopiperazine, mainly isolated from fungi and bacteria (for a review, see Borthwick 2012).

Up to now, the bioactive products identified and characterized from *Lysobacter* members mainly belong to the cyclodepsipeptides (lysobactin), cyclic lipodepsipeptides (WAP-8294A2), cephem type β -lactams (cephabacins) and polycyclic tetramate macrolactam (Xanthobaccin A) families (Ono *et al.* 1984; O'Sullivan *et al.* 1988; Kato *et al.* 1997b; Nakayama *et al.* 1999; Zhang *et al.* 2011; Xie *et al.* 2012). Our study thus provides the first evidence on the production of a 2,5-diketopiperazine from a member of the *L. capsici* species adding a new compound to the high number of bioactive natural-product families synthesized by *Lysobacter* genus. We showed moreover, for the first time, that this molecule has toxic effects against the sporangia of both *P. infestans* and *Pl. viticola*.

Looking at the distribution of 2,5-diketopiperazines within micro-organisms, cyclo(L-Pro-L-Tyr) and its isomer cyclo(D-Pro-L-Tyr) are known to be produced by different bacterial strains belonging mainly to the genus *Bacillus* and *Pseudomonas* (Jayatilake *et al.* 1996; De Rosa *et al.* 2003; Guo *et al.* 2007; Nishanth Kumar *et al.* 2012). Cyclo(L-Pro-L-Tyr) is also produced by *Aristobacter necator* 679-2 together with two compounds identified as pyrrolnitrin and banegasine (Cain *et al.* 2003). Moreover, cyclo(L-Pro-L-Tyr) isolated from *Pseudomonas aeruginosa* PAO1 and *Pseudomonas putida* WCS358 positively interfered with the quorum sensing system based on the *N*-acylated L-homoserine lactone signals (Holden *et al.* 1999; Degrassi *et al.* 2002). Recently, Ortiz-Castro *et al.* (2011) showed that the production of cyclo(L-Pro-L-Tyr) in *Ps. aeruginosa* PAO1 is positively controlled by the LasI quorum sensing system and, moreover, the cyclo-dipeptide acts as an auxin signal mimic on *Arabidopsis thaliana*.

Cyclo(L-Pro-L-Tyr) was also isolated from different fungal species such as *Alternaria alternata*, *Fusarium nivale* and *Leptographium qinlingensis* (Tatsuno *et al.* 1971; Stierle *et al.* 1988; Li *et al.* 2012). The cyclo(L-Pro-L-Tyr) compound produced by *Al. alternata* (also known as maculosin) was found to be toxic against spotted knapweed (*Centaurea maculosa*), and these evidences lead to the development of a safe and environmentally friendly herbicides against knapweed (Stierle *et al.* 1988; Bobylev

et al. 1996). Interestingly, an *Al. alternata* endophytic strain isolated from the grapevine produced three proline derivatives of 2,5-diketopiperazines [cyclo(L-Phe-*trans*-4-hydroxy-L-Pro); cyclo(L-Leu-*trans*-4-hydroxy-L-Pro); and cyclo(L-Ala-*trans*-4-hydroxy-L-Pro)] that when applied as mixtures was found to be effective in reducing *Pl. viticola* sporulation (Musetti *et al.* 2006, 2007). It is worth noting that these results achieved previously and the results reported in our work are indicating that the group of the proline derivatives of 2,5-diketopiperazine are active against *P. infestans* and *Pl. viticola*; thus, they can also be considered as good candidates for the development of new eco-friendly products that can be applied for a more sustainable control of plant pathogenic oomycetes.

However, on the basis of the results obtained on the application of different doses of cyclo(L-Pro-L-Tyr) on detached tomato leaf, we cannot rule out that other compounds sharing anti-oomycete activity are produced by *L. capsici* AZ78. These data showed in fact that the cell-free culture filtrates of *L. capsici* AZ78 were more active than cyclo(L-Pro-L-Tyr) when evaluated at the concentrations found in the growth medium ($10\ \mu\text{g}\ \text{ml}^{-1}$). Other potential compounds that can contribute to the control of the plant pathogenic oomycetes tested are the β -glucanases, already characterized in other *Lysobacter* members that are known to have effects against plant pathogenic oomycetes (Palumbo *et al.* 2003, 2005; Ko *et al.* 2009) and/or by other substances with antibiotic properties. During this work, we detected a genomic region that shared high sequence identity with a hybrid polyketide synthase and nonribosomal peptide synthetase in *L. enzymogenes* C3 and other bacterial strains belonging mainly to the genus *Streptomyces*. In *Lysobacter*, nonribosomal peptide synthetase is responsible for the biosynthesis of several antibiotic compounds such as lysobactin, WAPS8294A2, cephabacin and the dihydromalthophilin heat-stable antifungal factor (HSAF) (Kimura *et al.* 1995, 1996; Bernhard *et al.* 1996; Yu *et al.* 2007; Hou *et al.* 2011; Zhang *et al.* 2011). In particular, the nucleotide sequence detected in *L. capsici* AZ78 showed high sequence identity with the nucleotide region coding for a hybrid polyketide synthase and nonribosomal peptide synthetase deputed to the synthesis of HSAF in *L. enzymogenes* C3 (Yu *et al.* 2007). On the basis of this sequence identity, it is reasonable to hypothesize that *L. capsici* AZ78 may produce some other secondary metabolites active against *P. infestans* and *Pl. viticola*.

This study provided sound evidences that *L. capsici* AZ78 can also be applied on tomato for the control of *P. infestans*, enlarging the number of plant pathogenic oomycetes that can be effectively controlled through the application of *Lysobacter* members. Furthermore, we showed that *L. capsici* AZ78 produces cyclo(L-Pro-L-Tyr) *in vitro* and this cyclic dipeptide is toxic against the spo-

rangia of *P. infestans* and *Pl. viticola*. In the next future, the production of this compound will be monitored *in planta* to assess the role that it plays in the biological control of both the plant pathogenic oomycetes.

However, further investigation will be also aimed to better identify and characterize all the bioactive natural products synthesized by this bacterial strain, and particular attention will be given to the determination of the molecular pathways that are controlling their biosynthesis in order to assess which parameters might be modulated with the final aim of making the future application of *L. capsici* AZ78 more effective in the field.

Acknowledgements

The authors thank D. Ressa and C. Sicher for technical assistance, D. Melck from the laboratory of Chimica Biomolecolare (CNR, Pozzuoli, Italy) for the NMR spectra and D. Irwin Inne and M. Reid (Syngenta Jealott's Hill International Research Centre Bracknell, Berkshire, England) for HRESIMS spectra. The research was supported by the EU-project CO-FREE (theme KBBE.2011.1.2-06, grant agreement number 289497).

Conflict of interests

No conflict of interest declared.

References

- Berger, S. and Braun, S. (2004) *200 and More Basic NMR Experiments: A Practical Course*, 1st edn. Weinheim: Wiley-VCH.
- Bernhard, F., Demel, G., Soltani, K., Döhren, H.V. and Blinov, V. (1996) Identification of genes encoding for peptide synthetases in the gram-negative bacterium *Lysobacter* sp. ATCC 53042 and the fungus *Cylindrotrichum oligospermum*. *DNA Seq* **6**, 319–330.
- Bobylev, M.M., Bobyleva, L.I. and Strobel, G.A. (1996) Synthesis and bioactivity of analogs of maculosin, a host-specific phytotoxin produced by *Alternaria alternata* on spotted knapweed (*Centaurea maculosa*). *J Agric Food Chem* **44**, 3960–3964.
- Borthwick, A.D. (2012) 2,5-Diketopiperazines: synthesis, reactions, medicinal chemistry, and bioactive natural products. *Chem Rev* **112**, 3641–3716.
- Cain, C.C., Lee, D., Waldo, R.H. III, Henry, A.T., Casida, E.J. Jr, Wani, M.C., Wall, M.E., Oberlies, N.H. et al. (2003) Synergistic antimicrobial activity of metabolites produced by a nonobligate bacterial predator. *Antimicrob Agents Chemother* **47**, 2113–2117.
- Christensen, P. and Cook, F.D. (1978) *Lysobacter*, a new genus of nonfruiting, gliding bacteria with a high base ratio. *Int J Syst Bacteriol* **28**, 367–393.
- De Rosa, S., Mitova, M. and Tommonaro, G. (2003) Marine bacteria associated with sponge as source of cyclic dipeptides. *Biomol Eng* **20**, 311–316.
- Degrassi, G., Aguilar, C., Bosco, M., Zahariev, S., Pongor, S. and Venturi, V. (2002) Plant growth-promoting *Pseudomonas putida* WCS358 produces and secretes four cyclic dipeptides: cross-talk with quorum sensing bacterial sensors. *Curr Microbiol* **45**, 250–254.
- Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using bootstrap. *Evolution* **39**, 783–791.
- Folman, L.B., Postma, J. and van Veen, J.A. (2001) Ecophysiological characterization of rhizosphere bacterial communities at different root locations and plant developmental stages of cucumber grown on rockwool. *Microb Ecol* **42**, 586–597.
- Folman, L.B., Postma, J. and van Veen, J.A. (2003) Characterization of *Lysobacter enzymogenes* (Christensen and Cook 1978) strain 3.1T8, a powerful antagonist of fungal diseases of cucumber. *Microbiol Res* **158**, 107–115.
- Folman, L.B., De Klein, M.J.E.M., Postma, J. and van Veen, J.A. (2004) Production of antifungal compounds by *Lysobacter enzymogenes* strain 3.1T8 under different conditions in relation to its efficacy as a biocontrol agent of *Pythium aphanidermatum* in cucumber. *Biol Control* **31**, 145–154.
- Fry, W. (2008) *Phytophthora infestans*: the plant (and R gene) destroyer. *Mol Plant Pathol* **9**, 385–402.
- Fry, W. and Goodwin, S. (1997) Re-emergence of potato and tomato late blight in the United States. *Plant Dis* **81**, 1349–1357.
- Gessler, C., Pertot, I. and Perazzolli, M. (2011) *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. *Phytopathol Mediterr* **50**, 3–44.
- Guo, Q., Guo, D., Zhao, B., Xu, J. and Li, R. (2007) Two cyclic dipeptides from *Pseudomonas fluorescens* GcM5-1A carried by the pine wood nematode and their toxicities to Japanese black pine suspension cells and seedling *in vitro*. *J Nematol* **39**, 243–247.
- Halfeld-Vieira, B.A., Romeiro, R.S., Mounteer, A. and Mizubuti, E.S.G. (2008) Efficiency of phylloplane bacteria in controlling aerial tomato diseases under field conditions. *Summa Phytopathol* **34**, 64–87.
- Hayward, A.C., Fegan, N., Fegan, M. and Stirling, G.R. (2010) *Stenotrophomonas* and *Lysobacter*: ubiquitous plant-associated gamma-proteobacteria of developing significance in applied microbiology. *J Appl Microbiol* **108**, 756–770.
- Holden, M.T.G., Chhabra, S.R., de Nys, R., Stead, P., Bainton, N.J., Hill, P.J., Manefield, M., Kumar, N. et al. (1999) Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other Gram-negative bacteria. *Mol Microbiol* **33**, 1254–1266.

- Homma, Y., Uchino, H., Kanzawa, K., Nakayama, T. and Sayama, M. (1993) Suppression of sugar beet damping-off and production of antagonistic substances by strains of rhizobacteria. *Ann Phytopathol Soc Jpn* **59**, 282.
- Hou, J., Robbel, L. and Marahiel, M.A. (2011) Identification and characterization of the lysobactin biosynthetic gene cluster reveals mechanistic insights into an unusual termination module architecture. *Chem Biol* **18**, 655–664.
- Islam, M.T., Hashidoko, Y., Deora, A., Itoi, T. and Tahara, S. (2005) Suppression of damping-off disease in host plants by rhizoplane bacterium *Lysobacter* sp. strain SB-K88 is linked to plant colonization and antibiosis against soilborne peronosporomycetes. *Appl Environ Microbiol* **71**, 3786–3796.
- Jayatilake, G.S., Thornton, M.P., Leonard, A.C., Grimwade, J.E. and Baker, B.J. (1996) Metabolites from an Antarctic sponge-associated bacterium, *Pseudomonas aeruginosa*. *J Nat Prod* **59**, 293–296.
- Kato, M., Mizubuti, E.S., Goodwin, S.B. and Fry, W.E. (1997a) Sensitivity to protectant fungicides and pathogenic fitness of clonal lineages of *Phytophthora infestans* in the United States. *Phytopathology* **87**, 973–978.
- Kato, A., Nakaya, S., Ohashi, Y. and Hirata, H. (1997b) WAP-8294A(2), a novel anti-MRSA antibiotic produced by *Lysobacter* sp. *J Am Chem Soc* **119**, 6680–6681.
- Kimura, M. (1983) *The Neutral Theory of Molecular Evolution*. Cambridge, UK: Cambridge University Press.
- Kimura, H., Suzuki, M. and Sumino, Y. (1995) Cloning and expression of the isopenicillin N synthase gene from *Lysobacter lactamgenus* YK90. *J Ferment Bioeng* **80**, 118–123.
- Kimura, H., Miyashita, H. and Sumino, Y. (1996) Organization and expression in *Pseudomonas putida* of the gene cluster involved in cephalosporin biosynthesis from *Lysobacter lactamgenus* YK90. *Appl Microbiol Biotechnol* **45**, 490–501.
- Kloepper, J.W., Ryu, C.M. and Zhang, S.A. (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* **94**, 1259–1266.
- Ko, H.-S., Jin, R.-D., Krishnan, H.B., Lee, S.-B. and Kim, K.-Y. (2009) Biocontrol ability of *Lysobacter antibioticus* HS124 against *Phytophthora* blight is mediated by the production of 4-hydroxyphenylacetic acid and several lytic enzymes. *Curr Microbiol* **59**, 608–615.
- Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Lamsal, K., Kim, S.W., Kim, Y.S. and Lee, Y.S. (2013) Biocontrol of late blight and plant growth promotion in tomato using rhizobacterial isolates. *J Microbiol Biotechnol* **23**, 897–904.
- Li, X.-J., Gao, J.-M., Chen, H., Zhang, A.-L. and Tang, M. (2012) Toxins from a symbiotic fungus, *Leptographium qinlingensis* associated with *Dendroctonus armandi* and their *in vitro* toxicities to *Pinus armandi* seedlings. *Eur J Plant Pathol* **134**, 239–247.
- Lourenço Júnior, V., Maffia, L.A., Romeiro, R.S. and Mizubuti, E.S.G. (2006) Biocontrol of tomato late blight with the combination of epiphytic antagonists and rhizobacteria. *Biol Control* **38**, 331–340.
- Mizubuti, E.S.G., Júnior, V.L. and Forbes, G.A. (2007) Management of late blight with alternative products. *Pest Technol* **1**, 106–116.
- Musetti, R., Vecchione, A., Stringher, L., Borselli, S., Zulini, L., Marzani, C., D'Ambrosio, M., di Toppi, L.S. *et al.* (2006) Inhibition of sporulation and ultrastructural alterations of grapevine downy mildew by the endophytic fungus *Alternaria alternata*. *Phytopathology* **96**, 689–698.
- Musetti, R., Polizzotto, R., Vecchione, A., Borselli, S., Zulini, L., D'Ambrosio, M., di Toppi, L.S. and Pertot, I. (2007) Antifungal activity of diketopiperazines extracted from *Alternaria alternata* against *Plasmopara viticola*: an ultrastructural study. *Micron* **38**, 643–650.
- Nakayama, T., Homma, Y., Hashidoko, Y., Mizutani, J. and Tahara, S. (1999) Possible role of xanthobaccins produced by *Stenotrophomonas* sp. strain SB-K88 in suppression of sugar beet damping-off disease. *Appl Environ Microbiol* **65**, 4334–4339.
- Nishanth Kumar, S., Mohandas, C., Siji, J.V., Rajasekharan, K.N. and Nambisan, B. (2012) Identification of antimicrobial compound, diketopiperazines, from a *Bacillus* sp. N strain associated with a rhabditid entomopathogenic nematode against major plant pathogenic fungi. *J Appl Microbiol* **113**, 914–924.
- Ono, H., Nozaki, Y., Katayama, N. and Okazaki, H. (1984) Cephabacins, new cephem antibiotics of bacterial origin. I. Discovery and taxonomy of the producing organisms and fermentation. *J Antibiot* **37**, 1528–1535.
- Ortiz-Castro, R., Díaz-Pérez, C., Martínez-Trujillo, M., del Río, R.E., Campos-García, J. and López-Bucio, J. (2011) Transkingdom signalling based on bacterial cyclopeptides with auxin activity in plants. *Proc Natl Acad Sci USA* **108**, 7253–7258.
- O'Sullivan, J., McCullough, J.E., Tymiak, A.A., Kirsch, D.R., Trejo, W.H. and Principe, P.A. (1988) Lysobactin, a novel antibacterial agent produced by *Lysobacter* sp. I. Taxonomy, isolation and partial characterization. *J Antibiot* **41**, 1740–1744.
- Palumbo, J.D., Sullivan, R.F. and Kobayashi, D.Y. (2003) Molecular characterization and expression in *Escherichia coli* of three beta-1,3-glucanase genes from *Lysobacter enzymogenes* strain N4-7. *J Bacteriol* **185**, 4362–4370.
- Palumbo, J.D., Yuen, G.Y., Jochum, C.C., Tatum, K. and Kobayashi, D.Y. (2005) Mutagenesis of beta-1,3-glucanase genes in *Lysobacter enzymogenes* strain C3 results in reduced biological control activity toward *Bipolaris* leaf spot of tall fescue and *Pythium* damping-off of sugar beet. *Phytopathology* **95**, 701–707.
- Park, J.H., Kim, R., Aslam, Z., Jeon, C.O. and Chung, Y.R. (2008) *Lysobacter capsici* sp. nov., with antimicrobial

- activity, isolated from the rhizosphere of pepper, and emended description of the genus *Lysobacter*. *Int J Syst Bacteriol* **58**, 387–392.
- Puopolo, G., Giovannini, O. and Pertot, I. (2014) *Lysobacter capsici* AZ78 can be combined with copper to effectively control *Plasmopara viticola* on grapevine. *Microbiol Res* **169**, 633–642.
- Raaijmakers, J.M. and Mazzola, M. (2012) Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Annu Rev Phytopathol* **50**, 403–424.
- Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sergeeva, V., Nair, N., Legendre, L., Darley, E. and Spooner-Hart, R. (2002) The use of fluorochromes to determine the effect of chlorine dioxide on survival of *Plasmopara viticola* on grapevine. *Australas Plant Pathol* **31**, 295–297.
- Sharma, K., Butz, A.F. and Finckh, M.R. (2010) Effects of host and pathogen genotypes on inducibility of resistance in tomato (*Solanum lycopersicum*) to *Phytophthora infestans*. *Plant Pathol* **59**, 1062–1071.
- Stierle, A., Cardellina, J.H. and Strobel, G.A. (1988) Maculosin, a host-specific phytotoxin for spotted knapweed from *Alternaria alternata*. *Proc Natl Acad Sci USA* **85**, 8008–8013.
- Tatsuno, T., Sato, M., Kubota, Y., Kubota, Y. and Tsunoda, H. (1971) Toxicological investigations of metabolic substances of *Fusarium nivale*. *Chem Pharm Bull* **19**, 1498–1500.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Res* **25**, 4876–4882.
- Tilcher, R., Wolf, G.A. and Brendel, G. (1994) Effects of microbial antagonists on leaf infestation, sporangia germination and zoospore behaviour of *Plasmopara viticola* (Berk. & Curtis) Berl. & de Toni. Mededelingen - Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Universiteit Gent 59, 919–929.
- Tilcher, R., Schmidt, C., Lorenz, D. and Wolf, G.A. (2002) About the use of antagonistic bacteria and fungi. In *10th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit-Growing and Viticulture. Proceedings to the Conference from 4th to 7th February* ed. Markus, B., pp. 142–145. Weinsberg/Germany.
- Tran, H., Ficke, A., Asimwe, T., Höfte, M. and Raaijmakers, J.M. (2007) Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*. *New Phytol* **175**, 731–742.
- Trouvelot, S., Varnier, A.L., Allègre, M., Mercier, L., Baillieul, F., Arnould, C., Gianinazzi-Pearson, V., Klarzynski, O. et al. (2008) A β -1,3 glucan sulfate induces resistance in grapevine against *Plasmopara viticola* through priming of defense responses, including HR-like cell death. *Mol Plant Microbe Interact* **21**, 232–243.
- Xie, Y., Wright, S., Shen, Y. and Du, L. (2012) Bioactive natural products from *Lysobacter*. *Nat Prod Rep* **29**, 1277–1287.
- Yan, Z., Reddy, M.S., Ryu, C.-M., McInroy, J.A., Wilson, M. and Kloepper, J.W. (2002) Induced systemic protection against tomato late blight elicited by plant growth-promoting rhizobacteria. *Phytopathology* **92**, 1329–1333.
- Yu, F., Zaleta-Rivera, K., Zhu, X., Huffman, J., Millet, J.C., Harris, S.D., Yuen, G., Li, X.-C. et al. (2007) Structure and biosynthesis of heat-stable antifungal factor (HSAF), a broad-spectrum antimycotic with a novel mode of action. *Antimicrob Agents Chemother* **51**, 64–72.
- Zhang, W., Li, Y., Qian, G., Wang, Y., Chen, H., Li, Y.Z., Liu, F., Shen, Y. et al. (2011) Identification and characterization of the anti-methicillin-resistant *Staphylococcus aureus* WAP-8294A2 biosynthetic gene cluster from *Lysobacter enzymogenes* OH11. *Antimicrob Agents Chemother* **55**, 5581–5589.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Disease incidence (percentage of symptomatic leaves) of *Phytophthora infestans* and *Plasmopara viticola* on tomato and grapevine plants, treated *Lysobacter capsici* AZ78 cells and its culture filtrate (c.f.).

Figure S1 Effect of the four organic fraction of *Lysobacter capsici* AZ78 culture filtrates extracted with ethyl acetate on viability of sporangia of *Phytophthora infestans* (a) and *Plasmopara viticola* (b).

Data S1 Identification of cyclo(L-Pro-L-Tyr).

Figure S2 ^{13}C NMR spectrum of cyclo(L-Pro-L-Tyr) was recorded at 600 MHz in monodeuterated chloroform.

Figure S3 Correlation spectroscopy (COSY) NMR spectrum of cyclo(L-Pro-L-Tyr) was recorded at 600 MHz in monodeuterated chloroform.

Figure S4 Heteronuclear single quantum coherence (HSQC) NMR spectrum of cyclo(L-Pro-L-Tyr) was recorded at 600 MHz in monodeuterated chloroform.

Figure S5 Nuclear overhauser enhancement spectroscopy (NOESY) NMR spectrum of cyclo(L-Pro-L-Tyr) was recorded at 600 MHz in monodeuterated chloroform.

Figure S6 Heteronuclear multiple bond correlation (HMBC) NMR spectrum of cyclo(L-Pro-L-Tyr) was recorded at 600 MHz in monodeuterated chloroform.

Figure S7 Liquid chromatography/high resolution (HR) electrospray ionization mass spectroscopy (ESI MS) spectrum of cyclo(L-Pro-L-Tyr).

Figure S8 Dose effect of cyclo(L-Pro-L-Tyr) on *Phytophthora infestans* disease incidence on detached tomato leaves.