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Lysobacter capsici AZ78 can be combined with copper to effectively control *Plasmopara viticola* on grapevine

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ABSTRACT

The bacterial genus *Lysobacter* represents a still underdeveloped source of biocontrol agents able to protect plants against pathogenic oomycetes. In this work the *L. capsici* strain AZ78 was evaluated with regard to the biological control of *Plasmopara viticola*, the causal agent of grapevine downy mildew. *L. capsici* AZ78 is able to resist copper ions and its resistance to this metal is probably due to the presence of genes coding for copper oxidase (*copA*) and copper exporting P_{IB}-type ATPases (*ctpA*). The presence of both genes was also detected in other members of the *Lysobacter* genus. Resistance to copper allowed *L. capsici* AZ78 to be combined with a low-dose of a copper-based fungicide, leading to more effective control of grapevine downy mildew. Notably, prophylactic application of *L. capsici* AZ78 alone to grapevine leaves reduced downy mildew disease to the same degree as a copper-based fungicide. Furthermore, *L. capsici* AZ78 persists in the phyllosphere of grapevine plants and tolerates environmental stresses such as starvation, freezing, mild heat shock and UV light irradiation. These traits suggest that *L. capsici* AZ78 could be a suitable candidate for developing a new biofungicide to be used in combination with copper to control grapevine downy mildew.

1. Introduction

Downy mildew is one of the most serious diseases of grapevine (*Vitis vinifera*) worldwide. It is caused by the biotrophic oomycete *Plasmopara viticola*, which can attack all green parts of the plant (Gessler et al. 2011). These days, control of downy mildew relies mainly on frequent applications of chemical fungicides in conventional agriculture or copper in organic production (Wong et al. 2001; Gessler et al. 2011). Growing concerns regarding the negative impact of copper in agricultural soils (Wightwick et al. 2008; Komarek et al. 2010) are giving rise to a search for new natural active ingredients against *P. viticola.* Naturally occurring microorganisms with favourable toxicological and ecotoxicological profiles offer a potential solution. Not only organic, but also conventional agriculture may benefit from the use of such low-impact biofungicides.

Although in recent years several bacterial strains have been selected for biological control of plant diseases caused by fungi and oomycetes (Lugtenberg and Kamilova 2009), none of them have reached the market for the control of *P. viticola*. The literature reports a strain of *Erwinia herbicola* showing inhibition of germination of *P. viticola* sporangia *in vitro* (Tilcher et al. 1994) and some bacterial strains belonging to *Erwinia*, *Pseudomonas* and other genera have yielded promising results (Tilcher et al. 2002), but no follow-up studies on these bacteria have been reported, in spite of enormous research efforts dedicated to finding alternatives to the use of copper against *P. viticola*. Some of the weaknesses of microbial fungicides regard their poor survival in the phyllosphere and their incompatibility with copper-based fungicides, so they cannot be integrated within a strategy aiming to reduce copper dosage (Dagostin et al. 2011).

The bacterial genus *Lysobacter* (Christensen and Cook 1978) includes species that may be potentially developed as biofungicides (Hayward et al. 2010). Some *Lysobacter* strains have already been shown to actively protect plants from attack by soil-borne oomycetes. For example *L. enzymogenes* strain 3.1T8 inhibits mycelial growth of *Phytophthora* (*Ph.*) *capsici*, *Pythium* (*Py.*) *ultimum* and *Py. aphanidermatum in vitro.* Production of extracellular proteases, lipases and unidentified biosurfactants and antifungal molecules by *L. enzymogenes* strain 3.1T8 is involved in the control of infections caused by *Py. aphanidermatum* on cucumber plantlets (Folman et al. 2003, 2004). The *Lysobacter* sp. strain SB-K88 synthesises Xanthobaccin A, B and C, macrocyclic lactams, which are highly effective *in vitro* against *Aphanomyces cochlioides, Ph. vignae* f. sp. *adzukicola* and *Py. ultimum* (Nakayama et al. 1996) and in suppressing damping-off of sugar beet caused by *Pythium* spp.

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in soil (Homma et al. 1993; Nakayama et al. 1996). Similarly *L. capsici* YC5194 inhibits the growth of *Py. ultimum* and other phytopathogenic fungi *in vitro* (Park et al. 2008). Another member of this species, the *L. capsici* strain PG4, reduces mycelial growth of several fungi and oomycetes *in vitro* and, when applied to tomato seeds, controls tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Puopolo et al. 2010). Recently a new *L. capsici* strain, named AZ78, isolated from the tobacco plant rhizosphere was characterised (unpublished). Thus, in order to find a new biofungicide which can replace copper, we assessed the efficacy of *L. capsici* AZ78 in controlling *P. viticola* on grapevine.

No studies exist regarding the possibility of combining bacterial strains with copper. For the first time we show that resistance to copper is a trait shared by *Lysobacter* species and associated with the presence of genes coding for copper oxidase (*copA*) and copper exporting P_{IB}-type ATPase (*ctpA*). We have demonstrated that resistance to copper in *L. capsici* AZ78 allows this bacterial strain to be applied with low doses of a copper-based fungicide and this combination led to an increase in the efficacy of grapevine downy mildew control. Since survival of microbial biocontrol agents in the plant phyllosphere is crucial for achieving consistent activity and as very little is known about the persistence of *Lysobacter* members in the plant phyllosphere (Giesler and Yuen 1998; Jochum et al. 2006) at the moment, we evaluated this trait in *L. capsici* AZ78. Furthermore, we also assessed *L. capsici* AZ78 tolerance to starvation, freezing, mild heat shock and UV light irradiation, which are important characteristics that may be helpful for the development of *L. capsici* AZ78 as a biofungicide for the control of grapevine downy mildew.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used in this work, *L. antibioticus* DSM 2044, *L. enzymogenes* DSM 2043, *L. gummosus* DSM 6980, *L. capsici* YC5194 (type strain), *L. capsici* AZ78, *L. capsici* M143 and *L. capsici* PG4, were stored at length in glycerol 40% at −80 °C and routinely grown on Luria Bertani Agar (LBA) in Petri dishes (90 mm diameter). The *L. capsici* AZ78 and M143 strains were respectively isolated from tobacco and tomato plant rhizosphere and identified on the basis of their gene coding for 16S rRNA, according to the procedure described by Puopolo et al. (2010). All the experiments were carried out at 27 °C except when otherwise indicated. In each experiment bacterial cell suspensions were prepared according to the following procedure: after 72 h growth, Petri dishes were flooded with 5 ml of sterile saline solution (0.85% NaCl) and cells were scraped from the medium surface using sterile spatulas. Bacterial cells were then collected in sterile 15 ml tubes. Bacterial cell suspensions were centrifuged (10,000 rpm, 5 min) and pellets were suspended in sterile distilled water to a final optical density at 600 nm ($A_{OD600nm}$) of 0.1 corresponding to 1×10^8 CFU ml^{-1} and used in all experiments, except when otherwise indicated.

2.2. Evaluation of resistance to copper in Lysobacter members

The survival of *L. capisici* AZ78 and the other *Lysobacter* strains reported above on agar medium amended with copper ions (Cu^{2+}) was assessed according to Ritchie and Dittanpongpitch (1991), with some modifications. Briefly, volumes of a filter-sterilized copper sulphate solution ($CuSO_4$, Sigma) were added to LBA then poured into Petri dishes in order to obtain the following final concentrations of $CuSO_4$: 100, 200, 300, 400 and 500 μg ml^{-1}. For each copper concentration, three Petri dishes were spot inoculated with three drops (30 μl) of a *Lysobacter* cell suspension and plates were incubated for 72 h. The development of macrocolonies on the medium reflected the ability of *Lysobacter* strains to tolerate increasingly high copper concentrations (Ritchie and Dittanpongpitch 1991).

To further characterise the level of resistance of *L. capsici* AZ78 to copper, 100 μl of a serial dilution (10^{-1} to 10^{-7}) of the cell suspension were spread onto LBA, amended with copper sulphate at the above mentioned concentrations. CFUs were counted after four days of incubation. Three replicates (Petri dishes) of each combination (dilution and copper concentration) were prepared. The survival ratio (SR) was obtained by dividing the CFUs grown on LBA amended with $CuSO_4$ by the CFUs grown on LBA (Stockwell et al. 2009).

2.3. Detection of genes associated with resistance to copper in Lysobacter members

The genomic DNA of *L. capsici* AZ78, *L. antibioticus* DSM 2044, *L. enzymogenes* DSM 2043, *L. gummosus* DSM 6980, *L. capsici* YC5194, *L. capsici* M143 and *L. capsici* PG4 was extracted with a Genomic DNA isolation Kit (Qiagen) and used as the template in PCR reactions aiming to detect the presence of genes involved in resistance to copper ions. The gene *copA* coding for a copper oxidase was detected following the method developed by Lejon et al. (2007), while the gene *ctpA* coding for copper P_{IB}-type ATPases was detected following the method of De la Iglesia et al. (2010) and Pavissich et al. (2010). The primer pairs copAUF (5′-GGT GCT GAT CAT CGC CTG-3′)/copAUR (5′-GGG CGT CGT TGA TAC CGT-3′), Coprun F2 (5′-GGS ASB TAC TGG TRB CAC-3′)/Coprun R1 (5′-TGN GHC ATC ATS GTR TCR TT-3′) and the same mixture composition and temperature cycles as reported respectively by Pavissich et al. (2010) and Lejon et al. (2007) were used in the PCR reactions. An additional primer pair (LctpA F: 5′-CTG TTG TTC GGT CAG CAC TG-3′/LctpA R: 5′-CGG CGT CCT TGA TCA GAA TG-3′) was employed in PCR reactions for the detection of *ctpA* homologs. In these PCR reactions, two microliters of genomic DNA was used as template in 25 μl reaction including 1X Dream Taq Green PCR Mastermix (Fermentas, Lithuania) and 0.2 μM of primer LctpA F/LctpA R. These PCR reactions 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 the manufacturer's instructions. Once purified, DNA amplicons were sequenced using BigDye Terminator v 3.1 and the resulting nucleotide sequences were analysed using BLASTN to find homologies with DNA sequences already deposited in GenBank.

2.4. Cloning of copA and ctpA genes from Lysobacter capsici AZ78 and phylogenetic analysis

Gene coding for CopA and CtpA were cloned in *L. capsici* AZ78 by using a set of different primer pairs and the PCR conditions reported in Table S1. PCR products were purified using Exo-Sap (Euroclone S.p.a., Italy) according to the manufacturer's instructions and the resulting purified DNA fragments were sequenced using BigDye Terminator v 3.1. Once sequenced, the nucleotide sequences were then assembled by using the software Geneious version 6.1.4 (Biomatters). The assembled sequences were analysed by BLASTN search to find homologies with DNA sequences in GenBank.

Several *copA* and *ctpA* genes belonging to other bacterial strains were collected from GenBank and used for phylogenetic analyses. These 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).

2.5. Evaluation of efficacy of Lysobacter capsici AZ78 in combination with copper for the control of Plasmopara viticola

Evaluation of the ability of *L. capsici* AZ78 to protect grapevine plants against *P. viticola* was carried out on two-year-old *V. vinifera* cv. Pinot Noir grapevine plants, grafted onto Kober 5BB. The plants were grown in a greenhouse under controlled conditions (20 ± 0.5 °C; 70 ± 10% relative humidity, RH) in 2.5 L pots containing a mixture of peat and pumice (3:1) for two months, until the plants had produced two shoots with at least nine leaves each.

P. viticola was isolated from an untreated vineyard in S. Michele all'Adige (Italy) in 2012 and maintained on grapevine plants by subsequent weekly inoculations. To obtain sporangia, plants showing oil spot symptoms were kept overnight in the dark at 20–21 °C and 100% RH. The inoculum of *P. viticola* was prepared by washing the freshly sporulating lesions on the abaxial leaf surface with cold (4–5 °C) distilled water. The sporangia suspension was then adjusted to a concentration of 2.5×10^5 sporangia ml^{-1} by counting with a haemocytometer under a light microscope.

L. capsici AZ78 was combined with a fungicide based on copper hydroxide containing 15% of copper (Kocide® 3000, Du Pont de Nemours, USA) at 1.25 and 0.6125 g L^{-1}. Kocide® 3000 was applied alone as a positive control at 2.5, 1.25 and 0.6125 g L^{-1} corresponding respectively to 375, 187.5 and 93.75 μg of copper ml^{-1}. The untreated control was treated with water. Treatments were applied on adaxial and abaxial leaf surfaces using a hand sprayer. *L. capsici* AZ78 cell suspension was applied 24 and 6 h before inoculation with *P. viticola*. Kocide® 3000 and water were applied six hours before inoculation. Each plant was sprayed with 40 ml of each treatment preparation.

P. viticola inoculum was sprayed onto the abaxial surface of each fully expanded leaf using a hand sprayer. Inoculated plants were subsequently 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.

Disease severity (percentage of abaxial leaf area covered with sporulating lesions) and disease incidence (percentage of leaves with visible sporulation) were evaluated seven days after *P. viticola* inoculation. Disease was assessed based on the EPPO standard scale (2004) and expressed as percentages. Each treatment was carried out on five plants (replicates).

2.6. Lysobacter capsici AZ78 persistence on grapevine leaves

The *L. capsici* AZ78 population on grapevine leaves was assessed one hour before and seven days after *P. viticola* inoculation using the dilution plating method. At these two time points, 5 g leaf sections were collected from the plants in each treatment. They were further cut and put individually into 100 ml bottles containing 45 ml of sterile saline solution and shaken (200 rpm) for 2 h at room temperature. Aliquots of the suspensions were serially diluted and spread onto the surface of LBA amended with kanamycin (25 μg ml^{-1}), since *L. capsici* AZ78, similarly to *L. capsici* PG4, is naturally resistant to this antibiotic (Puopolo et al. 2010). Plates were then incubated for 72 h and colonies resistant to kanamycin with *L. capsici* AZ78 colony morphology were counted to estimate the number of bacterial cells g^{-1} on the grapevine leaves.

Additional greenhouse experiments were carried out to monitor *L. capsici* AZ78 persistence on grapevine leaves at two RH levels. Ten grapevine plants were sprayed with *L. capsici* AZ78 suspension following the procedure described above and maintained at 25 °C. Half of the AZ78-treated plants were kept at 70 ± 10% RH and the other half at 90 ± 10% RH. At 1, 3, 6, 8 and 10 days after application, *L. capsici* AZ78 persistence on grapevine leaves was assessed using the dilution plating method described above.

2.7. Biofilm production

L. capsici AZ78 was evaluated for its ability to form biofilm on polystyrene microtitre plates using a modified version of the procedure described by Maddula et al. (2006). A volume of 1.5 μl of *L. capsici* AZ78 cell suspension (1×10^7 CFU ml^{-1}) was inoculated into 150 μl per well of three liquid media, LB, King's B (KB) and Nutrient Broth (NB), in 96-well polystyrene plates. Plates were incubated for 60 h without shaking and final cell densities were determined ($A_{OD600nm}$). Unattached cells were removed by inverting the plate and tapping it onto absorbent paper. The remaining adherent bacterial cells were fixed to the plates for 20 min at 50 °C and then stained for 1 min with 150 μl per well of crystal violet solution (0.1% in sterile distilled water). Excess stain was removed by inverting the plate, then washing twice with distilled water (each wash 250 μl per well). Adherent cells were decolorized with an acetone/ethanol (20%/80%) solution (200 μl per well) for 5 min to release the dye into the solution. A volume of 100 μl was transferred from each well to another 96-well plate and the amount of dye (proportional to the density of adherent cells) was quantified ($A_{OD540nm}$). A 96-well polystyrene plate was used for each time point in the time-course experiment. Twenty wells were filled with each of the tested growth media in each microtitre plate. *L. capsici* AZ78 cells were simultaneously inoculated into half of the wells containing the growth media, while the other half was not inoculated (negative control). Cell density and biofilm formation were determined at time zero and at 12 h intervals until 60 h after inoculation (six time points). $A_{OD540nm}$ values (adherent cells) were divided by $A_{OD600nm}$ values (bacterial growth) in order to obtain the specific biofilm formation value (SBF).

2.8. Lysobacter capsici AZ78 tolerance to environmental stresses

Stress response experiments were carried out on suspensions of *L. capsici* AZ78 according to Stockwell and Loper (2005) and Stockwell et al. (2009) with some modifications. Briefly, tolerance to starvation stress was assessed by inoculating *L. capsici* AZ78 into 15 ml sterile tubes containing 5 ml of sterile potassium phosphate buffer (1 mM, pH 7) and 0.8% NaCl to obtain the final concentration of 1×10^8 CFU ml^{-1}. Inoculated tubes were maintained at 27 °C for 15 days on a rotary shaker at 200 rpm. *L. capsici* AZ78 cell density was assessed using the dilution plating method at 0, 3, 6, 9, 12 and 15 days post-inoculation. SR was calculated by dividing the population reached at days 3, 6, 9, 12 and 15 by the population registered at the beginning of the experiment (0 days). Three tubes (replicates) were inoculated at each time point.

Tolerance to mild heat shock was measured by incubating sterile 1.5 ml microfuge tubes containing 100 μl of *L. capsici* AZ78 cell suspension for 20 min at the following temperatures: 30, 33, 36, 39 and 42 °C. After this period of time, a volume of 900 μl of sterile potassium phosphate buffer (1 mM, pH 7) was added and the suspension was mixed in a vortex for 30 s prior to dilution plating. SR was calculated by dividing the *L. capsici* AZ78 population after exposure to each temperature by the population after exposure to 27 °C. Three microfuge tubes (replicates) were used for each temperature.

Tolerance to freezing was assessed by transferring 100 μl aliquots of *L. capsici* AZ78 cell suspension into sterile 1.5 ml microfuge tubes that were maintained at −20 °C for 24 h. The viability of *L. capsici* AZ78 cells was assessed at 6, 12, 18 and 24 h. At these time points, a volume of sterile 900 μl of 10 mM phosphate buffer (pH 7) was added to each tube immediately after being removed from the freezer. Samples were gently mixed by pipetting and serially diluted in sterile saline solution (10^{-1} to 10^{-7}). Volumes of 100 μl of these dilutions were spread onto LBA and enumerable colonies were counted after four days of incubation. Three microfuge tubes (replicates) were used for each time point. SR was calculated by dividing the population after exposure to −20 °C by the population which was not exposed to freezing.

In order to evaluate tolerance to ultraviolet irradiation, dilutions from 10^{-1} to $10^{-7}$of *L. capsici* AZ78 cell suspension were spread onto LBA and immediately exposed to UV irradiation (λ 254 nm) at the following doses: 20, 40, 60, 80 and 100 J m^{-2}. Following exposure, plates were incubated in the dark for four days, after which colony forming units were counted. Three replicates (Petri dishes) were used for each dilution. SR was calculated by dividing the population after exposure to UV irradiation by the population of *L. capsici* AZ78 cells which were not exposed to UV.

2.9. Statistical analysis

Each experiment was carried out twice, except the efficacy trial in the greenhouse, which was carried out three times. As the experiment factor was not significant, the data of the repeated experiments were pooled and analysed by ANOVA using Statistica 7.1 (StatSoft, Tulsa, OK, USA) and means were compared with Tukey's test ($\alpha = 0.01$). SR values and disease incidence and severity were $\log_{10}$ and arcsin transformed beforehand.

3. Results

3.1. Evaluation of resistance to copper of Lysobacter capsici AZ78 and detection and cloning of genes associated with resistance to copper

Phylogenetic relations among the *Lysobacter* strains used in this work according to analysis of their 16S rRNA genes are reported in Fig. S1. During this work, these bacterial strains were evaluated for the resistance to copper. When spotted onto LBA amended with this metal at concentrations ranging from 100 to 500 μg ml^{-1} both *L. capsici* AZ78 and all the remaining *Lysobacter* strains developed macrocolonies. Amendment of the LBA with copper sulphate up to 300 μg ml^{-1} did not affect the survival of *L. capsici* AZ78. A slight decrease in SR was noticed at 400 μg ml^{-1} of copper (log value -0.42 ± 0.16), while a tenfold reduction was present at a concentration of 500 μg ml^{-1} (Fig. 1). *L. capsici* AZ78 released a brown pigment into the medium when grown on LBA amended with $CuSO_4$ at 400 and 500 μg ml^{-1} concentrations. The release of a brown pigment was also observed in the case of *L. capsici* M143, but not for the other *Lysobacter* strains evaluated (Fig. 2).

A nucleotide region of ≈1000 bp was amplified by using the primer pair specific for *copA* (CopRun F2/R1) gene in all the *Lysobacter* strains, the resulting nucleotide sequences showed the highest sequence identity with the gene coding for a copper oxidase from the complete genome of the bacterial strains *Pseudoxanthomonas suwonensis* 11-1, *Stenotrophomonas malthophilia* D457 and JV3. The partial sequences of *copA* gene from *Lysobacter* members tested in this work formed two clusters, one containing sequences of *L. antibioticus* and *L. capsici* strains and the other containing the sequences from *L. enzymogenes* and *L. gummosus* strains (Fig. S2A).

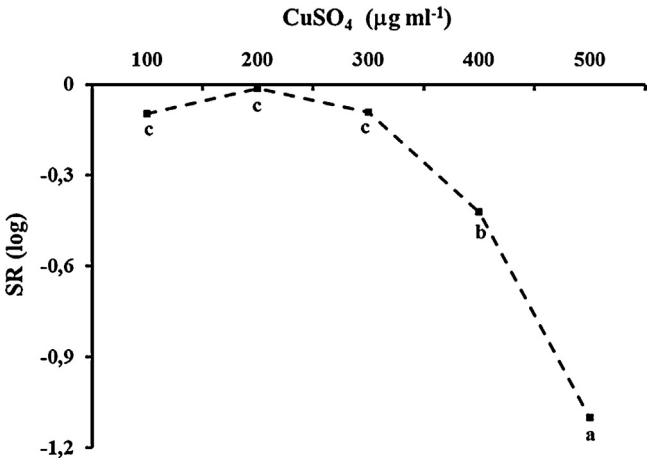


Fig. 1. *Lysobacter capsici* AZ78 resistance to copper ions at different concentrations. AZ78 resistance to copper ions is expressed as the logarithmic value of the survival ratio (SR log). Survival ratio (SR) was calculated as the ratio of the AZ78 CFU developed on LBA amended with copper sulphate at different concentrations to the AZ78 CFU developed on LBA. Points with the same letters do not differ significantly according to Tukey's test ($\alpha = 0.01$).

PCR reactions using primer pair CopAUF/R to detect the DNA region associated with the copper P_{IB}-type ATPase resulted in amplification of an 726 bp amplicon in *L. capsici* AZ78 only, which showed the highest sequence identity value with the *ctpA* gene from the complete genome of *P. suwonensis* strain 11-1. The *ctpA* gene from *P. suwonensins* strain 11-1 was thus used to design another primer pair (LctpAF/R) in order to detect the presence of the *ctpA* gene in all *Lysobacter* strains. The use of this new primer pair allowed amplification of an 840 bp region of the *ctpA* gene in all the *Lysobacter* strains used in this work. On the basis of BlastN analyses, the amplicons showed the highest sequence identity with the gene coding for a copper P_{IB}-type ATPase from bacterial strains *Burkholderia gladioli* BSR3, *Methylibium petroleiphylum* PM1 and *P. suwonensis* strain 11-1. All *ctpA* sequences originated from *Lysobacter* strains were located in a single cluster with the exclusive exception of the *ctpA* sequence of *L. antibioticus* DSM 2044, which clustered with *B. vietnamiensis* G4 (Fig. S2B).

On the basis of sequence identy, new primer pairs were designed in order to obtain the complete nucleotide sequence of *copA* and *ctpA* from *L. capsici* AZ78. Once determined the complete nucleotide sequences, phylogenetic analysis of these two genes indicated that the *copA* sequence of *L. capsici* AZ78 clustered with gene homologs from *Azotobacter vinelandi* strain CA6, *Pseudomonas fluorescens* strain PfO-1 and *P. stutzeri* strain CCUG 29243, bacterial species that are phylogenetically distant from the genus *Lysobacter* (Fig. 3A). Similarly, the *ctpA* gene sequence from *L. capsici* AZ78 was clustering with gene homologs from the *B. cenocepacia* strain J2315 and the *B. gladioli* strain ABSR3 and was distant from *ctpA* homologs of closely related bacterial genera, such as *Pseudoxanthomonas*, *Stenotrophomonas* and *Xanthomonas* (Fig. 3B).

3.2. Evaluation of the efficacy of Lysobacter capsici AZ78 in combination with copper for the control of Plasmopara viticola on grapevine

In the light of the results obtained *in vitro*, the efficacy of *L. capsici* AZ78 in controlling *P. viticola* was evaluated alone and in combination with a low dose of a copper-based fungicide (copper hydroxide), under controlled conditions (greenhouse). All the concentrations of copper hydroxide and *L. capsici* AZ78 reduced incidence of downy mildew as compared to the untreated control. Incidence ranged from $63 \pm 16\%$ to $73 \pm 11\%$ (average ± standard

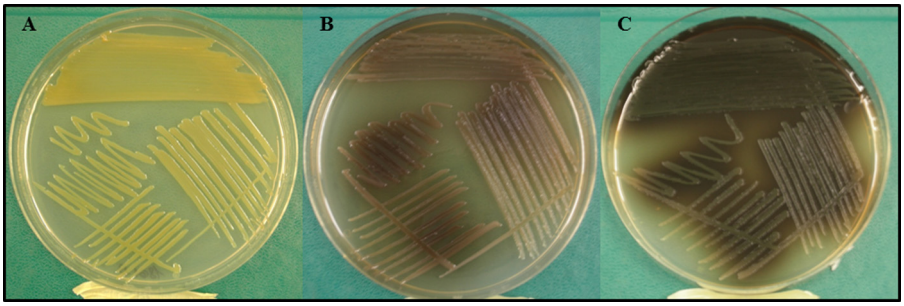


Fig. 2. Release of a brown pigment in LBA growth medium amended with $CuSO_4$ 400 μg ml^{-1}. (A) *Lysobacter capsici* YC5194; (B) *Lysobacter capsici* AZ78; (C) *Lysobacter capsici* M143.

deviation) in copper treatments, and was 63 ± 9% and 100 ± 3% in *L. capsici* AZ78 treated plants and the untreated control respectively. The combination of *L. capsici* AZ78 with copper further reduced the incidence of the disease (30 ± 14% and 39 ± 13% with the addition of 1.25 and 0.6250 g L^{-1}), indicating an additive effect (Table 1). *L. capsici* AZ78 strongly reduced the severity of downy mildew on leaves, similarly to copper at all the tested dosages. In particular when plants were treated with *L. capsici* AZ78 severity was 5%, whereas it was 67% in the untreated control (Table 1). The combination of *L. capsici* AZ78 with copper did not further reduce severity, probably because it was already very low.

3.3. Survival of Lysobacter capsici AZ78 cells in the grapevine phyllosphere

At neither of the two time points (1 h before and 7 days after pathogen inoculation) *L. capsici* AZ78 cells were isolated from untreated control plants or treated with the different copper doses alone, but they were recovered from leaves of plants treated with the bacterium. The bacterial cell population recovered from leaves collected one hour before *P. viticola* inoculation was 5.07 ± 0.16 $\log_{10}$ CFU g^{-1} of leaf, while at the end of trial the AZ78 cell population reached 5.22 ± 0.02 $\log_{10}$ CFU g^{-1} of leaf. In plants treated with a combination of *L. capsici* AZ78 and copper, a reduction of one order of magnitude in the bacterium population size was recorded: at the end of the experiments the population was 4.36 ± 0.12 and at 4.28 ± 0.14 $\log_{10}$ CFU g^{-1} of leaf on plants treated with copper at doses of 1.25 and at 0.6125 g L^{-1} respectively.

In the experiments assessing *L. capsici* AZ78 persistence on grapevine leaves over ten days, *L. capsici* AZ78 was recovered one day after its application at 5.41 ± 0.07 and 6.57 ± 0.50 $\log_{10}$ CFU g^{-1} of leaf for plants kept at normal (60–80%) and high (80–99%) relative humidity respectively (Fig. 4). AZ78 persisted at a constant rate for 10 days on plants exposed to high humidity (6.47 ± 0.16 $\log_{10}$ CFU g^{-1} of leaf after ten days) while at normal humidity the AZ78 population was constant until the 6th day post-inoculation and then decreased to 2.39 ± 0.04 $\log_{10}$ CFU g^{-1} of leaf after 8 days; AZ78 was still present after ten days, but at a low concentration (2.24 ± 0.24 $\log_{10}$ CFU g^{-1} of leaf).

Table 1
Biocontrol of *Plasmopara viticola* through prophylactic application of *Lysobacter capsici* AZ78 on grapevine leaves. Disease incidence is expressed as the percentage of symptomatic leaves while disease severity is expressed as the percentage of leaf area covered with sporulating lesions.

Treatments	Disease incidence[b] (%)	Disease severity[b] (%)
Untreated[a]	100 ± 3 a	67 ± 27 a
Kocide® 3000 (2.5 g L^{-1})	63 ± 16 b	3 ± 2 b
Kocide® 3000 (1.25 g L^{-1})	66 ± 10 b	8 ± 22 b
Kocide® 3000 (0.6125 g L^{-1})	73 ± 11 b	5 ± 5 b
L. capsici AZ78 + Kocide® 3000 (1.25 g L^{-1})	30 ± 14 c	2 ± 2 b
L. capsici AZ78 + Kocide® 3000 (0.6125 g L^{-1})	39 ± 13 c	1 ± 1 b
L. capsici AZ78	63 ± 9 b	5 ± 5 b

[a] Untreated: plants treated with water only.
[b] Mean values ± standard deviations are reported for each treatment. The same letters indicate values which do not differ significantly according to Tukey's test (α = 0.01). Data originating from three independent experiments were pooled.

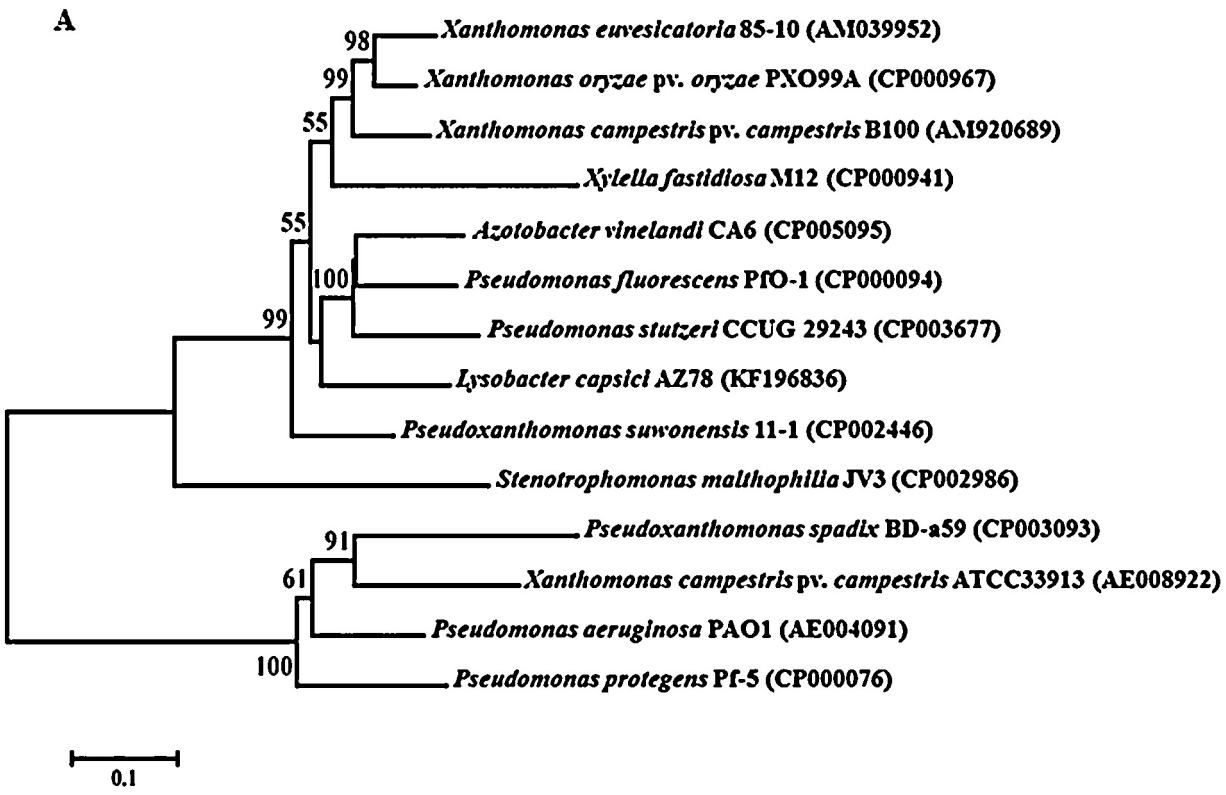
3.4. Assessment of the ability of Lysobacter capsici AZ78 to form biofilm and resist environmental stress

The ability of *L. capsici* AZ78 to form biofilm on inert surfaces was investigated in this work. *L. capsici* AZ78 grew differently in the three media used in the biofilm production assay and KB was found to be the medium sustaining the highest cell production (Fig. 5A). Bacterial growth in the other two liquid media (LB and NB), was almost identical and after 60 h *L. capsici* AZ78 reached similar $A_{OD600nm}$ values (Fig. 5A). Nonetheless, at this time point the SBF value registered in NB was higher than the quantity reached in LB (Fig. 4B). Biofilm was not produced in LB until 48 h and it attained its highest value (SBF = 2.62 ± 0.04) after 60 h; *L. capsici* AZ78 started to form biofilm between 24 and 36 h in NB (Fig. 5B); the highest SBF value in KB was reached at 12 h and thereafter decreased (Fig. 5B).

We also wished to investigate whether the ability of *L. capsici* AZ78 to persist on grapevine leaves was associated with resistance to different abiotic stresses, so a series of experiments were carried out *in vitro*. In the experiments aimed at assessing resistance to starvation, when *L. capsici* AZ78 was incubated in phosphate buffer and the concentration was monitored over fifteen days, its viability slowly decreased and by the end of the experiments (15th day) the total reduction was 0.70 ± 0.13 $\log_{10}$ (Fig. S3A). When mild heat shock was induced by exposing *L. capsici* AZ78 cell suspension to temperatures ranging from 30 to 42 °C for twenty minutes, exposure to 30 and 33 °C did not result in any loss of cell viability, whereas exposure to 36, 39 and 42 °C reduced viability by $\log_{10}$ −0.19 ± 0.06, −0.12 ± 0.08 and −0.35 ± 0.18 respectively (Fig. S3B). Incubation at −20 °C slightly decreased the viability of *L. capsici* AZ78 cells, although by less than one order of magnitude at all the time points in this experiment, and the differences were not significant (Fig. 6A). With respect to *L. capsici* AZ78 tolerance to UV light irradiation, the survival ratio drastically decreased when the strain was exposed to 60, 80 and 100 J m^{-2}, the reduction being almost six-tenfold, while exposure to 20 and 40 J m^{-2} reduced viability by $\log_{10}$ −1 and $\log_{10}$ −3 respectively (Fig. 6B).

4. Discussion

Identification of new microorganisms that can effectively control *P. viticola* may play an important role in the development of biofungicides helping to reduce the use of copper against downy mildew on organic grapevines. Previous studies have shown that other *Lysobacter* strains can control soil-borne plant



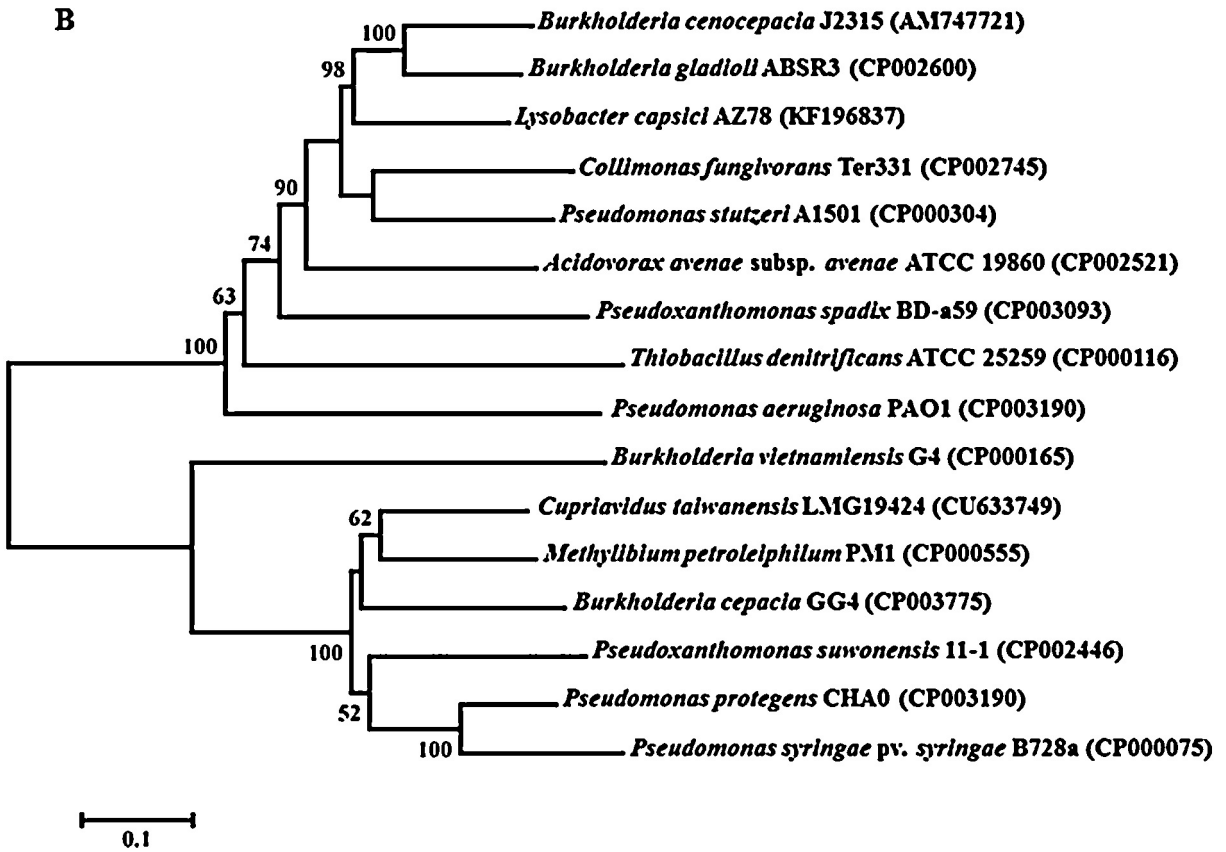


Fig. 3. Phylogenetic trees deriving from analysis of the *copA* (A) and *ctpA* (B) gene from *Lysobacter capsici* AZ78. The trees were obtained using the neighbour-joining method and evolutionary distances were calculated using Kimura two parameter model (Kimura 1983). Bootstrap values (Felsenstein 1985) higher than 50 are shown at the branch points.

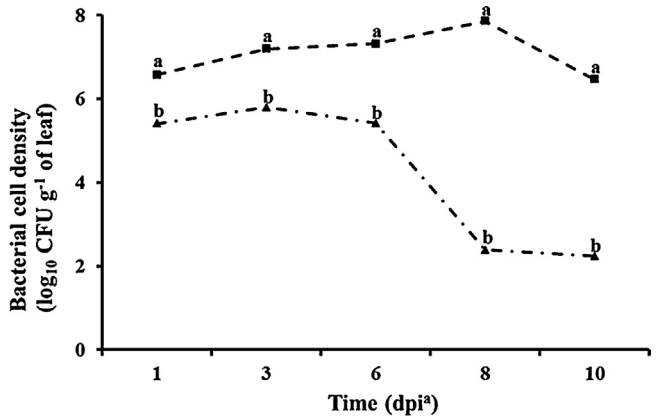


Fig. 4. *Lysobacter capsici* AZ78 persistence on grapevine leaves. The line with triangles represents persistence on plants maintained at 25 °C with 70 ± 10% RH; the line with squares represents persistence on plants maintained at 25 °C with 90 ± 10% RH. Bacterial cell density is expressed as $\log_{10}$ CFU g^{-1} of leaf. Points with the same letters do not differ significantly according to Tukey's test ($\alpha = 0.01$). [a] dpi = days post inoculation.

pathogenic oomycetes such as *A. choclioides* and *Py. aphanidermatum* (Nakayama et al. 1996; Folman et al. 2001, 2003, 2004; Islam et al. 2005). However to the best of our knowledge, this is the first evidence of the biological control of grapevine downy mildew achieved through the application of a member of genus *Lysobacter*. Moreover, *L. capsici* AZ78 is one of the few bacterial strains identified so far showing efficacy against this disease (Tilcher et al. 1994, 2002). Interestingly, application of *L. capsici* AZ78 cells to grapevine leaves resulted in a reduction of *P. viticola* incidence and severity comparable to the application of a copper-based fungicide. In order

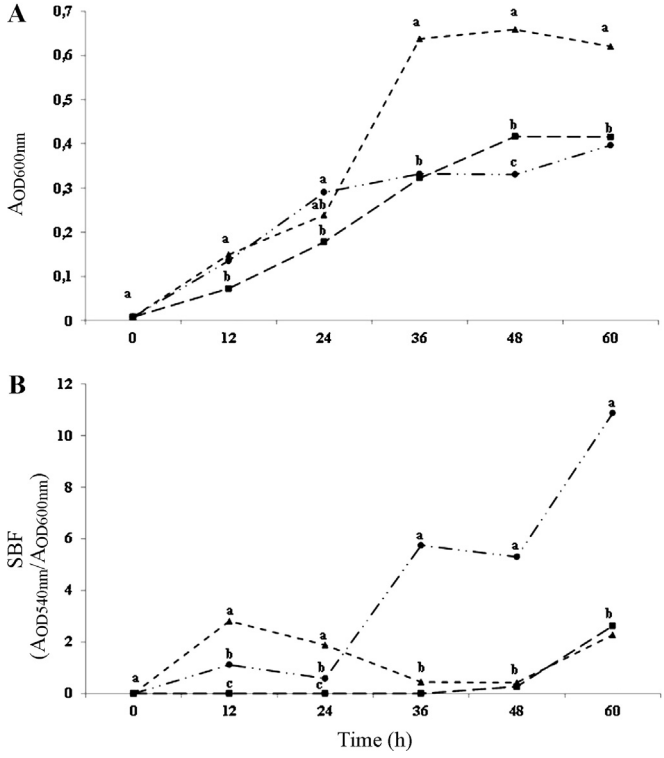


Fig. 5. Ability of *Lysobacter capsici* AZ78 to produce biofilm in different growth media. (A) Bacterial cell density was monitored by scoring the $A_{OD600nm}$ value every twelve hours. (B) Specific Biofilm Formation (SBF) was calculated as the ratio of adherent cells ($A_{OD540nm}$ value) to bacterial cell density ($A_{OD600nm}$ value). The liquid media were: KB (triangles), LB (squares) and NB (circles). Points with the same letters do not differ significantly according to Tukey's test ($\alpha = 0.01$).

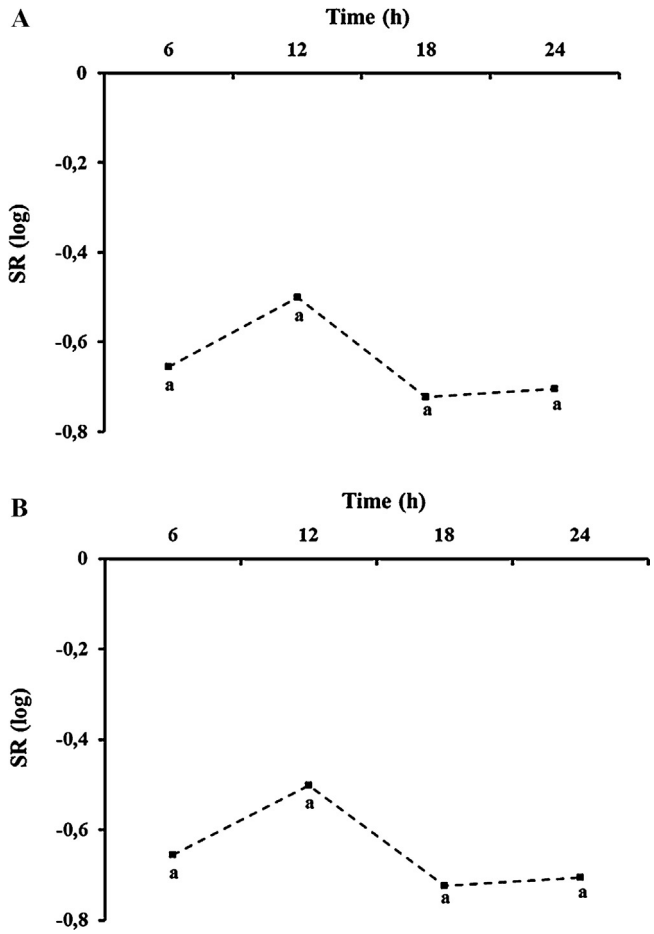


Fig. 6. *Lysobacter capsici* AZ78 tolerance to abiotic stress. (A) Tolerance to 24 h exposure to −20 °C. (B) Survival of AZ78 cells exposed to increasing UV light irradiation. The ability of AZ78 to tolerate abiotic stress is expressed as the logarithmic value of the survival ratio (SR log). Survival ratio (SR) was calculated as the ratio of AZ78 treated cells to AZ78 untreated cells. Points with the same letters do not differ significantly according to Tukey's test ($\alpha = 0.01$).

to develop a commercial biofungicide future research should confirm the high level of efficacy attained under controlled conditions and identify the mechanism of action of *L. capsici* AZ78 involved in controlling grapevine downy mildew.

However, building on this knowledge, our study provides the first evidence of copper-resistance in members of the *Lysobacter* genus. This biological trait is probably associated with the presence of nucleotide regions having high sequence homology with genes coding for copper oxidase (*copA*) and copper P_{IB}-type ATPase (*ctpA*). The latter protein belongs to the heavy metal transporter ATPases, ubiquitous membrane proteins deputed to the efflux of copper ions in various microorganisms (Fu et al. 1995; Ge et al. 1995; Petersen and Møller 2000; Arguello et al. 2007), while copper oxidase is deputed to the oxidation of copper ions and this activity protects periplasmic enzymes from copper-induced damage (Solomon et al. 1996; Grass and Rensing 2001). *Escherichia coli* strains sharing the multi-copper oxidase gene (*pcoA*) on the plasmid pRJ1004 produced brown colonies when grown on growth media containing $CuSO_4$ (Tetaz and Luke 1983). Similarly, *L. capsici* AZ78 and M143 released a brown pigment on LBA plates amended with $CuSO_4$ 400 μg ml^{-1} although this behaviour was not observed in the strain *L. capsici* YC5194.

It is worth noting that *copA* and *ctpA* genes from *L. capsici* AZ78 clustered with the genes of bacterial strains belonging to species that are phylogenetically distant from the genus *Lysobacter* (*i.e. P.*

fluorescens). Both *copA* and *ctpA* could be located on the bacterial chromosome or on plasmids, as in the case of *copABCD* operon in *P. syringae*, contained in the plasmid pPT23D (Cha and Cooksey 1991). From an ecological point of view, it would be of great interest to assess whether *copA* and *ctpA* in *L. capsici* AZ78 are located on the chromosome or on plasmids, in order to assess whether horizontal gene transfer events have occurred between a *Lysobacter* member and other bacterial species.

On the other hand and from a practical point of view, resistance to copper is a highly desirable trait, because copper is routinely applied in the control of *P. viticola* in organic agriculture, and the existence of a biofungicide which tolerates copper ions opens up the possibility of combining it with low doses of copper and gradually reducing its use (Dagostin et al. 2011). We found that concurrent application with *L. capsici* AZ78 was more effective as compared to the application of *L. capsici* AZ78 or copper alone. Moreover, the application of copper to *L. capsici* AZ78-treated plants reduced the bacterial population by only one order of magnitude, showing that the decrease in cell viability observed *in vitro* is partially conserved *in planta*.

The *L. capsici* AZ78 populations on grapevine leaves under controlled conditions were similar at the beginning and at the end of the experiments. In concurrent experiments aimed at monitoring the persistence of *L. capsici* AZ78 cells on grapevine leaves, we showed that the *L. capsici* AZ78 population remained constantly high until six days after application on plants maintained at 25 °C with a 60–80% RH. However, there were some discrepancies between the degree of persistence measured in the greenhouse efficacy trial and that measured in the experiments specifically designed to assess *L. capsici* AZ78 persistence. In the latter experiments, the *L. capsici* AZ78 population decreased over 8 days from 10^5 to 10^2 cells per gram of leaf, while after the same period 10^5 cells per gram of leaf were recovered from plants in the greenhouse efficacy trials. This discrepancy was probably due to the different conditions the bacterium encountered during the two experiments. While humidity remained constant over the ten days of the persistence experiments, during the biocontrol experiments relative humidity was increased twice, upon infection and upon sporulation of *P. viticola*. This hypothesis is confirmed by the constant persistence of *L. capsici* AZ78 on plants maintained at high HR for the entire duration of the persistence experiments. Humidity is known to influence bacterial growth (Leben 1988; Wilson et al. 1999; Cooley et al. 2003), so high humidity contributes towards sustaining a large *L. capsici* AZ78 population on leaves.

It is also worth noting that *L. capsici* AZ78 survives well in the grapevine phyllosphere, although it was isolated from the rhizosphere of tobacco plants. At the moment, most of the *Lysobacter* strains evaluated for biological control of plant diseases have been isolated from the soil or rhizosphere of cultivated plants, with the single exception of *L. enzymogenes* C3, which was obtained from the phylloplane of Kentucky bluegrass (Giesler and Yuen 1998). Interestingly, only this strain has been evaluated for biocontrol of plant pathogens attacking parts of the plant that grow above ground (Kilic-Ekici and Yuen 2003; Kobayashi and Yuen 2005; Jochum et al. 2006), while most of the *Lysobacter* strains have been evaluated for biocontrol of soil-borne pathogenic fungi and oomycetes (Nakayama et al. 1996; Rondon et al. 1999; Folman et al. 2003; Postma et al. 2008; Puopolo et al. 2010). The results of our study suggest that soil-borne *Lysobacter* species might also be assessed, at least under controlled conditions, for biological control of pathogenic microorganisms attacking the aerial parts of the plants.

Little is known about biofilm formation by *Lysobacter* strains. Islam et al. (2005) showed that strain SB-K88 forms dense microcolonies on the rhizoplane of sugar beet plantlets grown from seeds coated with this bacterium and they also reported that SB-K88 adheres to the plant surface by forming fimbriae. Since it is well documented that bacteria survive on plant surfaces by forming large aggregates indicated as biofilm (Morris et al. 1997; Dulla and Lindow 2008) and because of the high persistence of *L. capsici* AZ78 on the leaf surface, we investigated its biofilm ability. The ability of *L. capsici* AZ78 to form biofilm was tested on inert surfaces and our results showed for the first time that a *L. capsici* strain can do this, at least *in vitro*. However, this ability depended on the composition of the growth medium, as shown by the fact that the KB medium sustained development of the greatest quantity of bacterial cells, without however leading to the formation of biofilm.

Since KB contains low amounts of available iron ions (King et al. 1954), it is reasonable to assume that this metal affects the formation of biofilm by *L. capsici* AZ78, as is reported in other Gram-negative bacteria such as *Acinetobacter baumannii* and *P. aeruginosa*, where biofilm formation is highly influenced by iron source, concentration and bioavailability (Tomaras et al. 2003; Banin et al. 2005). Moreover, it has previously been shown that the other *L. capsici* member, strain PG4, is unable to produce siderophores and that supplementing the KB medium with $FeCl_3$ enhances its antibacterial properties (Puopolo et al. 2010). On the basis of these data, we surmise that iron availability may play an important role in terms of persistence in the environment and the biocontrol potential of *L. capsici* members.

When bacteria form a biofilm they become more resistant to various environmental factors that affect their persistence (Ophir and Gutnick 1994; Perrot et al. 1998; Elasri and Miller 1999). The most frequently investigated limiting environmental factors for bacterial persistence in the phyllosphere are starvation, temperature and exposure to UV light irradiation (Wilson et al. 1999; Stockwell et al. 2009). Since little information is available regarding the ability of *Lysobacter* species to survive following exposure to these factors, we investigated how AZ78 responds to them.

The viability of *L. capsici* AZ78 cells was not negatively affected by stress caused by lack of nutrients (starvation) nor did they suffer following exposure to increasing temperatures (mild heat shock). Interestingly, *L. capsici* AZ78 could tolerate exposure to UV irradiation and freezing temperatures (−20 °C). The survival ratio of *L. capsici* AZ78 cells after exposure to these environmental stresses was comparable with that of the epiphytic strain *P. fluorescens* 122 and the soil-borne strain *P. fluorescens* Pf5, as reported by Stockwell et al. (2009).

L. capsici AZ78 cell viability was not negatively affected by the lowest UV irradiation dose tested in this study (20 J m^{-2}). Sundin and Jacobs (1999) reported that the minimum inhibitory dose for the UV-sensitive strain *P. aeruginosa* PAO1 was 5 J m^{-2}, from which we can conclude that the sensitivity threshold of strain *L. capsici* AZ78 is higher than that of a sensitive bacterial strain. Recently, Wang et al. (2013) have shown that *L. enzymogenes* strain OH11 synthesises a yellow pigment that shows similarity with xanthomonadin produced by Xanthomonadaceae members. These xanthomonadin-like aryl polyene metabolites have been shown to be involved in the protection of OH11 cells against UV irradiation and H_2O_2 (Wang et al. 2013), thus showing for the first time that members of the *Lysobacter* genus have evolved molecular mechanisms associated with resistance to these factors.

The results presented here explain some aspects of the biology and ecology of *L. capsici* AZ78. We show for the first time that resistance to copper associated with the presence of genes coding for CopA and CtpA is a common biological trait in four *Lysobacter* species and that at least one member of this bacterial genus, *L. capsici* AZ78, can tolerate abiotic stresses such as starvation, cold temperature, mild heat shock and UV irradiation. All these characteristics make *L. capsici* AZ78 a suitable candidate for developing new sustainable strategies for controlling downy mildew in the grapevine, since it is resistant to copper and can establish

itself in the grapevine phyllosphere, can tolerate environmental stress and, most importantly, drastically reduces the severity of downy mildew. The next steps will be the development of a suitable formulation for *L. capsici* AZ78 in order to evaluate it in open field conditions and to assess its efficacy against downy mildew in vineyards, in combination or alternation with low doses of copper-based fungicides.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2013.09.013.

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