OXFORD
FEMS JOURNALS investing in science

FEMS Microbiology Letters, 363, 2016, fnv243

doi: 10.1093/femsle/fnv243
Advance Access Publication Date: 21 December 2015
Research Letter

RESEARCH LETTER – Environmental Microbiology

Monitoring *Lysobacter capsici* AZ78 using strain specific qPCR reveals the importance of the formulation for its survival in vineyards

Guillem Segarra, Gerardo Puopolo*, Elena Porcel-Rodríguez, Oscar Giovannini and Ilaria Pertot

Department of Sustainable Agro-Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via Edmund Mach 1, 38010 San Michele all'Adige, Trento, Italy

***Corresponding author:** Department of Sustainable Agro-Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach (FEM), 38010 San Michele all'Adige (TN), Italy. Tel: +39-0461-615502; Fax: +39-0461-615500; E-mail: gerardo.puopolo@fmach.it
One sentence summary: Improving *Lysobacter capsici* survival in the phyllosphere.
Editor: Paolina Garbeva

ABSTRACT

Survival in the phyllosphere is a critical feature for biofungicides based on non-spore forming bacteria. Moreover, knowledge of their persistence on plants is important to design effective formulations and application techniques. With this scope, the aim of this work was to develop a specific method to monitor the fate in the environment of *Lysobacter capsici* AZ78, a biocontrol agent of *Plasmopara viticola,* and to evaluate the contribution of formulation in its persistence on grapevine leaves. A strain-specific primer pair derived from REP-PCR fingerprinting was used in quantitative PCR experiments to track the evolution of *L. capsici* AZ78 population in vineyards. The population reached between 5 and 6 $\log_{10}$ cells gram of leaf^{-1} after application and decreased by more than 100 times in one week. Multiple regression analysis showed that unfavourable temperature was the main environmental factor correlating with the decrease of *L. capsici* AZ78 persistence on grapevine leaves. Importantly, the use of formulation additives protected *L. capsici* AZ78 against environmental factors and improved its persistence on the leaves by more than 10 times compared to nude cells. Formulation and the knowledge about the persistence of *L. capsici* AZ78 in vineyards will be useful to develop commercial biofungicides for foliar application.

Keywords: quantitative PCR; *Lysobacter capsici* AZ78; grapevine; REP-PCR; leaf colonization; formulation

INTRODUCTION

Public concerns about the side effects of synthetic chemical pesticides on health and the environment are pushing modern agriculture towards the use of alternative plant protection products with environmentally friendly profiles, such as biopesticides based on microbial biocontrol agents (mBCA; Cook 1993; Fravel 2005). The bacterial strain *Lysobacter capsici* AZ78 (AZ78) is a non-spore forming Gram-negative bacterium able to control grapevine downy mildew (Puopolo, Giovannini and Pertot 2014) caused by *Plasmopara viticola,* one of the most important phytopathogenic oomycetes worldwide (Gessler, Pertot and Perazzolli 2011; Kamoun *et al.* 2014). Members of the *L. capsici* species are typically soil inhabitants (Park *et al.* 2008; Postma, Schilder and van Hoof 2011), and when applied to the phyllosphere they can be easily killed by detrimental environmental factors such as UV radiation and desiccation (Lindow and Brandl 2003). Formulation by adding protective compounds can enhance the persistence of the mBCA in the target environment (Fravel,

Received: 7 October 2015; **Accepted:** 16 December 2015
© FEMS 2015. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from http://femsle.oxfordjournals.org/ by guest on January 14, 2016

Table 1. Bacterial strains used to develop specific primers for *L. capsici* strain AZ78.

Species	Strain	Origin	Reference
L. capsici	AZ78	Tobacco rhizophere	(Puopolo *et al.* 2014)
L. capsici	M143	Tomato rhizophere	(Puopolo *et al.* 2014)
L. capsici	DSM 19286^T	Pepper rhizosphere	DSMZ
L. capsici	10.4.5	Clay soil (grass crop)	(Postma *et al.* 2011)
L. capsici	1.3.3	Clay soil (grass-clover crop)	(Postma *et al.* 2011)
L. capsici	55	Clay soil (cauliflower crop)	(Postma *et al.* 2011)
L. capsici	6.2.3	Clay soil (grass crop)	(Postma *et al.* 2011)
L. enzymogenes	DSM 2043^T	Soil	DSMZ
L. antibioticus	DSM 2044^T	Soil	DSMZ
L. gummosus	DSM 6980^T	Soil	DSMZ
L. brunescens	DSM 6979^T	Lake water	DSMZ
L. ximonensis	DSM 23410^T	Soil	DSMZ
L. daejeonensis	DSM 17634^T	Greenhouse soil	DSMZ
L. oryzae	DSM 21044^T	Rhizosphere of rice	DSMZ
Stenotrophomonas maltophilia	DSM 50170^T	Oropharyngeal region	DSMZ
Xanthomonas campestris	DSM 3586^T	*Brassica oleracea*	DSMZ

^{T}Type strain; DSMZ, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures.

Connick and Lewis 1998). Recently, a first prototype formulation of AZ78 was designed and evaluated under controlled conditions and in small-scale field trials (Segarra *et al.* 2015).

Assessment of the impact of the formulation, application techniques and environmental conditions on the ecological fitness of mBCAs represents a critical step in the development of these products as biofungicides (Montesinos 2003). For this reason, methods to monitor mBCA population trends after their introduction in the field are indispensable. The use of culture-based methods to follow mBCA populations is time-consuming and often inadequate because of the lack of strain-specific growth media. This is why quantitative PCR (qPCR), based on highly specific DNA markers for the target mBCA, is the most common molecular method chosen to trace their fate in the environment (Schena *et al.* 2004; Smith and Osborn 2009). Repetitive sequence-based PCR such as Repetitive Extragenic Palindromic-PCR (REP-PCR) provides fingerprint profiles that can reveal discriminatory fragments (Versalovic *et al.* 1994). Specific primers can be designed, based on the DNA sequences of such discriminatory genome regions and used in qPCR methods to specifically detect the target mBCA (Pujol *et al.* 2006). REP-PCR in particular is a technique with a good discriminatory capacity for genetically related *Stenotrophomonas* and *Xanthomonas* genera (Rademaker *et al.* 2005; Lin *et al.* 2008). Moreover, it has already been successfully used to develop a TaqMan PCR protocol to quantify *L. enzymogenes* 3.1T8 on cucumber roots (Nijhuis, Pastoor and Postma 2010).

The aim of this work was to study the persistence and fate of AZ78 formulated and nude cells after their application on grapevine leaves through a specific quantification method based on qPCR.

Rainfall, relative humidity, solar radiation and temperature were monitored during the season and correlations with AZ78 persistence on grapevine leaves were analysed to determine the most critical factors for its fate in vineyards.

MATERIALS AND METHODS

Bacterial strains and development of specific primers for *L. capsici* AZ78

Specific primers for AZ78 were developed following the method of Nijhuis, Pastoor and Postma (2010). Briefly, REP-PCR was performed on the bacterial strains listed in Table 1, the banding pattern of AZ78 was compared to that of the other strains, and discriminating bands were excised and sequenced. Suitable primers for qPCR were designed for the sequences and the specificity of the primer pairs and the size of the resulting products was confirmed in the above-mentioned collection of bacterial strains using PCR.

Lysobacter capsici AZ78 cell production and prototype formulation for field applications

A five-litre fermenter controlled with a Biostat B unit (Sartorius Stedim Systems, Germany) was used to produce sufficient quantities of AZ78 cells to be used in field applications. The growth medium was made up of peptone 10 g L^{-1}, yeast extract 5 g L^{-1}, KH_2PO_4 1.4 g L^{-1} and $MgSO_4 \times 7H_2O$ 1 g L^{-1}. In addition, 0.75 mL L^{-1} of the antifoaming Silfoam SE2 (Wacker Chemie AG, Germany) was added to the fermenter. The temperature was set at 27°C, pH at 7, and at least 30% of pO_2 was ensured (Segarra *et al.* 2015). After 24 h, the content of the fermenter was centrifuged at 2500 *g* for 10 min to collect the AZ78 cells and remove the spent medium. Pelleted AZ78 cells were suspended in distilled water and stored at 4°C until application. AZ78 was formulated by adding 0.1% (w/v) of each of the following additives: corn steep liquor, polyethyleneglycol and lignosulfonate (Segarra *et al.* 2015).

Population dynamics of formulated and non-formulated *L. capsici* AZ78 on grapevine leaves and in soil

Field trials were carried out in two nearby vineyards in northern Italy (San Michele all'Adige) in 2014. The first vineyard was located at N 46.1845, E 11.1244, at 228 m above sea level and was planted in 2003 with the cv. Pinot gris SMA 514, grafted onto SO4 rootstock, with a double pergola trentina training system (5.50 × 0.66 m; distance between rows × distance between plants in the row). The second vineyard was located at N 46.1807, E 11.1259, at 250 m above sea level and was planted in 1997 with the cv. Mitter Vernatsch grafted onto Teleki 5C rootstock with a pergola trentina training system (3.00 × 0.66 m; distance between rows × distance between plants in the row).

Downloaded from http://femsle.oxfordjournals.org/ by guest on January 14, 2016

The treatments included untreated plants, plants treated with AZ78 at a final concentration of 10^8 cells mL^{-1} and plants treated with AZ78 at the same concentration plus the additives (formulated AZ78). AZ78 and formulated AZ78 were suspended in water and sprayed with a Solo 450 motorized backpack mist blower (Solo, Germany) using a spray volume of 550 L ha^{-1}. In both vineyards, each treatment consisted of four plots (replicates) with eight plants each. The plots were arranged in a completely randomized block design.

To study the population dynamics of AZ78 under different weather conditions during the crop cycle, the treatments were repeated on three different dates: 16 May, 19 June and 16 July 2014 on both the vineyards. AZ78 populations on grapevine leaves were monitored 1, 24 and 168 h after each treatment, while the soil was sampled 168 h after each application. In addition, samples of leaves and soil were collected before the first treatment and two months and one year after the last treatment. Grapevine leaves were collected randomly to obtain 100 g of sampled leaves from each of the four replicate plots for all treatments in both vineyards. Similarly, in both vineyards, 100 g of soil samples were taken under the canopy with a shovel at a distance of 0.8 m from the trunk and at a depth of 0.2 m in each plot for all the treatments. Grapevine leaves were washed by shaking them in a saline solution (0.85% NaCl; Sigma-Aldrich, USA) containing 0.01% of Tergitol (Sigma-Aldrich, USA) for 2 h at 100 rpm. The resulting washing solution was decanted, and the supernatant was centrifuged at 10 000 *g* for 10 min. Soil samples were air-dried and sieved at 2 mm to remove coarse material.

DNA was subsequently extracted from soil samples and pellets deriving from leaf wash with the FastDNA Spin Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instructions. qPCR was performed with the extracted DNA using the designed primers. The qPCR reactions contained a 10 μL mixture including 1 μL of the extracted DNA, 1X reaction buffer of the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, USA), 0.2 μM of each primer. The qPCR reactions were carried out on a Roche Light Cycler 480 (Roche Diagnostics, Germany) using the standard program (5 min at 50°C, 3 min at 95°C, followed by 40 cycles in a series of 15 s at 95°C and 45 s at 60°C). For each time point, three technical replicates were used for each of the four blocks from each treatment and vineyard.

To estimate the quantity of AZ78 cells on grapevine leaves and soil from vineyards, standard curves were produced by spiking known amounts of AZ78 cells in soil samples or in the washing buffer from grapevine leaves, in a similar way to the method described previously (Nijhuis, Pastoor and Postma 2010).

On June 2014, samples coming from vineyards were diluted and plated on Luria Bertani Agar (Sigma, USA) amended with kanamycin (25 mg L^{-1}, Sigma, USA), cycloheximide (100 mg L^{-1}, Sigma, USA) and $CuSO_4$ (250 mg L^{-1}, Sigma, USA) based on the reported resistance of AZ78 to kanamycin and $CuSO_4$ (Puopolo, Giovannini and Pertot 2014). Once inoculated, the Petri dishes were incubated at 27°C for 72 h and colony forming units (CFUs) with a colony morphology identical to AZ78 were counted.

Monitoring of environmental factors and analysis of their effect on *L. capsici* AZ78 persistence

Data of environmental factors [radiation, rainfall, relative humidity (RH), and temperature] were collected using an automatic weather station located near the experimental vineyards (http://meteo.iasma.it/meteo/index.php) from May to September 2014 (Fig. S1, Supporting Information).

For each sampling event, the effect of environmental factors on AZ78 persistence was assessed by calculating the accumulated values for conditions considered to be unfavourable for AZ78 survival. Accumulated radiation and rainfall were calculated as the addition of the hourly recordings of radiation (MJ m^{-2}) and rainfall (mm) according to previous results (Segarra *et al.* 2015). Accumulated non-optimal RH was calculated as the hourly addition of the difference between 100% and the actual RH according to previous results (Puopolo, Giovannini and Pertot 2014). Accumulated non-optimal temperature was calculated as the hourly addition of degrees below 15°C and over 25°C according to Puopolo *et al.* (2015).

Statistical analysis

The AZ78 population ($\log_{10}$ AZ78 cells gram of leaf^{-1}) was analysed using three-way Analysis of Variance (ANOVA), including the factors of vineyard, treatment and hours after application. The experiment was performed three times (May, June and July). The factor vineyard was found not to be significant. When statistical differences were found, Tukey's test ($\alpha = 0.05$) was used to separate the means. The Analysis of Covariance (ANCOVA) test was used to study the difference in slopes for treatments in terms of the correlations between environmental factors and the AZ78 population. A multiple regression analysis was performed on the environmental factors, and the AZ78 population to determine the significance of each separate factor. The correlations consisted of 18 values per treatment: three time points after the treatment (1, 24 and 168 h) $\times$ 3 experiments (May, June and July) $\times$ 2 vineyards. All tests were performed using Statistica 7.1 (StatSoft, USA).

RESULTS

Quantification of formulated and non-formulated *L. capsici* AZ78 on grapevine leaves and in soil samples

Electrophoresis of REP-PCR products revealed band patterns that were different for each tested bacterial strain (Fig. S2, Supporting Information). A discriminating band of 815 bp for AZ78 was excised from the gel and sequenced (GenBank accession number KP881481). Based on the sequence of the fragment, primer pair combinations were designed (Table 2). When tested against the collection of bacterial strains, primer set Lc122 showed a clear band of the expected size of 122 bp only for AZ78, while the other primer pairs were not specific for AZ78 and showed amplification in other bacterial strains (Table S1, Supporting Information).

The slope of the standard curve was –3.16 and R^2 was 0.99. The equation for quantification was $\log_{10}$ AZ78 cells gram of leaf^{-1} = 9.914 –0.316 Ct. The background Ct of leaf samples without added AZ78 was 31.4 $\pm$ 0.2 (mean value $\pm$ standard error). The slope of the standard curve for the soil was –3.21 and R^2 was 0.99. The equation for quantification was $\log_{10}$ AZ78 cells gram of leaf^{-1} = 12.139–0.312 Ct. The background Ct of soil samples without added AZ78 was 35.5 $\pm$ 0.3. Considering that it is usually recommended that the Ct value of the most diluted sample in the standard curve should be at least $\log_{10}$-fold (3.3 cycles) lower than the Ct value of the non-template controls (Smith and Osborn 2009), the detection limit of our technique can be considered to be $\sim$1 and 2 $\log_{10}$ AZ78 cells gram of leaf^{-1} and soil^{-1}, respectively.

AZ78 was applied on grapevine plants as nude and formulated cells. Soon after the application of AZ78 on grapevines

Downloaded from http://femsle.oxfordjournals.org/ by guest on January 14, 2016

Table 2. Primer pairs for qPCR of *L. capsici* AZ78 and their relative position in the sequence of a discriminating band from REP-PCR.

	Forward	Reverse	Position[a]
Lc111	CGAAAGCGGACATACAGACC	CCAAACGACAGACCTGAAGC	25–135
Lc122	GCTTCAGGTCTGTCGTTTGG	GGTAGAACTGCAGCTTCCCA	116–237
Lc158	CCGTAGTCCTGGTCGAACTC	ATCAACTACACCCACAGCGA	592–749
Lc192	GGGAAGCTGCAGTTCTACCA	GAAGAAACCGGGTCGAAAGG	219–410

[a]Position in the sequence GenBank accession number KP881481 obtained from REP-PCR.

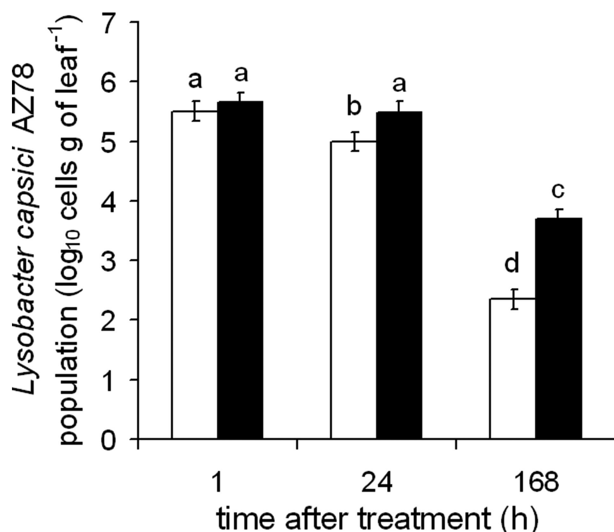

Figure 1. *Lysobacter capsici* AZ78 population on grapevine leaves treated with AZ78 (empty bars) or formulated AZ78 (filled bars) 1, 24 and 168 h after the application. The detection limit of the technique was ~1 log_{10} AZ78 cells gram of $leaf^{-1}$. Different letters show significant differences according to Tukey's test ($P < 0.05$). Data shown are the average of three experiments performed in three consecutive months at two different vineyards.

(1 h), the population was 5.5 log_{10} cells gram of $leaf^{-1}$, decreasing to 4.9 and 2.4 log_{10} cells gram of $leaf^{-1}$ after 24 and 168 h, respectively (Fig. 1). Plants treated with formulated AZ78 had significantly higher levels of AZ78 after 24 ($P = 0.0001$) and 168 h ($P = 0.0013$) than plants treated with AZ78 without additives (Fig. 1); in particular, after 168 h, the AZ78 population was more than ten times higher (Fig. 1). AZ78 cells were detected on untreated (control) grapevine plants located at 5.5 (Pinot gris) or 3 (Mitter Vernatsch) m from the AZ78 treated grapevine plants 1 and 24 h after treatment, while AZ78 was not detected after one week (Table S2, Supporting Information).

During the experiments carried out on June 2014, plate counting was carried out. At 1 and 24 h after the treatments, leaves treated with AZ78 showed a concentration of 3.54±0.29 and 1.49±0.08 AZ78 CFU g of $leaf^{-1}$, respectively. A concentration of 3.37±0.21 and 2.42±0.47 AZ78 CFU g of $leaf^{-1}$ was registered on leaves 1 and 24 h after the treatment with formulated AZ78. In both the cases, no CFU grew on plates from leaves sampled 168 h after the treatments. A significant correlation ($P < 0.0001$; $R^2 = 0.6074$) was obtained by plotting the population of AZ78 calculated from qPCR compared to that obtained from CFU counts on plates.

AZ78 and formulated AZ78 could not be detected in leaves before the beginning of the treatments, at two months and one year after the last application, nor could it be detected in any of the soil samples (data not shown).

Evaluation of environmental influences on *L. capsici* AZ78 persistence on grapevine leaves

The population dynamics of AZ78 after its application on grapevine leaves in relation to weather conditions are shown in Fig. 2. Accumulated non-optimal temperatures, accumulated radiation, accumulated non-optimal RH and accumulated rainfall correlated negatively and significantly with the log_{10} cells g of $leaf^{-1}$ concentration of AZ78 on leaf samples treated with AZ78 without additives, with the following R^2 values: 0.772, 0.809, 0.594 and 0.479 respectively (P values were < 0.0001, <0.0001, 0.0002 and 0.0014) and 0.678, 0.593, 0.386 and 0.395, respectively, on leaf samples treated with formulated AZ78 (P values were <0.0001, 0.0002, 0.0059 and 0.0052) (Fig. 2). The ANCOVA test revealed that the slope of the regressions was significantly different (less steep) in plants treated with formulated AZ78, compared to AZ78 for accumulated non-optimal temperatures ($P = 0.0477$) (Fig. 2A) and accumulated radiation ($P = 0.0210$) (Fig. 2B). When multiple regression analysis was performed considering the effect of the four factors together (accumulated radiation, rainfall, non-optimal RH and non-optimal temperature) on AZ78 population, the factor non-optimal temperature was significant for AZ78 and formulated AZ78 treated samples ($P =$ 0.0094 and 0.0066, respectively).

DISCUSSION

Monitoring the population of an mBCA on leaves in the field may help to understand how environmental factors can modulate its survival during the crop cycle. This applies above all to the *Lysobacter* genus since there is increasing interest in it as a source of novel mBCAs, and little is known about its ecology (Hayward *et al.* 2010). To our knowledge, this is the first time that the population of a *Lysobacter* member has been applied in field conditions as a prototype formulation and monitored using qPCR on plant leaves. Indeed, the majority of the *Lysobacter* strains appearing in the literature are soil inhabiting microorganisms. Indigenous populations of *L. antibioticus, L. capsici* and *L. gummosus* were monitored in various agricultural soils with TaqMan qPCR and populations ranged from <4.0–6.95 log gene copy numbers g^{-1} soil (Postma, Schilder and van Hoof 2011). *Lysobacter enzymogenes* C3 is the only case of a *Lysobacter* used on aerial parts of the plants in field conditions (Zhang and Yuen 1999; Jochum, Osborne and Yuen 2006). AZ78 populations decreased with time and this could putatively be related to desiccation, UV radiation and wash-off due to rain, as all these environmental factors had adverse effects on the bacterial population *in vitro* (Segarra *et al.* 2015). In addition, optimal temperatures for AZ78 have been shown to be between 15°C and 25°C (Puopolo *et al.* 2015). The negative correlations between bacterial population and accumulated radiation, rainfall, non-optimal RH and non-optimal temperatures also point in this direction. However, the results of the multiple regressions showed that only accumulated non-optimal temperatures had a significant effect on the AZ78

Downloaded from http://femsle.oxfordjournals.org/ by guest on January 14, 2016

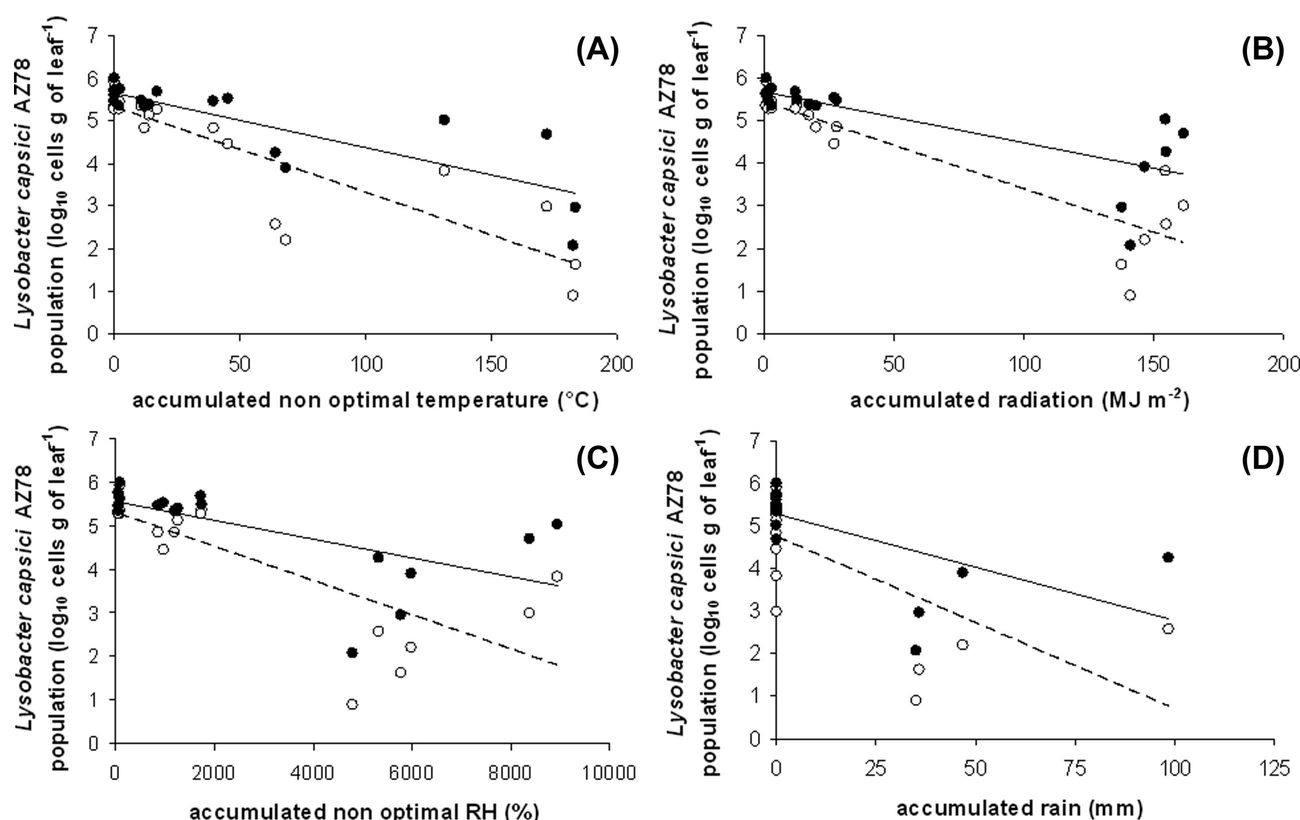

Figure 2. Effect of accumulated non-optimal temperature (**A**), solar radiation (**B**), accumulated non optimal relative humidity (RH,**C**) and rain (**D**) on *L.capsici* AZ78 population on grapevine leaves treated with AZ78 (empty circles) or formulated AZ78 (filled circles). The x-axis represents the hourly accumulated values. Data originated from three experiments performed in three consecutive months at two different vineyards were analysed.

population. For comparison, the *L. enzymogenes* C3 population, studied by dilution plating, declined by nearly 1 $\log_{10}$ unit 3 days after application to the aerial parts of tall fescue (Zhang and Yuen 1999) and declined to <2 $\log_{10}$ CFU g^{-1} at low daily temperatures (10°C –15°C) when applied to wheat heads (Jochum, Osborne and Yuen 2006). These results are also in line with what has previously been reported in relation to the sensitivity of other non-spore forming Gram-negative bacteria to environmental factors. For instance, the population of *Pantoea agglomerans* CPA-2 on orange fruit decreased by one order of magnitude when stored at 43% RH for 48 h and almost 1000 times after 4 h of sunlight exposure (Cañamás *et al* 2008). In the same way, the *Pseudomonas fluorescens* EPS62e population started at 10^7 cells g^{-1} and decreased progressively to 5×10^3 cells g^{-1} after one month on apple trees in field conditions (Pujol *et al.* 2006). Rain events have been also associated with the washing of the *P. agglomerans* strain C9-1S from apple blossoms (Johnson *et al.* 2000).

As a non-spore forming Gram-negative bacterium, the addition of additives to AZ78 cells can protect the bacterium from environmental factors. Indeed, our results showed that the use of additives significantly improved AZ78 persistence on leaves and specifically provided a certain degree of protection from radiation and unfavourable temperatures. On the basis of the results achieved, it is conceivable that treating in the afternoon and avoiding high temperature values could improve AZ78 persistence in the field. Previous results have shown the benefits of this particular additive mix in terms of UV radiation and desiccation protection, as well as the improved rain fastness of AZ78 (Segarra *et al.* 2015). In the same way, the use of an edible coating based on derivatives of fatty acids and polysaccharides in alcohol solution significantly improved the persistence of *P. agglomerans* CPA-2 (Cañamás *et al.* 2008).

The effect of competition with other phyllosphere inhabitants on AZ78 survival cannot be ruled out based on the experiment setup. However, in favourable greenhouse conditions the survival of AZ78 is higher, particularly at 25°C with high RH (Puopolo, Giovannini and Pertot 2014). Based on this, it can be hypothesized that the environmental conditions have a major role compared to the competition.

From a practical point of view, the study also provides important information regarding the fate of this mBCA in the environment, which is crucial knowledge for registration of a bacterium as a plant protection product. AZ78 was not naturally present on the leaves before the treatments, while on the day of treatment a slight presence (up to 3 $\log_{10}$ cells gram of leaf^{-1}) could be detected on untreated leaves, suggesting that cross-contamination occurred, probably due to the drift caused by wind during the application. The fact that AZ78 populations on untreated plants were not detectable after one week indicates that while contamination can occur, AZ78 is not able to establish itself on non-targeted plants and populations quickly decrease after treatment. We assessed the survival of AZ78 in the soil, since potential recolonization from soil to leaves may have occurred. In this sense, AZ78 was never detected in the soil of treated fields, confirming the low potential of this mBCA to spread outside the target area. Conversely, in previous greenhouse experiments where *L. enzymogenes* strain 3.1T8 was applied to the substrate of cucumber plants, it was found on non-inoculated control plants at a much lower concentration than the treated plants (Nijhuis, Pastoor and Postma 2010). The authors suggested that it could

Downloaded from http://femsle.oxfordjournals.org/ by guest on January 14, 2016

be due to transportation by insects or water splash from inoculated plants to non-inoculated plants (Nijhuis, Pastoor and Postma 2010).

In conclusion, a strain-specific procedure based on qPCR was designed to monitor the fate of AZ78 cells applied in vineyards. Results showed that the current formulation significantly improved AZ78 persistence on grapevine leaves in under field conditions. However, strategies should be designed to protect better AZ78 against non-favourable temperature which was identified as the main detrimental environmental factor in the field.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Joeke Postma, Plant Research International Business Unit Biointeractions and Plant Health, Wageningen (The Netherlands) for providing the *L. capsici* 10.4.5, *L. capsici* 1.3.3, *L. capsici* 55 and *L. capsici* 6.2.3 strains.

FUNDING

This work was supported by the EU INNOVA project [FP7-People-2012-IAPP, grant agreement 324416] and European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 289497 (project CO-FREE, theme KBBE.2011.1.2-06).

Conflict of interest. None declared.

REFERENCES

Cañamás TP, Viñas I, Usall J *et al.* Control of postharvest diseases on citrus fruit by preharvest application of the biocontrol agent *Pantoea agglomerans* CPA-2: Part I. Study of different formulation strategies to improve survival of cells in unfavourable environmental conditions. *Postharvest Biol Tec* 2008;**49**:86–95.

Cook RJ. Making greater use of introduced microorganisms for biological control of plant pathogens. *Annu Rev Phytopathol* 1993;**31**:53–80.

Fravel DR. Commercialization and implementation of biocontrol. *Annu Rev Phytopathol* 2005;**43**:337–59.

Fravel DR, Connick WJ, Jr, Lewis JA. Formulation of microorganisms to control plant diseases. In: Burges HD (ed.). Formulation of Microbial Biopesticides: Beneficial Microorganisms, Nematodes and Seed Treatments. Dordrecht: Kluwer Academic Publishers, 1998, 187–202.

Gessler C, Pertot I, Perazzolli M. *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. *Phytopathol Mediterr* 2011;**50**: 3–44.

Hayward AC, Fegan N, Fegan M *et al. Stenotrophomonas* and *Lysobacter*: ubiquitous plant-associated gamma-proteobacteria of developing significance in applied microbiology. *J Appl Microbiol* 2010;**108**:756–70.

Jochum CC, Osborne LE, Yuen GY. Fusarium head blight biological control with *Lysobacter enzymogenes* strain C3. *Biol Control* 2006;**39**:336–44.

Johnson KB, Stockwell VO, Sawyer TL *et al.* Assessment of environmental factors influencing growth and spread of *Pantoea agglomerans* on and among blossoms of pear and apple. *Phytopathology* 2000;**90**:1285–94.

Kamoun S, Furzer O, Jones JDG *et al.* The Top 10 oomycete pathogens in molecular plant pathology. *Mol Plant Pathol* 2014;**16**:413–34.

Lin CW, Chiou CS, Chang YC *et al.* Comparison of pulsed-field gel electrophoresis and three rep-PCR methods for evaluating the genetic relatedness of *Stenotrophomonas maltophilia* isolates. *Lett Appl Microbiol* 2008;**47**:393–8.

Lindow SE, Brandl MT. Microbiology of the phyllosphere. *Appl Environ Microbiol* 2003;**69**:1875–83.

Montesinos E. Development, registration and commercialization of microbial pesticides for plant protection. *Int Microbiol* 2003;**6**:245–52.

Nijhuis EH, Pastoor R, Postma J. Specific detection of *Lysobacter enzymogenes* (Christensen and Cook 1978) strain 3.1T8 with TaqMan-PCR. *J Appl Microbiol* 2010;**108**:1155–66.

Park JH, Kim R, Aslam Z *et al. Lysobacter capsici* sp. nov., with antimicrobial activity, isolated from the rhizosphere of pepper, and emended description of the genus *Lysobacter*. *Int J Syst Evol Micr* 2008;**58**:387–92.

Postma J, Schilder MT, van Hoof RA. Indigenous populations of three closely related *Lysobacter* spp. in agricultural soils using real-time PCR. *Microb Ecol* 2011;**62**:948–58.

Pujol M, Badosa E, Manceau C *et al.* Assessment of the environmental fate of the biological control agent of fire blight, *Pseudomonas fluorescens* EPS62e, on apple by culture and real-time PCR methods. *Appl Environ Microb* 2006;**72**:2421–7.

Puopolo G, Giovannini O, Pertot I. *Lysobacter capsici* AZ78 can be combined with copper to effectively control *Plasmopara viticola* on grapevine. *Microbiol Res* 2014;**169**:633–42.

Puopolo G, Palmieri MC, Giovannini O *et al.* Impact of temperature on the survival and the biocontrol efficacy of *Lysobacter capsici* AZ78 against *Phytophthora infestans*. *BioControl* 2015;**60**:681–9.

Rademaker JLW, Louws FJ, Schultz MH *et al.* A comprehensive species to strain taxonomic framework for *Xanthomonas*. *Phytopathology* 2005;**95**:1098–111.

Schena L, Nigro F, Ippolito A *et al.* Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *Eur J Plant Pathol* 2004;**110**:893–908.

Segarra G, Puopolo G, Giovannini O *et al.* Stepwise flow diagram for the development of formulations of non spore-forming bacteria against foliar pathogens: the case of *Lysobacter capsici* AZ78. *J Biotechnol* 2015;**216**:56–64.

Smith CJ, Osborn AM. Advantages and limitations of quantitative PCR(Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol Ecol* 2009;**67**:6–20.

Versalovic J, Schneider M, de Bruijn FJ *et al.* Genomic fingerprinting of bacteria using repetitive sequence based-polymerase chain reaction. *Method Cell Mol Biol* 1994;**5**:25–40.

Zhang Z, Yuen GY. Biological control of *Bipolaris sorokiniana* on tall fescue by *Stenotrophomonas maltophilia* strain C3. *Phytopathology* 1999;**89**:817–22.

Downloaded from http://femsle.oxfordjournals.org/ by guest on January 14, 2016