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Stepwise flow diagram for the development of formulations of non spore-forming bacteria against foliar pathogens: the case of *Lysobacter capsici* AZ78

Abbreviated running headline: Stepwise design of mBCA formulations

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Highlights

- A stepwise flow diagram to develop formulations for biopesticides is presented.
- It includes the selection of protecting additives and validation under field conditions.
- A formulation for *Lysobacter capsici* AZ78 was developed following this procedure.

Abstract

The formulation is a significant step in biopesticide development and is an efficient way to obtain consistency in terms of biological control under field conditions. Nonetheless, there is still a lack of information regarding the processes needed to achieve efficient formulation of non spore-forming bacterial biological control agents. In response to this, we propose a flow diagram made up of six steps including selection of growth parameters, checking of minimum shelf life, selection of protective additives, checking that the additives have no adverse effects, validation of the additive mix under field conditions and choosing whether to use additives as co-formulants or tank mix additives. This diagram is intended to provide guidance and decision-making criteria for the formulation of non spore-forming bacterial biological control agents against foliar pathogens. The diagram was then validated by designing an efficient formulation for a Gram-negative bacterium, *Lysobacter capsici* AZ78, to control grapevine downy mildew caused by *Plasmopara viticola.* A harvest of 10^{10} *L. capsici* AZ78 cells ml^{-1} was obtained in a bench top fermenter. The viability of cells decreased by only one order of magnitude after one year of storage at 4°C. The use of a combination of corn steep liquor, lignosulfonate, and polyethyleneglycol in the formulation improved the survival of *L. capsici* AZ78 cells living on grapevine leaves under field conditions by one order of magnitude. Furthermore, the use of these additives also guaranteed a reduction of 71% in *P. viticola* attacks. In conclusion, this work presents a straightforward stepwise flow diagram to help researchers develop formulations for biological control agents that are easy to prepare, stable, not phytotoxic and able to protect the microorganims under field conditions.

Keywords: Formulation; *Lysobacter capsici*; shelf life; UV irradiation; rainfastness; desiccation.

1. Introduction

Increasing concerns about the negative impact of synthetic chemical pesticides on health and the environment is fostering the development of more sustainable alternatives. Biopesticides based on microbial biological control agents (mBCA) are a promising option (Cook 1993). However, different factors need to be taken into account when developing a commercial biopesticide, such as the cost of production (Glare *et al.* 2012). The period of time for which the product can be stored in its packaging before the microorganism dies (shelf-life) and its survival on the plant/soil when applied in the field are crucial aspects (Fravel *et al.* 1998; Fravel 2005). During the process of selection and initial screening, microorganisms are evaluated mainly in the laboratory or under controlled conditions, where environmental stressors are absent (Montesinos 2003). However, mBCAs have to withstand harsh and hostile conditions when applied to leaf surfaces. For instance, desiccation could negatively impact their survival and consequently their biocontrol efficacy (Lindow and Brandl 2003). In addition, mBCA also have to resist sunlight, and in particular ultraviolet irradiation, which has a strong germicidal effect (Lahlali *et al.* 2011). Rain and wind may physically remove mBCA from the phyllosphere and prevent colonization of the ecological niche of plant pathogens, thus nullifying their biocontrol activity (Jones and Burges 1998).

The formulation of mBCA may offer a solution to these problems. A formulated product is composed of the active ingredient (microorganism) and co-formulants, which are additives that improve its survival and efficacy without any direct effect on the pathogen (Fravel *et al*. 1998). These additives can be incorporated into microbial biopesticides before, during or after fermentation, or added later to the spray tank mixes (Burges 1998; Ravensberg 2011). The additives must ensure conditions that maintain viability throughout production, distribution and storage, aid handling and product application. Importantly, they must also ensure the persistence and activity of the mBCA at the target site (Rhodes 1993; Fravel *et al.* 1998). For instance, humectants improve the adverse effects of fluctuating humidity on the leaf surface on microorganisms by absorbing

water during peak night humidity and losing it during low daytime humidity (Burges 1998). The adverse effect of sunlight on the mBCA may be hindered by UV light protectants that act by reflecting or absorbing radiation (Lahlali *et al.* 2011). Stickers improve adherence of microorganisms to foliage and persistence in the event of wind and rain (Schisler *et al*. 2004).

Despite the crucial role of the formulation for the commercial success of a microbial biopesticide, it is often a neglected topic in science. This is in part due to the fact that the formulation is often developed by companies and protected by confidentiality, but also to a failure to acknowledge the importance of the subject within the scientific community (Fravel 2005; Ravensberg 2011). As a consequence, the majority of authors have focused on optimizing the whole screening process, as in the case of Köhl *et al.* (2011) who proposed a stepwise screening program for the production of biopesticides. Similarly, Slininger and Schisler (2013) proposed a high-throughput assay based in microwell plates for speeding up the screening process. Guidelines or indications for the development of formulations, especially for Gram-negative bacteria, are absent in the literature. To address this issue, we propose a stepwise flow diagram that could be useful for designing mBCA formulations, in particular, non spore-forming bacteria targeting foliar pathogens. This scheme consists of several steps, which includes selecting parameters for cell mass production, selection of protective additives and validation of the additive mix under field conditions. It is intended to provide guidance, and the decision-making criteria are also given. In addition, we validated our scheme by designing an efficient formulation for a Gram-negative bacterium, *Lysobacter capsici* AZ78 (AZ78) based on its high efficacy against grapevine downy mildew caused by *Plasmopara viticola* (Puopolo *et al.* 2014a,b).

2. Material and methods

2.1. Overview of the stepwise flow diagram

The flow diagram that we propose is made up of six steps, as shown in Fig. 1. The first step involves selecting growth conditions for the candidate mBCA, specifically, pH, temperature, pO_2 and fermentation time. These parameters have to be considered not only in terms of cell growth but also in terms of economic feasibility. The first step also includes selection of a growth medium that must not have any economic constraints and lead to acceptable cell mass production in a bench scale fermenter. A starting point for the selection of growth parameters and the medium could be the parameters recommended for the type strain of the mBCA. Useful information can also be obtained from the published literature and patents. The second step consists of verifying the attainment of a minimum desirable shelf life for the cells produced in the best medium. The third step focuses on the selection of additives for the protection of the mBCA against deleterious environmental conditions such as desiccation, UV light and wash off. To develop this step, it is first necessary to determine the effective concentration of the mBCA in a relevant pathosystem under controlled conditions. It is also necessary to determine the stress conditions that can reduce the mBCA concentration below an effective level. Once this information has been acquired, additives with protective ability are tested in parallel for three (or more) different stress factors or conditions to save time. The practical concentration at which the additives can be tested depends on the volume of tank mix used per unit of crop surface and the rate at which the products are usually used for target pathogen and crop. Additives are considered effective when they are able to protect the microorganism up to the minimum effective concentration.

In the fourth step, the side effects of the most effective additives against the mBCA, pathogen and the plant are tested in laboratory conditions and additives with undesired effects are eliminated from the pipeline. The fifth step involves validation of the combined additives on the efficacy and persistence of the mBCA, performed under field conditions on the target pathosystem. In the sixth step, a decision is made as to whether or not the additives should be marketed together with the mBCA as co-formulants or only as tank mix additives, because they may interfere with its shelf-

life. If at any point in the procedure one of the steps leads to unsatisfactory results it is advised to go back and modify the conditions of the previous step.

In this work, the proposed diagram was used to develop a formulation for AZ78 intended for use against *P. viticola* in vineyards.

2.2. First step: selection of growth conditions and medium

Suitable pH and temperature conditions for *L. capsici* AZ78 cell growth were identified using a Synergy 2 (Biotek, Winooski, VT, USA) agitated multiwell plate reader. Several temperatures (24, 27 and 30°C) and pH (6, 7 and 8) were assayed on LB medium inoculated with a AZ78 cell suspension produced as described by Puopolo *et al.* (2014a) in order to obtain an initial concentration of 1×10^7 cells ml^{-1}. Non-inoculated LB medium was used as a control. Growth was measured as absorbance at 600 nm every hour for 48 h. For each condition, twelve wells were inoculated and the experiment was repeated. The best parameters were subsequently used in the fermenter for selection of a growth medium.

A 5 l fermenter controlled by a Biostat B unit (Sartorius Stedim Systems, Guxhagen, Germany) was used to compare four different growth media in terms of harvested cells: MYM (Molasses 20 g l^{-1} and Yeast Extract 5 g l^{-1}), PYKM (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}), PYNM (Peptone 10 g l^{-1}, Yeast Extract 5 g l^{-1} and NaCl 5 g l^{-1}) and SYM (Sucrose 10 g l^{-1} and Yeast Extract 5 g l^{-1}). To prevent the formation of foam during fermentation, 0.75 ml l^{-1} of antifoaming Silfoam SE2 from Wacker Chemie AG (Munich, Germany) was added to each medium. PYNM derives from the commonly used LB medium, where peptone was used instead of tryptone. PYKM was developed based on the assumption that K, P, S and Mg could represent limiting factors for growth in other media (Spaargaren 1996). SYM and MYM were tested because they are relatively inexpensive as compared with PYKM and PYNM (Costa *et al.* 2001).

The fermenter was filled with 2 l of each medium, autoclaved and subsequently inoculated with an AZ78 cell suspension to obtain an initial concentration of 1×10^7 cells ml^{-1}. The temperature was set at 27°C, and pH was kept at 7 with automatic pumping of acetic acid (30% w/v) or ammonium hydroxide (30% w/v). Agitation and air flow were variable and automatically regulated by the Biostat B unit to maintain at least a dissolved oxygen of 30% of saturation. This last parameter was adjusted based on the content commonly used in the literature (Delvigne *et al.* 2011) and already used for the fermentation of another *Lysobacter* strain (*Lysobacter* sp. XL1; Kulaev *et al.* 2006). Samples (50 ml) were taken 24 h after inoculation, serially diluted and plated onto LBA. Once inoculated, LBA dishes were incubated at 27°C for 72 h, and then Colony Forming Units (CFU) were counted to determine the concentration of AZ78 cells ml^{-1}. The medium was considered acceptable if it was able to produce at least 1×10^{10} cells ml^{-1} (Köhl *et al.* 2011).

2.3 Second step: assessment of the shelf life of the harvested cells

The shelf life of AZ78 cells deriving from fermentation on the optimal medium was evaluated as follows: after the 24 h fermentation process, the content of the fermenter was centrifuged at 2500 ***g*** for 10 min to collect the cells and remove the spent medium; pelleted cells were resuspended in 50 ml of sterile distilled water (SDW, bacterial cell stocks) and stored at 4 and 25°C; three 100-µl samples were taken just after the preparation of the bacterial cell stocks and after 7, 14, 41, 70, 84, 112, 168 and 365 days of storage and serially diluted and plated onto LBA to determine the concentration of viable AZ78 cells. The shelf life was considered acceptable if the viability showed a maximum decrease of one order of magnitude after one year.

2.4. Third step: selection of additives to protect *Lysobacter capsici* AZ78

2.4.1 Setting the conditions for the selection of additives

The minimum effective dose of AZ78 was determined as follows. Potted grapevine plants, grown as described in Puopolo *et al.* (2014a) were treated with AZ78 cells at the following concentrations: 1 × 10^4, 10^5, 10^6, 10^7 and 10^8 cells ml^{-1}. Treatments were applied on adaxial and abaxial leaf surfaces using a hand sprayer (100 ml per plant). The following day, *P. viticola* inoculum (2.5 × 10^5 sporangia ml^{-1}) produced as described in Puopolo *et al.* (2014a) 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. Once sporulated, disease severity was evaluated as the percentage of leaf area covered by *P. viticola* sporulation.

To determine the conditions required for the testing of additives, the susceptibility of AZ78 to desiccation, UV light and wash off was assessed as follows. For desiccation, five 2-µl drops of AZ78 suspension (1 × 10^8 CFU ml^{-1}) were applied to the inner part of sterile 15 ml tube caps. Subsequently, caps were kept face up under a Bio II A flow hood (Telstar, Terrassa, Spain) at 15% RH and 40 m s^{-1} wind speed. Caps bearing AZ78 cells were placed on the corresponding sterile 15 ml tubes containing 10 ml of sterile saline solution (SSS, NaCl 0.85%) after 30, 60 and 120 min. Subsequently, the tubes were shaken (150 rpm) for 1 h at room temperature to wash the bacterial cells from the caps. The number of viable AZ78 cells was determined using the dilution plating method on LBA as reported above.

To assess tolerance to UV light irradiation, 100 µl of AZ78 cell suspensions (1 × 10^3-10^4 CFU ml^{-1}) were spread onto LBA and immediately exposed to UV-B light (280-360 nm, peak at 306 nm) for 10, 20 , 30 and 60 s to obtain respectively 130, 260, 390 and 780 J m^{-2} using Sankyo Denki bulbs (Hiratsuka, Japan). Following exposure, dishes were incubated in the dark for 4 days at 27°C, after which the CFU were counted. The ability of AZ78 cells to resist wash off was evaluated by mimicking the rain on leaf disks. Leaf disks (1.9 cm of diameter) were excised from leaves of grapevine plants grown in the greenhouse as above. The disks were homogeneously sprayed (5 µl

cm^{-2}) on the abaxial surface with AZ78 cell suspension (1×10^8 CFU ml^{-1}). Treated disks were incubated on water-soaked filter paper and placed in Petri dishes (90 mm) at 25°C. After 24 h, the leaf disks were placed on a plastic sheet having a slope of 45%, and 5 or 25 ml of SDW (corresponding to simulated rain of 18 and 88 mm respectively) were applied to the abaxial surface of the leaf disks using drop dispensing bottles (Thermo Fisher Scientific, Waltham, MA USA) placed 25 cm above the disks. The leaf disks were then put into 50 ml sterile tubes containing 10 ml of SSS and the suspension serially diluted onto LBA. In all the experiments, the Survival Ratio (SR) was calculated as the ratio of AZ78 CFU developed from cells exposed to the deleterious agent to AZ78 CFU from non-treated samples.

2.4.2. Selection of additives protecting against desiccation, UV light and wash off

All the additives were tested at a concentration of 0.1 % (w/v), which is assumed to be the highest practical concentration. This is based on the fact that for practical reasons (transportation, packaging, etc.) the common amount of formulated plant protection products applied in vineyards ranges from 1 to 4 kg ha^{-1} (http://www.magrama.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/ registro/menu.asp, accessed on 27 January 2015). Considering application rates of up to 1000 l of tank mixture ha^{-1}, a concentration of 0.1% will result in a final quantity of 1 kg ha^{-1} (1 g of additive l^{-1}× 1000 l ha^{-1} = 1000 g ha^{-1}) for each additive.

Based on the critical time of exposure to desiccation assessed in the above mentioned experiment (30 min), the following additives were evaluated for protection of AZ78 cells against desiccation: arabic gum, chitosan, carboxymethylcellulose, corn steep liquor, gelatin, glycerol, molasses, paraffin, pinolene (Nu Film containing 96% pinolene; CBC Europe, Grassobbio, Italy), polyacrylate, polyethyleneglycol, polyvinylalcohol, polyvinylpyrrolidone, skimmed milk, sodium alginate, sorbitol, starch and xanthan gum. The aforementioned additives were added to AZ78 cell suspensions. The resulting suspensions were treated as stated above.

Based on the critical UV-B irradiation dose assessed in the above mentioned experiment (780 J m^{-2}), the following additives were evaluated for their ability to protect AZ78 cells against UV-B irradiation: ascorbic acid, bentonite, fluorescent brightener 28, folic acid, kaolin, lignosulfonate, molasses, skimmed milk, titanium oxide, and zinc oxide. AZ78 suspensions containing these additives were spread onto LBA and treated as described above.

Based on the critical amount of simulated rain assessed in the above mentioned experiment (25 ml), the following additives were evaluated for their ability to protect AZ78 cells against simulated rain on grapevine leaf disks: arabic gum, chitosan, carboxymethylcellulose, corn steep liquor, gelatin, molasses, pinolene, polyacrylate, polyvinyl alcohol, polyvinylpyrrolidone, skimmed milk, sodium alginate, starch, and xanthan gum. AZ78 cell suspensions containing these additives were sprayed onto leaf disks and treated as described above. In all experiments, AZ78 cell suspensions without additives were used as controls.

Additives were considered effective if they were able to protect the mBCA up to the minimum effective concentration after the deleterious treatment.

2.5. Fourth step: evaluation of the side effects of the best additives on *Lysobacter capsici* AZ78 cells, *Plasmopara viticola* and leaf disks from grapevine plants

The possible side effects of the best additives (arabic gum, fluorescent brightener, corn steep liquor, glycerol, lignosulfonate, molasses, polyethyleneglycol, titanium oxide and xanthan gum) on AZ78 cells, *P. viticola* and the grapevine were determined. Solutions containing 0.1% (w/v) in SDW of the above mentioned compounds with or without AZ78 cell suspension (1×10^8 CFU ml^{-1}) were applied to the abaxial surface of leaf disks prepared as reported above. AZ78 and SDW were used alone as controls. Subsequently, the treated leaf disks were incubated on water-soaked filter paper contained in Petri dishes. After 24 h, *P. viticola* inoculum (2.5×10^5 sporangia ml^{-1}) was sprayed onto the abaxial surface of the treated leaf disks. Once inoculated, leaf disks were kept on water-

soaked filter paper contained in Petri dishes at 25°C with a 16/8-h day/night light regime. Seven days after *P. viticola* inoculation, disease severity was evaluated as the percentage of leaf disk area covered by *P. viticola* sporulation. Non-inoculated leaf disks were examined to find any phytotoxic effect of the treatment. As mentioned previously, additives with undesired effects were eliminated from the pipeline.

2.6. Fifth step: validation of the combined additives on the efficacy and persistence of *Lysobacter capsici* AZ78 against *Plasmopara viticola* under field conditions

The best additives not showing undesirable side effects were combined in tertiary combinations. In our particular example, one additive was chosen to provide rain fastness (corn steep liquor), two to provide protection against UV light (titanium oxide and lignosulfonate) and two to protect against desiccation (glycerol and polyethyleneglycol). This resulted in the following four combinations: corn steep liquor, polyethyleneglycol and titanium oxide (CPT), corn steep liquor, glycerol and lignosulfonate (CGL), corn steep liquor, polyethyleneglycol and lignosulfonate (CPL), corn steep liquor, glycerol and titanium oxide (CGT). Each compound was used at 0.1% (w/v).

Field experiments were carried out to validate the efficacy of these combinations in increasing AZ78 cell survival and control of *P. viticola*. Potted grapevine plants, grown as described previously, were used to standardize the experiments. Plants were treated with AZ78 cells formulated (1×10^8 CFU ml^{-1}) with the four combinations of additives. Grapevine plants treated with SDW, AZ78 nude cells, the four combinations alone, and a copper based fungicide [2 g l^{-1} of Coprantol HiBio, 25% $Cu(OH)_2$, Syngenta, Basel, Switzerland] were used as controls. Treatments were applied as described above, and the plants were kept under field conditions for 24 h. Radiation, rainfall, relative humidity and temperature data were collected by an automatic weather station located near the experimental site (Table S1). The plants were moved into a greenhouse to carry out the inoculation with *P. viticola*, and the experiment was performed as reported above.

Leaves were collected from treated grapevine plants an hour before pathogen inoculation. Subsequently, the leaf samples were processed as follows: 10 g of leaves were washed using agitation (200 rpm) in 100 ml of SSS and tergitol (0.01%) for 2 h. The resulting suspension was serially diluted and plated on LBA amended with kanamycin (25 mg l^{-1}), cycloheximide (100 mg l^{-1}) and $CuSO_4$ (250 mg l^{-1}), based on the reported resistance of AZ78 to kanamycin and $CuSO_4$ (Puopolo *et al.* 2014a). Once inoculated, Petri dishes were incubated at 27°C for 72 h, and CFU were counted to determine the AZ78 populations surviving on the grapevine leaves.

2.7. Sixth step: choosing whether to use additives as co-formulants or tank mix additives

The additives need to be compatible with the microorganism at the concentration found in the tank mix (in our case 0.1%). However, the stock concentration may be too high to be compatible with the cells. The compatibility of AZ78 cells with the concentrated mix of additives included in the combinations CPL and CGL was tested. Pelleted AZ78 cells were re-suspended in concentrated CPL stock containing 25% corn steep liquor, 25% polyethyleneglycol and 25% lignosulfonate and concentrated CGL stock containing 25% corn steep liquor, 25% glycerol and 25% lignosulfonate. Pelleted AZ78 cells re-suspended in SDW only were used as a control. After seven days, the shelf life of AZ78 cells was assessed as described previously.

2.8. Statistical analysis

Three batches were fermented for each medium in the production experiments and three bacterial cell stocks were prepared for each storage temperature in shelf life evaluation. Four replicates per treatment consisting of caps and LBA dishes were used respectively in the desiccation and UV-irradiation experiments. Four leaf disks (replicates) were used in rainfastness experiments and to determine side effects. Greenhouse and field experiments were carried out on four plants (replicates) for each treatment. All experiments were repeated, except for the greenhouse and field

experiments, which were carried out three times. Before statistical analysis, all the data obtained in the experiments were $\log_{10}$ (CFU and SR values) and arcsin-transformed (disease severity). The data attained in all the experiments were subjected to ANOVA according to a randomized block design, where each independent repetition of an experiment is a block. The effect of the block was not significant in any of the experiments and hence data from repeated experiments were pooled. Mean comparisons were performed with Tukey's test (α = 0.05). Data were analyzed using Statistica 7.1 (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Selection of growth conditions and medium for production of *L. capsici* AZ78 and assessment of the shelf life of the stored cells

Incubation for 24 h at 27°C and pH 7 determined the highest AZ78 cell growth (Fig. S1). Thus, these parameters were used to select the growth medium. There were no significant differences between PYNM, MYM and SYM media in the cells harvested after fermentation and they yielded 9.15 ± 0.09, 9.03 ± 0.18 and 9.08 ± 0.14 $\log_{10}$ cells ml^{-1} respectively after 24 h (mean value ± standard error; Fig. 2). On the other hand, the use of PYKM resulted in significantly higher yields of AZ78 cells (10.19 ± 0.15 $\log_{10}$ cells ml^{-1}; Fig. 2). This medium was therefore selected and used in all the experiments for the production of AZ78 cells.

AZ78 cells stored in SDW maintained their viability for up to half a year, but had a significant decrease in viability, by one order of magnitude, after one year of storage at 4°C (from 9.73 ± 0.10 to 8.70 ± 0.11 $\log_{10}$ cells ml^{-1}). Conversely, viability at 25°C was reduced by more than three orders of magnitude after the same storage time (from 9.73 ± 0.10 to 6.24 ± 0.11 $\log_{10}$ cells ml^{-1}; Fig. 3).

3.2. Selection of the best additives to increase *Lysobacter capsici* AZ78 tolerance to desiccation, UV-B light irradiation and wash off

The application of 1×10^8 AZ78 cells ml^{-1} resulted in a significant reduction in the disease severity of *P. viticola* on grapevine leaves as compared with control plants (Fig. S2). Although using 1×10^7 cells ml^{-1} also led to a significant reduction in disease severity, the protection achieved was however significantly lower than that obtained with the highest concentration (Fig. S2). Lower concentrations did not provide any disease reduction (Fig. S2). The minimum effective concentration of AZ78 was considered to be 1×10^7 cells ml^{-1}, while the optimal concentration was 1×10^8 cells ml^{-1}.

Exposure of AZ78 cells to 15% RH and 40 m s^{-1} wind speed was highly deleterious for AZ78 cells, significantly reducing viability by $\log_{10}$ -2.49 ± 0.21, -3.03 ± 0.31 and -5.93 ± 0.22 after 30, 60 and 120 min respectively (Fig. S3). With the sole exception of paraffin, addition of all the compounds at 0.1% (w/v) to the AZ78 cell suspensions provided a range of protection against these conditions for 30 min (Fig. 4 a). Arabic gum, carboxymethylcellulose, corn steep liquor, gelatin, glycerol, polyacrylate, polyethyleneglycol, polyvinyl alcohol, polyvinylpyrrolidone, skimmed milk, starch and xanthan gum significantly preserved the viability of cells after the stress (Fig. 4 a). Interestingly, polyethyleneglycol gave an SR of $\log_{10}$ 0.03 ± 0.09, meaning that it was able to effectively protect the viability of AZ78 cells, and it was significantly better than all the other compounds (Fig. 4 a).

Exposure of LBA dishes inoculated with AZ78 to UV-B light irradiation produced deleterious effects that depended on the dose. While 130, 260 and 490 J m^{-2} produced reductions of $\log_{10}$ -0.24 ± 0.06, -0.28 ± 0.02 and -0.62 ± 0.06 respectively, the dose of 780 J m^{-2} reduced the viability of AZ78 cells by $\log_{10}$ -1.90 ± 0.07 (Fig. S3). The addition of various compounds at 0.1% (w/v) to the AZ78 cell suspensions provided a range of protection compared to SDW when they were treated with 780 J m^{-2} of UV-B light irradiation (Fig. 4 b). All the tested compounds, except ascorbic acid,

folic and skimmed milk, had a significantly beneficial effect on AZ78 cell viability after exposure to UV-B light irradiation. AZ78 cell suspensions containing fluorescent brightener had the highest viability after UV-B light treatment, showing an SR of $\log_{10}$ -0.79 ± 0.04, more than one order of magnitude higher than SDW (Fig. 4 b). However, kaolin, lignosulfonate, molasses, and titanium oxide and zinc oxide provided levels of protection that were not significantly different from fluorescent brightener (Fig. 4 b).

Simulated rain resulted in a reduction in $\log_{10}$ -0.69 ± 0.06 and $\log_{10}$ -1.03 ± 0.04 of AZ78 cell concentration due to wash-off from leaf disks after 5 and 25 ml of simulated rain respectively (Fig. S3). The addition of various additives at 0.1% (w/v) to the AZ78 cell suspensions sprayed on the leaf disks provided protection against 25 ml of the simulated rain (Fig. 4 c). Corn steep liquor, arabic gum, molasses, polyvinyl alcohol, carboxymethylcellulose, polyvinylpyrrolidone and starch had significantly beneficial effects on AZ78 cell rainfastness compared to SDW (Fig. 4 c). Interestingly, leaf disks treated with AZ78 cell suspensions containing corn steep liquor and arabic gum showed increased populations of AZ78 compared to unwashed control disks, with SR of $\log_{10}$ 0.37 ± 0.02 and 0.15 ± 0.03 respectively (Fig. 4 c).

3.3. Assessing the side effects of the best additives on the grapevine, *Lysobacter capsici* AZ78 cells and the pathogen

Spraying the leaf disks with 0.1% (w/v) arabic gum, corn steep liquor, fluorescent brightener, glycerol, lignosulfonate, molasses, polyethyleneglycol, titanium oxide or xanthan gum, with or without AZ78 cells, did not determine signs of phytotoxicity on grapevine leaf disks. Leaf disks treated with SDW and inoculated with *P. viticola* showed a 64 ± 7% of disease severity (mean value ± standard error; Fig. 5). Spraying the leaf disks with most of the selected additives did not have any significant effect on disease development (Fig. 5). On the contrary, the fluorescent brightener significantly reduced the development of disease, achieving a level of disease control that was not

significantly different from that of AZ78 (2 ± 1%; Fig. 5). For this reason, fluorescent brightener was not used in subsequent experiments. Spraying the leaf disks with AZ78 cell suspensions containing 1×10^8 CFU ml^{-1} provided a significant reduction in disease severity - 88% compared to leaf disks treated with SDW. The addition of 0.1% (w/v) of the selected additives did not have any significant negative effect on the ability of AZ78 to control the disease (Fig. 5).

3.4. Validation of the combined additives on the efficacy and persistence of *Lysobacter capsici* AZ78 against *Plasmopara viticola* under field conditions

Plants treated with SDW showed a disease severity of 39 ± 4%, with no significant difference as compared to plants treated with CGL, CGT, CPL, and CPT (Fig. 6 a). In the field, the application of AZ78 cells, alone and in combination with CPT and CGT, had no significant effect on disease severity as compared to control plants (Fig. 6 a). Conversely, the efficacy of AZ78 amended with CGL and CPL was not different from that obtained with the copper treatment, with significant disease reduction of 71 and 63% respectively (Fig. 6 a).

In the field trials, AZ78 cells were not recovered from control plants treated with SDW, CGL, CGT, CPL, CPT and copper. On AZ78 treated plants, 1.94 ± 0.39 $\log_{10}$ CFU g^{-1} of leaf were recovered 24 h after the treatment (Fig. 6 b). The addition of CPT and CGT did not affect AZ78 population on the leaf, while plants treated with AZ78 with the addition of CPL and CGL showed significantly higher populations of AZ78 on the leaves, being 3.05 ± 0.48 and 2.92 ± 0.43 $\log_{10}$ CFU g^{-1} on the leaf respectively (Fig. 6 b).

3.5. Shelf life of *Lysobacter capsici* AZ78 cells formulated with the best combinations of additives

The combination of AZ78 cells with concentrated additives of the CPL and CGL resulted in a 2.65 and 2.49 $\log_{10}$ reduction in AZ78 cells ml^{-1} respectively after one week of storage at 4°C (Table S2).

In addition, a $\log_{10}$ reduction of 3.03 (CPL) and 2.69 (CGL) was registered at 25°C, (Table S2). On the other hand, the viability of AZ78 cells suspended in SDW only decreased by $\log_{10}$ 1.33 ± 0.06 after one week storage at 25°C, while it remained unaffected when the bacterium was stored at 4°C (Table S2).

4. Discussion

The stepwise flow diagram described here is the first protocol specifically dealing with the formulation of non-spore forming bacteria targeting foliar pathogens. In general, the existing literature only provides lists of additives or examples of formulation recipes without describing logical step-by-step decision-making criteria (Burges 1998; Schisler *et al.* 2004; Bashan *et al.* 2014). The diagram can provide guidance and decision-making criteria that will help other researchers to optimize their choices in this area. However, any formulation should be evaluated on a strain basis (Bashan *et al.* 2014), and on protocols adapted to specific needs. With this in mind the settings and cut-off criteria described here can be adjusted and modified to best fit the scope (i.e. growth media, harvesting technique, storage conditions, list of additives, etc.). In addition, decisions related to the use of one kind of harvesting process or additive need to be sound in terms of their economic feasibility (Ravensberg 2011). Despite such limitations, this research offers a straightforward stepwise flow diagram that can help researchers developing formulations, particularly for non-spore forming bacteria, in ways that are easy to prepare, stable, can protect bacteria in field conditions and are not phytotoxic. Following the proposed scheme, we were able to develop an effective formulation to preserve the biological control performance of AZ78 under field conditions. To our knowledge, this is the first time that a formulation for a member of the Xanthomonadaceae family has been developed.

As regards the cell mass production step, it is worth noting that yeast extract was used in all four media, since the high final growth of this substance (5 g l^{-1}) obtained at low concentrations could indicate a possible industrial application (Costa *et al.* 2001). The use of PYKM allowed us to reach concentrations of AZ78 cells that were approximately ten times higher than those achieved with other media. The concentration of AZ78 cells achieved with PYKM (10.19 ± 0.15 $\log_{10}$ CFU ml^{-1}) is also acceptable, since a threshold of 1×10^{10} CFU ml^{-1} is indicated as the minimum concentration that a bacterium should reach to be considered as a potentially useful strain for future development as an mBCA (Köhl *et al.* 2011).

In this work, we opted for a cell suspension in SDW when shelf life at 4°C was satisfactory. Cell harvesting was achieved by centrifugation, and bacterial cells were re-suspended in SDW. Removing the spent medium by centrifugation has been suggested as beneficial in terms of shelf life in various Gram-negative bacterial strains, when compared with keeping cells in the liquid from the fermenter (Slininger and Schisler 2013). It is also worth noting that the shelf life of AZ78 cells obtained with the method suggested in this work was interestingly similar to that achieved with freeze-drying, a more sophisticated and energy consuming technique. For instance, freeze-dried *Pseudomonas fluorescens* EPS62e cells with lactose as a lyoprotectant showed a decrease in concentration from 3×10^{11} to 1×10^{10} CFU g^{-1} after one year at 4°C (Cabrefiga *et al.* 2014). The physiological adaptation of *P. fluorescens* EPS62e cells during cultivation under salt-amended medium (osmoadaptation) increased shelf life, with the concentration being 9×10^{10} CFU g^{-1} after one year (Cabrefiga *et al.* 2014). However, there is still room for improvement of AZ78 cell shelf life by using other techniques in the future.

Exposing AZ78 cells to desiccation, UV-B light irradiation and rain simulation showed the susceptibility of AZ78 cells to agents likely to be encountered in field conditions. Sensitivity to low RH and sunlight has previously been reported for Gram-negative bacteria. For instance, the *Pantoea agglomerans* CPA-2 population on orange fruit showed a decrease of one order of magnitude when stored at 43% RH for 48 h, and a reduction of almost three orders of magnitude after 4 h of sunlight

exposure (Cañamás *et al.* 2008). Similarly, the population of *P. fluorescens* EPS62e on apple leaves experienced a sharp decline within a few days of application (Pujol *et al.* 2006).

The screening of compounds for their protective action in laboratory trials allowed us to identify compounds that might be used as additives for spray applications. The use of polyethyleneglycol resulted in a 107% protection against desiccation for the initial amount of AZ78 cells. It has already been reported that polyethyleneglycol acts as a protectant against desiccation when used to improve the survival of *Candida oleophila* O on apple fruit surface (Lahlali and Jijakli 2009). Interestingly, corn steep liquor allowed recovery of 156% of the initial amount of cells after simulated rain. To our knowledge, the use of corn steep liquor as a sticker has never been reported in the literature, while corn derivatives including starch have been reported to improve *Bacillus thuringiensis* field rainfastness (McGuire *et al.* 1996).

As regards tolerance to UV-B light irradiation, fluorescent brightener was the best performing additive, although it preserved the viability of only 16% of AZ78 cells. Fluorescent brightener 28 has been already used successfully to improve *Pseudoplusia includens* nuclear polyhedrosis virus activity (Zou and Young 1996). However, the results in the leaf disks assay showed that fluorescent brightener had a direct effect on the pathogen in the absence of AZ78. We therefore had to exclude fluorescent brightener from the formulation, since Regulation (EC) 1107/2009 indicates that additives must be inactive against phytopathogenic microorganisms.

AZ78 alone had no efficacy in the field trials, and this may be explained by its susceptibility to environmental factors such as temperature (Puopolo *et al.* 2015). Indeed, temperature values below the optimal temperature for AZ78 efficacy (25°C; Puopolo *et al.* 2015) occurred during the 24 h in which the experiments took place. Moreover, other environmental conditions such as UV light and desiccation negatively affected the persistence of AZ78 cells on grapevine leaves.

Interestingly, under the same conditions, AZ78 mixed with CPL and CGL additive combinations provided significant disease control, similar to that of copper. The relationship between microbial agent establishment and biocontrol efficacy was reported by Cañamás *et al.* (2008), who stated that

it is necessary to have a minimal antagonist population level on the target surface to obtain efficient control. Our results showed that the populations of AZ78 living on grapevines sprayed with the pilot CPL and CGL formulation were ten times higher than those recovered from plants treated with only AZ78 or with the addition of CGT or CPT. Thus, it is conceivable that the combination CPL and CGL guaranteed a concentration of AZ78 cells on grapevine leaves capable of controlling *P. viticola* attacks.

As mentioned previously, throughout this work we choose an additive concentration of 0.1%, based on the most common amount of commercial plant protection product applied to vineyards. Some authors have worked with concentrations much higher than those proposed here. For instance, Lahlali *et al.* (2011) used concentrations of up to 1% of additives to protect yeasts from UV light. However, storage and transport logistics and cost are practical aspects that have to be taken into account when designing a combination of additives (Ravensberg 2011). Similarly, McGuire *et al.* (1996) reported that use of a 4% mixture of flour and sucrose provided protection against environmental agents by *B. thuringiensis* on cabbage leaves. However, the authors also admitted that the required quantities limited the usefulness of that particular formulation (McGuire *et al.* 1996). The same kind of considerations also applies to the concentration of additives used in the formulation of AZ78 cells.

Since the working concentration of the bacteria is 1×10^8 cells ml^{-1}, the stock has to contain 1×10^{11} cells ml^{-1}, which agrees with the concentrations found in other commercial products based on Gram-negative bacteria (Stockwell and Stack 2007). Given that the concentrated additive stock is not compatible with AZ78 cells, we suggest that the prototype commercial product could be based on two separate bottles intended to be mixed in the spray tank: one for the additive mix and the other for bacterial cells.

To summarize, we present a stepwise flow diagram that was useful for developing a formulation for *L. capsici* AZ78 used to protect the grapevine against downy mildew. Furthermore, this stepwise

flow diagram, with the relevant strain-specific fine tuning, has the potential to help researchers to develop formulations for other mBCAs, especially for non-spore forming bacteria.

Conflict of interest

No conflict of interest declared

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Figure Captions

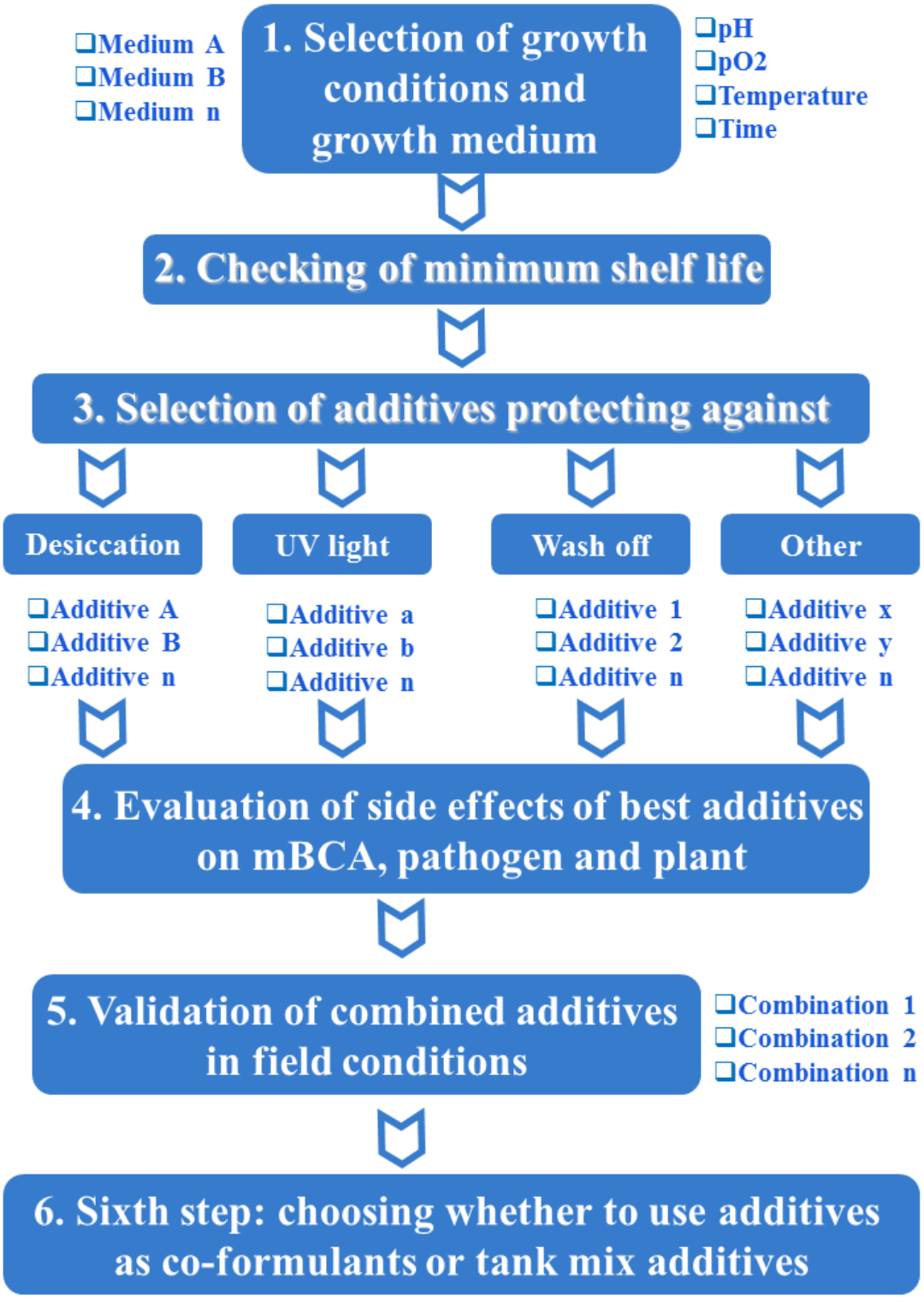

Figure 1 Stepwise flow diagram for the development of formulations of non spore-forming bacteria against foliar pathogens

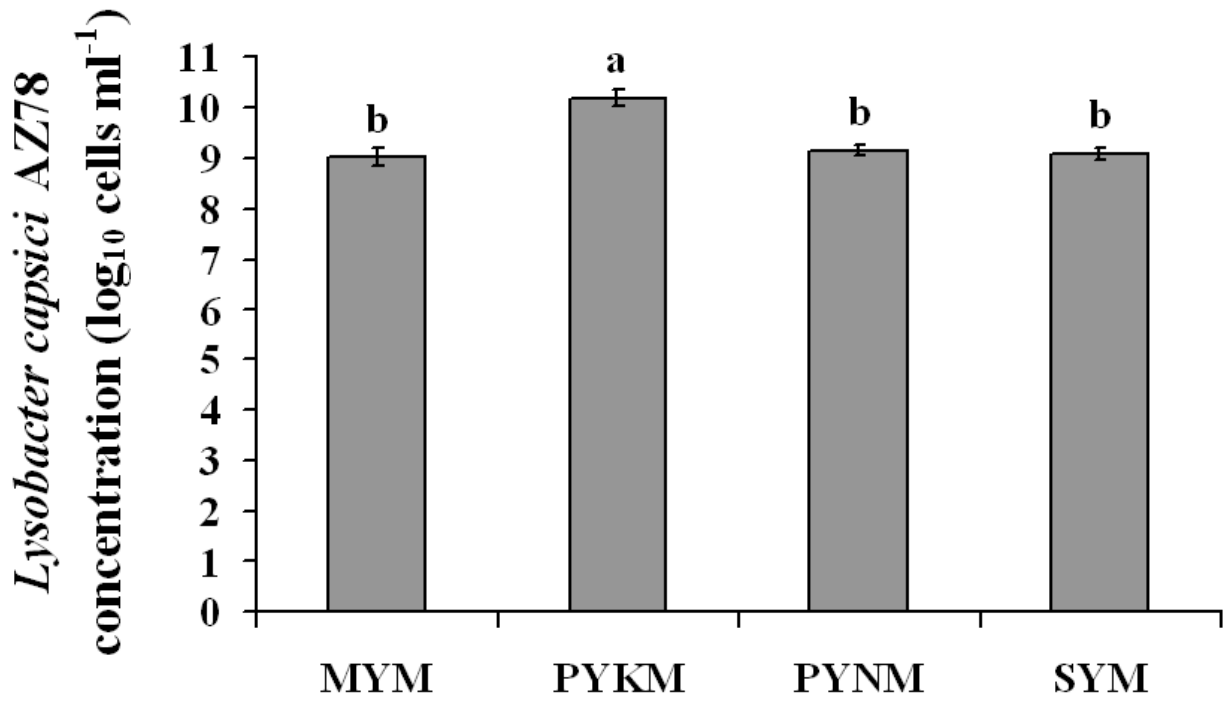

Figure 2 Production of *Lysobacter capsici* AZ78 cells in 4 different media after 24 h of incubation in a 5 L fermenter. MYM (Molasses 20 g l^{-1} and Yeast Extract 5 g l^{-1}), PYKM (Peptone 10 g l^{-1}, Yeast Extract 5 g l^{-1}, KH_2PO_4 1.4 g l^{-1} and $MgSO_4 \times 7H_2O$ 1 g l^{-1}), PYNM (Peptone 10 g l^{-1}, Yeast Extract 5 g l^{-1} and NaCl 5 g l^{-1}), SYM (Sucrose 10 g l^{-1} and Yeast Extract 5 g l^{-1}). Three replicates were performed for each medium and data originating from two independent experiments were pooled. Different letters show significant differences, $\alpha = 0.05$, in Tukey's test. Data shown are means ± SE.

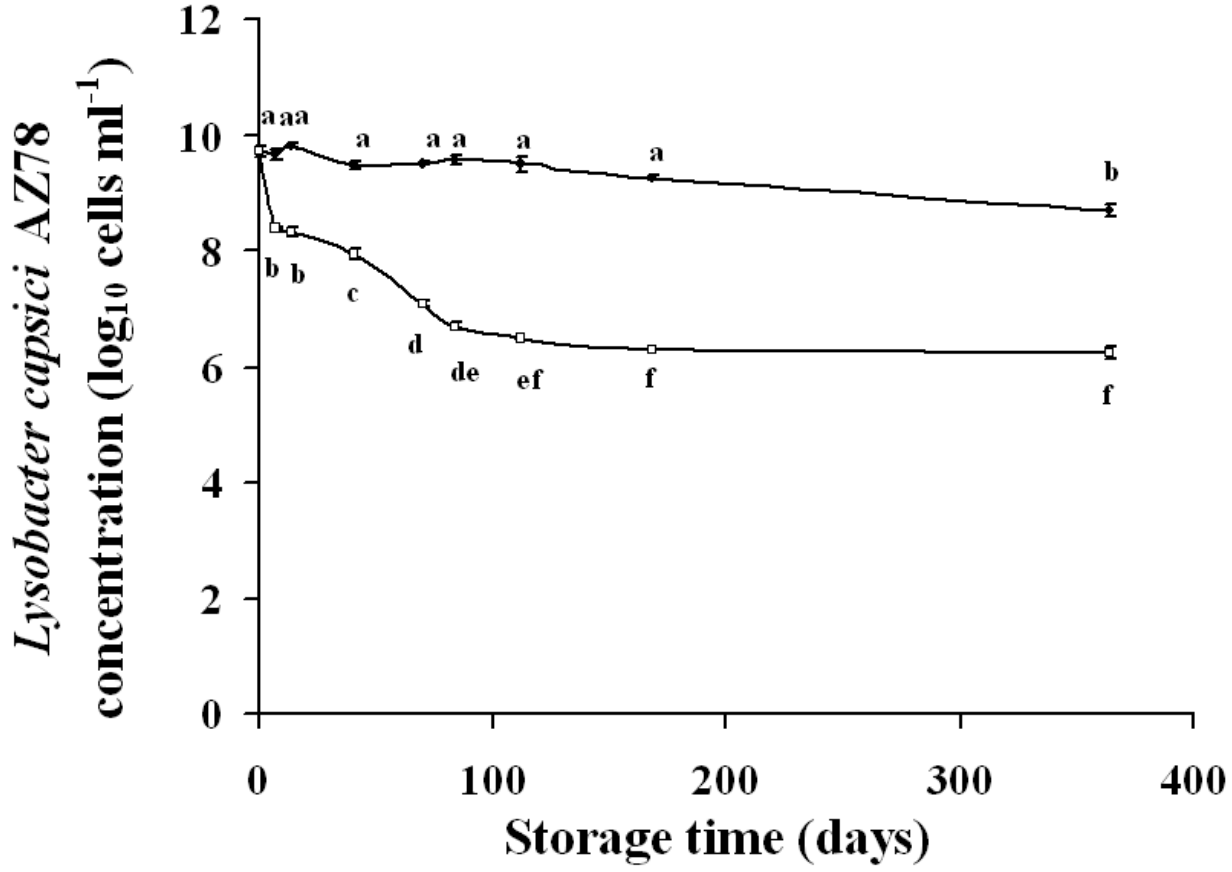

Figure 3 Survival of *Lysobacter capsici* AZ78 in sterile distilled water stored at 4 (●) or 25°C (□). The initial concentration of cell stocks was 9.73 ± 0.10 $\log_{10}$ cells ml^{-1}. Three bacterial cell stocks were prepared for each storage temperature and data originating from two independent experiments were pooled. Different letters show significant differences, $\alpha = 0.05$, in Tukey's test. Data shown are means ± SE.

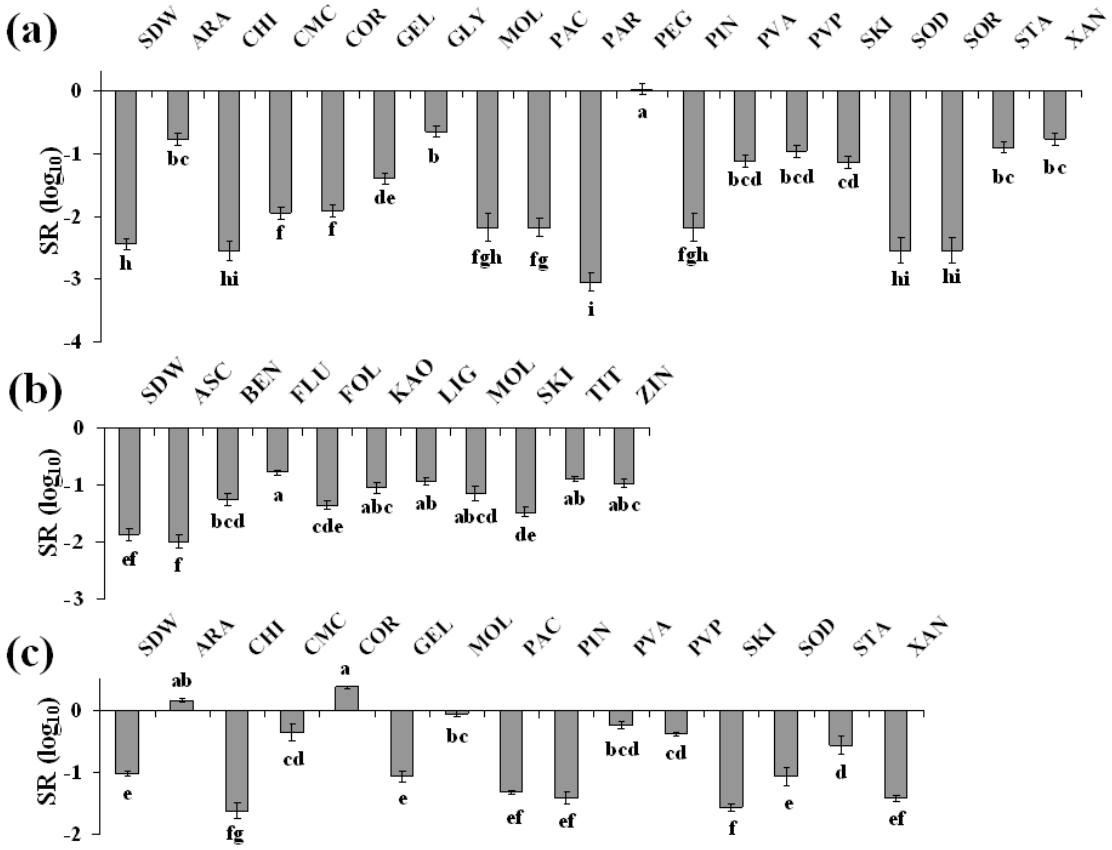

Figure 4 Effect of various additives in protecting *Lysobacter capsici* AZ78 cells against **(a)** desiccation (15% RH and 40 m s^{-1} wind speed for 30 min), **(b)** UV-B light irradiation (780 J m^{-2}) and **(c)** cell washing-off (25 ml of simulated rain). Additives were added to the AZ78 cell suspension at a final concentration of 0.1% (w/v). Survival ratio (SR) was calculated as the ratio of the AZ78 CFU developed from treated samples to the AZ78 CFU from non treated samples and expressed as $\log_{10}$. SDW, sterile distilled water; ARA, arabic gum; ASC, ascorbic acid; BEN, bentonite; CHI, chitosan; CMC, carboxymethylcellulose; COR, corn steep liquor; FLU, fluorescent brightener; FOL, folic acid; GEL, gelatine, GLY, glycerol; KAO, kaolinite; LIG, lignosulfonate; MOL, molasses; PAC, polyacrylate; PAR, paraffin; PEG, polyethyleneglycol; PIN, pinolene; PVA, polyvinylalcohol; PVP, polyvinylpyrrolidone; SKI, skimmed milk; SOD, sodium alginate; STA, starch TIT, titanium oxide; ZIN, zinc oxide and XAN, xanthan gum. Each treatment included four replicates and data originating from two independent experiments were pooled. Different letters show significant differences, $\alpha = 0.05$, in Tukey's test. Data shown are means ± SE.

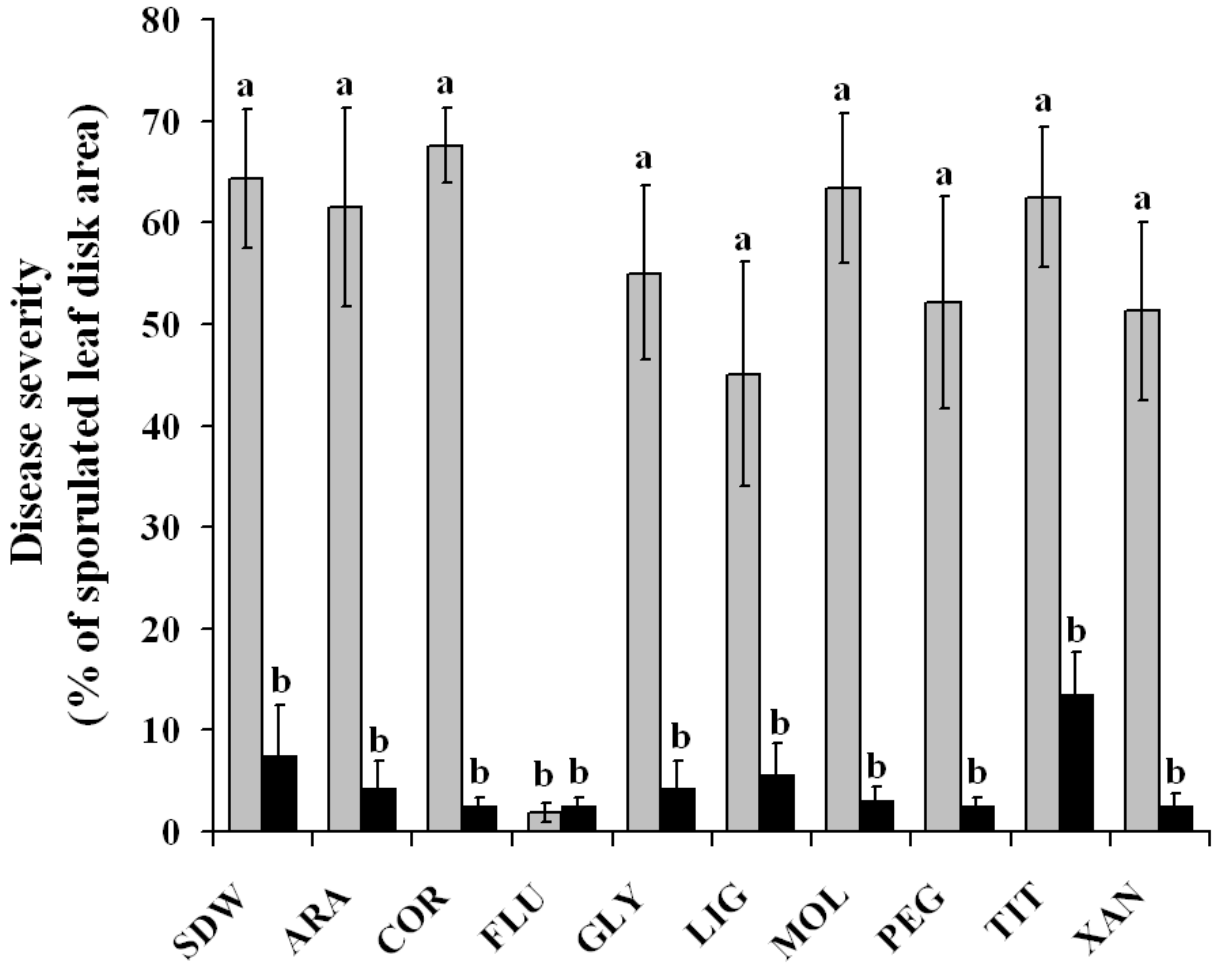

Figure 5 Effect of various additives used at 0.1% (w/v) on the disease severity caused by *Plasmopara viticola* on grapevine leaf disks with (black bars) or without (gray bars) *Lysobacter capsici* AZ78. SDW, sterile distilled water; ARA, Arabic gum; COR, corn steep liquor; FLU, fluorescent brightener; GLY, glycerol; LIG, lignosulfonate; MOL, molasses; PEG, polyethyleneglycol; TIT, titanium oxide and XAN, xanthan gum. Each treatment included four replicates and data originating from two independent experiments were pooled. Different letters show significant differences, $\alpha = 0.05$, in Tukey's test. Data shown are means ± SE.

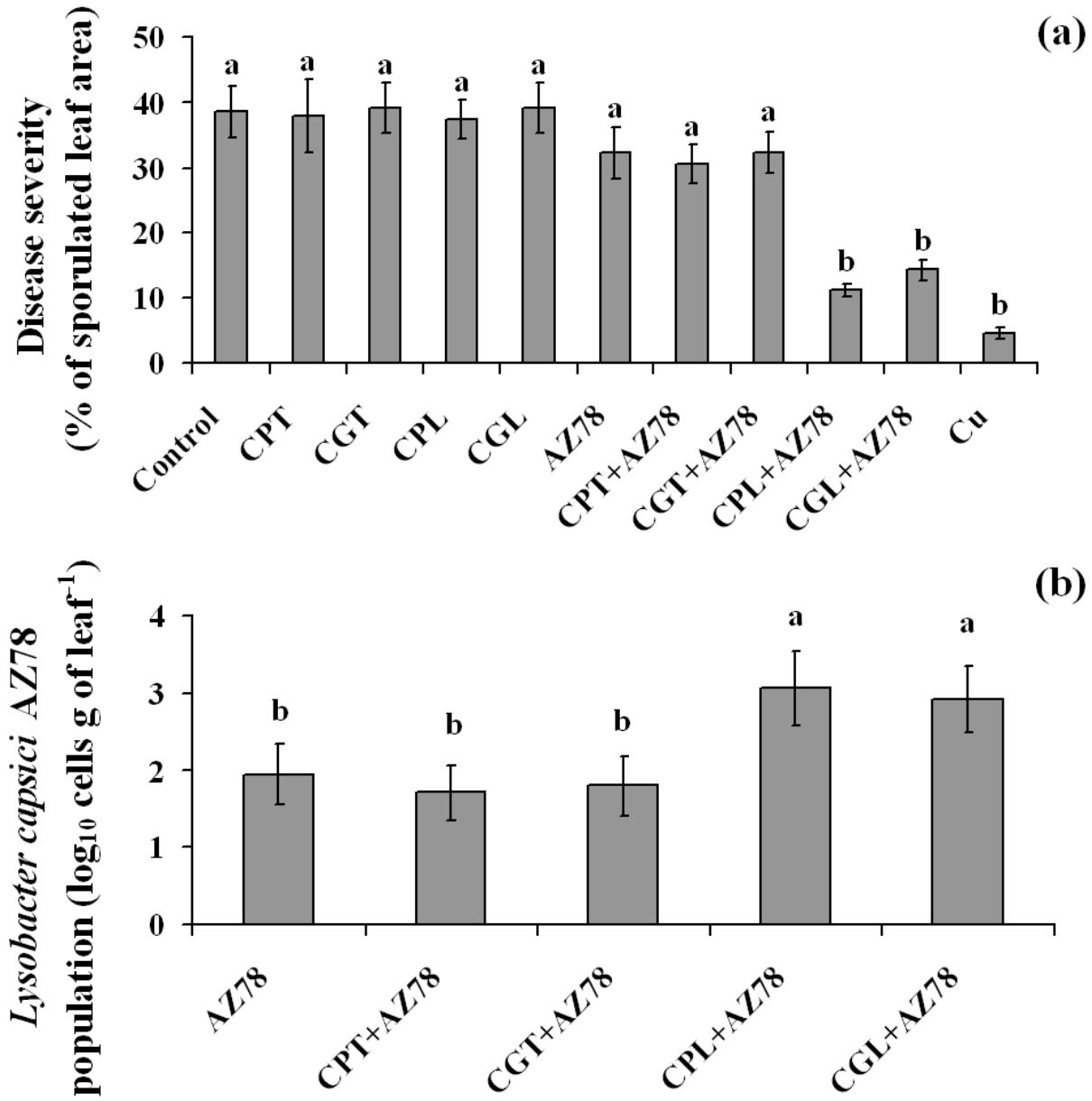

Figure 6 Effect of four combinations of additives on the ability of *Lysobacter capsici* AZ78 to **(a)** control *Plasmopara viticola* and **(b)** colonize leaves of potted grapevine plants in field conditions. Control, plants treated with water; AZ78, treated with 10^8 cells ml^{-1} of AZ78; CPT, plants treated with 0.1% of corn steep liquor, polyethyleneglycol and titanium oxide; CGT, plants treated with 0.1% corn steep liquor, glycerol and titanium oxide; CPL, plants treated with 0.1% corn steep liquor, polyethyleneglycol and lignosulfonate; CGL, plants treated with 0.1% corn steep liquor, glycerol and lignosulfonate; CPT+AZ78, plants treated with CPT plus AZ78; CGT+AZ78, plants treated with CGT plus AZ78; CPL+AZ78, plants treated with CPL plus AZ78; CGL+AZ78, plants

treated with CGL plus AZ78; Cu, plants treated with 2 g l^{-1} of Coprantol HiBio composed of 25% $Cu(OH)_2$. Each treatment included four replicates and data originating from three independent experiments were pooled. Different letters show significant differences, $\alpha = 0.05$, in Tukey's test. Data shown are means ± SE.