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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

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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.

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; 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

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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.

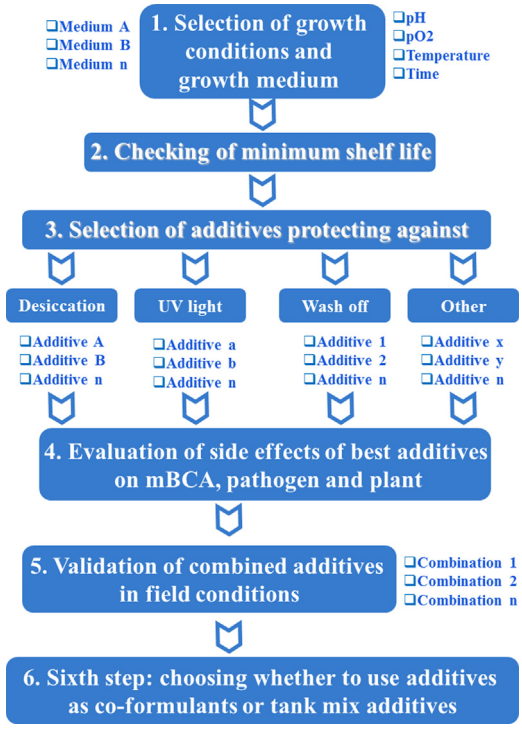

Fig. 1. Stepwise flow diagram for the development of formulations of non spore-forming bacteria against foliar pathogens.

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