ABSTRACT

Biopesticides are an interesting solution in integrated pest management (IPM). Developing a microbial antagonist to a usable product is a long path with many production, formulation and legislative hurdles. During the production and formulation steps, the efficacy against pests and pathogens, storability and ease of application have to be kept in mind to deliver a product that is accepted by the end consumer.

In this report the quality of the bacterium *Pseudomonas fluorescens* Pf153 (Pf153) was optimized by developing appropriate production technologies. Freeze-drying was chosen as conservation process and it was shown that the freezing rate and the added cryo-protectant agent (CPA) influence the survival of *Pseudomonas* spp. CPA can increase the viability during the process and during storage and influences the efficacy of the formulated bacterium but also the pathogen activity. In particular for Pf153 the survival after freeze-drying and storage could be increased by using 0.04-0.12 °C/min as freezing-rate, 30 °C for the drying process and lactose as CPA. Since production and formulation are closely linked, the fermentation parameters have also to be optimized for high biomass production with high survival during formulation, storage and with a high efficacy. The tested media, fermentation temperatures, mild heat shocks and pH changes influenced the viability of Pf153 after freeze-drying with survival rates varying between 28% and 100%. Efficacy against *Botrytis cinerea* on *Vicia faba* leaves was increased only when the bacterium was fermented at 20 °C. This temperature "stabilized" the survival after freeze-drying, increased the storage viability at 25 °C for 12 weeks and showed a slightly better efficacy against five diverse *B. cinerea* strains in dual culture tests.

Different behaviour under diverse conditions is a big issue in the performance of formulated bioproducts. Trichostar®, RhizoVital® 42 fl. and *Metarhizium brunneum* Ma43 (Ma43) were tested, in greenhouse and field trials, on various growth parameters and yield of strawberries in the presence of soil pathogens. In greenhouse trials, Trichostar® had a poor performance compared to the control sample without treatment, when the strawberry plants were inoculated with *Phytophthora cactorum* and *Verticillium dahliae*. On the other hand, it increased the studied parameters when no pathogen was inoculated. RhizoVital® 42 fl. showed a better performance than Trichostar® in the presence of the pathogens in greenhouse trials. In commercial fields, the two selected products showed a good performance with an increased strawberry yield of about 8% for Trichostar® and 6% for RhizoVital® 42 fl., in two consecutive years on two different fields. The soil properties

influenced the performance more than the weather conditions, however the application schedule should be ameliorated. Ma43 was tested in greenhouse and field controlled trials. In foregoing trials it increased strawberry yield, but in the trials presented here, its positive influence could not be confirmed. The contemporaneous application of the Trichostar® and RhizoVital® 42 fl. with or without Ma43 did not show an increase in their efficacy. The compatibility of the micro-organisms with chemical pesticides (12 fungicides, one insecticide and one acaricide used in strawberry production) showed their potential in IPM.

The results presented here, show the potential of biopesticides and their dependence from production and formulation parameters. Storage and efficacy are influenced by biotic and abiotic factors and not all of them can be controlled. Field application tests with the end product are urgently needed, because until now lab results could not predict the performance at its application site. The choice of a bioproduct has to be rational and judicious since more selection parameters have to be kept in mind in reference to chemical ones. Bioproducts can help to increase production, as shown for strawberries, with less depletion and pollution and without great changes for the grower.

ZUSAMMENFASSUNG

Biopestizide sind im integrierten Pflanzenschutz (IPM) eine interessante Lösung. Die Entwicklung eines mikrobiellen Antagonisten zu einem verwendbaren Produkt ist ein viele Disziplinen umfassender Prozess. Die Produktions- und Formulierungsschritte müssen erforscht werden, die Wirksamkeit gegen Krankheitserreger und Schädlinge und die Lagerfähigkeit und einfache Anwendung der Produkte müssen erarbeitet und erhöht werden.

In diesem Bericht wurde die Qualität des Bakteriums *Pseudomonas fluorescens* Pf153 (Pf153) durch die Entwicklung geeigneter Produktionstechnologien optimiert. Die Gefriertrocknung wurde als Trocknungsmethode gewählt und diverse *Pseudomonas* spp. zeigten unterschiedliche Überlebensraten, bei unterschiedlichen Gefrierraten aber nicht so bei den Trocknungstemperaturen die weniger stammabhängig waren. Die Lebensfähigkeit während des Trockungsprozesses und der Lagerung wurde mit der Zugabe von Kryoschutzmitteln (CPA) verbessert. Das Überleben nach Gefriertrocknung und Lagerung konnte durch die Verwendung der angepassten Gefrierrate 0.04-0.12 °C/min, die Trocknung bei 30 °C und Laktose als CPA für Pf153 erhöht werden. Die Wirksamkeit der gefriergetrockneten Zellen wurde in diversen Biotests gezeigt und diese war mit der Wirksamkeit von frischen Zellen vergleichbar. Diverse Fermentationsparameter wurden getestet um die Lebens- und Lagerfähigkeit und die Wirksamkeit von Pf153 nach Gefriertrocknung zu erhöhen: unterschiedliche Medien, Fermentationstemperaturen, milde Hitzeschocks und pH-Änderungen. Die Überlebensraten variierten zwischen 28% und 100%. Die Wirksamkeit wurde durch Änderungen der Fermentationstemperatur erhöht, jedoch konnte der Einfluss des Zellalters auf die zunehmende Wirksamkeit nicht ausgeschlossen werden. Niedrigere Temperaturen (20 °C) erhöhten die Lagerfähigkeit bei 25 °C für 12 Wochen und zeigten die beste Wirkung gegen fünf verschiedene *B. cinerea*-Stämme in Doppelkultur-Tests gegenüber Zellen, die bei 28 °C gezüchtet und in derselben Phase geerntet wurden.

Unterschiedliche Wirksamkeit unter verschiedenen Bedingungen ist ein großes Problem bei der Leistungsfähigkeit der formulierten Produkte. Trichostar®, RhizoVital® 42 fl. und *Metarhizium brunneum* Ma43 (Ma43) wurden in Gewächshaus- und Feldversuchen auf verschiedene Wachstumsparameter getestet und der Ertrag von Erdbeeren in Gegenwart von Krankheitserregern ausgewertet. In. Gewächshausversuchen zeigte das Trichostar® im Vergleich zur unbehandelten Kontrollprobe eine schlechtere Leistung;

wenn die Erdbeerpflanzen mit *Phytophthora cactorum* und *Verticillium dahliae* beimpft wurden. Andererseits erhöhte Trichostar® die untersuchten Parameter, wenn kein Erreger geimpft wurde. RhizoVital® 42 fl., zeigte eine bessere Leistung als Trichostar® in Anwesenheit der Pathogene in Gewächshausversuchen. Einen erhöhten Erdbeerertrag von etwa 8% für Trichostar® und 6% für RhizoVital® 42 fl., wurde im kommerziellen Bereich, in zwei aufeinander folgenden Jahren auf zwei verschiedenen Feldern erzielt. Es scheint, dass die Bodenbeschaffenheit die Leistung stärker beeinflusste als die Witterungsbedingungen. Ma43 wurde ebenfalls getestet. In früheren Versuchen konnte der Erdbeerertrag erhört werden, aber sein positiver Einfluss konnte hier nicht bestätigt werden. Die gleichzeitige Anwendung aller drei Bioprodukte zeigte keine Steigerung deren Wirksamkeit. Die Verträglichkeit der Mikroorganismen mit im Erdbeeranbau gängigen chemischen Pflanzenschutzmitteln (12 Fungizide, ein Insektizid und ein Akarizid) zeigt ihr Anwendungspotenzial im IPM.

Die vorgestellten Ergebnisse zeigen das Potenzial von Biopestiziden und ihre Abhängigkeit von Produktions- und Formulierungsparametern. Lagerung und Wirksamkeit werden durch biotische und abiotische Faktoren beeinflusst die nicht alle kontrolliert werden können. Feldanwendungstests mit dem Endprodukt sind dringend erforderlich, da die Laborergebnisse bisher die Leistung am Einsatzort nicht vorhersagen können. Die Wahl eines Bioprodukts muss rational und vernünftig sein, und es müssen mehr Auswahlparameter berücksichtigt werden als bei chemischen Produkten. Bioprodukte können dazu beitragen, die Produktion zu steigern, wie es bei den Erdbeeren der Fall war, mit weniger Umweltverschmutzung ohne dass der Landwirt große Veränderungen vornehmen muss.

CHAPTER 1

GENERAL INTRODUCTION

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New challenges and incentives in agriculture

Modern farming is confronting an increasing demand for steady supply and high-quality fresh goods (Berninger et al., 2018; Emmert and Handelsman, 1999; Gamliel, 2010). Nowadays, the way to make and provide healthy products is to increase the efficient use of available resources (Colla and Rouphael, 2015; Parnell et al., 2016). Maximizing profit by increasing production led to an intensive use of the existing fields (Chellemi et al., 2016) resulting in an increase in infestation and damage by various pests (Gamliel, 2010). Plant disease control is therefore still a "pressing need" for agriculture in this century; controlling diseases that reduce crop yield is required (Emmert and Handelsman, 1999). Chemical pest management in crops is becoming a challenging task, due to the strong requirement to minimize or avoid the use of pesticides (Gamliel, 2010; Singh and Singh, 2009). In addition, resistance to synthetic pesticides is also an increasing problem (Chandler et al., 2011; Droby et al., 2016; Grabke and Stammler, 2015; Lefebvre et al., 2015; Mnif and Ghribi, 2015; Siegwart et al., 2015). Indeed, the awareness regarding environmental degradation and health risk associated with chemical pesticides, increasing production costs and crop losses due to diseases even by increased use of pesticides, opened the door to alternative approaches to pest management (Chellemi et al., 2016; Ji et al., 2006; Siegwart et al., 2015).

In Europe, conventional agriculture based on chemical pesticides is currently in transition to integrated pest management (IPM) due to changes in the European legislation which has as objective human health and environment protection (Lamichhane et al., 2017). The European Directive 2009/128/EC on sustainable use of pesticides was an important step in the reduction of utilization of chemical pesticides. This Directive requires, starting 2014, the implementation of the general principles of IPM in which non chemical methods must be preferred and pesticides should have the smallest possible impact on non target organism and the environment (Colla et al., 2012). The Food and Agriculture Organization of the United Nations (FAO) defines IPM as "the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms" (FAO, 2018). In Annex III of Directive 2009/128/EC the general principles are explained (European Commission, 2009a). IPM practice helps to reduce insecticide use, but for the management of soil pathogens, its application is more difficult (Chellemi et al., 2016). Against soil pathogens, chemical fumigation has provided great benefits to agricultural production for many years (Gamliel, 2010), but after the withdrawal of methyl bromide, in countries that used it very intensive, some phytopathological problems became difficult to manage (Colla et al., 2012).

The Directive 2009/128/EC implies the need to apply diverse biological and cultural strategies to reduce synthetic pesticides. Diverse techniques are currently available and the ones generally used in organic farming become of increasing interest also in conventional farming. Good agricultural practices help to prevent or reduce disease symptoms, however the use of fungicides, soil fumigants and disease resistant varieties, hybrids or rootstocks, still constitute the major effective tools to manage plant diseases (Tjamos et al., 2010). In IPM the decision to apply plant protections measures is based on threshold levels of harmful organisms based on monitoring results (European Commission, 2009a). Therefore diverse strategies can be applied to control pests to reduce the use of conventional pesticides, going from cultural and physical controls to host resistance including transgenic plants (Eilenberg et al., 2001). Well known are, for example, crop rotation, the use of resistant or tolerant cultivars, mulching, mix cultures, minimum tillage, biocontrol agents, but also hygienically rules and computer supported models for pest alert, which are very important to reduce or to curtail pest (Colla et al., 2012; Gamliel, 2010; Koike and Gordon, 2015). For soil-borne disease management, where all sources of inoculum among the entire disease cycle have to be considered, measures have to include actions such soil disinfestation and sanitation (Gamliel, 2010).

Biological control or biocontrol agents (BCAs) are in line with the principles listed in Annex III (Matyjaszczyk, 2015) and have the potential to become one main pillar of IPM practice (Lamichhane et al., 2017). BCAs may provide an integration or an alternative to chemical pesticides to manage plant disease (Ji et al., 2006; Lamichhane et al., 2017; Mathivanan et al., 2005; Tjamos et al., 2010). Beneficial micro-organisms received increasing levels of attention since the middle of the 1990ties as biofertilizers, to improve plant growth, and as BCAs, to manage plant disease (Mathivanan et al., 2005) because of consumers' concerns regarding the residues of chemical pesticides (Ji et al., 2006; Liu et al., 2014; Nehra and Choudhary, 2015) and the environmental pollution (Mathivanan et al., 2005; Nehra and Choudhary, 2015). Ease to handle and application with standard machinery are important features for growers (Bashan et al., 2014; Berninger et al., 2018).

Plant growth-promoting rhizobacteria viz. micro-organism that can influence plants and their environment

BCAs are part of the biological control system where living organisms are used to suppress pest population or their damaging impact (Eilenberg et al., 2001). BCA are often found in the well known group of plant growth-promoting rhizobacteria (PGPR). PGPR are soil free-living bacteria that inhabit the rhizosphere (zone around the root), the rhizoplane (root surface) and/or live within the root (endophytes) (Ruzzi and Aroca, 2015). These micro-organisms can benefit from the organic compounds released by the roots as carbon and energy source (Chauhan et al., 2015). Generally PGPR (and root colonizing plant beneficial fungi) exert their mode of action: by changes in plant hormonal balances; through production of volatile organic compounds; by increasing the nutrient availability and favour nutrient uptake by plants; by influencing the abiotic tolerance of plants (Ruzzi and Aroca, 2015); by competitive colonization of plant roots; by stimulation of plant growth and/or reduction of plant disease incidence (Haas and Défago, 2005) and by modulating the root architecture (Vacheron et al., 2016; Verbon and Liberman, 2016). Their mode of action classifies them into three bioproducts: biostimulants, biofertilizer and biocontrol agents. **Biostimulants** are defined as substances or micro-organisms, that enhance nutrition efficiency uptake, tolerance to abiotic stress and/or crop quality traits when applied to plants (Chojnacka, 2015; du Jardin, 2015). **Biofertilizers** increase the availability of nutrients and their utilization by the plants (Chojnacka, 2015; du Jardin, 2015; Nehra and Choudhary, 2015). These micro-organisms can be seen as living fertilizers (Chojnacka, 2015) that act for example through nitrogen fixation or phosphate solubilisation (Haas and Défago, 2005). In this case, the deleterious effects of plant pathogens is reduce or even prevented by the indirect plant growth promotion of PGPR, by producing antagonistic substances or by inducing resistance (Beneduzi et al., 2012).

BCAs are living organisms that protect plants against enemies (du Jardin, 2015) and are comprised in the biopesticide group (Chojnacka, 2015; Haas and Défago, 2005). BCA exert their action directly through: antibiotics; by production of enzymes; by secretion of volatile toxic metabolites; by competition for nutrient resources and essential micronutrients; by interfering with the pathogenesis mechanism; by hyperparasitismus and/or indirectly by inducing resistance (Bardin et al., 2015; Beneduzi et al., 2012; Bhattacharjee and Dey, 2014; Robinson-Boyer et al., 2009; Tjamos et al., 2010). It has been found, that BCA can selective compensate the impact of a pathogen on the plant-associated microbiota. This could be originate by direct impact on the microbiota or by indirect impact on the pathogen (Massart et al., 2015a). The most representative species under the PGPR are *Pseudomonas* and *Bacillus* spp. (Nehra and Choudhary, 2015), which are antagonists of recognized root pathogens.

***The bacterial genus* Pseudomonas**

Members of the genus *Pseudomonas* are rod-shaped motile aerobic Gram-negative bacteria characterized by metabolic versatility (Haas and Défago, 2005; Mnif and Ghribi, 2015), due to the presence of a complex enzymatic system (Mnif and Ghribi, 2015), and a genomic G+C content of 59–68% (Haas and Défago, 2005). Fluorescent pseudomonades, effective rhizosphere bacteria, exert beneficial effect on plant growth promotion in addition to disease control (Commare et al., 2002; Mathivanan et al., 2005). Known to survive in both the rhizosphere and the phyllosphere, they can be used as BCA for the management of foliar infection (Rabindran and Vidhyasekaran, 1996) and as effective strategy against soil-borne disease (Nandakumar et al., 2001). To control soil-borne diseases, pseudomonades produce secondary metabolites and antifungal compounds, compete for nutrients, or produce lytic enzymes that act on fungal cell wall components (Commare et al., 2002). Additionally, they can induce systemic resistance and thus protect the leaves when foliar diseases are controlled by application of the bacteria as seed, soil or root treatments (Vidhyasekaran et al., 1997).

Among fluorescent pseudomonades, *Pseudomonas fluorescens* play an important role in biological control of pathogens. They dominate in the rhizosphere and possess several properties that allow them to be considered the prime candidates for biological control. *P. fluorescens* has excellent root-colonizing abilities, grow rapidly and is able to exploit root exudates, and colonise plant roots of several crops. They can significantly increase yield and enhance plant growth which is often accompanied by reductions of pathogenic fungi and bacteria in the root zone population (Gade and Armarkar, 2011). *P. fluorescens* are interesting bacteria also because of their catabolic versatility, and their capacity to produce diverse antifungal metabolites (Mukherjee and Babu, 2013).

Pseudomonas derived bioactive compounds include: 1. biosurfactants like lipopeptide and rhamnolipid with their efficient antifungal activities; 2. mycolytic enzymes e.g. protease, lipase and glucanase; 3. secondary bioactive metabolites like hydrogen cyanide, salicylic acid and iron chelating siderophores with the ability to reduce plant fungi growth and infection; 4. bacteriocine like the lectin-like bacteriocin produced by *P. fluorescens* Pf-5 and active against a wide variety of phytopathogenic fungi and bacteria. In addition *Pseudomonas* derived biosurfactants and secondary metabolites like phenazines and siderophores can induce systemic resistance in plants (Mnif and Ghribi, 2015). It is also reported that *Pseudomonas* biocontrol strains were observed at the root surface, forming micro-colonies or discontinued biofilms, capable of endophytic behaviour in the intercellular spaces of the epidermis and the cortex (Maurya et al., 2014).

Diverse *P. fluorescens* products were developed and tested in recent years. Two *Pseudomonas* strains are listed in the European Union (EU) as active substances: *P. chlororaphis* MA 342 and *Pseudomonas* spp. strain DSMZ 13134. *P. chlororaphis* MA342 is authorized at a national level in 14 nations, DSMZ13134 in 15 nations and in one the authorization is in progress (EU, 2018). In Germany, both are approved as fungicide (Table 2). *P. chlororaphis* MA 342 is sold in two products, as emulsion or flowable concentrate for seed treatment, for spelt, barley, rye, triticale and wheat. It is used against *Tilletia caries*, *Tilletia foetida, Fusarium* spp., *Pyrenophora graminea*, *Pyrenophora teres* and *Septoria nodorum*. *Pseudomonas* spp. strain DSMZ 13134 is sold as wettable powder for potatoes against *Rhizoctonia solani.*

Production and formulation of bioproducts

The production and formulation of bioproducts is a challenge. Many potential highly useful strains are described in the scientific literature, but just a few arrive on the commercial market (Bashan et al., 2014). In 2005, Haas and Défago stated that to find an effective biocontrol PGPR strain is time consuming because no *in vitro* diagnostic kits were available. Nowadays, through the easier access to molecular technologies, screening can be almost made *in vitro*. Beside the classical methods in which the production of secondary metabolisms are tested, polymerase chain reaction (PCR) targeting relevant genes involved in defence traits can be used (Hol et al., 2013; Vacheron et al., 2016). The discovery of the adequate micro-organism is just the first step in the BCA research (Bashan et al., 2014). The following steps to produce a micro-organism ready for commercial use, involve collaboration of diverse disciplines (see figure 1) and generate high costs (Droby et al., 2016; Köhl et al., 2011). Different issues have to be considered for a commercial product: it has to be compatible with field routine practice, storable, adaptable to different

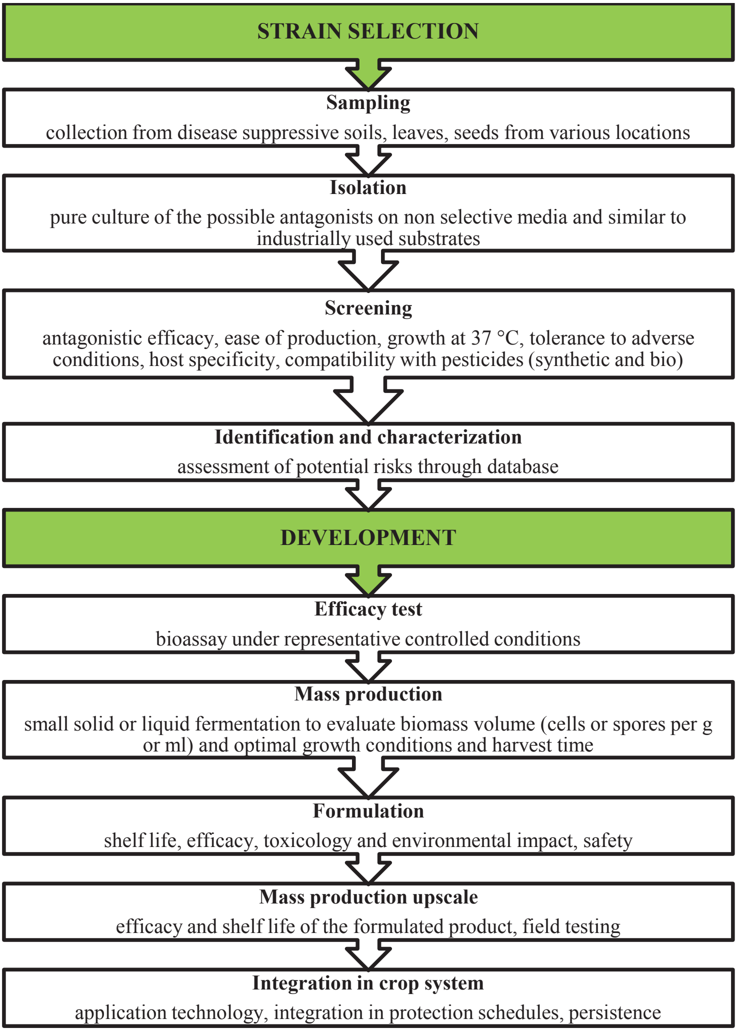

Figure 1: Steps from screening to development of a microbial pesticide for a target crop and disease. For the industry the costs for development are also important but not considered here. Costs depend on the market size, presence of competitive products, availability of the selected micro-organism (patents), regulations and registration costs (Jackson, 1997; Jensen et al., 2016; Köhl et al., 2011; Montesinos, 2003).