ENDURE

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Glossary

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Summary

For the development of new biocontrol products against plant diseases high numbers of candidate antagonists have to be evaluated in screening programs. Antagonists for commercial use have to fulfil many different requirements. Besides being active control agents against the specific targeted plant pathogens, they must be safe, suitable for registration and cost effective. Important criteria besides efficacy in disease control are market size, ecological characteristics, production costs, safety, environmental risks and possibilities for protection of intellectual property rights. A stepwise screening considering these very different aspects is proposed.

The present document results from the collective effort of researchers from three European research institutions (DLO, INRA and CNR) together with the International Biocontrol Manufacturers Association (IBMA).

The organisation and edition of this document was coordinated by J. Köhl (DLO).

1. Introduction

Antagonism between microorganisms is a common phenomenon. Also plant pathogenic fungi and bacteria can be affected by fungal and bacterial antagonists (Cook and Baker, 1883). Such a naturally occurring interference between beneficial microorganisms and plant pathogens contributes to the natural buffering of cropping systems, thus preventing or limiting disease development. This is most obvious in suppressive soils where an antagonistic potential has been built up in the presence of the pathogen population (Weller *et al.*, 2002). One of the starting points of research on biological control was the identification of the role of antagonistic microorganisms responsible for such a natural buffering, for example saprophytes preventing colonization of cereal stubble by *Fusarium roseum* 'Culmorum' (Cook and Bruehl, 1968; Cook, 2008). In a next step, the use of antagonistic microorganisms has been studied in inundation biological control. Potential antagonists are multiplied in the laboratory and applied to crops for disease control (Eilenberg *et al.*, 2001). During the last decades, antagonists have been successfully targeted at diseases of seedlings, roots, leaves and fruits (Paulitz and Bélanger, 2001; Sharma *et al.*, 2009; Smilanick, 1994; Weller, 1998; Whipps, 2001). Seed and soil treatments have been developed as well as spray applications for treatments of leaves or fruits. Post-harvest applications were also successfully used to protect fruit commodities from decay during transportation and storage.

After experimental research demonstrated the successes of inundation biological control of plant pathogens, the development of biological control for practical use was investigated. First commercial products for use by growers were introduced to the market, e.g. BINAP T in 1976 in France (Ricard and Ricard, 1997) and in 1989 with the United States Environmental Protection Agency (EPA) (Fravel, 2005). In 2005, 26 microbial products for biological disease control with proven effects were marketed by commercial companies in the U.S. market (Fravel, 2005). In Europe, 14 microbial products for disease control were registered in 2008 and listed on Annex 1 of the Directive 91/414 EEC (Ehlers, 2010). The biocontrol industry dealing with microbials developed to a professional business especially during the last decade and manufacturers are now organized in the International Biocontrol Manufacturers' Association (IBMA), Basle, Switzerland, and international business meetings are organized, such as the Annual Biocontrol Industry Meeting (ABIM), Lucerne, Switzerland (International Biocontrol Manufacturers' Association, 2010).

Biological control products based on microbials are considered as plant protection products in most countries. Consequently, governmental regulations for the registration and use are applied in the same way as for synthetic chemical plant protection products. Detailed toxicological studies have to be undertaken to guarantee that there are no risks for producers, users, and consumers when products are used. Furthermore, studies are needed to ensure that no environmental risks will occur after use. Besides the toxicological profile of an antagonist, industries will also consider technologies for production and formulation and their costs, genetic stability of the antagonist, market size for the biocontrol product and the possibilities of patent protection for the application (Whitesides *et al.*, 1994). It is a significant step from the isolation of a microorganism showing antagonistic property to the commercial marketing of an economically viable biological control product (Blum, 2007). Numerous

isolates of microorganisms can be found showing antagonism in model systems. But amongst those only very few may fulfil requirements for commercial use. Consequently, knowing and considering such requirements when a screening program is initiated will help to select candidates which fit better into commercial use. A good example for such a screening which included commercial aspects in an early stage is the selection of bacterial antagonists against *Gibberella pulicaris* causing dry rot of stored potatoes (Schisler and Slininger, 1997). The authors divided the screening process into three categories: (1) choosing an appropriate pathosystem; (2) choosing an appropriate method for microbe isolation; and (3) conducting an appropriate isolate characterization and performance evaluation. Main emphasis was laid, besides bioefficacy, on the favourable growth kinetics of candidates in commercially feasible liquid media. Another example, how industrial needs can already be considered during the early screening steps is given by Validov *et al*. (2007) who selected bacteria for use against *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Drying processes are often detrimental for mass produced and formulated antagonistic bacteria, thus limiting the opportunities for their commercial use. As an initial screening step, Validov *et al*. (2007) freeze- and spray dried rhizosphere samples before potential bacterial antagonists were isolated from such samples. It was assumed that these pre-selected bacteria were also tolerant to industrial drying processes and thus suitable for commercial production systems. When antagonists against the apple scab pathogen *Venturia inaequalis* were selected in a recent study, the antagonist candidates that were pre-selected fulfilled basic criteria regarding commercial production and registration of plant protection products, as well as ecological needs for applications to leaves (Köhl *et al*., 2009; Köhl, 2010). Only those isolates with sufficient spore production on cereal-based solid media, which did not grow at human body temperature and are tolerant to low temperatures and humidity were selected in a first step before their efficacy was tested.

However, reviewing the literature on biological control leads to the conclusion that the majority of screening programs primarily focus on efficacy, tested *in vitro* or *in planta*, as the main criterion. In nine research papers published between 2006 and 2010 on selection of new antagonists for biological control of plant pathogens, antagonism shown *in vitro* or in plant assays is reported as the selection criterion (Card *et al*., 2009; Grosch *et al*., 2006; Hynes *et al*., 2008; Kalogiannis *et al*., 2006; Pliego *et al*., 2007; Pugliese *et al*., 2008; Rocha *et al*., 2009; Rubio-Pérez *et al*., 2008; Thomashow *et al*., 2007). Pliego *et al.* (2007) and Thomashow *et al*. (2007) included rhizosphere competence and Hynes *et al.* (2008) plant growth promotion as an additional selection criterion. However, in none of the publications other characteristics of the candidates relevant for commercial use have been considered during screening. Although strong novel antagonists are selected in the model systems in this way, such candidates may not be suitable for commercial use. For example, mass production may not be cost effective, shelf life of produced inoculum may be too short or a targeted market too small to allow an implementation of a new biological control product.

The objective of the present paper is to propose a systematic, stepwise antagonist screening. Different categories of criteria all relevant for commercial development are discriminated. They include marketing, microbial ecology, mass production, safety, protection of intellectual property rights (IPR), environmental risks, and biocontrol efficacy. Such criteria may be considered when new programs for biological control development are initiated by industries and scientific institutions. Examples for

protocols are given as well as examples of key decisions that are needed for the selection of the most promising candidates.

2. Screening steps

The screening of new microorganisms for commercial use in biocontrol of plant pathogens is a complex process. Besides the antagonistic efficacy many other criteria are evaluated - ranging from ecological characteristics needed for good field performance to toxicological profiles, growth in fermenters for mass production and aspects of legal property rights and marketing (Blum, 2007). Some criteria can easily be defined as exclusive, e.g. a fungal candidate antagonist producing only few spores on an agar plate will not be assessed further since mass production is expected to be very costly or even impossible. However, many criteria are more quantitative and lead to a ranking of candidates, e.g. the antagonistic efficacy against a pathogen measured in a bio-assay may range between 0 and 100 % for different candidates. In such cases it is essential not to select the very best candidates regarding a single criterion for further assessments, but to rank various quantitative results at the same time, e.g. on efficacy in a bioassay, estimated mass production costs and more criteria. This leads to the selection of candidates with an optimum combination of characteristics, often a compromise regarding a single criterion.

Testing the various criteria is costly and time-consuming. Some criteria can be evaluated at low costs per isolate, e.g. the growth of a candidate at a certain temperature in a simple experiment on agar or the search of a possibly existing patent protection for the envisaged application by database mining. Such criteria can thus be evaluated for a huge number of candidates. Other criteria can only be evaluated in complex and expensive experiments, e.g. the field performance of a candidate antagonist under different environmental conditions or basic toxicological tests. Consequently, such criteria will only be considered for a few, pre-selected biocontrol candidates. This implies to plan a screening process in different steps: from simple, cheap evaluations of many candidates to complex evaluations with few candidates.

In the proposed concept for antagonist screening, various criteria have been arranged in this way in nine screening steps (Fig. 1). Within each step, the evaluation criteria entail similar approaches at comparable costs per candidate, e.g. in rapid throughput screening experiments (Step 3) or data mining in various data bases (Step 4). In Step 1, the targeted crop, disease and market is studied so that specific screening criteria can be defined. Thereafter, origin and isolation techniques of candidates are considered and microorganisms are collected (Step 2). This collection of microorganisms is preliminarily screened tested in rapid through put systems (Step 3) and, after identification of the selected candidates at genus or even at species level, data from data bases on the specific genus or species are evaluated (Step 4). The antagonistic potential of suitable candidates selected during these early evaluations steps is tested then in bioassays (Step 5) and the feasibility of mass production of thus selected candidates is assessed (Step 6). For a limited number of candidates pilot formulations are then developed and tested again in bioassays. In parallel, costs and opportunities for registration are preliminarily estimated (Step 7). In Step 8 the production of a few candidates is scaled up and pilot formulations are subsequently tested under field conditions. The

most promising formulation of the best candidate is selected and then tested in crops at different locations and seasons with full integration in existing crop protection strategies or those under development (Step 9).

Besides the antagonistic efficacy of the candidate microorganisms, many other important aspects (grouped into different categories such as production, safety or marketing) are already considered in early screening steps (Tables 1 – 9). Therefore, complementary expertise, skills and facilities are needed in the single steps. Collaborative projects combining industrial expertise in research and development and fundamental and applied microbiology, plant pathology and agronomy are thus a pre-requisite for a powerful and targeted screening of new biocontrol agents.

Following the screening steps described here, industries will decide on dossier forming including the compulsory studies on human toxicology and environmental risks, registration and market introduction. Scientific research will focus on detailed studies on the mode-of action and the genetic determinants of the antagonism as well as on field performance including population dynamics and further integration in cropping systems. Both types of activities are beyond the scope of this proposal on screening steps and are not described here.

2.1. Step 1: Assessment of targeted crop, disease and markets

Commercial exploitation of biological control agents depends on a sufficiently large potential market size. In Step 1, the potential markets for biological control in targeted crop and diseases are evaluated (Table 1). Specific marketing skills are needed for this study estimating which entails future market sizes but also considering competing products and specific governmental regulations on the use of microbial plant protection products.

Once the targeted market is defined, the envisaged application for the control of defined diseases in relevant crops can be described. Such a detailed analysis of the targeted cropping systems and the types of diseases in this first step is essential for the definition of relevant screening criteria in later steps. Thorough knowledge on the life cycle of the targeted pathogen is needed to identify stages during which application of biocontrol agents is feasible and pathogen populations are most vulnerable. Such critical stages for pathogen populations might be the attraction of pathogens by host exudates in the rhizosphere (Thomashaw *et al.*, 2007), spore germination on the host (Guetsky *et al.*, 2002), multiplication on affected host tissue (Heijwegen, 1989; Kiss, 2003), or survival in crop residues (Köhl and Fokkema, 1998). A rational identification of such key stages is essential because specific stages will require antagonists with specific ecological characteristics and a specific mode-of-action. There is also a great impact of the targeted epidemiological stage on the marketing of the final biocontrol product. An example for successful practical use of biological control is the product Contans containing spores of the antagonistic fungus *Coniothyrium minitans* which destroys sclerotia of *Scerotinia sclerotiorum* (Whipps *et al.*, 2008; McQuilken and Chalton, 2009). Results of treatments often cannot directly be seen in the treated crop after the treatments but in subsequent crops (Gerlagh *et al.*, 1999). It is important to communicate this disease control strategy carefully to farmers who are used to observe direct control effects during the growing season when fungicides are applied.

The identification of suitable control points at certain epidemiological stages has thus a strong impact on selection criteria for antagonists as well as on the feasibility of commercial use.

During the last decades, epidemiological knowledge has often been developed to support the use of chemical control. New insights in epidemics will help to identify promising opportunities for biological control and specific epidemiological field experiments may be needed to support the decision making process. For example, the role of conidia production within an onion crop on the progression of onion leaf spot disease has been studied in a field trial (Köhl *et al*., 1995a). Results confirmed that suppression of pathogen sporulation by antagonists is a valid biocontrol strategy. Especially for new diseases with unknown epidemiology, the development of the proper biocontrol strategy is risky if not combined with epidemiological research.

Biological control differs fundamentally from the conventional chemical crop protection concept. The latter is focused on eradicating pathogens while the use of biocontrol agents aims at protecting plant health. Therefore, the efficacy of biocontrol methods has often to be considered within a mix of non-chemical measures, which require to be integrated to provide the anticipated results. The envisaged biological control system should not simply follow disease control strategies conceived for fungicides, e.g. protecting the targeted plant tissue from infection. With such a restriction many alternative opportunities for biocontrol in a cropping system are missed, since biological control can target all vulnerable stages during the pathogen life cycle. Examples are applications of antagonists to destroy sclerotia as survival structures of *Sclerotinia minor* in peanuts (Partridge et al., 2006), to reduce multiplication of the tan spot pathogen *Pyrenophora tritici-repentis* on crop residues of wheat as the local primary inoculum source of the disease (Adee and Pfender, 1989; Pfender and Wootke, 1988) and to slow down the summer epidemic of apple scab by suppressing the conidiation of the pathogen (Köhl *et al*., 2009).

2.2. Step 2: Origin and isolation of candidate antagonists

After the targeted crop, pathogen and epidemiological stage of the disease have been defined, a collection of candidate antagonists is built up (Table 2). Possible restrictions for the international exploitation of organisms due to the Convention of Biological Diversity (CBD) (1992) have to be considered and rights of donor countries have to be respected. Samples for antagonist isolation are collected from the adequate niches to obtain ecologically adapted microorganisms, for example from soil and crop residues of fields where the disease has been present (Pfender and Wootke, 1988.) or the rhizosphere of plants grown in rockwool (Validov *et al*., 2007). It is important to sample in a broad range of geographical regions or from crops managed at very different levels of intensity to ensure that the collection represents a broad biodiversity common in the targeted niche. Isolation of candidate antagonists from samples only limited to few locations or specific cropping systems leads to a biased collection and many opportunities may be missed. Also the applied isolation techniques have impact on the characteristics of the collected microorganisms. Selective conditions favour organisms with biased characteristics. The use of nutrient rich media, synthetic media or even specific media preferring certain microbial groups as well as the choice of a high incubation temperature to enhance

microbial growth may lead to such an undesired bias. On the other hand, a careful choice of isolation conditions can also pre-select organisms with favourable characteristics, such as the ability to grow on industrially used inexpensive media (Schisler and Slininger, 1997) or at specific temperature ranges which occur in the targeted cropping systems. Another example is the freeze-drying of samples before bacteria are isolated to obtain candidates which will survive drying processes during product formulation (Validov *et al*., 2007).

In Step 2, expertise in microbiology and plant pathology is applied and the common basic equipment of a microbiological laboratory is needed.

2.3. Step 3: Preliminary assessments in rapid throughput screening systems

Characteristics of a high number of candidate antagonists are preliminarily assessed in Step 3 using a rapid throughput approach with standardized, simple experiments (Table 3). Candidates are grown on liquid or solid media similar to industrially used inexpensive substrates to assess their biomass production and to identify known toxigenic fungal species. Suspensions of spores or cells are prepared and subsamples are stored for DNA isolation in the following screening step (Step 4). Spores or cells are counted to estimate the productivity of the candidate and particle sizes are measured. Candidates are undesirable if they produce spores or cells below a minimum threshold amount, e.g. 10^5 spores per agar plate. The higher risk of deep lung deposition (Millage *et al*., 2010) for candidates producing particles sized <5 µm with may also be considered. Suspensions are then used to inoculate a series of microtiter plates which are subsequently incubated under various conditions to assess different characteristics determining their ecological fitness (Alabouvette *et al*., 2009), such as tolerance to low or high temperatures, or drought (Köhl et al, 2009). Tolerance to UV-irradiation or low pH values can also be screened in this way. Candidates able to grow at human body temperature are excluded because this characteristic may hinder registration (Berg *et al*., 2005).

Without much extra efforts, the growth of candidates on microtiter plates containing agar media amended with pesticides commonly used in the targeted cropping system can already be assessed. First information on the compatibility of antagonists with agrochemicals and thus their potential use in integrated cropping systems is obtained in this step. Fungicides under development and not yet on the market can also be included to anticipate on future cropping situations.

With this rapid throughput screening approach, a huge number of isolates can be tested at low cost per isolate. Candidates which do not fulfil minimum requirements, e.g. in biomass production or ecological characteristics, are excluded in this initial screening step. Consequently, higher costs for more expensive experiments, e.g. efficacy trials, are avoided for such candidates. Expertise in microbiology is applied in this screening step. Experiments are carried out in a microbiological laboratory under safe conditions as common for experiments with pure cultures of non-identified microorganisms with unknown characteristics.

2.4. Step 4: Identification of candidate antagonists and database mining

Candidate antagonists which passed screening Step 3 are identified preferably at species level based on DNA sequence analysis or morphology. Information on the identified species (or genus if a reliable identification at species level is impossible) is screened in various databases and is evaluated in screening Step 4 (Table 4). In medical and microbiological databases e.g. German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de), and regulations (European Commission 2000) information on safety is searched to judge on potential risks regarding human pathogenicity, allergenicity and toxicity (Brimner and Boland, 2003). In case that the targeted disease occurs also in feed crops, information on farm animals will also be screened. Patent searches are done to judge on options for the protection of intellectual property rights for isolates of the given species in combination with the envisaged application. Furthermore, information on possible plant pathogenicity and geographical distribution is screened in relevant databases. Based on the overall information, species are excluded from further screening if indicated potential risks for humans, crops, animals or the environment do not allow the exploitation as biocontrol product or excessive registration costs are expected because expensive toxicological studies may be required for risk assessments. Candidates potentially fulfilling the criteria for low risk active substances are preferred since incentives are foreseen for the placing of low-risk plant protection products on the European markets (European Regulation EC/1107/2009, European Commission, 2009; Ehlers, 2010).

Depending on the type of assessment, expertise in medicine, marketing, microbiology and plant pathology is needed in this screening step. Costs for the identification of a considerable huge number of candidates are significant. However, the subsequent screening costs per isolate are low once the infrastructure for database mining has been established. Exclusion of toxicologically suspicious species or already patent-protected applications at this early stage of product development by a systematic data-mining will avoid unnecessary high costs during later screening steps with significantly higher costs of tests per isolates. However, a database mining on toxicological information gives only a first indication on the safety of candidates and helps to exclude known microorganisms with major risks. It will not replace the detailed toxicological evaluation at strain level as required for the registration of a biological control product.

2.5. Step 5: Efficacy testing against pathogens on plants

After the number of candidate antagonists has been significantly reduced during screening Steps 3 and 4, complex, cost intensive and time-consuming experiments are carried out on the remaining microorganisms. A bioassay is designed to assess the antagonistic potential of candidates against the targeted pathogen (Table 5). The main requirement for such a bioassay is that host or host tissue, pathogen and antagonist are interacting under controlled conditions which are representative for the targeted epidemiological stage and environmental conditions in the crop. Various examples for such bioassays are described in literature. Folman et al. (2003) used an assay with young cucumber plants

in nutrient solution which had been inoculated with zoospores of *Pythium aphanidermatum*. In this assay the antagonistic potential of bacteria was tested for use in hydroponic cropping systems. Antagonists of biotrophic powdery mildews can be selected on detached leaves of the host which are inoculated with the pathogen and incubated under controlled conditions. An example is the selection of isolates of *Verticillium lecanii* on detached cucumber leaves for control of *Sphaerotheca fuliginea* at different humidities (Verhaar *et al*., 1998). Antagonists of necrotrophic *Botrytis* spp. were selected on segments of dead onion leaves for their ability to suppress sporulation of the pathogens under controlled alternating moisture conditions representative for the field situation (Köhl *et al*., 1995b). Sclerotia as resting structures of several important plant pathogenic fungi are an attractive target for biocontrol. Antagonists have been selected directly on sclerotia by assessing their ability to kill sclerotia of pathogens such as *Sclerotinia sclerotiorum* (Jones and Stewart, 2000), *Sclerotium cepivorum* (Clarkson *et al*., 2002) or *Botrytis cinerea* (Köhl and Schlösser, 1989). Post-harvest decay of fruit caused by pathogens such as *Monilinia fructicola*, *Botrytis cinerea*, *Penicillium* spp., or *Rhizopus stolonifer* can potentially be controlled by antagonists applied to fruit surfaces before or after harvest (Sharma *et al*., 2009). Such antagonists can be selected on wound inoculated fruits incubated under controlled conditions close to those applied in commercial stores Smilanick, 1994).

Bioassays used for antagonist screening must be robust and reproducible. Experimental conditions are controlled and a set of different conditions can be applied. For example, bioassays may be conducted at a series of relevant temperature and humidity regimes. The aim of this selection step is to find a group of moderate to superior antagonists. Therefore, it is important to create a moderate disease level allowing the differentiation between antagonists with low and moderate to high efficacy. The development of a powerful bioassay usually is time demanding and depends on thorough expertise in plant pathology. In most cases, high standard climate and greenhouse facilities are needed. If several screening programs against different pathogens are run in parallel, candidates assessed in Step 5 may also be tested in separate bioassays against the alternative targets. Such a procedure results in more cost effective screening programs and may allow the early detection of candidates effective against multiple targets.

After a set of antagonists has been selected, these candidates are tested in repeated assays under similar and under different combinations of conditions. This may include a variety of constant or fluctuating regimes of temperature and humidity, growth stages of the host and different pathogen strains allowing insight in the ecological demands and weaknesses of the candidates. It also provides information on the stability of the antagonistic potential of a given microorganism after the isolate has been stored, sub-cultured and produced in different batches. The end result of this step is the selection of a group of antagonists which showed moderate to high antagonism under a range of representative environmental conditions without high variation between replicate experiments or different batches of inocula. It is important in this screening step not to search for a single superior antagonist since besides efficacy; various other criteria still have to be evaluated. Possible effects of antagonists on host growth are also evaluated in pot experiments in this screening step. Growth promotion of host plants by microorganisms may be observed, adding extra value to a candidate.

2.6. Step 6: Preliminary tests on mass production

For the set of antagonists selected at the end of Step 5, pilot experiments on mass production will follow (Table 6). Biomass production and spore production is measured in solid state fermentation, commonly used for filamentous fungi, or liquid fermentation, commonly used for bacteria and yeasts. Media consisting of inexpensive substrates and growth conditions commonly applied in industrial fermenters are evaluated.

In this step, expertise in microbiology has to be linked to expertise in industrial biotechnology. The results allow a first estimation of production costs. Isolates with low or variable yields or depending on very specific growth conditions are excluded if high production costs are expected.

Investments in the assessment of candidate antagonists are considerably high in screening steps 5 and 6. To lower such costs, experiments on efficacy in Step 5 and on mass production in Step 6 are preferably run in parallel. In this way, candidates which fail to produce sufficient inocula in fermenters can be excluded from further experiments in which their efficacy in disease control is tested under different conditions and *vice versa*.

2.7. Step 7: Development and testing of a pilot formulation and estimation of registration costs

Steps 5 and 6 resulted in the selection of a subset of candidates which showed reproducible moderate to superior disease control under defined environmental conditions in bioassays and sufficient inoculum production in the first fermentation experiments. In screening Step 7, these microorganisms are evaluated more thoroughly. Inoculum production is further studied in experimental small scale fermenters in liquid or solid state fermentation (Table 7). Growth conditions and growth substrate are optimized. Produced spores or cells are separated from the growth substrate after fermentation and formulated dry as powers or granules or moist as concentrated emulsions. A main criterion is the viability of the inoculum after the several down streaming steps. Antagonists are excluded after this screening step if no cost effective system can be developed for production, separation and formulation. For a variety of differently produced and formulated inocula with sufficient viability, shelf life studies are initiated. Inocula are stored at commercially acceptable temperatures, e.g. at room temperature, in common refrigerators and in freezers, for at least 12 months and viability is tested at regular intervals. Experimental stress conditions may also be applied to study the stress tolerance of inocula in short term experiments to predict the outcome of the long-term shelf life studies. Such studies on production, formulation and quality control of inocula under conditions mimicking industrial processes require sophisticated fermentation technologies and specialized expertise.

The set of antagonists for which inocula can be produced and formulated successfully is then tested again in bioassays under controlled conditions, now using differently produced, formulated and stored inocula in comparison with fresh inoculum produced as in Step 4 on agar medium or in liquid culture. In bio-assays the combined or alternated use of candidate antagonists and fungicides is

assessed on plants. This re-assessment of fungicide compatibilities of antagonists is needed because the preliminary *in vitro* tests (Step 2) may not always be representative for *in planta* conditions.

In parallel, the preliminary toxicological assessments done in Step 4 are complemented with more detailed assessments of available data in public databases, scientific publications and dossiers after the number of organisms tested in this step has been limited to a few candidates. The objective is to identify and to exclude antagonists which most likely will not fulfil the criteria for registration as plant protection substances (Regulation EC/1107/2009, European Commission, 2009). Antagonists fulfilling the criteria for low risk active substances will be preferred. Additional to data mining, preliminary toxicological experiments are carried out to allow a first insight in the toxicological profiles of the selected individual isolates. In this step, non-animal (vertebrate) test methods are preferred, e.g. using *Caenorhabditis elegans* as model organism (Zachow *et al*., 2009). The main objective of the early toxicological assessments in screening Steps 4 and 7 is, besides early exclusion of unwanted candidates, the estimation of amount and costs of additional experiments needed for dossier forming during registration as plant protection substance. Such toxicological tests are expensive and time consuming. Furthermore, animals are used in such experiments which thus should be reduced to minimum numbers. For that reason, detailed experiments on human toxicology and ecotoxicology are generally not carried out during the screening of antagonists but will follow during product development as the results constitute part of the information presented in the registration dossier. Experimental field testing of candidate antagonists has to be permitted by the governmental authorities. Depending on the country, an evaluation of existing information in data bases on possible risks may be sufficient. In other countries, basic toxicity tests are compulsory and have to be carried out first in Step 7 before a first field testing can be envisaged in Step 8.

2.8. Step 8: Field testing and up-scaling mass production

Pilot-formulated inocula of two to four selected antagonists are subsequently tested in Step 8 for the first time in the field under conditions conducive for disease development (Table 8). If needed, the pathogen is artificially inoculated in the field and micro-climatic conditions are manipulated to ensure disease development. Experiments focus on the interaction between pathogen and antagonists during the targeted epidemiological stage, e.g. reducing the numbers of surviving sclerotia, but experiments do not need to cover the whole cropping period, e.g. the effect of reducing numbers of sclerotia on disease development in a crop. Compared to whole season experiments, such short trials allow, with the similar input of resources, the testing of many more different treatments. For example, different antagonists, formulations, application times and intervals and concentrations are tested. Possible phytotoxic or growth stimulating effects of formulated inocula on plant development are also assessed at this stage.

Candidates which gave promising results in the first field evaluation are subsequently mass produced on inexpensive media in fermenters such as those used for commercial industrial production to assess the up-scaling of production processes and their costs. Inocula are separated from the growth media after fermentation, dried and formulated. This process follows industrial procedures and

is adapted to the needs of the envisaged way of application, e.g. as water dispersible granule for spray applications. Again, costs are evaluated and possibly several alternative treatments are investigated. Subsamples of the inocula are used for shelf life studies. Storage of inocula under stress conditions simulating long-term storage allows first insight in storability. These studies on production and formulation are expensive and need highly specialized expertise in industrial biotechnology.

The best one or two pilot-formulated candidate antagonists are then further studied in large scale field experiments and their potential in disease control is quantified and compared to common fungicide treatments. The success of such field experiments depend on the presence and level of the disease in a particular field plot during the trial, and may thus vary between locations and seasons. Furthermore, the control efficacy of biocontrol agents is often not consistent but depends on environmental conditions. Consequently, biocontrol agents have to be tested under various conditions in the field to allow a final judgment on their efficacy and consistency in disease control. For these reasons, experiments are needed at multiple locations and have to be repeated in different seasons.

Both product development and field trials are time consuming and extremely expensive. Only the most promising candidates achieve this evolution step close to implementation. Additional studies on markets, safety and registration issues are carried in parallel to indicate possible limitations for commercial exploitation.

2.9. Step 9: Integration in cropping systems

In a last screening step, the application of formulated inocula of the best one or two candidates is fully integrated into the existing cropping systems (Table 9). Complete plant protection schedules are developed. The biocontrol product is applied as stand-alone treatment and in combination with compatible fungicides. Sophisticated decision support systems, if available, are used to decide on the timing of applications of biological and chemical control depending on forecasted risks for infections by the pathogen and environmental factors. Also the use of fungicides or other control methods targeting diseases not controlled by the biocontrol agent is integrated. Preferably, field experiments are carried following the relevant governmental regulations so that results can be used for dossier preparation if registration as plant protection product is envisaged. Such large scale field experiments are expensive and often last a whole season followed by possible post-harvest assessments on product quality. Results depend on the occurrence of the disease and complex interactions between the pathogen, antagonists, crops and environmental factors. Consequently, such experiments have to be repeated during several growing seasons at multiple locations. Depending on the crop, it can be relevant to include different cropping systems and varieties in field trials. More than for chemical control, the success of biological control often depends on the integrated use of several disease control measures reducing the disease pressure stepwise as in hurdle concepts. Therefore, it is important to assess the integrated use of the biological agent together with non-chemical and prevention methods already existing or under development in large scale field experiments. This may open novel options for disease control which are not detected if such methods are applied as stand-alone options. Because field trials are carried out at multiple sites and different control methods are integrated in different

cropping systems, an excellent network with expertise in applied plant pathology and agronomy has to collaborate in this step with the biocontrol industry.

In parallel to the large field trials on control of the primarily targeted disease, the biocontrol agent is tested in smaller scaled field trials in other crops against other pathogens. Possibly, new options can be found for use of the antagonist which will enlarge the potential market for a product.

Field samples collected during and after the trials will be used to assess possible environmental risks. Especially the persistence in the field and possible problems of biovigilance are considered during the registration procedure (Regulation EC/1107/2009, European Commission, 2009). Specific methods for quantification of the applied inoculum are used to monitor the dynamics of the antagonist population and their natural background in treated and untreated plots after applications in field trials for periods of several months.

3. Conclusions

The presented framework for screening programs considers various selection criteria aiming at commercial use of biological control products. The proposed stepwise screening (Tables 1 – 9) includes 62 screening criteria which can be grouped into eight categories: efficacy, ecology, production, safety, environmental risks, targeted diseases, marketing and IPR protection. This list of selection criteria is not exhaustive and may not be suitable for all possible types of diseases. For specific selection programmes, additional criteria may be added and some criteria may not be valid.

The objective of the paper was to identify screening criteria and organise them according to a pragmatic approach. Depending on different categories into which screening criteria can be divided, different highly specialised expertises and corresponding research facilities are needed to conduct the necessary experiments and assessments. This shows that a screening programme for biological control agents is a very complex process. It also demonstrates that only a multi-disciplinary approach will lead to successes. Combined efforts are needed of fundamental and applied scientific expertise in plant pathology, microbiology, agronomy and biotechnology together with industrial know how. Expertise in human and environmental toxicology, industrial biotechnology, economy, marketing and patenting is also essential in the process. A pre-requisite for a successful program is the willingness and ability of the different disciplines to collaborate. Furthermore, an adequate funding of the work is essential. Funding agencies and industries must be convinced that a screening considering relevant aspects in a broad way as exemplified in Tables 1-9 should be preferred. In this way, the goal of commercialisation of biological control agents may be achieved more often than with projects focused primarily on phytopathological research. Overall, fewer resources may also be needed for the development of a biocontrol product using such a multi-disciplinary approach because it will reduce the number of expensive field experiments currently carried out with candidates which eventually do not fulfil basic requirements for commercialisation.

Efficacy testing of candidate antagonists is an essential part in each screening programme. The supposed selection steps (Steps 5, 8 and 9) consisting of bioassays on plants or plant parts under controlled conditions followed by applications in crops are time-consuming and expensive. Because efficacy testing in bioassays and under field conditions requires significant resources and time, the

antagonistic potential of candidates has often been tested in a first screening round under the *in vitro* conditions of agar plates allowing a rapid throughput with clearly discriminating results. Such a testing in dual cultures of pathogen and candidate antagonist will result in the selection of those candidates which produce toxins secreted into the medium and cause growth inhibition of the pathogen. Some hyperparasites invading the host tissue may also be found. There are significant limitations for such a selection strategy. Firstly, it may be strongly biased and as a result, the selected microorganisms may be restricted primarily to antagonists that have toxin secretion as a mode-of-action. The full range of potential modes-of-action (Elad, 2003), expressed alone or in complex combinations by an individual antagonist cannot be evaluated in studies relying on dual culture *in vitro* studies, e.g. competition or induced resistance. Consequently, many opportunities to find antagonists are missed. Secondly, there is no guarantee that antagonists selected for their ability to secrete potential toxins under artificial *in vitro* conditions will also produce and secrete the toxin in the crop environment. The production of such secondary metabolites often depends on specific environmental and nutritional conditions. Several studies have shown that there may be no correlation between antagonism assessed under *in vitro* conditions and on the plant (Knudsen et al., 1997). Although *in vitro* assays can be used to study the mode-of-action of known antagonists, pre-screening under *in vitro* conditions on agar plates or culture broths should thus be avoided. However, further research efforts are needed to develop novel valid *in vitro* selection systems for rapid throughput screening. Such an efficacy testing under *in vitro* conditions should not bias preferring single mechanisms such as toxin production and should preferably allow the detection of candidates with promising combinations of different mode-of-actions or even new mode-of-action not yet known.

The proposed stepwise screening aiming at commercial use of antagonists will always depend on results of fundamental and applied research on the principles of biological control of plant pathogens and on specific antagonists. Such knowledge is crucial to improve selection procedures of antagonists as well as their application in crops.

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Appendix: Figures and Tables

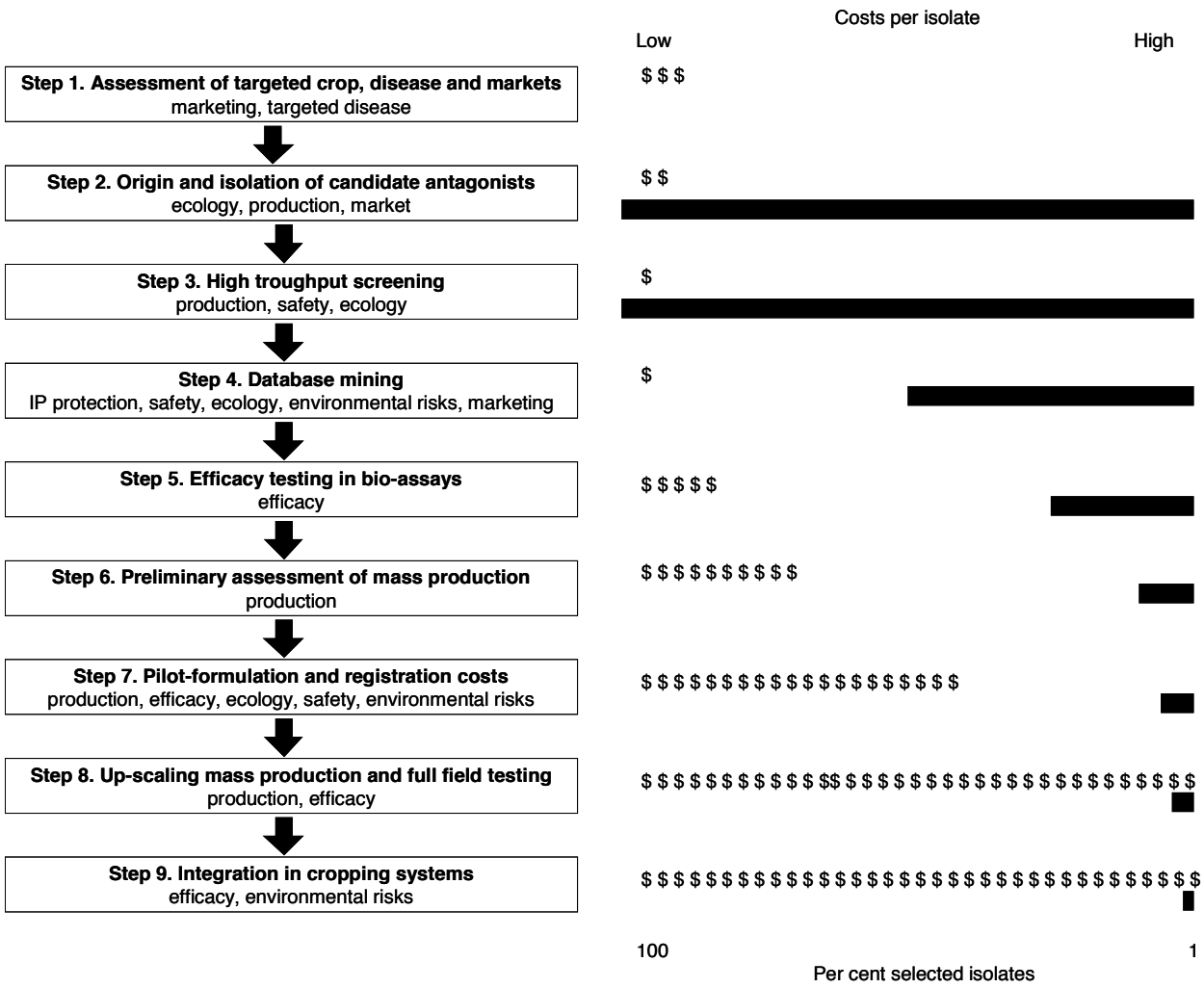

Fig. 1. Stepwise screening of microorganisms for commercial use in biological control of plant pathogens. For each screening step, specific categories of selection criteria are considered (for details see Tables 1-9), screening costs per isolate are estimated ($) and the percentage of assessed isolates is given (■).

Table 1. Assessment of targeted crops, diseases and markets for a microbial plant protection product.

Criterion	Category	Motivation	Example for decision	Skills needed
Crop characteristics	Targeted disease	Different solutions needed for bulk crops and high value crops	Consider needs together with crop experts	Agronomy
Life cycle of pathogen	Targeted disease	Knowledge on vulnerable stages allows specific targeting	Define targeted stage of pathogen life cycle and define relevant characteristics of antagonists	Plant pathology
Affected and targeted plant part	Targeted disease	Antagonists needed with specific ecological characteristics	Define specific selection criteria for screening	Microbiology / Plant pathology
Use of genetically modified organisms	Targeted disease	Strategies depending on genetically modified organisms cause additional costs during registration	Consider advantages versus additional costs	Microbiology / Product development
Market size	Marketing	Minimum market size needed to allow return of investments	No go for small markets unless supported by specific regulations for minor uses	Product development
Competing products	Marketing	Existing chemical, biological and other non-chemical methods restrict exploitation	No go if market needs no new solutions	Product development
Regulations	Marketing	Present or future regulations may affect marketing position and costs	Go / no go depending on (expected) regulations and costs	Product development

Table 2. Origin and isolation of candidate antagonists.

Criterion	Category	Motivation	Example for protocol	Example for decision	Skills needed
Origin of isolates	Ecology	Adaptation to relevant ecological niche	Collect samples for isolation of potential antagonists from adequate niche, e.g. leaves, seeds, or (suppressive) soils from various locations	Prefer candidates from relevant niche	Microbiology
Growth on media	Ecology	Ensure high variation between isolates	Isolate candidates on non-selective media	Do not prefer specific microbial groups	Microbiology
Growth conditions on media	Ecology	Ecological characteristics	Isolate candidates after incubation at 18℃ or lower depending on envisaged applications	Avoid isolates favoured by high temperatures	Microbiology
Growth on media	Production	Mass production costs	Isolate candidates on media similar to industrially used inexpensive substrates	Exclude isolates depending on specific nutrient conditions	Microbiology
Convention of biological diversity	Marketing	Restrictions in international exploitation narrow markets	Assess possible restrictions for use depending on origin and biodiversity regulations	Avoid isolates with restrictions in use	Product development

Table 3. Stepwise assessment of biomass production, safety and ecological characteristics of candidate antagonists in rapid throughput screening systems.

Criterion	Category	Motivation	Example for protocol	Example for decision	Skills needed
Spore production on agar (filamentous fungi)	Production	Mass production costs	Grow fungus on oat meal agar	Exclude isolates producing less than $1x10^5$ spores per plate	Microbiology
Cell production in broth (bacteria, yeasts)	Production	Mass production costs	Grow bacterium or yeast in nutrient broth	Exclude isolates producing less than a threshold amount of cells per ml	Microbiology
Mycotoxin risks (filamentous fungi)	Safety	Possible mycotoxin production; additional costs during registration	Recognize fungi belonging to toxigenic species, e.g. of *Aspergillus*, *Penicillium*, *Fusarium*	Prefer isolates not belonging to genera with toxigenic species	Microbiology
Particle size	Safety	Risk of deep lung deposition of particles sized < 5 µm	Determine particle size microscopically	Consider extra costs for workers protection during production	Microbiology
Germination and growth at 37℃	Safety	Possible risks for humans (skin, lungs); additional costs during registration	Incubate inocula[1] on agar in microtiter plates at 37℃	Consider extra costs during registration for risk assessments if spore germination and colony growth within 14 days at 37℃	Microbiology
Cold tolerance	Ecology	Temperature of leaves during leaf wetness periods or in soil often low; cold periods critical for disease development	Incubate inocula[1] on agar in microtiter plates at 5℃	Go if spore germination and colony growth within 14 days	Microbiology
Growth at pH 5 and pH 8 (control of soil pathogens)	Ecology	Many arable soils are acid or alkine	Incubate inocula[1] on agar with pH 5 and pH8 in microtiter plates	Go if spore germination and colony growth within 14 days	Microbiology
Drought tolerance (control of aboveground diseases)	Ecology	Long dry periods on surfaces of aboveground plant parts	Incubate inocula[1] on agar with low water potential (-10 MPa) in microtiter plates	Go if spore germination and colony growth within 14 days	Microbiology

Survival of UV-irradiation (control of aboveground diseases)	Ecology	Exposure of surfaces of aboveground plant parts to sunlight	Incubate inocula[1] on agar in microtiter plates; expose several times during incubation to UV-A and UV-B-irradiation at relevant intensity of sunlight	Go if spore germination and colony growth within 14 days	Microbiology
Compatibility with fungicides against target pathogen	Ecology	Combined or alternated applications with fungicides against target pathogen will broaden market size	Incubate inocula[1] in microtiter plates on agar amended with fungicide at 10% of recommended dose	Consider consequences for market size	Plant pathology
Compatibility with pesticides against non target pathogens	Ecology	Integration in spray schedules against other pathogens pre-requisite for practical use	Incubate inocula[1] in microtiter plates on agar amended with pesticides at 10% of recommended dose	Consider perspectives for practical spray schedules	Plant pathology

[1] Spores of fungi or actinomyctes or cells of bacteria or yeasts.

Table 4. Assessment of potential risks for use and patent positions of candidate antagonists. All candidates are identified at genus or species level based on DNA sequence analysis and available information is collected for relevant species by database mining.

Criterion	Category	Motivation	Example for decision	Skills needed
Envisaged application already protected by patents	IPR protection	No commercial use possible without permission of patent owner	Exclude if patent protected	Marketing
Envisaged application already published	IPR protection	No patent protection possible if published	Consider commercial value without IPR protection	Marketing
Risks for humans: human pathogenicity	Safety	Additional costs for toxicological studies during registration; high risk of no go decision	Exclude suspicious species with significant records in medical literature	Microbiology / Medicine
Risks for humans: allergies	Safety	Additional costs during registration; additional costs for mass production, formulation and application	Consider additional costs for workers protection and registration	Microbiology / Medicine
Risks for humans: toxins	Safety	Additional costs for toxicology studies during registration; high risk of no go decision	Exclude suspicious species with significant records in medical literature	Microbiology / Medicine
Pathogenicity to plants	Ecology	Additional costs for studies during registration; high risk of no go decision or restrictions for use	Consider costs and possible restrictions in use	Plant pathology
Natural occurrence in continent	Environmental risks	Higher costs for risks studies for exogenous microorganisms	Exclude exogenous species	Microbiology
Availability of data relevant for registration in dossiers or in publications	Marketing	Lower costs if information available	Prefer species with recorded information	Microbiology / Medicine / Product development
Overall toxicological profile	Marketing	Possible incentives may stimulate placing of low-risk plant protection products on the European markets	Prefer species expected to fulfil criteria of low risk active substance	Product development

Table 5. Efficacy testing of candidate antagonists in bio-assays on plants or part of plants.

Criterion	Category	Motivation	Example for protocol	Example for decision	Skills needed
Disease control on plants	Efficacy	Main criterion for exploitation	Replicated bio-assays under controlled conditions representative for commercial growing conditions considering: • Reproducibility • Time intervals between antagonist and pathogen application • Inoculum amount used for antagonist and pathogen inoculations • Different constant environmental conditions, e.g. temperatures or humidities • Fluctuating environmental conditions, e.g. interruptions of leaf wetness	Select best 25% candidates in disease control; avoid isolates showing high variation between repeated tests	Plant pathology
Plant growth stimulation	Efficacy	Larger market size	Replicated seedling assays under controlled conditions	Prefer plant growth stimulating organisms	Plant pathology

Table 6. Preliminary assessments of mass production.

Criterion	Category	Motivation	Example for protocol	Example for decision	Skills needed
Spore production in solid state fermentation (filamentous fungi)	Production	Mass production costs	Grow fungus on different inexpensive media in < 1L fermenter	Exclude isolates producing less than 10^9 spores per gram of substrate	Biotechnology
Spore production in solid state fermentation (filamentous fungi)	Production	Mass production costs	Grow fungus on selected inexpensive medium at different growth conditions and time periods in < 1L fermenter	Optimise conditions for mass production and assess feasibility and costs	Biotechnology
Cell production in liquid fermentation (bacteria, yeasts)	Production	Mass production costs	Grow bacterium or yeast in different inexpensive media in < 1L fermenter	Exclude isolates producing less than 10^{10} cells per ml of substrate	Biotechnology
Cell production in liquid fermentation (bacteria, yeasts)	Production	Mass production costs	Grow bacterium or yeast on selected inexpensive medium at different growth conditions and time periods in < 1L fermenter	Optimise conditions for mass production and assess feasibility and costs	Biotechnology

Table 7. Development and testing of a pilot formulation and estimation of registration costs.

Criterion	Category	Motivation	Example for protocol	Example for decision	Skills needed
Spore production in solid state fermentation (filamentous fungi)	Production	Mass production costs	Grow fungus on inexpensive medium in 10L fermenter	Exclude isolates producing less than 10^9 spores per gram	Biotechnology
Cell production in liquid fermentation (bacteria, yeasts)	Production	Mass production costs	Grow bacterium or yeast in inexpensive medium in 10L fermeter	Exclude isolates producing less than 10^{10} cells per ml	Biotechnology
Choice of formulants	Marketing	Registration costs	Evaluate status of candidate adjuvants, co-formulants, safeners and synergists regarding their approval for use in plant protection	Prefer already approved formulants; exclude formulants for which additional toxicological studies are needed	Product development
Ease of downstreaming and formulation	Production	Downstreaming and formulation costs	Formulate pilot products following industrial procedures	Consider ease and costs	Biotechnology
Shelf life of spores or cells in formulated product	Production	Shelf life demands depending on market	Store formulated pilot-product during short period under stress conditions simulating long-term storage	Prefer viability > 80%	Biotechnology
Shelf life of spores or cells in formulated product	Production	Shelf life demands depending on market	Store formulated pilot-product at room temperature, 5℃ and -18℃; determine viability at monthly intervals	Prefer viability > 80% for 12 months at room temperature or at 5℃	Biotechnology
Disease control under controlled	Efficacy	Main criterion for exploitation	Apply pilot-formulated inocula in repeated bio-assays under controlled conditions representative	Select pilot-formulations with best disease control	Plant Pathology

condition			for commercial growing conditions		
Compatibility with fungicides against target pathogen	Ecology	Combined or alternated applications with fungicides against target pathogen will broaden market size	Re-evaluate compatibility of candidates with key-fungicides in bio-assays on plants	Consider consequences for market size	Plant Pathology
Human toxicity	Safety	Registration demands for safe plant protection products	• Assess acute and chronic toxicity and allergenicity in preliminary tests • Re-assess available data on toxicology in dossiers, databases and publications • Estimate amount and costs of experimental data needed for registration in respect to available data	Exclude candidates with expected toxicity not fulfilling criteria for plant protection products; prefer expected low risk active substances	Toxicology / Product development
Environmental risks	Safety	Registration demands for safe plant protection products	• Re-assess available data on toxicology in dossiers, databases and publications • Estimate amount and costs of experimental data needed for registration in respect to available data	Exclude candidates with expected environmental risks not fulfilling criteria for plant protection product; prefer expected low risk active substances	Toxicology / Product development

Table 8. Up-scaling mass production and full field testing.

Criterion	Category	Motivation	Example for protocol	Example for decision	Skills needed
Disease control in crops	Efficacy	Main criterion for exploitation	Apply pilot-formulated inocula in repeated small scale field experiments; compare several candidates after assessment of relevant disease parameters	Select best 2-4 candidates in disease control	Plant pathology
Phytotoxicity	Environ-mental risks	Limits opportunities for use	Assess crop for possible symptoms	Consider relevance of possible phytotoxicity	Plant pathology
Inocula production in solid state or liquid fermentation	Production	Mass production costs	Grow antagonist on inexpensive medium in commercial scale fermenter	Consider production costs of inoculum amount needed per ha	Biotechnology
Ease of downstreaming and formulation	Production	Downstreaming and formulation costs	Formulate pilot products following industrial procedures	Consider ease and costs	Biotechnology
Shelf life of inocula in formulated product	Production	Shelf life demands depending on market	Store formulated inocula under stress conditions simulating long-term storage	Prefer viability > 80% after simulated long-term storage	Biotechnology
Shelf life of inocula in formulated product	Production	Shelf life demands depending on market	Store formulated inocula at room temperature, 5℃ and -18℃, plate on agar at monthly intervals and determine CFU	Prefer viability > 80% for 12 months at room temperature or at 5℃	Biotechnology
Disease control in crops	Efficacy	Main criterion for exploitation	Apply formulated inocula of 2 candidates in repeated large-scale field experiments at different locations; assess disease, yield and quality	Select best formulated candidate in disease control	Plant pathology

Registration costs	Marketing	Lower costs and better predictability of registration procedure	Re-assess toxicological status of species or isolate as active substance and of formulants; evaluate options to avoid duplicate testing and share tests involving vertebrate animals	Prefer candidates with expected low risk profiles and low and predictable assessment costs	Product development

Table 9. Integration in cropping systems.

Criterion	Category	Motivation	Example for protocol	Example for decision	Skills needed
Disease control in crops with complete common plant protection schedules	Efficacy	Main criterion for exploitation	Apply formulated inocula of 1-2 candidates in repeated large-scale field experiments at multiple sites and seasons	Consider integrated application schedules	Applied plant pathology
Disease control in crops combined with non-chemical control or prevention methods already existing or under development	Efficacy	Main criterion for exploitation	Apply formulated inocula of 1-2 candidates in repeated large-scale field experiments at multiple sites and seasons	Consider integrated application schedules	Applied plant pathology
Disease control of other diseases in other crops	Efficacy	Larger market size	Apply formulated inocula of 1-2 candidates in repeated small-scale field experiments in multiple crops	Consider additional markets	Applied plant pathology
Persistence in environment	Environ-mental risks	Persistent establishment in environment may hinder registration and cause biovigilance problems	Sampling of soil and crop residues 1, 2 and 6 months after application; specific quantification of applied organism and of the natural background	Consider consequences for registration if introduced organism is persistently established	Microbiology