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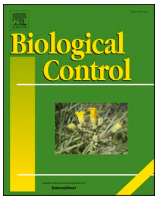

Screening criteria for microbial bioprotectants for seed coating to protect seeds and seedlings from diseases

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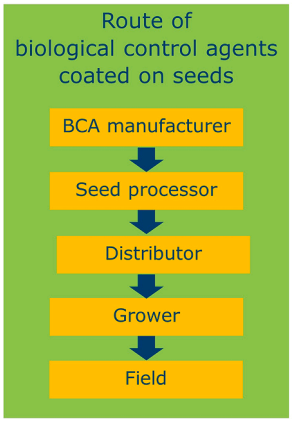
HIGHLIGHTS

- Systematic analysis of seed processing and seed handling steps.
- Identification of seed coating processes detrimental for microorganisms.
- Identification of seed handling steps detrimental for coated microorganisms.
- Bioassays for testing antagonist survival during seed processing steps needed.
- High throughput viability assays needed for antagonist selection.

GRAPHICAL ABSTRACT



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ABSTRACT

Biological control of seedborne pathogens and soilborne seedling pathogens is an alternative to chemical seed treatments. Limited survival and shelf life is one of the major bottlenecks for a broader implementation of seed treatments with microbial biological control agents (MBCA). Microbial inocula are typically coated on seed lots that have been dried and cleaned before. After coating, seeds are dried, stored, handled for packaging, distributed and used by the grower. During these processes, conditions challenge survival of the coated MBCA.

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Antagonists
Seed coating

Systematic analysis of seed technologies including various seed processing steps and seed handling, identified various detrimental conditions for microorganisms during seed coating and thereafter when MBCA-coated seeds are dried, stored and distributed. Screening systems for new antagonists for seed applications should consist of various bioassays simulating very different stress components on the applied microorganisms. Proposed bioassays have in common that viability of the tested microorganisms has to be assessed after exposure to differential conditions. Improved methodology for high throughput viability testing will allow larger screening programs and will support the development of new MBCAs for seed applications.

1. Introduction

Seeds can carry seedborne plant pathogens on their surface or internally (Neergaard, 1979). Such seedborne pathogens may damage seeds, seedlings or infect crops later during their development. Examples are *Xanthomonas campestris* in *Brassica* spp. and various smut pathogens of cereals. Germinating seeds are also exposed to pre-emergence diseases caused by soilborne pathogens and seedlings are exposed to post-emergence soilborne diseases. Examples are damping-off diseases of many crops caused by *Pythium* spp. and *Rhizoctonia solani*.

Fungicide treatments of seeds of agricultural and horticultural crops are common practices to protect seeds and seedlings from such seed- and soilborne pathogens (Lamichhane et al., 2020; Mancini and Romanazzi, 2014). Although the applied amounts on seeds per ha are low compared to spray applications in crops, routine applications on seed lots may result in a substantial fungicide use. Such general preventative seed treatments in many cases may not be cost-effective, especially under conditions with low pathogen incidences. Fungicide resistance of pathogens and negative effects of fungicides commonly used for seed treatments on non-target organisms have been reported and the use of several fungicides commonly used for seed applications has already or will be phased out in the European Union (EU) (Lamichhane et al., 2020). During the recent years, authorizations for seed applications of the following fungicides were withdrawn in the European Union: Carbendazim in 2006, Iprodione in 2018, Thiram stepwise in 2012–2019, and Metalaxyl-M in 2020 (EU, 2020). This resulted in limited choices for seed treatments with fungicides - especially of combinations of different fungicides - to achieve protection against a broad pathogen spectrum including *Pythium* spp.. Also seed treatments with insecticides such as neonicotinoids have been phased out and currently only few insecticides are available for seed treatment in the EU. Furthermore, options for commercial applications of fungicides and insecticides are missing for many vegetable seeds since procedures for authorization for minor use applications are considered to be too time consuming and expensive.

Biological control of seedborne pathogens and soilborne seedling pathogens through living antagonists applied to seeds is an alternative to seed treatments with synthetic fungicides or microbial metabolites (Bisen et al., 2020). An example is the commercial application of *Pseudomonas chlororaphis* (Johnsson et al., 1998), with isolate MA342 formulated as products Cedomon and Cerall for seed applications in cereals and vegetables (Anderson & Kim, 2018). The global value of seed treatments with biological control agents for control of pests and diseases was estimated at 268 Million US dollar for 2018 with an expected Consolidated Annual Growth Rate (CAGR) of 18 % for the period from 2015 to 2025 (Trimmer, 2021). Strong growth is expected especially in seed applications with row crops. Commercial seed treatments with microbial biostimulants are also used to protect crops from abiotic stresses and improve nutrient uptake, e.g. plant growth promoting bacteria, mycorrhizal fungi and *Rhizobium* spp. on legume seeds (Rocha et al., 2019; Cardarelli et al., 2022).

Objective of our study was to conduct a systematic analysis of applied seed technologies including various seed processing steps and seed handling. This analysis, in combination with a literature review, resulted in (1) identification of various conditions detrimental for microorganisms during seed coating and thereafter when seeds coated with microbial biological control agents (MBCA) are dried, stored and distributed; and (2) screening criteria for microbial bioprotectants for seed coating to protect seeds and seedlings from diseases.

2. Research on biological control application

Seed applications of a high number of microorganisms have been investigated in research on biological control of seed- and soilborne plant pathogens (O'Callaghan, 2016; Bisen et al., 2020). This includes, amongst many others, bacterial isolates of *Pseudomonas fluorescens*, *P. chlororaphis*, *Bacillus subtilis*, *B. amyloliquefaciens*, *Lysobacter gummosus*, *Paenibacillus polymyxa*, *Serratia plymuthica*, *Streptomyces* spp., and fungi belonging to *Trichoderma* spp., *Clonostachys* spp., *Verticillium isaacii* and *Pythium oligandrum* (Deketelaere et al., 2020; Ferrigo et al., 2020; Hökeberg et al., 1997; Jensen et al., 2004; Koch et al., 2006, Mastouri et al., 2010). However, commercial applications of seed treatments for control of seed- or soilborne plant diseases are still very limited (Bisen et al., 2020).

Different technologies for application of microorganisms to seeds have been reviewed by McQuilken et al. (1998), O'Callaghan (2016) and Ali et al. (2019), and have been evaluated in specific crops, e.g. oilseed rape (Müller and Berg, 2008; Abuamsha et al., 2011). Application technologies such as bio-priming, film-coating, slurry-coating, pelleting and encapsulation of microorganisms before adding to seeds aim at the maintenance of high viability of microbial inocula distributed on the seeds at sufficient densities. Use of powder formulations or liquid formulation in combination with stickers for on-farm applications by growers are also described. Despite the long history of seed inoculation with rhizobia, poor survival remains a significant problem (O'Callaghan, 2016). Whereas fungal spores and especially endospore-forming bacteria are less sensitive, survival of non-spore forming bacteria during desiccation and storage are seen as a major bottleneck in seed applications (McQuilken et al., 1998). Seed inoculation techniques are often not feasible at a commercial scale and major challenges regarding viability of microbial inocula during seed treatment processes and storage have to be solved (O'Callaghan, 2016). This would allow the use of more environmentally sensitive potential seed inoculants.

As all MBCA, microbial inoculants on seeds must show high efficacy against the targeted pathogens under field conditions but require additional specific physiological and ecological characteristics. Obviously, survival of microbial inoculants during seed treatments and shelf life during seed storage and distribution are crucial success factors since a sufficiently high number of living propagules must be able to grow rapidly after seeding to protect seeds and seedlings from plant pathogens. Limited survival and shelf life is one of the major bottlenecks for a broader implementation of microbial seed treatments with biological control agents or biostimulants and often led to a focus on gram-positive bacteria and exclusion of many other alternative microbial groups.

3. Screening approaches of new antagonists for seed applications

The microbial diversity of microbiota on and in plants and seeds, and in soil is huge and rather unexplored (Berg et al., 2017). Members of the microbial communities may contribute to the resilience of seeds and seedlings against damage by plant pathogens or may be antagonistic to plant pathogens. Such beneficial microorganisms are potentially

candidates for the development of new MBCA. Besides their ability to protect seeds and seedlings against seed- and soilborne pathogens, candidates must fulfil many other criteria, e.g. must show a suitable ecotoxicological risk profile to pass authorization processes and must show suitable production and storage characteristics to allow an economic mass production and marketing (Köhl et al., 2011).

Criteria for the selection of microorganisms for biological control of plant pathogens have been described by Köhl et al. (2011). They proposed a stepwise screening approach to consider relevant screening criteria in an efficient fast and cost-effective process for the development of new biological control products against plant pathogens. Such products are typically produced by biocontrol manufacturers, marketed through plant protection market chains and used by growers as the end-users for applications in their crops, usually through spray applications. Products must be economically feasible, authorized for use, effective, and formulated ready for use by grower with sufficient shelf life during storage at the productions site, distribution and storage at the farm. A preliminary set of relevant screening criteria has been proposed and can be considered during screening programs to select new biological control agents fitting for this purpose. Such strategies have been applied successfully in screening programs for control of diseases in field crops such as apple scab (Köhl et al., 2009), Botrytis grey mold (Calvo-Garrido et al., 2018), powdery mildew (Köhl et al., 2019), European fruit canker (Elena et al., 2022), and the selection of growth-promoting rhizobacteria (Vasseur-Coronado et al., 2021).

The application of MBCAs to seeds differs considerably from their field application in crops. Biological control products for field application, typically containing spores of fungi or cells of bacteria or yeast-like fungi, are produced by biocontrol manufacturers in bioreactors in solid or liquid media, separated from the growth substrate, dried and formulated and stored at the company. During distribution, the product is transported and stored again at one or several storage facilities before finally reaching the grower. At the growers site, the product is again stored for a certain period. Spraying suspensions of the spores or cells are prepared and sprayed to crops using conventional application equipment usually shortly after suspensions have been prepared. During this entire chain viability of the spores or cells is a major issue. Their shelf life in the formulated product depends on the biology of the microorganism and on the downstream process, formulation and packaging at the manufacturers site. Shelf life also depends on the duration of storage periods and storage conditions, especially storage temperature and temperature fluctuations, at the sites of the manufacturer, the distributor and the grower until the product reaches the field. Processes and handling might be optimized and adapted to improve shelf life if needed. Since shelf life strongly depends on the ecological and physiological characteristics of the used species and strain, aspects of shelf life should already be a selection criterion during screening programs. For example, screening assays can address tolerance of spores or cells to commonly used drying processes and the survival of dried spores or cells during storage at different temperatures, including 'room temperature' and even higher temperatures that may occur during transportation of the product during distribution. Selected strains with an appropriate shelf life potential are used by biocontrol manufacturer who will optimize technologies including formulation and define required storage conditions to achieve optimum survival and thus finally optimum field performance.

The route of a biocontrol product from production to the field via coated seeds is longer, more variable and more demanding compared to products marketed for spray applications in the field. Production, downstreaming, formulation and storage at the biocontrol manufacturers site may not differ much from the way products for field spray applications are handled. Once in the field, demands for biocontrol products applied via seed coating, soil application and canopy applications are again similar: efficacy against the targeted plant pathogens depends on survival, germination and growth of the applied antagonist inoculum under favorable and often unfavorable environmental conditions.

However, biological control agents for seed applications are not marketed by the biocontrol manufacturer directly to the grower as end-user. They are applied to seeds by seed processing industries and marketed through the seed distribution chains. During this route, biocontrol agents are exposed to different technological processes during seed processing and seed handling. The applied antagonist is not anymore protected in its optimized package environment but on a surface of a seed, or within a seed if biopriming techniques are applied. Handling, storage conditions and time spans during seed processing and distribution always must guarantee highest seed quality, germination and vigor and should fit into regular seed marketing procedures. After application to seeds, the coated and dried seeds may be seeded shortly after being treated with the microbial inoculants. However, in the majority of cases, treated seeds will be stored. High value seeds such as seeds of many vegetables or ornamentals are stored at storage warehouses under optimum storage conditions for seeds, usually at low relative humidity (RH) and cool temperatures, e.g. 15 °C, 30 % RH. For the majority of arable crops, seeds are not stored at low temperature or precisely controlled RH. After storage, seeds are distributed through a marketing chain to the grower as end-user. During that period, seeds may be exposed to various uncontrolled environmental conditions. The route for biological control agents via seed treatment is thus more demanding and complex compared to the routes for agents applied by crop sprays. Antagonists applied to seeds must fit to this purpose and be able to survive crucial additional processing and handling steps.

The description of seed processing steps and their possible effects on MBCAs allows to identify ecological requirements for seed inocula and to propose selection criteria and selection assays for the screening of new antagonist for seed applications. It also allows to identify possible bottlenecks for seed applied biocontrol agents and needs to adapt seed processing and handling procedures to support the broader use of biocontrol through seed applications.

4. Seed processing, selection criteria for microbial seed inoculants and assays needed

Seeds generally are produced in seed production crops at specific production sites fulfilling specific hygiene and climatic requirements. Harvested seeds are dried and cleaned from debris and shipped to seed processors. Major steps in the further seed processing are sorting, drying, testing, cleaning, packaging, and storing (for more details see: Buitink and Leprince, 2022; Dadlani & Yadava, 2023). To control possible seed pathogens, seeds may be treated by chemical or physical methods. Seed may also be coated with chemical products to protect seeds from seedborne and soilborne diseases of seeds and seedlings. Insecticides and other compounds may also be added to the seed coatings. After treatment, drying and a storage period, seeds are distributed through a distribution chain, stored again at the grower facilities and finally seeded with machineries by the grower. Certified seeds have to fulfill legal requirements according to the Organisation for Economic Co-operation and Development (OECD) and national regulations (OECD, 2021). The aim of all seed processing steps is to achieve seed lots of a particular cultivar free from other seeds or sclerotia, with high viability, vigor and uniform germination, to establish resilient crops without losses due to seedborne or seedling pathogens.

Microbial inocula are typically coated on dried, cleaned and tested seeds. After coating, seeds are dried, stored, handled for packaging, distributed and used by the grower. During these processes, conditions challenge survival of the coated MBCA. Inoculation of seeds by slurry coating and pelleting consists of several steps inducing particular potential stress factors for the applied fungal or bacterial cells (Table 1). MBCA are added as pure spores or cells or encapsulated in microscopic gel capsules to the slurries and pelleting materials, usually mixtures of several compounds. Such compounds may be inert for the applied MBCA or may interfere with their metabolism, e.g. if fungicides are added to the mixtures or biocides had been added to some compounds as

Table 1
Screening criteria for microbial biological control agents (MBCA) adapted to seed coating processes.

Seed coating process				
Process	Conditions	Possible effects on MBCA[1]	Type of screening assays[2]	Decision
Adding MBCA to coating materials	Exposure to mixes of compounds	Toxic effects of compounds and their combinations	Exposure of inocula to single components and final mixes of coating materials	Exclude most sensitive isolates; modify compound mixture
Storage of coating materials containing MBCA	Long-term exposure to compounds	Toxic effects of compounds and their combinations	Exposure of inocula to single components and final mixes of coating materials	Exclude most sensitive isolates; modify compound mixture and exposure duration
	Long-term exposure to constant or fluctuating moderate temperature and high water activity	Temporal activation of metabolism	Exposure of inocula to single components and final mixes of coating materials under different temperature conditions during time	Exclude most sensitive isolates; modify compound mixture and exposure conditions
Coating process	Movement, bouncing and abrasions of coated seeds	Cell wall damage due to mechanical forces	Simulation of mechanical forces on inocula on coated seeds	Exclude most sensitive isolates; modify exposure to mechanical forces
Drying process	Exposure to high temperatures or rapid change of water activity	Stress of unprotected cells during desiccation	Exposure of inocula on coated seeds to different drying temperatures and drying processes	Exclude most sensitive isolates; modify drying process or add protectants

[1] Resulting in stress or reduced viability.
[2] Viability of inocula assessed in all assays.

preservatives. Different metabolic active compounds may have synergistic effects. Slurries contain water so that added inocula take up water and their metabolism is activated and indigenous energy reserves are metabolised. Slurries with added MBCA are used for seed coating processes during a certain time span, e.g. during a working day, so that the MBCA are exposed to the conditions in slurries before coated on seeds during such time spans. Slurries may be cooled or, more common, may stay at ambient temperature. Slurries with added MBCA may also be stored for further use for hours or days under different storage conditions including room temperature. Slurries for seed treatments may be even kept for several months ready for use at seed processors.

MBCA on the seed surface may be exposed to mechanical forces during the further seed coating process with tumbling motions of the seeds in coating equipment turning at different rotation speed depending on type of equipment and coating. The resulting mechanical forces may lead to cracking or possibly abrasions of cell walls of the applied MBCA. Coating processes are most often followed by a drying step to remove the added water again to maintain high vigor and germination ability of the seeds during subsequent storage. Temperatures of 38–40 °C may be applied for several minutes to hours or even up to 70 °C for few minutes for seed drying after the seed coating processes. In the environment, microorganisms develop different ways to protect themselves from desiccation such as storage of trehalose or the formation of biofilms. Under the artificial production systems for MBCA, such protective mechanisms may not be activated. During the drying processes at the biocontrol manufacturer site often specific protectants are added to maintain viability during the drying process. At the seed processing site, such protectants may not be part of the slurry recipes. Inocula may thus be exposed to particular stresses through desiccation, especially since this is the second artificial desiccation process for such inocula after the first processing at the biocontrol manufacturers site. In more complex seed pelleting processes with several layers added on seeds, several coating-drying cycles may be needed increasing possible stresses for the added inocula.

The different processes during seed coating can lead to stress or even reduce viability of the MBCA inocula. Stressed inocula may have higher sensitivity to further stresses during seed processing and handling, finally leading to reduced viability. Candidates in screening programs can be tested in adequate bioassays mimicking the different steps during seed coating, e.g. testing the effect of single compounds included in the slurry mixes or mimicking mechanical stresses. Alternatively, the entire coating process can be executed in more complex assays with each candidate. Obviously, the relevant assessment in all such bioassays is the determination of the viability of the tested inocula. This can be done by commonly applied plating techniques for colony forming unit counts after serial dilutions. Specific plating media and incubation conditions have to be applied for different taxonomic groups. Plating is laborious and costly especially if hundreds of candidates have to be tested under various conditions in high throughput screening (HTS). Use of molecular techniques combining quantification of DNA by qPCR and nucleic acid intercalating dyes such as propidium monoazide (PMA) or ethidium monoazide (EMA) is an alternative to plating techniques (Elizaquível et al., 2014). In HTS with bacterial isolates, generic bacterial 16S pimers can be used for such a viability qPCR, for fungal isolates generic ITS primers. Later in the screening program, after exclusion of the majority of isolates, few isolates or a single superior isolate can be tested more in details using plating techniques or strain-specific qPCR in combination with nucleic acid intercalating dyes. Testing the effect of various stresses on the vigor of biocontrol inocula is rather uncommon in screening programs, whereas vigor testing of seeds is standard practice in seed technology. Vigor assays for MBCAs may include the speed of germination or the sensitivity to additional stresses, e.g. reduced water activities, possibly resulting in reduced viability. The latter can be measured by viability qPCR. The assessment in various assays mimicking different components of seed coating processes allows the exclusion of isolates most sensitive to the exposed conditions. Results can also be used to advise modifications of the seed coating and drying processes including adaptations of the applied slurries as long as seed quality is not affected.

MBCA-coated seeds have to be stored for days to months or even few years, packed, transported for distribution, stored again at sites of the distribution chain and at the end-user facilities, and finally seeded using sowing machineries. During these processes, specific circumstances may cause stress to the MBCA on the seeds, leading to reduced vigor and finally reduced viability (Table 2). Storage of seeds at the seed processing site will be under optimum conditions for seed vigor and seed germinability, e.g. at 15 °C and 30 % RH, for high value seeds but will be less controlled for the majority of seed commodities. Although the low storage relative humidity will favour survival of coated microorganisms, the relatively high storage temperature may be sub-optimum and viability will be reduced during storage duration. Especially extensive storage periods for longer than one or even two years, causing no quality losses in many seeds, will be detrimental for coated microorganisms. An important issue during storage are possible interferences of secondary metabolites, volatile or non-volatile, that are present in the seeds and released to the coating or the air. Such metabolites may affect viability of sensitive microbial groups and reduce their viability during longer storage periods or even already when coated onto the seeds. Seed

Table 2
Screening criteria for microbial biological control agents (MBCA) adapted to handling processes of MBCA-coated seeds.

Seed handling process				
Process	Conditions	Possible effects on MBCA[1]	Type of screening assays[2]	Decision
Storage of coated seeds	Exposure to secondary metabolites of seeds	Toxic effects of volatile or non-volatile metabolites	Exposure of inocula to single metabolites or to seeds	Exclude most sensitive isolates; modify coating layers for critical plant species and varieties
	Sub-optimum temperature > 10 °C and water activity $a_w > 0.75$	Loss of energy reserves	Simulation of storage conditions with coated seeds	Exclude most sensitive isolates; implement adapted storage protocol
	Extensive storage of 2 year or longer under controlled conditions (e.g. 15 °C, 30 % RH, dark)	Viability loss after long term exposure	Simulation of long-term storage conditions with coated seeds	Exclude most sensitive isolates; implement adapted storage protocol
Handling of coated seed lots (e.g. removal from storage)	Temperature changes resulting in condensation on seed surfaces	Stress by temporal activation of metabolism	Simulation of temporally wet surface conditions with coated seeds	Exclude most sensitive isolates; implement adapted handling protocol
Packaging	Movement, bouncing and abrasions of coated seeds	Cell wall damage due to mechanical forces	Simulation of mechanical forces with coated seeds	Exclude most sensitive isolates; adapt coating; modify exposure to mechanical forces
Transportation	Uncontrolled temperature	Stress by high and fluctuating temperature	Simulation of transportation conditions with coated seeds	Exclude most sensitive isolates; implement instructions for transportation
Unintended storage under uncontrolled conditions	Extreme temperatures in containers (e.g. −20 °C or +50 °C)	Stress by extreme temperatures	Simulation of extreme conditions with coated seeds	Indicate unacceptable conditions
Storage at grower	Controlled cooled or at ambient temperature, opened package with uncontrolled increasing humidity	Stress by high temperature and high water activity	Simulation of storage conditions with coated seeds	Exclude most sensitive isolates; implement instructions for use
Seeding machineries	Uncontrolled temperature and humidity; mechanical forces, e.g. during pneumatic precision drilling	Cell wall damage due to mechanical forces	Simulation of mechanical forces with inocula on coated seeds	Exclude most sensitive isolates; adapt coating

[1] Resulting in stress or reduced viability.
[2] Viability of inocula assessed in all assays.

handling for sorting and packaging may also result in stresses for coated MBCA. Moving seeds from the controlled environmental conditions of the storage warehouse to seed processing facilities will expose seeds to temperature changes, resulting in condensation of water on seed surfaces if seeds are not packed hermetically. This may stimulate metabolism of coated microorganisms for periods of high water availability before seed surface will be dry again. Microorganisms will use part of their endogenous nutrients during this short wetness events for metabolic activity. Movement of seeds during packaging may cause mechanical stresses for the coated microorganisms. In the distribution chain, seeds may be exposed to fluctuating, uncontrolled temperatures. Since transportation commonly is not done in a cool chain, temperatures will be sub-optimum or even detrimental for the coated MBCA. Especially in unintended situations, e.g. if containers with seeds get stuck for few days outside warehouses, extreme temperatures can occur, e.g. temperatures of −20 or +50 °C have been measured in such situations. Finally, coated seeds arrive at the growers location where seeds may be stored in cold rooms or – in the majority of cases - ambient temperature. Storage of open bags may result in increased humidity of seeds. Mechanical stresses may occur during the seeding process, e.g. during pneumatic drilling.

The effect of the described stresses for coated microorganisms can be assessed in adequately designed bioassays simulating certain stress situations separately or their combinations. Such assays can be used to select superior microbial isolates with the ecological appropriate characteristics. Bioassays can also be used to optimise the different seed processing steps with the aim to allow better survival of the coated microbials. Results will also support decisions on modifications of seed processing technologies and conditions during storage and distribution detrimental to coated MBCA so that extreme situations will be identified and avoided. Common to such bioassays is that vigor and viability of the coated microorganism needs to be quantified. As show above for testing effects of coating processes on microorganisms, assessments potentially can be done through plating, but a more reliable, cost-effective and faster technology will be the application of generic fungal and bacterial viability qPCR. This will allow high throughput assessments of candidate antagonists and the testing of various simulated conditions.

5. Conclusions and outlook

Seed treatment with MBCA protecting seeds and seedlings from damage by pathogens have a huge potential in agriculture and horticulture. Currently, a very limited range of microorganisms is commercially applied on seeds, e.g. endospore-forming *Bacillus* spp. are often preferred because of their ability to survive various environmental stresses. Consequently, the full potential of the broad microbial diversity is not utilized in biological control of seed and seedling diseases.

Systematic analysis of seed technologies including various seed processing steps and seed handling, identified various conditions detrimental for microorganisms during seed coating and thereafter when MBCA-coated seeds are dried, stored and distributed. Although different causal factors and different detrimental effects on MBCAs potentially limit their utilization, all described stresses are leading to decreased vigor of the applied inocula and finally to reduced viability. Survival and improved shelf life of microbial inocula is thus a key factor to utilize the potential of biological control through seed treatments. To achieve better survival and shelf life, adapted microorganisms with the appropriate ecological characteristics need to be selected. Where possible and needed, seed processing technologies and seed handling has to be adapted to support survival of the coated inocula.

The main conclusion of our analysis is that screening systems for new antagonists for seed applications should include various bioassays mimicking very different stress components on the applied microorganisms, e.g. the potentially detrimental effects of seed volatiles, components of the used seed coating mixes, the drying treatments, the duration of storage at conditions needed to maintain high seed qualities, exposure to unintended situations with uncontrolled environmental conditions or mechanical forces during seed handling etc. Such a system

of bioassays addressing different stress components cannot simply be replaced by a single shelf life assay conducted under a selected set of environmental conditions. However, the various proposed bioassays have in common that viability of the tested microorganisms has to be assessed after exposure to differential conditions. Improved methodology for high throughput viability testing will thus allow larger screening programs and will support the development of new MBCAs for seed applications.

CRediT authorship contribution statement

Jürgen Köhl: Conceptualization, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Patrick Butterbach:** Conceptualization, Investigation. **Ralf-Udo Ehlers:** Conceptualization, Investigation. **Typhanie Gaildry:** Conceptualization, Investigation. **Lia Groenenboom-de Haas:** Conceptualization, Investigation. **Steven P.C. Groot:** Conceptualization, Funding acquisition, Investigation. **Liesbeth van der Heijden:** Conceptualization, Investigation. **Ilse Houwers:** Conceptualization, Investigation. **Ezra de Lange:** Conceptualization, Investigation. **Giovanny Lopez:** Conceptualization, Investigation. **Anita van Nieuwenhoven:** Conceptualization, Investigation. **Martje Notten:** Conceptualization, Investigation. **Mirjam Storcken:** Conceptualization, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abuamsha, R., Salman, M., Ehlers, R.-U., 2011. Effect of seed priming with *Serratia plymuthica* and *Pseudomonas chlororaphis* to control *Leptosphaeria maculans* in different oilseed rape cultivars. Eur. J. Plant Pathol. 130, 287–295. https://doi.org/10.1007/s10658-011-9753-y.

Ali, M.A., Ilyas, F., Arshad, M., Hussain, S., Iqbal, M., Ahmad, S., Saboor, A., Mustafa, G., Ahmed, N., 2019. Microbial inoculation of seeds for better plant growth and productivity. In: Hasanuzzaman, M., Fotopoulos, V. (Eds.), Priming and Pretreatment of Seeds and Seedlings. Springer Nature Singapore Pte Ltd. https://doi.org/10.1007/978-981-13-8625-1_26

Anderson, A.J., Kim, Y.C., 2018. Biopesticides produced by plant-probiotic *Pseudomonas chlororaphis* isolates. Crop Protect. 105, 62–69. https://doi.org/10.1016/j.cropro.2017.11.009.

Berg, G., Köberl, M., Rybakova, D., Müller, H., Grosch, R., Smalla, K., 2017. Plant microbial diversity is suggested as the key to future biocontrol and health trends. FEMS Microbiol. Ecol. 93 https://doi.org/10.1093/femsec/fix050.

Bisen, K., Singh, V., Keswani, C., Ray, S., Sarma, B.K., Singh, H.B., 2020. Use of biocontrol agents for the management of seed-borne diseases. In: Kumar, R., Gupta, A. (Eds.), Seed-Borne Diseases of Agricultural Crops: Detection, Diagnosis & Management. Springer, Singapore. https://doi.org/10.1007/978-981-32-9046-4_22.

Buitink, J., Leprince, O., 2022. Advances in Seed Science and Technology for More Sustainable Crop Production. Burleigh Dodds Science Publishing Limited, Sawston, UK.

Calvo-Garrido, C., Haidar, R., Roudet, J., Gautier, T., Fermaud, M., 2018. Pre-selection in laboratory tests of survival and competition before field screening of antagonistic bacterial strains against Botrytis bunch rot of grapes. Biol. Control 124, 100–111. https://doi.org/10.1016/j.biocontrol.2018.05.006.

Cardarelli, M., Woo, S.L., Rouphael, Y., Colla, G., 2022. Seed treatments with microorganisms can have a biostimulant effect by influencing germination and seedling growth of crops. Plants 11, 259. https://doi.org/10.3390/plants11030259.

Dadlani, M., Yadava, D.K., 2023. Seed Science and Technology - Biology, Production, Quality. Springer Nature Singapore Pte Ltd, Singapore.

Deketelaere, S., Spiessens, K., Pollet, S., Tyvaert, L., De Rooster, L., Callens, D., França, S. C., Höfte, M., 2020. Towards practical application of *Verticillium isaacii* vt305 to control verticillium wilt of cauliflower: exploring complementary biocontrol strategies. Plants 9, 1–23. https://doi.org/10.3390/plants9111469.

Elena, G., Groenenboom-de Haas, B.H., Houwers, I., de Lange, E., Schnabel, S.K., Köhl, J., 2022. Systematic stepwise screening of new microbial antagonists for biological control of European canker. Biol. Control 174. https://doi.org/10.1016/j.biocontrol.2022.105009.

Elizaquível, P., Aznar, R., Sánchez, G., 2014. Recent developments in the use of viability dyes and quantitative PCR in the food microbiology field. J. Appl. Microbiol. 116, 1–13. https://doi.org/10.1111/jam.12365.

EU, 2020. Commission Implementing Regulation (EU) 2020/617 of 5 May 2020 renewing the approval of the active substance metalaxyl-M, and restricting the use of seeds treated with plant protection products containing it, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending the Annex to Commission Implementing Regulation (EU) No 540/2011. Off. J. Euro. Union L 143, 6–10.

Ferrigo, D., Mondin, M., Ladurner, E., Fiorentini, F., Causin, R., Raiola, A., 2020. Effect of seed biopriming with *Trichoderma harzianum* strain INAT11 on Fusarium ear rot and Gibberella ear rot diseases. Biol. Control 147, 104286. https://doi.org/10.1016/j.biocontrol.2020.104286.

Hökeberg, M., Gerhardson, B., Johnsson, L., 1997. Biological control of cereal seed-borne diseases by seed bacterization with greenhouse-selected bacteria. Eur. J. Plant Pathol. 103, 25–33. https://doi.org/10.1023/A:1008681608400.

Jensen, B., Knudsen, I.M.B., Madsen, M., Jensen, D.F., 2004. Biopriming of infected carrot seed with antagonist, *Clonostachys rosea*, selected for control of seedborne Alternaria spp. Phytopathology 94, 551–560. https://doi.org/10.1094/PHYTO.2004.94.6.551.

Johnsson, L., Hökeberg, M., Gerhardson, B., 1998. Performance of the *Pseudomonas chlororaphis* biocontrol agent MA 342 against cereal seed-borne diseases in field experiments. Eur. J. Plant Pathol. 104, 701–711. https://doi.org/10.1023/A:1008632102747.

Koch, E., Weil, B., Wächter, R., Wohlleben, S., Spiess, H., Krauthausen, H.-J., 2006. Evaluation of selected microbial strains and commercial alternative products as seed treatments for the control of *Tilletia tritici*, *Fusarium culmorum*, *Drechslera graminea* and *D. teres*. J. Plant Dis. Prot. 113, 150–158. https://www.jstor.org/stable/44754742.

Köhl, J., Goossen-van de Geijn, H., Groenenboom-de Haas, L., Henken, B., Hauschild, R., Hilscher, U., Lombaers-van der Plas, C., van den Bosch, T., Wikström, M., 2019. Stepwise screening of candidate antagonists for biological control of *Blumeria graminis* f.sp. *tritici*. Biol. Control. https://doi.org/10.1016/j.biocontrol.2019.104008.

Köhl, J., Molhoek, W.M.L., Groenenboom-de Haas, B.H., Goossen-van de Geijn, H.M., 2009. Selection and orchard testing of antagonists suppressing conidia production of the apple scab pathogen Venturia inaequalis. Eur. J. Plant Pathol. 123, 401–414. https://doi.org/10.1007/s10658-008-9377-z.

Köhl, J., Postma, J., Nicot, P., Ruocco, M., Blum, B., 2011. Stepwise screening of microorganisms for commercial use in biological control of plant pathogenic fungi and bacteria. Biol. Control 57, 1–12. https://doi.org/10.1016/j.biocontrol.2010.12.004.

Lamichhane, J.R., You, M.P., Laudinot, V., Barbetti, M.J., Aubertot, J.-N., 2020. Revisiting sustainability of fungicide seed treatments for field crops. Plant Dis. 104, 610–623. https://doi.org/10.1094/PDIS-06-19-1157-FE.

Mancini, V., Romanazzi, G., 2014. Seed treatments to control seedborne fungal pathogens of vegetable crops. Pest Manag. Sci. 70, 860–868. https://doi.org/10.1002/ps.3693.

Mastouri, F., Björkman, T., Harman, G.E., 2010. Seed treatment with *Trichoderma harzianum* alleviates biotic, abiotic, and physiological stresses in germinating seeds and seedlings. Phytopathology 100, 1213–1221. https://doi.org/10.1094/PHYTO-03-10-0091.

McQuilken, M.P., Halmer, P., Rhodes, D.J., 1998. Application of microorganisms to seeds. In: Burges, H.D. (Ed.), Formulation of Microbial Biopesticides. Kluwer Academic Publishers, the Netherlands, pp. 255–285.

Müller, H., Berg, G., 2008. Impact of formulation procedures on the effect of the biocontrol agent *Serratia plymuthica* HRO-C48 on Verticillium wilt in oilseed rape. BioControl 53, 905–916. https://doi.org/10.1007/s10526-007-9111-3.

Neergaard, P., 1979. Seed Pathology. MacMillan, London, UK.

O'Callaghan, M., 2016. Microbial inoculation of seed for improved crop performance: issues and opportunities. Appl. Microbiol. Biotechnol. 100, 5729–5746. https://doi.org/10.1007/s00253-016-7590-9.

OECD, 2021. OECD Seed Schemes 2021 - OECD Schemes for the Varietal Certification or the Control of Seed Moving in International Trade. <http://www.oecd.org/agriculture/code/seeds.htm> (visited 11.10.2023).

Rocha, I., Ma, Y., Souza-Alonso, P., Vosátka, M., Freitas, H., Oliveira, R.S., 2019. Seed coating: a tool for delivering beneficial microbes to agricultural crops. Front. Plant Sci. 10, 1357. https://doi.org/10.3389/fpls.2019.01357.

Trimmer, M.C., 2021. Microbial bioprotectants and the marketplace. In: Köhl, J., Ravensberg, W.J. (Eds.), Microbial Bioprotectants for Plant Disease Management. Burleigh Dodds Science Publishing Limited, Sawston, Cambridge, UK, pp. 223–244.

Vasseur-Coronado, M., du Boulois, H.D., Pertot, I., Puopolo, G., 2021. Selection of plant growth promoting rhizobacteria sharing suitable features to be commercially developed as biostimulant products. Microbiol. Res. 245, art. no. 126672. 10.1016/j.micres.2020.126672.