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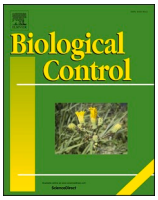

Generic viability qPCR for monitoring shelf life of microbial biological control agents coated on seeds based on the nucleic acid intercalating dyes EMA and PMAxx

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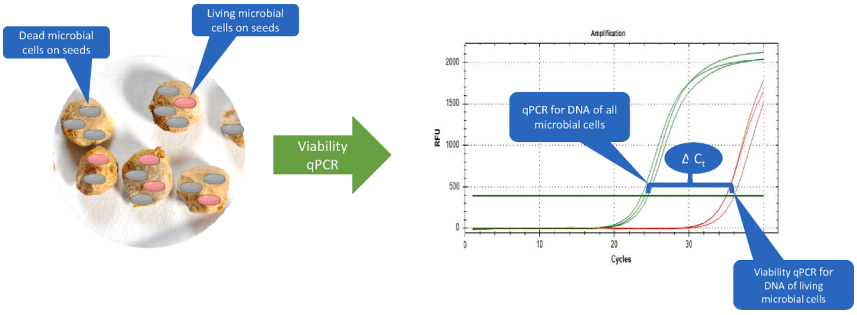
HIGHLIGHTS

- Biological control of pathogens feasible through microbial seed treatments.
- Poor survival of microbial inocula on seeds is a main bottleneck for implementation.
- Viability of microorganisms coated on seeds assessed by viability (v)-qPCR.
- V-qPCR will support screening of antagonists with better survival on seeds.
- V-qPCR will support development of seed technologies for microbial seed coating.

GRAPHICAL ABSTRACT



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ABSTRACT

Biological control of seedborne pathogens and soilborne seedling pathogens through antagonists applied on seeds is an alternative to chemical seed treatments. Information on the viability of inocula on treated seeds is essential for any development and use of beneficial fungi or bacteria on seeds. Generic fungal and bacterial qPCR assays were combined with the nucleic acid intercalating dyes ethidium monoazide (EMA) and propidium monoazide (PMAxx) for the quantification of viable cells of fungi and bacteria on seeds. The applied protocols for generic fungal viability qPCR (v-qPCR) in combination with EMA and PMAxx and for generic bacterial v-qPCR in combination with PMAxx allowed the viability quantification of fungal and bacterial isolates representing a broad range of species with the exception of fungal species with highly melanized conidia. A first application of v-qPCR to coated seeds of onion and spinach indicated a differential plant species effect on survival of a coated fungus and a yeast with a generally better survival on seeds of spinach compared to seeds of onions and a similar good survival of the bacterium *L. enzymogenes* 3.1T8 on both seed types. The v-qPCR protocols can be applied in screening assays aiming at the selection of new antagonists with higher survival potentials and the development of new seed processing technologies compatible with coated antagonists.

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1. Introduction

Biological control of seedborne pathogens and soilborne seedling pathogens through antagonists applied on seeds is an alternative to chemical seed treatments (Bisen et al., 2020; Lamichhane et al., 2020). Examples are the 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), and the use of *Trichoderma* spp. in cereals and vegetables (Mastouri et al., 2010; Ferrigo et al., 2020). Different technologies are used for seed applications such as pelleting, coating and bio-priming (Müller & Berg, 2008; Abuamsha et al., 2011).

Information on the amount of living inoculum on treated seeds is essential for any use of microbial inocula on seed (Köhl et al., 2024). Techniques for shelf life assessments have to be applied during the screening of new strains, the development of seed application and storage technologies as well as for quality control of the marketed treated seeds. A commonly used technique to assess viability is plating of the inoculants after removal from the seeds. This technique depends on suitable plating media, microbial labs, equipment and expertise. The preparation of dilution series is time and labour consuming. Results will be available after several incubation days. Specific species- or even strains-specific qPCR assays can also be applied to quantify the applied DNA of microbial inoculants. However, no distinction between DNA of living or dead cells can be made therefore obtained data are not relevant for shelf life studies.

For the quantification of viable cells of bacteria and fungi, qPCR has been combined with nucleic acid intercalating dyes such as propidium monoazide (PMA) or ethidium monoazide (EMA) (Nogva et al., 2003; Nocker et al., 2006). The principle of this viability quantitative PCR (v-qPCR) is that the viability dyes penetrate only in cells with compromised cell membrane (PMA) or are actively pumped out of an intact cell (EMA) (Codony et al., 2015). Viability stains within the cells intercalates covalently into the DNA after exposure to strong visible light, so that DNA amplification of the blocked DNA in qPCR assays is hampered (Elizaquível et al., 2014).

The combination of qPCR with the nucleic acid-binding dyes PMA and EMA (v-qPCR) has been reported for the quantification of several foodborne human pathogens such as *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica,* for infectious viruses such as Poliovirus and Norovirus and for foodborne yeasts and hyphal fungi such as *Zygosaccharomyces bailii*, and *Alternaria* sp. (Elizaquível et al., 2014). Besides the important advantages of using v-qPCR in food for the detection of viable harmful microorganisms, several factors may limit the application of v-qPCR based on PMA and EMA. First, the complex matrices that may affect the efficiency of v-qPCR by adsorption and other mechanisms (Elizaquível et al., 2014), second the need to detect very low levels in the case of foodborne pathogens, and third the occurrence of false positive signals observed in several studies with membrane-compromised dead bacterial cells. Other recent applications of v-qPCR using PMAxx in combination with EMA is the quantification of viable but not culturable cells (VBNC) of *Listeria monocytogenes* in process wash water of fresh-cut produce including a validation in an industrial processing (Truchado et al., 2020) and in ready-to-eat salad (Bernardo et al., 2021). V-qPCR has also been applied to assess viability of plant pathogenic *Xylella fastidiosa*, *Fusarium* spp. in soil and *Candidatus* Liberibacter solanacearum on carrot seeds (Baró et al., 2020; Chen et al., 2022; Othmen et al., 2023).

Examples of the use of v-qPCR in biological control of plant diseases are still limited. *Pantoea agglomerans* CPA-2, antagonistic to several post-harvest pathogens of citrus and pome fruit, has been monitored on orange peels in the post-harvest condition (Soto-Munoz et al., 2015). The combination of a strain-specific qPCR with a pre-treatment with PMA demonstrated a low persistence of *P. agglomerans* CPA-2 in situations where genomic DNA of the antagonist was still detected in high amounts by the qPCR without addition of PMA. A v-qPCR specific for the biological control agent *Lactobacillus plantarum* PM411 has been developed and applied for population monitoring on aerial plant surfaces (Daranas et al., 2018). Under conducive conditions for bacterial development on plant surfaces, qPCR assays and such assays in combination with the nucleic acid-binding dye PEMAX gave similar results. However, under stressful conditions, v-qPCR assays showed lower amounts compared to qPCR assays due to cell death of a part of the applied bacterial population. In literature different DNA blocking dyes are used like PMA, PMAxx and PEMAX. PMAxx is the new and improved version of PMA from BIOTIUM (Fremont, California). PEMAX is the double dye technology from GenIUL (Terrassa, Spain) and no more available in 2022. Using v-qPCR in ecological studies of field-released populations of biological control agents offers important new opportunities to improve strain selection, product formulation and application strategies. Applications of v-qPCR for fungal antagonists have not been reported yet.

The objective of the study was to contribute to the development of a method allowing quantification of viable microbial seed inoculum based on generic quantification of bacterial or fungal DNA in combination with DNA nucleic acid-binding dyes to enumerate viable cells. This will allow fast and standardized monitoring of shelf life of microbial seed inoculants for selection of new candidate strains, development of improved seed coating and storage technologies and quality control of microbial-coated seed lots. If available, the generic primers can be substituted by strain-specific primers allowing more focussed shelf life monitoring and quality control of specific inocula. Protocols were developed for the combined use of generic primers, qPCR and nucleic acid-binding dyes for v-qPCR of bacteria and fungi. The protocols were tested for a panel of bacterial and fungal isolates and seeds of different vegetable and grass species and applied in a first pilot experiment for seeds of onion and spinach coated with bacterial or fungal inocula.

2. Materials and methods

2.1. Micro-organisms

Fungal and bacterial isolates used in the study are from the culture collection of our institute, stored as single isolates at −80 °C (Table 1). The seed-producing companies provided seed lots of Brassica vegetables, carrots, onion, perennial ryegrass and spinach that were mechanically cleaned from dirt but not further processed or coated before use.

2.2. Development of v-qPCR

2.2.1. Suspensions of fungal and bacterial spores or cells and their lethal treatment

Spore suspensions (or cell suspension of yeasts) were prepared from fungal isolates grown in Petri dishes on potato dextrose agar (PDA) for 21 days at 18 °C in the dark. Colonies were flooded with sterile water containing 0.01 % Tween 80. After gently rubbing with a sterile rubber spatula, the obtained suspensions were filtered through nylon gauze (200 μm mesh). Spore concentrations were determined using a haemocytometer and adjusted with water containing 0.01 % Tween 80 to a maximum of 1×10^8 spores or cells ml^{-1}. In some cases lower concentrations were reached, e.g. 3×10^6 spores ml^{-1} for *Alternaria* sp. BN115. Tubes with suspensions were kept in ice water.

An aliquot of 2,000 μl of the suspensions was mixed in 10 ml-tubes with 5,140 μl isopropanol (96 %) to obtain a final concentration of isopropanol of 70 % to kill spores and cells in the suspension. In the untreated control treatment 2000 μl of the suspensions were mixed with 5,140 μl water containing 0.01 % Tween 80. Tubes were shaken on a Tube roller for 10 min. at room temperature. Thereafter, suspensions were centrifuged at 3828 g at 4 °C for 10 min, supernatants were discarded and pellets were resuspended in 2 ml water containing 0.01 % Tween 80.

The germinability of fungal spores and yeast cells after the isopropanol treatment was assessed before spores were treated with nucleic

Table 1
Origin of fungal and bacterial isolates used in the study.

Name	Isolate number	Host/substrate	Origin	Year
Yeasts and yeast-like fungi				
Aureobasidium pullulans	BN072	Apple wood	Netherlands	2019
Cryptococcus albidus	733	Potato leaf	Netherlands	2004
Cryptococcus macerans	M6a9b	Spinach seed endophyte	Netherlands	2012
Cryptococcus macerans	M5d1	Spinach seed endophyte	Netherlands	2012
Cystobasidium laryngis	BN151	Apple wood	Netherlands	2019
Rhodotorula glutinis	BN093	Apple wood	Netherlands	2019
Rhodotorula sp.	M6a3	Spinach seed endophyte	Netherlands	2012
Rhodotorula sp.	M6e1	Spinach seed endophyte	Netherlands	2012
Sporobolomyces metaroseaus	BN155	Apple wood	Sweden	2019
Hyphal fungi				
Alternaria brassicae	Z164	Brassica seed	Netherlands	1991
Alternaria sp.	BN115	Apple wood	Germany	2019
Boeremia exigua	BN012	Apple wood	Netherlands	2019
Chalaropsis thielavioides	Z350	Carrot	Unknown	Before 2000
Cladosporium cladosporioides	H39	Apple leaf	Netherlands	2003
Cladosporium ramontenellum	BN031	Apple wood	Netherlands	2019
Clonostachys rosea *	J1446	Soil	Finland	Before 1998
Coniothyrium minitans	1143	Soil	UK	Before 2011
Didymella macrostoma	BN007	Apple wood	Netherlands	2019
Fusarium sp.	6180–12	Carnation	Netherlands	1992
Fusarium oxysporum	E32b2	Spinach seed endophyte	Netherlands	2012
Isaria javanica *	FE9901	*Bemisia tabaci*	India	Before 2012
Metarhizium brunneum *	F52	Scarab larvae	USA	Before 1988
Penicillium sp.	Z114	Brassica seed	Netherlands	1991
Trichoderma viride	004	Beech bark	Germany	1985
Trichoderma viride	226	Spruce bark	Germany	1985
Ulocladium atrum	385	Onion leaf tip	Netherlands	1991
Ulocladium cucurbitae	742	Unknown	Unknown	Before 2005
Verticillium dahliae	P15a	Spinach seed endophyte	Netherlands	2012
Gram-bacteria				
Bacteroides sp.	M12	Potato	Netherlands	Before 2012
Lysobacter enzymogenes	3.1T8	Cucumber roots	Netherlands	1996
Pantoea vagans	E20a	Spinach seed endophyte	Netherlands	2012
Pseudomonas chlororaphis *	MA342	Crawberry roots	Sweden	Before 1991
Pseudomonas fluorescens	PE75-1	Potato	Netherlands	2000
Pseudomonas (fluorescens group)	C17	Chrysanthemum	Netherlands	2000
Pseudomonas sp.	P4a	Spinach seed endophyte	Netherlands	2012
Rahnella aquatilis	E22b1	Spinach seed endophyte	Netherlands	2012
Rhizobium sp.	E6d	Spinach seed endophyte	Netherlands	2012
Serratia marcescens	A2	Arabidopsis	Netherlands	2000
Serratia plymuthica	HROC48	*Brassica napus* rhizosphere	Germany	1994
Xanthomonas anxonopodis	D17	Potato	Netherlands	2000
Gram + bacteria				
Arthrobacter sp.	P9c	Spinach seed endophyte	Netherlands	2012
Bacillus pumilus	4.42	Soil bulk	Netherlands	2003
Bacillus sp. *cereus/ thurigiensis*	P17a1	Spinach seed endophyte	Netherlands	2012
Paenibacillus peoriae	2.3.2	Soil bulk	Netherlands	2003
Paenibacillus polymyxa	P13a	Spinach seed endophyte	Netherlands	2012
Streptomycetes				
Streptomyces galilaeus	12.2.5	Soil rhizosphere	Netherlands	2003
Streptomyces griseoviridis *	K61	Peat	Finland	1975
Streptomyces lateritius	2.3.8	Soil rhizosphere	Netherlands	2003
Streptomyces platensis	7.3.8	Soil rhizosphere	Netherlands	2003
Streptomyces pseudovenezuelae	14.4.2	Soil rhizosphere	Netherlands	2003

* Isolates from commercially available plant protection products kindly provided by e-nema, Schwentinental, Germany.

acid-binding dyes. Suspensions (100 µl containing 10^5 spores or cells ml^{-1}) were plated on 1/10 malt extract agar (MA) and plates were incubated for 20 h at 25 °C in the dark. Germinability of fungal spores were determined microscopically for 50 spores. Conidia were considered germinated when the germ tube was at least as long as the shortest diameter of the conidium. Fifty yeast cells were assessed and counted as vital if divided at least into two cells after incubation.

Streptomycetes were grown on PDA amended with 100 mg l^{-1} Delvocid for 28 days at 25 °C. All other bacteria were grown on tryptic soy agar (TSA) for 24 h at 25 °C. Suspensions of bacteria were prepared by flooding colonies with Ringer solution (2 ¼ strength tablets l^{-1} of tap water; Sigma Aldrich) at room temperature and scratching colonies with an inoculation loop. Concentrations were determined with haemocytometer and adjusted using Ringers solution to 10^8 cells ml^{-1}. Aliquots of the cells suspensions were treated with isopropanol as described for fungi but Ringer salts were added to maintain the osmotic values during the treatments. The suspensions were centrifuged at room temperature for 10 min at 3828 g.

Viability of the bacterial cells was tested by plating 100 ul suspension on TSA except Streptomycetes that were tested on PDA with Delvocid. For untreated bacterial suspensions, concentrations of the suspensions were 10^6, 10^7 and 10^8 cells ml^{-1}, for isopropanol-treated cells concentrations were 10^2, 10^3 and 10^4 cells ml^{-1}. Developed colonies were counted per plate after incubation for 24 h at 25 °C for bacteria and 6 days at 25 °C for Streptomycetes. Number of colonies were multiplied by the corresponding dilution factor to calculate the amount of culturable bacterial cells ml^{-1}.

2.2.2. Treatment with DNA-binding dyes

PMAxx reagent (Biotium) was diluted in HyPure water (nuclease free HyClone™ Molecular Biology-Grade Water; VWR) to obtain a stock solution of 500 µM which was stored at −20 °C in the dark. For EMA (Biotium) a 250 µM solution was prepared using HyPure water. The solution was kept at 4 °C in the dark. Treatments of fungal and bacterial cells were performed in DNeasy 96 plates (Qiagen). Both untreated and isopropanol-treated cells were treated in triplicate with DNA-binding

dyes or HyPure water. For hyphal fungi and yeasts 215 µl of suspensions containing 10^8 spores or cells ml^{-1} were mixed with 25 µl PMAxx and 10 µl EMA stock solution. For bacteria including Streptomycetes 225 µl 10^8 cells ml^{-1} were mixed with 25 µl EMA stock solution. 35 µl or 25 µl HyPure water was added to fungal or bacterial suspensions not treated with the dyes. Thereafter, samples were incubated for 30 min in the dark at 20 °C in an heat block with plate adapter (Eppendorf, Hamburg, Germany) at 650 rpm. Subsequently, samples were photoactivated for 10 min and 30 % light intensity [equals 5870 µmol m^{-2} s^{-1} as measured using a UPRtek PG200N spectrophotometer] with the 96 Wells LED module (PhenoVation, Wageningen, Netherlands). Cells were collected by centrifugation at 5796 g for 10 min and 200 µl supernatant was pipetted off. Remaining samples were freeze-dried and DNA was extracted immediately.

2.2.3. DNA extraction

Isolation of genomic DNA from fungal and bacterial cells was performed in 96-well format using the Sbeadex maxi plant kit (LGC, Teddington, UK). Lyophilized tissue was kept in liquid nitrogen for 30 s and thereafter disrupted using the TissueLyser II (Qiagen) and 90 mg 1.0 mm Silicon Carbide Beads (BioSpec Products, Bartlesville, USA) for 30 s at 30 Hertz. After disruption 250 µl lysis solution was added to each sample and further DNA extraction was done with the KingFisher™ Flex Purification System pipetting robot (Thermo Fischer Scientific, Waltham, MA, USA) according to the protocol supplied by the manufacturer with one modification; the incubation time at 65 °C was prolonged from 10 min to one hour. Negative extraction controls without any added DNA or cells of fungi or bacteria were included in each qPCR assay run. For experiments done to determine the dynamic range of v-qPCR assays and the effects of seed washings on v-qPCR, DNA was extracted using the Molgen PurePrep Seed Kit, expected to be free of bacterial DNA, according to the protocol of the manufacturer.

2.2.4. qPCR

The generic fungal assay targets the internal transcribed spacer (ITS) region found in rRNA genes, using ITS1 (TCCGTAGGTGAACCTGCGG) as forward primer and 5.8 s (CGCTGCGTTCTTCATCG) as reverse primer (Fierer et al., 2005). The generic bacterial qPCR assay targets 16S rRNA genes, using Eub338 (ACTCCTACGGGAGGCAGCAG) as forward primer and EUb518 (ATTACCGCGGCTGCTGG) as revers primer (Fierer et al., 2005). Both primer sets were synthesized by IDT (Integrated DNA Technologies, Leuven, Belgium).

The qPCR assays were conducted in 384-well format in a CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). For each PCR assay, 1 µl sample was mixed with 9 µl reaction mix containing 5 µl 2X TB Green Premix Ex Taq™ (Takara BIO Europe) and 200 nM of each forward and reverse primer for fungi and 100 nM primers for bacteria. The reaction conditions were: 95 °C for 2 min; 40 cycles of 95 °C for 15 s followed by amplification for 30 s at 59 °C for fungi and 63 °C for bacteria. Analysis of the data was done by automatic threshold calculation within the Biorad CFX Manager software version 1.0 (Bio-Rad Laboratories Inc.). Melting curve analysis of the PCR products was performed in each assay to confirm that the fluorescence signal originated from specific PCR products and not from primer-dimers or other artifacts.

Strain-specific TaqMan qPCR assays were applied for *L. enzymogenes* 3.1T8 (Nijhuis et al., 2010) and *C. cladosporioides* H39 (Köhl et al., 2009), using forward primer Fw 2_590.820 (GAGCACACCCAAGTCTTTG), reverse primer Rv 2_590820 (GCGTCTGCTTTGCGTT) and probe probe 590.820 (Ctgcgaa-GACctcgacc) labelled at the 5′end with a fluorescein label 6-FAM and at the 3′end with BHQ1, capital letters are LNA nucleotides (Köhl and Groenenboom-de Haas, unpublished).

For each TaqMan qPCR of *L. enzymogenes* and *C. cladosporioides*, 1 µL sample was mixed with 9 µL reaction mix containing 5 µL Quanta PerfeCTa®qPCR Toughmix™ (Quantabio), 100 nM fluorescein (FAM)-labelled probe and 300 nM of each forward and reverse primer. The reaction conditions were: 95 °C for 2 min; 40 cycles of 95 °C for 15 s followed by amplification for 1 min at 60 °C. Analysis of the data was done by automatic threshold calculation within the Biorad CFX Manager software version 1.0 (Bio-Rad Laboratories Inc.).

2.2.5. Determination of the dynamic range of v-qPCR assays

Conidial suspensions of *C. cladosporioides* H39 at concentrations from 10^4 to 10^8 conidia ml^{-1} and spore suspensions of *L. enzymogenes* 3.1T8 at concentrations from 10^5 to 10^9 cells ml^{-1} were prepared. Untreated suspensions or suspensions treated with isopropanol were mixed with EMA/PMAxx or PMAxx as described above and DNA was extracted using the Molgen PurePrep Seed Kit. qPCR assays were conducted in triplicate using the generic fungal primer set ITS1 and 5.8 s and the *C. cladosporioides* H39 strain-specific primer-probe combination for suspensions of *C. cladosporioides* H39 and the generic bacterial primer set Eub338 and EUb518 and the *L. enzymogenes* 3.1T8 strain-specific primer-probe combination for suspensions of *L. enzymogenes* 3.1T8. Calibration curves were fitted for the measured C_t values below the obtained negative extraction controls (NEC).

2.2.6. Effect of seed washings on v-qPCR

Seeds of two seed lots of Brassica vegetables (180 seeds), carrots (140 seeds), onion (150 seeds), perennial ryegrass (90 seeds) and spinach (45 seeds for lot 1 with smaller seeds and 27 seeds of lot 2 with larger seeds) were shaken in 7 ml tap water containing 0.01 % Tween 80 or Ringers in 50 ml Greiner tubes on a Vortex for 5 s followed by shaking for 1 min on a Vortex Genie 2 with a horizontal tube holder for 6 tubes (level 5) (Scientific Industries Inc., New York, U.S.A.). Obtained solutions were left for 10 min and 1170 µl of each washing with 0.01 % Tween were mixed with 130 µl of conidial suspensions of *C. cladosporioides* H39 (2×10^8 conidia ml^{-1}; untreated or treated with isopropanol). Washing with Ringers were mixed with cell suspensions of *L. enzymogenes* 3.1T8 (2×10^9 cells ml^{-1}; untreated or treated with isopropanol) in the same way. Immediately thereafter, EMA/PMAxx or PMAxx solution or HyPure water as control was added, DNA was extracted using the Molgen PurePrep Seed Kit and *C. cladosporioides* H39 and *L. enzymogenes* 3.1T8 strain-specific qPCR assays were conducted as described above.

2.2.7. Application of v-qPCR on coated seeds

Seed treatment. *Cryptococcus albidus* 733 and *Coniothyrium minitans* 1143 were grown on PDA at 18 °C for 5 and 14 days, respectively. *Lysobacter enzymogenes* 3.1T8 was grown on TSA for 1 day at 25 °C. Suspensions were produced as described above, dissolved in saline or Ringer solution to a final volume of 6 ml per isolate and added to the commercially available and used seed polymers Polyselect 527C containing blue pigment and Polyselect 539C containing red pigment to simulate commercial seed coating using 5.5 ml microbial suspension, 2 ml tap water and 7.5 ml Polyselect 527C for coating 86 g (7.500 seeds) spinach seeds, and 2.1 ml microbial suspension and 0.7 ml Polyselect 539C for coating 18.6 g (5.000 seeds) onion seeds. Batches of onion seeds were coated by adding the coating mix with a syringe to a polyethylene bag with seeds while mixing the bag by hand to reach even coverage. Batches of spinach seeds were treated using a minicoater (HR160/Hoopman, Aalten, Netherlands). Based on the concentration of the applied suspensions and the number of treated seeds the estimated amount of *C. albidus* 733 cells was 4.0×10^4 cells per onion seed and 3.5×10^5 cells per spinach seed. For *C. minitans* 1143 estimated amounts of spores were 2.0×10^4 spores per onion seed and 1.8×10^5 spores per spinach seed. *L. enzymogenes* 3.1T8 was applied with approximately 5.6×10^5 cells per onion seed and 4.7×10^6 cells per spinach seed. The treated seeds were stored dry at room temperature for 4 days.

Coated fungal isolates *C. albidus* 733 and *C. minitans* 1143 were removed from three sub-samples each consisting of 100 onion or 50 spinach seeds and 1 ml water containing 0.01 % Tween 80 was added to 100 onion seeds in 1.5 ml Eppendorf vials and to 50 spinach seeds in 2

ml vials. For removal of *L. enzymogenes* 3.1T8 from seed samples, Ringer solution was used instead. Tubes were shaken for 1 min at 1000 rpm using the Thermomixer (Eppendorf, Hamburg, Germany). Immediately thereafter aliquots of the supernatant were processed and treated separately for the three replicates per isolate with EMA/PMAxx or PMAxx as described above or remained untreated. DNA was extracted as described above. For each generic fungal or bacterial qPCR a separate amplification control (AC) with a green fluorescent protein (GFP) qPCR assay was conducted with the fungal or bacterial DNA extracts, to assess possible PCR inhibition (Klercks et al., 2004). If measurements of the AC indicated inhibition, measurements of fungal or bacterial DNA without and with EMA/PMAxx or PMAxx treatments were repeated after 2-fold dilution of the sample.

2.2.8. Statistics

Per each experiment, C_t values obtained from the v-qPCR assays were compared to determine significant differences within the following combinations of treatments (ΔC_t): Isopropanol/EMA/PMAxx or PMAxx – Untreated/EMA/PMAxx or PMAxx; Isopropanol/Untreated – Untreated/Untreated; Untreated/EMA/PMAxx or PMAxx – Untreated/Untreated; Isopropanol/EMA/PMAxx or PMAxx – Isopropanol/Untreated. T-tests were performed to detect statistically significant differences between C_t value means using the Data Analysis tool of Microsoft® Excel® for Microsoft 365 MSO (Version 2308 Build 16.0.16731.20542) 32-bit.

3. Results

3.1. Protocol development for EMA/PMAxx treatments

Isopropanol treatments were effective in killing fungal spores, and cells of yeasts and bacteria as proven by plating (data not presented). Untreated spores or cells of all fungal isolates germinated for >95 % on nutrient agar. Isopropanol-treated fungal spores or cells did not germinate for most isolates and for <3 % for several isolates. Also bacteria were strongly affected by isopropanol treatments. No colonies were found on plates for most bacteria. Some isopropanol-treated bacteria produced few colonies, but <0.01 % of the CFU as produced by untreated bacterial cells, meaning that >99.99 % of the bacterial cells had been killed.

C_t values were >36 for negative extraction controls for all runs done with the generic primers for fungi (Table 2). However, for qPCR assays done with the generic primers for bacteria, C_t values were lower than 40 with $C_t = 28.0$ for run 1, $C_t = 27.6$ for run 2 and $C_t = 27.5$ for run 3 (Table 3). Additional assessments confirmed that bacterial DNA is present in the used Sbeadex maxi plant kit (data not presented).

All tested fungal and bacterial isolates were detected by the qPCR assays using the generic primer sets. Measured C_t values for fungal DNA not treated by isopropanol or EMA/PMAxx ranged between 11.1 and 25.4 for the different fungal isolates tested. For bacterial DNA quantification, C_t values ranged between 13.3 and 25.3 for the different isolates of bacteria.

C_t values for fungal isolates non-treated with isopropanol but with EMA/PMAxx showed similar values as for isolates non-treated with the dyes (Table 2). The resulting ΔC_t values for fungal isolates non-treated with isopropanol were all low, in most cases close to ΔC_t values = 0, indicating that DNA of living cells had not been blocked by the combined dyes. Similar results were obtained for bacterial isolates with PMA treatments.

C_t values for fungal isolates non-treated with EMA/PMAxx which had been treated by isopropanol were generally higher compared to C_t values for isolates non-treated with isopropanol. The ΔC_t values mostly were below 3, but in 7 out of the 28 cases even higher with a maximum ΔC_t values of 14.4 for *Clonostachys rosea* J1446 (Table 2). Although for fungi results indicated a lower measured concentration of DNA after isopropanol treatment, for bacteria negative ΔC_t values indicated that after propanol treatment there was a trend to measure higher DNA concentrations (Table 3).

The comparison of EMA/PMAxx-treated fungal isolates non-treated with isopropanol versus propanol-treated generally revealed statistically significant differences resulting in high ΔC_t values above 7 till a maximum of 16 indicating that the dyes were highly efficient in binding to DNA of dead fungal cells (Table 2). These values were distinctly higher than ΔC_t values found when isolates non-treated with isopropanol versus isopropanol-treated were compared without EMA/PMAxx treatment. In three exceptional cases, the dye combination only weakly indicated dead fungal spores with ΔC_t value = 1.8 for *A. brassicae* Z164, ΔC_t value = 0.3 for *U. atrum* 385 and ΔC_t value = 2.7 for *U. curcurbitae* 742. These three fungi are dematiaceous hyphomycetes producing spores with distinctly thick and highly melanized cell walls that may not allow penetration of the dyes or penetration of sufficient light needed for photoactivation. However, *Alternaria* sp. BN155, also belonging to the same group fungal group, revealed an ΔC_t values of 8.3.

Similar results were obtained for bacteria (Table 3). The generally high ΔC_t value above 5 indicated that DNA of dead bacterial cells after isopropanol treatment had been blocked efficiently by PMA. However, the differences were less pronounced as for fungal isolates since the low C_t values for the NEC of approximately $C_t = 27$ did not allow C_t values above that level for measurement of dead bacterial cells. *Streptomyces pseudovenezuelae* 14.4.2 showed a ΔC_t value close to zero with 0.8. C_t values for S. *pseudovenezuelae* 14.4.2 were already high for cells not treated by isopropanol indicating low concentration of cells which is confirmed by the results of plating the suspension on agar medium. Due to the observed low C_t value for NEC, it was technically not possible to detect lower values for isopropanol treated cells and thus to obtain a larger ΔC_t value for S. *pseudovenezuelae* 14.4.2.

ΔC_t values for isopropanol-treated fungal isolates with or without EMA/PMAxx treatments were generally about ΔC_t values = 10, confirming that the combined dyes efficiently blocked DNA of dead fungal cells (Table 2). There were three significant exceptions with *A. brassicae* Z164, *U. atrum* 385 and *U. curcurbitae* 742. This is in line with the low ΔC_t values found for these isolates after EMA/PMAxx treatment of spores non-treated by isopropanol versus isopropanol-treated spores. For bacterial isolates, also high ΔC_t values indicated that bacterial DNA efficiently has been blocked by PMA treatment. The lower ΔC_t values compared to fungal isolates can be explained by the lower NEC values for bacterial isolates.

3.2. Dynamic range of v-qPCR assays

The dynamic ranges for both qPCR assays with *C. cladosporioides* H39 ranged from $2 * 10^3$ to $2 * 10^5$ conidia ml^{-1} (Table 4A, Fig. 1A, B). As generally found for fungal DNA of spores treated with isopropanol (Table 2), C_t values were higher than for untreated spores. Distinctly higher C_t values for isopropanol-treated conidia were found at all concentrations within the dynamic range after EMA/PMAxx treatments. Lines were not fitted to these values since measured C_t values generally were close to or similar with the NEC due to the expected considerably low concentration of vital conidia in such suspensions. Primer-dimer formation in the generic fungal qPCR occurred for isopropanol treated spores at concentrations below $2 * 10^3$ conidia ml^{-1} (Table 4A). EMA/PMAxx treatments had no effect on the dynamics of the qPCR reactions for suspensions not treated with isopropanol.

The dynamic range of generic bacterial qPCR was limited to 10^6 to 10^8 cells ml^{-1} since measurements of concentrations lower than 10^6 cells ml^{-1} already reached the level of the low NEC due to bacterial DNA presence in the used DNA extraction kit (Molgen PurePrep Seed Kit) (Table 4B; Fig. 1 C). Application of PMAxx had no effect on the dynamics of the assays with cells not treated with isopropanol. Distinctly higher C_t values for isopropanol-treated cells were found at all concentrations after PMAxx treatments. However, the variation between replicates was

Table 2
C_t values of generic fungal qPCR assays with primer set ITS1 and 5.8 s conducted without or with pre-incubation with EMA/PMAxx of untreated fungal spores or cells in comparison to isopropanol-treated spores or cells. Effects of isopropanol treatment and EMA/PMAxx treatments are indicated by ΔC values.

Isolate	Concentration (spores or cells ml^{-1})	qPCR assessment								
			C_t values [b]				ΔC_t values [b]			
		NEC[c]	Untreated		Isopropanol-treated		Isopropanol-treated EMA/PMAxx + − Untreated EMA/PMAxx +	Isopropanol-treated EMA/PMAxx − − Untreated EMA/PMAxx −	Untreated EMA/PMAxx+ − Untreated EMA/PMAxx-	Isopropanol-treated EMA/PMAxx+ − Isopropanol-treated EMA/PMAxx-
			EMA/PMAxx −	EMA/PMAxx +	EMA/PMAxx −	EMA/PMAxx +				
Yeast-like fungi										
Aureobasidium pullulans BN072	$8.9x10^7$	>36	11.9 ±0.1	12.7 ±0.1	14.4 ±0.8	21.9 ±0.8	9.2±0.9**	2.5±0.8†	0.7±0.1*	7.4±0.1***
Cryptococcus macerans M6a9b	$1.0x10^8$	>36	11.1 ±0.0	11.9 ±0.1	12.3 ±0.4	23.9 ±2.2	12.0±2.2*	1.5±0.5	0.7±0.0**	11.5±1.9*
Cryptococcus macerans M5d1	$1.0x10^8$	>36	11.6 ±0.3	11.8 ±0.1	14.8 ±1.0	25.6 ±1.3	13.8±1.4**	3.1±0.9†	0.2±0.3	10.9±1.5*
Cystobasidium laryngis BN151	$8.9x10^7$	>36	12.0 ±0.3	12.8 ±0.2	13.8 ±0.6	24.3 ±2.0	11.5±1.8*	1.8±0.8	0.8±0.3	10.5±1.7*
Rhodotorula glutinis BN093	$1.0x10^8$	>36	12.1 ±0.2	12.3 ±0.1	17.0 ±0.9	29.2 ±0.7	16.9±0.8**	5.0±1.0*	0.3±0.2	12.2±0.4**
Rhodotorula sp. M6a3	$1.0x10^8$	>36	12.3 ±0.2	12.7 ±0.4	19.6 ±1.6	29.4 ±1.8	16.6±1.6**	7.3±1.4*	0.5±0.2	9.8±1.4*
Rhodotorula sp. M6e1	$1.0x10^8$	>36	12.5 ±0.1	13.0 ±0.3	20.1 ±1.5	30.6 ±1.5	17.6±1.6**	7.6±1.5*	0.5±0.3	10.6±2.9†
Sporobolomyces metaroseaus BN155	$1.0x10^8$	>36	12.1 ±0.0	12.2 ±0.1	17.8 ±0.8	29.9 ±0.6	17.6±0.6**	5.7±0.7*	0.2±0.0*	12.1±0.3***
Hyphal fungi										
Alternaria brassicae Z164	$2.2x10^7$	>36	16.1 ±0.1	16.2 ±0.1	16.3 ±0.5	18.0 ±0.6	1.8±0.6†	0.2±0.4	0.1±0.1	1.7±0.6†
Alternaria sp. BN115	$3.4x10^6$	>36	16.4 ±0.4	15.9 ±0.2	19.5 ±0.1	24.2 ±0.3	8.3±0.2**	3.1±0.4*	−0.5±0.2	4.7±0.3**
Boeremia exigua BN012	$8.5x10^7$	>36	11.7 ±0.1	12.0 ±0.1	14.8 ±0.4	26.0 ±0.1	14.0±1.1**	3.1±0.3**	0.3±0.1	11.3±1.2*
Chalaropsis thielavioides Z350	$1.1x10^8$	>36	20.1 ±0.8	22.6 ±2.4	22.7 ±0.9	25.9 ±1.5	3.3±3.6	2.6±1.3	2.5±2.8	3.2±2.4
Cladosporium cladosporioides H39	$1.0x10^8$	>36	14.0 ±0.1	14.6 ±0.1	16.8 ±0.1	30.0 ±1.5	15.4±1.5**	2.8±0.1**	0.5±0.1·	13.2±1.3*
Clado. ramontenellum BN031	$8.6x10^7$	>36	17.4 ±0.2	16.7 ±0.3	18.0 ±0.6	24.4 ±0.1	7.7±0.4**	0.7±0.4	−0.7±0.5	6.4±0.5**
Clonostachys rosea J1446	$1.1x10^8$	>36	21.0 ±0.4	20.9 ±0.2	35.4 ±0.4	37.0 ±0.4	16.1±0.6**	14.4±0.8**	−0.2±0.3	1.6±0.4†
Didymella macrostoma BN007	$3.8x10^7$	>36	13.1 ±0.2	13.2 ±0.2	15.4 ±0.2	26.2 ±1.3	13.0±1.2**	2.3±0.3*	0.1±0.2	10.8±1.1**
Fusarium sp. 6180–12 (1) [a]	$1.0x10^8$	>36	20.3 ±0.0	21.1 ±0.3	22.4 ±0.3	34.8 ±0.4	13.7±0.7**	2.1±0.3*	0.8±0.3·	12.5±0.7**
Fusarium sp. 6180–12 (2) [a]	$6.0x10^7$	>36	21.0 ±0.1	21.3 ±0.1	23.6 ±0.4	33.8 ±1.4	12.5±1.4*	2.6±0.4*	0.3±0.2	10.2±1.8*
Fusarium oxysporum E32b2	$1.4x10^7$	>36	25.4 ±0.4	25.1 ±0.3	27.2 ±0.5	35.6 ±0.7	10.5±0.5**	1.9±0.9	−0.2±0.3	8.4±1.0*
Isaria javanica FE9901	$1.1x10^8$	>36	23.0 ±0.2	22.9 ±0.2	24.9 ±0.7	35.2 ±1.3	12.3±1.2**	1.9±0.6†	−0.2±0.2	10.3±1.2*
Metarhizium brunneum F52	$1.8x10^7$	>36	24.4 ±0.1	24.9 ±0.4	31.0 ±0.5	35.2 ±2.0	10.3±1.6*	6.6±0.6**	0.5±0.5	4.2±1.6
Penicillium sp. Z114	$1.0x10^8$	>36	17.4 ±0.2	17.8 ±0.1	20.6 ±0.7	33.2 ±1.7	15.4±1.6*	3.2±0.8†	0.4±0.1†	12.6±1.8*
Trichoderma viride 004 (1) [a]	$1.0x10^8$	>36	21.9 ±0.1	21.6 ±0.1	23.1 ±0.1	26.8 ±0.3	5.3±0.3**	1.1±0.2*	−0.4±0.1†	3.7±0.3**
Trichoderma viride 004 (2) [a]	$1.1x10^8$	>36	22.5 ±0.2	22.1 ±0.2	23.9 ±0.1	30.0 ±0.3	7.9±0.1***	1.3±0.1**	−0.4±0.1*	6.1±0.2***
Trichoderma viride 226	$1.1x10^8$	>36	21.7 ±0.2	22.7 ±0.3	24.3 ±0.2	30.3 ±0.6	7.6±0.8*	2.5±0.5	0.8±0.6	6.1±0.7*
Ulocladium atrum 385	$6.4x10^6$	>36	13.0 ±0.2	13.0 ±0.2	14.1 ±0.1	13.3 ±0.0	0.3±0.1	1.1±0.2*	0.0±0.3	−0.8±0.0**
Ulocladium cucurbitae 742	$5.8x10^7$	>36	14.7 ±0.1	14.8 ±0.1	15.8 ±0.3	17.5 ±0.4	2.7±0.4*	1.1±0.3†	0.1±0.1	1.7±0.6†
Verticillium dahliae P15a	$1.1x10^8$	>36	21.6 ±0.2	22.8 ±0.2	25.7 ±0.7	33.6 ±1.2	10.7±0.9**	4.2±0.7*	1.3±0.3†	7.8±0.6**

[a] Isolates tested twice in independent experiments.
[b] Mean±standard error of the mean for three replicated measurements of the same suspension. Differences statistically significant according to t-tests for treatments

expressed by ΔC_t −values are indicated with (†) if $p < 0.1$, (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$.
[c] Negative extraction control, one measurement.

Table 3
C_t values of generic bacterial qPCR assays with primer set Eub338 and EUb518 conducted without or with pre-incubation with PMA of untreated cells in comparison to cells treated with isopropanol. Effects of isopropanol treatment and PMAxx treatments are indicated by ΔC_t-values.

Isolate	qPCR assessment								
		C_t values [a]				ΔC_t values [a]			
	NEC [b]	Untreated		Isopropanol-treated		Isopropanol-treated PMAxx + − Untreated PMAxx +	Isopropanol-treated PMAxx − − Untreated PMAxx −	Untreated PMAxx+ − Untreated PMAxx-	Isopropanol-treated PMAxx+ − Isopropanol-treated PMAxx-
		PMAxx −	PMAxx +	PMAxx −	PMAxx +				
Gram-bacteria									
Bacteroides sp. M12	27.6	22.1 ±1.2	18.8 ±0.2	16.5 ±0.2	27.0 ±0.1	8.2±0.3**	−5.6±1.4†	−3.3±1.4	10.5±0.3***
Pantoea vagans E20a	28.0	18.2 ±1.1	18.1 ±0.7	16.6 ±0.5	27.2 ±0.1	9.1±0.6**	−1.6±0.6	−0.1±0.5	10.6±0.5**
Pseudomonas chlororaphis MA342	27.6	24.2 ±1.7	20.9 ±0.5	16.2 ±0.2	27.1 ±0.1	6.3±0.5**	−8.1±1.6*	−3.3±1.7	11.0±0.2***
Pseudomonas fluorescens PE75-1	27.6	17.2 ±0.1	17.9 ±0.3	16.5 ±0.2	26.6 ±0.3	8.7±0.1*	−0.7±01*	0.7±0.2†	10.1±0.1**
Pseudomonas (fluorescens group) C17	27.6	21.7 ±0.3	22.0 ±0.7	18.3 ±0.4	26.7 ±0.1	4.7±0.8*	−3.4±0.3**	0.3±0.8	8.4±0.4**
Pseudomonas sp. P4a	28.0	20.3 ±3.8	16.7 ±0.2	15.9 ±0.1	27.2 ±0.2	10.5±0.4**	−4.4±3.9	−3.6±3.7	11.3±0.3***
Rahnella aquatilis E22b1	27.6	19.2 ±0.5	19.1 ±0.5	21.1 ±0.5	27.9 ±0.1	8.8±0.4**	1.9±0.1**	−0.1±0.1	6.8±0.4**
Rhizobium sp. E6d	28.0	17.8 ±0.4	19.2 ±0.0	16.5 ±0.3	26.7 ±0.6	7.5±0.6**	−1.3±0.6	1.4±0.5†	10.2±0.5**
Serratia marcescens A2	27.6	22.0 ±0.5	21.8 ±0.1	22.2 ±0.2	27.3 ±0.2	5.5±0.1***	0.2±0.7	−0.2±0.4	5.1±0.3**
Serratia plymuthica HROC48	27.6	21.2 ±3.2	18.7 ±0.2	16.8 ±0.2	26.9 ±0.1	8.1±0.2***	−4.4±3.4	−2.4±3.4	10.1±0.3***
Xanthomonas arxonopodis D17	27.6	20.7 ±0.4	20.2 ±0.1	18.9 ±0.1	27.1 ±0.2	6.9±0.2**	−1.7±0.5†	−0.4±0.4	8.2±0.3**±
Gram + bacteria									
Arthrobacter sp. P9c	27.5	18.4 ±0.2	19.3 ±0.0	17.7 ±0.2	27.2 ±0.1	7.9±0.1***	−0.7±0.3	1.0±0.2*	9.5±0.2***
Bacillus pumilus 4.42	27.5	16.5 ±0.1	18.4 ±0.3	17.0 ±0.1	26.8 ±0.3	8.4±0.4**	0.6±0.1†	1.9±0.2*	9.7±0.2***
Bacillus sp. *cereus/thurigiensis* P17a1	27.5	18.3 ±0.7	19.2 ±1.1	15.5 ±0.3	23.7 ±1.1	4.5±0.1***	−2.8±0.5*	0.9±0.5	8.1±0.8**
Paenibacillus peoriae 2.3.2	27.5	15.9 ±0.2	18.6 ±0.3	16.1 ±0.3	24.9 ±1.1	6.3±1.0*	0.3±0.5	2.7±0.4*	8.7±0.9*
Paenibacillus polymyxa P13a	27.5	13.3 ±0.1	15.7 ±0.2	13.7 ±0.2	25.0 ±0.8	9.3±1.0*	0.4±0.2	2.3±0.1**	11.3±0.8**
Streptomycetes									
Streptomyces galilaeus 12.2.5	28.0	18.4 ±0.1	18.1 ±0.0	17.8 ±0.3	27.3 ±0.1	9.3±0.2***	−0.6±0.3	−0.3±0.0*	9.5±0.3**
Streptomyces griseoviridis K61	28.0	21.9 ±0.6	21.4 ±0.3	21.9 ±0.1	27.1 ±0.3	5.7±0.3**	0.0±0.6	−0.5±0.8	5.2±0.4**
Streptomyces lateritius 2.3.8	28.0	17.9 ±0.2	18.7 ±0.3	17.5 ±0.1	27.0 ±0.2	8.3±0.3**	−0.4±0.1	0.9±0.1*	9.5±0.3**
Streptomyces platensis 7.3.8	28.0	20.6 ±0.5	21.1 ±0.1	21.1 ±0.4	25.9 ±0.4	4.7±0.4**	0.5±0.9	0.5±0.5	4.7±0.2**
Streptomyces pseudovenezuelae 14.4.2	28.0	25.3 ±0.1	26.6 ±0.1	24.3 ±0.2	27.5 ±0.2	0.9±0.3†	−1.0±0.2*	1.3±0.3*	3.2±0.1***

[a] Mean±standard error of the mean for three replicated measurements of the same suspension. Differences statistically significant according to t-tests for treatments expressed by ΔC_t −values are indicated with (†) if $p < 0.1$, (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$.
[b] Negative extraction control, one measurement.

higher for the measurements of isopropanol-treated cells using PMAxx at the expected low concentrations of living cells in such suspensions and values in many cases reached the level of NEC. ΔC_t values for cells not treated with isopropanol versus isopropanol-treated cells were considerably lower because of the low NEC due to the presence of bacterial DNA in the used DNA extraction kit. The dynamic range for the strain-specific qPCR assay with *L. enzymogenes* 3.1T8 ranged from 10^5 to 10^8 cells ml^{-1} with and without use of PMAxx with distinctly higher C_t values for isopropanol-treated cells found at all concentrations after PMAxx treatments (Table 4B; Fig. 1 D).

3.3. Effects of seed matrix on viability qPCR

Washings of two seed lots of Brassica vegetables, carrots, onion,

Table 4
C_t values of generic fungal and bacterial qPCR assays and strain-specific qPCR assays for conidial suspensions of *Cladosporium cladosporioides* H39 (A) and cell suspensions of *L. enzymogenes* 3.1 T8 (B). Assays were conducted without or with pre-incubation with EMA/PMAxx of untreated suspensions in comparison to isopropanol-treated suspensions. Effects of isopropanol treatment and EMA/PMAxx treatments are indicated by ΔC_t values.

A *Cladosporium cladosporioides* H39

Concentration (conidia ml^{-1})	qPCR assessment							
	C_t values [a]				ΔC_t values [a]			
	Untreated		Isopropanol-treated		Isopropanol-treated EMA/PMAxx + / − Untreated EMA/PMAxx +	Isopropanol-treated EMA/PMAxx − / − Untreated EMA/PMAxx −	Untreated EMA/PMAxx+ / − Untreated EMA/PMAxx-	Isopropanol-treated EMA/PMAxx+ / − Isopropanol-treated EMA/PMAxx-
	EMA/PMAxx −	EMA/PMAxx +	EMA/PMAxx −	EMA/PMAxx +				
Generic qPCR								
$2 * 10^5$	14.2±0.1	14.5±0.1	18.9±0.9	31.7±0.8	17.1±0.9**	4.7±1.0*	0.4±0.2	12.8±1.1**
$2 * 10^4$	17.6±0.1	18.4±0.2	24.1±0.2	33.1±1.1	14.7±1.2**	6.5±0.3**	0.8±0.1**	9.0±0.9*
$2 * 10^3$	21.5±0.2	22.4±0.1	28.6±0.5	(35.5 ±0.5)[b]	13.1±0.4**	7.0±0.6**	0.8±0.1*	–
$2 * 10^2$	25.3±0.3	25.8±0.3	(34.4 ±0.4)	(35.6 ±0.9)	9.8±1.1*	9.1±0.2***	–	–
$2 * 10^1$	31.2±1.5	31.1±0.2	(35.4 ±0.2)	(36.6 ±1.0)	5.5±1.0*	4.1±0.2	–	–
Strain-specific qPCR								
$2 * 10^5$	24.6±0.2	25.1±0.1	27.7±0.6	36.0±0.1	10.9±0.1***	3.1±0.6*	0.5±0.2†	8.3±0.5**
$2 * 10^4$	27.6±0.1	27.9±0.3	31.2±0.1	38.1±0.1	10.1±0.4**	3.5±0.2**	0.3±0.2	6.9±0.1***
$2 * 10^3$	31.2±0.1	31.5±0.1	34.2±0.3	40.0±0.0	8.4±0.1***	3.0±0.3*	0.4±0.2	5.8±0.3**
$2 * 10^2$	35.1±0.3	34.8±0.2	39.0±0.5	39.8±0.2	5.0±0.3**	4.0±0.8*	−0.3±0.3	0.8±0.7
$2 * 810^1$	39.1±0.8	38.9±0.6	40.0±0.0	40.0±0.0	1.1±0.6	0.8±0.8	−0.3±0.9	0.0±0.0

B *Lysobacter enzymogenes* 3.1T8

Concentration (cells ml^{-1})	qPCR assessment							
	C_t values [a]				ΔC_t values [a]			
	Untreated		Isopropanol-treated		Isopropanol-treated PMAxx + / − Untreated PMAxx +	Isopropanol-treated PMAxx − / − Untreated PMAxx −	Untreated PMAxx+ / − Untreated PMAxx-	Isopropanol-treated PMAxx+ / − Isopropanol-treated PMAxx-
	PMAxx −	PMAxx +	PMAxx −	PMAxx +				
Generic qPCR								
10^8	20.8 ±0.4	19.0 ±0.0	20.4 ±0.3	25.1 ±0.3	6.1±0.3**	−0.4±0.4	−1.8±0.4*	4.8±0.5*
10^7	24.3 ±0.3	22.2 ±1.0	23.4 ±0.2	28.2 ±0.2	6.0±1.2*	−0.9±0.3†	−2.1±0.6†	4.9±0.3**
10^6	25.9 ±0.1	24.1 ±0.1	26.2 ±0.2	28.6 ±0.0	4.5±0.1***	0.3±0.1	−1.7±0.1**	2.5±0.3*
10^5	27.8 ±0.1	27.5 ±0.2	28.1 ±0.1	28.5 ±0.1	1.0±0.3†	0.3±0.1†	−0.4±0.2	0.4±0.0*
10^4	28.8 ±0.3	27.6 ±1.9	28.7 ±0.0	28.7 ±0.4	1.1±2.2	−0.2±0.3	−1.3±2.2	0.0±0.4
Strain-specific qPCR								
10^8	23.0 ±0.3	21.6 ±0.1	22.6 ±0.2	27.9 ±0.2	6.3±0.2**	−0.3±0.2	−1.3±0.5*	5.3±0.3**
10^7	26.5 ±0.4	24.7 ±0.7	25.6 ±0.2	35.0 ±2.6	10.2±3.2†	−0.9±0.3†	−1.7±0.4*	9.4±2.6†
10^6	28.9 ±0.2	27.1 ±0.0	28.5 ±0.2	37.9 ±1.1	10.8±1.1**	−0.3±0.2	−1.8±0.1**	9.3±1.1*
10^5	32.1 ±0.2	31.0 ±0.3	32.0 ±0.3	39.0 ±0.5	8.1±0.5**	−0.1±0.2	−1.1±0.4†	7.1±0.7*
10^4	35.6 ±0.4	32.5 ±2.9	36.0 ±0.4	40.0 ±0.0	7.5±2.9	0.5±0.6	−3.1±5.7	4.0±0.4*

[a] Mean±standard error of the mean for three replicated measurements of the same suspension. Differences statistically significant according to t-tests for treatments expressed by ΔC_t −values are indicated with (†) if $p < 0.1$, (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$.
[b] Primer-dimer formation observed, values excluded from further analysis.
C_t value of negative extraction control was >36.
[a] Mean±standard error of the mean for three replicated measurements of the same suspension. Differences statistically significant according to t-tests for treatments expressed by ΔC_t −values are indicated with (†) if $p < 0.1$, (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$.
C_t value of negative extraction control was 28.

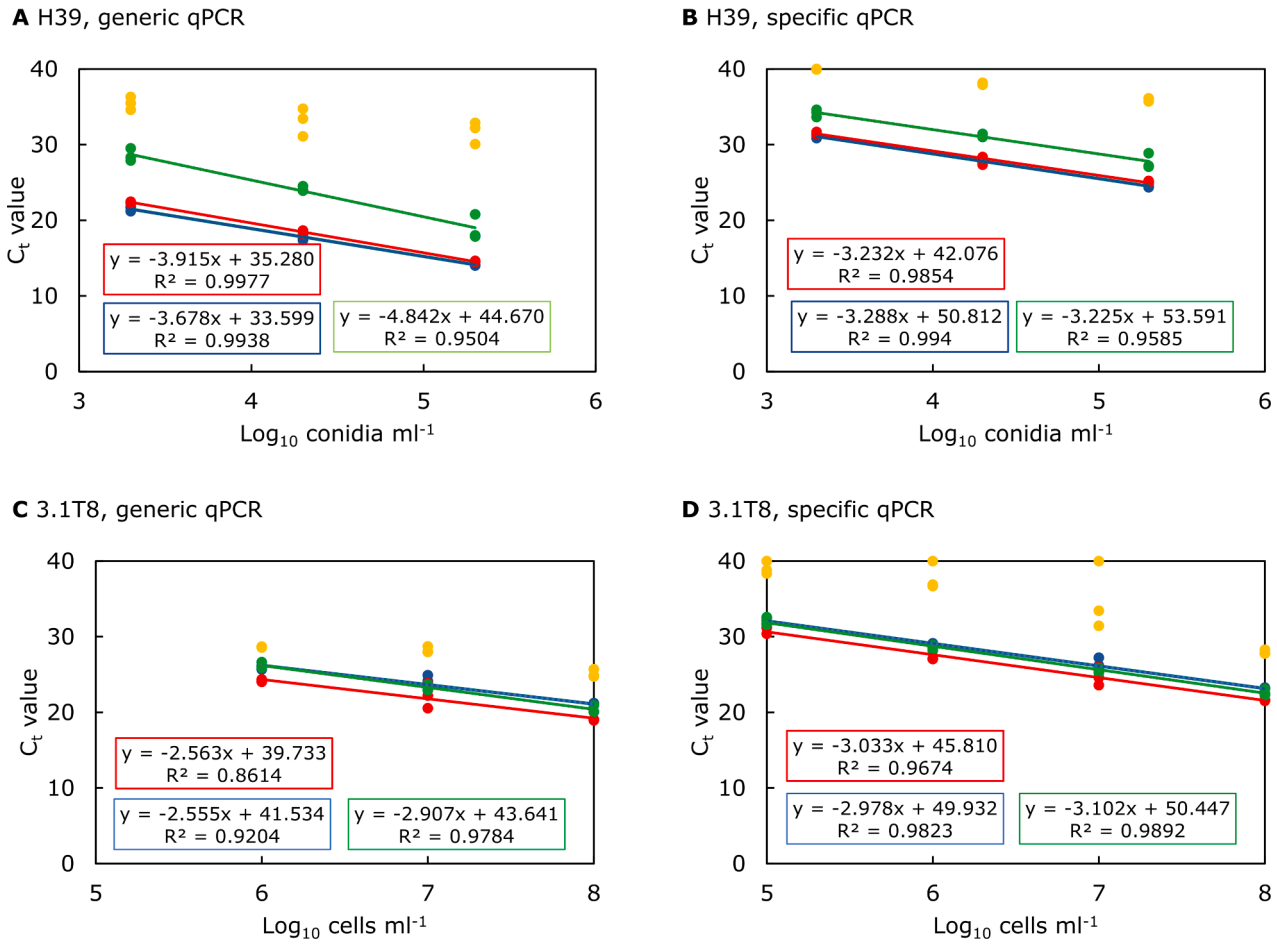

Fig. 1. C_t values of generic fungal qPCR assays (A), generic bacterial PCR assays (C) and strain-specific qPCR assays (B, D) conducted with *Cladosporium cladosporioides* H39 (A, B) and *Lysobacter enzymogenes* 3.1T8 (C, D). Non-treated with isopropanol, without EMA/PMAxx (Blue); Non-treated with isopropanol, with EMA/PMAxx (Red); Treated with isopropanol, without EMA/PMAxx (Green); Treated with isopropanol, with EMA/PMAxx (Yellow).

Table 5
C_t values of *Cladosporium cladosporioides* H39 strain-specific qPCR assays for conidia of *C. cladosporioides* H39 spiked to washings of different seed lots or to a water control without washings. Assays were conducted without or with pre-incubation with EMA/PMAxx of untreated conidia of *C. cladosporioides* H39 in comparison to isopropanol-treated conidia. Effects of isopropanol treatment and EMA/PMAxx treatments are indicated by ΔC_t values.

Seed lot	qPCR assessment							
	C_t values [a]				ΔC_t values [a]			
	Untreated		Isopropanol-treated		Isopropanol-treated EMA/PMAxx + − Untreated EMA/PMAxx +	Isopropanol-treated EMA/PMAxx − − Untreated EMA/PMAxx −	Untreated EMA/PMAxx+ − Untreated EMA/PMAxx-	Isopropanol-treated EMA/PMAxx+ − Isopropanol-treated EMA/PMAxx-
	EMA/PMAxx −	EMA/PMAxx +	EMA/PMAxx −	EMA/PMAxx +				
Brassica 1	27.1±0.2	27.7±0.1	28.0±0.1	36.2±0.0	8.5±0.1**	0.9±0.1†	0.5±0.3	8.2±0.2*
Brassica 2	26.6±0.2	27.8±0.1	27.4±0.3	36.0±0.5	8.2±0.6*	0.8±0.1†	1.2±0.3	8.7±0.2*
Carrot 1	27.3±0.1	26.2±0.2	27.7±0.2	30.7±0.4	4.5±0.3*	0.4±0.3	−1.2±0.1*	3.0±0.6
Carrot 2	26.8±0.1	26.7±0.7	27.5±0.4	32.3±0.1	5.6±0.8†	0.7±0.6	−0.1±0.8	4.8±0.6†
Onion 1	26.5±0.1	27.1±0.1	26.7±0.1	36.3±1.6	9.2±1.6	0.2±0.2	0.6±0.2	9.7±1.6
Onion 2	26.8±0.1	27.5±0.2	26.7±0.1	36.8±0.6	9.2±0.8†	−0.1±0.2	0.8±0.3	10.1±0.7*
Ryegrass 1	26.8±0.1	27.2±0.2	27.0±0.2	34.8±0.6	7.6±0.4*	0.3±0.1	0.4±0.0*	7.8±0.4*
Ryegrass 2	26.8±0.1	27.1±0.1	26.9±0.0	35.3±0.2	8.2±0.2*	0.2±0.1	0.3±0.2	8.4±0.2*
Spinach 1	26.9±0.1	27.5±0.6	27.8±0.0	34.7±0.2	7.2±0.8†	0.9±0.0*	0.6±0.5	6.9±0.2*
Spinach 2	27.0±0.3	27.1±0.0	27.6±0.1	37.1±1.1	10.0±1.1†	0.5±0.2	0.1±0.4	9.5±0.9†
water control	26.6±0.1	27.3±0.1	27.0±0.0	36.7±0.5	9.0±0.6**	0.5±0.1†	0.7±0.1*	9.2±0.6**

[a]Mean±standard error of the mean for two replicated measurements of the same suspension with washings and three replicated measurement of the water control. Differences statistically significant according to t-tests for treatments expressed by ΔC_t −values are indicated with (†) if $p < 0.1$, (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$.
C_t value of negative extraction control was > 40.

perennial ryegrass and spinach were spiked with spore suspensions of spores of *C. cladosporioides* H39 non-treated or treated with isopropanol. HyPure water without seed washing served as control. The strain-specific qPCR for *C. cladosporioides* H39 indicated no differences in C_t values between the control measurements or measurements of the fungus in any of the seed washings for conidia non-treated by isopropanol without or with prior treatment with EMA/PMAxx (Table 5). Also for isopropanol-treated conidia without prior EMA/PMAxx treatment no differences of C_t values were found. For isopropanol-treated spores with EMA/PMAxx treatment, similar ΔC_t values were obtained for all seed washings except for conidia spiked to washings of the two carrot seed lots where ΔC_t values were smaller with 5.6 and 4.5 compared to the water control with 9.0. However, also in these cases ΔC_t values still indicated a viability of <5 % of the isopropanol-treated conidia in carrot seed washings. Washings of carrot seeds had a brownish colour whereas washings from other seeds were clear.

3.4. Application of v-qPCR on coated seeds

Several qPCR assays were conducted for suspensions washed off from onion and spinach seeds coated with *Lysobacter enzymogenes* 3.1T8, *Coniothyrium minitans* 1143 or *Cryptococcus albidus* 733 (Supplement Figs. 1 and 2). C_t values of qPCRs assays conducted with EuB338/Eub518 for seeds coated with fungi ranged between 21.5 and 24.8, representing bacterial DNA naturally present on the seeds and possibly also some plantal DNA (Table 6A). C_t values for *L. enzymogenes* 3.1T8-coated seeds were 15.8 for onion seeds and 15.9 for spinach seeds representing a high concentration of *L. enzymogenes* 3.1T8 DNA with considerable low variation between replicated seed samples or seed types. The differences between C_t values for *L. enzymogenes* 3.1T8-coated seeds and seed coated with fungi of >7 indicate that <1 % of the measured DNA for *L. enzymogenes* 3.1T8-coated seeds belonged to bacterial background populations or possibly also to the plant. C_t values for qPCRs assays with EuB338/Eub518 after treatment with PMAxx were higher for seeds treated with fungi. The obtained ΔC_t (C_t value PMAxx-treated – C_t value water-treated) of 3.1 for onion seeds (mean for *C. minitans* 1143- and *C. albidus* 733-treated seeds) and of 2.3 for spinach seeds indicated that approximately 10 % of the measured bacterial (or plantal) DNA washed off from onion seeds and 20 % from spinach seeds was obtained from living cells. For *L. enzymogenes* 3.1T8-coated seeds ΔC_t (C_t value PMAxx-treated – C_t value water-treated) was 0.6 for onion seeds and 0.9 for spinach seeds. Considering that C_t values of qPCR assays represented for >99 % DNA of *L. enzymogenes* 3.1T8 and less than 1 % DNA of background bacterial populations, the low ΔC_t values indicate that the more than 50 % of *L. enzymogenes* 3.1T8 cells washed off from the coated seeds were living.

C_t values of qPCRs conducted with ITS1-5.8s for seeds coated with *L. enzymogenes* 3.1T8 was 25.9 for onion seeds and 27.0 for spinach seeds, representing fungal DNA naturally present on the seeds (Table 6B). C_t values for *C. minitans* 1143-coated seeds were lower with 22.8 for onions seeds and 19.8 for spinach seeds so that it can be assumed that for *C. minitans* 1143-coated seeds less than 10 % of the measured DNA on onions seeds and 1 % on spinach seeds belonged to fungal background populations. For *C. albidus* 733-coated seeds C_t values were 20.7 for onion seeds and 23.0 for spinach seeds. The estimated background of fungal populations was 5 % for onion seeds and 10 % for spinach seeds. The ΔC_t (C_t value EMA/PMAxx-treated – C_t value water-treated) for *L. enzymogenes* 3.1T8-coated seeds of onions (ΔC_t = 5.2 and spinach (ΔC_t = 3.3) indicated that approximately <5 % of the fungal background DNA had been obtained from living cells for onions seeds and 10 % for spinach seeds. ΔC_t was also high with 2.8 for *C. minitans* 1143-coated onions seeds and 3.4 for *C. albidus* 733-coated onions seeds. From these values it can be estimated that more than 90 % of the fungal cells coated on onion seeds did not survive under these conditions. For spinach seeds, ΔC_t values were low with 0.4 for *C. minitans* 1143 and 0.3 for *C. albidus* 733-coated seeds, indicating that with estimated 80 % viable cells the majority of the spores or yeast cells were alive on the coated seeds.

Table 6
C_t values for qPCR assays using generic primers for bacteria (EuB338/Eub518) or fungi (ITS1-5.8s) for suspensions washed from onion and spinach seeds coated with *Lysobacter enzymogenes* 3.1T8, *Coniothyrium minitans* 1143 or *Cryptococcus albidus* 733. Suspensions were treated with water (control), PMAxx or EMA/PMAxx before DNA extraction.

A C_t values and ΔC_t values of qPCR assays with primers EuB338/Eub518.

	C_t values [a]		ΔC_t [b]
	Water-treated	PMAxx-treated	
Onion			
L. enzymogenes 3.1 T8	15.8±0.4	16.4±0.4	0.6±0.4
C. minitans 1143	23.2±0.8	26.8±0.3	3.6±0.9†
C. albidus 733	24.8±0.2	27.4±0.1	2.7±0.3**
Spinach			
L. enzymogenes 3.1 T8	15.9±0.2	16.8±0.2	0.9±0.5
C. minitans 1143	21.5±0.5	23.6±0.3	2.1±0.2**
C. albidus 733	23.2±1.2	25.8±1.2	2.6±0.1***

B C_t values and ΔC_t values of qPCR assays with primers ITS1-5.8s.

	C_t values [a]		ΔC_t [c]
	Water-treated	EMA/PMAxx-treated	
Onion			
L. enzymogenes 3.1T8	25.9±0.3	31.1±0.5	5.2±0.2**
C. minitans 1143	22.8±0.3	25.5±0.3	2.8±0.3**
C. albidus 733	20.7±0.3	24.1±0.6	3.4±0.3**
Spinach			
L. enzymogenes 3.1T8	27.0±0.4	30.28±1.7	3.3±2.1
C. minitans 1143	19.8±0.2	20.16±0.3	0.4±0.1†
C. albidus 733	23.0±0.8	23.29±0.7	0.3±0.1

[a] Means and standard errors of the means for three replicate samples from each coated seed batch. Differences statistically significant according to t-tests for treatments expressed by ΔC_t-values are indicated with (†) if $p < 0.1$, (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$.
[b] ΔC_t = C_t value PMAxx-treated − C_t value water-treated.
[c] ΔC_t = C_t value EMA/PMAxx-treated − C_t value water-treated.

4. Discussion

The applied protocols for generic fungal qPCR in combination with the DNA nucleic acid-binding dyes EMA and PMAxx and for generic bacterial qPCR in combination PMAxx allowed the quantification of fungal and bacterial isolates representative of a broad range of species within defined concentration ranges. ΔC_t values for spore or cell suspensions obtained from cultures on growth media and the same suspensions after treatment with isopropanol to kill the cells indicated that the assay differentiated between living and dead cells.

Measured C_t values of the generic qPCR for fungal or bacterial DNA were different between the individual tested isolates. The differences of C_t values can be explained by different amounts of extracted DNA due to different concentrations of spores or cells in the original suspensions prepared per isolate, in different amounts of cells per spore, or different amounts of nuclei and DNA per cells. Furthermore, variation of C_t values between the different species can be explained by possibly different extraction efficiency of the DNA extraction kit or a bias of the applied generic primers for certain species. Another source of variation may be due to insufficient reproducibility of measurements. However, for the two isolates *Fusarium* sp. 6180-12 and *T. viride* 004 which had been tested in independent experiments using different cultures and DNA extracts, C_t values differed between the repeated measurements for less than one C_t value. This expected bias in quantification of different species will not allow the comparison between species. However, the main purpose of the study was to measure differences between living and dead

cells within the same isolate. The obtained results demonstrate that this application is possible for the broad range of tested fungal and bacterial species. An exception are fungal species with highly pigmented dark spores. Highly melanized spores, e.g. of *Alternaria* spp., *Chalaropsis* spp. and *Ulocladium* spp. may not allow appropriate photoactivation needed for efficient binding of the dye to DNA. Increase of light intensity for measurement of such highly pigmented spores may result in heating up spores or even the entire suspensions and as a consequence possibly the death of the targeted spores. Several isolates of such species showed low ΔC_t values in this study indicating low efficiency in blocking of DNA of dead conidia, whereas dead conidia of another isolate of *Alternaria* sp. were detected. Carefully developed protocols are also needed for endospore-forming bacteria. Depending on used DNA extraction methods, only DNA of vegetative cells may be evaluated but not DNA of endospore. In such a case, v-qPCR may strongly underestimate the viability of inocula of endospore-forming bacteria. DNA extractions including mechanical lysis steps as used in this study generally result in extraction also of DNA of endospores (Knüpfer et al., 2020).

The evaluation of the dynamic ranges of the generic and strain-specific qPCR used in this study revealed several limitations, e.g. limited ranges of only two orders of magnitude, presence of primer-dimer formation or the slightly lower efficiency of generic primers for specific species and the expected resulting bias in quantifications. A major concern is the presence of bacterial DNA in both commercially available DNA extraction kits used in this study. Interestingly, such DNA contents also varied between batches of the same extraction kit (data not presented). Adaptions of the protocols to overcome such limitations are needed. Careful monitoring of applied commercial kits or the development of in-house protocols guaranteeing the use of DNA-free agents for DNA extraction are of key importance. However, the main purpose of this study was the development of applications of v-qPCR assays to individual isolates in the framework of antagonist screening for applications on seeds, monitoring of effects of seed technologies on viability of coated inocula and improvement of specific coating, handling and storage technologies to achieve superior survival of inocula. Such applications are less affected by the found possible limitations. Tested concentrations can be increased and adapted to the adequate concentration ranges to overcome limitations due to limited detection levels (Elizaquível et al., 2014). An important finding is that standardized concentrations of the DNA binding dyes are sufficient for the broad concentration ranges of targeted cells.

Complex matrices often can limit the application of v-qPCR technologies (Elizaquível et al., 2014). Chemical adsorption of the dyes, prevention of photoactivation due to organic compounds or unsuitable turbidity may limit photoactivation (Elizaquível et al., 2014). Applications of the v-qPCR protocols in the background of seed washings were successful in this study. A possible limitation was found for carrot seeds that caused a brownish staining of seed washings potentially interfering with photoactivation step. However, the turbidity of the carrot seed washings did not affect the final conclusion since artificially killed cells were measured as non-viable. Dilution of such washings can easily overcome possible more severe limitations by higher turbidity caused by seeds or possibly also by coating materials.

Coated seeds of spinach and onion were used for a first successful pilot application of the v-qPCR protocols with generic fungal and bacterial primers to assess viability of inocula of the hyphal fungus *Coniothyrium minitans* 1143, the yeast *Crypptococcus albidus* 733 and the bacterium *Lysobacter enzymogenes* 3.1T8. These first results indicated a differential effect of seed type on survival of coated fungi with a generally better survival on seeds of spinach compared to seeds of onions and a similar good survival of *L. enzymogenes* 3.1T8 on both seed types.

We foresee many potential uses of v-qPCR protocols in biological control applications on seeds (Köhl et al., 2024). Antagonist survival during and after coating is – besides high efficacy against the targeted disease – the main success factor of seed applications of antagonists. With the exception of endospore-forming bacteria such as *Bacillus* spp., currently known antagonists generally lack sufficient shelf life potential for commercial seed applications. Selection of a new generation of antagonists with superior shelf life characteristics is thus essential to achieve progress in microbial seed treatments. The application of generic v-qPCR protocols will allow high-throughput screening assays of isolates belonging to various fungal and bacterial species with potentially long shelf life. Adaptation of candidate antagonists to specific processes during seed processing can also be tested. For example, survival in specific coating materials, during drying processes, during storage under different conditions and after mechanical treatments and handling can be assessed. Effects of seeds themselves on antagonist survival, e.g. through formation of volatile compounds, can be tested at species level or at cultivar level. Besides screening for antagonists with high adaptation to seed processing and handling, also technologies can be adapted to the requirements of microbial inocula. The effects of various treatments aiming at such adaptations on potential antagonists, e.g. of seed bio-priming (Müller & Berg, 2008; Abuamsha et al., 2011), can be tested by v-qPCR assays. If specific antagonists have been selected and are further integrated in seed processing technologies, the development of strain-specific v-qPCR is a potential option to enhance technology adaptations to achieve optimum use of microbial biocontrol products. We expect that new bioassays targeting the various essential challenges in microbial applications in biocontrol on seeds will be developed and used that include the use of v-qPCR assays for fast and reliable monitoring of antagonist survival.

CRediT authorship contribution statement

Jürgen Köhl: Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Lia Groenenboom-de Haas:** Writing – original draft, Methodology, Investigation. **Ilse Houwers:** Methodology, Investigation. **Georgina Elena:** Methodology, Investigation. **Ezra de Lange:** Methodology, Investigation. **Patrick Butterbach:** Methodology, Investigation, Conceptualization. **Liesbeth van der Heijden:** Methodology, Investigation, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2024.105569.

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