



Application of viability-qPCR to assess survival of *Cladosporium cladosporioides* H39 and *Lysobacter enzymogenes* 3.1T8 in slurries used for seed coating

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Abstract

Seed coating technologies are used to cover seeds with external materials including propagules of microbial biological control agents (MBCA). Protection of MBCA during seed processing and handling is essential to achieve long shelf-life of coated MBCA. The viability of MBCA added to slurries prepared before use for seed coating should thus not be affected. The survival of *Cladosporium cladosporioides* H39 and *Lysobacter enzymogenes* 3.1T8 in slurries provided by seed companies was quantified by previously designed strain-specific viability-qPCR assays. The effects of storage temperature and storage duration of slurries on survival of the added microorganisms were also quantified. Viability of added inocula decreased by more than 90% within a few days in most slurries, even if stored at low temperature (5°C). However, for certain slurry-MBCA combinations, reduction in viability was less than 90% during the initial days of storage. Selection of MBCA with long shelf-life on coated seeds and adaptations of seed processing technologies to protect MBCA during seed processing and handling are essential for a change from chemical to biological seed treatments for control of seedling diseases.

Keywords: microbial biological control agents, seed coating, slurries, survival, viability-qPCR

Introduction

Seed coating is the practice of covering seeds with external materials with the aim to improve seed handling, to protect seeds and seedlings from damage by pests and diseases, and to improve germination and seedling establishment (Halmer, 2008; O'Callaghan, 2016; Pedrini *et al.*, 2017; Afzal *et al.*, 2020; Javed *et al.*, 2022). Seeds are typically coated

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to improve flowability of seeds in sowing machines and to apply compounds to the seed surface. Such compounds can be chemical or biological crop protection products, or biostimulants and fertilizers. Besides active components, colorants, adjuvants, binders and fillers are added to coating materials (Afzal *et al.*, 2020). Scientific research and scientific publications in the field of seed coating are rare. Coating technologies are usually developed by the seed industry, details are not disclosed and published scientific information is scarce and scattered (Pedrini *et al.*, 2017). Seed coating slurries are the liquid mixtures of coating materials applied to seeds in specialised equipment. Commonly, batches of slurries with added compounds are used by seed processors during an entire seed processing campaign. Rules for storage conditions and storage duration of amended slurries may differ considerably between companies and are not disclosed.

The current system of seed health is based on production of pathogen-free seeds by combining the use of fungicide applications and other hygiene measures in the seed production crops with seed processing technologies (Du Toit, 2004). This system guarantees pathogen-free seeds but also can have detrimental effects on non-target organisms including the beneficial plant and seed microbiome. A paradigm shift is needed in the whole seed chain to become less dependent or even independent from chemical pesticides which are increasingly being phased out (European Union, 2009, 2020; Lamichhane *et al.*, 2020). Alternatively, microbial biological control agents (MBCA) can be used for disease control. MBCA function through antagonism against the targeted pathogen or enhancement of the resistance of treated plants against the pathogen (Köhl *et al.*, 2019). Seeds can be inoculated with MBCA by seed soaking, seed coating techniques such as seed dressing, film coating, pelleting and encrusting, or by biopriming (McQuilken *et al.*, 1998; Rocha *et al.*, 2019). Beneficial microorganisms with specific characteristics have to be selected for such applications (Köhl *et al.*, 2024a). Poor survival resulting in reduced shelf-life is often experienced as a disadvantage of microbial seed inoculations. Selection of MBCA with long shelf-life on coated seeds and adaptations of seed processing technologies to protect MBCA during seed processing and handling are essential for the change from chemical to biological seed treatments for control of seedling diseases.

Recently, generic and strain-specific viability-qPCR assays based on the nucleic acid intercalating dyes EMA and PMAxx have been developed for the purpose of screening individual beneficial microbial isolates with long shelf-life for applications on seeds, monitoring the effects of seed technologies on viability of coated inocula and improvement of specific coating, handling and storage technologies to achieve superior survival of inocula (Köhl *et al.*, 2024b). Antagonist survival during and after coating is – besides high efficacy against the targeted disease – the main success factor of seed applications of antagonists.

The objective of our study was to (1) assess the potential of recently developed strain-specific viability-qPCR assays for the quantification of the survival of added microorganisms in seed coating slurries, (2) to assess potential effects of different slurries on survival of added microorganisms, and (3) to test the potential effects of storage conditions and storage duration of amended slurries on survival of the added microorganisms. Project partners provided ten slurries to the laboratory of Wageningen University & Research for two independent experiments on survival of *Cladosporium*

cladosporioides (Fresenius) de Vries (strain H39) and *Lysobacter enzymogenes* Christensen and Cook 1978 (strain 3.1T8) in slurries. Inocula were produced on agar, added to slurries and their viability was assessed by viability-qPCR.

Materials and methods

Microorganisms

The fungal isolate *Cladosporium cladosporioides* H39 originates from a scabbed apple leaf collected in a Dutch apple orchard. The isolate *C. cladosporioides* H39 is a strong antagonist of *Venturia inaequalis* causing apple scab (Köhl *et al.*, 2015) and has a broader pathogen range as potential targets (Köhl, 2009). The bacterial isolate *Lysobacter enzymogenes* 3.1T8 originates from root tips of cucumber plants grown on *Pythium*-suppressive rockwool (Folman *et al.*, 2003). The isolate produces several lytic enzymes and has antagonistic activity against several fungi and oomycetes (Folman *et al.*, 2003). Efficacy of the isolate to control *Pythium aphanidermatum* was demonstrated in several greenhouse experiments with cucumber plants (Postma *et al.*, 2009). *Cladosporium cladosporioides* H39 is stored on potato dextrose agar (PDA) and *L. enzymogenes* 3.1T8 on tryptic soy agar (TSA) at 10°C in the dark.

Commercial slurries used for seed coating

Each of five seed companies participating in the project provided two slurries. Slurries typically contained polymers, mixes of various agents for stabilisation, colorants, preservatives and additional water. Specific polymers or slurries were obtained from suppliers of commercial seed coating materials. Detailed information on the composition of commercially applied slurries for seed coating is generally confidential and cannot be disclosed. Slurries 1 to 8 are used for seed coating with synthetic plant protection products (table 1). Slurry 9 is a variant of slurry 8 but without preservatives, several additional ingredients and pigment. Slurry 8, commonly used for coating seed with synthetic pesticides, has also been used successfully at the company for seed coating with microorganisms. Slurry 10 has been developed for seed coating with microorganisms. Slurries 4, 8 and 9 include polymers containing micro-plastics whereas the remaining slurries are free of (intentionally added) micro-plastic polymers (as obligatory within the European Union by October 2028; EU, 2023). Slurries 6 and 10 are certified for use in organic farming. Biocidal compounds, added as preservatives to the polymers or the finally composed slurries, are present in slurries 1, 2, 3, 5 and 8. For slurries 6 and 10 information on possible biocides added to the polymers by the supplier is not available. Slurries were free of synthetic fungicides or insecticides except slurry 1, containing the fungicides fludioxonil and mefenoxam, and slurry 2, containing fludioxonil.

Production of suspensions of C. cladosporioides H39 conidia and L. enzymogenes 3.1T8 cells

Cladosporium cladosporioides H39 was grown on PDA for 14 days at 20°C in the dark. *Lysobacter enzymogenes* 3.1T8 was grown on TSA overnight for 24 hours at 25°C in the dark. *C. cladosporioides* H39 colonies were flooded with sterile water containing 0.01%

Tween 80. After gently rubbing with a sterile rubber spatula, the obtained suspension was filtered through nylon gauze (200 µm mesh). Conidial concentration was determined using a haemocytometer and adjusted with water containing 0.01% Tween 80 to 2.7×10^8 conidia ml⁻¹. *L. enzymogenes* 3.1T8 suspension was prepared by flooding colonies with Ringer solution at room temperature and scratching colonies with an inoculation loop. Concentration was determined with a haemocytometer and adjusted using Ringers solution to 2×10^8 cells ml⁻¹. The fungal suspension was kept at 4°C and the bacterial suspension at room temperature until use within one hour. The germination of *C. cladosporioides* H39 conidia was assessed by plating 100 µl of a suspension containing 10⁵ conidia ml⁻¹ on 1/10 malt extract agar (MA) and incubation for 20 hours at 25°C in the dark. Germinability was determined microscopically for 50 conidia. Conidia were considered germinated when the germ tube was at least as long as the shortest diameter of the conidium. In both experiments, 99 – 100% of the conidia germinated. Viability of *L. enzymogenes* 3.1T8 was tested by plating 100 µl suspension on TSA, concentrations of the suspensions were 10⁴, 10³ and 10² cells ml⁻¹. Developed colonies were counted per plate after incubation for 24 hours at 25°C. The number of colonies were multiplied by the corresponding dilution factor to calculate the amount of culturable bacterial cells ml⁻¹. CFU counts showed that more than 95% of the plated bacterial cells were viable.

Table 1. Characteristics of commercial slurries used in the study.

Slurry number	Slurry developed for seed coating with	Slurry contains			Certified for use in organic farming
		Synthetic PPP*	Preservatives	Micro-plastic	
1	Synthetic PPP	Fludioxonil, mefenoxam	Yes	No	No
2	Synthetic PPP	Fludioxonil	Yes	No	No
3	Synthetic PPP	None	Yes	No	No
4	Synthetic PPP	None	No	Yes	No
5	Synthetic PPP	None	Yes	No	No
6	Synthetic PPP	None	Unknown	No	Yes
7	Synthetic PPP	None	No	No	No
8	Synthetic PPP	None	Yes	Yes	No
9	**	None	No	Yes	No
10	Microorganisms	None	Unknown	No	Yes

* Plant protection products.

** Experimental version of slurry 8.

Experimental design, and preparation and storage of suspensions of C. cladosporioides H39 conidia and L. enzymogenes 3.1T8 cells in slurries

Two independent experiments were conducted using the same experimental design. Six ml of each of the ten slurries were mixed with 6 ml suspensions of *C. cladosporioides* H39 conidia (2.7×10^8 cells ml⁻¹) or *L. enzymogenes* 3.1T8 cells (2×10^8 cells ml⁻¹). For each slurry-microbe combination, subsamples were made by pipetting 250 µl into a 1 ml Eppendorf tube. Three subsamples were further processed directly without storage (T=0) and 36 subsamples were stored at 4, 10 or 20°C in the dark for 1, 2, 8 or 28 days, with three replicates per slurry-microbe combination.

Quantification of viable C. cladosporioides H39 conidia and L. enzymogenes 3.1T8 cells in slurries by viability-qPCR

Suspensions of *C. cladosporioides* H39 conidia and *L. enzymogenes* 3.1T8 cells in slurries were diluted 1:100 after storage for 0 to 28 days in two steps. A subsample of the suspension (150 µl) was mixed with 1350 µl water containing 0.01% Tween 80 or Ringers solution. From this dilution, 150 µl were again mixed with water containing 0.01% Tween 80 or Ringers solution to obtain a final 1:100 dilution. The diluted suspensions (215 µl for *C. cladosporioides* H39 and 225 µl for *L. enzymogenes* 3.1T8) were treated with DNA binding dyes, DNA was extracted and strain-specific viability-qPCR assays were conducted as described by Köhl *et al.* (2024b). In short, PMAxx reagent (Biotium) was diluted in nuclease free HyPure water (HyClone™; VWR) to obtain a stock solution of 500 µM that 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 conidia of *C. cladosporioides* H39 and cells of *L. enzymogenes* 3.1T8 in the diluted suspensions were performed in DNeasy 96 plates (Qiagen). For *C. cladosporioides* H39, 215 µl of suspensions were mixed with 25 µl PMAxx and 10 µl EMA stock solution. For *L. enzymogenes* 3.1T8, 225 µl were mixed with 25 µl PMAxx stock solution. The three subsamples per slurry-microbe combination were treated immediately (T=0) with DNA binding dyes after 60 or 45 minutes at 20°C for *C. cladosporioides* H39 and after 75 or 60 minutes at 20°C for *L. enzymogenes* 3.1T8, respectively, in the first and second experiment because of technical reasons. Thereafter, samples were incubated for 30 minutes in the dark at 20°C in a heat block with plate adapter (Eppendorf, Hamburg, Germany) at 650 rpm. Subsequently, samples were photoactivated with the 96 Wells LED module (PhenoVation, Wageningen, Netherlands) for 10 minutes and 30% light intensity [equals 5870 µmol m⁻² second⁻¹ as measured using a UPRtek PG200N spectrophotometer]. Cells were collected by centrifugation at 5796 g for 10 minutes and 185 µl supernatant was pipetted off. Remaining samples were freeze-dried and thereafter DNA was extracted immediately.

Isolation of genomic DNA from cells of the two microorganisms was performed in 96-well format using the Molgen PurePrep Seed Kit (Molgen, Veenendaal, Netherlands) according to the protocol of the manufacturer. Lyophilised tissue was kept in liquid nitrogen for 30 second and thereafter disrupted using the TissueLyser II (Qiagen) and 90 mg 1.0 mm silicon carbide beads (BioSpec Products, Bartlesville, USA) for 30 seconds at 30 Hertz. After disruption, 500 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). Negative extraction controls without any added DNA or cells of the two microorganisms were included in each qPCR assay run.

An initial Taqman PCR assay with 0.8 pg gfp encoding DNA per reaction was performed using gfp specific primers (Klerks *et al.*, 2004) as internal amplification control to detect possible PCR inhibition caused by slurries. One slurry per company was selected and tested in two replicates in two dilutions (1:1 and 1:100) with added conidial suspension of *C. cladosporioides* H39 and with and without treatment with PMAxx and EMA. Thereafter, strain-specific Taqman qPCR assays were applied for *C. cladosporioides*

H39, using forward primer Fw 2_590.820 (GAGCACACCCAAGTCTTTG), reverse primer Rv 2_590820 (GCGTCTGCTTTGCGTT) and probe 590.820 (CtgcgaaGACctcgacc) labelled at the 5' end with a fluorescein label 6-FAM and at the 3' end with BHQ1, capital letters of the probe sequence are LNA nucleotide (Köhl and Groenenboom-de Haas, unpublished) and for *L. enzymogenes* 3.1T8 (Nijhuis *et al.*, 2010). The qPCR assays were conducted in 384-well format in a CFX384 real-time PCR detection system (Bio-Rad Laboratories Inc.). For each TaqMan qPCR of *C. cladosporioides* H39 and *L. enzymogenes* 3.1T8, 1 μ L sample was mixed with 9 μ L reaction mix containing 5 μ L 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 two minutes; 40 cycles of 95°C for 15 seconds followed by amplification for one minute 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.).

Results

The initial gfp Taqman qPCR assays with gfp encoding DNA added as internal amplification control resulted in similar C_t values for the HyPure water serving as control and all tested slurries. There was no indication of PCR inhibition by any of the slurries with complex (and unknown) composition. Strain-specific viability-qPCR was first used to study the survival of *C. cladosporioides* H39 and *L. enzymogenes* 3.1T8 in five slurries diluted 1:1 and further diluted 1:100 (resulting in a 1:200 dilution of the original slurries). Measurements in 1:1 diluted slurries failed, most probably because pigments interfered during photoactivation of EMA and PMA. In slurries diluted 1:200, photoactivation in fungal and bacterial cells was not blocked allowing the differentiation between dead and living cells. The same dilution was used for subsequent assays.

Generally, C_t values of approximately 28 for *C. cladosporioides* H39 and 25 for *L. enzymogenes* 3.1T8 at the beginning of the experiment confirmed that viable cells were added to the slurries (figures 1 and 2). Based on the measured initial percentage of viable conidia of *C. cladosporioides* H39 and cells of *L. enzymogenes* 3.1T8 and the obtained C_t values, the numbers of viable conidia or cells ml^{-1} was estimated (figures 3 and 4). During the course of the experiments, measured C_t values consistently increased indicating that conidia of *C. cladosporioides* H39 had reduced viability after exposure to the slurries (figures 1 and 3). Similar results were obtained for cells of *L. enzymogenes* 3.1T8 (figures 2 and 4). Loss of viability was slower for slurries stored at 4°C compared to slurries stored at 10°C and most rapid during storage at 20°C. Standard errors of the mean for C_t values obtained for the three replicates within each of the two experiments were small and results of the two independent experiments showed similar trends for *C. cladosporioides* H39 indicating a high reproducibility of the applied viability-qPCR assay and of the effects of slurries on the fungus. Also, for *L. enzymogenes* 3.1T8, similar results were obtained in the two experiments in most cases. However, there were striking differences in C_t values at the beginning of the experiment ($T = 0$) with C_t values of approximately 38 in the first experiment and approximately 28 in the second experiment

in slurries 1, 9 and 10 (figure 2A, I, J; figure 4A, I, J). These differences were observed especially in slurries in which *L. enzymogenes* 3.1.T8 lost viability rapidly during the further course of the experiments. Time spans between adding *L. enzymogenes* 3.1.T8 to the slurries and adding PMA to the slurries were 75 minutes in the first and 60 minutes in the second experiment, so that slurries with strong detrimental effects on *L. enzymogenes* 3.1.T8 potentially had an even slightly shorter period during the second experiment to affect the bacterial cells before the first measurements.

There were clear differential effects of the individual slurries on *C. cladosporioides* H39 and on *L. enzymogenes* 3.1.T8. A rapid increase of C_t values during two days storage at 20°C by more than 6, indicating that approximately 99% of *C. cladosporioides* H39 conidia lost viability, was found for slurries 2, 3 and 10 (figure 1B, C, J; figure 3B, C, J). Conidia of *C. cladosporioides* H39 survived better when slurries were stored at 4°C. For several slurries, e.g. slurries 4, 6, 7, 8 and 9, C_t values showed only slight increase during the 28 storage period (figure 1D, F-I; figure 3D, F-I) at 4 or 10°C, whereas for slurry 10, an increased C_t value of approximately 36 indicated that conidia did not survive during such storage conditions. C_t values obtained for slurries 1, 2, 3, 5 and 8, all known to contain biocides, showed no obvious differences from values obtained for the other slurries (figure 1A-C, E, H). C_t values for slurry 1, containing the fungicides fludioxonil and mefenoxam, did not differ from values obtained for the majority of the other slurries whereas slurry 2, containing fludioxonil, showed strong effects on *C. cladosporioides* H39 viability (figure 1A, B; figure 3A, B). Despite the general increasing effects of storage time and storage temperature on C_t values, indicating decreasing viability, several slurries, e.g. slurry 4 and 6 showed only slight effects on the viability of *C. cladosporioides* H39 conidia (figure 1D, F). Strongest detrimental effects on viability were found for slurry 10, which has been developed for seed coating of microbial inocula (figure 1J; figure 3J). A major observation for all slurries is that C_t values for *C. cladosporioides* H39 were consistently low and stable during the first one to two days after being added to slurries if slurries were stored at 4 or 10°C (figure 1).

Different to *C. cladosporioides* H39, *L. enzymogenes* 3.1.T8 showed high sensitivity to several slurries as indicated by C_t values above 36 after one day or even already at the beginning of the experiment in experiment 1 (figure 2). It can be concluded that more than 99% of the cells of *L. enzymogenes* 3.1.T8 lost viability in slurries 1, 4, 5, 7, 9 and 10 during the first 24 hours of the experiments, irrespective the storage temperature (figure 2A, D, E, G, I; figure 4A, D, E, G, I). Also, for the remaining slurries 2, 3, and 6, detrimental effects on viability were observed during the course of the experiments (figure 2B, C, F; figure 4B, C, F). Exceptional survival of *L. enzymogenes* 3.1.T8 was found in slurry 8 (figure 2H; figure 4H). After an initial increase of the C_t values from approximately 28 to approximately 30 during the first day, C_t values remained stable during the following storage period irrespective the storage temperature. Interestingly, this was not found for slurry 9, which consists of the same polymer as slurry 8 but without several additional ingredients, biocide and pigment. Obviously, slurry 8 contains a biocide not affecting *L. enzymogenes* 3.1.T8 and ingredients protecting bacterial cells during the course of the experiments.

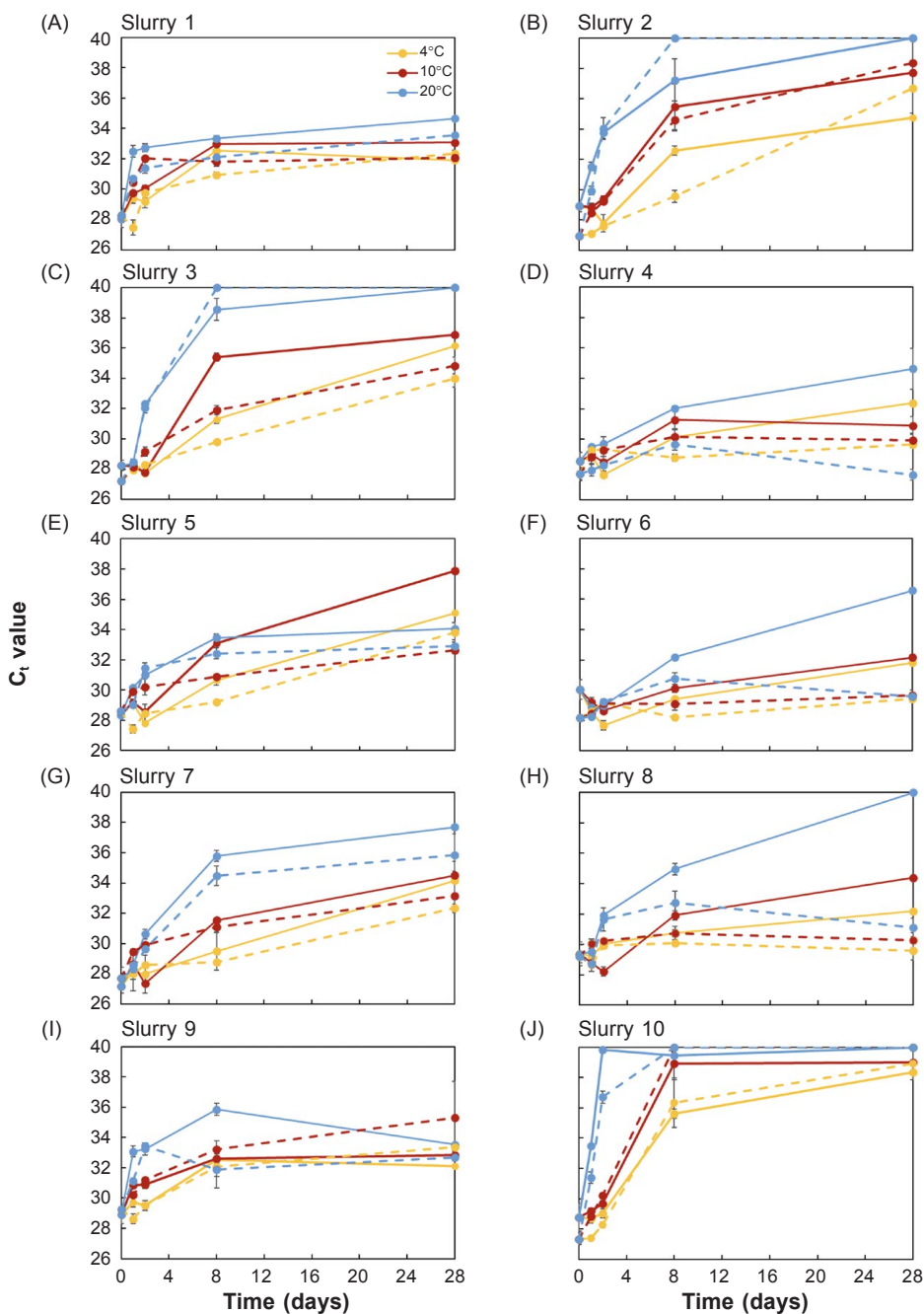


Figure 1. C_t values of strain-specific viability-qPCR for *Cladosporium cladosporioides* H39 added as conidial suspensions to ten slurries (A-J). Slurries with added *C. cladosporioides* H39 conidia were stored at 4, 10 or 20°C for 28 days. Means of three replicates of experiment 1 (solid lines) and experiment 2 (dashed lines). Bars show standard errors of the mean.

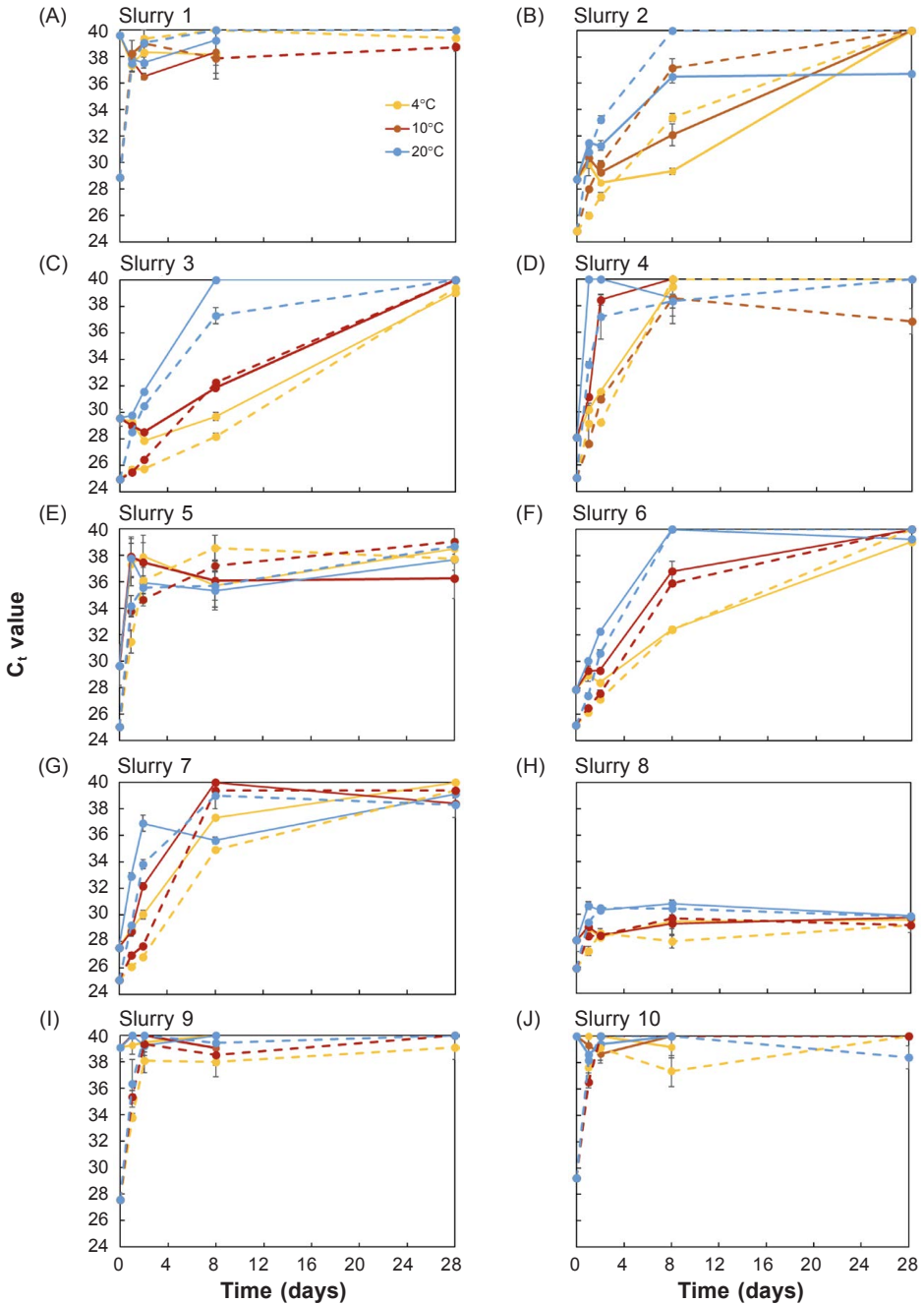


Figure 2. C_t values of strain-specific viability-qPCR for *Lysobacter enzymogenes* 3.1.T8 added as cell suspensions to ten slurries (A-J). Slurries with added *L. enzymogenes* 3.1.T8 cells were stored at 4, 10 or 20°C for 28 days. Means of three replicates of experiment 1 (solid lines) and experiment 2 (dashed lines). Bars show standard errors of the mean.

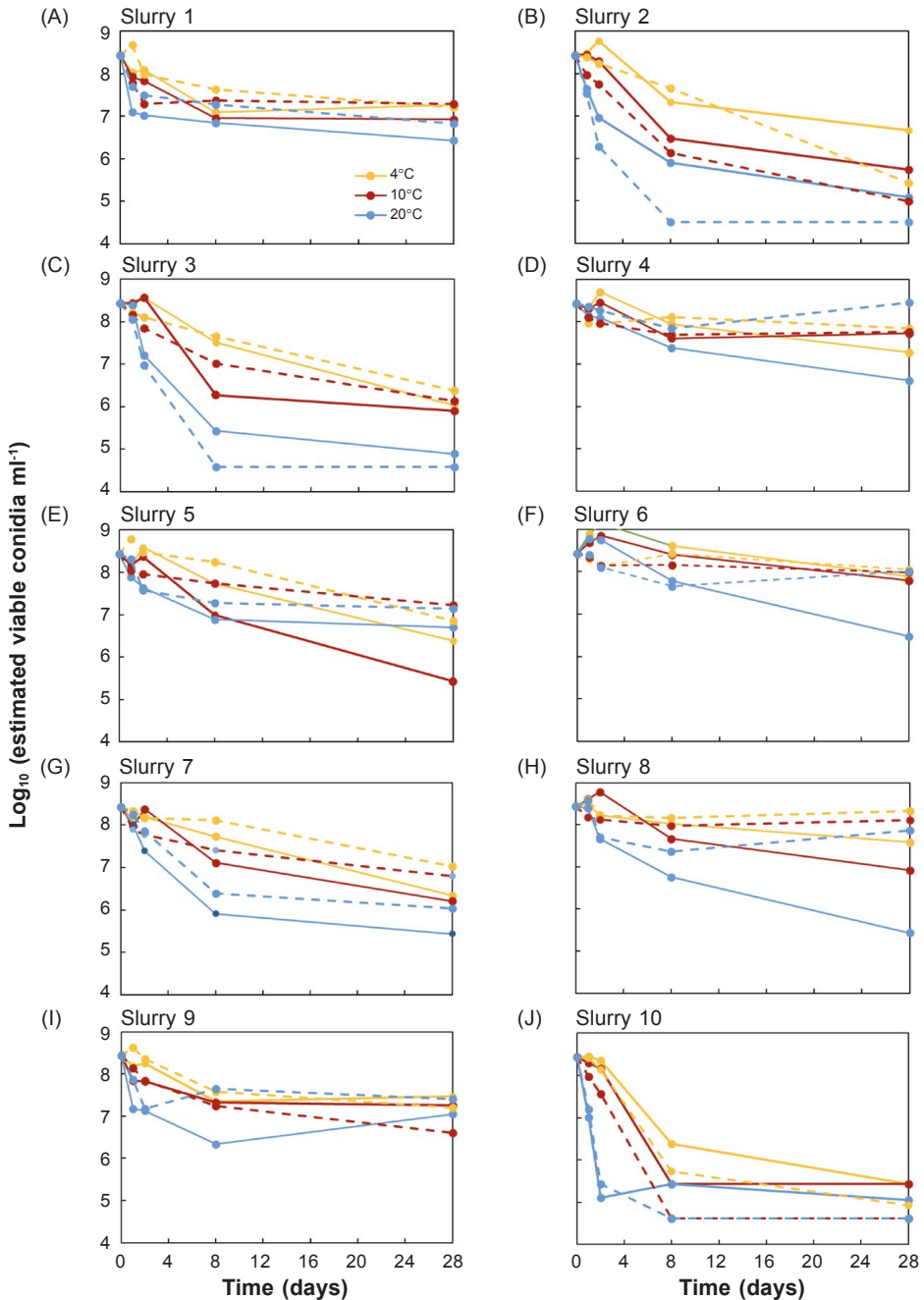


Figure 3. Number of viable conidia of *Cladosporium cladosporioides* H39 added as conidial suspensions to ten slurries (A-J) as estimated from C_i values of strain-specific viability-qPCR. Slurries with added *C. cladosporioides* H39 conidia were stored at 4, 10 or 20°C for 28 days. At the beginning of the experiment, 100% of the added conidia were viable.

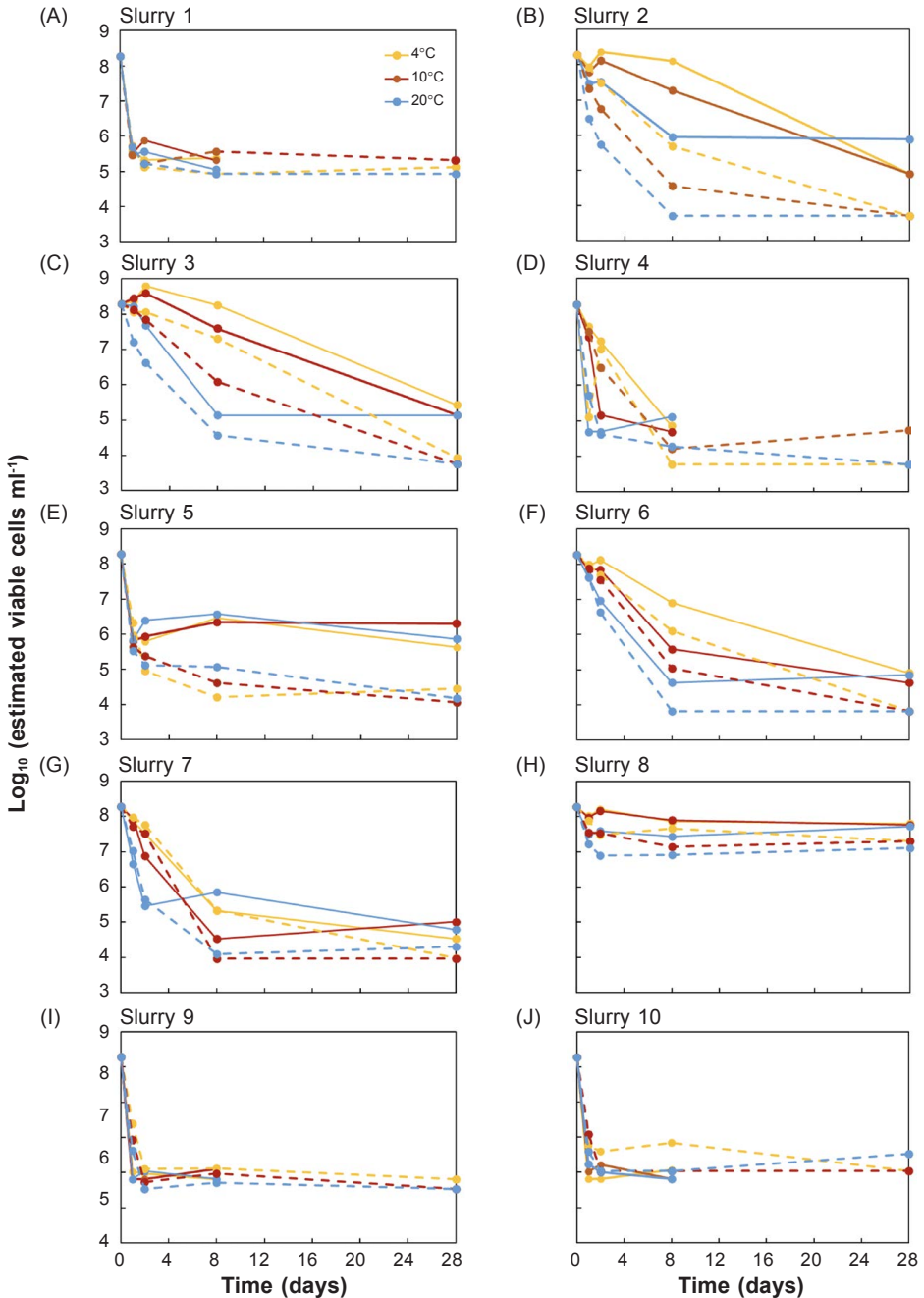


Figure 4. Number of viable cells of *Lysobacter enzymogenes* 3.1.T8 added as cell suspensions to ten slurries (A-J) as estimated from C_v values of strain-specific viability-qPCR. Slurries with added *L. enzymogenes* 3.1.T8 cells were stored at 4, 10 or 20°C for 28 days. At the beginning of the experiment, 95% of the added cells were viable.

Discussion

Recently developed strain-specific viability-qPCR assays (Köhl *et al.*, 2024b) were successfully used to quantify viability of microbial inocula in commercial slurries commonly used for seed coating. Once the methodology is established in a laboratory, viability-qPCR measurements would be reliable and fast, allowing high-throughput applications with potential to replace laborious plating techniques. A further advantage is that, besides strain-specific viability-qPCR as used in our study, generic viability-qPCR assays are also available and can be applied if viability of different fungal or bacterial inocula are tested, e.g. during a screening program for new antagonists (Köhl *et al.*, 2024a). The possible disadvantage for testing commercially used slurries is that they are often amended with pigments used for recognition (Halmer, 2008; Afzal *et al.*, 2020). Such pigments may absorb light and consequently may block photoactivation of DNA intercalating dyes. Sufficient dilution of the slurries before application of the dyes and their photoactivation counteracted this possible limitation in our study.

The viability of conidia of *C. cladosporioides* H39 and cells of *L. enzymogenes* 3.1T8 was affected by most of the tested slurries (figures 1-4). Interestingly, slurry 10, marketed as slurry for microbial seed treatments and certified for use in organic farming, also reduced the survival of the two model antagonists. Slurries consist of different components including polymers. Polymers that contain biocides added for preservation were used for most of the studied slurries. Further information on the biocidal ingredients and their concentrations was not disclosed. Thus, the possibly differential effects of certain biocides at the relevant concentrations on the added antagonists could not be tested in our study. Interestingly, slurry 8, was less detrimental for the antagonist inocula although this slurry contains a polymer with added biocide.

Once MBCA are added to slurries for seed coating, the availability of water may switch inocula from a dormant stage to onset of germination and growth. At the same time, components present in the slurries may affect both dormant or physiologically active inocula. After the coating process, coated seeds are dried again and MBCA need to become dormant again and to remain in a dormant stage again during seed storage and transportation until seeds are sown. Both the onset of physiological activity and the possibly detrimental effects of slurry components may have adverse effects on immediate viability and long-term effects on longevity during further storage of MBCA-coated seeds. Slurry composition as well as seed processing thus need to be optimised for MBCA requirements and sensitivities to certain environmental conditions, chemical components and their various interactions. The results of our study clearly demonstrate that storage of slurries with already added MBCA is detrimental for the inocula in most of the tested slurries. These adverse effects are increasing in time and are more serious when slurries are stored at room temperature compared to storage at 4°C. The general conclusion is that MBCA should be exposed to the watery environment containing also possibly toxic substances for as short as possible and to use slurries amended with MBCA immediately after MBCA are added and to keep amended slurries cooled during handling. Bailly *et al.* (2020) proposed adding MBCA to slurries only a few minutes before the coating process or to develop coatings with a lipid phase to avoid rehydration and the

subsequent dehydration step. The specific needs for MBCA handling in slurries demand specific protocols for seed coating for the seed processor. Commonly, slurries applied with added chemical plant protection products can be handled differently since storage during several days, also at room temperature, commonly is possible. Changing from chemical to biological seed treatments, thus needs adaptation of protocols, work flows and training of the personnel to avoid failures due to viability losses.

Our study focussed on the use of MBCA during a specific step of seed processing, amending slurries with microbial inocula and handling such amended slurries before use for seed coating. Many other steps of seed handling and seed processing need similar attention with detailed studies on survival of different groups of potential inocula applied for biological control. A similar development is needed for applications of microbial biostimulants and biofertilizers. It will be a substantial challenge to identify the crucial steps for microbial inocula survival along the entire seed processing lines from adding MBCA to slurries and subsequent seed coating and drying processes, to storage under environmentally controlled conditions, packaging and transportation and storage at growers facilities under less controlled environmental conditions. Seed quality parameters such as germination and vigour will always have the highest priority during this essential re-thinking of many seed handling processes aiming at optimised viability and longevity of the applied MBCA. If specific antagonists have been selected and are further integrated in seed processing technologies, the application of generic and strain-specific viability-qPCR assays 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 viability-qPCR assays for fast and reliable monitoring of antagonist survival.

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Conflicts of Interest declaration

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.

Ethical standards

This manuscript does not involve human and/or animal experimentation, human data or human tissue. No special ethics approval was therefore required.

Author' contributions

JK, LG, IH and EL designed the study. PB, TG, LH, AN and MN gave conceptual advice. JK, LG, IH and EL performed the experiment and collected data. JK analysed data. JK and LG drafted the manuscript. JK, PB, TG, LG, LH, IH, EL, AN and MN revised drafts of the manuscript. All authors read and approved the final manuscript.

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