Research Article

Assessment of microbial biocontrol agent (BCA) viability to mechanical and thermal stress by simulating spray application conditions

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Abstract

Background: In order to improve the biological control agent (BCA) efficacy, stress factors threatening the viability of microorganisms during spray application need to be determined. The effect of spray mixture temperature and exposure time on *Trichoderma harzianum* T 22 and *Bacillus amyloliquefaciens* QST713 viability were tested. Concurrently the combined effect of mechanical and thermal stress effect on BCA viability were tested at two initial spray mixture temperatures (14 and 25 °C) by simulating a spray application using airblast sprayers featured by different tank capacity and a spray liquid circuit (without and with hydraulic agitation system). To assess the BCA microorganism viability, spray mixture samples were collected at time intervals along trials and plated to count the colony forming units (CFU).

Results: The critical temperature threshold that inhibited BCA viability was 35 °C with 30 min of exposure. The sprayer type, the initial temperature of the spray mixture and the temperature increment during the trials significantly decreased the number of CFU recovered. When simulating a spray application, the spray mixture temperature increase rate was determined mainly by the residual amount of spray mixture in the tank. Even if the tank capacity does not substantially affect the final temperature reached by the spray mixture, the higher residual spray mixture in bigger tanks can expose the BCAs for a longer time to critical temperatures.

Conclusions: Experimental trials allowed us to identify the effect of factors affecting the viability of tested BCAs, providing information about the actual chance to guarantee the biological efficacy of BCA treatments.
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Keywords: integrated pest management; airblast sprayers; spray application; bio plant protection products; antagonist BCAs; colony forming unit

1 INTRODUCTION

Vineyards worldwide are subject to a variety of fungal diseases, of which gray mold (*Botrytis cinerea*), powdery mildew (*Uncinula necator*) and downy mildew (*Plasmopara viticola*) have the highest incidence in Europe. Besides significant crop yield losses, these diseases also can reduce wine quality, further contributing to extensive economic losses.[1] Traditionally, management of these fungal diseases relied on viticultural practices, such as pruning and defoliation, and specific training systems to improve ventilation and air circulation to reduce canopy humidity and inoculum sources, and using synthetic fungicides.[1,2] However, the use of synthetic fungicides is not considered sustainable owing to recurring rapid development of resistant strains of the pathogens and because of adverse effects of the fungicides on the environment and human health.[3] The presence of residues can furthermore

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negatively affect wine quality.[4] These concerns have led to the search for alternatives to the use of synthetic fungicides. In organic viticulture, copper (Cu)-based products have been used extensively, leading to accumulation of Cu in soils, causing phytotoxic effects and negative impacts on the wine quality.[2,5] In this regard, EU regulatory bodies have strengthened the legislation on Cu-based products, limiting the use to 28 kg ha^{-1} Cu until the year 2025.[6] Therefore, the use of nonpathogenic microorganisms as biological control agents (BCAs) is increasingly considered as a promising alternative.[7,8] Research in this field hs been very intense during the last two decades.[9,10] Despite the difficult introduction of BCAs in the market, some commercial products based on fungal or bacterial genera are available in Europe for biological control. Bacteria belonging to the genus *Bacillus* and fungi belonging to the genus *Trichoderma* are two of the most important groups of microorganisms used to control fungal pathogens in numerous crops.[11–13] Although BCAs were successfully implemented, their efficacy is generally more variable compared to synthetic fungicides.[2,3] This is not surprising, given that BCAs are living organisms dynamically interacting with the target pathogen, the host plant and the microbial communities present on the host surface in a changing physical environment. For example, (micro)climatic conditions, such as temperature and relative humidity, have a major effect on the efficacy of BCAs.[14] The lack of consistency in the field is, however, a significant barrier in the adoption of biocontrol products.[2] To improve the efficacy of the bio-fungicides, stress factors threatening the viability of the microorganisms under field conditions need to be determined. Few studies have focused on the factors that influence the efficacy of BCAs during and after spray application,[14–16] but none have been performed on the effect of the spray application process under different temperatures and mechanical characteristics of the equipment used, except for entomopathogenic nematodes (EPN).[17–19] In fact, during spray application, the sprayer tank and the liquid circuit can be inhospitable environments for microorganisms which can negatively affect the efficacy of BCAs in controlling the pathogen. For example, liquid temperature within a spray tank increases during pump recirculation and can produce conditions that are detrimental for EPN. It is therefore recommended to avoid temperatures >30 °C within the pump, the tank and the nozzles when applying EPN.[20] Similar bottlenecks are expected for other BCAs. Even if previous studies have shown an *in vitro* effect of air temperature on the efficacy of BCAs,[16] the efficacy observed under field conditions may be different from their performance *in vitro*. Identifying the BCA requirements and selecting suitable and efficient application techniques and conditions is of utmost importance to safeguard the viability and the efficiency of the BCAs in controlling the pathogens.

This study focused on the viability of two BCAs in two commercial formulations, that is, *Trichoderma harzianum* T-22 (fungus) and *Bacillus amyloliquefaciens* (former *subtilis*) QST713 (bacteria). Both are broad-spectrum antagonists, registered in several European countries for use on grapes, vegetables, cereals, and fruit crops. Furthermore, they are of prime interest as they are partially compatible with synthetic fungicides such as Cu-based products.[21]

The objectives of this study were (i) to evaluate the effect of spray liquid temperature and exposure time on the viability of the two BCAs under laboratory conditions, and (ii) to determine the combined effect of mechanical and thermal sprayer stress on the microorganisms viability by simulating spray application conditions.

2 MATERIALS AND METHODS

Viability trials were conducted with two commercial formulations of BCAs, that is, Trianum-P® (Koppert B.V., Berkel en Rodenrijs, the Netherlands) containing *T. harzianum* Rifai strain KRL-AG2 (T-22) and Serenade® ASO (Bayer Crop Science S.r.l., Milano, Italy) containing *B. amyloliquefaciens* (formerly *subtilis*) strain QST 713. Trials were carried out in two phases. The first phase was performed under laboratory conditions and aimed (i) to determine the baseline colony forming units (CFU) of the microorganisms in the tested formulations and (ii) to evaluate the effect of thermal stress on the microorganisms viability for a wide range of temperatures and exposure times. The second phase consisted of static airblast sprayer trials conducted in the workshop, aiming (iii) to evaluate the combined effect of mechanical stress and thermal stress on the microorganisms viability by simulating spray application conditions.

2.1 Laboratory trials

2.1.1 Baseline CFU in the commercial formulations

The number of CFU present in Trianum-P® was determined by suspending 5 g formulation in 45 mL sterile deionized water containing 0.01% of polysorbate (Tween 20; Sigma-Aldrich, Milan, Italy). The suspension was shaken for 30 min using an orbital shaker (Advanced 3500; VWR Avantor, Milan, Italy) and 15 serial dilutions (1:10) were made. Then, 1 mL of the 1×10^{-11} to 1×10^{-15} dilutions were plated on 9 mL potato dextrose agar (PDA; VWR Avantor) containing 25 mg L^{-1} of streptomycin sulphate (VWR Avantor). This step was conducted under a laminar flow chamber (TC72 S; Gelaire, Sydney, Australia). Five plates per dilution were made and incubated at room temperature (RT, 20–22 °C) for ≈60 h before conducting the plate count of the colonies as described by Dhingra and Sinclair.[22] The same protocol was adopted for Serenade® ASO, except that 1 mL of the 1×10^{-6} to 1×10^{-10} dilutions were plated on 9 mL lysogeny broth (LB) agar (Luria Bertani Broth; VWR Avantor).

2.1.2 Effect of liquid temperature and exposure time on the BCA viability

In order to determine the effect of liquid temperature and exposure time on the viability of the microorganisms, sterile deionized water was heated in a climatic chamber (C.I.F.L.U.D.; Cavallo S.r.l., Milan, Italy) to 15, 20, 25, 30, 35 and 40 C°. Then, 5 g Trianum-P® was suspended in 45 mL water of each temperature using the orbital shaker (VWR Advanced 3500) and serial dilutions (1:10) were made. The 10^{-13} to 10^{-15} dilutions were kept at the six different water temperatures. After 0, 10, 20, 30 and 60 min of exposure, 1 mL of the 10^{-13} to 10^{-15} dilutions were plated by inclusion in 9 mL PDA containing 25 mg L^{-1} streptomycin sulfate. Per dilution–temperature–exposure time combination, five plates were made and incubated at RT (20–22 °C) for ≈60 h before conducting plate count analysis. For Serenade® ASO, the same protocol was adopted, except that the dilutions from 10^{-8} to 10^{-10} were prepared and plated on LB agar.

2.2 Static airblast sprayer trials

2.2.1 Technical sprayer characteristics

The combined effect of mechanical and thermal stress on the microorganism viability by simulating vineyard spray applications was tested using two airblast sprayers which differed in hydraulic circuit and in level of technology, that is, basic *versus* high-tech.

The basic sprayer was the Virgola mounted airblast sprayer (Dragone S.r.l., Castagnole Lanze, Italy). This sprayer represents a

conventional sprayer as currently used by most winegrowers in south Europe.[23] The sprayer is equipped with a 300-L polyethylene tank and six conventional nozzle holders on each sprayer side. Conventional hollow cone nozzles ATR 80 yellow VK (Albuz® CoorsTek, Monchengladbach, Germany) were mounted on the sprayer. The hydraulic circuit of the Dragone Virgola sprayer was fed by an APS 51 membrane pump (Comet S.p.a., Reggio Emilia, Italy) characterized by maximum flow rates of 53.9 L min^{-1} at 0.0 MPa and 50.7 L min^{-1} at 4.0 MPa at 550 ¯rpm. The spray tank was not equipped with a dedicated hydraulic agitation system [Fig. 1(a)]. A by-pass pipeline at the spray control unit provided a liquid return to the tank for the amount of liquid exceeding the total flow rate of the activated nozzles.

As high-tech sprayer, the prototype developed in the framework of the H2020 OPTIMA project, that is, Smart Synthesis (Caffini S.p.a., Palù, Verona, Italy), was used. This trailed sprayer with a 1000-L polyethylene tank and an innovative, electrically driven axial fan[24] was equipped with a DynaJet® Flex 7140 PWM system (TeeJet Technologies/ Spraying Systems Co., Wheaton, IL, USA). Each sprayer side contained a vertical boom with eight single-nozzle holders coupled with a PWM solenoid valve. The PWM valves allowed variation of the duty cycle of the pulse signals to change the nozzle flow rates at a frequency of 20 Hz.[25] The sprayer was equipped with standard flat fan nozzles TeeJet XR 80 02 VS (Spraying Systems Co.). The hydraulic circuit of the Caffini Smart Synthesis sprayer was fed by an IM 312 membrane pump (Imovilli pompe S.r.l., Mancasale, Italy) characterized by maximum flow rates of 130.0 L min^{-1} at 0.0 MPa and 122.0 L min^{-1} at 4.0 MPa at 550 ¯rpm. The spray tank was

equipped with a dedicated hydraulic agitation system for the spray mixture consisting of two pipelines directly connected to the pump [Fig. 1(b)]. One pipeline fed eight venturi nozzles (Ø 1.2 mm; Tecomec S.r.l., Reggio Emilia, Italy) ad hoc distributed in the tank at different positions and orientations to maintain the agitation of the mixture throughout the spray application, irrespective of the amount of liquid inside the tank. The second pipeline of the agitation system fed a single venturi nozzle (Ø 2.0 mm, Tecomec S.r.l.) installed at the bottom of the tank to guarantee a good level of agitation, even at low liquid levels. The by-pass pipeline at the spray control unit guarantees the return of the exceeding flow rate to the main tank.

2.2.2 Performance of the spray tank agitation systems

The capability of the hydraulic systems of both sprayers to keep the liquid properly agitated was characterized following ISO 5682-2[26] before the viability trials. Briefly, the sprayer tank was filled up to its nominal capacity with a suspension of water and copper oxychloride (35% (w/w)) (Patrol 35 WP; Certis Europe B.V., Varese, Italy) at 1 g L^{-1} concentration. The sprayer agitation system was maintained as active throughout the whole tank-filling duration and continued for 10 min after the tank was filled. The Dragone Virgola sprayer was operated at 2.0 MPa and the Caffini Smart Synthesis sprayer at 1.5 MPa for the agitation of spray liquid. Then, three reference samples (100 mL each) were simultaneously collected from the tank at 10%, 50%, and 90% of nominal tank capacity using an ad hoc designed device which also was used to transfer the liquid in different glass laboratory bottles. Subsequently the sprayer agitation system was turned off to allow
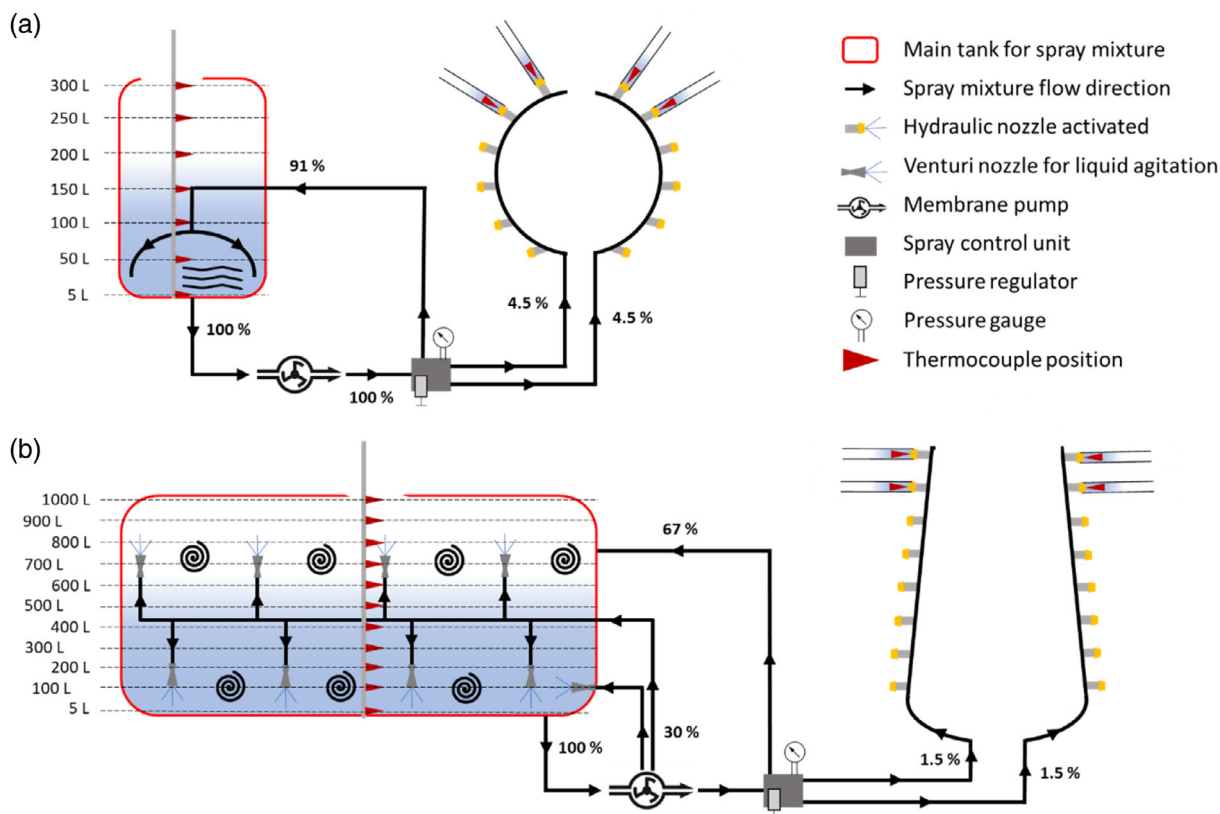
Figure 1. Schematic of the hydraulic circuit (a) of the Dragone Virgola airblast sprayer and (b) of the Caffini Smart Synthesis airblast sprayer, including liquid flow direction and rates, and position of the thermocouples in the tank and at the outlet of the activated nozzles.

the liquid suspension to settle for 16 h. After the settlement period, the sprayer agitation system was turned on for 10 min. The re-agitated spray mixture was sampled from the tank following the same procedure as described above for the collection of reference samples. Immediately after the tank sampling, the nozzles were activated, and the spray mixture agitation was kept active. Then samples were collected from two nozzles (the one placed at the end of the pipeline/boom-section per sprayer side) at the beginning of the trials and after every 50 or 100 L of tank emptying for the 300- and 1000-L tank sprayer, respectively. The last sample was taken just before the pump ran dry by checking the spray pressure drop. In all cases, combination of nozzles and tank emptying frequency, three samples were taken. The Dragone Virgola sprayer was operated at 1.4 MPa. All 12 Albuz ATR 80 yellow VK nozzles were activated providing a total flow rate of 14.52 L min^{-1}. Likewise, the Caffini Smart Synthesis sprayer was operated at 1.0 MPa during spraying. All 16 TeeJet XR 80 02 VS nozzles were activated resulting in a total flow rate of 23.36 L min^{-1}. Both sprayers were set up to comply with the requirements of ISO 5682-2.[26]

The copper oxychloride concentration of each sample was determined by evaporating the liquid from the sample.[27] Based on the tank copper oxychloride concentration after initial agitation (reference samples) the percentage deviation of tank and spraying concentrations after spray mixture re-agitation was calculated following the ISO 5682-2[26] methodology. According to the ISO 16119-3 no deviations higher than ±15% from the reference concentration are allowed for the re-suspension of copper oxychloride after 16 h of sedimentation in the sprayer tank with the agitation system turned off.[28] This ±15% deviation threshold guarantees the capability of the agitation system to keep the spray mixture in the main tank homogeneous.

2.2.3 Viability trial set-up

Before the viability trials, the hydraulic circuits of both sprayers were sanitized using a solution of water and sodium hypochlorite (PA-PUREX; Carlo Erba Reagents S.A.S., Val de Reuil, France) at 10 g L^{-1} concentration. For the Dragone and Caffini sprayer, respectively 20 and 60 L solution was placed in the tank, in line with the sprayer tank dimensions. Then, the tractor power take-off (PTO) was turned on allowing the solution to pass through the hydraulic circuit pipelines. Finally, the hydraulic circuit was washed with pure water in three cleaning cycles. Based on preliminary trials, this procedure was necessary to minimize cross-contamination and to avoid the growth of unwanted microorganisms potentially affecting the results of the CFU counting of the viability trials.

Once the sprayer tanks were sanitized, they were filled with water taken directly from the public water supply pipeline. The viability trials were performed at two different initial temperatures of the spray liquid, that is, at 14 ± 1 °C (tap water) and at 25 ± 1 °C (heated tap water simulating rainwater stored in big tanks during spring/summer conditions). The exact amount of water used to fill the tanks was measured using an electronic flow meter PRO-FLOW 12 V (Polmac S.r.l., Mirandola, Italy). To heat the liquid to 25 °C, a customized heating system was designed and constructed by Lorenzoni S.r.l. (Nove, Italy). The device was composed of an *ad hoc* dimensioned submersible electric heater (12 kW rated power) with a stainless-steel flange for the connection to the sprayer tank opening. The heater included a control device with a temperature probe used to stop the heating once the required water temperature was achieved.

To homogenize the water temperature in the tank during the heating process, the liquid return of the Dragone Virgola sprayer was activated and manual agitation using an oar-shaped mast was performed simultaneously. For the Caffini Smart Synthesis sprayer the agitation system was activated every 10 min for 3 min. Starting from 14 °C, the heating process to reach 25 °C took about 20 and 35 min for the tank water in the Dragone Virgola and Caffini Smart Synthesis sprayers, respectively.

The formulations were added to the tanks at 2.5 g L^{-1} for Trianum-P® and at 4 g L^{-1} for Serenade® ASO according to their label indications. At 12 h before the trials, the formulations, which were stored at 4 °C in a refrigerator, were brought to RT (between 16 and 18 °C) to avoid thermal shock of the microorganisms during addition and mixing in the tank. During addition of the formulation to the tank and for 5 min in total, the spray liquid was mixed through the by-pass line and the agitation system of the sprayer (if present). After 5 min of agitation, the pipelines feeding the nozzles were activated for 1 min with all nozzles activated, that is, 12 and 16 nozzles for the Dragone Virgola and the Caffini Smart Synthesis sprayers, respectively, filling the hydraulic circuit with the actual tank mixture.

2.2.4 Combined effect of mechanical and thermal sprayer stress on the BCA viability

Spray application simulations were performed indoor at the Crop Protection Technology laboratory (DiSAFA, UNITO). The trials with the Dragone Virgola sprayer were performed at 1.4 MPa spray pressure, resulting in a nominal flow rate of the Albuz ATR 80 yellow hollow cone nozzles of 1.21 L min^{-1}. The trials using the Caffini Smart Synthesis sprayer were performed at 0.4 MPa and 100% duty cycle, resulting in a nominal flow rate of 0.92 L min^{-1} of the standard flat fan nozzles TeeJet XR 80 02. The spray setting was defined based on the results obtained by Grella et al.[29] To maximize the stress on the microorganisms in the spray mixture the longest spray duration was simulated by activating only two nozzles per sprayer. This situation corresponds to a spray application performed in trellised vineyards at the beginning of the growing season. Total flow rates of 4.84 L min^{-1} with the Dragone Virgola sprayer and of 3.68 L min^{-1} with the Caffini Smart Synthesis sprayer were achieved. In both cases, the tractor PTO was kept constant at 540 ¯rpm. This means that when operating with the Dragone Virgola sprayer ≈9% of the pump flow rate was directed to the nozzles, whereas 91% returned to the tank generating some tank mixing [Fig. 1(a)]. When operating with the Caffini Smart Synthesis sprayer, ≈3% of the pump flow rate was directed to the nozzles, 30% was directed to the two pipelines dedicated to the agitation system and 67% to the main tank through the by-pass pipeline on the spray control unit [Fig. 1(b)].

At different time intervals, 15 mL spray liquid was collected at the nozzle outlets and in the tank. The samples were taken at three positions and with three replicates per position: the positions accounts for three depths in the residual spray mixture in the tank and three nozzles. The sampling times were selected in accordance with the capacity of the sprayer tank and the spray settings. At the selected spray settings, the Dragone Virgola sprayer took 63 min to empty the tank, whereas the Caffini Smart Synthesis took 269 min. Specifically, samples were taken for the Dragone Virgola sprayer after 0, 30 and 63 min, and for the Caffini Smart Synthesis sprayer samples after 0, 30, 60, 120 and 269 min. In addition to the spray mixture samples, a blank sample of pure water was taken directly from the tank and nozzles before adding the formulations. All samples were collected in laboratory tubes

and kept in an ice box for transport to the Plant Pathology laboratory (DiSAFA, UNITO). Immediately after arrival at the laboratory, serial dilutions and plating procedures were performed according to the protocols described above (see Baseline CFU in the commercial formulations section above). In total, 288 Dragone Virgola samples were analyzed [2 BCA × 2 spray mixture initial temperatures (14 and 25 °C) × 2 sampling locations (tank and nozzle) × 3 positions (different depth for the tank and different nozzles) × 4 exposure times (blank, 0, 30, and 63 min) × 3 replicates]. In addition, 432 Caffini Smart Synthesis samples were analyzed [2 BCA × 2 spray mixture initial temperatures × 2 sampling locations × 3 positions × 6 exposure times (blank, 0, 30, 60, 120, and 269 min) × 3 replicates].

The temperature of the spray mixture was monitored continuously during the trials in the tank and at the nozzle outlets. To this end, a set of calibrated thermocouples was installed on a vertical mast at different liquid levels inside the sprayer tanks from 5 L up to the maximum level of the tank (i.e. 300 or 1000 L). The thermocouples in the Dragone Virgola sprayer were placed at intervals of 50 L, whereas those in the Caffini Smart Synthesis sprayer were placed at intervals of 100 L (Fig. 1). Distance between thermocouples depended on the size and shape of the tank and were determined based on progressive tank-filling at intervals of 50 and 100 L depending on the sprayer type, using the electronic flow meter PRO-FLOW 12 V (Polmac S.r.l.). For both sprayers an additional set of four thermocouples was used to measure the spray liquid temperature at the outlet of the activated nozzles (Fig. 1). A membrane was installed on the nozzle to create a small chamber at the outlet in which the thermocouple was placed. The small chamber ensured the thermocouple to be immersed in the sprayed liquid. All thermocouples were connected to a HD32.8.16 datalogger (Delta OHM S.r.l., Caselle di Selvazzano, Italy) and data were recorded at 0.5 Hz for the entire duration of the trials.

Air temperature and relative humidity in the workshop were recorded during the trials using a TESTO 625 thermo-hygrometer (Testo S.p.a., Settimo Milanese, Italy).

2.3 Data analysis

Concerning the laboratory trials (see Laboratory trials section above), two linear models were carried out to assess the effects of liquid temperature and exposure time on the viability of the two microorganisms (*T. harzianum* T-22 and *B. amyloliquefaciens* QST 713) contained in the two BCA commercial formulations (Trianum-P® and Serenade® ASO). Liquid temperature, exposure time and their interaction were considered as fixed factors. When the interaction was not significant, the model was run without the interaction term.

Concerning the static airblast sprayer trials (see Static airblast sprayer trials section above), for each combination of BCA formulation, sprayer type (Dragone Virgola or Caffini Smart Synthesis), and initial spray mixture temperature in the tank (14 or 25 °C), the CFU measurements from the tank and at the nozzle outlet were compared by Student's *t*-test or Wilcoxon test for independent samples (depending on whether the requirements of the assumptions of the parametric statistics were met or not). The tests showed that the CFU values measured in the tank and at the nozzle outlets were comparable. The values of both sampling locations were therefore combined and considered as a unique sample unit for the further data analysis. Separately for the two BCAs formulations, the temperature inside the tank was related to the duration of the experiment (63 min for Dragone Virgola and 269 min for Caffini Smart Synthesis) to ascertain the combined effect of mechanical and thermal stress caused by spraying. A third-degree polynomial was used to mathematically describe these relationships. Furthermore, the temperature–time derivative ($\Delta T/\Delta t$, °C min^{-1}) was calculated to evaluate the temperature increments rate over the trials. Then, the relationship between the temperature–time derivative (°C min^{-1}) and the residual amount of spray mixture in the tank (l) was used to compare the effect of the sprayer type and the initial spray mixture temperature on the residual spray mixture temperature in the tank. For each BCA, the effect of temperature measured during the experiment, initial temperature (14 and 25 °C), and sprayer type (Dragone Virgola and Caffini Smart Synthesis) on the number of CFU was tested using a linear model. The three factors and their two- and three-way interactions were considered as fixed factors. The model was run to test how much the temperature variation, resulting from combined mechanical and thermal stress, affects the CFU throughout the duration of the experiment in addition to the initial temperature and sprayer type.

In all cases, the linear models were carried out with the function "lm" in the R environment.[30] The assumptions of homogeneity of variances, normality and independence of the residuals were graphically checked. The assessment of the significance of the fixed and interacting factors in linear models were carried out with the "join_tests," of the emmeans package.[31] The significance threshold was set to $P < 0.05$.

3 RESULTS AND DISCUSSION

3.1 Laboratory trials

3.1.1 Baseline CFU and viability in the commercial formulations of the BCAs

In the commercial formulation, a concentration of 1×10^{13} CFU g^{-1} of *T. harzianum* T-22 was found. This baseline was 1×10^4 CFU higher than the minimum stated on the formulation label, that is, $>1 \times 10^9$ CFU g^{-1}. For *B. amyloliquefaciens* QST 713, a baseline concentration of 1×10^{12} CFU g^{-1} was found in the formulation, corresponding well with the minimum concentration reported on the label ($>1 \times 10^{12}$ CFU g^{-1}). These results show that in both cases the BCAs contained in the commercial formulation tested were viable and suitable for the experimental trials. The obtained information is essential when considering that not all BCAs can survive the conditions imposed during the formulation process at large-scale production, even if in most cases protective substances are added to the formulation medium to improve the stability.[32] Additionally, the literature reported a strong effect of packaging (flasks *versus* bags) and storage conditions, especially temperature, on the shelf life of BCAs.[33–36] To exploit a BCA as a commercial product, important concentrations of microorganism biomass should be produced, stabilized, packaged and correctly stored/managed to be transported to its end-user.[32] This is one of the reasons why their use is still limited and contains hurdles. Due to the above-mentioned constraints the evaluation of baseline CFU in the commercial formulation is essential before the evaluation of BCA viability under combined mechanical and thermal stress in order to avoid misinterpretation of results.

3.1.2 Effect of liquid temperature and exposure time on the BCA viability

The viability of the microorganisms *T. harzianum* T-22 and *B. amyloliquefaciens* QST 713 (expressed as log$_{10}$ CFU mL^{-1}) as a function
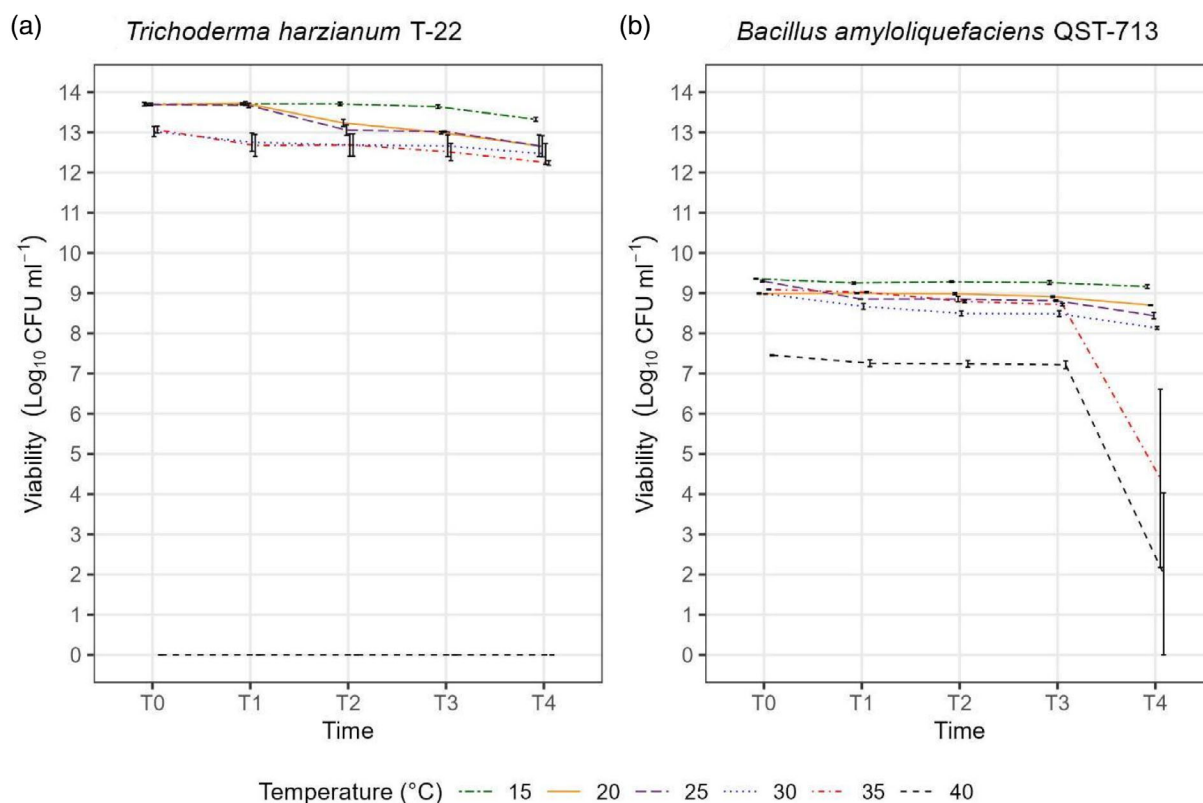
of liquid temperature and exposure time under laboratory conditions is presented in Fig. 2. Liquid temperature ($P < 0.001$) and exposure time ($P < 0.001$) significantly affected the viability of *T. harzianum* T-22. No significant interaction between liquid temperature and exposure time ($P < 0.057$) was observed (Table 1). At 35 °C the viability was slightly reduced compared to the viability at 15 °C, whereas at 40 °C a complete and immediate (T0) loss of viability was observed. In general, increasing the exposure time slightly decreased the viability. After 20 min of exposure to temperatures >15 °C, the initial number of CFU mL^{-1} of both BCAs decreased and tended to further diminish with exposure time. The viability of *B. amyloliquefaciens* QST 713 was significantly affected by liquid temperature ($P < 0.001$), exposure time ($P < 0.001$) and their interaction ($P < 0.001$) (Table 1). Thus, the effect of exposure time depended on the liquid temperature. Indeed, after 60 min of exposure to temperatures of 35 and 40 °C a considerable decrease in number of CFUs was observed (from 10^8 to 10^4 CFU mL^{-1} at 35 °C and from 10^7 to 10^2 CFU mL^{-1} at 40 °C), whereas no such drop was found at the other liquid temperatures. At 15, 20, 25 and 30 °C the viability tended to decrease slightly in relation to exposure time. At all exposure times, the number of CFUs was considerably lower at 40 °C compared to the other temperatures. The concentrations tended to decrease with increasing temperature, except at 35 °C. These results suggest a mixture temperature threshold for BCA viability at 35 °C. Higher temperatures resulted in a considerable or complete loss of BCA viability, especially when associated with the long exposure time (60 min). The technical efficacy of BCAs depends on several factors, such as the biology of the BCA, the pathogen, the environment and the interactions with other microorganisms.[37–39] The BCA must remain viable through the operation chain from production to application. The effect on viability of temperatures during storage was studied for *Trichoderma viride*, which lost viability after 3 months storage at 25 °C,[40] but to date no information is available in the literature on the effect of temperature and exposure time on BCA viability during their field application through airblast sprayers. Below 35 °C, irrespective of exposure time, the CFU counts of both BCAs were not substantially different even if factors resulted statistically significant. Indeed, in this last case all the CFU mL^{-1} value variations were ≈1.5 log$_{10}$ CFU mL^{-1} thus without potentially compromising the biocontrol efficacy of BCAs as demonstrated by other authors.[41–43]

3.2 Static airblast sprayer trials

3.2.1 Performance of the sprayer tank agitation systems

For both sprayers, the deviation of the initial concentration of copper oxychloride in the sprayer tanks at different sampling levels and sprayed amounts of liquid, as a measure for performance of the agitation system, is shown in Fig. 3. According to Badules et al.[44] who investigated an alternative CFD-based method for the evaluation of sprayer tank agitation systems, the ISO 5682-2[26] is the most reliable method to date. For the Dragone Virgola sprayer the deviation ranged from −64% at the top of the sprayer tank to −18% at the bottom of the sprayer tank, and from −21% for a full spray tank (0 L sprayed) to −3% for the empty spray tank (300 L sprayed). With the Caffini Smart Synthesis sprayer, the concentrations deviated −9% from the initial concentration at the top of the sprayer tank to −7% at the bottom, and −13% with the full spray tank (0 L sprayed) to −11%


Figure 2. Viability of biological control agent (BCA) (log$_{10}$ CFU mL^{-1}) for *Trichoderma harzianum* T-22 and *Bacillus amyloliquefaciens* QST 713 at different liquid temperatures (C°) and exposure times (T0 = 0, T1 = 10, T2 = 20, T3 = 30, and T4 = 60 min) (mean ± standard error of mean).

Table 1. Linear models describing the factors associated with the BCA viability ($\log_{10}$ CFU mL^{-1}) for *T. harzianum* T-22 and *B. amyloliquefaciens* QST 713 as measured during the laboratory trials

Model term	*T. harzianum* T-22				*B. amyloliquefaciens* QST 713			
	d.f.1	*F*-ratio	*P*-value	Significance[a]	d.f. 1	*F*-ratio	*P*-value	Significance[a]
Main effects								
BCA mixture temperature (T)	4	16.403	<0.0001	***	4	14.216	<0.0001	***
Exposure time (E)	5	6297.30	<0.0001	***	5	20.011	<0.0001	***
Interactions								
T × E	20	1.711	0.0568	NS	20	3.727	<0.0001	***

Abbreviation: BCA, biological control agent.
[a] Statistical significance level: NS, $P > 0.05$; *,$P < 0.05$; **,$P < 0.01$; ***,$P < 0.001$.
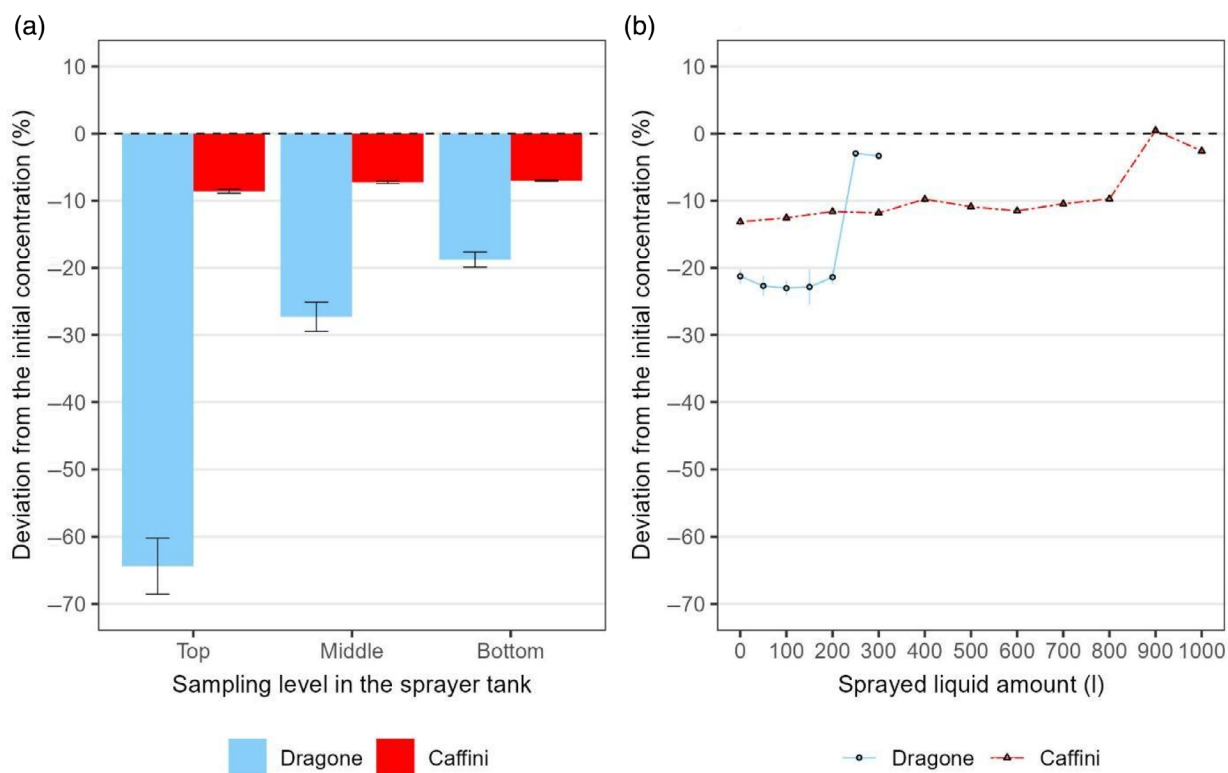
Figure 3. Deviation (%) from the initial concentration of copper oxychloride (dashed black line) for (a) the three sampling levels in the sprayer tank (top = 90%, middle = 50%, and bottom = 10% of nominal tank capacity) and for (b) the sprayed amount of liquid (L) during emptying of the tank for Dragone Virgola sprayer and Caffini Smart Synthesis sprayer (mean ± standard error of mean).

for the empty spray tank (1000 L sprayed). Not surprisingly, the Dragone Virgola sprayer, which relies solely on a by-pass line for agitation, resulted in poorer agitation when compared to the Caffini Smart Synthesis sprayer. In addition, the Dragone Virgola sprayer did not meet the criterion for adequate agitation set by the ISO 16119-3 standard – that is, maximum 15% deviation from the initial concentration of copper oxychloride after 16 h of sedimentation in the sprayer tank with the agitation system turned off.[28] The Caffini Smart Synthesis sprayer fulfilled this requirement. These results illustrate the capability of the tank agitation system installed in the Caffini Smart Synthesis sprayer to create enough turbulence in the tank to maintain a homogeneous spray mixture. A homogeneous spray mixture throughout the spray application helps avoid under- and over-dosage which

is essential for an efficient and efficacious spray application. Indeed, the proper number of venturi nozzles, their position and orientation, together with the liquid circuit pressure were demonstrated to play a key role in the proper agitation of the spray mixture in the tank.[45,46] However, the Dragone Virgola sprayer without a dedicated tank agitation system was not able to guarantee a homogeneous and constant copper oxychloride dosage throughout the trials. It must be mentioned that the chemical and physical properties of PPP formulations strongly affect the performance of the agitation system. Hence, copper oxychloride has been used for a long time as a standard test material as, owing to its chemical and physical properties, it is challenging to keep it suspended in the tank and to re-suspend when sedimented. Therefore, it allows discrimination between

tank agitation systems capable or not at effectively homogenizing the spray mixture in the tank. The Dragone Virgola sprayer was not able to keep the copper oxychloride in suspension and furthermore showed a large amount of sedimentation on the bottom of the tank at the end of the trials. In case of the viability trials with the BCA formulations a visual assessment of the bottom of the tank was done at the end of the trials and in all cases no BCA sedimentations were noticed. This suggests that the tested BCA formulations were easier to maintain suspended than the copper oxychloride and a good final BCA agitation can be hypothesized. This hypothesis was confirmed by the laboratory analysis that did not show anomalous CFU data. The adverse effect of a well-functioning tank spray agitation system is that it exposes the BCAs to more mechanical stress potentially affecting their viability. To this extent, the high-tech sprayer Caffini Smart Synthesis could be more harmful compared to the basic Dragone Virgola sprayer.

3.2.2 Combined effect of mechanical and thermal sprayer stress on BCA viability

The effect of workshop air temperature and relative humidity on the spray mixture temperature variability among trials can be considered negligible as environmental conditions throughout the experiments were quite steady ranging from 17.4 to 20.1 °C and from 40.8% to 52.2%.
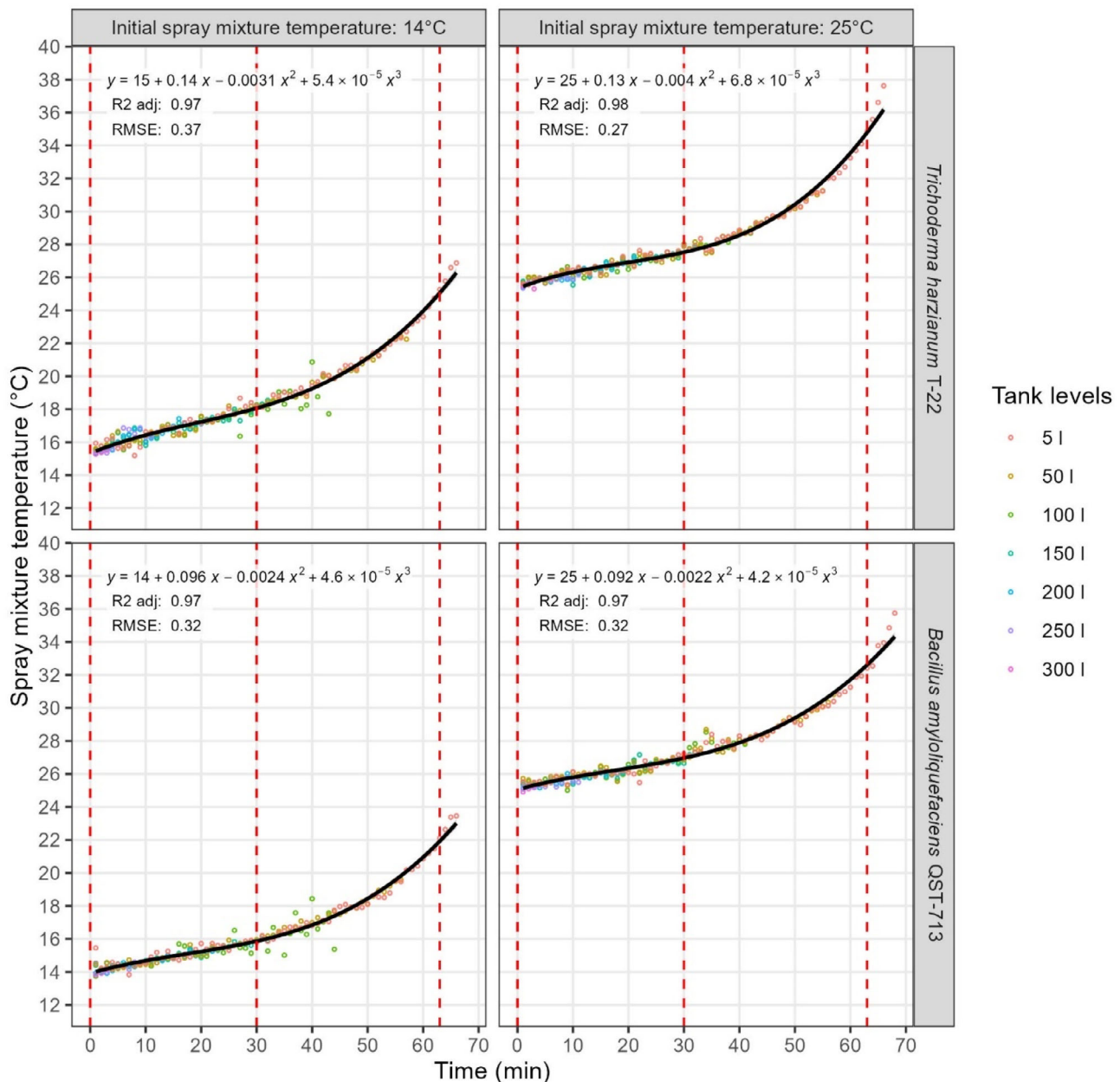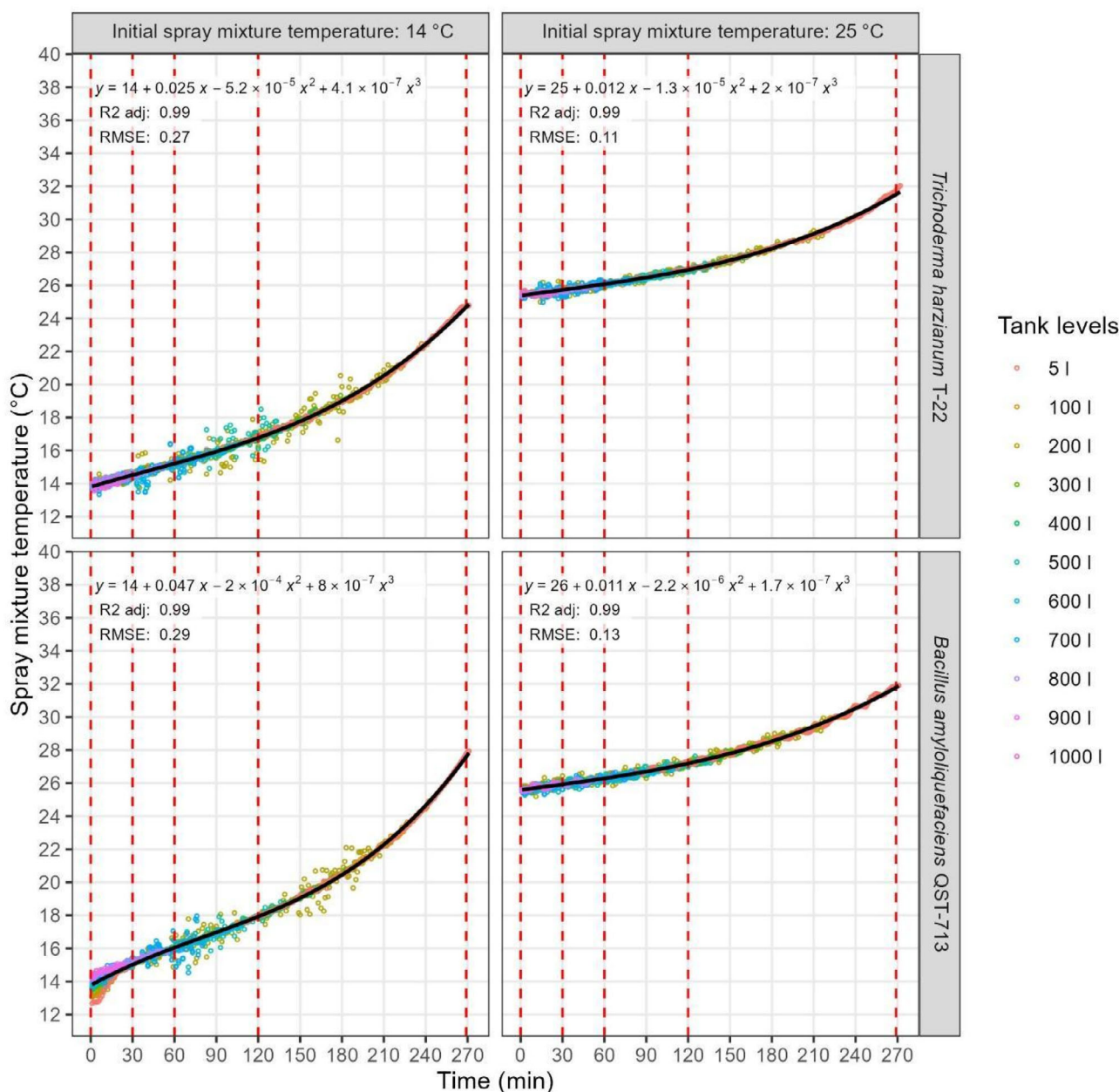

Figure 4. Third-degree polynomial relationships (dark solid line) between the spray mixture temperature (°C) and duration of the trials (min) for the Dragone Virgola sprayer, per biological control agent (BCA) [*Trichoderma harzianum* T-22 (top) and *Bacillus amyloliquefaciens* QST 713 (bottom)] and initial spray mixture temperature [14 °C (left) and 25 °C (right)]. Red dashed lines indicate the spray mixture sampling times. Colored dots indicate the tank levels of the samples.

3.2.2.1. Monitoring of spray mixture temperature throughout the trials. The temperature of the spray mixture inside the tank increased over the course of the viability trials, as shown in Fig. 4 for the Dragone Virgola sprayer and in Fig. 5 for the Caffini Smart Synthesis sprayer. After 63 min of spraying, the initial temperature of 14 °C in the Dragone Virgola sprayer tank rose to 26.1 and 23.7 °C during the trials with *T. harzianum* T-22 and with *B. amyloliquefaciens* QST 713, respectively. At an initial water temperature of 25 °C, the spray liquid temperature reached 37.8 °C during the experiments with *T. harzianum* T-22 and 35.8 °C with *B. amyloliquefaciens* QST 713.

During the trials with the Caffini Smart Synthesis sprayer, the recorded maximum temperatures were 24.7 and 27.9 °C starting from an initial water temperature of 14, 32.1, and 31.9 °C at 25 °C with the *T. harzianum* T-22 and *B. amyloliquefaciens* QST 713, respectively. Therefore, at an initial water temperature of 25 °C, the temperature of the spray liquid inside the tank almost reached the critical point that was shown to affect the viability of both microorganisms under laboratory conditions (i.e. *c.* 35 °C). The increase in temperature (difference between initial and final temperature) was similar to that found by Fife et al.[47] using a similar diaphragm pump as in this study.


Figure 5. Third-degree polynomial relationships (dark solid line) between the spray mixture temperature (°C) and duration of the trials (min) for the Caffini Smart Synthesis sprayer, per biological control agent (BCA) [*Trichoderma harzianum* T-22 (top) and *Bacillus amyloliquefaciens* QST 713 (bottom)] and initial spray mixture temperature [14 °C (left) and 25 °C (right)]. Red dashed lines indicate the spray mixture sampling times. Colored dots indicate the tank levels of the samples.

For both initial water temperatures, the Caffini Smart Synthesis sprayer showed a more gradual temperature increase than the Dragone Virgola sprayer (Figs 4 and 5) as a consequence of the bigger amount of water in the tank. The larger the amount of water, the greater the energy required to heat it (water specific heat capacity is 4187 J kg^{-1} K^{-1}). Temperature increases are most likely caused by the extensive recirculation of the tank mixture through the sprayer hydraulic circuits. Because the sprayer hydraulic circuit is a closed circuit, friction in the pipes and the pump causes the temperature of the water inside the sprayer to rise. The energy to heat the spray mixture was mainly transferred by the pumps to the spray mixture by recirculating action throughout the duration of the trials (63 and 269 min for the Dragone Virgola and Caffini Smart Synthesis sprayers, respectively). According to Fife et al.,[47] different type of pumps (i.e. centrifugal, diaphragm and roller pumps) exert different effects on spray mixture temperature. In all cases (i.e. combination of sprayer, BCA formulation and initial spray mixture temperature) the increase of the spray liquid temperature in the tank was successfully described by cubic functions showing adjusted $r^2 > 0.97$ (Figs 4 and 5). Concurrently, the root mean square errors (RMSE) were calculated and in the worst case they were equal to 0.37, underlining the high capability of the model (third-degree polynomial) to accurately predict the spray mixture temperature data (Figs 4 and 5).

The relationships between the ratio $\Delta T/\Delta t$ (°C min^{-1}) and the residual amount of spray mixture in the tank (l) are shown for *T. harzianum* T-22 in Fig. 6(a) and for *B. amyloliquefaciens* QST 713 in Fig. 6(b). In general, the rate of temperature increase of the spray mixture in the tank depended on the sprayer type and especially on the residual amount of spray mixture in the tank (Fig. 6). The temperature increase rate was higher for the Dragone Virgola sprayer, equipped with the tank of lower capacity, than that for the Caffini Smart Synthesis sprayer. Even if the two tested sprayers were equipped with different pumps, the results show that the residual amount of spray mixture predominantly determines the spray mixture temperature increase rate. Concurrently, for both BCAs tested, the tank capacity does not substantially affect the final temperature achieved by the spray mixture at the end of the trials when the initial temperature was either 14 or 25 °C (3.2 and 0.2 °C difference between sprayers in the worst and best case, respectively) (Figs 4 and 5). This indicates that sprayers with a bigger tank capacity can be exposed to a certain temperature level for a longer time because the rate of increase is smooth (e.g. the spray mixture temperature stayed in the range 30–32 °C for nearly 10 and 30 min when the Dragone and Caffini sprayers, respectively, were tested). Additionally, the rate of temperature increase is slightly lower when the cold water (14 °C initial temperature) was used to prepare the spray mixture. Under these conditions also the curves did not substantially differ for the BCAs (Fig. 6).

3.2.2.2.   Evaluation of CFUs of tested BCAs. The results of the preliminary data analysis—which aimed to compare the CFU values (log$_{10}$ CFU mL^{-1}) obtained from the spray mixture sampled directly from the tank and from the nozzle outlet—is displayed in Fig. 7. Significant differences in CFU were observed between samples taken from the tank and at the nozzle outlets for BCA
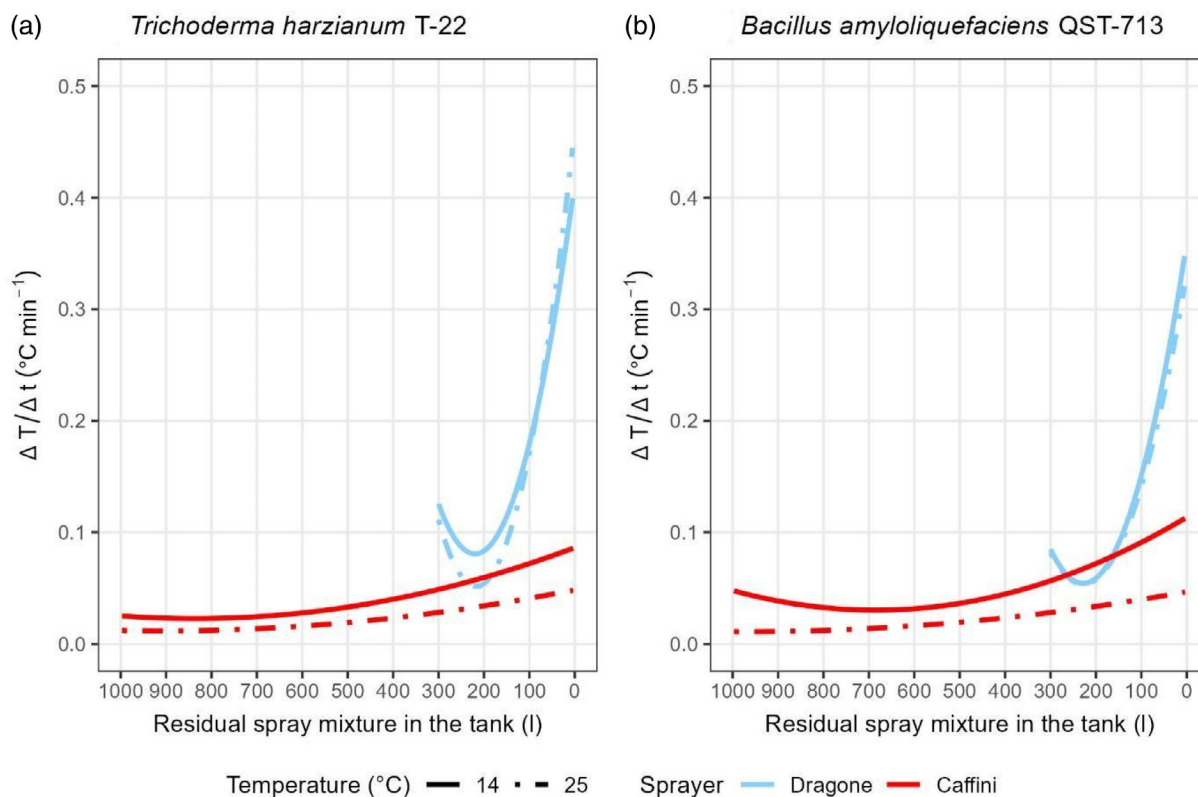
Figure 6. Relationship between the ratio $\Delta T/\Delta t$ (°C min^{-1}) and the residual amount of spray mixture in the tank (l) for (a) *Trichoderma harzianum* T-22 and (b) *Bacillus amyloliquefaciens* QST 713. Curves are displayed for the combination of initial spray mixture temperature (14 °C = solid lines, 25 °C = dotdash lines) and sprayer (Dragone Virgola sprayer = light blue, Caffini Smart Synthesis sprayer = red).
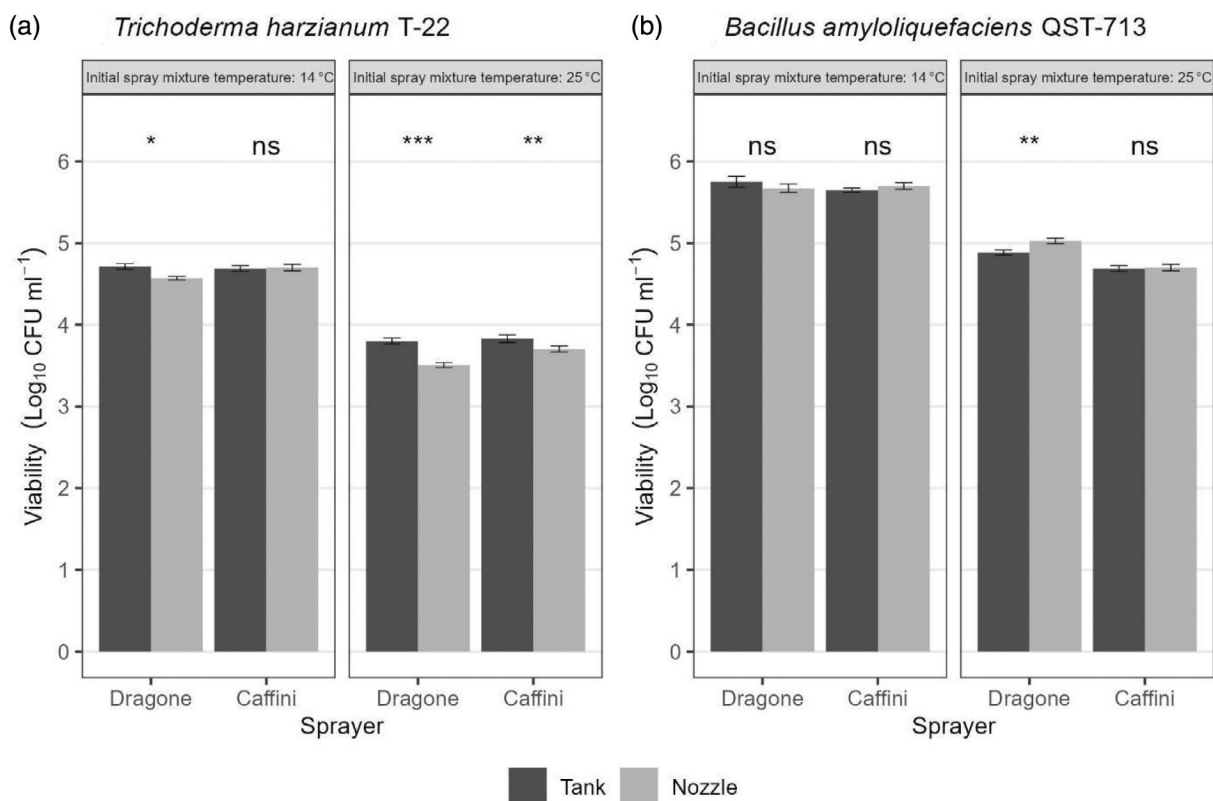
Figure 7. Average biological control agent (BCA) viability ($\log_{10}$ CFU mL^{-1}) obtained from samples taken in the tank and at the nozzle outlet of Dragone Virgola and Caffini Smart Synthesis sprayers for *Trichoderma harzianum* T-22 and for *Bacillus amyloliquefaciens* QST 713 during the trials carried out using different initial spray mixture temperature (14and 25 °C). The differences are defined by Student's *t*-test or Wilcoxon test for independent sample (statistical significance level: NS, $P > 0.05$; *,$P < 0.05$; **,$P < 0.01$; ***,$P < 0.001$) (mean ± standard error of mean).

formulations containing *T. harzianum* T-22 when trials were carried out with an initial spray mixture temperature of 25 °C, for both sprayers, and when an initial spray mixture temperature of 14 °C was adopted in combination with the Dragone Virgola sprayer [Fig. 7(a)]. Concerning the BCA formulation containing *B. amyloliquefaciens* QST 713, significantly higher CFUs were detected at nozzles outlet testing the Dragone Virgola sprayer in combination with initial spray mixture temperature 25 °C [Fig. 7 (b)]. In general, data in Fig. 7 show no or very limited differences between the two sampling positions for the CFU (<2.0 and 0.5% in the worst cases, corresponding to a Δ CFU equal to 0.28 and 0.11 $\log_{10}$ CFU mL^{-1} for *T. harzianum* T-22 and *B. amyloliquefaciens* QST 713, respectively). This is expected to have very few practical implications from the biological point of view.[16,48] Based on this preliminary data analysis, the CFU values measured in the tank and at the nozzle outlet can be considered fully comparable. Therefore, these values are reported as a unique sample unit in the following results showing the effect of the tested parameters on the BCA viability. Results are in line with those of Fife et al.[17] where entomopathogenic nematode (EPN) viability reduction was observed only when nozzles characterized by ISO 01 orifice size were tested. For bigger sized nozzles, like those used in these experimental trials, no EPN viability reduction was observed.

Per sprayer type and initial water temperature, the concentration of CFUs of *T. harzianum* T-22 and *B. amyloliquefaciens* QST 713 is presented as a function of measured spray mixture temperature in Figs 8 and 9, respectively. The viability of *T. harzianum* T-22 was significantly affected by the three-way interaction between

spray mixture temperature, initial water temperature and sprayer type (Table 2). In general, both sprayers showed similar concentrations at the start of the trials. For both sprayers, the concentration of CFUs, and thus the viability of the BCAs, was lower at 25 °C when compared to 14 °C initial water temperature. In addition, the concentrations decreased with increasing spray mixture temperature, except for the Dragone Virgola sprayer at 25 °C initial water temperature. The three-way interaction between spray mixture temperature, initial water temperature and sprayer type also significantly affected the viability of *B. amyloliquefaciens* QST 713 (Table 2). Similar to *T. harzianum* T-22, the concentration of CFUs was lower at an initial water temperature of 25 °C than at 14 °C for both sprayers. However, the initial concentration at 25 °C appeared to be lower for the Caffini Smart Synthesis sprayer than with the Dragone Virgola sprayer. With increasing spray mixture temperature, the concentration slightly reduced, except with the Dragone Virgola sprayer at 25 °C initial water temperature, which is the same as for *T. harzianum* T-22. When considering the optimal and critical thresholds for efficient biological control against fungal pathogens as defined by Montesinos and Bonaterra,[48] the concentration of *T. harzianum* T-22 never went below the optimal threshold of 4 $\log_{10}$ CFU mL^{-1} with both sprayers at an initial temperature of 14 °C (Fig. 8). Even at the initial temperature of 25 °C, the concentration stayed above the critical threshold of 3 $\log_{10}$ CFU mL^{-1} for fungus–fungus biocontrol systems. For what concerns *B. amyloliquefaciens* QST 713, at an initial temperature of 14 °C, the CFU concentration also stayed in between the optimal value of 6 $\log_{10}$ CFU mL^{-1} and the critical value of 5 $\log_{10}$ CFU

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mL^{-1} for bacteria–fungus biocontrol systems according to the findings of Tut et al.[16] However, at the higher initial temperature of 25 °C, the concentration dropped below this critical value posing risk for the efficacy of the treatments (Fig. 9).

These trials permitted identification of some practical implications related to the sprayer features and their use on the viability of BCA during their spray application. In general, spray mixture temperature affects BCA viability. Good practice should therefore be to use cold water from the public water supply pipeline when mixing BCAs to guarantee their viability along the entire spray application process irrespective of the sprayer used. Indeed, when warm water (25 °C initial temperature) was used, suboptimal and/or critical CFU values were noticed in all cases from the beginning of the trials, although the laboratory trials did not show 25 °C as being detrimental for the tested BCAs (Fig. 2). Comparison with the reference thresholds for different microorganisms[16,48] suggests that the efficacy of the treatment cannot be guaranteed as the BCAs can exit the sprayer nozzles with compromised viability. This is of particular interest considering that, according to Johnson et al.[49] the water temperature in the storage tanks can reach temperatures that correspond to air temperatures. In Mediterranean areas, during summer period, water temperature used for preparing spray mixture can potentially reach peaks of ≥35 °C. The temperature of water used to prepare the spray mixture was indeed demonstrated to vary
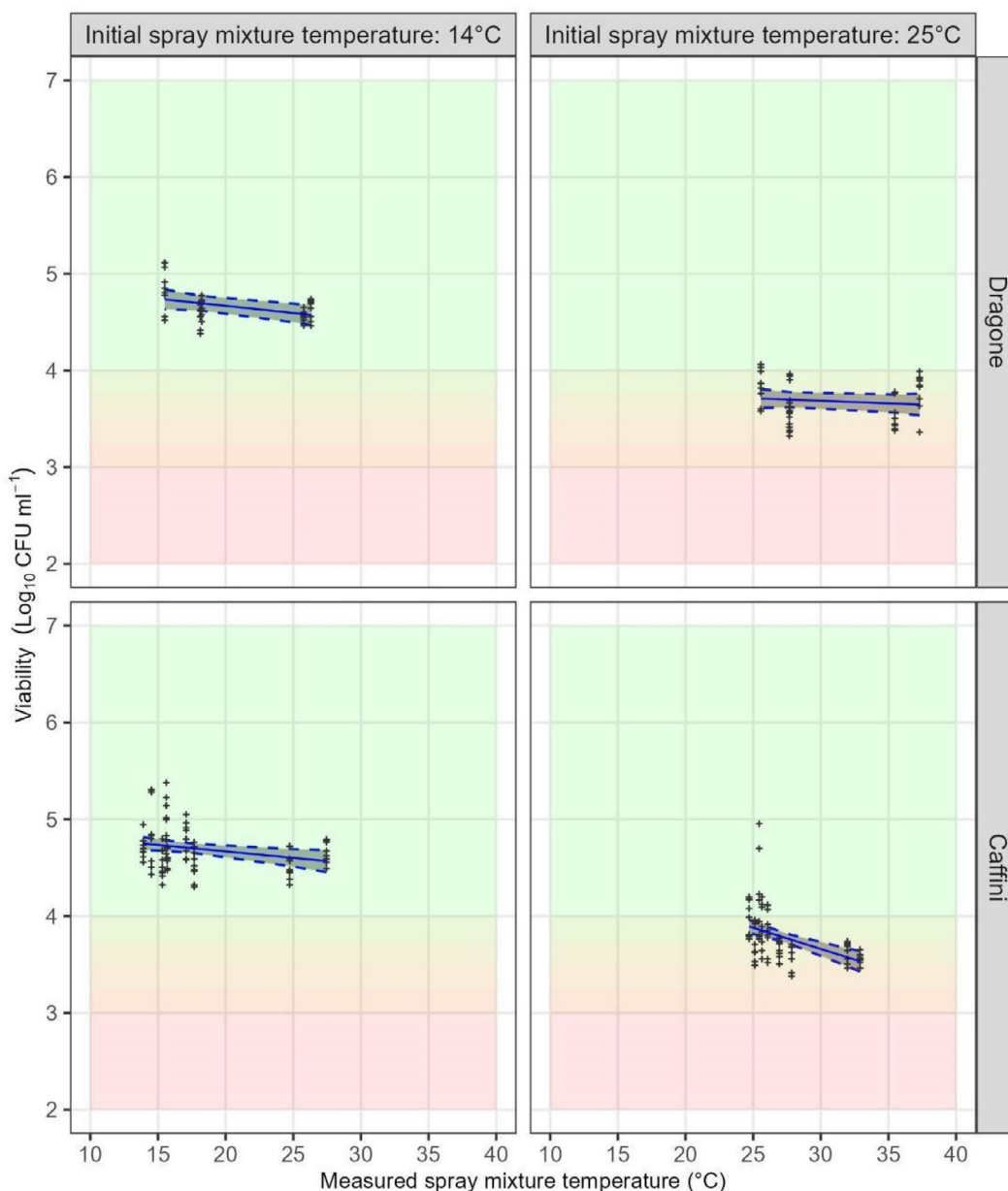
Figure 8. Viability of *Trichoderma harzianum* T-22 (log$_{10}$ CFU mL^{-1}) at different spray mixture temperatures throughout the duration of the trials for the two sprayers [Dragone Virgola sprayer (top) and Caffini Smart Synthesis (bottom)] and two initial spray mixture temperatures [14 °C (left) and 25 °C (right)]. Solid blue lines show the mean viability predicted by the model, dashed blue lines display the 95% confidence intervals of the predictions. Background color changes from green (optimal) to red (critical) represent the colony forming units (CFU) thresholds considered for *T. harzianum* T-22 efficacy according to Montesinos and Bonaterra.[48]
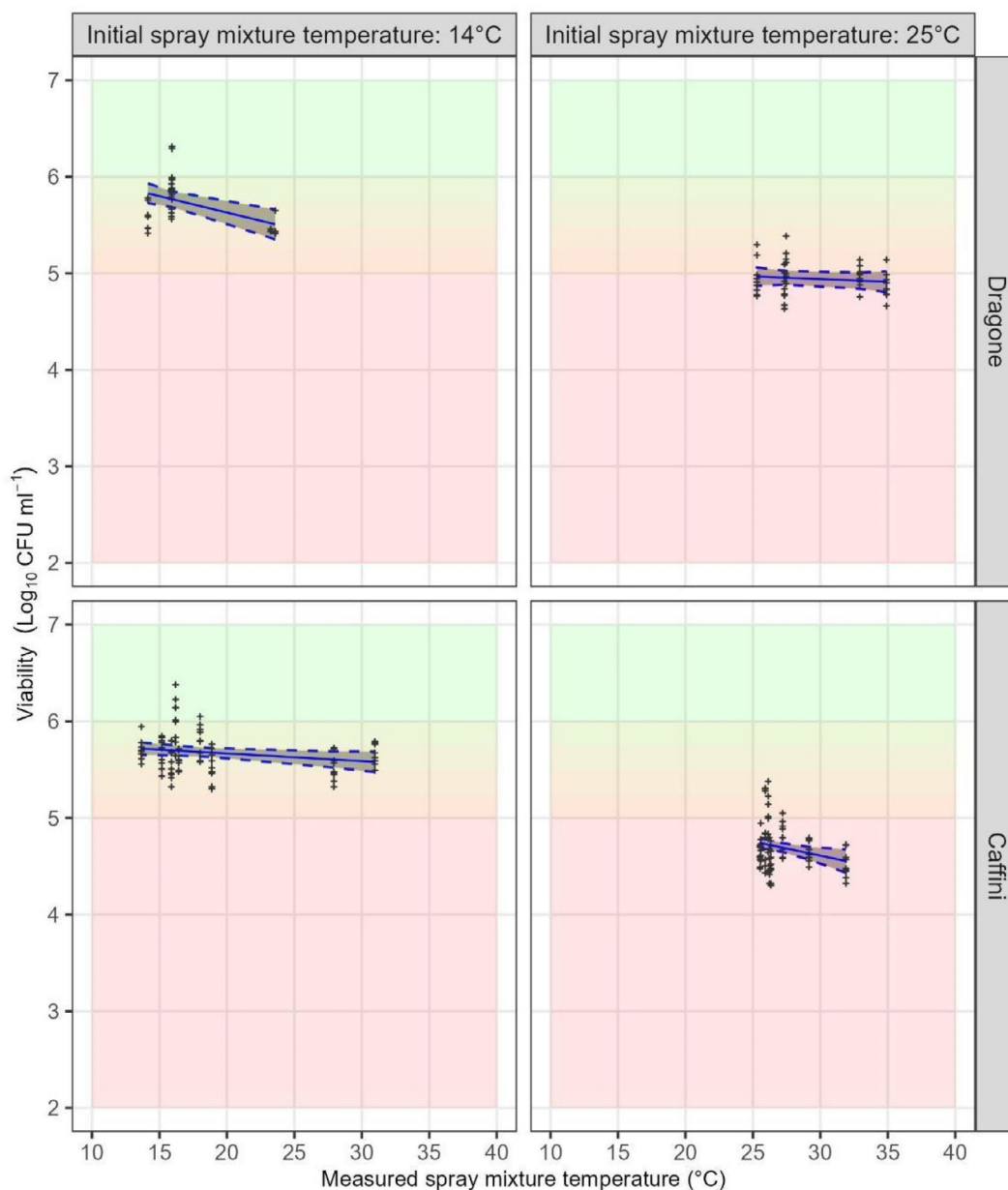
Figure 9. Viability of *Bacillus amyloliquefaciens* QST 713 ($\log_{10}$ CFU mL^{-1}) at different spray mixture temperatures throughout the duration of the trials for the two sprayers [Dragone Virgola sprayer (top) and Caffini Smart Synthesis (bottom)] and two initial spray mixture temperatures [14 °C (left) and 25 °C (right)]. Solid blue lines show the mean viability predicted by the model, dashed blue lines display the 95% confidence intervals of the predictions. Background color changes from green (optimal) to red (critical) represent the colony forming units (CFU) thresholds considered for *B. amyloliquefaciens* QST 713 efficacy according to Tut et al.[16]

widely according to the season, location and storage conditions, and this variability can affect the PPP chemical-based formulation performances.[49] Thus, an inhospitable environment for BCAs is likely to be created. In this latter scenario, the probability of spraying inactive BCAs is realistic. Also, the exposure time is relevant, especially when associated with high temperatures.

The effect of exposure time was first demonstrated by the laboratory trials (Fig. 2) and afterwards confirmed by the sprayer trials (Figs 8 and 9) where in all cases a CFU decreasing trend along trial duration was noticed. However, in general, the importance of exposure time was not as great as shown by the slight slope of the CFU linear models (Figs 8 and 9). In conclusion, based on

experimental data there was evidence about the mechanical effect of pump recirculation on spray mixture temperature increase and BCA viability even if the CFU critical biological viability threshold was never reached. Furthermore, no actual effect on the BCA viability was demonstrated for both sprayers featured by different liquid circuits, even if the one installed on the Caffini Smart Synthesis is supposed to be more stressful for BCAs by creating more liquid recirculation (Figs 1 and 3). Therefore, it can be stated that the effect of mechanical sprayer stress on the tested BCA viability is less important than the effect of thermal stress. Results are in line with the findings of Fife et al.[17,47] for EPNs. Indeed, those authors demonstrated the detrimental mechanical

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Table 2. Linear models describing the factors associated with the BCA viability (log$_{10}$ CFU ml^{-1}) for *T. harzianum* T-22 and *B. amyloliquefaciens* QST 713 as measured during the sprayer trials

	T. harzianum T-22				*B. amyloliquefaciens* QST 713			
Model term	d.f.1	*F*-ratio	*P*-value	Significance[a]	d.f.1	*F*-ratio	*P*-value	Significance[a]
Main effects								
BCA spray mixture temperature (T)	1	27.919	< 0.0001	***	1	16.854	0.0001	***
BCA initial spray mixture temperature (IT)	1	320.071	< 0.0001	***	1	141.621	< 0.0001	***
Sprayer (S)	1	2.801	0.0995	NS	1	0.149	0.6999	NS
Interactions								
T × IT	1	2.072	0.1514	NS	1	0.132	0.7170	NS
T × S	1	6.502	0.0114	*	1	0.013	0.9085	NS
IT × S	1	2.303	0.1304	NS	1	8.918	0.0031	**
T × IT × S	1	7.470	0.0067	**	1	7.125	0.0081	**

Abbreviation: BCA, biological control agent.
[a] Statistical significance level: NS, *P* > 0.05; *,*P* < 0.05; **,*P* < 0.01; ***,*P* < 0.001.

effect of small-sized nozzles whereas pump did not substantially affect EPNs viability.[17,47] For this reason, the selection of a properly sized sprayer tank plays a key role for BCA viability during spray application as the tank capacity indirectly influences the increment of spray mixture temperature as residual tank volume is the primary factor influencing the temperature increase.

4 CONCLUSIONS

Experimental trials permitted identification of the effect of factors on the viability of tested BCAs (i.e. *T. harzianum* T-22 and *B. amyloliquefaciens* QST 713) through the CFU count method. Laboratory trials underlined the effect of both mixture temperature and exposure time on the viability of both BCAs, thus, individuating the critical thresholds. In general, temperatures <35 °C in combination with a maximum exposure time of 30 min, are recommended. A spray application was simulated using sprayers with a different liquid circuit and tank capacity (Dragone Virgola 300 L and Caffini Smart Synthesis 1000 L) while spray mixture temperature was monitored. The spray mixture temperature increased continuously during the trials reaching in the worst case (25 °C initial spray mixture temperature) values close to the individuated threshold, but the critical values were not reached. For this reason, the BCA viability during the spray simulation never dropped abruptly, and just a slight CFU decrement was observed in all cases. However, with an initial water temperature of 25 °C, lower CFU values were detected at all sampling times compared to an initial water temperature of 14 °C. Nevertheless, it cannot be excluded that in real field conditions (e.g. summer periods in Mediterranean areas) higher mixture temperatures can be reached as a result of solar radiation, leading to an important decrease in BCA viability or to full mortality. Furthermore, the analysis of the temperature increase rate along the trials underlined that the residual amount of spray mixture in the tank is the main factor affecting the spray mixture temperature (increase). Even if the experimental data show the combined effect of mechanical and thermal stress on the BCA viability, there was only clear evidence about the mechanical effect of pump recirculation on spray mixture temperature increase. In line with findings of other authors, the mechanical stress by itself appears to be less relevant than the thermal stress created by the consistent temperature increase of the spray mixture during the spray application.

For this reason, the selection of properly sized spray tanks plays a key role for BCA viability as the residual volume in the tank is the primary factor influencing temperature increase. A higher tank capacity can lead to a lower final temperature even if the lower temperature-increase rate can cause a longer time of exposure to critical temperatures especially toward the end of spray application when the residual spray mixture in the tank is limited. To control and/or modify the spray mixture temperature increase rate, sprayer liquid circuits need to be improved. Possible options consist of: (i) selecting adequate pump size (currently oversized in commercial sprayers) according to the sprayer tank capacity and the liquid circuit features to minimize the pump recirculation while guaranteeing the requested flow rate for the nozzles and for the spray agitation system; (ii) installing active systems able to cool the spray mixture, to maintain the ideal temperature according to the BCA characteristics; and (iii) exploiting the current possibility of machinery electrification. To this end, dedicated electrical pumps can be installed on the sprayer so that a first pump provides the nozzles with the exact amount of spray mixture while a second one would manage spray mixture agitation, thus limiting the spray mixture over-recirculation.

This research provides the basis for further investigation about the effect of spray application process on BCA viability and therefore about the actual chance to guarantee the biological efficacy of BCA treatments using sprayers with different features and technology levels. In future, other BCAs as well as a wide range of sprayer types and settings deserve to be investigated.

CONFLICT OF 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 STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1 Rodríguez JAC, González-Fernández E, Fernández-González M, Vázquez-Ruiz RA and Aira MJ, Fungal diseases in two north-West Spain vineyards: relationship with meteorological conditions and predictive aerobiological model. *Agronomy* **10**:219 (2020). https://doi.org/10.3390/agronomy10020219.

2 Jacometti MA, Wratten SD and Walter M, Review: alternatives to synthetic fungicides for *Botrytis cinerea* management in vineyards. *Aust J Grape Wine Res* **16**:154–172 (2010). https://doi.org/10.1111/j.1755-0238.2009.0067.x.

3 Mundy DC, Elmer P, Wood P and Agnew R, A review of cultural practices for botrytis bunch rot management in New Zealand vineyards. *Plan Theory* **11**:3004 (2022). https://doi.org/10.3390/plants11213004.

4 Barba B, Oliva J and Payá P, Influence of fungicide residues in wine quality, in *Fungicides*, ed. by Carisse O. InTech, London, UK, pp. 421–440 (2010) Available from: http://www.intechopen.com/books/fungicides/influence-of-fungicide-residues-in-wine-quality Accessed 15 Mar 2023.

5 Perria R, Ciofini A, Petrucci WA, D'arcangelo MEM, Valentini P, Storchi P et al., A study on the efficiency of sustainable wine grape vineyard management strategies. *Agronomy* **12**:392 (2022). https://doi.org/10.3390/agronomy12020392.

6 European COMMISSION, COMMISSION IMPLEMENTING REGULATION (EU) 2018/1981 of 13 December 2018, Renewing the approval of the active substances copper compounds, as candidates for substitution, in accordance with regulation (EC) No 1107/2009 of the European Parliament and of the council concerning the placing of plant protection products on the market, and amending the annex to Commission Implementing regulation (EU) No 540/2011. *Official Journal of the European Union* **317**:16–20 (2018).

7 Brusselman E, Beck B, Pollet S, Temmerman F, Spanoghe P, Moens M et al., Effect of the spray application technique on the deposition of entomopathogenic nematodes in vegetables. *Pest Manag Sci* **68**:444–453 (2012). https://doi.org/10.1002/ps.2290.

8 Beck B, Brusselman E, Nuyttens D, Moens M, Temmerman F, Pollet S et al., Improving the biocontrol potential of entomopathogenic nematodes against Mamestra brassicae: effect of spray application technique, adjuvants and an attractant. *Pest Manag Sci* **70**:103–112 (2014). https://doi.org/10.1002/ps.3533.

9 Pal KK and McSpadden Gardener B, Biological control of plant pathogens. *Plant Health Instructor* **2006**:1–25 (2006). https://doi.org/10.1094/PHI-A-2006-1117-02.

10 Köhl J, Kolnaar R and Ravensberg WJ, Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. *Front Plant Sci* **10**:845 (2019). https://doi.org/10.3389/fpls.2019.00845.

11 Ons L, Bylemans D, Thevissen K and Cammue BPA, Combining biocontrol agents with chemical fungicides for integrated plant fungal disease control. *Microorganisms* **8**:1930 (2020). https://doi.org/10.3390/microorganisms8121930.

12 Calvo H, Roudet J, Gracia AP, Venturini ME and Fermaud M, Comparison of efficacy and modes of action of two high-potential biocontrol *bacillus* strains and commercial biocontrol products against *Botrytis cinerea* in table grapes. *OENO One* **55**:228–243 (2021). https://doi.org/10.20870/oeno-one.2021.55.3.4688.

13 Dimopoulou A, Theologidis I, Varympopi A, Papafotis D, Mermigka G, Tzima A et al., Shifting perspectives of translational research in biobactericides: reviewing the bacillus amyloliquefaciens paradigm. *Biology* **10**:1202 (2021). https://doi.org/10.3390/biology10111202.

14 Fedele G, Brischetto C and Rossi V, Biocontrol of *Botrytis cinerea* on grape berries as influenced by temperature and humidity. *Front Plant Sci* **11**:1232 (2020). https://doi.org/10.3389/fpls.2020.01232.

15 Calvo-Garrido C, Vinas I, Usall J, Rodriguez-Romera M, Ramos MC and Teixido N, Survival of the biological control agent Candida sake CPA-1 on grapes under the influence of abiotic factors. *J Appl Microbiol* **117**:800–811 (2014). https://doi.org/10.1111/jam.12570.

16 Tut G, Magan N, Brain P and Xu X, Critical evaluation of two commercial biocontrol agents for their efficacy against *B. cinerea* under in vitro and in vivo conditions in relation to different abiotic factors. *Agronomy* **11**:1868 (2021). https://doi.org/10.3390/agronomy11091868.

17 Fife JP, Ozkan HE, Derksen RC, Grewal PS and Krause CR, Viability of a biological pest control agent through hydraulic nozzles. *Transact ASAE* **48**:45–54 (2005). https://doi.org/10.13031/2013.17939.

18 Brusselman E, Moens M, Steurbaut W and Nuyttens D, Evaluation of hydraulic, pneumatic and mechanical agitation for the spray application of *Steinernema carpocapsae* (Rhabditida: Steinernematidae). *Biocontrol Sci Technol* **20**:339–351 (2010). https://doi.org/10.1080/09583150903484144.

19 Beck B, Spanoghe P, Moens M, Brusselman E, Temmerman F, Pollet S et al., Improving the biocontrol potential of Steinernema feltiae against Delia radicum through dosage, application technique and timing. *Pest Manag Sci* **70**:841–851 (2014). https://doi.org/10.1002/ps.3628.

20 Wright DJ, Peters A, Schroer S and Fife JP, Application technology, in *Nematodes as Biocontrol Agents*, ed. by Grewal PS, Ehlers R-U and Shapiro-Ilan DI. CABI Publishing, Wallingford, UK, pp. 91–106 (2005).

21 Bardin M and Pugliese M, Biocontrol agents against diseases, in *Integrated pest and disease management in greenhouse crops*, ed. by Gullino ML, Albajes R, Nicot P and van Lenteren JC. Springer, Dordrecht, The Netherlands, pp. 385–407 (2019).

22 Dhingra OD and Sinclair JB, *Basic Plant Pathology Methods*, 4th edn. CRC Press, Inc, Boca Raton, Florida. U.S.A, p. 355 (1987).

23 Marucco P, Balsari P, Grella M, Pugliese M, Eberle D, Gil Moya E et al., OPTIMA EU project: main goal and first results of inventory of current spray practices in vineyards and orchards, in *Proceeding of SUPROFRUIT 2019-15th Workshop on Spray Application and Precision Technology in Fruit Growing*. NIAB EMR, East Malling, UK, pp. 99–100 (2019). https://doi.org/10.18174/494871

24 Grella M, Marucco P, Gioelli F, Balsari P, Athanasakos L, Mylonas N et al., Airblast sprayer electrification for real-time, continuous fan-airflow adjustment according to canopy density during pesticide application in 3D crops, in *VDI-Berichte: LAND.TECHNIK 2022-the Forum for Agricultural Engineering Innovations*, Vol. **2395**. VDI Wissensforum GmbH, Dusseldorf, D, pp. 389–395 (2022). https://doi.org/10.51202/9783181023952-389

25 Grella M, Gioelli F, Marucco P, Zwertvaegher I, Mozzanini E, Mylonas N et al., Field assessment of a pulse width modulation spray system applying different spray volumes: duty cycle and forward speed effects on vines spray coverage. *Precision Agric* **23**:219–252 (2022). https://doi.org/10.1007/s11119-021-09835-6.

26 International Standard Organization, ISO 5682-2:1997, Equipment for crop protection, in *Spraying equipment–part 2: test methods for hydraulic sprayers*. International Standards Organization, Geneva, Switzerland, pp. 1–9 (1997).

27 Grella M, Allochis D, Marucco P and Balsari P, Assessment of external sprayer cleaning efficiency by comparing different cleaning devices, sprayer tank materials and operators, in *Safety, health and welfare in agriculture and agro-food systems. SHWA 2020. Lecture notes in civil engineering*, Vol. **252**, ed. by Biocca M, Cavallo E, Cecchini M, Failla S and Romano E. Springer, Cham, pp. 127–136 (2022). https://doi.org/10.1007/978-3-030-98092-4_14

28 International Standard Organization, *ISO 16119-3:2013: agricultural and forestry machinery—environmental requirements for sprayers—Part 3: sprayers for bush and tree crops*. International Organization for Standardization, Geneva, Switzerland, pp. 1–12 (2013).

29 Grella M, Marucco P, Zwertvaegher I, Gioelli F, Bozzer C, Biglia A et al., The effect of fan setting, air-conveyor orientation and nozzle configuration on airblast sprayer efficiency: insights relevant to trellised vineyards. *Crop Prot* **155**:105921 (2022). https://doi.org/10.1016/j.cropro.2022.105921.

30 R Core Team, *R: a language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria (2021).

31 Length R, emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.7.4–1 (2022). Available from: https://CRAN.R-project.org/package=emmeans Accessed 3 Oct 2022.

32 Teixidó N, Usall J and Torres R, Insight into a successful development of biocontrol agents: production, formulation, packaging, and shelf life

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Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

15

as key aspects. *Horticolturae* **8**:305 (2022). https://doi.org/10.3390/horticulturae8040305.

33 Prasad RD, Rangeshwaran R, Anuroop CP and Phanikumar PR, Bioefficacy and shelf life of conidial and chlamydospore formulations of *Trichoderma harzianum* Rifai. *J Biol Control* **16**:145–148 (2002).

34 Mareeswaran J and Radhakrishnan B, Shelf life of bacterial and fungal biocontrol agents in different formulations. *Indian Phytopathol* **69**: 422–423 (2016).

35 Carbó A, Teixidó N, Usall J, Solsona C and Torres R, Shelf life improvement of the biocontrol agent *Candida sake* CPA-1 by suitable package and storage conditions. *BioControl* **64**:435–446 (2019). https://doi.org/10.1007/s10526-019-09944-2.

36 Gotor-Vila A, Usall J, Torres R, Solsona C and Teixidó N, Enhanced shelf-life of the formulated biocontrol agent *Bacillus amyloliquefaciens* CPA-8 combining diverse packaging strategies and storage conditions. *Int J Food Microbiol* **290**:205–213 (2019). https://doi.org/10.1016/j.ijfoodmicro.2018.10.013.

37 Schisler DA, Slininger PJ, Behle RW and Jackson MA, Formulation of bacillus spp. for biological control of plant diseases. *Phytopathology* **94**:1267–1271 (2004). https://doi.org/10.1094/PHYTO.2004.94.11.1267.

38 Legget M, Leland J, Kellar K and Epp B, Formulation of microbial biocontrol agents–an industrial perspective. *Can J Plant Pathol* **33**: 101–107 (2011). https://doi.org/10.1080/07060661.2011.563050.

39 Wei F, Hu X and Xu X, Dispersal of *Bacillus subtilis* and its effect on strawberry phyllosphere microbiota under open field and protection conditions. *Sci Rep* **6**:22611 (2016). https://doi.org/10.1038/srep22611.

40 Manandhar S, Baidya S, Manandhar C and Pant B, Viability study of *Trichoderma viride* in different formulations at different temperatures. *J Plant Protect Soc* **5**:148–154 (2018). https://doi.org/10.3126/jpps.v5i0.47125.

41 Elad Y and Kirshner B, Establishment of an active *Trichoderma* population in the phylloplane and its effect on grey mould (*Botrytis cinerea*). *Phytoparasitica* **20**:S137–S141 (1992). https://doi.org/10.1007/BF02980425.

42 Berger F, Li H, White D, Frazer R and Leifert C, Effect of pathogen inoculum, antagonist density, and plant species on biological control of Phytophthora and Pythium damping-off by *Bacillus subtilis* Cot1 in high-humidity fogging glasshouses. *Phytopathology* **86**:428–433 (1996). https://doi.org/10.1094/Phyto-86-428.

43 Singh A, Srivastava S and Singh HB, Effect of substrates on growth and shelf life of *Trichoderma harzianum* and its use in biocontrol of diseases. *Bioresour Technol* **98**:470–473 (2007). https://doi.org/10.1016/j.biortech.2006.01.002.

44 Badules J, Vidal M, Boné A, Gil E and García-Ramos FJ, Biphasic computational fluid dynamics modelling of the mixture in an agricultural sprayer tank. *Molecules* **25**:1870 (2020). https://doi.org/10.3390/molecules25081870.

45 Ucar T, Fox RD, Ozkan HE and Brazee RD, Simulation of jet agitation in sprayer tanks: comparison of predicted and measured water velocities. *Transact ASAE* **44**:223–230 (2001). https://doi.org/10.13031/2013.4678.

46 Tamagnone M, Balsari P, Bozzer C and Marucco P, Assessment of parameters needed to design agitation systems for sprayer tanks. *Aspects Appl Biol* **114**:167–174 (2012).

47 Fife JP, Ozkan HE, Derksen RC and Grewal PS, Effects of pumping on entomopathogenic nematodes and temperature increase within a spray system. *Appl Eng Agric* **23**:405–412 (2007). https://doi.org/10.13031/2013.23480.

48 Montesinos E and Bonaterra A, Dose-response models in biological control of plant pathogens: an empirical verification. *Phytopathology* **86**:464–472 (1995). https://doi.org/10.1094/Phyto-86-464.

49 Johnson B, Whitford F, Young BG, Devkota P and Legleiter T, Water temperature and herbicide performance: a first look at new research. *Purdue Extension* **112**:1–6 (2016).