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Influence of abiotic factors on kinetics of viable populations of biocontrol agents in the phyllosphere of lettuce and strawberry leaves

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Abstract

There is little information on the effect of temperature, relative humidity (RH) and vapour pressure deficit (VPD) on the viable populations of two commercial biocontrol strains, *Bacillus subtilis* QST 713 (recently classified as *B. velezensis*) and *Gliocladium catenulatum* J1446 (syn. *Clonostachys rosea*). The PMAxx™-qPCR molecular assay was used to quantify the viable BCA populations on fully extended lettuce and strawberry leaves under a range of temperature and relative humidity combinations. Overall, there was a small decline in the population size of viable cells for the two biocontrol organisms on strawberry and lettuce leaves within 10 days of application. However, for most experimental runs which contained general UK agronomy climates, such a decline was not statistically significant. Moreover, for a few runs, the viable populations increased significantly in optimal BCA growth temperatures with high RH. Only temperature (ambient) and dew point significantly affected the rate of temporal changes in the viable biocontrol population size. Thus, an increasing temperature led to decreased daily mortality. It should be noted

that much of the variability in the estimated daily mortality rates remains unaccounted for, thus unless under extreme conditions, the biocontrol efficacy in practice is less likely to be affected by the survival of biocontrol microbes but more likely to be affected by other factors that influence the density of biocontrol cells in the phyllosphere: especially dilution due to rapid host leaf expansion, spray coverage, and rain wash-off.

Keywords: Biological control; temperature; relative humidity; VPD; qPCR; PMA; phyllosphere; lettuce; strawberry; viable cells; *Bacillus subtilis* QST 713; *Gliocladium catenulatum*

1 INTRODUCTION

There is significant interest in the use of biocontrol agents in commercial systems to improve the sustainability of horticultural crop production systems and reduce the use of chemical inputs, especially of fungicides. Biocontrol products based on formulated *Bacillus subtilis* and *Gliocladium cantenulatum* are now used in commercial production of many horticultural crops (Keswani et al., 2016). The bacterial biocontrol organism *B. subtilis* synthesizes a variety of biologically active compounds with a broad range of activity against phytopathogens (Shafi *et al.*, 2017), and are capable of inducing host systemic resistance (Bais *et al.*, 2004; Butcher *et al.*, 2007; Aliye *et al.*, 2008). It has the potential of forming multicellular structures and biofilms (Branda *et al.*, 2001; Bais *et al.*, 2004) and can also compete for iron and nutrients through siderophore synthesis (de Boer *et al.*, 2003; Saha *et al.*, 2015). Biocontrol mechanisms of *G. catenulatum* J1446 involves secretion of cell wall degrading enzymes (Chatterton *et al.*, 2009), mycoparasitism (Mcquilken *et al.*, 2001), and competition for space and nutrients (Chatterton *et al.*, 2008; Chatterton & Punja, 2011; Helyer *et al.*, 2014).

Recent studies with these two commercial BCAs (*B. subtilis* QST 713 [recently re-classified as *B. velezensis* (Pandin *et al.*, 2018)] and *G. cantenulatum* [syn. *Clonostachys rosea*]) showed, using dose-response relationships, that a high population density of viable cells or propagules of the BCAs was required for effective control of the pathogen, *Botrytis cinerea*, and how their relative viability was affected by biotic and abiotic factors (Tut *et al.*, 2021b). Identifying the optimum population density required for biocontrol is an important step in optimization of BCA formulations and application to control economically important plant diseases, especially in the phyllosphere (Gotor-Vila *et al.*, 2017). The key bottleneck in foliar applications of BCAs is effective establishment of the sufficient viable inoculum load of the BCA for control of the target pathogen, especially under naturally fluctuating abiotic factors that affects their establishment in the phyllosphere (Magan, 2020). Indeed, the establishment of BCAs under field conditions has been a major hurdle for achieving high biocontrol efficacies because under fluctuating abiotic conditions there can be a rapid loss of viability of introduced inoculum populations of the BCA, resulting in poor pathogen control (Magan, 2006; Liu *et al.*, 2013; Sui *et al.*, 2015). There is a significant lack of data on these aspects to develop appropriate timings of the application of commercial available BCAs.

For a BCA to be reliable, consistent and effective, establishment in the phyllosphere is essential, and can only be successful if the inoculum has the required resilience over the range of climatic regimes where the pathogen infection is a significant problem (Andrews, 1992, Andrews and Hirano, 2012, Liu et al., 2013, Magan, 2020, Sui et al., 2015, Xu and Jeger, 2012). Previously, the inconsistent efficacy of BCAs has been attributed to the lack of establishment of the chosen BCA when applied to the phyllosphere of horticultural crops under naturally fluctuating abiotic conditions, over the ecological range conducive to pathogen infection (Elad, 1996; Cray *et al.*, 2013; Liu *et al.*, 2013; Sui *et al.*, 2015; Spadaro & Droby, 2016). Thus, resilience under changing abiotic

factors is critically important to produce ecologically competent BCAs that can become effectively established at the sufficient threshold levels to have an impact on the pathogen.

Information on the effect of temperature, relative humidity (RH) and vapour pressure deficit (VPD) changes on *B. subtilis* and *G. catenulatum* viable populations in the phyllosphere of horticultural crops is not available, although many studies have suggested that abiotic factors were predominantly responsible for the loss of biocontrol efficacy (Andrews, 1992, Andrews and Hirano, 2012, Liu et al., 2013, Magan, 2020, Sui et al., 2015, Xu and Jeger, 2012). The optimum inoculum potential range of these two BCAs for control of *B. cinerea* was identified recently (Tut *et al.*, 2021b). However, there is a need to understand the changes in viable BCA populations under abiotic conditions of temperature, RH and VPD relevant to UK lettuce and strawberry production.

Thus, the objectives of this study were to (a) examine the impact of temperature (10-34^{o}C), RH (65-95%) and VPD (0.3-1.6 kPa) on viable populations of *B. subtilis* QST 713 and *G. catenulatum* J1446 in the phyllosphere of lettuce and strawberry crops using the previously developed PMAxx™-qPCR molecular assay (Tut *et al.*, 2021a), and (b) develop a model from the data for predicting BCA fate in relation to climatic conditions.

2 MATERIALS AND METHODS

2.1 Summary of the procedures for studies with lettuce and strawberry leaves

Experiments under controlled conditions followed seven common steps: (1) propagation of the plants; (2) culture and preparation of BCAs for spray applications; (3) spraying of plants with one of the two BCAs in a glasshouse compartment to run-off; (4) moving plants into controlled environment (CE) cabinets 1 h after spraying to allow drying; (5) sampling two leaves from one treated plant initially prior to moving them to the CE cabinet and then on days 2, 4, 6, 8 and 10 after

spraying; (6) imaging each individual sampled leaf (Panasonic DMC-SZ3) and then immediately placing leaves into a falcon tube containing 10 ml of maximum recovery diluent (Sigma) sealed and shaken on a rotary shaker at 100 rpm for 30 mins at 10^{o}C; (7) surface washing, filtering through wet muslin cloth (four layers) and pelleting the cells by centrifugation at 2000 × g for 15 minutes at 4°C. The supernatant was decanted and the cell pellet supplemented with maximum recovery diluent solution and transferred into a 1.5 ml Eppendorf, and stored at 4°C; and (8) quantifying viable population sizes with the PMA-based qPCR method (Tut *et al.*, 2021a).

2.2 Culture and preparation of BCAs for spray applications

Serenade® ASO (Serenade, an aqueous formulation of *B. subtilis* strain QST 713) and Prestop (a dry formulation of *G. catenulatum* strain J1446) were purchased from Fargro Ltd (Arundel, West Sussex, UK). For both formulations, culture conditions, growth and formulation components were unknown due to industrial proprietary. Serenade was stored at room temperature, while Prestop was stored in a cool dry location at < 8°C and, once opened, frozen at −20°C. The batches used were less than 6 months old. Up to $\log_{10}$ 1.0 CFUs or conidia/mL were found to be non-viable in the formulations when counted in a haemocytometer and plated on agar media.

Since preliminary studies showed that the commercial formulation of Serenade interfered the PMA-based qPCR quantification of *B. subtilis* propagules, cell concentrate of *B. subtilis* QST 713 was sprayed onto the phyllosphere. The cell concentrate was prepared using the following steps. *B. subtilis* QST 713 was first isolated from the formulation and grown *in vitro* on culture media by serially diluting Serenade thrice into maximum recovery diluent (Sigma Aldrich, Gillinham, Dorset, UK), and then spread-plating 10 µL onto nutrient agar (NA, Oxoid Ltd) and incubating at 30 °C for three days. The *B. subtilis* QST 713 cultured colonies on nutrient agar were divided into four equal parts and transferred to a 1 L vacuum filter flask containing pre-autoclaved tryptone soya broth

(Sigma) and grown on a rotary shaker (110 rpm) at 20–25 °C for 10 days. The concentrate was stored at 20 °C. The commercial formulation Prestop was used for applying *G. catenulatum* J1446 to the phyllosphere; 5 g Prestop were mixed with 1 L water and then shaken vigorously for 15s. Prior to spray application the concentration of both BCAs was determined by plate counts on three replicate nutrient agar for *B. subtilis*, and malt extract agar in 9 cm Petri plates for *G. catenulatum.*

2.3 Plant propagation

Berryplants (Romney Marsh, England) provided strawberry tray plants of cv. Malling Centenary, and Premier Plants (West Drayton, England) provided lettuce seedling plants of cv. Carter in peat blocks. Plants were grown in pots (9 cm x 9 cm x 10 cm) using Miracle-Gro All Purpose Premium Compost (Evergreen Garden Care Ltd., Frimley, Surrey, UK) in a glasshouse compartment, and hand watered daily. Strawberry plants were at their early flowering stage, while lettuce plants were in early head development with about 4–6 bottom leaves just before experimentation.

For field trial sampling, a commercial lettuce planting of Laurence J Betts (West Malling, England) was used and irrigated from an overhead watering system when needed. An experimental strawberry plot at NIAB East Malling was used for field sampling of strawberry flowers; plants were planted in coir bags, each with 10 plants, inside a polythene tunnel, irrigated and fertilised by drippers. No pesticides were applied to the lettuce and strawberry plants during the experimental period.

2.4 Environmental treatments and experimental design for controlled studies

Twenty climatic (temperature and RH) conditions (Table 1) were selected to represent the range of UK agronomic climatic conditions for strawberry and lettuce production for studying their effects on the population dynamics of *B. subtilis* and *G. catenulatum*. The rationale in selecting these conditions were to (1) cover a wide range of UK growing climates, and (2) have several sets of

common VPD (kPa) values, allowing the examination of the effects of temperature under the same evaporative demand. Experiments were conducted in CE cabinets. For the same environmental conditions, both strawberry and lettuce plants were kept in the same CE cabinet and treated with the two biocontrol organisms (a single plant receiving only one of the two organisms). Lettuce and strawberry plants were separated into different blocks in a cabinet. All plant foliar parts received biocontrol sprays until run-off in a glasshouse compartment; there were five plants per combination of BCA and crop species for each replicate. Plants were sampled six times to estimate viable population sizes on leaves: D0 (ca. 1 h after spraying, taken before plants were moved to the cabinet), D2, D4, D6, D8 and D10. On each sampling occasion, two leaves were sampled from a single plant. Thus, a different plant was sampled at each time point. Only fully expanded leaves (at the time of spraying) were sampled to minimise host growth dilution effect. The experiments also contained a negative control (i.e., plants that did not receive any BCA sprays).

Each climatic (temperature and humidity combination) treatment was carried out at least twice. The exact temperature and RH conditions might differ slightly amongst repeats (Table 2) because of difficulties in maintaining the exact temperature/humidity over the entire study duration and were recorded via a temperature and RH dual logger placed inside the cabinet. Experiments were carried out in two climatic chambers (Panasonic model MLR-352, and Sanyo format 650). Prior to experimentation climate chambers were calibrated using external data loggers (EasyLog EL-USB-2 standalone USB temperature and RH, dew point data logger). The same data loggers were used for monitoring the temperature, RH and dew point in each chamber throughout the experimental periods. Climatic conditions were randomly selected for experimentation at a given time for specific CE cabinet. All climatic treatments had the same light:dark cycles (14 h light and 10 h dark): 3 h at

30 % light, 3 hrs at 50 % light, 4 hrs at 70 % light, 2 h at 100% light, followed by 2 h at 10 % light, and 10 h of dark.

2.5 Treatments and experimental design for commercial plantings

Similar experiments were carried out over time using plants in commercial plantings, except that strawberry flowers (instead of leaves) were sampled. A major route for both pre- and post-harvest fruit rotting due to *B. cinerea* is through infection of strawberry flowers by the pathogen, establishing latent infection and manifesting rot symptoms when fruits are ripening (Xu *et al.*, 2012). Thus protecting strawberry flowers from infection by *B. cinerea* is a key component of grey mould management in strawberry. On each sampling occasion, four older lettuce leaves were collected from two lettuce plants, and 10 strawberry flowers were collected (one from each strawberry plant). Three repeat experiments were carried out over time. A soil removal step was used which involved samples being spun at 100 x g for 1 min at 4^{o}C. This was followed by filtration using muslin cloth.

2.6 Leaf Imaging (Panasonic DMC-SZ3)

During the sampling period of BCA's from leaves an image was acquired next to standard square paper with a known area. Subsequently the pixel area of a single square at three different locations on the standard was measured with image J, following this the total area of leaves were measured with image J. In succession the following formula was utilised to calculate a given total area.

$$\text{Total Area} = \frac{\text{Total area of leafs in pixel}}{\text{Mean area of standard square in pixel}}$$

$$= \text{Number of standard squares in leaf area} \times \text{Area of a single sqaure}$$

After calculating the total area, the following formula was used to calculate CFU/mm^2

$$\mathrm{CFU/mm2} = \frac{\text{Total quantified viable population of BCA with PMAqPCR}}{\text{Total Area}}$$

2.7 Quantification of viable cells with PMA-qPCR

2.7.1 PMAxx™ treatment

This followed a recently published protocol (Tut *et al.*, 2021a). PMAxx™ was diluted in DEPC water to produce a 2.5 mM concentration and stored at −20°C; 25 µM PMAxx were used for *B. subtilis* and 50 µM for *G. catenulatum*, respectively. This was followed by an incubation stage in which BCA suspensions were encased in aluminium foil and placed into a lightproof sealable container, which was shaken on a rocker at 35 rpm for 15 min. In a cold room (4–8°C) a photo-activation system was assembled with a rocker fitted with an icebox that contained ca. 2 L ice, with the interior covered by two layers of aluminium foil (reflective side), and two light sources with a light output of 800 W fitted on the top at an angle of ca. 45°. After incubation, cell suspensions were transferred into the system at a distance of 20 cm from the light source to initiate photo-activation. Photo-activation consisted of 1 min light treatment followed by 2 min cooling for 30 cycles with constant agitation at 35 revs/min. After photo-induced cross-linking of PMAxx™ and exposed DNA in dead cells, BCA suspensions were centrifuged at 5000× *g* for 10 min at 4°C, and the supernatant discarded.

2.7.2 Grinding of *G. catenulatum* J1446 cells

Briefly, after PMA treatment, cells were pelleted by centrifugation at 5,000 × g for 10 minutes at 4°C. The supernatant was decanted and conidia suspended in maximum recovery diluent solution with a final volume of 1 ml. After slow pipetting for homogenisation, five stainless steel beads (6 mm) were transferred into each sample, and *G. catenulatum* cells were ground with the use of the

genome grinder 2000 set at 1750 rpm for 20 minutes. After samples were ground the steel beads were removed with a magnet and sterilized with 5 % bleach and 70 % ethanol.

2.7.3 DNA extraction and qPCR

DNA from pellets was extracted with TRI Reagent® (Sigma–Aldrich) following the manufacturer's protocol. Extracted DNA was filtered with Millex-VV syringe filter unit 0.1 µm (PVDF, 33 mm and gamma sterilized) and the purity and concentration determined on a NanoDrop spectrometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, Delaware, U.S.A.). The integrity of the DNA was determined by electrophoresis on a 1.5% agarose gel run at 60 V for 90 min within TAE buffer solution and stained with GelRed (Biotum, Hayward, California, U.S.A.). The DNA was quantified with the BIO-RAD CFX96™ real time PCR detection system (BIO-RAD). Reactions were prepared in a white/green semi-skirted 96 well qPCR plate (BIO-RAD) and sealed with an adhesive cover. The final volume in each reaction for both BCAs was 44 µL which contained 10 µL SensiFAST™ SYBR® No-ROX Kit (Bioline Meridian Bioscience, London, U.K.). The target eDNA concentration was 150ng for both BCAs. Each reaction well contained 400 ng/µL of bovine serum albumin (Schrader et al., 2012, Schriewer et al., 2011). *B. subtilis* reactions contained 434 nM of Bs_dnaK1154 forward primer (5'-ACACGACGATCCCAACAAGC-3'), and 434 nM of Bs_dnaK1254 reverse primer (5'-AGACATTGGGCGCTCACCT-3') (Hertwig et al., 2015). *G. catenulatum* reactions contained 344 nM of Gc1-1 forward primer (5'-CCGTCTCTTATCGAGCCAAGAT-3'), and 344 nM of Gc3-2a reverse primer (5'-GCCCATTCAAAGCGAGGCATTA-3') (Paavanen-Huhtala et al., 2000). The PCR conditions used for both BCAs were 95 °C for 3 min followed by 40 cycles of 45 s at 95°C, 60 s at 61°C and 60 s at 72°C. Each qPCR run included three replicates of controls without a template (NTC). The controls included 5 µL of DEPC water instead of DNA. CFX Manager™ Software version 3.1 (BIO-RAD) was used to analyse and calculate the Ct values automatically. All samples that reached fluorescence values

above the threshold were treated as a positive reaction, which was determined by the software.

Each PMA-qPCR run was followed by a melt curve analysis, and this generated melt curve profiles of the standard curves and the tested samples from the phylloplane, which were compared to each other to confirm the presence of the target biocontrol agent only. Where a melt-curve profile was absent the sample was not quantified.

2.7.4 Minimisation and suppression of qPCR inhibitors

For improving the dissolution of the extracted eDNA into 8 mM sodium hydroxide, five steel beads (6 mm) were transferred into each sample which contained a total volume of 1 ml and the DNA pellets were ground using the genome grinder 2000, set at 500 rpm for 1 minute followed by a cool down period of 2 minutes, the cycle was repeated fifteen times. After the grinding phase the DNA was pelleted at 1500 × g for 5 minutes at 4°C and the supernatant (clear DNA suspended in 8 mM sodium hydroxide) was diluted into 8 mM sodium hydroxide. The dilution depended on the clarity of the DNA sample. Filtration of the diluted DNA was completed with a Millex-VV Syringe Filter Unit 0.1 µm (PVDF, 33 mm and gamma sterilized), and the DNA purity and concentration was measured on a Nanodrop spectrometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE).

2.8 Statistical analyses

Leaf area was estimated from the image with imageJ (available from imagej.nih.gov/ij/download/), and then the estimated total number of viable population cells (CFUs) was expressed as the CFUs per mm^2 of leaf area, and log_{10} transformed before statistical analysis. As the exact temperature and RH values varied slightly between repeat experiments of the same environmental treatment, a linear model was fitted to the data set for each repeat separately to describe the relationship of

viable CFUs with time – the slope estimate from this linear model represents the temporal increase or decrease of viable CFUs (the observed data did not fit other non-linear relationships) Occasionally, the PMA-qPCR method failed for specific samples, and a viable population quantification Ct cut-off value of 35 was thus applied to all samples. In exceptional cases, where data sets had fewer than six data points only a linear regression was applied where at least four temporal points were available. Analysis of variance (ANOVA) was then carried out to assess whether the slope varied with plant species and biocontrol organisms; in the ANOVA, individual repeat experiments were used as a blocking factor since all BCA and plant species combinations were contained in each of the repeated experiments. Finally, regression modelling was used to assess whether the slope estimate was related to ambient temperature, RH, VPD or dew point in addition to the BCA and plant species. Statistical analysis was carried out in R version 3.4.1 (R Core Development Team, 2019).

3 RESULTS

3.1 Experiments in controlled environment cabinets

The dynamics of viable CFUs over time of all CE studies are shown in Figures 1 (*B. subtilis* on strawberry), 2 (*G. catenulatum* on strawberry), 3 (*B. subtilis* on lettuce), and 4 (*G. catenulatum* on lettuce). Several observations could be made from these data. Consideration of all the statistical analysis as a whole suggests that the viable CFUs of both BCAs in both crops was strongly influenced by temperature, RH and VPD. However, the relationship between viable CFU changes and the three factors in the phyllosphere was complex. The BCAs appeared to prefer specific climatic regimes (temperature and RH combinations). For both BCAs in both crops, as temperature was increased, the viable CFUs increased but this was restricted to theoretical sub-optimal and optimal plant

transpiration rates. Firstly, in most cases viable CFUs decreased with time. However, the viable CFUs remained relatively stable or in some cases increased in a few specific abiotic conditions. Secondly, there were considerable differences amongst repeat experiments and some showed the opposite trend (increase or decrease). Finally, the overall temporal dynamics of viable CFUs were similar between *B. subtilis* and *G. catenulatum*, and between strawberry and lettuce leaves. The difference between the initial viable CFUs of *B. subtilis* and *G. catenulatum* was expected due to formulation type. Also, as shown in Fig 1 and 3 for *B. subtilis* and Fig 2 and 4 for *G. catenulatum* adherence of initial viable BCA CFUs were impacted by the leaf type. In all conducted CE experiments the negative plant controls lacked a cell pellet, and thus PMA treatment and qPCR could not be performed.

In total, there were 165 experiments where there were at least four time points. The linear slope estimates (describing the rate of temporal increase or decrease in the viable CFUs per mm^2 leaf area) varied greatly amongst the individual studies (Figure 5). The average slope was -0.069 with a standard error of 0.008. The slope range appeared to be narrower for *G. catenulatum* than for *B. subtilis* (see Figure 5); it ranged from -0.549 to 0.192 for *B. subtilis* and from -0.241 to 0.098 for *G. catenulatum*. Table 3 provides the summary of slope estimates for each BCA and plant combination. In 28 out of the 165 cases, the slope was statistically significant ($P < 0.05$). Only for three of the 28 cases was the slope estimate positive: two for *G. catenulatum* on lettuce leaves (slope = 0.074 and 0.095) and the other for *B. subtilis* on strawberry leaves (slope = 0.189).

ANOVA of all the slope estimates showed that the overall slope estimate of the two biocontrol organisms did not differ significantly on either of the two plant species. The slope estimate differed between the temperature and humidity combinations ($P < 0.01$), but only accounted for ca. 20% of the total variability. There was no discernible pattern of relationships with VPD and RH, which was confirmed by statistical analysis. Regression analysis indicated that the slope was only significantly

294 (P < 0 05) affected by ambient temperature or dew point. However, only a small proportion of
295 variability in the slope estimates was accounted for by temperature (6.5%) or dew point (7.9%). The
296 linear effect of temperature or dew point on the slope did not differ between the two host species
297 for the same biocontrol organism, but appeared to differ between the two biocontrol organisms (P
298 in range of 0.04 and 0.07): *G. catenulatum* appeared to be less affected by temperature than *B.*
299 *subtilis* (Figure 6).
300

301 ## 3.2 Field samples

302 For *G. catenulatum* on lettuce leaves, one repeated experiment failed to generate data for more
303 than three sampling points. We suspect this was because of rainfall on day 2 (0.5-1mm), day 4 (7-
304 8mm), and day 6 (5-6mm). As found in the CE study, both biocontrol organisms appeared to show
305 an overall decline trend but with some variability amongst replicate experiments (Figure 7). In only
306 three of the seven field experiments was the linear relationship of temporal viable CFUs per mm^2
307 leaf area close to being statistical significant (P in the of range of 0.05 and 0.07): two for *G.*
308 *catenulatum* on strawberry flowers and *B. subtilis* on lettuce leaves.
309

310 # 4 DISCUSSION

311 This is the first study to investigate the impact of a wide range of temperature and RH combinations
312 on viable populations of these two BCAs in the phyllosphere of lettuce and strawberry crops. The
313 development of the PMAxx™-qPCR allowed the sensitive tracking and quantification of the viable
314 populations of these two BCAs in the phyllosphere of these two crops. Interestingly, under some
315 temperature x RH conditions there was an increase in viable populations. However, in many of the
316 treatment conditions there was a significant, albeit small, decrease in the viable biocontrol

317 populations over time. Overall, increasing ambient temperature or dew point led to a decreasing
318 mortality rate over time for the two biocontrol organisms. However, most of the variability in the
319 viable population sizes of the two organisms remains unexplained. Viable population sizes of *G.*
320 *catenulatum* appeared to be less affected by the treatment conditions than *B. subtilis.* The present
321 study specifically used (fully expanded) older leaves to avoid the dilution factor due to the natural
322 expansion of younger leaf areas that would result in a decrease in the actual spatial nature of the
323 viable populations.

324 The major hurdle in successful establishment of BCAs in the phyllosphere has indeed been
325 relative resilience of the BCAs and the formulations used under different interacting conditions of
326 temperature, microclimate RH and host status (Magan, 2020). Prestop, the formulated commercial
327 product of *G. catenulatum* was available as a dry formulation, and *B. subtilis* was freshly prepared
328 and remained in liquid form. Therefore, the shown differences in initial viable CFUs post spraying
329 and the observed dynamic differences between the BCAs may be related to formulation (Cumagun,
330 2014, Keswani et al., 2016, Melin et al., 2007). These will all influence the viable population
331 establishment for competing effectively with target pathogens. In the majority of UK commercial
332 growing climates represented by temperature and RH combinations, significant, albeit small,
333 differences in the introduced viable populations of both BCAs in lettuce and strawberry
334 phyllospheres were found. Of the abiotic factors studied, the viable population sizes of the two
335 biocontrol organisms varied primarily with the ambient temperature. Temperature has usually been
336 suggested to be the most important factor influencing the development of an organism. *B. subtilis*
337 cell growth becomes limited at < 11 °C (Price, 2000) and is optimal over the range 25-37°C (Cook,
338 1996). The fungal BCA, *G. catenulatum* is active at 5-34 °C, but optimal temperatures for growth are
339 between 15-25 °C (Helyer *et al.*, 2014). Overall, temperature appeared to be a major abiotic hurdle

for the establishment and development of the BCA population in both strawberry and lettuce phyllospheres. Previous studies with bacterial, yeast and filamentous fungal inoculants (Kessler *et al.*, 2003) all concluded that temperature significantly influenced the development and survival of their BCAS. However, none of these studies examined interactions between temperature and RH of the phyllosphere of their target crops. Present results suggested that under the most conditions experienced in the UK, increasing ambient temperature led to decreased mortality, assisting the initial establishment of biocontrol organisms in the phyllosphere. However, it should be noted that most variability in the viable population size of the two BCAs remained unaccounted for. Of course, the effect of extended periods in unfavourable temperatures, especially on foliar surfaces, may deviate from the general trend as shown in the present study. This has also been found with some other BCAs (Melent'ev *et al.*, 2000; Kessler *et al.*, 2003).

Reduced BCA mortality with increasing dew point suggests that increasing ambient humidity at a given ambient temperature is expected to result in improved establishment of biocontrol organisms in the phyllosphere. This is consistent with the general knowledge that often RH of >95% is required for microbial population establishment, especially in the phyllosphere of crops (Hallsworth & Magan, 1999; Magan, 2006, 2020). The present study is one of the first to have demonstrated the impact of RH interacting with temperature on the viable populations of a bacterial and fungal BCA. This study has shown that for effective phyllosphere establishment, RH is an important factor together with temperature. Thus, appropriate formulation of the BCA to maintain the required threshold concentrations under a wide range of temperature x RH conditions can considerably affect the initial establishment of biocontrol organisms in the phyllosphere of different target crops. This may need to include desiccation protectants and other adjuvants to improve BCA survival (Barbosa-Cánovas *et al.*, 2008).

In outdoor horticultural crop production systems, they are exposed to rainfall, and sometimes to overhead irrigations and/or dew, which was not considered in the present research. These events can lead to removal of fungicides or microbial organisms on the leaf surface (Wei et al., 2016, Xu et al., 2008). Thus, the actual level of viable biocontrol population size are expected to be lower under open-field conditions than predicted by the present research. The present findings may also explain why biocontrol has generally been more successful for post-harvest horticultural applications where no host-expansion or less wash-off is expected.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

Data availability statement

Data will be available upon request to Xiangming Xu (xiangming.xu@niab.com)

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FIGURE LEGENDS

FIGURE 1 Temporal dynamics of viable cells of *Bacillus subtilis* (log_{10} CFUs mm^2) on strawberry leaves exposed to a range of climatic conditions (see Tables 1 and 2). Points with different colours represent different repeated experiments and the lines are the fitted linear models.

FIGURE 2 Temporal dynamics of viable cells of *Gliocladium catenulatum* (log_{10} CFUs mm^2) on strawberry leaves exposed to a range of climatic conditions (see Tables 1 and 2). Points with different colours represents different repeated experiments and the lines are the fitted linear models.

FIGURE 3 Temporal dynamics of viable cells of *Bacillus subtilis* (log_{10} CFUs mm^2) on lettuce leaves exposed to a range of climatic conditions (see Tables 1 and 2). Points with different colours represent different repeated experiments and the lines the fitted linear models.

FIGURE 4 Temporal dynamics of viable cells of *Gliocladium catenulatum* (log_{10} CFUs mm^2) on lettuce leaves exposed to a range of climatic conditions (see Tables 1 and 2). Points with different colours represent different repeated experiments and the lines the fitted linear models.

FIGURE 5 Density plot of the slope estimates, describing the rate of temporal dynamics of viable CFUs, for each combination of BCA and plant species.

FIGURE 6 Plot of the slope estimates, describing the rate of temporal dynamics of viable CFUs, against (a) dew point and (b) ambient temperature for *G. catenulatum* and *B. subtilis* on strawberry and lettuce leaves exposed to a range of temperature and relative humidity combinations.

FIGURE 7 Temporal dynamics of viable cells of *Bacillus subtilis* and *Gliocaldium catenulatum* (Log_{10} CFUs per mm^2) on strawberry flowers (in a polyethylene tunnel) and lettuce leaves of commercial field plantings. Points with different colours represent different repeated experiments and the lines are the fitted linear models.

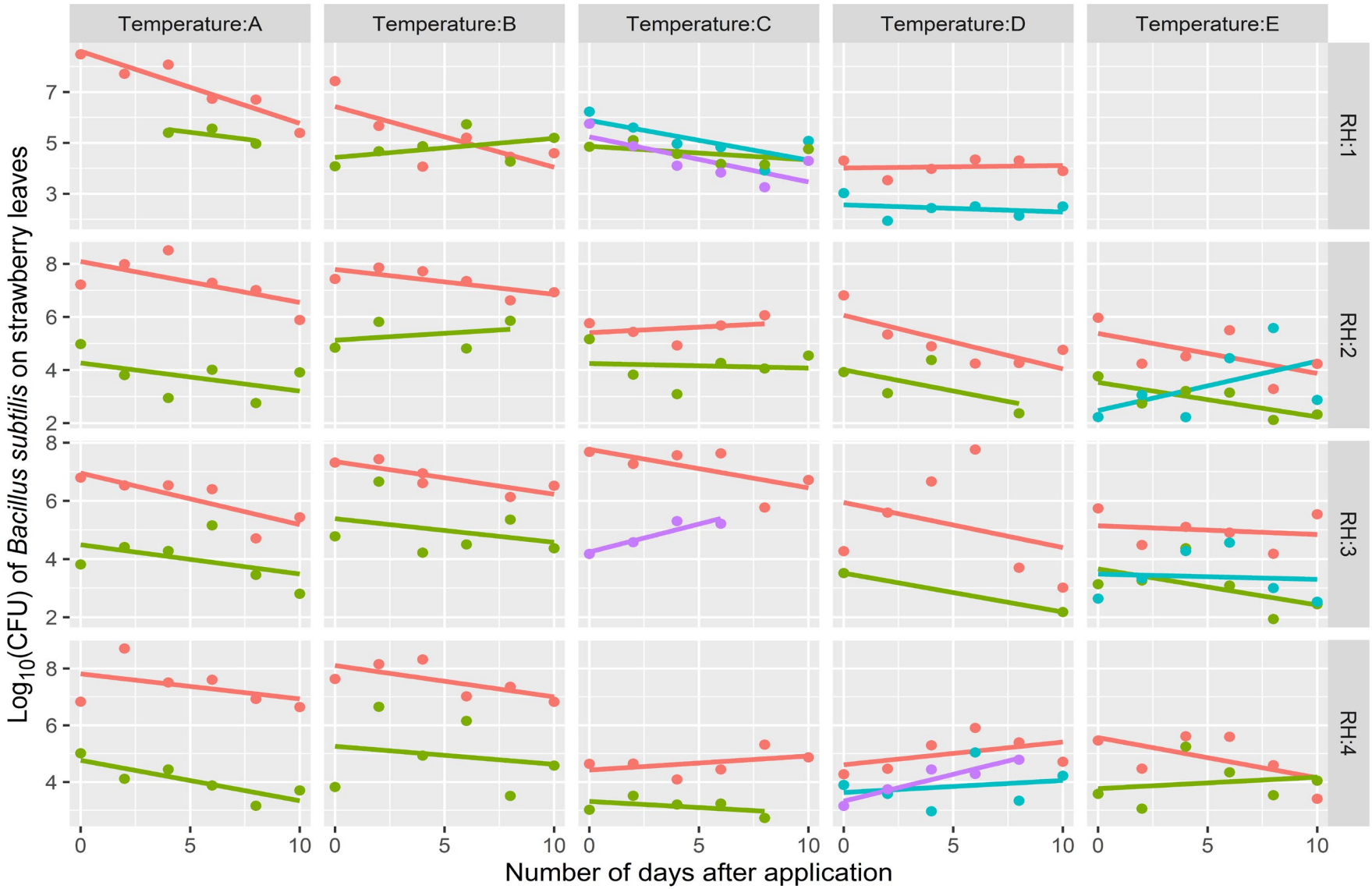
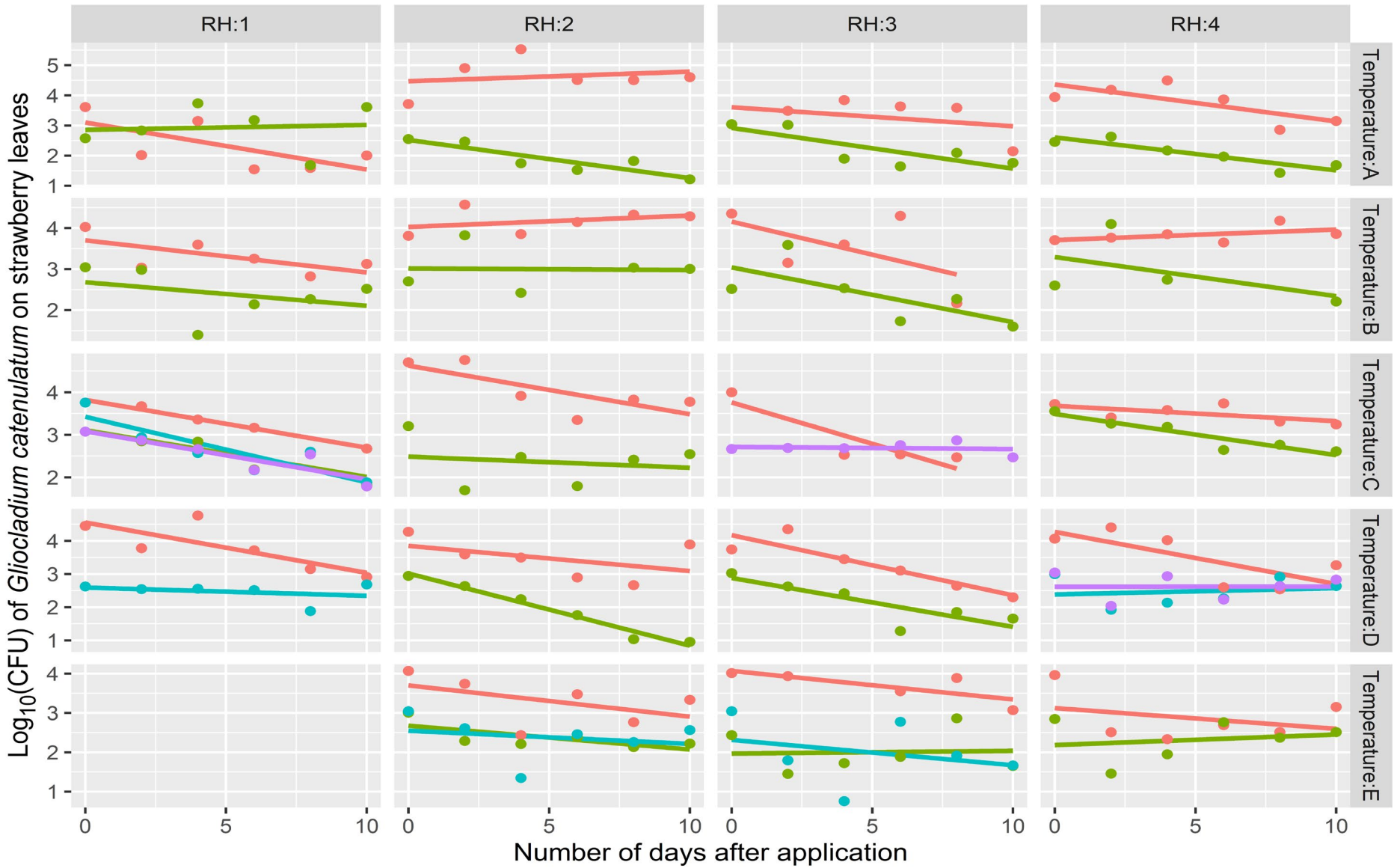

Temperature:A
Temperature:B
Temperature:C
Temperature:D
Temperature:E
RH:1
RH:2
RH:3
RH:4
Log10(CFU) of Bacillus subtilis on strawberry leaves
Number of days after application

Figure 1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RH:2
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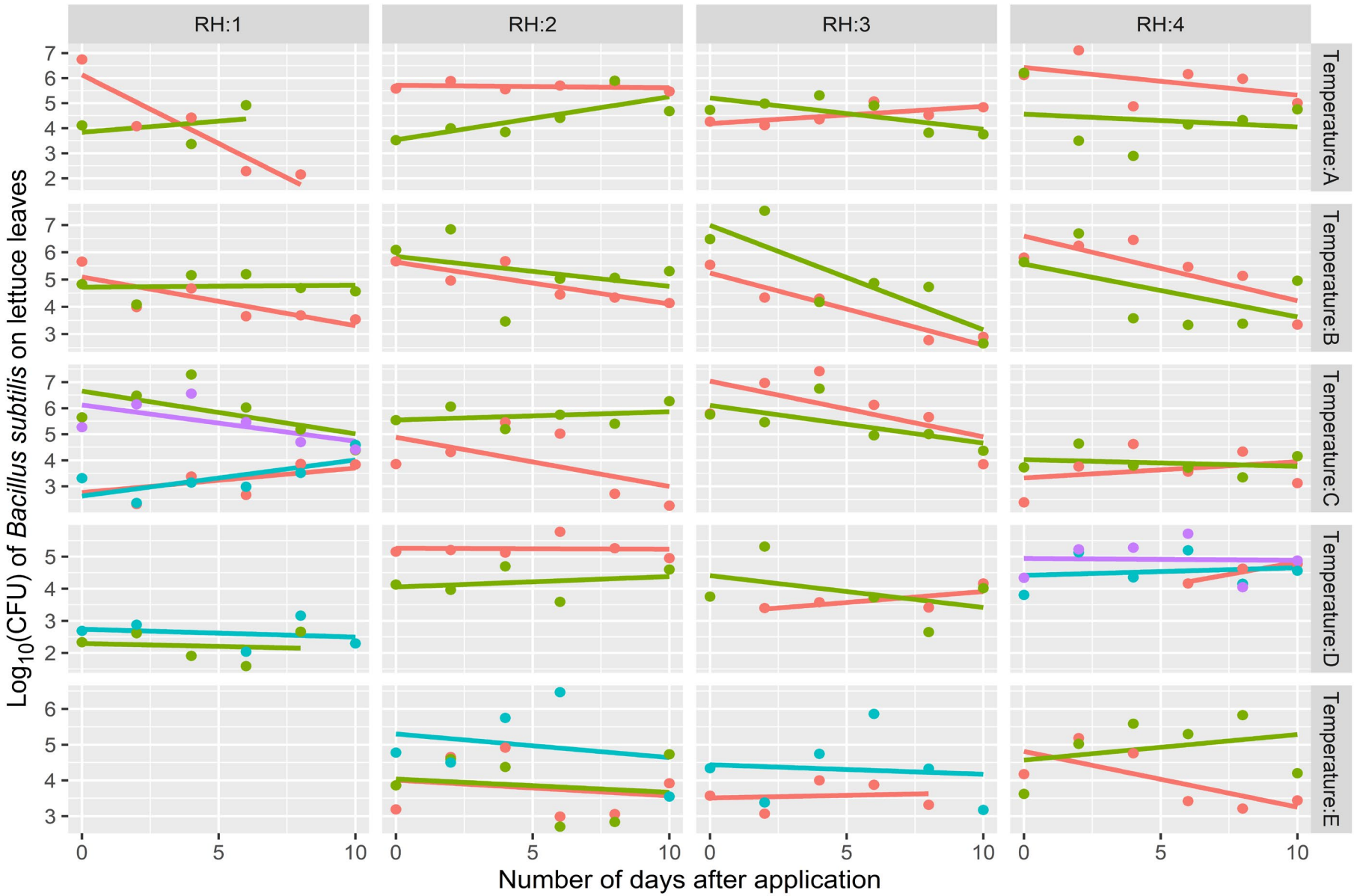
Temperature:A
Temperature:B
Temperature:C
Temperature:D
Temperature:E
Log10(CFU) of Gliocladium catenulatum on strawberry leaves
Number of days after application

Figure 2


RH:1
RH:2
RH:3
RH:4
Temperature:A
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Temperature:C
Temperature:D
Temperature:E
Log10(CFU) of Bacillus subtilis on lettuce leaves
Number of days after application

Figure 3

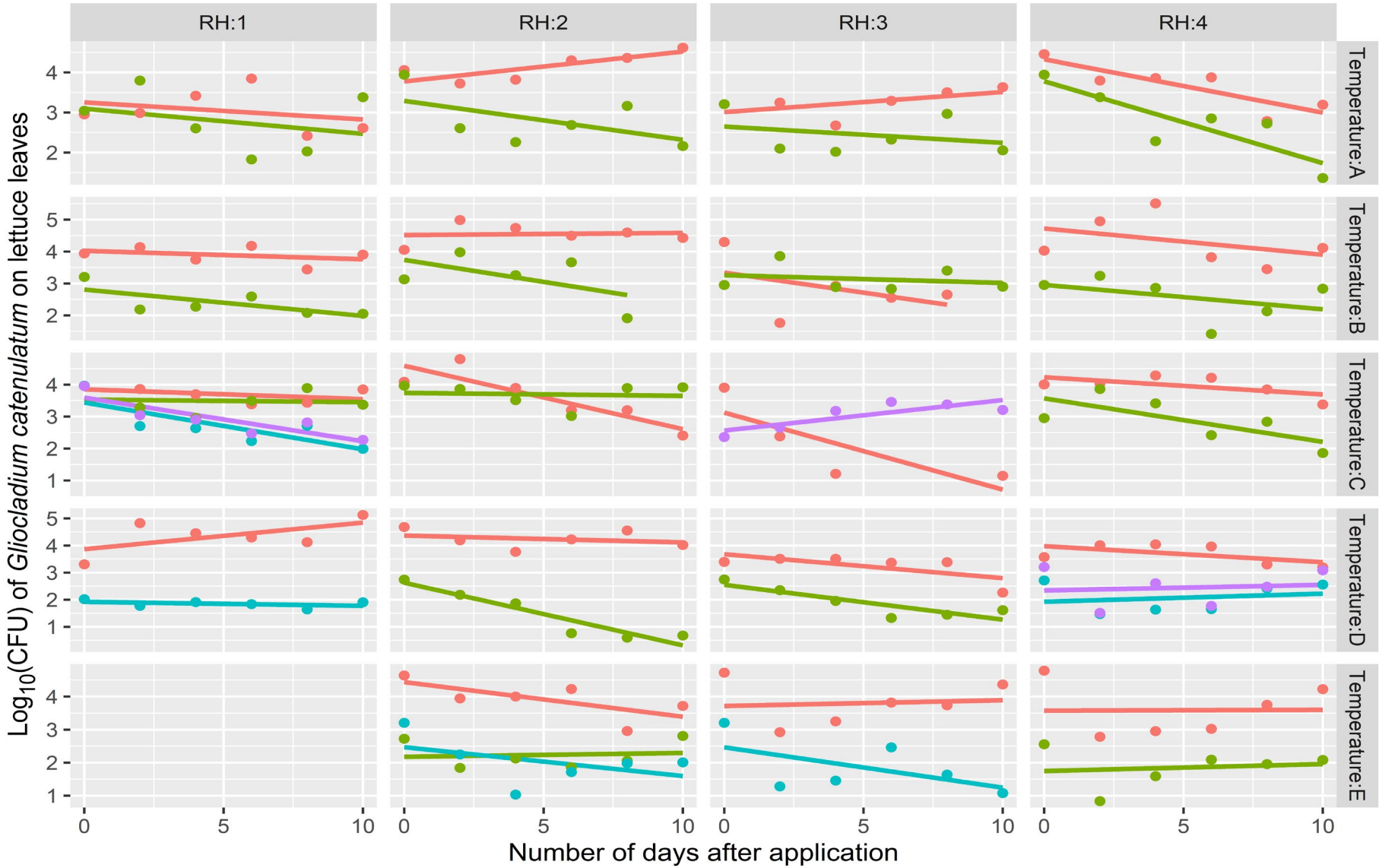
RH:1
RH:2
RH:3
RH:4
Temperature:A
Temperature:B
Temperature:C
Temperature:D
Temperature:E
Log10(CFU) of Gliocladium catenulatum on lettuce leaves
Number of days after application
0
5
10

Figure 4

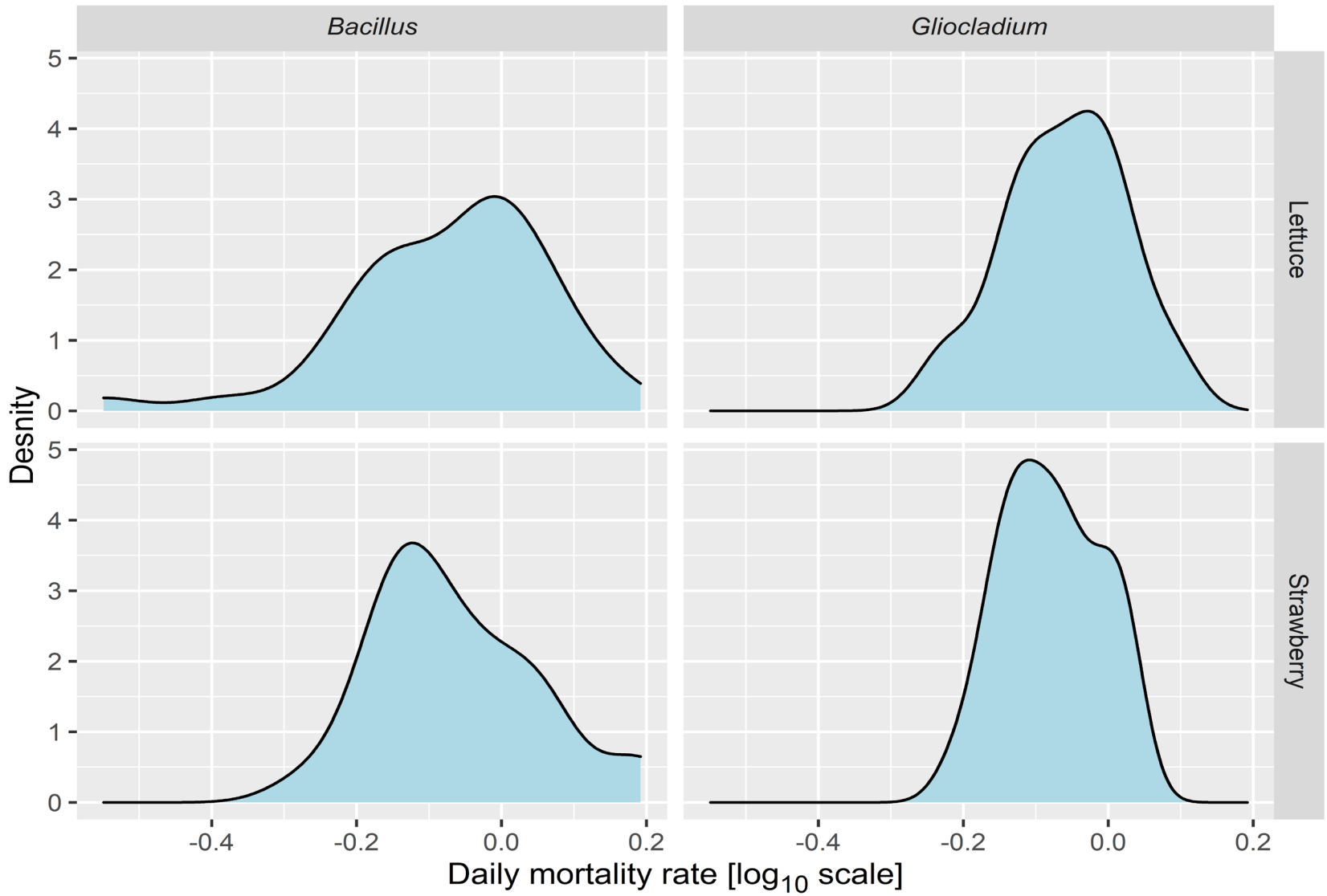
Bacillus
Gliocladium
Lettuce
Strawberry
Desnity
0
1
2
3
4
5
-0.4
-0.2
0.0
0.2
Daily mortality rate [log10 scale]

Figure 5

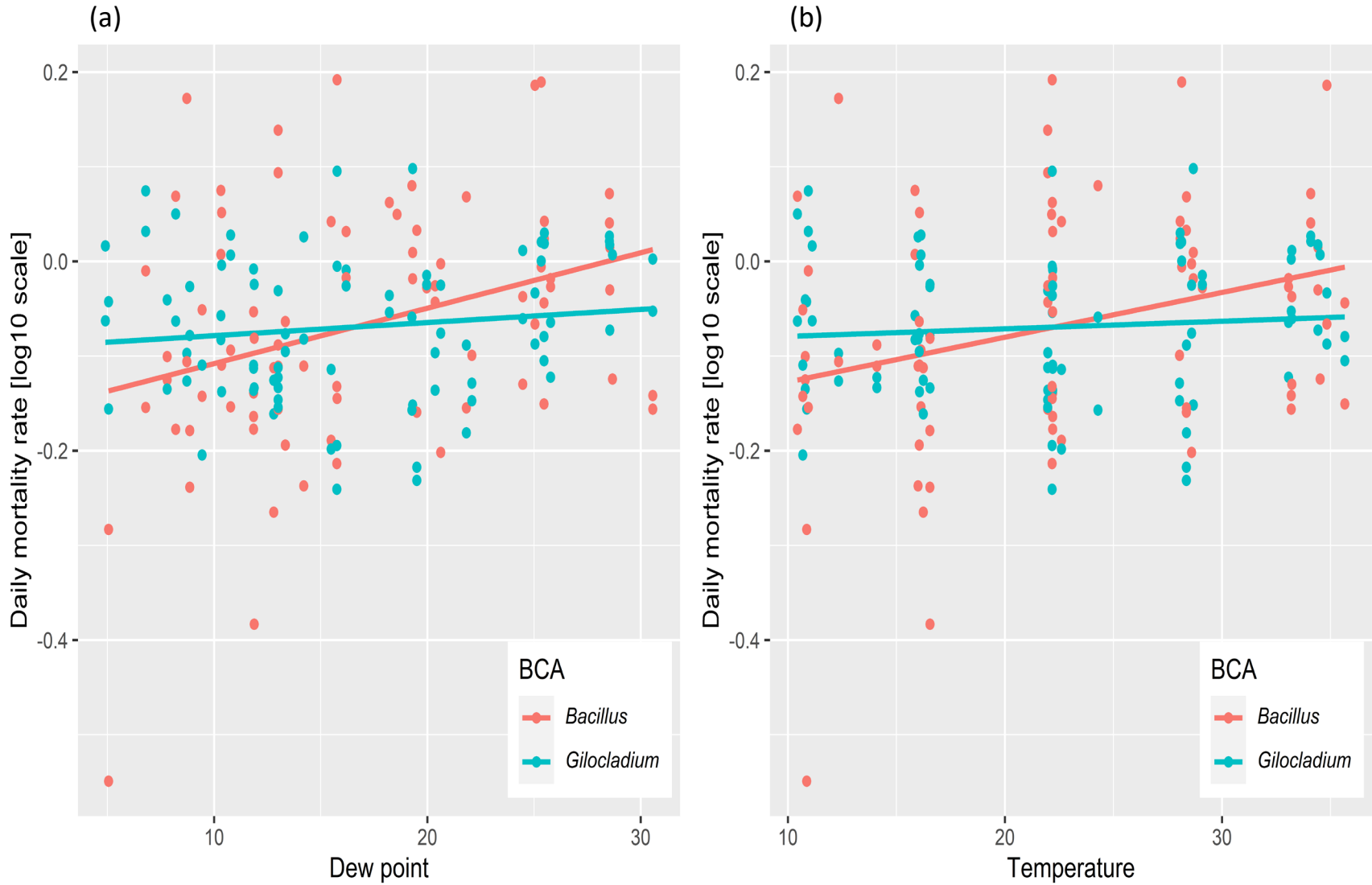
(a)
(b)
Daily mortality rate [log10 scale]
0.2
0.0
-0.2
-0.4
10
20
30
Dew point
Temperature
BCA
Bacillus
Gilocladium

Figure 6

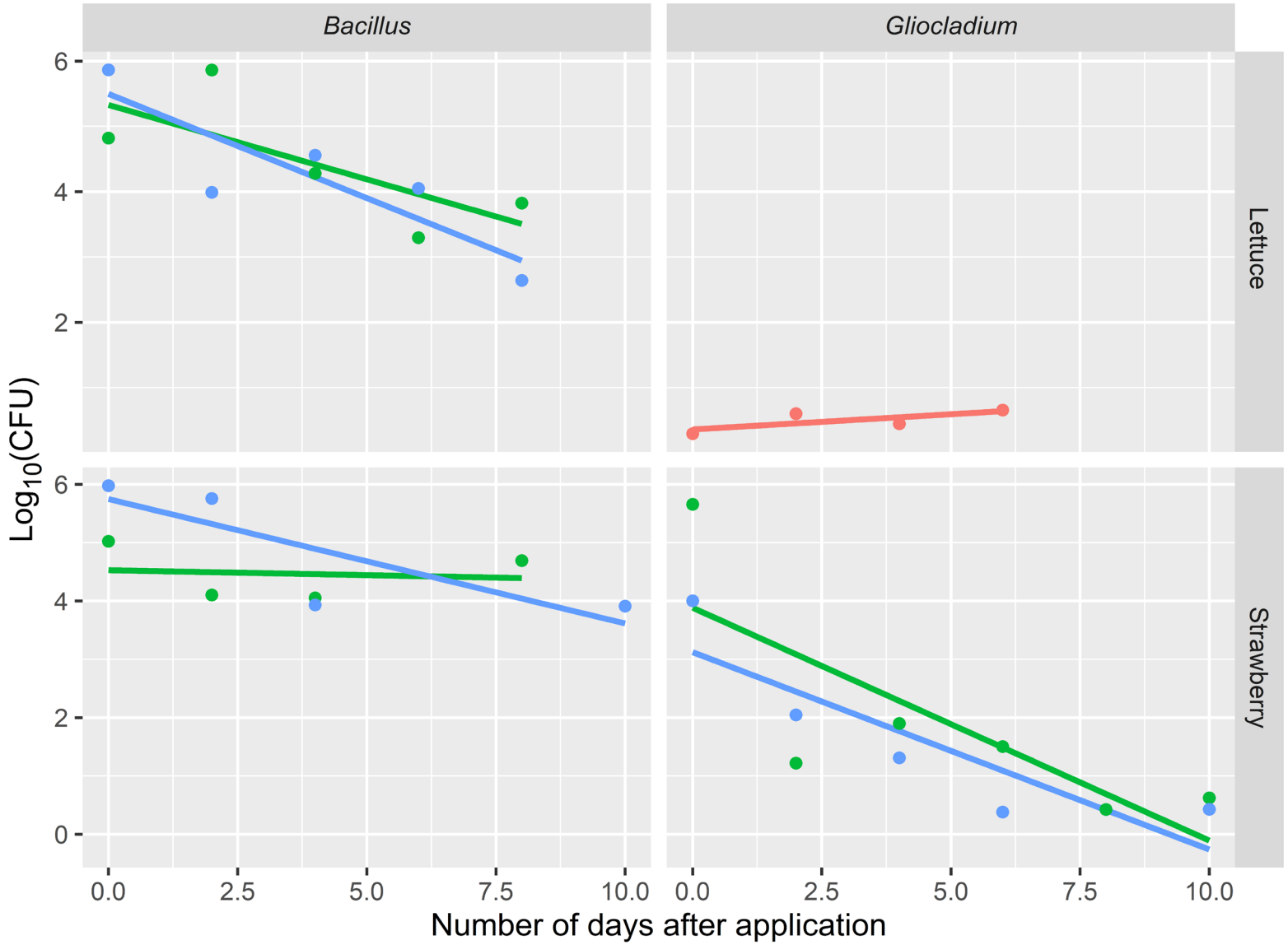
Bacillus
Gliocladium
Lettuce
Strawberry
Log10(CFU)
6
4
2
0
0.0
2.5
5.0
7.5
10.0
Number of days after application

Figure 7