

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 PMAxxTM-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

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

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

30

31 **1 INTRODUCTION**

32 There is significant interest in the use of biocontrol agents in commercial systems to improve the
33 sustainability of horticultural crop production systems and reduce the use of chemical inputs,
34 especially of fungicides. Biocontrol products based on formulated *Bacillus subtilis* and *Gliocladium*
35 *catenulatum* are now used in commercial production of many horticultural crops (Keswani et al.,
36 2016). The bacterial biocontrol organism *B. subtilis* synthesizes a variety of biologically active
37 compounds with a broad range of activity against phytopathogens (Shafi et al., 2017), and are
38 capable of inducing host systemic resistance (Bais et al., 2004; Butcher et al., 2007; Aliye et al.,
39 2008). It has the potential of forming multicellular structures and biofilms (Branda et al., 2001; Bais
40 et al., 2004) and can also compete for iron and nutrients through siderophore synthesis (de Boer et
41 al., 2003; Saha et al., 2015). Biocontrol mechanisms of *G. catenulatum* J1446 involves secretion of
42 cell wall degrading enzymes (Chatterton et al., 2009), mycoparasitism (Mcquilken et al., 2001), and
43 competition for space and nutrients (Chatterton et al., 2008; Chatterton & Punja, 2011; Helyer et
44 al., 2014).

45 Recent studies with these two commercial BCAs (*B. subtilis* QST 713 [recently re-classified as
46 *B. velezensis* (Pandin *et al.*, 2018)] and *G. cantenulatum* [syn. *Clonostachys rosea*]) showed, using
47 dose-response relationships, that a high population density of viable cells or propagules of the BCAs
48 was required for effective control of the pathogen, *Botrytis cinerea*, and how their relative viability
49 was affected by biotic and abiotic factors (Tut *et al.*, 2021b). Identifying the optimum population
50 density required for biocontrol is an important step in optimization of BCA formulations and
51 application to control economically important plant diseases, especially in the phyllosphere (Gotor-
52 Vila *et al.*, 2017). The key bottleneck in foliar applications of BCAs is effective establishment of the
53 sufficient viable inoculum load of the BCA for control of the target pathogen, especially under
54 naturally fluctuating abiotic factors that affects their establishment in the phyllosphere (Magan,
55 2020). Indeed, the establishment of BCAs under field conditions has been a major hurdle for
56 achieving high biocontrol efficacies because under fluctuating abiotic conditions there can be a rapid
57 loss of viability of introduced inoculum populations of the BCA, resulting in poor pathogen control
58 (Magan, 2006; Liu *et al.*, 2013; Sui *et al.*, 2015). There is a significant lack of data on these aspects
59 to develop appropriate timings of the application of commercial available BCAs.

60 For a BCA to be reliable, consistent and effective, establishment in the phyllosphere is
61 essential, and can only be successful if the inoculum has the required resilience over the range of
62 climatic regimes where the pathogen infection is a significant problem (Andrews, 1992, Andrews
63 and Hirano, 2012, Liu *et al.*, 2013, Magan, 2020, Sui *et al.*, 2015, Xu and Jeger, 2012). Previously, the
64 inconsistent efficacy of BCAs has been attributed to the lack of establishment of the chosen BCA
65 when applied to the phyllosphere of horticultural crops under naturally fluctuating abiotic
66 conditions, over the ecological range conducive to pathogen infection (Elad, 1996; Cray *et al.*, 2013;
67 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°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

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

98 **2.2 Culture and preparation of BCAs for spray applications**

99 Serenade® ASO (Serenade, an aqueous formulation of *B. subtilis* strain QST 713) and Prestop (a dry
100 formulation of *G. catenulatum* strain J1446) were purchased from Fargro Ltd (Arundel, West Sussex,
101 UK). For both formulations, culture conditions, growth and formulation components were unknown
102 due to industrial proprietary. Serenade was stored at room temperature, while Prestop was stored
103 in a cool dry location at < 8°C and, once opened, frozen at –20°C. The batches used were less than
104 6 months old. Up to log₁₀ 1.0 CFUs or conidia/mL were found to be non-viable in the formulations
105 when counted in a haemocytometer and plated on agar media.

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

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

119 **2.3 Plant propagation**

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

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

132 **2.4 Environmental treatments and experimental design for controlled studies**

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

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

149 Each climatic (temperature and humidity combination) treatment was carried out at least
150 twice. The exact temperature and RH conditions might differ slightly amongst repeats (Table 2)
151 because of difficulties in maintaining the exact temperature/humidity over the entire study duration
152 and were recorded via a temperature and RH dual logger placed inside the cabinet. Experiments
153 were carried out in two climatic chambers (Panasonic model MLR-352, and Sanyo format 650). Prior
154 to experimentation climate chambers were calibrated using external data loggers (EasyLog EL-USB-
155 2 standalone USB temperature and RH, dew point data logger). The same data loggers were used
156 for monitoring the temperature, RH and dew point in each chamber throughout the experimental
157 periods. Climatic conditions were randomly selected for experimentation at a given time for specific
158 CE cabinet. All climatic treatments had the same light:dark cycles (14 h light and 10 h dark): 3 h at

159 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
160 10 h of dark.

161

162 **2.5 Treatments and experimental design for commercial plantings**

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

172

173 **2.6 Leaf Imaging (Panasonic DMC-SZ3)**

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

$$\begin{aligned} \text{Total Area} &= \frac{\text{Total area of leafs in pixel}}{\text{Mean area of standard square in pixel}} \\ &= \text{Number of standard squares in leaf area} \times \text{Area of a single sqaure} \end{aligned}$$

180 After calculating the total area, the following formula was used to calculate CFU/mm²

181
$$\text{CFU/mm}^2 = \frac{\text{Total quantified viable population of BCA with PMAqPCR}}{\text{Total Area}}$$

182 **2.7 Quantification of viable cells with PMA-qPCR**

184 **2.7.1 PMAxx™ treatment**

185 This followed a recently published protocol (Tut *et al.*, 2021a). PMAxx™ was diluted in DEPC water
186 to produce a 2.5 mM concentration and stored at –20°C; 25 µM PMAxx were used for *B. subtilis* and
187 50 µM for *G. catenulatum*, respectively. This was followed by an incubation stage in which BCA
188 suspensions were encased in aluminium foil and placed into a lightproof sealable container, which
189 was shaken on a rocker at 35 rpm for 15 min. In a cold room (4–8°C) a photo-activation system was
190 assembled with a rocker fitted with an icebox that contained ca. 2 L ice, with the interior covered
191 by two layers of aluminium foil (reflective side), and two light sources with a light output of 800 W
192 fitted on the top at an angle of ca. 45°. After incubation, cell suspensions were transferred into the
193 system at a distance of 20 cm from the light source to initiate photo-activation. Photo-activation
194 consisted of 1 min light treatment followed by 2 min cooling for 30 cycles with constant agitation at
195 35 revs/min. After photo-induced cross-linking of PMAxx™ and exposed DNA in dead cells, BCA
196 suspensions were centrifuged at 5000× *g* for 10 min at 4°C, and the supernatant discarded.

197 **2.7.2 Grinding of *G. catenulatum* J1446 cells**

198 Briefly, after PMA treatment, cells were pelleted by centrifugation at 5,000 × *g* for 10 minutes at
199 4°C. The supernatant was decanted and conidia suspended in maximum recovery diluent solution
200 with a final volume of 1 ml. After slow pipetting for homogenisation, five stainless steel beads (6
201 mm) were transferred into each sample, and *G. catenulatum* cells were ground with the use of the

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

204 **2.7.3 DNA extraction and qPCR**

205 DNA from pellets was extracted with TRI Reagent[®] (Sigma–Aldrich) following the manufacturer’s
206 protocol. Extracted DNA was filtered with Millex-VV syringe filter unit 0.1 µm (PVDF, 33 mm and
207 gamma sterilized) and the purity and concentration determined on a NanoDrop spectrometer
208 (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, Delaware, U.S.A.). The integrity of the
209 DNA was determined by electrophoresis on a 1.5% agarose gel run at 60 V for 90 min within TAE
210 buffer solution and stained with GelRed (Biotum, Hayward, California, U.S.A.). The DNA was
211 quantified with the BIO-RAD CFX96[™] real time PCR detection system (BIO-RAD). Reactions were
212 prepared in a white/green semi-skirted 96 well qPCR plate (BIO-RAD) and sealed with an adhesive
213 cover. The final volume in each reaction for both BCAs was 44 µL which contained 10 µL SensiFAST[™]
214 SYBR[®] No-ROX Kit (Bioline Meridian Bioscience, London, U.K.). The target eDNA concentration was
215 150ng for both BCAs. Each reaction well contained 400 ng/µL of bovine serum albumin (Schrader et
216 al., 2012, Schriewer et al., 2011). *B. subtilis* reactions contained 434 nM of Bs_dnaK1154 forward
217 primer (5′-ACACGACGATCCCAACAAGC-3′), and 434 nM of Bs_dnaK1254 reverse primer (5′-
218 AGACATTGGGCGCTCACCT-3′) (Hertwig et al., 2015). *G. catenulatum* reactions contained 344 nM of
219 Gc1-1 forward primer (5′-CCGTCTCTTATCGAGCCAAGAT-3′), and 344 nM of Gc3-2a reverse primer
220 (5′-GCCCATTCAAAGCGAGGCATTA-3′) (Paavanen-Huhtala et al., 2000). The PCR conditions used for
221 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.
222 Each qPCR run included three replicates of controls without a template (NTC). The controls included
223 5 µL of DEPC water instead of DNA. CFX Manager[™] Software version 3.1 (BIO-RAD) was used to
224 analyse and calculate the Ct values automatically. All samples that reached fluorescence values

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

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

230

231 **2.7.4 Minimisation and suppression of qPCR inhibitors**

232 For improving the dissolution of the extracted eDNA into 8 mM sodium hydroxide, five steel beads
233 (6 mm) were transferred into each sample which contained a total volume of 1 ml and the DNA
234 pellets were ground using the genome grinder 2000, set at 500 rpm for 1 minute followed by a cool
235 down period of 2 minutes, the cycle was repeated fifteen times. After the grinding phase the DNA
236 was pelleted at $1500 \times g$ for 5 minutes at 4°C and the supernatant (clear DNA suspended in 8 mM
237 sodium hydroxide) was diluted into 8 mM sodium hydroxide. The dilution depended on the clarity
238 of the DNA sample. Filtration of the diluted DNA was completed with a Millex-VV Syringe Filter Unit
239 0.1 μm (PVDF, 33 mm and gamma sterilized), and the DNA purity and concentration was measured
240 on a Nanodrop spectrometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE).

241

242 **2.8 Statistical analyses**

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

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

260

261 **3 RESULTS**

262 **3.1 Experiments in controlled environment cabinets**

263 The dynamics of viable CFUs over time of all CE studies are shown in Figures 1 (*B. subtilis* on
264 strawberry), 2 (*G. catenulatum* on strawberry), 3 (*B. subtilis* on lettuce), and 4 (*G. catenulatum* on
265 lettuce). Several observations could be made from these data. Consideration of all the statistical
266 analysis as a whole suggests that the viable CFUs of both BCAs in both crops was strongly influenced
267 by temperature, RH and VPD. However, the relationship between viable CFU changes and the three
268 factors in the phyllosphere was complex. The BCAs appeared to prefer specific climatic regimes
269 (temperature and RH combinations). For both BCAs in both crops, as temperature was increased,
270 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 mm² 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 mm²
307 leaf area close to being statistical significant (P in the 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

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

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

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

370

371

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374 **Conflict of interest statement**

375 The authors declare that they have no conflict of interest.

376 **Data availability statement**

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

378

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383

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501 **FIGURE LEGENDS**

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

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

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

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

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

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

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

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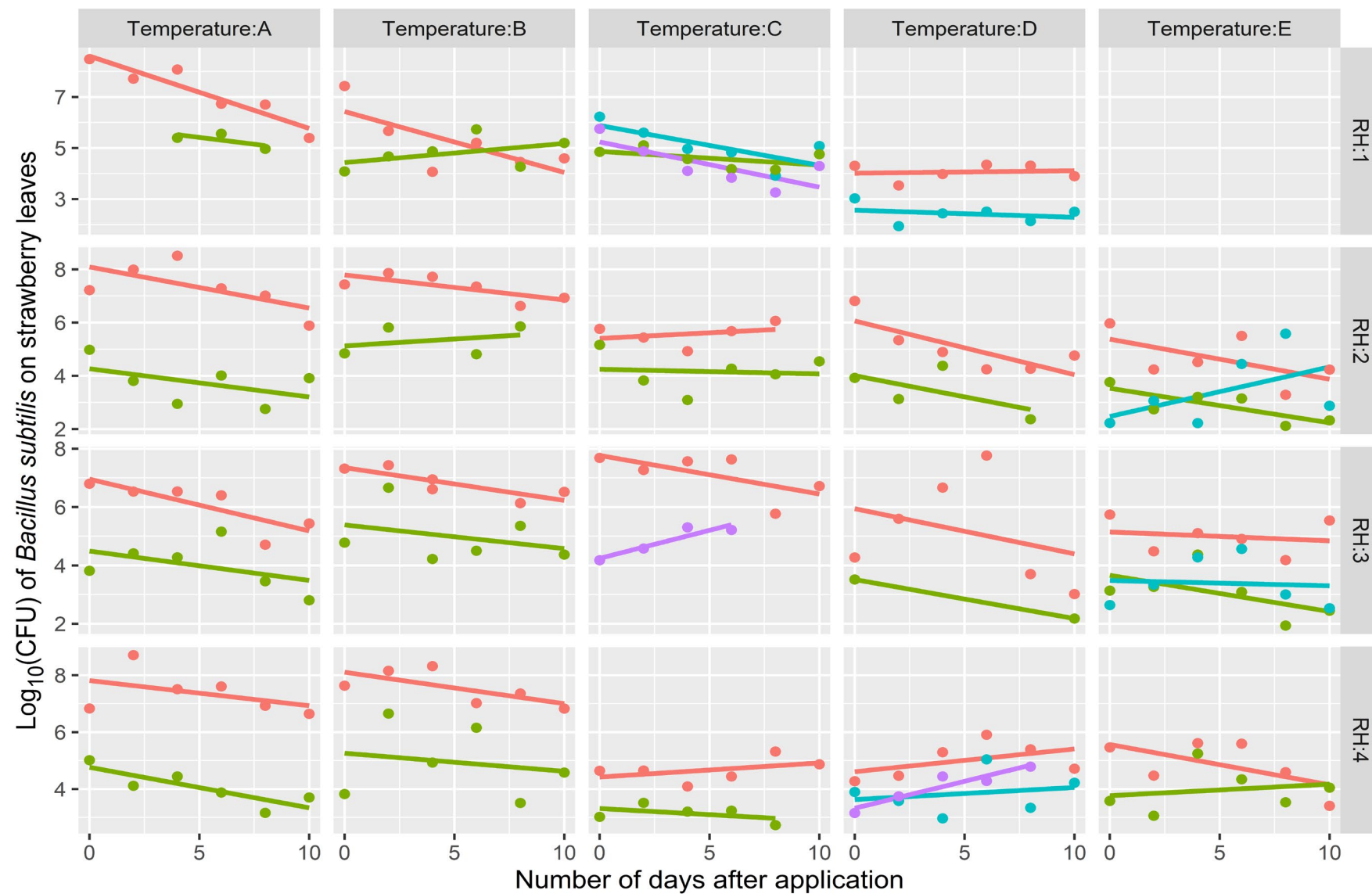


Figure 1

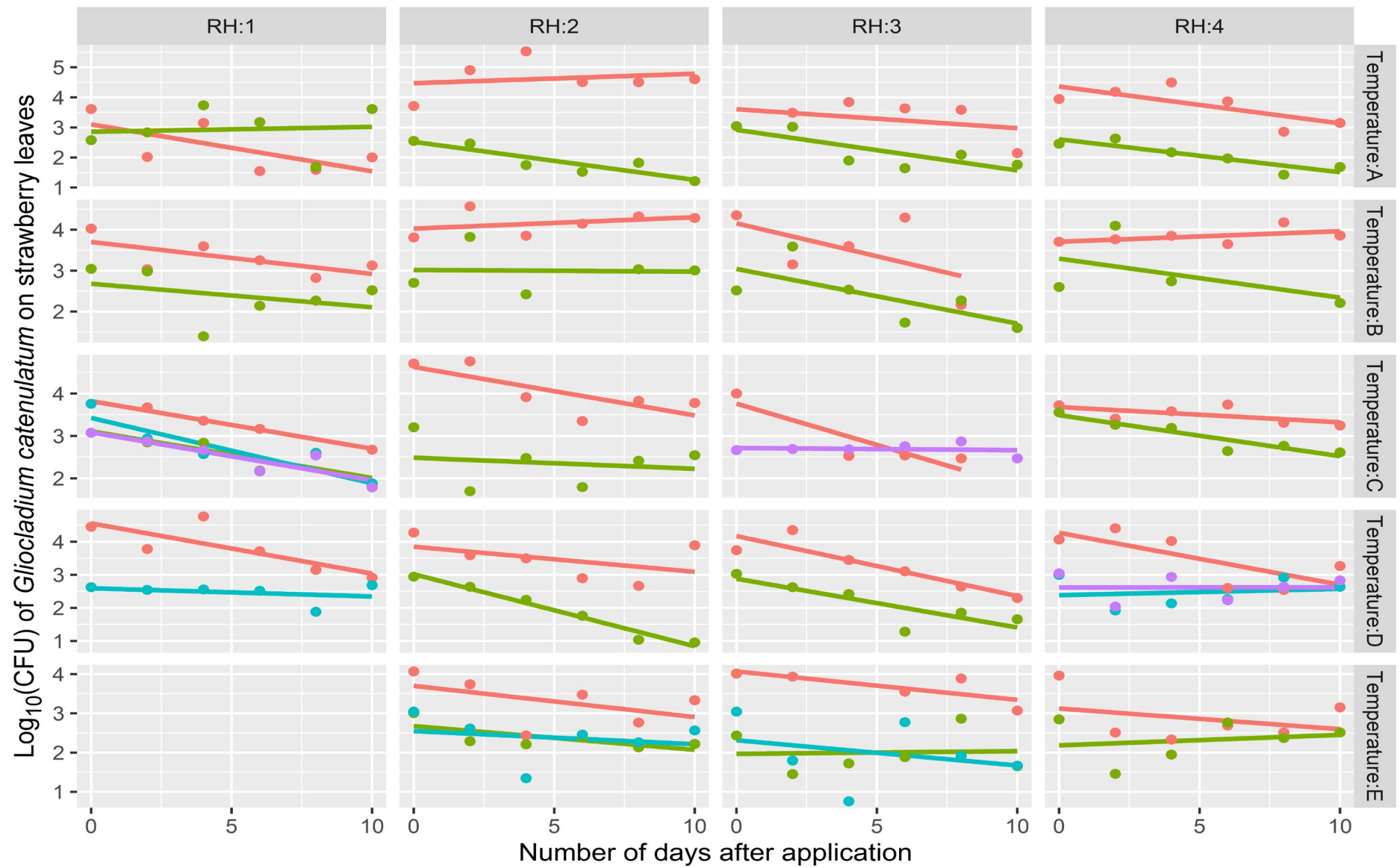


Figure 2

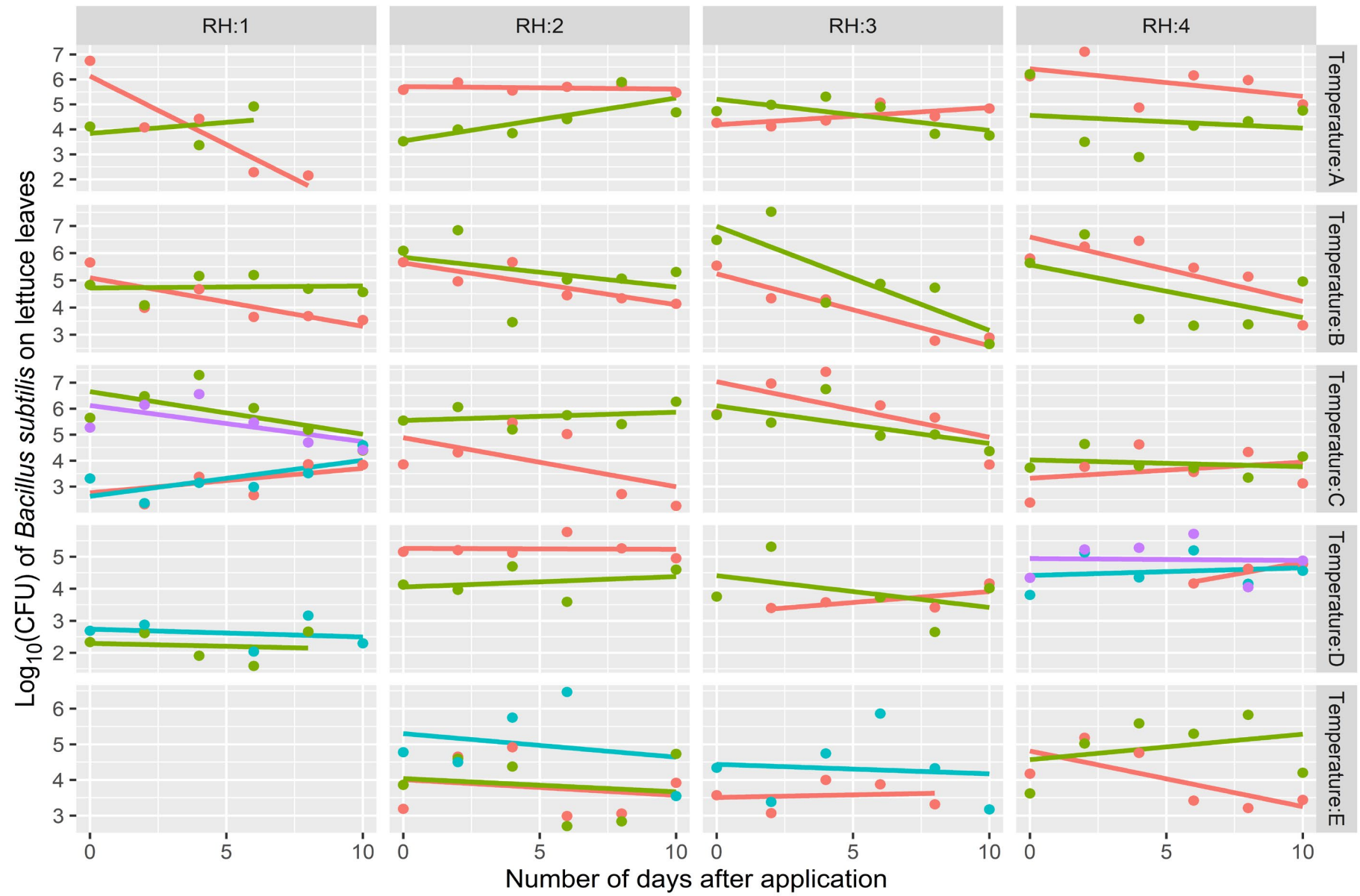


Figure 3

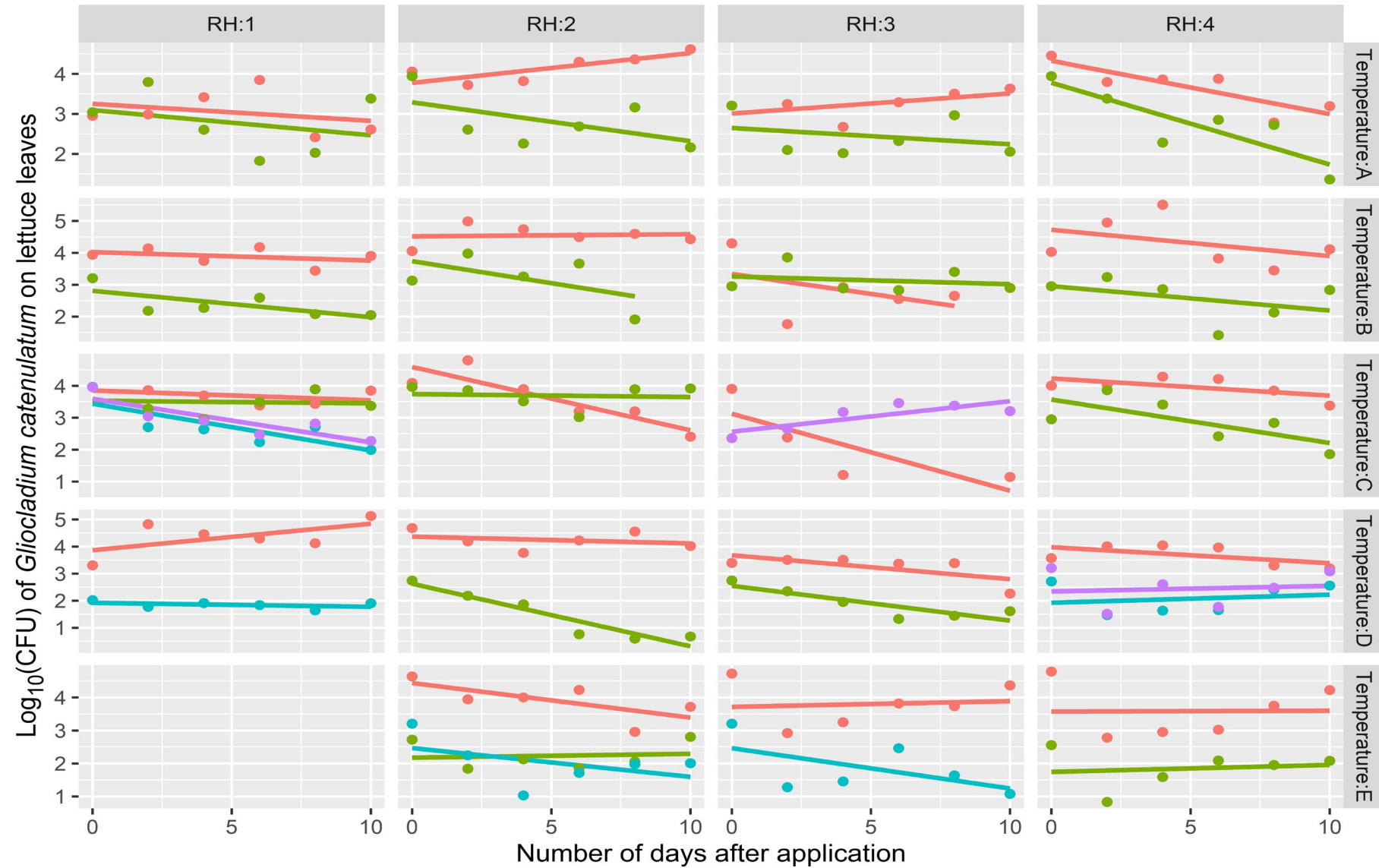


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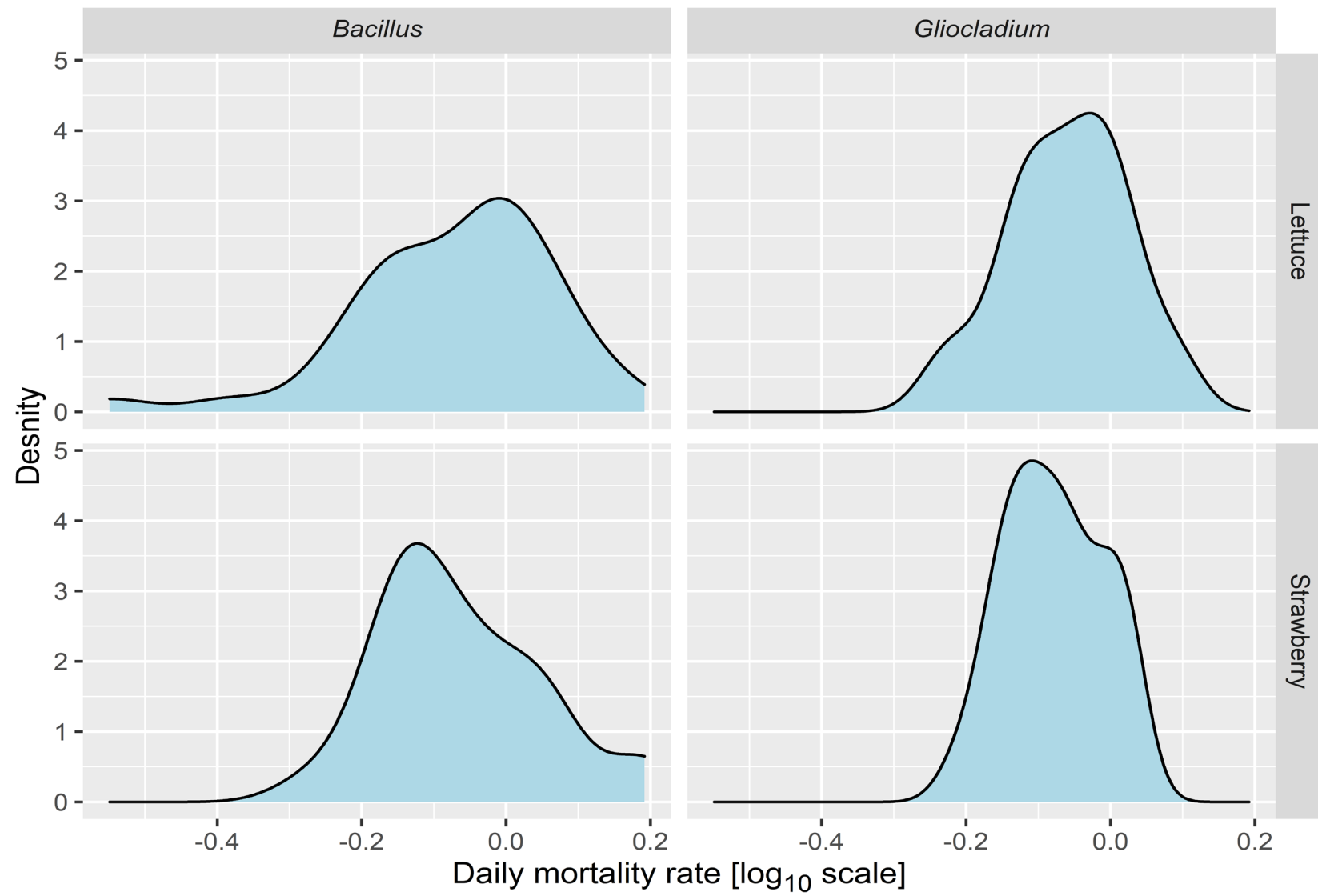


Figure 5

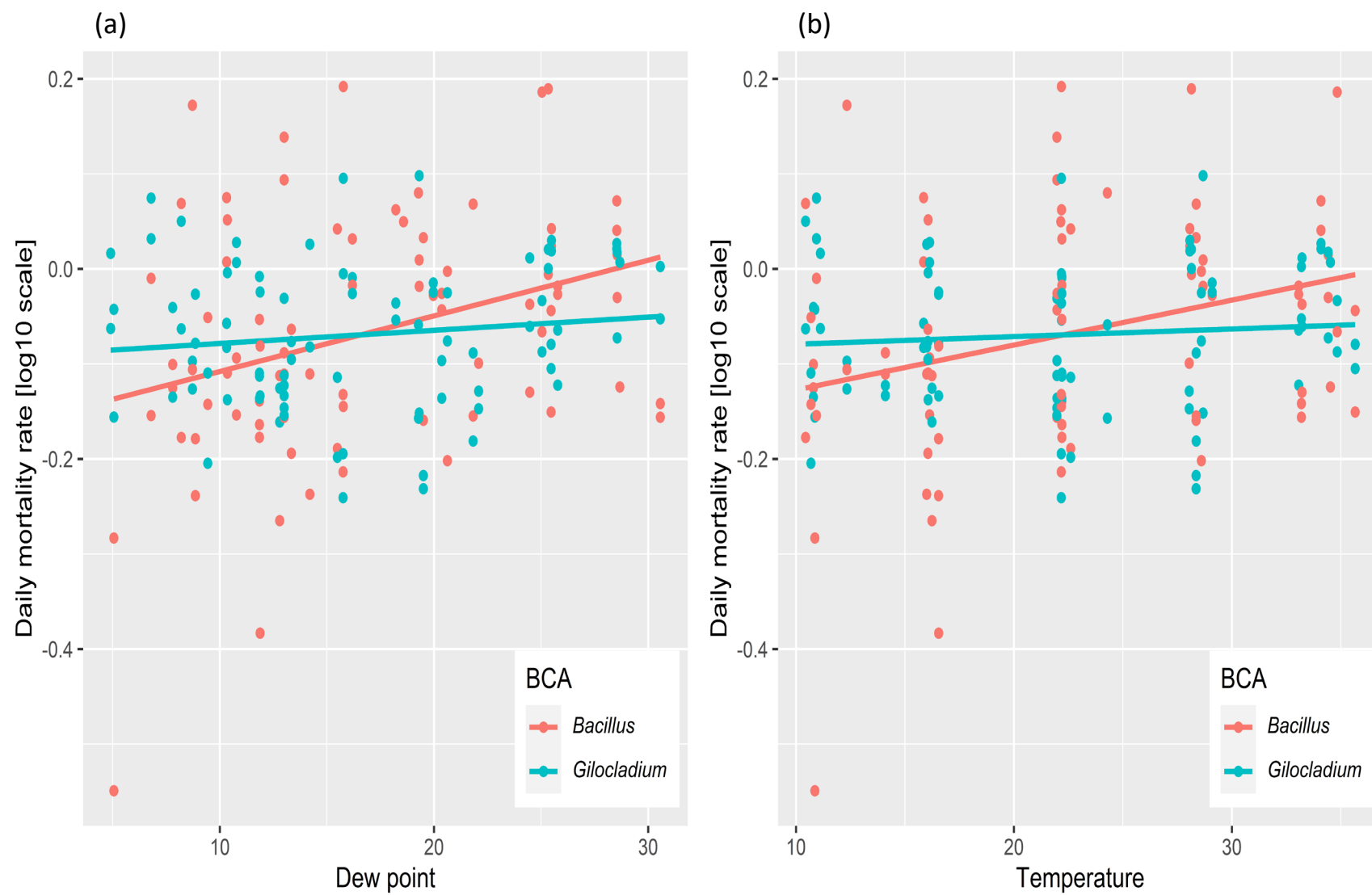


Figure 6

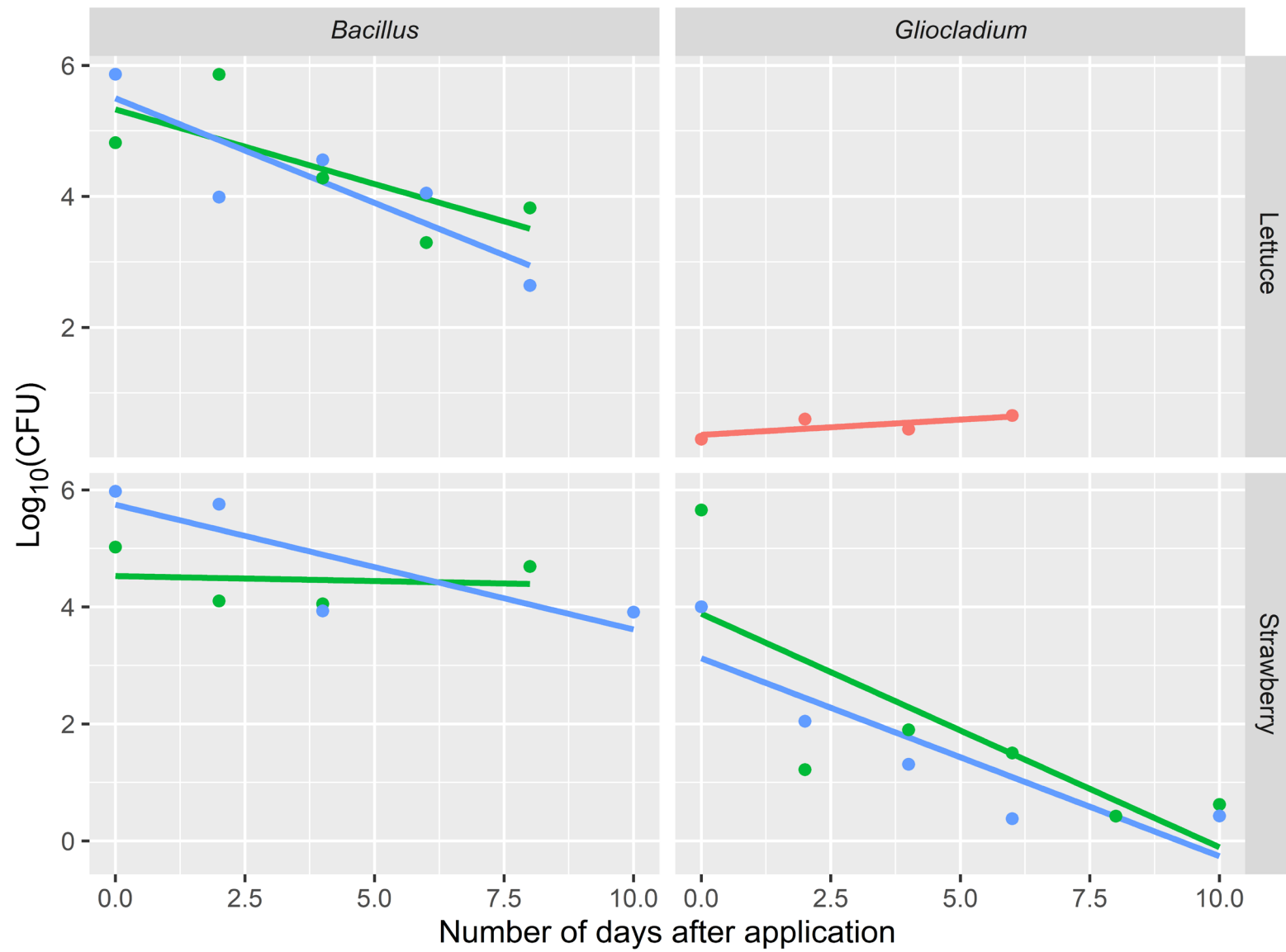


Figure 7



DSPACE

<https://dspace.org/>

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

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