Single-cell versus population-level reproductive success of bacterial immigrants to pre-colonized leaf surfaces

Mitja NP Remus-Emsermann[1,*,#], George A Kowalchuk[1,2,3], and Johan HJ Leveau[1,4]

[1]Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Droevendaalsesteeg 10, 6708 PB Wageningen, The Netherlands; [2]Deparment of Ecological Science, VU University Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands; [3]Institute of Environmental Biology, Faculty of Science, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands; [4]Department of Plant Pathology, University of California, One Shields Avenue, Davis, CA 95616, USA

[*]Current address: Institute of Microbiology, Eidgenössische Technische Hochschule Zürich, Wolfgang-Pauli-Strasse 10, 8093 Zürich, Switzerland

[#]Corresponding author: Dr. Mitja NP Remus-Emsermann, Institute of Microbiology, Eidgenössische Technische Hochschule Zürich, Wolfgang-Pauli-Strasse 10, HCI F 435, 8093 Zürich, Switzerland; E-mail: remus-emsermann@micro.biol.ethz.ch; Tel.: (+41) 44 632 38 30; Fax: (+41) 44 633 94 43.

Running Title: Individual bacterial experience on pre-colonized leaves

Summary

We assessed how preemptive inoculation of plant leaves with bacteria affected the establishment of secondary colonizers. We quantified the latter in two ways: 1) at the population level, i.e. as counts of colony-forming units and 2) at the level of single cells by tracking the reproductive success of individual bacteria. Both analyses showed that the ability of secondary immigrants to establish on the leaf was negatively correlated with the level of pre-population by primary colonizers. This effect was best described by an inverse dose-response curve with an apparent half-point inhibition efficacy of approximately 10^6 cells of primary colonizers per gram leaf. This efficacy was the same whether calculated from population- or average single-cell data. However, single-cell data revealed that even under conditions of heavy pre-population with primary colonizers, a small fraction of secondary immigrants still produced offspring, although the corresponding population measurement showed no increase in total population size. This observation has direct relevance for biocontrol strategies that are based on the principle of preemptive exclusion of foliar bacterial pathogens: even at seemingly saturating levels of primary inoculum, some secondary colonizers may still be able to reproduce and possibly reach a quorum to trigger behaviors that enhance survival or virulence.

Keywords: Phyllosphere, biological control, BCA, *Erwinia herbicola*, *Pantoea agglomerans*, *Phaseolus vulgaris*, preemptive colonization, biocontrol

46 **Introduction**

47 The plant leaf surface, or phyllosphere, is an extreme and uninviting microbial environment
48 due to abiotic stress conditions such as drought and exposure to UV radiation (Beattie and
49 Lindow 1999; Andrews and Harris 2000; Leveau and Lindow 2001; Lindow and Brandl
50 2003; Leveau 2006; Vorholt 2012). Nevertheless, a variety of microbes, including bacteria,
51 archaea, yeasts, and filamentous fungi, is well adapted to life in the phyllosphere, and they
52 can have a wide array of interactions with their hosts, ranging from mutualism to
53 commensalism and pathogenicity (Leveau 2006; Vorholt 2012). Of constant and considerable
54 concern are foliar plant pathogens that can cause a significant decrease in plant productivity
55 and crop yield and are a threat to food security (Savary, Willocquet et al. 2000; Anderson,
56 Cunningham et al. 2004). Another growing concern is the contamination of leafy greens with
57 human pathogenic bacteria such as *Escherichia coli* O157:H7 and *Salmonella*, which can
58 cause outbreaks from consumption of fresh produce (Beuchat 1996; Brandl 2008; Whipps,
59 Hand et al. 2008; Frank, Faber et al. 2011).

60 Many strategies have been proposed and tested to prevent or mitigate the
61 establishment of plant or human pathogens on leaf surfaces. One such strategy is the use of
62 bacterial biocontrol agents (BCAs) (Andrews 1992; Snyder, Ballard et al. 2004), that are
63 generally defined as nonpathogenic bacterial species that have the potential to reduce
64 pathogen load or activity (Compant, Duffy et al. 2005; Pilkington, Messelink et al. 2010).
65 Numerous examples exist in the literature demonstrating the effectiveness of bacteria with
66 biocontrol activity towards preventing growth of plant- (Elad 2003; Fernando, Ramarathnam
67 et al. 2007) or human- (Cooley, Chao et al. 2006; Lopez-Velasco, Tydings et al. 2012)
68 pathogens on leaf surfaces. One mechanism by which some bacterial BCAs operate is
69 preemptive exclusion where they effectively compete with the pathogen for space and/or
70 resources (Lindow 1987; Wilson and Lindow 1994; Wilson and Lindow 1995; Wilson, Savka

71 et al. 1995; Monier and Lindow 2005; Stockwell, Johnson et al. 2011). In effect, this
72 mechanism constitutes a case of resource monopolization of a newly colonized habitat (De
73 Meester, Gomez et al. 2002). A practical example is the preemptive inoculation of apple
74 blossoms and leaves with high-density suspensions of *Pantoea vagans* (Smits, Rezzonico et
75 al. 2010; Stockwell, Johnson et al. 2010), which minimizes the chance of secondary
76 colonization by the plant pathogenic bacterium *Erwinia amylovora,* the causal agent of
77 fireblight (Venisse, Barny et al. 2003). It has been argued (Wilson and Lindow 1994) that
78 preemptive exclusion works best when niche overlap between the BCA and pathogen is
79 maximal. A case in point is the successful use of near-isogenic ice nucleation-deficient
80 *Pseudomonas syringae* strains to reduce population sizes of ice nucleation-active *P. syringae*
81 strains in order to prevent frost injury to the plant foliage (Lindow 1987).

82 Numerous studies have dealt with biocontrol by preemptive exclusion, mostly via top-
83 down population-based approaches, e.g. by investigating the effect of bacterial BCA pre-
84 inoculation on the colony forming unit (CFU) counts of a subsequently introduced plant
85 pathogen or on the development of disease symptoms (Lindow 1987; Wilson and Lindow
86 1994; Braun-Kiewnick, Jacobsen et al. 2000; Nix, Burpee et al. 2009; Stockwell, Johnson et
87 al. 2010; Xu, Salama et al. 2010). BCA applications sometimes fail to achieve desired disease
88 suppression (Andrews 1992; Kinkel, Newton et al. 2002), and the reasons for this failure are
89 often not clear. In one study (Kinkel, Newton et al. 2002), it was suggested that a highly
90 aggregated distribution of resources on the leaf surface makes it difficult for a bacterial BCA
91 to avoid 'pathogen escape', i.e. even high-density applications of a bacterial BCA may not be
92 sufficient to fill all resource-rich patches on a leaf and to prevent pathogens from foliar
93 establishment. Recent studies, many of which featured bioreporter technology (Leveau and
94 Lindow 2002), have lent support to the notion that phyllosphere resources occur aggregated
95 (Leveau and Lindow, 2001) and that there is considerable variation in the exploitation of

96 these resources by bacterial immigrants to the leaf (Remus-Emsermann and Leveau 2010;
97 Remus-Emsermann, Tecon et al. 2012).

98 Here, we tested the hypothesis of 'pathogen escape' on pre-colonized leaves by
99 approaching it from a single-cell perspective through the use of a recently established
100 bioreporter for bacterial reproductive success, *Pa*299R$_{\mathrm{CUSPER}}$ (Remus-Emsermann and
101 Leveau 2010; Remus-Emsermann, Tecon et al. 2012). It is based on the bacterium *Pantoea*
102 *agglomerans* (formerly *Erwinia herbicola*) 299R (*Pa*299R) which was originally isolated
103 from pear leaves (Brandl, Clark et al. 1996) and has become a model strain for the study of
104 (non)pathogenic bacteria with an epiphytic phase as part of their life cycle. The
105 *Pa*299R$_{\mathrm{CUSPER}}$ bioreporter was designed so that it can be loaded with green fluorescent
106 protein (GFP) and its reproduction can be followed at the single-cell level by determining the
107 dilution of the fluorescent signal, which occurs at each cell division. In our experiments, we
108 used *Pa*299R$_{\mathrm{CUSPER}}$ as the secondary (or 'pathogen') colonizer, after prior inoculation and
109 colonization of leaves with a red fluorescent derivative of *P. agglomerans* 299R, i.e.
110 *Pa*299R$_{\mathrm{dsRed}}$, which served as the primary colonizer (or 'BCA'). Because *Pa*299R$_{\mathrm{CUSPER}}$ and
111 *Pa*299R$_{\mathrm{dsRed}}$ are nearly isogenic (they only differ in the color of fluorescent protein that they
112 produce), they can be considered each other's most effective resource competitors (Lindow
113 1987; Wilson and Lindow 1994). Establishment of the secondary colonizer (*Pa*299R$_{\mathrm{CUSPER}}$,
114 or 'pathogen') as a function of the population density by the primary colonizer (*Pa*299R$_{\mathrm{dsRed}}$,
115 or 'BCA') was then measured by CFU counts and the reproductive success of individual
116 cells.

117

118 **Results and Discussion**

119 *Changes in population size of Pa299R$_{CUSPER}$ cells on bean leaves pre-colonized with*
120 *Pa299$_{dsRed}$*

121 Fully expanded cotyledon leaves of two-week-old *Phaseolus vulgaris* plants (green snap
122 bean, variety Blue Lake Bush 274) were each inoculated by airbrush with 500 µL cell
123 suspension of red fluorescent *Pa*299R$_{dsRed}$ (Tecon and Leveau 2012) in phosphate-buffered
124 saline at concentrations of 10^6, 10^7, and 10^8 CFU per mL, as approximated by optical density.
125 After 24 hours of incubation under high relative humidity and constant illumination at room
126 temperature, these primary colonizers had reached population sizes of 2.09×10^6, 6.31×10^6,
127 and 6.76×10^7 CFU per gram leaf, respectively. Subsequent inoculation of such pre-colonized
128 leaves with approximately $5x10^5$ cells of *Pa*299R$_{CUSPER}$ (Remus-Emsermann and Leveau
129 2010; Remus-Emsermann, Tecon et al. 2012) revealed that the increase in population size of
130 this secondary colonizer over the next 24 hours was inversely correlated with the level of pre-
131 colonization by *Pa*299R$_{dsRed}$ (Figure 1A). The data fit a downward-sloping response-curve
132 (Figure 1B), with an approximate threshold of 10^5 CFUs of *Pa*299$_{dsRed}$ per gram of leaf
133 tissue. In other words, below this population density, the secondary colonizer *Pa*299R$_{CUSPER}$
134 would do as well as when the leaves were not first sprayed with *Pa*299$_{dsRed}$. The effective
135 range of *Pa*299R$_{dsRed}$ extended to approximately 10^8 CFUs per gram of leaf, with an apparent
136 50% inhibitory concentration or IC50 value of 1.31×10^6 CFUs of *Pa*299R$_{dsRed}$ per gram leaf
137 tissue. This finding is in good overall agreement with the idea that in situations where
138 primary and secondary colonizers use the exact same resources, secondary colonizers are
139 expected to be less successful in the colonization of a pre-populated environment (Wilson
140 and Lindow 1994).

141

142 *Single-cell experience of Pa299R$_{CUSPER}$ after arrival on leaves pre-colonized with different*
143 *densities of Pa299$_{dsRed}$*

144 The level of pre-colonization by *Pa*299R$_{dsRed}$ had a clear impact on the reproductive success
145 of individual secondary immigrants of *Pa*299R$_{CUSPER}$ (Figure 2). The higher the population

146 of *Pa*299R$_{dsRed}$ bacteria, the lower was the average reproductive success of *Pa*299R$_{CUSPER}$
147 cells, resulting in a shift to the left in the corresponding histograms (Figure 2). By
148 reconstructing the increase in population size from single-cell measurements (as was done in
149 Remus-Emsermann, Tecon et al. 2012), we were able to correlate relative population growth
150 as a function of CFUs of *Pa*299R$_{dsRed}$ at time t_0 (Figure 3). The resulting pattern was
151 similar to that presented in figure 1B, i.e. the data fit a downward-sloping response curve
152 (Figure 3). The IC50 value for this curve was 1.59x10^6 CFUs of *Pa*299R$_{dsRed}$ per gram leaf.
153 Despite the apparent agreement between the population-based (Figure 1) and average
154 single-cell-based (Figure 3) data, a closer inspection of the individual cell data (Figure 2)
155 revealed several instances where we found a small proportion of the cells that reproduced and
156 formed offspring in the absence of a total increase in population size. For example, 3% of the
157 *Pa*299R$_{CUSPER}$ cells that immigrated onto leaves which were heavily pre-populated at
158 6.31x10^6 CFUs per gram leaf were able to reproduce three times to create 8 offspring each
159 (Figure 2, column 4, row 3). In other words, a small fraction of the secondary inoculum
160 landed in areas of the leaf that allowed for growth, likely because they were devoid of
161 primary colonizers or because of the inability of the primary colonizer to completely
162 monopolize all resources (De Meester, Gomez et al. 2002). However, the increase in
163 population size that resulted from the growth of this small fraction was not sufficiently large
164 to be detected through CFU counts. This is a highly relevant observation if one considers that
165 for foliar pathogens such as *Pseudomonas syringae* the quorum size, i.e. the number of cells
166 in a local population to trigger a common behavior by signaling molecules, can be as low as
167 13 cells or even fewer (Dulla and Lindow 2008). Thus, a microcolony of 8 cells might be
168 sufficiently large to trigger quorum sensing behavior, which has been shown for *P. syringae*
169 to be important for virulence (Quiñones, Dulla et al. 2005). This observation supports the
170 notion that bacterial reproduction or the expression of pathogenicity factors on leaf surfaces

171 may not always be predicted accurately from population-based measurements. This pertains
172 not only to plant pathogens, but possibly also to other unwanted contaminants of leaf
173 surfaces, such as the human pathogen *Escherichia coli* O157:H7. Several studies have
174 documented the rapid decline of O157:H7 or proxy isolates on leafy greens (Delaquis, Bach
175 et al. 2007; Moyne, Sudarshana et al. 2011), and this decline is typically measured at the
176 population level as CFUs that can be washed off leaf surfaces. Our study suggests that,
177 masked by this population decline, there might be some cells that are able to survive, or even
178 reproduce into microcolonies that offer advantages for survival (Monier and Lindow 2005).
179 The ability of some bacterial immigrants to be highly successful on leaf surfaces, while most
180 others are not, can be explained by the highly aggregated distribution of resources on the leaf
181 surface, i.e. the existence of "oases" of relative abundant nutrients (Lindow and Brandl
182 2003). It would thus appear that the challenges for biocontrol strategies that depend on the
183 principal of preemptive exclusion lie in 1) the occupation of all such nutritional oases and 2)
184 the complete exhaustion of available resources in each of these oases.
185
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323

324 **Figure Legends**

325

326 **Figure 1.** A) Changes in population size of bacterial strain *Pa*299R$_{\mathrm{CUSPER}}$ as a function of
327 time, following inoculation at time *t*=0 onto leaves that were pre-colonized by *Pa*299R$_{\mathrm{dsRed}}$
328 cells at densities of on average 0 (●), 2.09x10^6 (■), 6.31x10^6 (▲), or 6.76x10^7 (▼) CFU per
329 gram leaf. Population sizes of *Pa*299R$_{\mathrm{CUSPER}}$ were estimated by spreading three independent
330 leaf washings (performed as described in Remus-Emsermann and Leveau 2010) onto LB agar
331 plates supplemented with 20 µg tetracycline mL^{-1}. B) Plot of *Pa*299R$_{\mathrm{CUSPER}}$ population sizes
332 after 24 hours of incubation as a function of different population sizes of *Pa*299R$_{\mathrm{dsRed}}$ at t_0.
333 The line shows the best-fitting dose response curve (value = best-fit ± std. error, log top-
334 response = 6.369 ± 0.033, log bottom-response = 4.773 ± 0.033, log(IC50) = 6.116 ± 0.049,
335 slope = Hill-slope, r^2 = 0.99, formula: y = bottom-response + (top-response – bottom-
336 response) / (1 + $10^{(x - \mathrm{logIC50})}$)). Error-bars represent standard deviations from the mean.
337 Dashed lines represent upper and lower 95% confidence intervals, respectively, of the fitted
338 dose response curve. Statistical analysis was performed in Prism 5.0c (GraphPad, La Jolla,
339 USA).

340

341 **Figure 2.** Distribution of the reproductive success among *Pa*299R$_{\mathrm{CUSPER}}$ cells that at *t* = 0
342 were inoculated onto bean leaves pre-colonized with *Pa*299R$_{\mathrm{dsRed}}$ at one of four different
343 densities (on average 0, 2.09x10^6, 6.31x10^6, or 6.76x10^7 cells per gram leaf). Reproductive
344 success was estimated after 0, 3, 6, 8, and 24 hours by fluorescence microscopy analysis of
345 cells recovered from three leaves per treatment by washing (Remus-Emsermann and Leveau
346 2010). Error-bars represent the standard deviation from the mean. GFP fluorescence of single
347 *Pa*299R$_{\mathrm{CUSPER}}$ cells was determined at 1000-fold magnification using an Axio Imager.M1
348 epifluorescent microscope (Zeiss, Oberkochen, Germany) coupled to a AxioCam MRm

349 CCD-camera (Zeiss, Jena, Germany). Images were acquired using the AxioVision 2.8.2
350 software package (Zeiss, Jena, Germany). Reproductive success of individual cells was
351 calculated from single-cell GFP fluorescence as explained elsewhere (Remus-Emsermann
352 and Leveau 2010).
353
354 **Figure 3**. The increase in population size of *Pa*299R_{CUSPER} as derived from single-cell
355 measurements at *t*=24 plotted against the population size of *Pa*299R_{dsRed} at t_0. The curve
356 through the points represents the fitted dose response (value = best-fit ± std. error, log top-
357 response = 1.38 ± 0.022, log bottom-response = 0.1835 ± 0.022, logIC50 = 6.204 ± 0.041,
358 slope = Hill-slope, r^2 = 0.99, formula: y = bottom-response + (top-response – bottom-
359 response) / (1 + $10^{(x - logIC50)}$)). Error-bars represent standard deviations from the mean.
360 Dashed lines represent upper and lower 95% confidence intervals, respectively, of the fitted
361 dose response curve.