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Colonization dynamics of *Pantoea agglomerans* in the wheat root habitat

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Summary

Plants are colonized by microbial communities that have diverse implications for plant development and health. The establishment of a stable plant–bacteria interaction depends on a continuous coexistence over generations. Transmission via the seed is considered as the main route for vertical inheritance of plant-associated bacteria. Nonetheless, the ecological principles that govern the plant colonization by seed endophytes remain understudied. Here we quantify the contribution of arrival time and colonization history to bacterial colonization of the wheat root. Establishing a common seed endophyte, *Pantoea agglomerans*, and wheat as a model system enabled us to document bacterial colonization of the plant roots during the early stages of germination. Using our system, we estimate the carrying capacity of the wheat roots as 10^8 cells g^{-1}, which is robust among individual plants and over time. Competitions *in planta* reveal a significant advantage of early incoming colonizers over late-incoming colonizers. Priming for the wheat environment had little effect on the colonizer success. Our experiments thus provide empirical data on the root colonization dynamics of a seed endophyte. The persistence of seed endophyte bacteria with the plant population over generations may contribute to the stable transmission that is one route for the evolution of a stable host-associated lifestyle.

Introduction

Plants are habitat to bacteria that colonize all plant tissues where the level of bacterial fidelity to a particular host species varies depending on the plant and bacterial species (Fitzpatrick *et al*., 2020). Bacteria that colonize plants may have consequences for various aspects of plant biology and have positive or negative effects on plant fitness. Examples include pathogenic bacteria, e.g. *Xylella fastidiosa* the causative agent of various plant diseases (e.g. citrus variegated chlorosis (Chang *et al*., 1993), Pierce's disease of grapevine (Hopkins *et al*., 1973), oleander leaf scorch, phony disease of peach (Purcell *et al*., 1999)), as well as beneficial symbionts, e.g. members of the rhizobia, which supply their host with fixed nitrogen (reviewed in Poole *et al*., 2018). Notwithstanding, most bacterial inhabitants of plants are considered as opportunistic (non-pathogenic) colonizers of the plant habitat (Bulgarelli *et al*., 2012; Edwards *et al*., 2018).

The establishment of a long-term stable bacterial association with the plant host largely depends on the interaction persistence over evolutionary time scales. The transmission of plant-associated bacteria over generations can be either by horizontal transmission where the bacteria are acquired from the environment or by vertical inheritance where the bacteria are transmitted from ancestor to descendants (Vandenkoornhuyse *et al*., 2015). Horizontal transmission via bacterial migration from the environment into the plant may occur via various tissues including the root (e.g. Amellal *et al*., 1998), leaves (e.g. Björklöf *et al*., 2000; Mercier and Lindow, 2000) or flowers (e.g. Mitter *et al*., 2017). The main road for vertically inherited bacteria in plants is considered to be transmitted via the seed (reviewed in Shade *et al*., 2017). Bacteria that can colonize the seed (i.e. seed endophytes) are considered as being able to persist with their plant host through the plant life cycle (Mundt and Hinkle, 1976; Okunishi *et al*., 2005; Truyens *et al*., 2015; Díaz Herrera *et al*., 2016). Notwithstanding,

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colonization of the host upon germination remains paramount for the bacteria vertical inheritance. Once bacteria enter the roots, they can potentially colonize other plant tissues (e.g. as in wheat (Ruppel *et al*., 1992) or rice (Chi *et al*., 2005; Kim *et al*., 2020)) and thus persist in the next life cycle stage. The presence of seed-borne bacteria in the roots of seedlings led to the suggestion that bacteria residing in the seed endosperm may have an advantage over bacteria migrating from the soil into the plant upon germination (Shade *et al*., 2017). Indeed, community ecology theory predicts that early incoming colonizers may exclude later arriving colonizers through a numerical priority effect. Priority effects in colonization may be enhanced under prior adaptation *sensu lato* to the environment, giving rise to monopolization of the habitat by specific colonizers having a fitness advantage over other colonizers (reviewed in De Meester *et al*., 2016). Notwithstanding, the effect of prior adaptation may depend on resource availability in the habitat; in conditions where the fitness differences between competing colonizers are small, numerical priority effects are expected to prevail (Grainger *et al*., 2019). Whether seed-borne bacteria have an advantage in plant colonization due to specific adaptations to the plant habitat or rather due to their high abundance during germination only (i.e. their presence in the right place and time) remains understudied. Here we study the early stages of plant colonization by seed endophytes with a focus on a specific plant colonizer species: *Pantoea agglomerans*. Members of the *Pantoea* genus are ubiquitous in diverse habitats (Dutkiewicz *et al*., 2015), where they lead diverse lifestyles ranging from free-living organisms to diverse associations with eukaryotic hosts including insects and plants (Nadarasah and Stavrinides, 2014). *Pantoea agglomerans* is a common epiphyte and endophyte and it has been shown to colonize plant surfaces, the xylem, and intercellular spaces of various cereals (Remus *et al*., 2000). While several *P. agglomerans* strains have been described as plant pathogens (reviewed in Barash and Manulis-Sasson, 2009), *P. agglomerans* is generally considered as a beneficial plant colonizer and its presence has been associated with an increased biomass production in a variety of plants, including sugarcane (Quecine *et al*., 2012), wheat (Remus *et al*., 2000), maize (Mishra *et al*., 2011) and peas (Höflich and Ruppel, 1994). Indeed, *P. agglomerans* harbours a wide repertoire of plant growth–promoting traits, including indole-3-acetic acid production and phosphorus solubilization. The presence of *P. agglomerans* has been shown to prime the plant immune system enabling a faster response to pathogen infection (Ortmann and Moerschbacher, 2006); additionally, it has antagonistic properties towards bacterial pathogens (e.g. *Pseudomonas syringae* (Braun-Kiewnick *et al*., 2000), *Erwinia amylovora* (Vanneste, 1996; Johnson and Stockwell, 1998) and fungal pathogens (e.g. *Fusarium graminearum* (Díaz Herrera *et al*., 2016)). The ubiquity of *P. agglomerans* as a plant colonizer suggests that a long-term association of *P. agglomerans* with plant hosts is well established. The finding of *P. agglomerans* as a seed endophyte (e.g. in *Eucaliptus* (Ferreira *et al*., 2008), Rice (Ruiza *et al*., 2011) and wheat (*Triticum aestivum*) (Links *et al*., 2014; Díaz Herrera *et al*., 2016; Ridout *et al*., 2019)) and the ability of seed-borne *P. agglomerans* isolates to colonize the roots of seedlings upon germination (e.g. in Rice (Ruiza *et al*., 2011) and Wheat (Díaz Herrera *et al*., 2016)) suggest a role of the vertical transmission in the evolution of *P. agglomerans* association with plants. Here we study *P. agglomerans* colonization dynamics of wheat seedlings during the early growth stages. Additionally, we study the effect of the order of arrival on the colonization success of competing colonizers in the root habitat. For that purpose, we established a well-controlled gnotobiotic system that enabled us to document the population size of a native *P. agglomerans* strain in the root habitat over time. Using our system, we assessed the consequences of preemptive *in planta* competition during the early colonization phase of the wheat roots.

Results

Isolation and establishment of seed-borne P. agglomerans *from wheat*

Aiming to perform our experiments with a native member of the wheat seed microbiota, we opted for the isolation of *P. agglomerans* strains from several cultivars of *Triticum aestivum*, including the cultivars Runal, Benchmark and Akteur, as well as a *T. aestivum* landrace from Turkey. The grains were surface sterilized and germinated under laboratory conditions for 5–6 days. *Pantoea* sp. could be isolated from the majority of grains as well as seedlings germinated in the laboratory. To further examine the phylogenetic position of our isolates within *P. agglomerans* species, we sequenced the genomes of selected isolates, including five German and six Turkish strains (Table S1). The genomic sequencing reveals that all sequenced isolates harbour the large *Pantoea* plasmid (LPP-1) that is common for endophytic *Pantoea* species (De Maayer *et al*., 2012).

A reconstruction of the isolated *Pantoea* strains phylogeny shows that the Turkish and German isolates branch in two different clades (Fig. 1). The clade containing the Turkish isolates is comprised of 23 strains, with a majority (17; 74%) of strains isolated from plants, and several strains isolated from soil/sediment or human. The clade including the German isolates comprises 40 strains, of which 28 (70%) were isolated from plants,

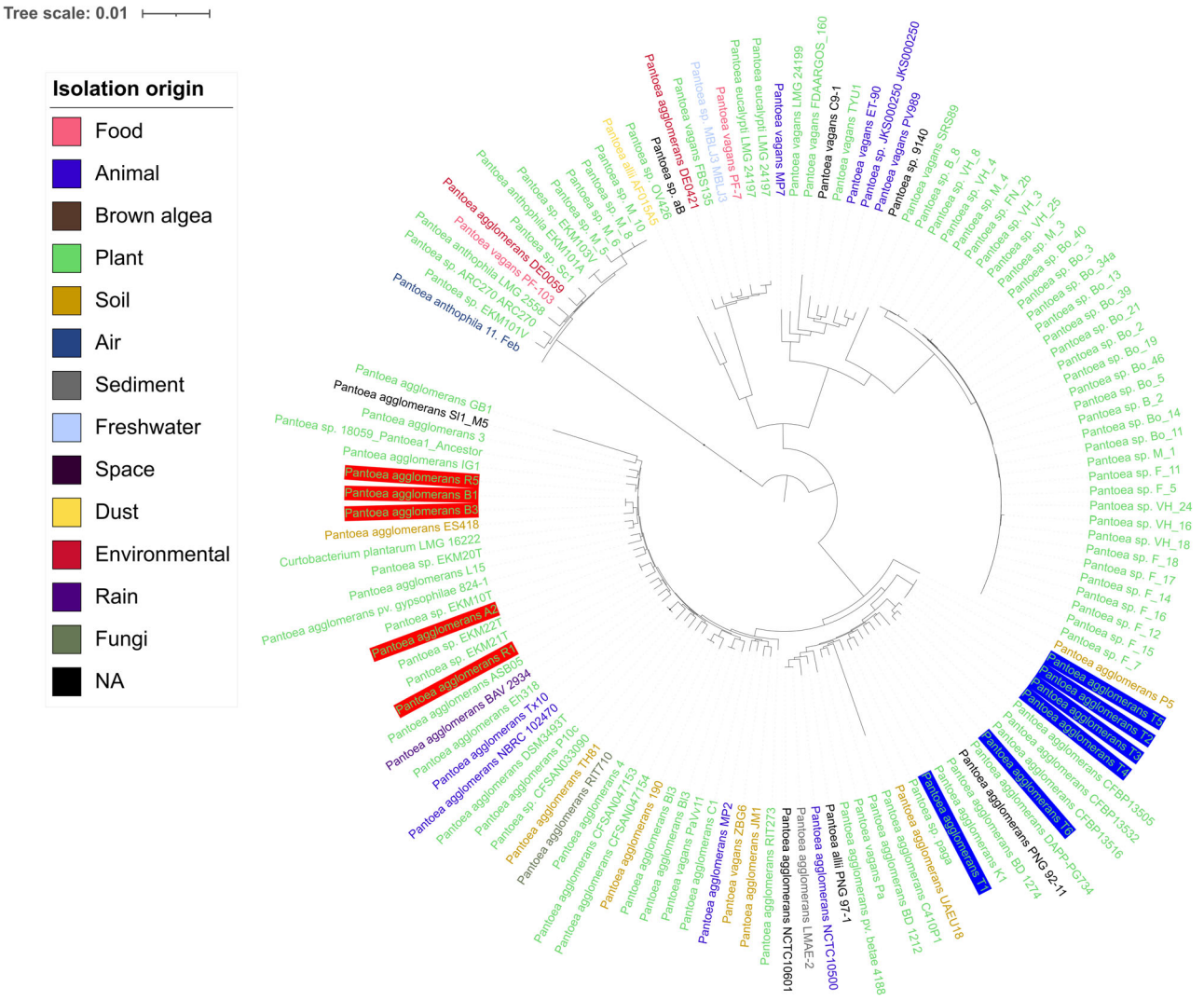


Fig. 1. Phylogenetic relations of *Pantoea agglomerans* isolates from wheat seeds. The phylogenetic tree was inferred from 932 universal single-copy proteins identified among 141 genomes of the genus *Pantoea* using maximum-likelihood (ML) (a broader phylogeny and selection criterion are presented in Fig. S1). Label colouring corresponds to the isolation origin according to BioProject metadata. For the presentation, 11 genomes belonging to *Pantoea* sp., *Pantoea deleyi*, *Pantoea conspicua*, *Pantoea brenneri* and *Pantoea agglomerans* were excluded from the tree as their external branches are long relative to the remaining genomes. Note that *P. agglomerans* isolated from Turkish wheat (blue background) and *P. agglomerans* isolated from German wheat (red background) are found in two diverged clades. All 11 isolates from this study are closely related to other *Pantoea* isolates associated with plants or soil habitats.

seven strains were isolated from soil/sediment and four isolated from other habitats. Consequently, we conclude that our isolates of *P. agglomerans* are close relatives of plant-associated *P. agglomerans*. Taking together with our isolation approach, the *P. agglomerans* isolates reported here are likely genuine members of the native wheat seed microbiota.

Pantoea colonization dynamics and the wheat roots carrying capacity

To study the dynamics of *Pantoea* colonization of the wheat roots, we followed the *Pantoea* population size in the roots over time. For that purpose, we transformed *P. agglomerans* strain R1 (hereafter termed *Pantoea*) with a reporter plasmid (pRL153-GFP; (Tolonen *et al*., 2006)) encoding the green fluorescent protein (GFP). We note that the reporter plasmid presence had a negligible effect on the *Pantoea* fitness (i.e. growth dynamics; Table S2). Germ-free seedlings were inoculated with *Pantoea* in the early growth stages. *Pantoea* population size in the wheat root habitat, which comprises the root surface and the endosphere, was determined by colony-forming units (CFUs) counts of plated root homogenates (Fig. 2A). Our results reveal that pRL153-GFP carrying *Pantoea* cells were already then present in the root sample already 5 h

post-inoculation (Fig. 2B). The *Pantoea* population increased in size rapidly in the first days of the experiment. Note that due to the low inoculum size, it is possible that the bacterial density within the root habitat was below our detection threshold in the early days. This possibility is supported by our observation that in later days, *Pantoea* was detected in most tested plants. After 120 h, the changes in *Pantoea* population size were minimal (Fig. 2B) and this was associated with a slower root mass increase (Table S3). At the end of the experiment, 168 h after inoculation, *Pantoea* reached a stable density with a median of 5.1×10^7 CFUs per gram root ($n = 6$) (Fig. 3C). The stable *Pantoea* density in the wheat root habitat over several days suggests that the *Pantoea* population size has reached the carrying capacity of the root habitat. We note that the *Pantoea* population size results obtained here for 44 independently sampled wheat seedlings form a typical bacterial growth curve similar to observations of growth dynamics for a single microbial population. The similarity among the *Pantoea* density measurements across individual plants serves as an internal validation for our standardized approach.

Throughout the experiment, *Pantoea* cells were observed in the media where their abundance was similar to that of the root system (Table S3). We note that *Pantoea* is unable to proliferate in fresh plant media, as it does not contain a significant carbon source. Thus, the presence of *Pantoea* in the media during the experiment depends on plant exudates as a carbon source. Our observation of *Pantoea* in spent media indicates a

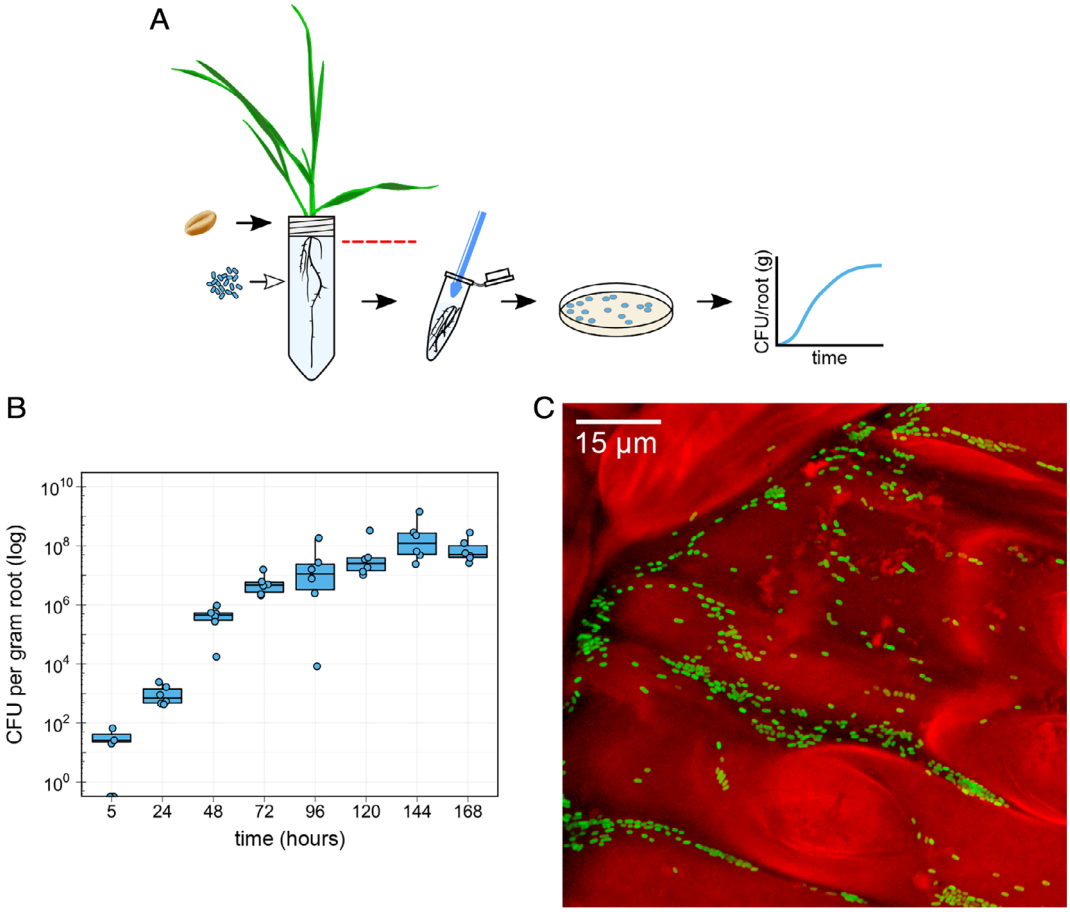


Fig. 2. *Pantoea* population growth on the wheat root.
A. Illustration of the experimental pipeline. Each plant was infected individually with *Pantoea* (*c*. 1800 cells per plant). For each replicate plant, the entire root system was homogenized and the *Pantoea* population size was evaluated by plating (see Materials and methods for further details).
B. Number of CFUs per gram root over the colonization experiment duration. Bacterial density in the root is calculated as the frequency of CFUs divided by the root tissue fresh weight (g, Fig. S1). The boxplots present the CFUs median and interquartile range (IQR). Each data point shows the CFUs from a single, independently sampled root system, hence, the variation in CFUs measurements with each sampling time reflects differences among individual plants.
C. Confocal laser scanning fluorescence micrograph (CLSM) of a longitudinal outer cell layer area of the primary wheat root (red, chlorophyll autofluorescence) visualized 72 h post-infection with fluorescently labelled *Pantoea* carrying plasmid pRL153-GFP (green, GFP). Immobile GFP-tagged cells cluster along naturally occurring root crevices.

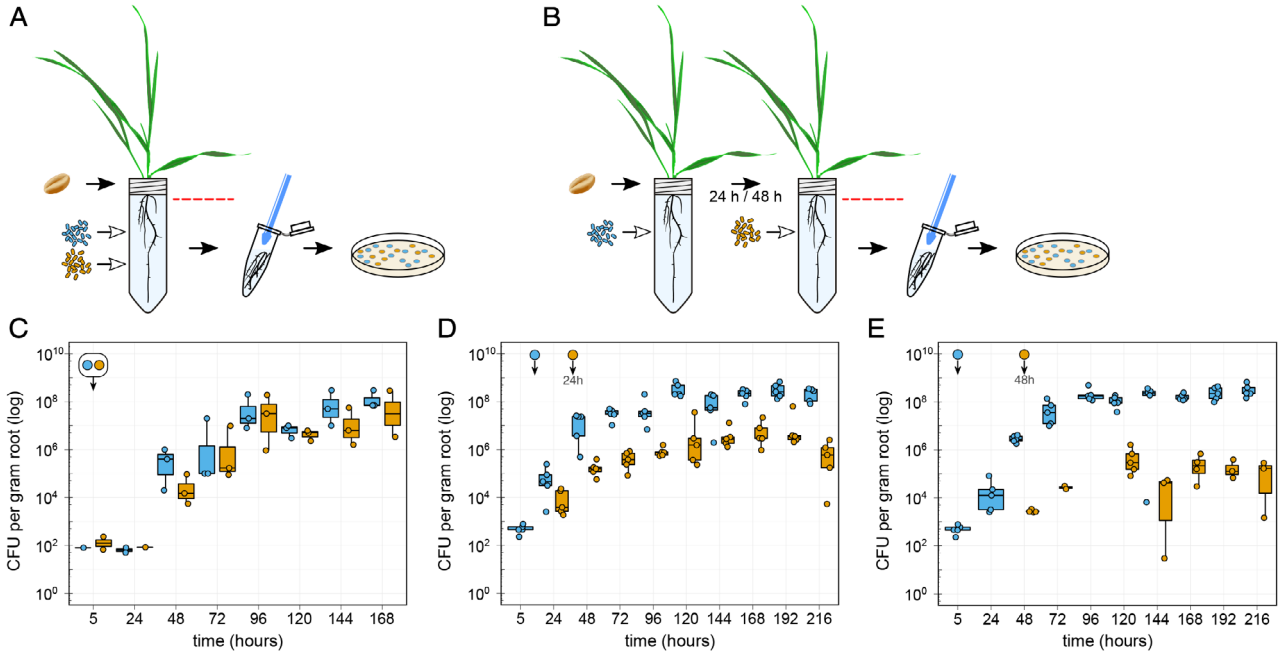


Fig. 3. Competition experiments reveal advantage for early incoming colonizers.
A. Illustration of the experimental pipeline for *in planta* competition between two colonizers. Both colonizing *Pantoea* strains were derived from the same clone (isolate R1) and differed only in the expression of the plasmid-encoded fluorophore.
B. Illustration of the experimental approach for testing the effect of consecutive arrival order on the colonization dynamics.
C. When both strains were introduced in co-infection, both colonizers remain equally abundant through the experiment (b = 0.007, $P_{t\text{-test}}$ = 0.27, $P_{Shapiro}$ = 0.33, using linear regression; see Methods for details). Nonetheless, we repeated the following experiments while alternating the roles of the tagged strains as the first and second colonizers, which showed that the choice of GPF- or mCherry-tagged strain as the first colonizer had a negligible effect on the results. The experiments presented here were selected to maximize the number of available replicates. Introduction of the colonizers with a lag of (D) 24 h (1st:GFP-tagged) or (E) 48 h (1st:mCherry-tagged) resulted in a higher density of the first incoming colonizer. The log ratio of 1st:2nd colonizer density had a positive trend in both lag experiments that was significant in 24 h-lag experiment (b = 0.011, $P_{t\text{-test}}$ = 0.028, $P_{Shapiro}$ = 0.51, using linear regression) and marginally significant in the 48 h-lag experiment (b = 0.011, $P_{t\text{-test}}$ = 0.057, $P_{Shapiro}$ = 0.17, using linear regression). Performing the experiment with the tagged clones switched in their order of arrival revealed a similar result (Fig. S4).

constant shedding of *Pantoea* from the root that is accompanied by proliferation of *Pantoea* in plant media sustained by plant exudates (Fig. S2).

To validate the presence of *Pantoea* in the roots we used confocal laser scanning microscopy (CLSM), which revealed a patchy distribution of *Pantoea* cells in the root, with clusters of bacterial cells most prevalent in crevices between the root cells (Fig. 2C). In addition, we estimated the *Pantoea* carrying capacity in the root using an alternative experimental setup where the seeds were inoculated prior to germination. This experiment revealed that *Pantoea* reached the root carrying capacity already after 24 h, likely due to the higher *Pantoea* density present already during germination; the population density remained stable over 7 days (Fig. S3).

Order of arrival is an important determinant of Pantoea colonization success

To further study the determinants of *Pantoea* colonization dynamics in the wheat root habitat, we tested for the effect of arrival time on the colonization success. For that purpose, we tagged *Pantoea* with an additional fluorophore (pTW1-mCherry (Wein *et al*., 2018)). Testing the fitness difference between the GFP- and mCherry-tagged strains using a competition experiment *in vitro* with serial transfer revealed a minor fitness advantage of the GFP-tagged strain over the mCherry-tagged strain (w = 2.5%; Table S2). The availability of the two different fluorophore tags on otherwise isogenic strains allowed us to perform *in planta* competition experiments while minimizing the impact of fitness difference between competing colonizers.

To test for fitness differences between the GFP- and mCherry-tagged *Pantoea in planta* we performed a competition experiment using the colonization documentation pipeline as described below, inoculating the roots with a 1:1 mixture of the two colonizers (Fig. 4A). The documentation of *Pantoea* population size in the daily sampled roots shows that the 1:1 ratio of the two tagged strains was maintained throughout the experiment. After 120 h, both colonizers reached a similar median density in the wheat root habitat: the GFP-tagged *Pantoea* reached a median density of 1.15×10^8 CFUs g^{-1} (n = 5) and the

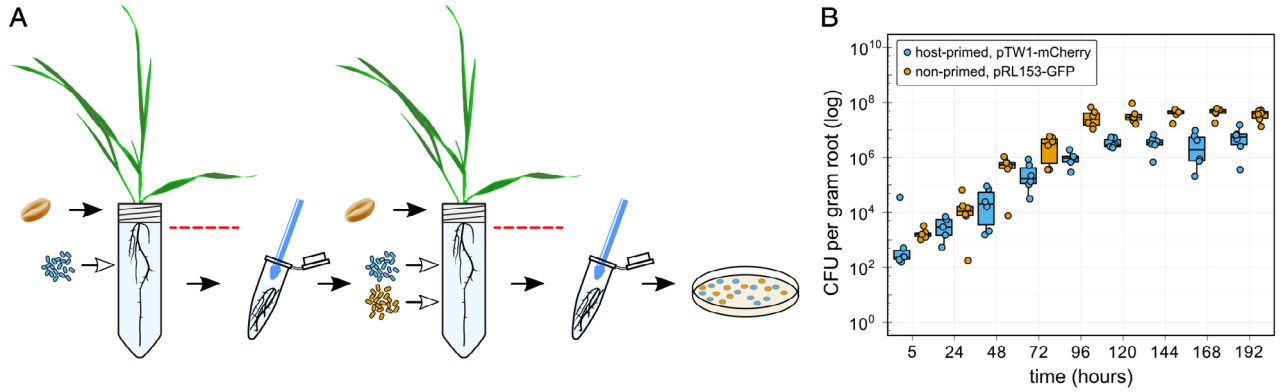


Fig. 4. Competition experiments reveal minor difference in colonization success of primed versus non-primed *Pantoea*.
A. Host-primed *Pantoea* cells tagged with mCherry were obtained directly from the root homogenate of three plants harbouring approximately 10^8 *Pantoea* cells per gram of root tissue after 7 days. Cells of the non-primed strain tagged with GFP were taken from a fresh overnight culture grown in R2A medium. Host-primed and non-primed cells were added to the media in order to infect experimental plants.
B. Quantification of growth and population size of host-primed and non-primed *Pantoea* in the wheat root habitat over time. All experiments were conducted with $n \geq 3$. The log ratio of the primed:non-primed had no significant trend ($b = -0.003$, $P_{t\text{-test}} = 0.12$, $P_{\text{Shapiro}} = 0.034$, using linear regression). Performing the experiment with the tagged clones in alternated roles revealed a similar result (Fig. S4).

mCherry-tagged *Pantoea* reached a median density of 1.25×10^8 CFUs g^{-1} ($n = 5$). Consequently, we conclude that no one of the tagged *Pantoea* had a fitness advantage over the other (Fig. 4C). Notably, the total *Pantoea* population in the roots at the end of the experiment reached a median density of 2.2×10^8 CFUs g^{-1} ($n = 5$), which is within the same order of magnitude of the bacteria density observed in the single colonizer experiment (Fig. 4C). The results of the *in planta* competition experiment thus support our estimate of the root habitat carrying capacity obtained for a single *Pantoea* colonizer (*c*. 5.7×10^8).

To test for the effect of preemptive competition in *Pantoea* colonization of the wheat roots, we conducted competition experiments where the two competitor strains (i.e. the two tagged strains) were inoculated sequentially with a delay of 24 or 48 h between the first and second incoming colonizers (Fig. 4B). Performing the experiment with a 24 h delay of the second incoming colonizer shows an initial relative density of the 1st:2nd colonizers of *c*. 12:1 (Fig. 4D) with the first colonizer median density of 4.6×10^4 CFUs g^{-1} ($n = 5$) and the second colonizer median density of 3.9×10^3 CFUs g^{-1} ($n = 5$). The difference in population size between the two colonizers increased with time and the ratio of the 1st:2nd colonizer density *in planta* had a significant positive trend over time ($b = 0.12$; $P = 0.028$, using *t*-test). At the end of the experiment, 216 h post the first colonizer inoculation, the first colonizer reached a median density of 2.8×10^8 CFUs g^{-1} ($n = 5$), while the second colonizer reached a median density of 5.9×10^5 CFUs g^{-1} ($n = 5$). The median of the total bacterial density at the end of the experiment was 2.8×10^8 CFUs g^{-1} ($n = 5$) with a relative 1st:2nd colonizer density of 475:1 (Fig. 4D).

Repeating the competition experiment with a 48 h delay of the second colonizer reveals slightly different dynamics. Directly after the inoculation, the population size of the second incoming colonizer was similar to that of the second colonizer in the 24 h experiment (median: 2.6×10^3 g^{-1} ($n = 5$); Fig. 4E). Nonetheless, the difference from the first incoming colonizer was much larger with a 1st:2nd ratio of 1030:1. The population size of both colonizers increased over the experiment duration and the ratio of 1st:2nd colonizer density had a positive trend over time (Fig. 4E). Notably, the second colonizer could not be observed in several plants during the experiment, while the first colonizer could be observed in all plants on all days (Table S3). At the end of the experiment, the first colonizer reached a median density of 2.3×10^8 g^{-1} ($n = 5$), while the second colonizer reached a median density of 1.5×10^3 g^{-1} ($n = 5$); hence, the final 1st:2nd colonizer median density ratio was 155 762:1. The log ratio of the 1st:2nd colonizers density had furthermore a positive trend over the experiment duration (Fig. 4E). Hence, the first colonizer had an advantage over the second incoming colonizer in the 48 h delay experiment. Since the ratio of both colonizer densities had a similar positive trend (i.e. slope) in the 24 and 48 h delay experiments, we conclude that a 24 h delay in second colonized arrival was sufficient for the manifestation of the first colonizer advantage in the root. Further comparison of the colonizers abundance in the media showed that the first colonizer was more abundant in comparison to the second colonizer in all competition experiments, albeit the difference between the two colonizers' abundance was smaller than what we observed in the roots (Fig. S4).

Our results show that the colonizer density in the root depends on the order of colonizer arrival where the time

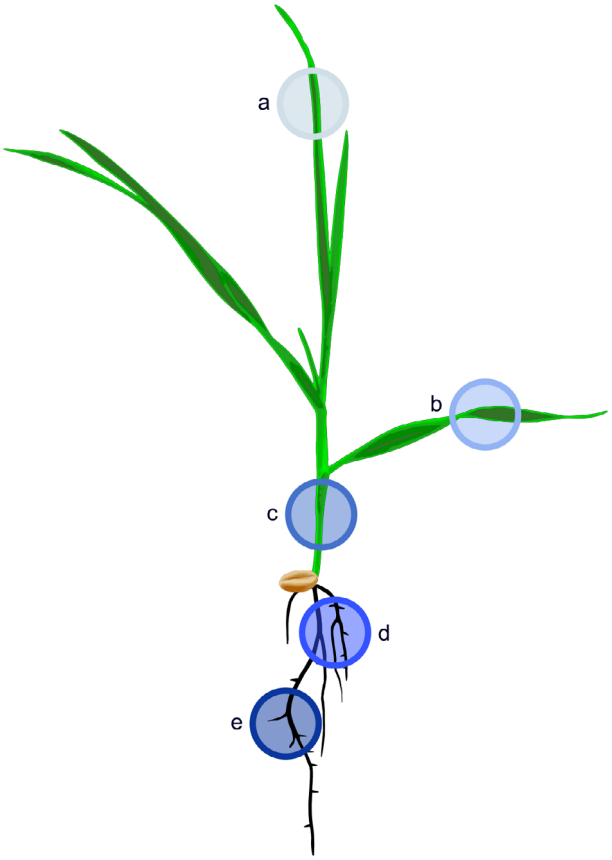
lag between consecutive colonizers is an important determinant of the colonization success. Since the root carrying capacity is finite (Fig. 3), the carrying capacity of an incoming colonizer largely depends on the root habitat occupancy. Bacterial occupancy of the seedling tissues is thus an important determinant of the establishment success of incoming colonizers.

Priming to the root habitat has little effect on the success of early incoming Pantoea *colonizers*

The stark differences we observed between the first and second incoming colonizers depending on the time of arrival may suggest the presence of short-term *Pantoea* response to the root habitat, which may contribute to the success of early incoming colonizers. To further test if the physical interaction with wheat leads to a fitness benefit of early incoming colonizers over the late incoming colonizers, we performed *in planta* competitions between *Pantoea* isolated from the root - i.e. primed for the root environment - and *Pantoea* cultivated in liquid media, i.e. non-primed to the root habitat. The priming of *Pantoea* was performed as in our standard inoculation procedure (e.g. Fig. 3A). Seven days post-inoculation the primed *Pantoea* cells were isolated from the roots and directly used in a mixture with non-primed *Pantoea* grown in liquid media to inoculate germ-free roots (Fig. 5A). The documentation of the colonizer population size directly after the inoculation shows that the initial ratio of primed to non-primed density was 1:7 (Fig. 5B). The population density of both colonizers increased over the duration of the experiment and stabilized 192 h post-inoculation. At the end of the experiment, the total population median density was 4.5×10^7 CFUs g^{-1} root ($n = 6$), which is similar to our estimate for the carrying capacity of the wheat roots. The ratio of primed and non-primed colonizers at the end of the experiment was similar to their initial ratio (1:7) and furthermore, there was no significant trend in the ratios of both colonizers over time (Fig. 5B). Furthermore, we compared the fitness of the primed and non-primed *Pantoea* in liquid media. The results of the competition experiments in media revealed a minimal growth difference between the primed and non-primed *Pantoea* that ranged between 1.2% and 1.5% (Table S3). Our results thus show that the competitive advantage of early incoming *Pantoea* colonizers unlikely to be strongly associated with short-term response to the root environment.

The pattern of long-term Pantoea *persistence in the plant reveals a preference for the root*

To further characterize the consequences of *Pantoea* colonizers in the wheat habitat, we tested for the presence



CFU per gram (fw)

location	38 days	46 days
a	70, 9×10^3	0, 4×10^5
b	0, 80	0, 1×10^5
c	1×10^6, 3×10^7	3×10^6, 3×10^8
d	2×10^8, 2×10^8	2×10^9, 2×10^9
e	6×10^7, 2×10^7	7×10^7, 2×10^7

Fig. 5. Long-term *Pantoea* persistence and density in wheat. *Pantoea* remains present within the plant host even at 40 days post-inoculation (46 days post-germination). Increased size of the host enabled sub-sampling as indicated by coloured circles (a, topmost; b, first (bottommost) leaf; c, stem (below the first leaf); d, crown roots; e, primary roots). Two plants per timepoint were sampled individually. *Pantoea* densities were determined for complete leaves and roots (see Materials and methods for details). High densities of the colonizer were found within stem and roots with especially high densities at the younger crown roots, whereas the leaves harboured notably fewer colonizer cells.

of *Pantoea* in the wheat tissues over longer time scales. For that purpose, we inoculated wheat seedlings with *Pantoea* and cultivated the plants up to 40 days post-inoculation using our standard protocol as in the colonization experiment (e.g. Fig. 2). Testing for *Pantoea* presence in the wheat tissues 32–40 days post-inoculation showed that *Pantoea* was present in the wheat plants where its abundance was variable across the plant tissues (Fig. 5). *Pantoea* was highly abundant in the stem,

while the lowest population density was observed in the leaves. The ability of *Pantoea* to migrate between plant tissues has been previously well documented (e.g. Ruppel *et al*., 1992), yet we cannot rule out aerial transfer of bacteria between the aboveground plant compartments. The highest *Pantoea* density was observed within the habitat of the primary and crown roots of the plants (Fig. 5D and E). Our results thus show that *Pantoea* is able to persist in wheat over long time-scales and is more likely to thrive in the root.

Discussion

Experimental ecology experiments performed in controlled laboratory conditions provide a rigorous framework to assess and quantify the basic ecological principles that govern the plant microbiota community assembly (Vorholt *et al*., 2017). In our study, we aimed to follow seed-borne bacteria colonization of the wheat root habitat over time. The ability to equip *Pantoea* with marker protein-expressing plasmids enabled the quantification of host colonization dynamics from single colonizer cells to growth-limited populations in real-time. Our results show that the carrying capacity of the wheat root as a habitat is finite, stable over time and has little variation among individual plants. Furthermore, the stable carrying capacity per gram root is maintained during the root growth, that is when the habitat is expanding. Notably, the bacterial densities observed in our experiments are within the range of the average bacterial density previously observed in the rhizosphere (10^6–10^9 CFU g^{-1}) and the root endosphere (10^4–10^8 CFU g^{-1}) (e.g. Wang *et al*., 2020; reviewed in Bulgarelli *et al*., 2013). Furthermore, the decreased *Pantoea* density in the aboveground plant tissues in comparison to the root is in line with other studies showing that the overall density of wheat leaf endophytes is significantly lower than the density of root endophytes (e.g. Robinson *et al*., 2016). Hence, our reductionist one host - one colonizer system appears realistic with regards to bacterial population size in the plant habitat.

The attachment and settlement process of bacteria on roots is a critical stage in the establishment of plant–microbe interactions. Bacterial chemotaxis and motility facilitate reaching the plant root surface, while cell surface structures like fimbriae, collagen-like proteins and exopolysaccharides can have key functions in the adhesion to the root surface (Castiblanco and Sundin, 2016). Our results show that the settlement process of *Pantoea* in the wheat root habitat is rapid, reproducible and robust across individual plants already 5 h after the inoculation. We note that our wheat-borne *P. agglomerans* isolate R1 exhibits swimming motility that potentially contributed to the fast attachment process we observed. The *Pantoea* root attachment process is probably driven by pili and attachment proteins (as in plant-colonizing pseudomonads (Wheatley and Poole, 2018)), likely in conjunction with or in response to chemical cues of the plant.

Our results reveal consistent *Pantoea* growth dynamics that culminate in a stable population size in the wheat root habitat over time. It is conceivable that the colonization process is orchestrated by bacterial quorum sensing (QS). Our observation of the high abundance of *Pantoea* in the plant media points to the constant shedding of cells from the roots. Bacterial shedding may be considered as a form of population density regulation in biofilm-like clusters on roots. Many plant-associated bacteria, including *Pantoea*, utilize N-acyl homoserine lactone–mediated QS to regulate traits involved in the establishment and fine-tuning of communal and host-associated lifestyles, including the formation, maturation and dispersion of biofilms (e.g. Beck von Bodman and Farrand, 1995; Koutsoudis *et al*., 2006). Hence, QS likely plays an important role in *Pantoea agglomerans* colonization and the maintenance of population structure in the host habitat. Indeed, all *P. agglomerans* isolates reported here encode an acylhomoserinelactone synthase belonging to the family of LuxI-protein and a homologue of the transcriptional regulator belonging to the LuxR-protein family (e.g. LuxI/LuxR homologues are found in the *Pantoea* isolate we used here under accession numbers WP_060681649 and WP_163799064 respectively).

Priority effects play an important role in the community assembly of horizontally transmitted bacteria in the plant microbiome (reviewed in Cordovez *et al*., 2019). For example, the order of colonizer arrival has been shown as an important determinant of the phyllosphere microbial community composition in *Arabidopsis* (Carlström *et al*., 2019). We suggest that the similar ecological principles apply for seed endophytes that migrate from the spermosphere to the plant tissue during germination. Indeed, the reduced density of *Pantoea* in the aboveground plant tissues (Fig. 5) and the variable presence of *P. agglomerans* in wheat seeds (e.g. Díaz Herrera *et al*., 2016) indicate a low fidelity in the *P. agglomerans*-wheat association over generations. At the same time, the phylogenetic reconstruction of the *P. agglomerans* we isolated from wheat seeds suggests the presence of genetic adaptation to the plant habitat in those isolates. The evolution of stable host–microbe interactions depends on the persistence of both partners over generations and hence on the transmission fidelity. Bacteria that are able to colonize the spermosphere may gain an advantage in the plant colonization upon germination due to numerical priority effects, which likely contributes to the maintenance of stable transmission. Such persistence of bacteria with the host population over generations constitutes a stable transmission that is one route for the evolution of symbiotic relations (Wein *et al*., 2019).

Materials and methods

Isolation of Pantoea *from wheat grains and seedlings*

Seeds of the winter wheat variety Runal were obtained from Delley Samen und Pflanzen AG, Delley, Switzerland. Seeds of the commercially cultivated winter wheat varieties Benchmark (I.G. Pflanzenzucht GmbH, Ismaning, Germany), and Akteur (Deutsche Saatveredelung AG, Lippstadt, Germany) were collected shortly before harvesting in August 2017 at the experimental farm Hohenschulen of Kiel University. Seeds of domesticated *T. aestivum* from Turkey were obtained from a local farm in Kışlak (Hatay Province) in 2017 (Özkurt *et al*., 2020). For the isolation of endophytically associated *P. agglomerans* a surface sterilization protocol was devised. First, wheat grains were submerged for 10 min in 3% sodium hypochlorite with occasional vortexing and then washed five times with deionized water. In order to assess the success of the sterilization, 100 μl supernatant of the final washing step was plated on non-selective R2A media plates. Grains were only considered surface-sterilized when no colony formation was observed after 48 h of incubation at 25°C. For the isolation of viable bacteria from the inside of grains, the surface-sterilized grains were incubated individually in 0.2 ml of saline amended with 0.1% Tween 20 and incubated with shaking for 3 h at 20°C. The supernatant was plated on R2A, LB and MacKonkey media plates and incubated at 20°C until colonies could be distinguished.

For the isolation of plant-borne *P. agglomerans*, seedlings were grown from surface-sterilized grains on agarose-solidified plant medium (Johnson *et al*., 2011). The surface-sterilized grains were individually incubated in 50 ml polypropylene tubes. The tubes were then placed with open lids allowing for air exchange in a phytochamber (Panasonic MLR-352H-PE) and incubated for 5–7 days under a light/dark regime (16 h light at 25°C; 8 h dark at 20°C) with 75% relative humidity. For the isolation of viable bacteria, no more than 300 mg (preferably 100–200 mg) fresh weight of plant tissue (roots and leave) were placed in a 1.5 ml Eppendorf tube and manually homogenized with a sterile pestle. After the homogenization, the pestle was washed with 0.2 ml of saline supplemented with 0.1% Tween 20 and the wash-off pooled with the homogenate. The homogenate was vortexed for at least 30 s, and serial dilutions of the homogenate were plated on R2A, LB and MacKonkey medium agar plates and incubated for 24 h at 25°C. Single colonies were purified by repeated streak-plating. Pure isolates were then characterized by 16S rRNA gene sequencing. PCR amplification of the 16S rRNA gene was performed using the primers 63F (Marchesi *et al*., 1998) and 1391R (Turner *et al*., 1999). The 16S rRNA gene PCR products were Sanger sequenced (Eurofins Genomics, Cologne, Germany) using the primers used for the amplification.

Genome sequencing and phylogenetic analysis

Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol with minor modifications. The genomes from *Pantoea agglomerans* isolated were sequenced using Illumina MiSeq2500 platform. In addition to that MinION sequencing was performed for two of the German isolates as well as the six Turkish isolates. The genomes of isolates for which both Illumina paired-end reads and MinION long-reads were sequenced were assembled using Unicycler version 0.4.4 (Wick *et al*., 2017). The genome of the remaining isolates was assembled with SPAdes version 3.9.0 (Bankevich *et al*., 2012). Assembly statistics were estimated using QUAST (Gurevich *et al*., 2013). Additionally, contigs were screened for the presence of plasmid-related sequences. To this end, we searched for homologies to the NCBI plasmids database (August 23th, 2019) using BLAST (Camacho *et al*., 2009). Hits with an *e*-value above 10^{-10} and nucleotide sequence identity of at least 80% were reported. Plasmids were identified whenever at least 59% of the total plasmid length is covered by hits. In addition, we identified the presence of the *Pantoea*-associated LPP-plasmid based on the size of the plasmid (281–794 kbp) and the presence of thiamine biosynthesis protein, as previously reported (De Maayer *et al*., 2012). The genomic assemblies have been deposited in NCBI under BioProject PRJNA578090. Phylogenies were initially reconstructed for all 322 genomes classified as *Pantoea* genus (NCBI Refseq version 10/04/2020; Table S4). Translated sequences of all CDSs in the genomes (>1.4M) were used to reconstruct protein families by sequence similarity. For that, we searched for sequence similarity across genomes by using BLAST (Camacho *et al*., 2009). Hits with an *e*-value above 10^{-10} were kept for further analyses. At the next step, we performed Needleman-Wunsch global sequence alignments of all sequence pairs (Rice *et al*., 2000) and those pairs with amino acid sequence identity ≥30% were clustered using mcl (Enright *et al*., 2002), with an inflation index of 2. We identified 50 universal single-copy proteins, performed multiple alignments with MAFFT (Katoh and Standley, 2013) and used the concatenated multiple alignment of these 50 proteins to reconstruct a splits network using SplitsTree (Huson and Bryant, 2006). To gain a clear visualization of Pantoea phylogeny, we excluded strains that are distantly related to our isolates from the analysis; only strains included in the network subsplit that contained the 11 isolates sequenced in this study and additional 130 genomes were retained for further analysis

(as shown in Fig. S1). We thereafter identified 932 universal single-copy proteins among these 141 genomes that were used to reconstruct a maximum likelihood phylogeny. The phylogeny was reconstructed by setting LG as the evolutionary model and using a partitioned strategy, where each protein can have a different evolutionary rate (Nguyen *et al*., 2015).

Growth conditions and plasmids

Pure cultures of *P. agglomerans* were routinely cultured at 25°C in R2A medium with aeration or on R2A agar plates. When required, kanamycin (15 μg ml^{-1}) was used to select for plasmid carriage. The plasmids pRL153-GFP (Tolonen *et al*., 2006) and pTW1-mCherry (Wein *et al*., 2018), both encoding resistance against kanamycin (*nptII*) and a fluorescent protein variant (GFPmut3.1 or mCherry respectively), were transferred into *P. agglomerans* strain R1 by electroporation as described (Dower *et al*., 1988).

Competitive fitness assays

The fitness of each plasmid-carrying R1 strain was determined in pairwise competition experiments *in vitro* relative to its plasmid-free ancestor counterpart, the wild-type isolate R1. For each experiment, overnight cultures grown from a single colony of the respective strain were diluted 1:100 in R2A medium and mixed in a 1:1 volumetric ratio. 0.5 ml of the mixture was then transferred into a 2 ml deep-well plate (approximately 2×10^6 cells of each competitor per ml) and the mixed cultures were incubated at 20°C overnight with shaking and afterwards diluted 1:100 following the same incubation regime for 7 days. At the onset of the experiment and each day, the ratio of the two competing strains was determined by non-selective plating.

Fitness of host-primed plasmid-carrying *Pantoea* was determined relative to the naïve (i.e. non-primed) R1 isolate carrying either pRL153-GFP or pTW1-mCherry. Primed *Pantoea* has acquired extraction from wheat roots, 7 days post-inoculation (plants cultivated according to the above-mentioned protocol). Non-primed competitors originated from liquid media culture, cultivated in parallel to plants with daily transfer.

Plasmid-carrying colonies were identified by their emitted fluorescence signal upon excitation with a green-blue LED flashlight (Nippon Genetics Europe) while wild-type colonies remained non-fluorescent. The selection coefficient s, which is equal to the selective difference between the two competing strains during each transfer, was calculated as $s = b/\ln(1/d)$ as previously described (Levin *et al*., 2000), where b is the slope of the LN ratio of the density of the competing strains as a function of transfer and d is the dilution factor (1:100). Wild-type fitness was set to 1.0, and the value of w (relative Darwinian fitness) for the plasmid-carrying strain was calculated as $w = 1 + s$.

Colonization experiments

All preparative steps and wheat colonization experiments were performed under a light/dark regime (16 h light at 25°C; 8 h dark at 20°C) with 60% relative humidity in a phytochamber. At the onset of the colonization experiments, wheat grains were submerged with gentle agitation in 50 ml of 12% sodium hypochlorite containing 0.05% Tween 20 for 30–60 min and then washed three times with 40–50 ml of sterile water. Aliquots of the last washing step were used for plating on R2A media plates in order to confirm the absence of viable bacteria. The wheat grains were then placed individually into 24-well plates and immersed (not completely submerged) in plant media containing streptomycin (25 μg ml^{-1}), spectinomycin (25 μg ml^{-1}), neomycin (15 μg ml^{-1}) and amphotericin B (3–8 μg ml^{-1}). After 2 days, the germinating seedlings were transferred individually into six-well plates and incubated in 5 ml of plant medium for approximately 4 days. When the roots had reached approximately 1.5 cm in length (usually 6–8 days after germination), the seedlings were moved onto plastic cell strainers containing a hole in the middle for the roots to pass through and mounted on 50 ml polypropylene tubes filled with plant medium with neomycin (10 μg ml^{-1}) and amphotericin B (3–5 μg ml^{-1}). All incubation steps included daily exchanges of the plant medium. For the start of the colonization experiments (day 0 or $t = 0$ h), individual plants were transferred into 5 ml plant medium containing no more than 2000 (i.e. 5×400 cells ml^{-1}) cells. The inoculation media was prepared by diluting fresh overnight cultures of *Pantoea* carrying either pRL153-GFP or pTW1-mCherry in plant medium to the desired density. The dilutions were prepared based on the optical density ($OD_{600\ nm}$) of the overnight cultures compared with a known standard (i.e. OD of representative overnight cultures of *Pantoea* with known viable titre). For each replicate the exact inoculum density was confirmed by plating on R2A media plates. Five hours post-inoculation and then every 24 h, the plants were transferred to new 50 ml tubes containing plant medium supplemented with neomycin (10 μg ml^{-1}) and amphotericin B (3–5 μg ml^{-1}), and three to six randomly chosen plants from the pool of individual plants were sampled. For the sampling, the roots were placed on sterile filter paper to absorb excess plant media and then separated at the junction to the grain/seed from the remainder of the plant using a sterile scalpel. The roots of individual plants were transferred into a 1.5 ml into 1.5 ml Eppendorf tubes, their sample mass recorded and manually homogenized using a

sterile pestle. The pestle was washed off with 0.1–1 ml saline and the volume pooled with the sample. Serial dilutions were plated on non-selective R2A agar plates to determine the CFUs of *Pantoea* per root system (i.e. per *planta*) and to distinguish between labelled populations (i.e. *Pantoea* colonizer) and potentially present other bacteria that had survived the sterilization procedure. After overnight incubation at 25°C fluorescent colonies were counted to estimate the population size of *Pantoea* sp. recovered from the plant tissue. Experiments were carried between 12 and 16 days post-inoculation.

For the long-time persistence experiment, plants were grown as described above for 38 and 46 days. For each plant, the first (bottommost), the third or fourth (topmost) leaves, the stem (between the roots and first leaf) and the roots (subdivided into primary and secondary crown roots) were sampled individually. The leaves were sampled as whole blades and cut off at the collar. Randomly sampled roots were cut off at their base and processed with their full length (i.e. they were not further cut/trimmed). Sample weight did not exceed 200 mg of raw material. Tissue homogenization and plating for determination of *Pantoea* abundance were performed as described above.

Fluorescence microscopy

Roots of wheat seedlings colonized by GFP-expressing *Pantoea* were harvested 10 days post-infection, fixed in 4% paraformaldehyde (15 min), washed in PBS and then mounted in PBS buffer using high-precision coverslips (Carl Zeiss Microscopy GmbH, Jena, Germany) and object slides with moulds. The visualizations were performed with an Airyscan detector of a ZEISS LSM 880 confocal laser scanning microscope system and an LD LCI Plan-Apochromat multi-immersion objective with a numerical aperture of 0.8 applying Immersol™ W 2010 with a refractive index of 1.334 as the immersion medium (Carl Zeiss Microscopy GmbH). The Airyscan detector was operated in the super-resolution mode. The GFP fluorescence of the bacteria was excited with 488 nm laser light and detected with a bandpass filter transmitting light with wavelengths of 495–550 nm. The root morphology was visualized using cell wall autofluorescence excited with 405 nm laser light and detected with a long pass filter transmitting light with wavelengths of 570 nm and longer. The system was operated by the software ZEISS Efficient Navigation 2, and the Airyscan raw data were processed with the automatic Airyscan processing function of this software.

Numerical priority experiments

Head-to-head *in planta* competition and preemptive competition experiments followed the colonization dynamics pipeline described above. For *in planta* competition experiments, the roots of germ-free seedlings were incubated with an inoculum comprised of an equal mixture of *Pantoea* cells carrying either pRL153-GFP or pTW1-mCherry (final inoculation density: pRL153-GFP: 42 cells per seedling; pTW1-mCherry: 58 cells per seedling). Cells used for the inoculum were obtained from fresh-overnight cultures grown under selection for plasmid carriage. For preemptive competition experiments, germ-free seedlings were first inoculated with *Pantoea* carrying pTW1-mCherry (inoculation density: 41 cells per seedling). The plants were incubated for 24 or 48 h (the 48 h incubation period followed the regime of daily exchange of the growth medium), and then transferred into fresh plant medium with *Pantoea* carrying pRL153-GFP (inoculation density: 24 h: 160 cells per seedling; 48 h: 200 cells per seedling). After 5 h of incubation, the plants were moved to a fresh medium and treated for the remainder of the experiment as described above.

Adaptive priority experiments

For the preparation of host-primed *Pantoea* cells, the roots of 6-day-old germ-free plants were inoculated with *Pantoea* carrying pTW1-mCherry and the plants were grown for another 6 days (120 h) until the bacterial population had reached a stable population size on the roots (estimated as described above). CFU counts per gram of root tissue obtained from five replicate plants were used to infer an estimate for the extraction of host-primed cells. For the extraction, the roots of five plants were processed. In parallel to the host-priming treatment, the second colonizer population (*Pantoea* carrying pRL153-GFP) was serially passaged (1:100, every 24 h) in a liquid R2A medium. Population size estimates for *in vitro* cultured cells were obtained by plating serial dilutions. An equal mixture containing host-extracted (i.e. primed) and *in vitro*-cultured (i.e. naïve) *Pantoea* colonizer populations was prepared and used for colonization experiments following the pipeline described above.

Experimental data analysis

Statistical analysis of the experimental data was performed using R (R Core Team, 2020). Linear regression analysis was performed using lm function with the time of measurement (in hours) as the explanatory variable and the log ratio of first to second colonizer (or primed to non-primed) as the response variable. The linear regression quality was further assessed by testing for a normal distribution of the residuals using the Shapiro–Wilk test of normality with the shapiro.test function.

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Data Availability Statement

Genome sequences of the *P. agglomerans* isolates described in this study have been deposited at NCBI GenBank under BioProject number PRJNA578090.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information.

Table S3. Bacterial counts and plant masses in all experiments.

Data supplied in a supplementary excel table.

Table S4. List of genomes used for the phylogenetic reconstruction.

Data supplied in a supplementary excel table.