

Establishment of a system for bacterial colonization experiments in wheat

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Declaration

I hereby declare that the thesis entitled "Establishment of a system for bacterial colonization experiments in wheat" has been carried out in the Institute of General Microbiology at the Christian-Albrechts University of Kiel, Kiel, Germany, under the guidance of Prof. Dr. Tal Dagan and Dr. Nils Hülter. The work is original and has not been submitted in part or full by me for any degree at any other University. I further declare that the material obtained from other sources has been duly acknowledged in the thesis. My work has been produced in compliance to the principles of good scientific practice in accordance with the guidelines of the German Science Foundation. I hereby assure that I have not been revoked any of my academic degrees.

Kiel, 17.03.2020

Ryszard Soluch

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1 Abstract

Eukaryotes, including plants, are hosts to microbes. These microbes can be recruited from the environment as well as transferred between the generations horizontally or vertically. We hypothesize that in wheat, due to the priority effects, vertically transferred microbiota has priority advantage over bacteria recruited from the environment.

Isolation efforts on *Triticum aestivum* seeds and seedlings have yielded few cultivable bacterial strains present both on and in the seed and seedling. *Pantoea agglomerans* was the species found in most of the samples, independent of the geographical origin (Germany, Turkey).

Here we show that the GFP-tagged strain of *Pantoea agglomerans*, originally isolated from wheat, maintains its ability to colonize germ-free wheat roots and to proliferate within the root. Independently of the inoculum size used, log-phase-like population growth within the root was observed. Afterward, while the population growth was still ongoing, any further increase in the population size was tied to the growth of the root habitat. Once the population count was standardized based on root fresh weight, the plateau was observed within 8 days post-inoculation, showing that the carrying capacity of the host is limited.

In planta competitions between GFP and mCherry tagged *Pantoea* strains were performed to check for numerical priority effects and adaptive priority effects. Competing strains were introduced with a time lag between them, or one of the strains was pre-cultivated in a plant, while the second was cultivated in liquid media.

Our results show a strong impact of the priority effect. The numerical advantage of primary colonizer led to its high relative abundance. No adaptive advantage was observed. Compared to our single colonizer experiments, total carrying capacity of the host was unchanged.

Persistence of *Pantoea* in the plant host was tested up to 46 days post-inoculation. Density of the population varies between the plant compartment, reaching highest CFU per gram of tissue in the roots, lowest in the leaves.

My observations support the hypothesis of vertical transfer of *Pantoea agglomerans* and its association with host lifecycle (ability to colonize the seedling from the seed while outcompeting soil-borne *Pantoea*).

This data provides the fundament for future experiments on mechanistic aspects of wheat colonization by *Pantoea agglomerans*, which can be performed by analysis of the bacterial genome and preparation of knock-outs. The established gnotobiotic system enables further competition and exclusion experiments.

2 Zusammenfassung

Eukaryoten, einschließlich Pflanzen, bewirten Mikroben. Diese Mikroben können sowohl aus der Umgebung rekrutiert als auch horizontal oder vertikal zwischen Generationen transferiert werden. In dieser Arbeit wird die Hypothese aufgestellt, dass vertikal transferierte Mikrobiota durch Prioritätseffekte einen Vorteil gegenüber aus der Umgebung rekrutierter Bakterien haben.

Isolationen auf *Triticum aestivum* ergaben kultivierbare Bakterienstämme, die sowohl auf Samen und Sämling vorhanden waren. Die Spezies, die dabei in den meisten Proben gefunden wurde, war unabhängig vom geografischen Ursprung (Deutschland, Türkei) *Pantoea agglomerans*.

In dieser Arbeit wird gezeigt, dass GFP-markierte Stämme von *Pantoea agglomerans*, welche ursprünglich von Weizen isoliert wurden, ihre Fähigkeit der Kolonisierung von keimfreien Weizen behalten und sich in der Wurzel stark vermehren. Unabhängig von der Größe des Inokulums wurde exponentielles Wachstum in der Wurzel festgestellt, welches in seiner Dauer an limitierende Eigenschaften der Wurzel gekoppelt war. Bei standardisierter Population in Relation zum Gewicht der Wurzel wurde das Plateau innerhalb von acht Tagen nach der Inokulation erreicht.

GFP- und mCherry-markierte *Pantoea*-Stämme wurden in planta verglichen, um numerische und adaptive Prioritätseffekte zu untersuchen. Dafür wurden verschiedene Stämme mit Zeitversatz oder mit unterschiedlichem Ursprung aufgetragen. So wurde der erste Stamm in einer Pflanze, der zweite in einem Flüssigmedium kultiviert.

Meine Ergebnisse zeigen einen starken Einfluss des Prioritätseffekts – der erste Kolonisierer hat eine wesentlich höhere Population als der zweite, während die Gesamtkapazität der Wirtspflanze unverändert bleibt. Es wurden keine adaptiven Vorteile festgestellt.

Die Persistenz von *Pantoea* in der Wirtspflanze wurde bis zu 46 Tage nach der Inokulation untersucht. Die Populationsdichte ist abhängig vom Gewebetyp, wobei der höchste CFU pro Gramm in den Wurzeln, der niedrigste in den Blättern gemessen wurde.

Die in dieser Arbeit angestellten Untersuchungen unterstützen die Hypothese des vertikalen Transfers von *Pantoea agglomerans*. Die Daten bilden das Fundament für Experimente zur Bestimmung weiterer Aspekte der Weizenkolonisierung durch *Pantoea agglomerans*, welche auf der Analyse von bakteriellen Genomen oder Knockouts basieren können. Der Bereich der gnotobiotischen Systeme ermöglicht weitere Wettbewerbs- und Exklusionsexperimente.

3 Introduction

3.1 Plant bacteria interactions

Plants are habitat to bacteria that can colonize their tissues (figure 1) where the level of persistence and fidelity varies depending on plant-bacteria-environment interplay. Interactions between plants and bacteria range from pathogenic interactions, e.g., as in the Olive-*Xyliella fastidiosa* (Redak et al. 2004) to beneficial symbiosis as in plant-bacteria interactions that are based on the supply of fixed nitrogen to the plant host (e.g., Azolla fern and its *Nostocaceae* symbiont (Strasburger 1873; Baker et al. 2003)), or *Bradyrhizobium japonicum* and Legum (Zahran 1999). Recent studies pointed out bacteria to have a role in growth promotion (e.g., (Spaepen et al. 2009)) or defense against pathogens (e.g., (Stockwell et al. 2010; Ruiz et al. 2011; Town and Dumonceaux 2016)). Most of the bacterial inhabitants of plants are opportunistic (and non-pathogenic) colonizers (Bulgarelli et al. 2013; Edwards et al. 2015).

The mode of bacterial colonization highly depends on both interaction partners. Bacterial colonization of the plant habitat may include migration from the environment (e.g., soil) into the plant tissues; root (e.g., (Amellal et al. 1998; Dance 2008)), leaves (e.g., (Mercier and Lindow 2000; Björklöf et al. 2000)) or flowers (e.g., (Mitter et al. 2017)). Alternatively, some bacterial organisms persist with their plant host along their life cycle (e.g., *Nostoc-Azola* association (Pereira 2017)). Bacteria that can colonize the seed (i.e., seed endophytes) are thought to possess the ability to persist with their plant host through their life cycle (Okunishi et al. 2005; Truyens et al. 2015; Díaz Herrera et al. 2016). Few examples of plant-bacteria interactions are summarized in table 1.

Some seed endophytes can have plant growth-promoting properties, for instance, *Pantoea agglomerans* isolated from switchgrass seeds (Kim-Dura et al. 2016) or members of *Paenibacillus* genus isolated from wheat seeds (Díaz Herrera et al. 2016). The aforementioned *Paenibacillus* and *Pantoea* isolates, furthermore show antagonistic activity against plant pathogens (Díaz Herrera et al. 2016). *Pantoea agglomerans* isolated from a variety of sources shows antipathogenic properties (Braun-Kiewnick et al. 2000; Ruiz et al. 2011; Smits et al. 2015). Bacterial presence in the seed is crucial for maintaining the vertical inheritance.

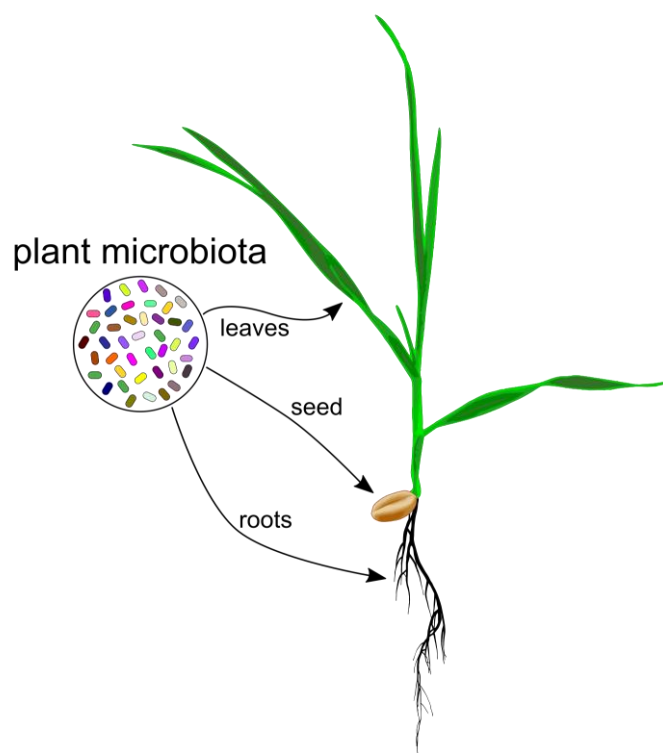


Figure 1. Plant associated bacteria are able to colonize various parts of the plant host.

The density of colonizing bacteria may vary among different plants and their tissues. For instance, the density of bacteria in the styrian oil pumpkin endorhiza is around 10^4 CFU/g (colony forming units per gram of tissue), in anthosphere (flowers) it reaches 10^7 CFU/g, in carposphere (fruit) it reaches 10^3 - 10^4 CFU/g and in spermosphere (seed) the bacterial density is less than 10^2 CFU/g (Fürnkranz et al. 2012). Bacteria colonization of the root is not uniform, as shown in seminal roots of winter wheat, where symbiotic *Pseudomonas fluorescens* reached 10^5 to 10^6 CFU per cm in the root near the seed, and 10^3 to 10^5 CFU per cm near the tip of the root (Weller 1984). The composition and abundance of the wheat microbiota, not only depends on plant tissue but also on plant age, soil nutrients and fertilizing (Robinson et al. 2016a; Kavamura et al. 2019).

The density of the bacterial colonizer of the plant may be also related to its function. For instance growth-promoting microorganisms are considered to be mainly soil- and rhizosphere-derived organisms, that can colonize plant roots in significant numbers (10^5 – 10^7 CFU per gram of fresh root) (Spaepen et al. 2009). The density in other parts of the host is different and stays within a low range of 10^3 - 10^4 CFU/g; in comparison, pathogens reach a high density, e.g., up to 10^7 - 10^8 CFU per gram of dead tissue (Spaepen et al. 2009).

Table 1. Examples of plant-bacteria interactions. Yet unknown characteristics are marked by n.a.

Host			Symbiont					References.
Organism	Dependency	Bacterium	Currency supplied	Dependency	Transmission mode	Localization	Trophic state	
<i>Arabidopsis thaliana</i>	Facultative	<i>Xanthomonas</i> , <i>Stenotrophomonas</i> , and <i>Microbacterium</i> spp.	Protection of the off spring	Facultative, recruited as a response to fungal pathogens	To daughter plants by soil	Root, extracellular	n.a.	(Berendsen et al. 2018)
Styrian oil pumpkin (<i>Cucurbita pepo</i> L. subsp. <i>pepo</i> var. <i>styriaca</i> Greb.)	Facultative	<i>Bacillus</i> sp., <i>Pseudomonas</i> sp.	Protection from <i>Pectobacterium carotovorum</i> , <i>Pseudomonas viridiflava</i> , <i>Xanthomonas cucurbitae</i> , <i>Didymella bryoniae</i>	Facultative	Possibility of vertical transfer	Endophyte (spermosphere, endorhiza, anthosphere, carposphere)	n.a.	(Fürnkranz et al. 2012)
Winter wheat (<i>Triticum aestivum</i> L.)	Facultative	<i>Pseudomonas fluorescens</i>	<i>Gaeumannomyces graminis</i> (pathogen) supression	Facultative	n.a.	Rhizosphere	n.a.	(Weller 1984)
<i>Azolla</i> fern	Obligatory	<i>Nostocaceae</i>	Fixed nitrogen	Facultative	Vertical	Endophyte	n.a.	(Baker et al. 2003)
Legume	Facultative, high impact on host fitness	<i>Rhizobium</i> sp.	Fixed nitrogen	Facultative	Vertical	Endophyte, root nodules, bacteroid inside symbiosome (endocellular)	N- depleted environment	(Zahran 1999)
<i>Oryza sativa</i>	Facultative	<i>Pantoea agglomerans</i>	Protection agains pathogens, improved germination rate	Facultative	Possible horizontal (present on the seed)	Rhizoplane (root surface)	n.a.	(Ruiz et al. 2011)
<i>Saccharum officinarum</i> L. (sugarcane)	Facultative	<i>Acetobacter diazotrophicus</i>	Nitrogen fixating	n.a.	n.a.	Endophyte (intercellular spaces of sugarcane)	n.a.	(Dong et al. 1994)

The process of root colonization is a very important step towards colonization of the host. Once the roots are colonized, bacteria can colonize other plant tissues, as shown in rice (Chi et al. 2005) and wheat (Ruppel et al. 1992). Root colonization is driven by rhizodeposition (Bulgarelli et al. 2013), meaning that host provides nutrients for incoming colonizers in form of root exudates. The process of colonization was observed in more detail in rice, where GFP-tagged *Azorhizobium caulinodans* ORS571 was tracked in plant tissues following inoculation on the root. Within 15 days post inoculation bacterial density within the root and rhizosphere was close to 10^7 CFU per gram of fresh weight of tissue, reaching lower densities in leave, and leaf sheaths. Population in roots remained stable throughout the experiment (60 days) (Chi et al. 2005).

3.2 The wheat microbiota

Early studies on the composition of endophytic communities in the seed, showed high similarity between the bacterial community of the plant and the soil, which led to the hypothesis of soil-borne bacteria internalizing into a plant through mechanical damage sites (Mundt and Hinkle 1976). These studies based on isolation and culturing failed to show the true picture of plant microbiota due to the technical limitations (low number of cultivable bacterial species and disproportion of bacterial growth on synthetic media compared to *in planta* growth). Only more recent studies, using a metagenomic approach, enabled more precise insight into the composition of the plant microbiota. Using this approach I have learned that most of the isolated plant bacteria belong to *Proteobacteria*; while *Firmicutes*, *Actinobacteria* and *Bacteroides* are less abundant (Reinhold-Hurek and Hurek 2011).

Wheat microbiota composition undergoes changes depending on many factors like plants' age, soil type, tissue type, time of the year, fertilizer, and other factors. While the exact composition shifts thought the year, roots appear to favor γ -proteobacteria while leaves show not only dominance of *Firmicutes* and *Actinobacteria* but harbor decisively lower number of bacteria (Robinson et al. 2016a; Kavamura et al. 2019). It is noteworthy that while seed embryo appears to be germ-free in healthy seeds, seeds themselves are inhabited by γ -proteobacteria (Robinson et al. 2016b). The transfer of microbiota from the seed to the seedling (especially to the aerial parts) is not well studied (Shade et al. 2017). I hypothesize that horizontal transfer of microbiota (figure 2) is still possible, if grain microbiota is first to colonize the seedling during the germination.

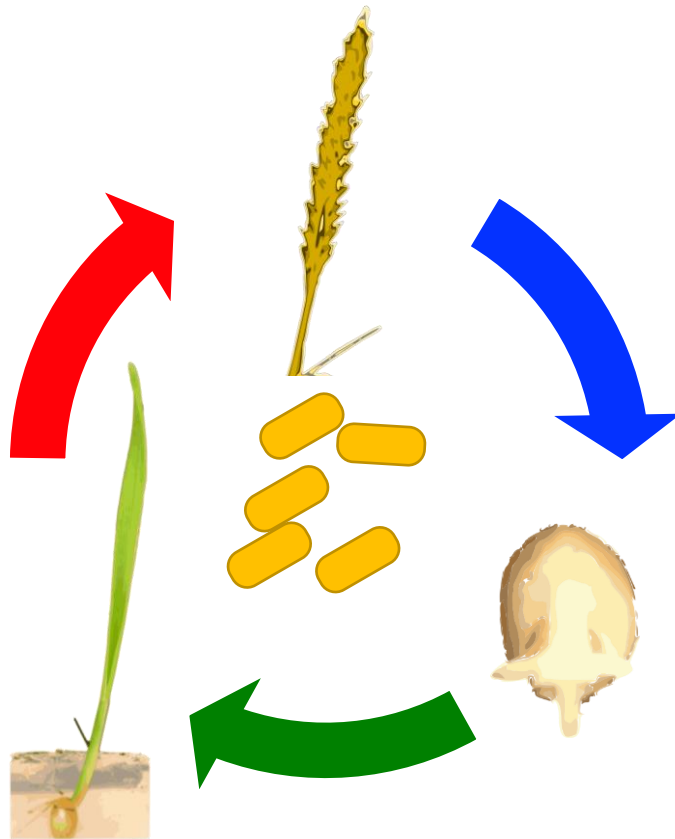


Figure 2. Wheat symbiotic bacteria are able to colonize the roots of the plant from the seed. In order to maintain vertical transmission, they must possess the ability to reach aerial the flowers, where seeds are formed. Then bacteria can be spread with the seed and colonize newly growing plants.

3.3 *Pantoea agglomerans*

In this thesis, I focus on one member of the genus: *Pantoea agglomerans* (figure 3), which is widely occurring epiphyte/endophyte of plants. In addition, it can be found abundantly in other habitats including water, soil, dust, air, animals, including humans (Dutkiewicz et al. 2015). One of the unique traits of this bacterium is reflected in its name: *agglomerans* ('forming into a ball'), refers to its ability to form symplasmata (figure 3b). These are bacterial agglomerates, which are considered, by some, a form of bacterial multicellularity (Achouak et al. 1996; Tecon and Leveau 2016).

Before exploring its biological significance and properties, it is worth mentioning that multiple changes in the taxonomic classification of *Pantoea agglomerans* took place over the years. At first described as *Bacillus agglomerans* by Beijerinck (Beijerinck 1888), *Pantoea agglomerans* had been known under different names: *Bacterium herbicola* (Löhnis 1911), *Erwinia herbicola* (Löhnis 1911; Dye 1964), *Bacillus milletiae* (Kawakami and Joshida 1920), *Pseudomonas herbicola* (Geilinger 1921; de'Rossi 1927), *Corynebacterium beticola* (Abdou) (Collins and Jones 1982), *Enterobacter agglomerans* (Ewing and Fife 1972), and other (Bergey et

al. 1957; Jabłoński 1971; The UniProt Consortium 2019). The complicated 'taxonomic history' of *Pantoea agglomerans* and, additionally, the use of metabolic phenotyping for identification (especially in clinical studies) led to difficulties in revealing the true identity of isolates. This includes isolates with pathogenic potential (Rezzonico et al. 2009; Rezzonico et al. 2012). As 16S rDNA sequencing became more affordable and widely used, researchers have turned to this method. The identification of *Pantoea agglomerans* using molecular methods is more trustworthy, unfortunately clinical diagnostics are still lagging behind (Analytical Profile Index – biochemical method of bacterial identification is still commonly used instead). While the existing body of literature cannot be easily updated, there are efforts to standardize the historical taxonomy of *Pantoea agglomerans* (Tindall 2014).

The genus *Pantoea* consists of yellow pigmented, Gram-negative, γ -proteobacteria, which are adapted to interact with various eukaryotic organisms, be it as pathogens, saprophytes or symbionts (Walterson and Stavriniades 2015). Wheat and rice, are just two of a plethora of hosts that are found in association with members of the genus *Pantoea*.

Pantoea originating from maize, was compared to other microbiome members of this plant and alongside of *Enterobacter* and *Pseudomonas*, showed the widest array of symbiotic and plant growth promoting traits. *Pantoea*'s symbiotic lifestyle is further exemplified in a study of bacteria associated with the maize cultivar Jala, which is described in the above mentioned paper, where *Pantoea ananatis* is proved to be the sole cultivable colonizer (99% of colonies) (Johnston-Monje and Raizada 2011).

Moreover, genus *Pantoea* is also prevalent in the example of the microbial composition of raw silage made from wheat. In the pre-ensiled plants, genus *Pantoea* makes 34.7% of the total bacterial population. After 6 hours of fermentation, its abundance is slightly increased (34.9%) only to decrease down to 0.16% at the final stage of ensiling (Keshri et al. 2019). This further shows that *Pantoea agglomerans* is abundant in wheat and it is adapted for association with a live host.

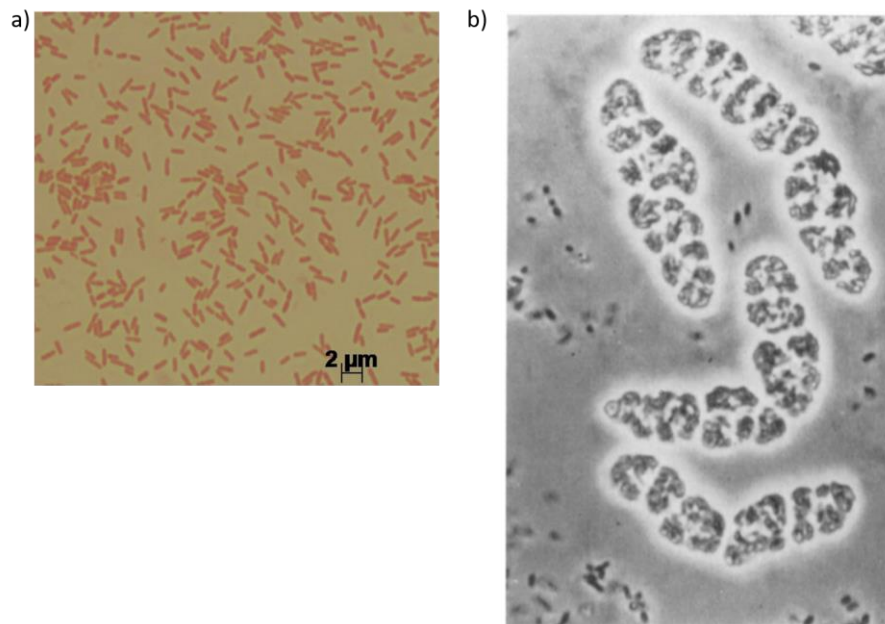


FIG. 3. Phase contrast microscopy of condensate of agar slant culture. Organisms are formed in characteristic elongated spheroidal aggregated masses ("symplasmata"). $\times 800$.

Figure 3. Micrographs of *Pantoea agglomerans*: a) Micrograph of *Pantoea agglomerans* MP2 stained with safranin. This strain was isolated from termite (Palmer et al. 2016); b) aggregate of *Pantoea agglomerans* is called symplasmata, cells are covered with polysaccharides (Bottone and Schneierson 1972)

Pantoea agglomerans can colonize plant surfaces, xylem and intercellular spaces (Remus et al. 2000). Some recognize it as a plant growth promoting rhizobacterium (PGPR). It is showing wide repertoire of PGPR-traits, including indole-3-acetic acid (IAA) production, phosphorus solubilization, 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)). *Pantoea agglomerans* also possesses the ability to prime plant immune system (Ortmann and Moerschbacher 2006).

It has been shown, that *Pantoea agglomerans* has a positive impact on seeds by improving germination rates of rice seeds (Ruiz et al. 2011). Also, it increases plant biomass production for a variety of plants, including sugarcane, wheat (increases yield of grains) and peas (Höflich and Ruppel 1994; Remus et al. 2000; Quecine et al. 2012). Higher biomass yield may be accompanied by improved quality of the final product and enrichment in desired compounds. This was observed in peppermint, where field inoculation with a mixture of *Pantoea agglomerans* and *Pseudomonas putida*, not only increased the biomass yield but also the quality of the crop, compared to phosphate fertilizer treatment (Seif Sahandi et al. 2019).

All these properties made the bacterium attractive to use for farming: some strains (such as *Pantoea agglomerans* P10c) are being used for biocontrol in modern agriculture (Town and

Dumonceaux 2016). Strain P5 is commercially available as bio-fertilizer under tradename PhosphoBarvar-2 (Shariati J. et al. 2017). *Pantoea* is registered by the United States Environmental Protection Agency as a microbial pesticide, and its residues are allowed on some foods (Environmental Protection Agency 2006). Possibility of use of a honeybee as a vector for spreading bio-fertilizer *Pantoea agglomerans* was investigated (Vanneste et al. 2002). Noteworthy, *Pantoea agglomerans* is capable of nitrogen fixing (both in pure culture and in association with wheat). Once radioactive isotopes of fixed nitrogen were tracked, it was shown, that only miniscule amounts were found in the plant (Merbach, Ruppel, Schulze 1998).

The safety regulations of *Pantoea agglomerans* usage remain unclear. Existing concerns go back to many cases where *Pantoea* was wrongly identified as a human pathogen, mostly due to lack of proper DNA-based identification method in a clinical environment (Rezzonico et al. 2009). For *Pantoea agglomerans* P5, the presence of potential outer-membrane protein secretion systems was confirmed. Of special interest are III, and VI secretion systems since these systems are often used for interaction with the host and may be related to pathogenicity (Shariati J. et al. 2017).

The presence of plasmids seems to play an important role in *Pantoea* genus. *Pantoea stewartii* can carry between 11 and 13 plasmids, which can make up even 25% of the total DNA of this bacterium (Coplin et al. 1981). The largest plasmid of *Pantoea*, is hypothesized to carry functions that have an impact on the bacterium host-associated lifestyle. The large *Pantoea* Plasmid 1 (abbreviated to LPP-1) carries genes responsible for versatile metabolism; host specificity, colonization and persistence, thus, it may be crucial for *Pantoea*'s ubiquitous presence in the environment. The plasmid is sized between 281 and 794 kb, which is between 5.6% and 12.6% of the total genome content of the bacterium. *Pantoea*'s LPPs are divided into three groups based on phylogeny of four housekeeping genes. All three groups of LPPs have a shared ancestor and the phylogeny of the plasmid closely resembles phylogeny of the bacterium itself (based on a chromosome) (De Maayer et al. 2012), implying, that the plasmid may not be easily transmissible. Noteworthy, outcome of host-bacterium interaction in maize varies from symbiotic to mildly pathogenic, even if LPP-1 has identical sequence (Sheibani-Tezerji et al. 2015). The LPP-1 from *Pantoea agglomerans* belongs to group I, alongside of LPPs from *Pantoea anthophilia*, *Pantoea eucalypti*, and *Pantoea vagans*. Flexible portion (non-conserved protein coding sequences) of the pan-plasmid in LPP-1 group I is 73.8% (709 coding sites), suggesting massive genetic diversification (De Maayer et al. 2012). There are still many unknowns regarding *Pantoea*'s plasmid role as a symbiont of plants.

Pantoea sp. and Triticum aestivum

Pantoea agglomerans can colonize wheat roots (Amellal et al. 1998) and possesses the ability to proliferate within bread wheat (*Triticum aestivum*) and spread to different plant compartments (Ruppel et al. 1992). Further experimental work showed that it can spread to the seedlings, grown from infected seeds (Ferreira et al. 2008).

Pantoea agglomerans has been frequently isolated from wheat, especially when the plant is grown in challenging conditions. For instance, wheat grown in Africa's irrigated field, characterized by their high salinity, was host to *Pantoea agglomerans* Pa. This strain is able to grow in temperatures up to 35°C, pH ranging from 5 up to 10, or in the presence of 30% polyethylene glycol, it tolerates up to 1 M of NaCl in growth media. This isolate has a variety of plant growth promoting traits, such as auxin biosynthesis, production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and ammonia and phosphate solubilization. These PGPR (plant growth promoting rhizobacterium) features were maintained in sodium chloride concentrations up to 400 mM. Overall, this isolate showed the best plant growth-promoting properties of all 22 wheat bacterial isolates tested during this study. Authors confirmed its endophytic lifestyle and ability to survive in non-sterile seeds (Cherif-Silini et al. 2019). *Pantoea agglomerans* wheat-originated isolate from Uzbekistan was shown to have growth-promoting properties as well (Egamberdieva et al. 2008). When another isolate of *Pantoea agglomerans* was screened for traits that could enable its use as a biofertilizer, on top of anticipated plant growth promoting traits, its ability to solubilize zinc, important micronutrient, was shown (Kamran et al. 2017).

As mentioned before, *Pantoea agglomerans*, can survive within the grain, which makes it activity against pathogenic *Fusarium graminearum* (Díaz Herrera et al. 2016). This is very important trait for cost-effective protection of wheat.

Pantoea agglomerans not only protects against pathogens by inhibiting their growth directly but also primes wheat defenses without eliciting the immune reaction itself. In other words, it prepares plants for contact with pathogens, in turn, making the oxidation burst and other defense reactions stronger. Noteworthy, the elicitation mechanism works differently between monocot and dicot plants (Ortmann et al. 2006; Ortmann and Moerschbacher 2006). I do not know enough to say whether these differences in elicitation levels are related to host specialization.

It has been shown, that not every wheat variety carries *Pantoea agglomerans*, hinting at possible host-related requirements for effective colonization (Comby et al. 2016) , or alternatively, that the *Pantoea* function in the wheat microbiome may be replaced by other genera.

4 Objectives

In this PhD thesis, I aimed to study the wheat (*Triticum aestivum*) root colonization dynamics during the early stages of germination. For that purpose, I focused on one member of the wheat microbiota, namely *Pantoea agglomerans*, that I isolated from wheat seeds. Here I study the bacterial colonization dynamics within an ecological framework with the main aim of evaluating the importance of priority effects in the *Pantoea* colonization of wheat roots.

Priority effects in colonization exist when the order of arrival of the colonizers influences the abiotic and biotic interactions within the habitat (De Meester et al. 2016). In the case of colonization of the wheat plant by bacteria from the grain, I anticipated either numerical or adaptive priority effect (figure 4), which could give seed-borne bacteria evolutionary advantage.

Numerical priority effect means that the early incoming (seed-borne) bacteria have to be at an advantage over the late (soil-borne) incomers by being present in the vicinity of the growing seedling first. This translates to faster access to the host, giving more time to proliferate and start benefiting from access to available resources (compared to soil-borne bacteria). Once a robust population is established, secondary colonizers will not be able to outcompete primary colonizer.

I hypothesize that the mechanism for adaptive priority effect would be based on the benefit of being pre-exposed to the plant host. 'Primed' bacteria would be able to colonize the seedling faster and more efficiently than soil-borne bacterial colonizer. Both effects assume that the host has a finite carrying capacity and stronger initial presence would benefit in stronger impact of priority effect.

Alternatively, colonization history or other factors such as host immune regulation may be involved in determining the community assembly process. In the latter case, the order of arrival is expected to have only a small effect on the microbiota composition. Here I establish system to follow the domesticated wheat and a seed-borne bacterium, *Pantoea agglomerans*, enabling its use as a model for observation of the root colonization.

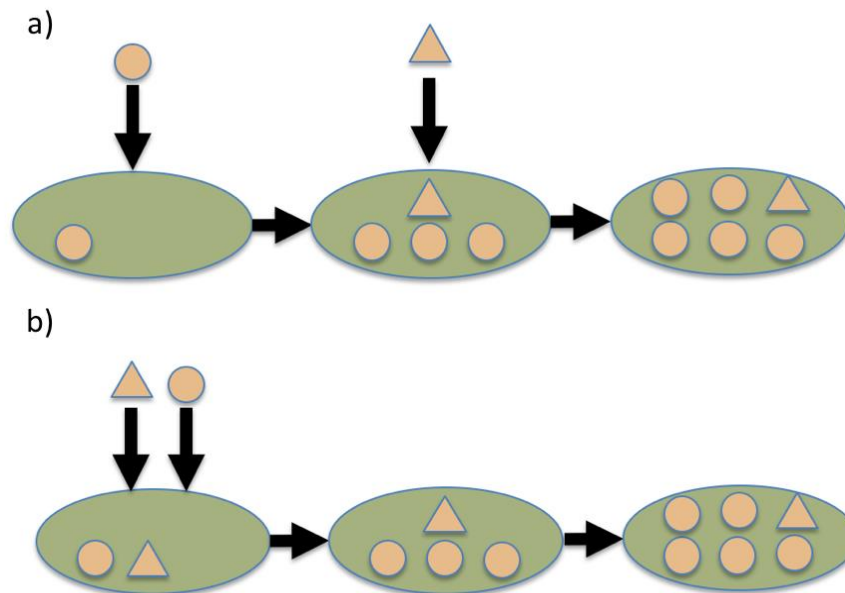


Figure 4: Priority effects. a) Numerical priority effect; first-arriving colonizer has time to proliferate and occupy (partially) the niche using up available resources, secondary colonizer is unable to out-compete primary colonizer. b) adaptive priority effect; one of the colonizers shows better adaptation or is preadapted to the niche; modified from (De Meester et al. 2016)

5 Methods

5.1 Isolation of total plant DNA for identification of endosymbionts and surface microbiota

To identify the wheat associated bacteria, up to 200 mg of fresh plant tissue (shoot or roots) was placed in an Eppendorf tube and manually homogenized using autoclaved plastic pestle. Total DNA was extracted from the fresh homogenate using the GeneJET Plant Genomic DNA Purification Kit (Thermo Scientific) according to the manufacturer's protocol. Briefly, 350 μ l of lysis buffer A was added to the homogenate, followed by 50 μ l of lysis buffer B and 20 μ l RNase. The solution was mixed and incubated at 65°C for 10 minutes. Afterwards 130 μ l of precipitation solution were added, mixed by inversion and incubated for 5 minutes on ice. The supernatant was collected by centrifugation for 5 minutes (20,000 x g), transferred into a clean Eppendorf tube and mixed with 400 μ l of 96% ethanol and 400 μ l of Plant gDNA Binding Solution. The solution was transferred onto a spin column, centrifuged for 1 minute (6,000 x g), washed with 500 μ l of Wash Buffer I and 500 μ l of Wash Buffer II. DNA elution was performed with 100 μ l of elution buffer or nuclease free water.

Amplification of bacterial 16S rDNA from plant samples

Isolated DNA was diluted with nuclease-free water (Roth) to a concentration of 100 ng/μl (concentration was estimated using ThermoScientific NanoDrop microvolume spectrophotometer). Primers used for PCR were designed for amplification of bacterial 16s rDNA from plant samples, 799F (Chelius and Triplett 2001), and 1114R (Simon et al. 2009) or 1391R (Schumann 1991; Turner et al. 1999). Usage of these primers generates two major PCR products: of bacterial and of plastid origin. These products can be distinguished by size, enabling separation by preparative gel electrophoresis.

Conventional PCR reactions were prepared as follows (for a single reaction volume of 50 μl): 29.6 μl of PCR water, 10 μl of High-Fidelity Buffer (5x), 2 μl of each primer (from 20 pmol/μl stock solution, final concentration 0.8 pmol), 1 μl of dNTPs (from a 2 mM stock, final concentration 0.04 mM) and 0.4 μl of Phusion polymerase (0.8 U per 50 μl reaction). When appropriate, the DreamTaq Green PCR Master Mix (2x) was used with exact same final primer concentration. Cycling conditions were set to the following protocol (with elongation and final elongation steps lasting three times longer for DreamTaq PCR Master Mix). All reactions were performed in PEQStar Thermocycler (PEQLAB)

96°C	3 min	
96°C	1 min	
56°C	20 sec	30x
72°C	20 sec	
72°C	3 min	

Following, PCR products were separated using agarose gel electrophoresis (2.5 - 3% gel) and fragments of interest excised and extracted from the gel using the GeneJET Gel Extraction Kit (Thermo Scientific) or the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. Purified products were used for sequencing, re-amplification, or stored at - 20°C.

5.2 Sampling, isolation and identification of culturable bacteria from grains, lab grown plants and field samples

Grain sampling

Samplings were performed in July 2017 Experimental Farm Hohenschulen of Kiel University (location: latitude: 54° 18' 46" N, longitude: 9° 59' 32" E).

Shortly before the harvest, whole ears were collected by cutting the stem approximately 2 cm below the ear with non-sterile scissors. Every 20 ears collected the sampling point was changed, all sampling points were at least 2 meters from the edge of the field. Each collected variety was packed in a separate cotton bag. All grains were manually extracted from the ears within 3 days post sampling. Afterwards, grains were placed in a small cardboard box and stored at room temperature at low humidity until processed.

Aseptic growth of plants in falcon tubes on gel medium

50 ml falcon tubes containing up to 10 ml agar-solidified plant media were prepared. Each grain was placed in a separate falcon tube filled with 10 ml of agar-solidified plant medium. The lid was loosely attached to enable gas exchange. The plants were grown in a phytochamber (Versatile Environmental Test Chamber MLR-352H, Panasonic) for 5-7 days (light/dark: 16h/8h at 25°C/20°C with 75% relative humidity).

Isolation of culturable bacteria

Isolation of bacteria from all plant tissue was performed according to the same protocol. No more than 300 mg (preferably 200 mg) fresh weight of plant tissue fragments were placed in a 1.5 ml Eppendorf tube and manually homogenized with a sterile pestle. After homogenization, the pestle was washed over a tube with 0.2 ml of saline supplemented with 0,1% Tween 20 (unless specified otherwise). Afterwards, tube was vortexed for 30 seconds before preparing dilutions (in saline) and plating aliquots on solidified R2A agar medium.

Identification of cultured bacteria and cryopreservation

Each isolate was streaked repeatedly (three to six times) for single colonies to ensure purity. Colonies that were assumed to be single clones were picked and transferred onto liquid media and grown overnight before cryopreservation. The same colony was used to set up the colony PCR by touching a colony with a pipette tip and washing it in the PCR reaction mixture.

PCR mix constituted of 10 µl HighFidelity buffer 5x), 2 µl of forward and 2 µl of reverse primers (20 pM stock), 1 µl of dNTPs (20 mM), 0.4 µl Phusion high fidelity polymerase (2,000 U/ml, New England BioLabs), filled up to 50 µl with PCR water (Roth). Alternatively, DreamTaq Green PCR Master Mix (2x) (Thermo Fisher Scientific) was used with identical final primers concentration.

Thermocycler program was as follows: 5 minutes 98°C, 30 cycles of 98°C for 30 seconds, 50°C for 20 seconds, 72°C for 30 seconds (for DreamTaq elongation time was extended to 2 minutes) followed by a final extension step in 72°C for 3 minutes (10 minutes for DreamTaq).

98°C	5 min	
98°C	30 sec	
50°C	20 sec	30x
72°C	30 seconds for Phusion 2 minutes for DreamTaq	
72°C	3 minutes 10 minutes for DreamTaq	

Bacterial isolates were identified by 16S rDNA sequencing (the service provided by Eurofins Genomics) using primers 63F (Marchesi et al. 1998) and 1319R (Schumann 1991; Turner et al. 1999) usually yielding around 700-900 bp long Sanger read; reads below 150 bp were discarded.

After washing pipette tip in PCR reaction mixture, the remaining bacteria were plated, resulting plates were used to prepare liquid cultures in R2A broth. In turn, these cultures were used for cryopreservation.

5.3 Genome sequencing, assembly and annotation

Genomic DNA was isolated using Qiagen protocol (Published in QIAGEN Genomic DNA Handbook, 2015) with minor changes. In short: overnight cultures of *Pantoea agglomerans* were centrifuged (3.000 x g, 2 minutes) and resuspended in 1 ml of Buffer B1 (Bacterial Lysis Buffer 1, consisting of 50 mM Tris-HCl pH 8.0; 50 mM EDTA pH 8.0; 0.5% Tween 20; 0.5% Triton-X100) supplemented with 2 µl RNase A. Following, 20 µl of proteinase K was added and the samples were incubated at 50°C for 30 minutes. Following, 350 µl of Buffer B2 (Bacterial Lysis Buffer 2, consisting of 3 M guanidine hydrochloride, 20% Tween 20) was added and the samples were incubated on ice for 10 minutes. Afterwards, the sample was centrifuged, and DNA was precipitated out of the solution using isopropanol. After centrifugation (20.000 g for at least 10 minutes at 4°C) the pellet was washed with 70% ethanol and left to dry. Once the ethanol has evaporated, the genomic DNA was re-suspended in nuclease-free PCR water.

Illumina sequencing was outsourced to MicrobesNG. Samples were processed according to the following protocol: three beads were washed with an extraction buffer containing lysozyme and RNase A, incubated for 25 min at 37°C. Proteinase K and additional RNaseA were added and

mix was incubated for 5 minutes at 65°C. The genomic DNA was purified using an equal volume of SPRI beads and resuspended in the EB buffer.

The DNA was quantified in triplicates with the quantitative dsDNA HS assay in an Ependorff AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol, with following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time was increased to 1 minute from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250bp paired end protocol.

Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 (Bolger et al. 2014).

For samples, where only HiSeq was performed, de novo assembly was performed on samples using SPAdes version 3.7 (Bankevich et al. 2012), and contigs were annotated using Prokka 1.11 (Seemann 2014).

Additionally, Minlon (Oxford Nanopore Technologies) sequencing was performed by Dr. rer. nat. Martin Fischer (AG Schmitz-Streit, CAU Kiel) following the manufacturer's protocol.

Hybrid genome assembly was performed with Unicycler software version 0.4.4 (Wick et al. 2017) using quality filtered reads from Illumina sequencing and Minlon. Default parameters were used.

The assembled genome contigs were annotated with Prokka version 1.11 (Seemann 2014). Program was run with following parameters: % prokka --kingdom Bacteria --outdir mydir --prefix mygenome contigs.fa

5.4 Establishment of phenotypically trackable *Pantoea agglomerans*

For the colonization assays, it is crucial to have the ability to distinguish easily between the species of interest and other bacterial species or strains and between different competing strains used for the experiments. For this purpose, a GFP-expressing and kanamycin/neomycin resistant strain of *Pantoea agglomerans* was prepared.

Pantoea agglomerans R1 was transformed with a derivative of the broad-host-range plasmid RSF1010, plasmid pRL153-GFP (Tolonen et al. 2006). This plasmid encodes a kanamycin resistance marker gene (*nptII*) and a version of a green fluorescent protein (*gfp*) optimized for prokaryotic expression (*gfpmut3.1*). The use of the phenotypic marker GFP enables one to co-cultivate the strain on non-selective media while maintaining the ability to differentiate between GFP-expressing colonies (glowing green under a blue or green light) and non-fluorescent

colonies formed by other bacteria. Kanamycin resistance, provided by *nptII* gene, enables selection of the plasmid carrying cells and allows the use of kanamycin or neomycin in a gnotobiotic system.

In order to enable tracking of second isogenic strain, plasmid pTW1-mCherry (Wein et al. 2018), was used. The only difference between pRL153-GFP and pTW1-mCherry is the presence of *mCherry* gene instead of *gfp*.

Henceforth, *Pantoea agglomerans* R1 belonging to GFP or mCherry tagged strains is called *Pantoea*.

Preparation and electroporation of electrocompetent *Pantoea agglomerans* cells

Electrocompetent cells were prepared as described by (Dower et al. 1988) with minor modifications. Briefly, an overnight culture of *Pantoea agglomerans* was diluted with R2A broth medium and grown until OD reached 0.6-0.7. Afterwards, the culture was chilled on ice with shaking for about 20 minutes, and the cells were harvested by centrifugation (6,000 rpm at 4°C). The cells were washed twice in 10% ice cold glycerol. Afterwards, the cells were re-suspended in 400 µl of 10% glycerol and aliquoted into 40 µl portions and stored at -80°C.

An aliquot of electrocompetent cells was mixed with 1 µl of plasmid DNA (123 ng/µl) and transferred into a pre-cooled cuvette (2 mm gap) and electroporated using the GenePulser Xcell (Bio-Rad) with the default *E. coli* preset (3,000 V, 100 Ω, and 25 µF). Afterwards, cells were resuspended in 1 ml R2A broth medium and transferred into an Eppendorf tube and allowed to recover for an hour (25°C with slow shaking). Transformed cells were selected for by plating on R2A agar supplemented with kanamycin (15 µg/ml). After overnight incubation at 25°C, colonies were checked for fluorescence.

Determination of relative plasmid effect on transformed *Pantoea agglomerans* fitness

The competition experiments were performed with eight biological replicates. 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. A volume of 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 seven days. At the onset of the experiment and each day, the ratio of the two competing strains was determined by non-selective plating. 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 $s=b/\log_{10}(1/d)$ as previously described (Nguyen et al. 1989), where b is the slope of the \log_{10} ratio of the density of the competing strains as a function of transfer, and d is the dilution factor (1:100). Wildtype fitness was set to 1.0, and the value of w for the plasmid-carrying strain was calculated as $w=1+s$. R-squared and P-value of linear regression used for slope estimation were calculated using R.

5.5 Colonization dynamics

Impact of inocula size on the colonization dynamics

Primarily, I tested the impact of inoculum size on the colonization dynamic. To achieve this, grains of Benchmark wheat variety were bleached. For bleaching, up to 10 ml volume of grains was placed in 50 ml falcon tube, and submerged for 20 minutes (unless stated otherwise) in 50 ml of 6% sodium hypochlorite with 0,05% sterile filtered Tween 20 (standard bleaching protocol). Seeds were occasionally agitated during the process to remove bubbles forming on the surface of the seed. This treatment is aimed to remove the majority of the surface microbiota.

Bleached grains were washed with autoclaved water until no foaming was apparent (not less than 3 times with 40 - 50 ml of water). Afterwards, seeds were placed in a deep well plate and inoculated with 50 μ l of diluted bacterial culture (of which the CFU/ml was estimated by plating). For this experiment six consecutive tenfold dilutions were used. The density and the final inoculum size were confirmed by plating. For negative controls sterile water was used instead of a bacterial suspension.

After 24 hours, germinating seeds were transferred into fresh deep well plates containing plant media. The plants were grown in a phytochamber (Versatile Environmental Test Chamber MLR-352H, Panasonic) under following conditions: 16h/8h (light/dark) at 25°C/20°C with relative 75% humidity. Plant media was replaced every three to four days. Plants were sacrificed on days six, fourteen and eighteen of the experiment. Each sampling comprised three biological replicates of each treatment plus one control (non-inoculated plant).

Before processing, roots were placed on an autoclaved filter paper to absorb excess plant media. Roots and leaves were homogenized, and dilutions were plated on selective media (R2A agar supplemented with 15 μ g/ml kanamycin). After overnight incubation at 25°C fluorescent colonies were counted to estimate the population size of *Pantoea agglomerans* recovered from the plant tissue.

Observation of colonization dynamics with daily samplings

Grains were bleached as described above. Overnight bacterial cultures of *Pantoea agglomerans* carrying pRL153-GFP were diluted, and the grains were inoculated with 750 or 75,000 CFU each (inoculum size first estimated using a Thoma counting chamber, later verified by plating).

After 24 hours, germinating seeds were transferred to fresh deep well plate containing plant media and grown in a phytochamber (Versatile Environmental Test Chamber MLR-352H, Panasonic) (16h/8h at 25°C/20°C with 75% relative humidity). Every 24 hours, plants were moved to a vessel with fresh plant media.

The samplings were performed daily, each sampling consisted of three biological replicates per treatment. Because of the small size of the germ, each replicate comprised three individuals, randomly picked plants and their root systems pooled prior to processing of the sample (i.e. values for each replicate represent the average of three root systems). Samples were processed as described previously.

Inoculation of grown root system

Plants were bleached and cultivated as described previously. Once most of the roots were over 1.5 cm long, the seedlings were transferred onto the lid of an Eppendorf tube. The tubes were modified by cutting a slit into the lid which allowed to keep the plant above the media, with the roots to be submerged in the media within the tube. In addition, this design limited the contact of the roots with the outside environment. Plants were grown in the phytochamber as described above.

Throughout the experiment fifteen samplings were performed, covering up to 75 hours post inoculation. All samplings comprised three biological replicates. Negative control (non-inoculated plants) were also sampled, though at different intervals. Samples were processed as described before.

5.6 Establishment of gnotobiotic system

This set of experiments was performed to improve the methodology for establishment of gnotobiotic (i.e. with controlled microbiota) system for the cultivation of plants in laboratory conditions. To be considered gnotobiotic all bacterial species on/in the plant host must be accounted for. With this system, my aim was the removal of all microbes followed by the inoculation of plant host with *Pantoea agglomerans*.

Preliminary experiments

The preliminary experiments on the gnotobiotic system aimed for optimization of the established protocols, especially finding conditions that enables reliable removal of the microbiota and contamination-free maintenance of germ-free plants. Not only the sterility of plants was tested, but impact on plant growth was assessed as well.

Plant media supplementation with antibiotic

Despite purposefully not providing a carbon source for bacteria, the plant medium used in previous experiments appeared to be environment hospitable for bacteria. Especially after bleaching treatments, the grains/young seedlings were very susceptible to fungal infections. In order to address this issue, the media was supplemented with antibiotics: 50 µg/ml kanamycin, streptomycin and spectinomycin 25 µg/ml each. In further experiments amphotericin B (3-7 µg/ml) was introduced to halt fungal contaminations. These modifications enabled the maintenance of germ-free plants.

Sterilization treatments

Benchmark seeds were divided into 3 batches, each underwent distinct treatment.

A: Mock Bleaching (as B but autoclaved water was used instead of sodium hypochlorite)

B: Standard bleaching: 20 minutes in 6% Sodium Hypochlorite with 0,05% Tween 20, periodically agitated, washed 3 times with sterile water

C: Stronger Bleaching treatment: washed with sterile water, one hour in 12% sodium hypochlorite with 0.1% Tween 20, periodically agitated.

Following the treatment, each batch was divided into three separate cultivation approaches (24 grains per treatment, per cultivation approach). Grains were transferred to 24-well plate (1 plate per treatment/cultivation method). Plant medium was added to each well (with or without antibiotic cocktail comprising kanamycin 50 µg/ml, spectomycin 25 µg/ml and streptomycin 25 µg/ml).

After four days, three plants from each plate were harvested, weights recorded, and root homogenates plated on R2A agar plates to recover bacteria. Plates were incubated for 48 hours at 20°C. Since the purpose of the experiment was to achieve germ-free plants, growth was evaluated in a presence/absence (0/1) manner.

As a result of this experiment, changes were introduced to default protocols used for treatment of the grains. This changed protocol was used for all of grown-root colonization experiments (priority effect experiments). In order to improve removal of viable bacteria following modifications were introduced: sodium hypochlorite concentration was increased from 6% to 12%, Tween 20 concentration was increased from 0.05% to 0.1%, and bleaching time was increased from 20 minutes to 60 minutes. Protocols were updated to include these changes.

Bleaching and maintenance of germ-free plants

To maintain the sterility and to further reduce the bacterial growth, an antifungal agent was added to the media. The final composition of the antibiotic additives for the plant media contains: 50 µg/ml spectomycin, streptomycin 25 µg/ml, neomycin 15 µg/ml, and 3 µg/ml amphotericin B.

Grains were divided into two batches. The first batch was bleach treated as in 5.5.1, the second batch underwent mock treatment, which replicates bleach treatment using autoclaved distilled water instead of sodium hypochlorite solution. On the starting day of the experiment, three grains from each treatment were percolated for three hours in 0.2 ml saline with 0,1% Tween 20. Supernatant and its dilutions were plated to estimate initial bacterial load.

Following the treatment, grains were moved to a twenty-four-well plate with plant media with antibiotic mix (for treated grains) or without antibiotic (mock-treated grains); this was maintained through whole experiment, except after day eight some treated plants were moved to antibiotic-free media to recover. For the first five days of the experiment, seeds were germinated and cultivated in this setup, and were transferred daily to fresh well-plate.

On day five, seedlings were moved on top of sieve (repurposed cell strainer) with a hole in the middle. This setup, allowed to keep the remaining grain and aerial parts of the plant above the media level, while keeping the roots submerged. Up to day eight, plants were kept on the sieve in six-well-plate.

On day eight, plants on the sieves were moved on top of falcon tubes filled with media. Some of the plants from treated batch were moved to antibiotic-free media in order to recover.

Samplings of roots and media were performed in triplicate on days 3, 5, 8, 10; for each biological replicate 3 technical replicates of plating were made. Samples were processed as in 5.5.1. For each sample mass of root and shoot was recorded.

This protocol was used to process all the grains used for priority effect experiments.

Colonization of germ-free seedlings

Bleached wheat grains were placed individually into 24-well plates and immersed (not fully submerged) in plant media containing streptomycin (25 µg/ml), spectinomycin (25 µg/ml),

neomycin (15 µg/ml), and amphotericin B (3-8 µg/ml). After two days, the germinating seedlings were transferred individually into six-well plates and incubated in 5 ml of plant medium for approximately four days. Once the roots had reached 1.5 cm in length, 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). 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. 400 cells per ml) cells from overnight cultures of either *Pantoea* carrying pRL153-GFP or pTW1-mCherry. For each replicate, the inoculum density was confirmed by plating on R2A media plates. After 5 hours of incubation with the cells, each plant was transferred (excess media from roots was soaked off with sterile filter paper) onto a 50 ml falcon tube with plant medium containing neomycin (10 µg/ml) and amphotericin B (3-5 µg/ml). Unless stated otherwise, three to six plants were sampled directly after inoculation and media from deep well plate was sampled alongside plants.

Every 24 hours, the plants were transferred to new tubes with plant medium, 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 rest of the plant using a sterile scalpel. The roots of individual plants were transferred into pre-weighted 1.5 ml Eppendorf tubes, their root mass recorded, and manually homogenized using a sterile pestle. The pestle was washed off with 0.1 to 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 labeled 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 agglomerans* recovered from the plant tissue. Experiments were carried between 12-16 days post germination.

Results of these experiments are represented as mean absolute deviation (abbreviated to MAD). Compared to more commonly used standard deviation, MAD is more efficient for data that do not adhere perfectly to normal distribution as well as it is more tolerant to extreme values in the majority of real-life situations (Gorard 2005). MAD is calculated in following steps: First, the mean of the results is calculated. Next, the deviation from the mean, for each data point, is calculated and their absolute values are summed up. Then, the sum of the absolute values of the deviations from the mean is divided by the number of measurements (number of observations). MAD is calculated using following equation:

$$MAD = n \sum |X_i - \bar{X}|$$

6 Results

6.1 Isolation of total plant DNA for identification of seed-borne bacteria

At the first stage, I aimed to identify members of the wheat microbiota that reside in seeds. To detect the most abundant bacteria in grains, PCRs with a high number of cycles were performed on total DNA extracted from seeds to amplify bacterial 16S ribosomal DNA (16S rDNA) gene sequences. Because of the nature of PCR, such conditions favor the amplification of the most abundant 16S rDNA sequence present in the sample. Using primers specific for bacterial 16S rDNA (799F (Chelius and Triplett 2001), and 1391R (Schumann 1991; Turner et al. 1999) or 1114R (Simon et al. 2009)) enabled selective amplification of 16S rDNA of bacterial origin only or generated bacterial and plant amplicons distinguishable by size. Occasionally, Sanger sequencing of the PCR amplicon provided high-quality sequence, meaning that one of the sequences was vastly outnumbering any other 16S rDNA sequences present in the source sample.

I further analyzed these results using BLAST (Altschul et al. 1990; Boratyn et al. 2012) and blasted against the 16S ribosomal RNA sequences database. Most similar hits, with 100% coverage and over 97% identity, matched to *Pantoea agglomerans*. This bacterium is a Gram-negative gammaproteobacterium of the family *Erwiniaceae*. It can be found widely on plants, though few strains may be pathogenic to certain hosts (Dutkiewicz et al. 2015). At first, I performed isolations using the winter wheat varieties Runal and Akteur collected in Schleswig-Holstein in the late summer of 2017. Later, varieties of Benchmark and Julius collected alongside of previously mentioned seeds were analyzed, yielding culturable *Pantoea agglomerans* among other bacterial species.

6.2 Sampling, isolation and identification of culturable bacteria from grains, lab grown plants and field samples

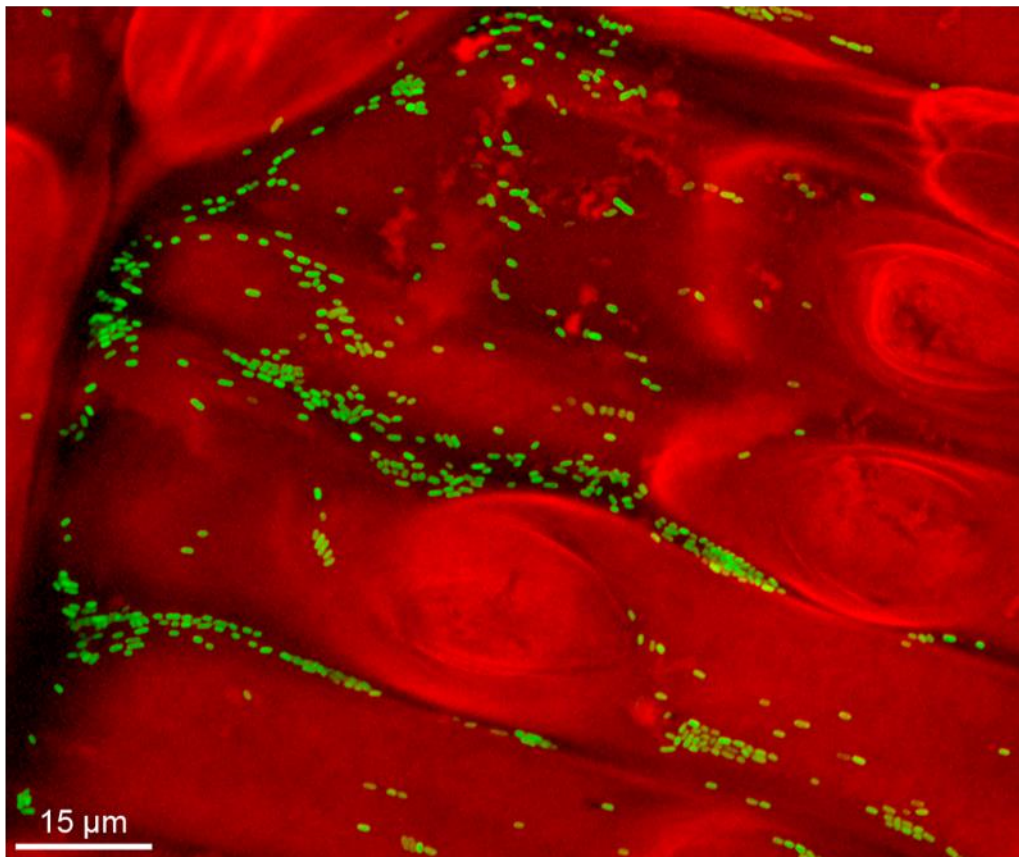
In parallel to the isolation of total plant DNA, I isolated viable bacteria directly by sampling seeds, germinated seeds, and young plants grown from surface treated grains. In addition, plating experiments were performed with crushed seeds and the supernatant from rinsed grains. My media of choice were the low-strength complex medium R2A agar, which is commonly used for the cultivation of environmental bacteria, LB, and MacConkey agar for the isolation of Gram-negative bacteria. Post-isolation platings were performed solely on R2A plates. Pure isolates were obtained through the streaking of single colonies. Colony PCR was performed in order to amplify the 16S rDNA used for identification of the isolates, which were later cryopreserved.

More field samplings were performed in spring of 2018. Ears with grains were processed, and grains were further used for isolation and cultivation of bacteria. Samples originated from the same location as grains of the Benchmark variety used for isolation of *Pantoea agglomerans*.

From grains and laboratory-grown plants (of Benchmark, Akteur and Julius) a total of seven bacterial species have been isolated and identified, some of which appear to be opportunistic species (for instance: *Moraxella osloensis*) and at least two more require sequencing of additional regions to confirm their taxa (table 2).

Pantoea agglomerans was isolated from the vast majority of plants cultivated in the lab. This observation, and the evidence in the literature for a common association of *Pantoea agglomerans* with wheat (Remus et al. 2000), led me to adopt this organism for our objectives. Five *Pantoea agglomerans* isolates were randomly selected for further work and genome sequencing (Table 3). A phylogenetic reconstruction of the isolated strains places my *Pantoea agglomerans* isolates in a cluster mostly consisting of other plant associated isolates (fig. 6).

a)



b)

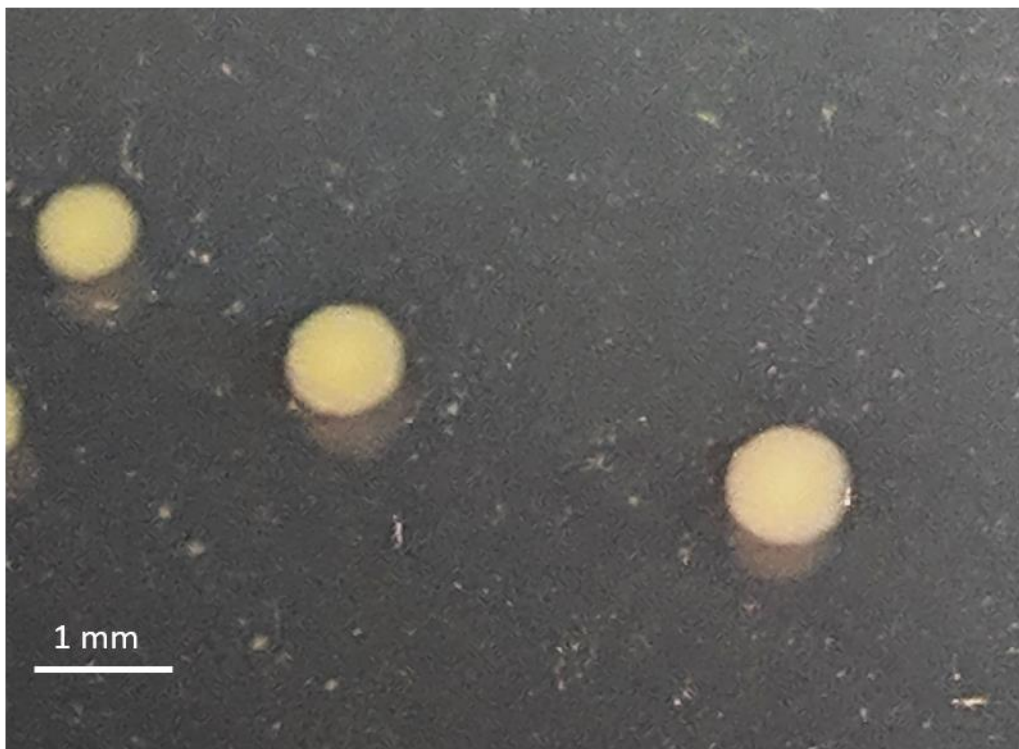
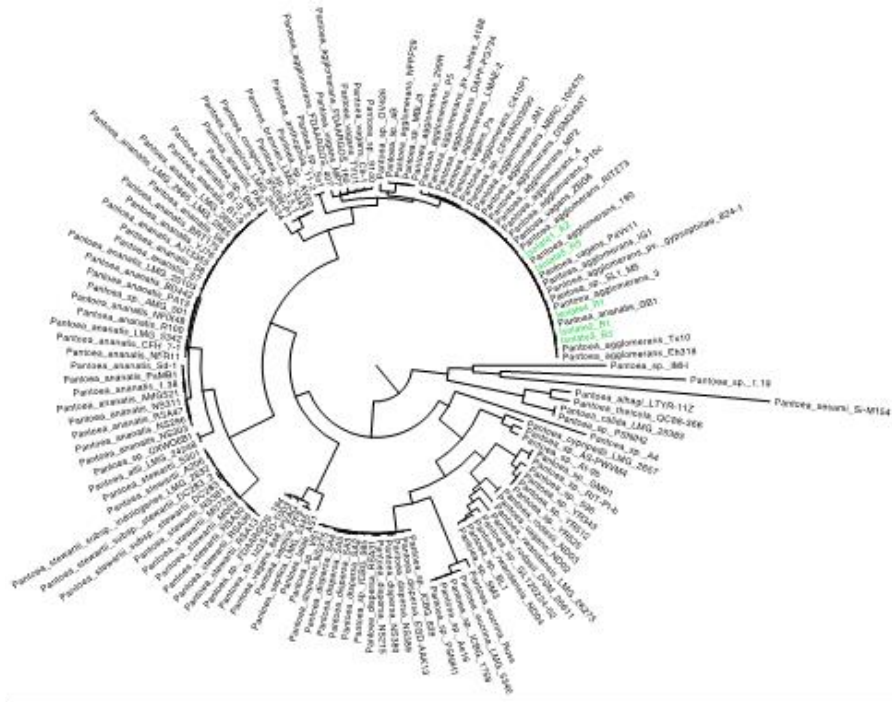


Figure 5. a) GFP-tagged *Pantoea agglomerans* seen colonizing wheat root. CLSM micrograph by Jan Michels (Zoological Institute, CAU Kiel). b) Macroscopic picture of *Pantoea agglomerans* R1 colonies grown overnight on R2A plate. Convex, smooth with uniform white-yellow coloring. Yellow pigmentation becomes more intense as colonies age.

Table 2. Representative bacterial isolates obtained from grains and young plants cultivated in laboratory conditions. Samples grouped by origin; german wheat varieties are Akteur, Benchmark and Julius varieties were used for isolation.

Species	Material used for isolation	Sampling location
<i>Micrococcus aloeverae</i>	Grain, leaves of a young plant	Schleswig-Holstein
<i>Bacillus cereus</i>	Grain, leaves of a young plant	Schleswig-Holstein
<i>Pantoea agglomerans</i>	Grain, shoot of seedling	Schleswig-Holstein
<i>Pantoea agglomerans</i>	Grain, shoot of seedling	Schleswig-Holstein
<i>Pantoea vagans</i>	Grain, shoot of seedling	Schleswig-Holstein
<i>Chryseobacterium psychrotolerans</i>	Grain, shoot of seedling	Schleswig-Holstein
<i>Chryseobacterium gregarium</i>	Grain, shoot of seedling	Schleswig-Holstein
<i>Sanguibacter inulinus</i>	Grain, shoot of seedling	Schleswig-Holstein
<i>Micrococcus yunnanensis</i>	Grain	Schleswig-Holstein
<i>Massilia sp.</i>	Grain, root of a young plant	Schleswig-Holstein
<i>Moraxella osloensis</i>	Grain	Schleswig-Holstein
<i>Pantoea agglomerans</i>	Grain	Antakya, Kışlak, Turkey
<i>Patnoea vagans</i>	Grain	Antakya, Kışlak, Turkey
<i>Pantoea agglomerans</i>	Grain	Antakya, Kışlak, Turkey
<i>Pseudomonas syringae</i>	Grain	Antakya, Kışlak, Turkey

a)



b)

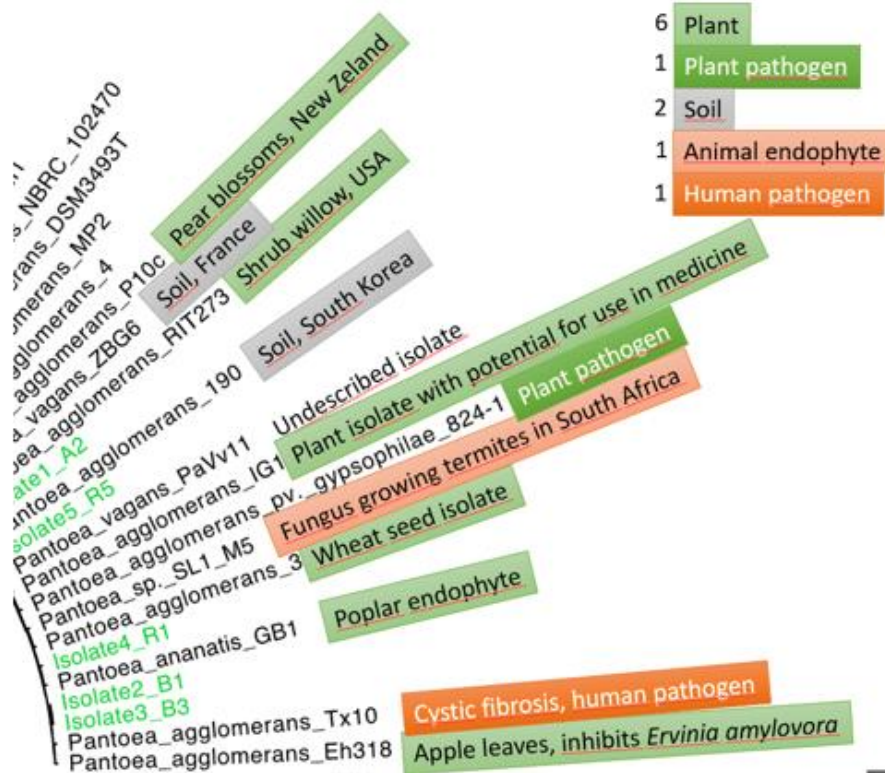


Figure 6. a) a phylogenetic reconstruction of *Pantoea* phylogenetic tree from 392 universal single copy gene families (analysis by T. Dagan). Strains isolated in this study are shown in green; b) origin of isolates that cluster together with local *Pantoea* isolates, most are plant associated.

Table 3. Summary of the assembly for *Pantoea agglomerans* genomes. All isolates contain LPP-1 plasmid. All assemblies have been published under provided GeneBank accession number (assembly and annotation assisted by DR Picazo).

Isolate	Coordinates	Replicon type	GenBank accession	Genome size (bp)	#Contigs	N50	G+C%	tmRNA	tRNA	rRNA	CDS	Sequencing platform
Actur2	54° 18' 46.194"N 9° 59' 31.89" E	Chromosome		4126280	18	518825	55.4	1	71	7	3762	MinION + Illumina
		Plasmid 1	WSSP00000000	166034	1	NA	52.7	0	0	0	148	
		LPP-1 plasmid		678173	1	NA	52.5	0	0	0	666	
Benchmark1	54° 18' 46.194"N 9° 59' 31.89" E	Chromosome		4444536	41	456717	54.9	1	73	9	4036	Illumina
		Plasmid 1	WSSQ00000000	203616	3	NA	51.9	0	0	0	179	
		LPP-1 plasmid		577164	1	NA	53.0	0	0	0	561	
Benchmark3	54°18' 46.194"N 9° 59' 31.89" E	Chromosome		4448667	54	456718	54.9	1	73	9	4035	Illumina
		Plasmid 1	WSSR00000000	203729	3	NA	51.9	0	0	0	178	
		LPP-1 plasmid		577499	2	NA	53.0	0	0	0	561	

Table 3. Summary of the assembly for *Pantoea agglomerans* genomes. All isolates contain LPP-1 plasmid. All assemblies have been published under provided GeneBank accession number (assembly and annotation assisted by DR Picazo).

Isolate	Coordinates	Replicon type	GenBank accession	Genome size (bp)	#Contigs	N50	G+C%	tmRNA	tRNA	rRNA	CDS	Sequencing platform
Runal1	54°18'46.194"N 9° 59' 31.89" E	Chromosome	WSSS000 00000	3972862	15	576160	55.5	1	73	5	3590	MinION + Illumina
		Plasmid 1		178415	1	NA	52.9	0	0	0	152	
		Plasmid 2		8401	1	NA	59.5	0	0	0	11	
		LPP-1 plasmid		558375	1	NA	53.1	0	0	0	540	
Runal5	54° 18' 46.194"N 9° 59' 31.89" E	Chromosome	WSST000 00000	4100957	43	574697	55.6	1	72	11	3740	Illumina
		Plasmid 1		187327	3	NA	52.1	0	0	0	168	
		LPP-1 plasmid		533500	1	NA	53.3	0	0	0	522	
Turkish1	35° 58' 21.8" N 36° 07' 41.8" E	Chromosome	WSSU000 00000	3996432	16	2259703	55.6	1	72	5	3592	MinION + Illumina
		LPP-1 plasmid		534313	1	NA	53.5	0	1	0	510	

Table 3. Summary of the assembly for *Pantoea agglomerans* genomes. All isolates contain LPP-1 plasmid. All assemblies have been published under provided GeneBank accession number (assembly and annotation assisted by DR Picazo).

Isolate	Coordinates	Replicon type	GenBank accession	Genome size (bp)	#Contigs	N50	G+C%	tmRNA	tRNA	rRNA	CDS	Sequencing platform
Turkish2	35° 58' 21.8" N 36° 07' 41.8" E	Chromosome	WSSV00000000	4271752	29	455407	55.2	1	76	3	3943	MinION + Illumina
		LPP-1 plasmid		551948	1	NA	53.4	0	0	0	529	
Turkish3	35° 58' 21.8" N 36° 07' 41.8" E	Chromosome	WSSW00000000	4163020	25	663084	55.3	1	76	3	3845	MinION + Illumina
		LPP-1 plasmid		551948	1	NA	53.4	0	0	0	529	
Turkish4	35° 58' 21.8" N 36° 07' 41.8" E	Chromosome	WSSX00000000	4234579	29	2192443	55.3	1	76	3	3912	MinION + Illumina
		Plasmid 1		166346	1	NA	52.4	0	0	0	151	
		LPP-1 plasmid		551948	1	NA	53.4	0	0	0	529	
Turkish5	35° 58' 21.8" N 36° 07' 41.8" E	Chromosome	WSSY00000000	4187578	23	488998	55.3	1	76	6	3853	MinION + Illumina
		LPP-1 plasmid		551962	1	NA	53.4	0	0	0	528	
Turkish6	35° 58' 21.8" N 36° 07' 41.8" E	Chromosome	WSSZ00000000	4202400	17	605029	55.2	1	69	6	3858	MinION + Illumina

Table 3. Summary of the assembly for *Pantoea agglomerans* genomes. All isolates contain LPP-1 plasmid. All assemblies have been published under provided GeneBank accession number (assembly and annotation assisted by DR Picazo).

Isolate	Coordinates	Replicon type	GenBank accession	Genome size (bp)	#Contigs	N50	G+C%	tmRNA	tRNA	rRNA	CDS	Sequencing platform
		Plasmid 1		819	1	NA	51.3	0	0	0	1	
		LPP-1 plasmid		538570	1	NA	53.9	0	0	0	533	

NA: N50 has not been estimated for plasmid sequences, as they are composed of few contigs, and here N50 is not informative.
 LPP-plasmid identification: size (281-794kbp) and presence of thiamine biosynthesis gene.

Grains originating from Turkey (provided by Ezgi Özkurt) were used to isolate Gram-negative bacteria on MacConkey agar. Isolation efforts were focused on colonies presenting morphology similar to *Pantoea* isolates, and confirmed the presence of *Pantoea* in the majority of tested grains.

Including field samples, a total of 56 species has been cultivated and identified; over 300 isolates have been cryopreserved in -80°C.

6.3 Establishment of phenotypically trackable *Pantoea agglomerans*

To verify the ability to colonize the host, *Gfp*-expressing, kanamycin and neomycin resistant *Pantoea agglomerans* R1 was prepared by transformation with pRL153-GFP plasmid (see methods). During *in planta* experiments, this tagged strain was easy to isolate using selective media and easy to distinguish from other wild-type bacteria (including wild type *Pantoea*) present on the seed and plant.

Electrocompetent cells were prepared from *Pantoea agglomerans* isolated from Runal wheat variety. Plasmid was electroporated into cells, which after recovery, were plated on selective media (R2A agar with kanamycin). After overnight incubation, expression of *gfp* was confirmed visually by exposing plates to blue light (all colonies were fluorescent). Plasmid harboring *mCherry* was introduced in the same manner. In further experiments, both strains proved to maintain the ability to colonize the plant via inoculation to the grain and media.

Assessing the fitness impact of phenotypical markers

Bacterial strains with higher fitness will produce more offspring, resulting in increased frequency over time (Wiser and Lenski 2015). Change in frequency caused by impact of plasmid (or different fluorophore encoded on the plasmid) could skew results of competition essays. This means, that strains with relatively similar fitness have to be used. To assess the fitness impact of pRL153-GFP/pTW1-mCherry plasmids on *Pantoea agglomerans* R1 harboring the plasmid, *ex vivo* (in liquid media) competition assays were conducted. Competitors were grown in R2A broth in 20 °C in a deepwell plate. Once competition essays were started, platings were performed every 24 hours, and aliquots of competition mixture were transferred to a freshly prepared deepwell plate, so each well contained a 100-fold dilution of the previous-day competition. The experiment lasted eight days.

Once data was collected, further analysis was performed based on methods available in literature (Nguyen et al. 1989). In brief, common logarithms of daily ratios of competitors were calculated, and the relative growth rate was acquired using the linear regression against time.

Relative fitness was calculated by adding one to the value of the slope divide by the daily dilution factor (100). Each competition was performed in 8 replicates, statistical hypothesis testing was performed using R.

This approach allowed me to compare head-to-head fitness of plasmid harboring, plasmid-free, and their primed counterparts in comprehensive way. If relative fitness is equal 1, both tested strains show exactly the same fitness; the more relative fitness deviates from 1, the stronger the fitness difference between the competitors.

As shown in table 4, both plasmids have a minor impact on bacterial fitness (when compared to wild-type). According to these competitions, strain expressing *gfp* shows 8.23% relative fitness cost, while *mCherry* expressing strain has 9.38% fitness cost (both compared to the wild-type).

When both plasmid harboring strains are compared directly in competition against one another, pTW1-*mCherry* has 2.5% fitness cost compared to the pRL153-GFP harboring strain.

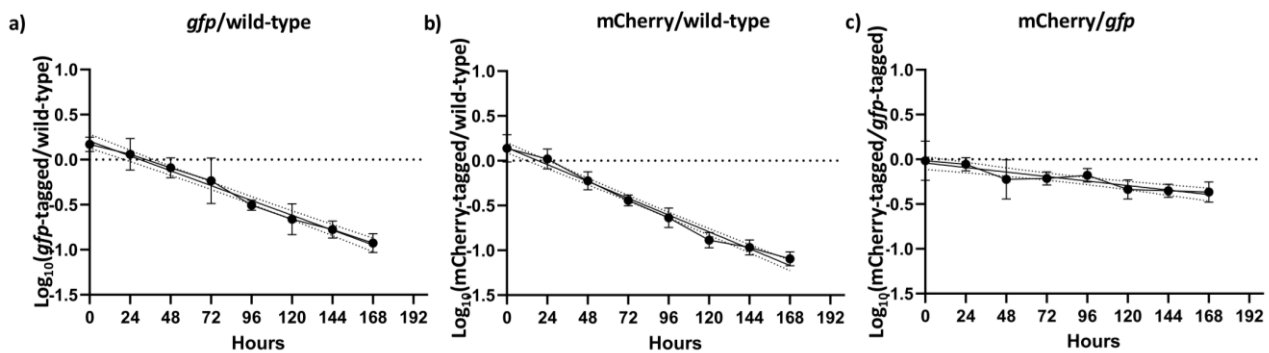


Figure 7. Competitions between *Pantoea* strains presented as change in common logarithm value of the ratio of the competitors. If there is no fitness difference between competitors, slope should be equal to zero; the steeper the slope, the higher the fitness difference between the competitors. Shown fitness differences, while relatively low, are statistically significant. a) and b) show fitness competition between fluorophore expressing strains and wild-type *Pantoea agglomerans* R1 strain.

Table 4. Relative fitness. Fitness cost calculated using mean of common logarithms of 1st competitor, against 2nd competitor. In all cases, carriage of plasmid causes significant fitness cost. Fitness difference between two plasmid harboring strains is lower than difference between wild-type and any of the plasmid harboring strains. The number of replicates in all experiments is n=8; all experiments were performed over 8 days.

*P-value of the regression.

a) R1 pRL153-GFP/ R1 WT				
Replicates	Slope (b)	P-value	FDR adjusted p-value	fitness (w) of strain A
1	-0.15541	0.000015	0.00625	0.92229
2	-0.15448	0.000174	0.0375	0.92276
3	-0.17606	0.027355	0.05	0.91197
4	-0.19582	0.000142	0.01875	0.90209
5	-0.13605	0.000296	0.04375	0.93197
6	-0.17118	0.000166	0.025	0.91441
7	-0.17999	0.000172	0.03125	0.91000
8	-0.14771	0.000139	0.0125	0.92614
Average				0.91771
Standard deviation				0.00977
Fitness cost				8.23%
Confidence Interval				0.00773
R1 pTW1-mCherry is significantly less fit than R1 WT				

b) R1 pTW1-mCherry/ R1 WT

Replicates	Slope (b)	P-value	FDR adjusted p-value	fitness (w) of strain A
1	-0.19720	0.000210	0.05	0.90140
2	-0.19811	0.000184	0.04375	0.90095
3	-0.19119	0.000019	0.01875	0.90441
4	-0.20866	0.000010	0.0125	0.89567
5	-0.16307	0.000142	0.03125	0.91846
6	-0.18711	0.000173	0.0375	0.90644
7	-0.19983	0.000022	0.025	0.90008
8	-0.15624	0.000004	0.00625	0.92188
Average				0.90616
Standard deviation				0.00925
Fitness cost				9.38%
Confidence Interval				0.00817

R1 pRL-153-GFP is significantly less fit than R1 WT

c) R1 pTW1-mCherry/R1 pRL153-GFP

Replicates	Slope (b)	P-value	FDR adjusted p-value	fitness (w) of strain A
1	-0.05041	0.011823	0.025	0.97479
2	-0.06352	0.002053	0.01875	0.96824
3	-0.04021	0.035005	0.03125	0.97990
4	0.00852	0.840364	0.05	1.00426
5	-0.09135	0.000644	0.00625	0.95432
6	-0.04814	0.060346	0.0375	0.97593
7	-0.03590	0.198915	0.04375	0.98205
8	-0.08198	0.001821	0.0125	0.95901
Average				0.97481
Standard deviation				0.01540
Fitness cost				2.52%
Confidence Interval				0.01287

R1 pTW1-mCherry is significantly less fit than R1 pRL153-GFP

6.4 Colonization dynamics

Impact of inoculum size on colonization dynamics

Our experiment was aimed at answering three important questions. Are fluorophore-tagged *Pantoea agglomerans* strains able to colonize wheat hosts? Does inoculum size impact colonization dynamics? And, is there a minimal inoculum size threshold required for successful colonization of the plant?

To answer these questions, I conducted several experiments where grains were sterilized and then colonized by a tagged bacterium. Bleached grains were inoculated with six tenfold dilutions of the bacterial culture and, after 24 hours, they were placed in the fresh plant media. Six days after inoculation, once the roots were grown, the sampling began. In each sampling, plants from each treatment were harvested. One or two biological replicates were analyzed, each biological replicate being a single plant. Roots were cut off from the shoot (since the whole rootsystem was sampled, each plant could be sampled only once), homogenized and CFU of *gfp*-tagged *Pantoea* estimated by plating six to eight tenfold dilutions of the homogenate. To check for the cross-contamination, a negative control comprising plants grown from bleached, non-inoculated seeds, was prepared yielding no fluorescent and kanamycin resistant colonies.

Within first six days, the absolute number of recovered tagged *Pantoea* was similar between all treatments, independently of the number of cells used for colonization experiment (figure 8). This hinted at the necessity to increase the resolution to document the early dynamics of wheat germ colonization. In the following days, slow growth of *Pantoea* population was observed. Tagged *Pantoea* was also recovered from several leaf samples, though the numbers were inconsistent. Because of the cultivation method used for the experiment, direct contact of aerial parts of the seedling with liquid media could not be avoided. This means that counts should not be used to monitor the process of colonization of the aerial parts of the plant. For the untreated grains used for negative control no tagged *Pantoea* cells were recovered from leaves nor roots, meaning that there was no cross-contamination between the treatments.

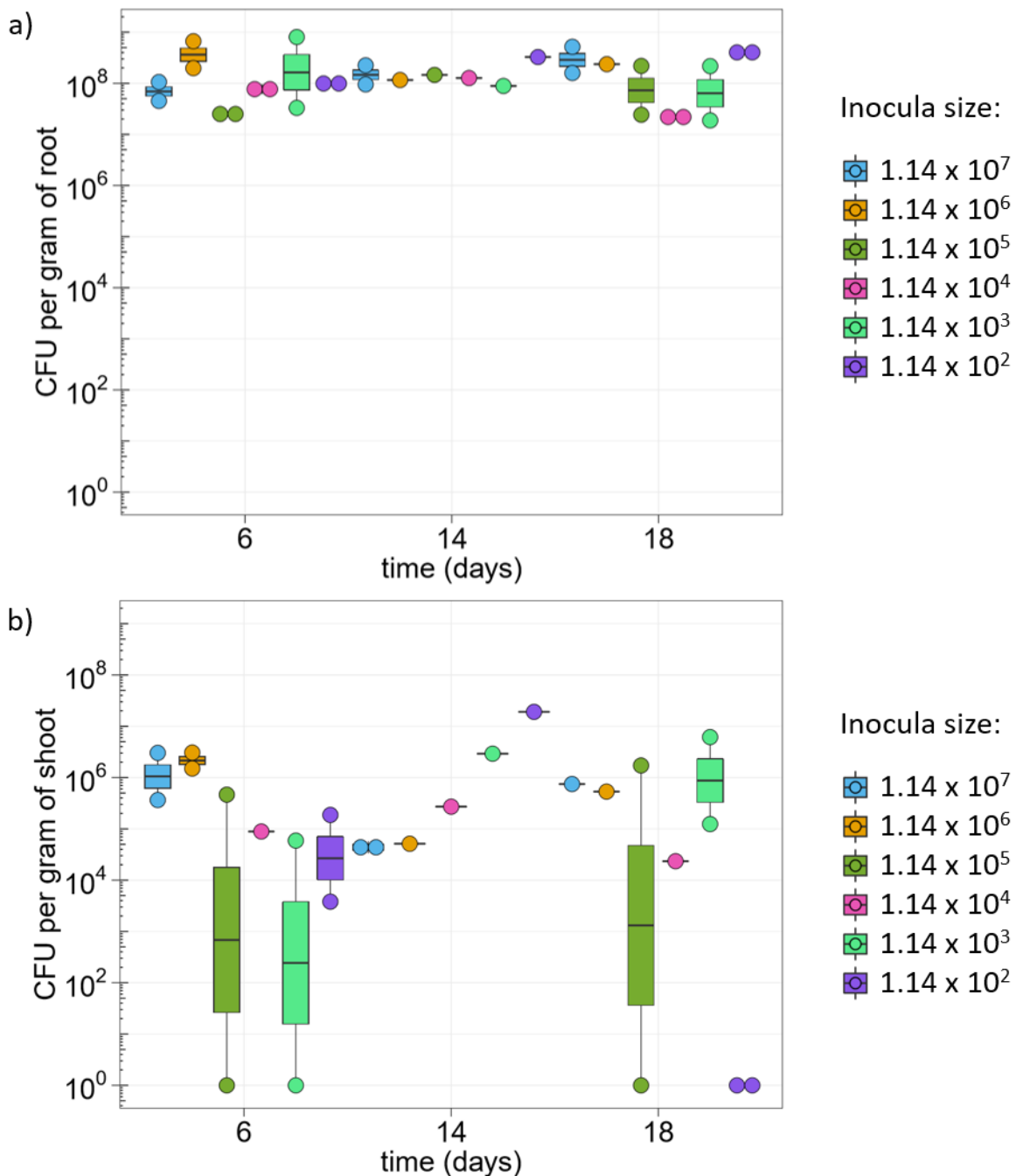


Figure 8. Estimated GFP-tagged *Pantoea agglomerans* population sizes per root system. Each color represents different inoculum size used or the treatment, each dot represents single sampled individual. Inocula sizes shown as estimated CFU of *Pantoea* used for inoculation of the grain, population density expressed as CFU per gram of root fresh weight. a) no correlation between inoculum size and population density on the root; by the time of first samplings all roots have reached the saturation point, b) density of *Pantoea* population is more variable, overall density in the shoot is lower than density in the root.

By day six, all experimental treatments yielded similar *Pantoea* population size per gram of root fresh weight. Density remained stable over time, suggesting that maximal bacterial population density has been reached. Final population size was independent of inoculum used. Some of the factors that could limit the bacterial population are lack of nutrients, a signal from the host or

quorum sensing; these factors are elaborated further in following experiments and in the discussion. As for the shoot, no clear trends in population density are visible, either in time nor in the relation to inoculum used.

This experiment showed that colonization, and following proliferation of *Pantoea* are more dynamic than thought, the process of population density increase has not been recorded in this experiment. While no usable data on early colonization dynamics were provided, maintained population was observed until the end of the experiment (18 days post inoculation).

Observation of colonization dynamics with daily samplings

In a follow up experiment, the resolution of the sampling was increased. Bleached seeds were inoculated with GFP-tagged *Pantoea* and transferred to plant media where these were germinated. First sampling took place 24-hours after the inoculation. Inoculum size was set to 750 and 75,000 CFU per grain by firstly counting cells in Thoma Chamber, later confirming the actual CFUs by plating. Due to the small size of 24-hours old seedling, each replicate consisted of three whole specimens pooled together. Each of the samplings comprised three biological replicates of each treatment (three isolations from three pooled seedlings, overall nine plants per inoculum size).

Both smaller and higher inocula reached *Pantoea agglomerans* cell counts higher than the number of cells inoculated on the grain, respectively within 24- and 48-hours. Thereby, *Pantoea agglomerans* proliferation was clearly confirmed (figure 9).

Overall, the growth of the *Pantoea* population continued during the whole experiment. Within 24-48 hours all the plants showed similar population density.

This suggests, that the most dynamic population growth happens before 48-hour mark. In addition, following the further increase of *Pantoea* population seems to follow root growth, suggesting that root-size-related factor is limiting further bacterial density increase, making the root mass a proxy for this not yet established factor. Even if a host's root system mass is not limiting *Pantoea* population directly, it could be looked into as an easily measurable proxy of such a limiting factor. If relationship between root mass and maximum carrying capacity is linear, once standardized per gram of the root system, plot of population density should reach plateau and oscillate within the same range afterwards.

During the experiment, no GFP-expressing *Pantoea* was recovered from negative control: germs/seedlings grown from non-bleached seeds.

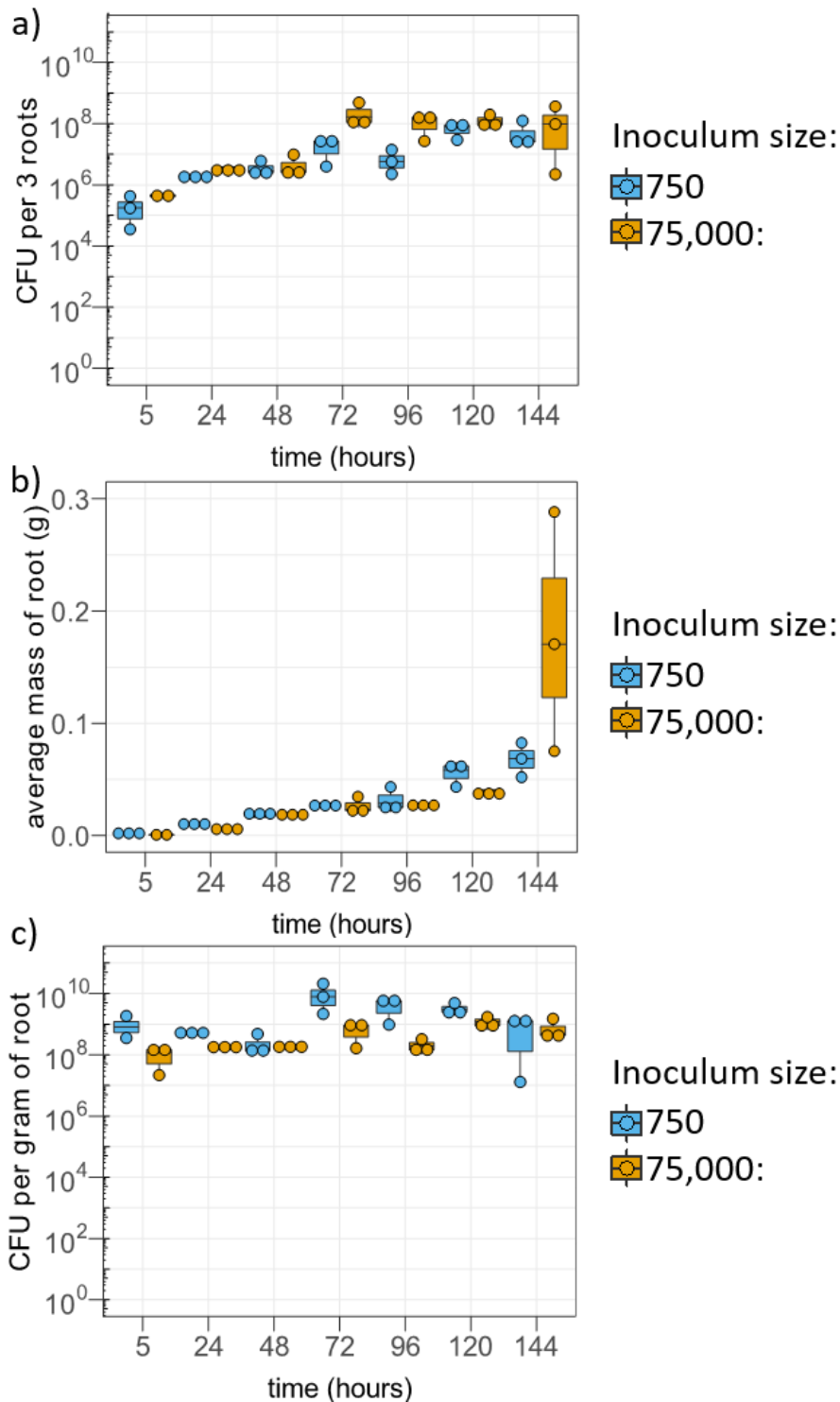


Figure 9. Samplings were performed every 24 hours, 3 root systems per replicate were sampled, homogenized, diluted and plated. Small inoculum (blue) equals 750 CFU, a large inoculum (orange) equals 75,000 CFU. a) Within first 48 hours both treatments (inoculum sizes) showed average *Pantoea* population higher than inoculum size, steadily growing until day 4. b) Average mass of root system of each replicate on the logarithmic scale. c) Once standardized per gram of fresh weight of root tissue, the population of *Pantoea* appears to saturate the host within first 24 hours independently of the inoculum size.

As shown above, once standardized to the fresh weight of root, the population of *Pantoea* appears to be stable, supporting role of the root mass as a proxy for factor limiting the bacterial growth. This appears to hold true throughout the experiment. This simple proxy may not remain accurate once the plant grows further and roots age, becoming less uniform. For example, older parts of roots have been shown to diffuse fewer nutrients that can be used by the root-associated bacteria (Sasse et al. 2018). Based on the standardized data, accommodating for host growth, it is clearly visible, that *Pantoea* population appears to be established as early as within first 24 hours. This highlights the necessity of even higher temporal resolution needed for early dynamics of colonization and supports the hypothesis of priority effect taking place.

Inoculation of grown root system

In order to further increase the resolution and observe even earlier colonization stages, a new experimental design was developed. Bleached grains were cultivated separately in sealed six-well plates in order to exclude external contaminations. Once the roots reached a length greater than 1.5 cm, seedlings were transferred from the wells onto the lid of an Eppendorf tube. The tube lids were equipped with a slot that allowed to place the roots inside the tubes filled with plant media inoculated with *Pantoea*. While risk of contamination from exogenous microbiota was limited, the drawback of this method is the persistence of bacteria that survived the bleaching protocol. The presence of such bacteria was visible in the previous experiments, though these did not appear to disturb the colonization process and their growth was hampered by antibiotics.

To put more emphasis on the early colonization stage, the number of bacteria used for the inoculation was further reduced (for this experiment it was ca. 160 CFUs of *Pantoea* or 40 CFUs of *E. coli* per plant). At every sampling time point, three replicates per treatment were harvested, homogenized, and bacterial load was estimated by plating on the selective media.

During this experiment, persisting environmental bacteria had an opportunity to proliferate on the root of the seedling before the inoculation with tagged *Pantoea*. In this context, it appears that 'being the first colonizer' of the growing host gives benefit over the incoming colonizers. When no such benefit was given to persisting environmental bacteria (inoculation of the grain—before the host began to grow), inoculated *Pantoea* was able to take over the host and no contaminants were observed. This observation led me to the hypothesis that priority effects are taking place during the colonization of the root: late colonizer (*Pantoea*) was unable to outcompete already present bacteria (contaminants).

Some surviving environmental bacteria could grow on selective media supplemented with kanamycin. It is common to find naturally occurring antibiotic-resistant bacteria in soil (Durso et al. 2016). Despite these difficulties, in this experiment I showed *Pantoea*'s ability to colonize the root

and its proliferation in the system, which contrasts with *E. coli* and its low abundance (figure 10). Additionally, I proved that plant media cannot sustain proliferation of *E. coli*, which is not plant colonizer. This suggests that root exudates may not be readily utilized or are not sufficient for *E. coli* growth.

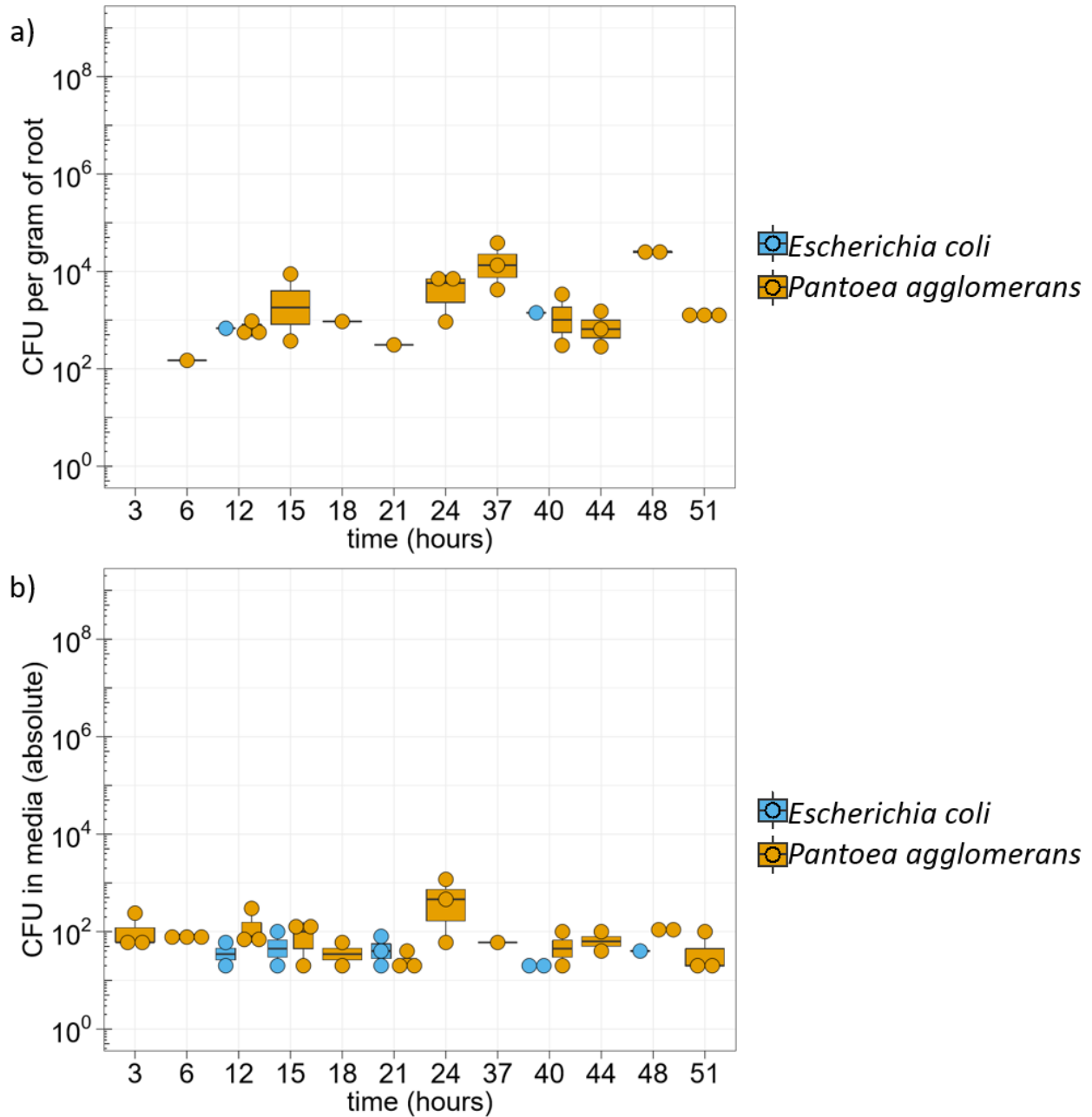


Figure 10. In this experiment roots, that were already pre-grown were colonized. Sampling began three hours post inoculation; each time three replicates were sampled. Missing point means that no tagged bacterium was detected. a) estimated bacterial populations on roots. Once inoculated proliferation was observed, though due to a strong priority effect *Pantoea* population did not reach density observed in previous experiments. High standard deviation is a result of variation between surviving wild type members of the microbiota. Shown CFUs are standardized per gram of root tissue (fresh weight). b) *Pantoea* was observed in the media, while *E. coli* was observed sporadically, though it was still observed in media originating from plants that had *E. coli* free-root. Values presented are absolute values: total population in whole media volume.

6.5 Establishment of gnotobiotic system

In order to further investigate the role of priority effects, the ability to introduce competitors in a controlled order was necessary. Without removing all the microbiota from the root and maintaining this state, observed results would be influenced by dynamics of multi-species assemblage. While being closer to natural microbiota assembly, it cannot be considered a feasible focus of this study.

Gnotobiotic system aimed at solving two main problems: preparation of plants free from bacterial colonizers and maintenance of germ-free plants (or plants with controlled microbiota). Once both elements were in place, further experiments researching the priority effect on *Pantoea agglomerans* colonization of wheat were possible.

Preliminary experiments

In the preliminary experiment two major changes, comparing to previously used methods, were introduced. First, bleaching was extended to one hour (compared to previous 20 minutes). Second, the plant medium was amended with an antibiotic cocktail consisting of kanamycin (50 µg/ml), spectinomycin (50 µg/ml), streptomycin (25 µg/ml).

To assess the efficiency of the changes in media composition and bleaching treatment, an experiment was performed. The experiment comprised the treatments described in table 5. After cultivation, roots were separated from the differently treated grains/plants in deep-well plates, homogenized and the supernatant plated to observe the growth of wild-type bacteria on non-selective media (R2A agar). Aliquots of plant medium from each treatment were plated as well. Three randomly chosen, four-days old plants from each treatment were used for plating.

Table 5. Treatments used for the preliminary experiment. Bacterial growth was observed in all the samples except for 60 minutes bleaching treatment paired with cultivation in plant media supplemented with antibiotic cocktail.

Bleaching method	Plant media	Bacterial growth
Mock	Without antibiotic	Present
Mock	With antibiotic	Present
20 minutes bleaching	Without antibiotic	Present
20 minutes bleaching	With antibiotic	Present
60 minutes bleaching	Without antibiotic	Present
60 minutes bleaching	With antibiotic	Absent

My results show that only the extended bleaching treatment, in combination with the addition of the antibiotic cocktail in the plant medium, provided samples free from cultivatable bacteria. No growth was observed after incubating R2A agar pates at 20° C for 48 hours. All the plants suffered from fungal growth, which was most pronounced on the plants originating from grains subjected to long bleaching treatment. For further experiments, an antifungal compound (amphotericin B) was introduced, resulting in a strong inhibition of fungal growth during the cultivation process.

6.6 Bleaching and maintenance of germ-free plants

After the pilot experiment, the protocol was further changed. Plant medium containing 50 µg/ml kanamycin, 25 µg/ml spectinomycin and streptomycin was further supplemented with 3 µg/ml amphotericin B to limit fungal growth. For growth in deep-well plate, breathable sealing covers were used in order to improve air circulation.

After germination and initial root growth, seedlings were moved from 24-wells plate onto a sieve (cell strainer) with a hole punctured in the middle (so that roots can grow through the hole while the grain remains on the sieve—above the media level; see figure 11. The sieves with plants were moved to 6-wells plates with plant medium (with inoculum) and later on top of 50 ml falcon tube with plant medium. This experimental set-up made it easier to maintain the plants (transferring to fresh media/vessels) while keeping the grain dry and above the media level. Keeping the grain dry reduced fungal growth and reduced the amount of grain borne exudates in the medium.

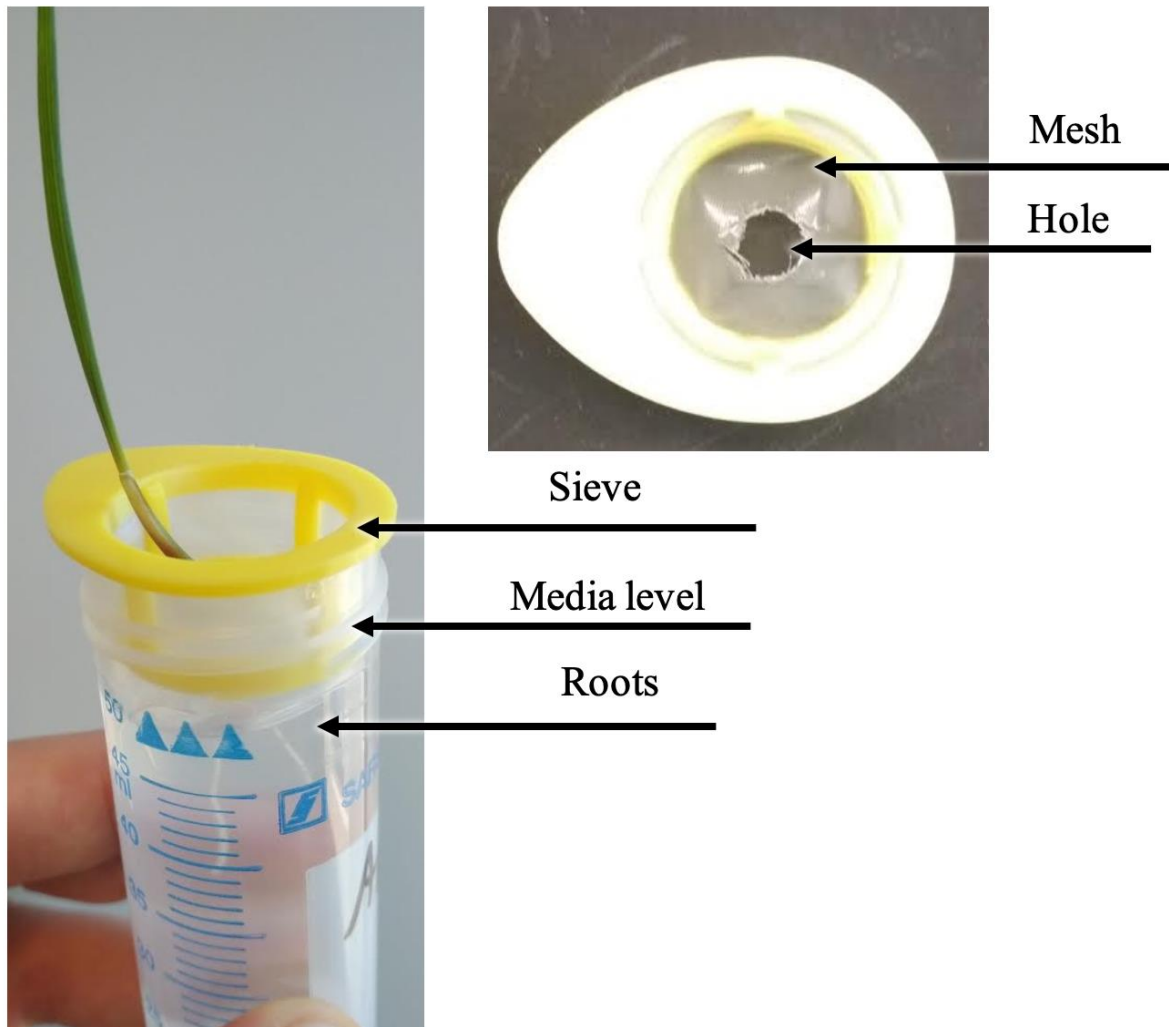


Figure 11. Seedling growing on top of falcon tube in the new setup. The plastic sieve holds grain above the medium level and provides the support for aerial parts of the plant. Roots are growing through the hole punched in the middle of the sieve, reaching the media.

The experiment began by looking into two treatments. Bleached grains (1-hour long bleaching) were germinated and cultivated in plant medium with antibiotics and fungicide. Mock-bleached (water treatment) grains were germinated and cultivated in plant medium without antibiotic and antifungal supplements. At day 8, plants which underwent the bleaching treatment were divided into two subgroups. One remained in media supplemented with antibiotics, the second was moved to antibiotic free media in order to allow plants to recover for two days from the antibiotic/antifungal treatment before the final sampling. During the 10 days long experiment, five samplings were performed (Figure 11).

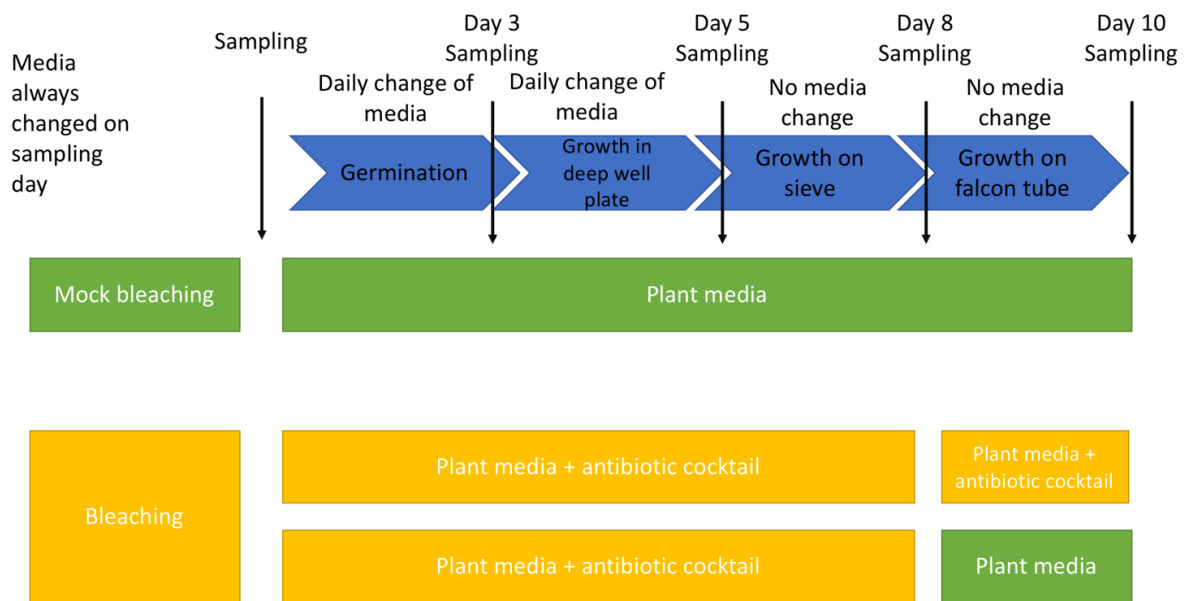


Figure 11. The timeline of the experiment. Plants were given two-day recovery period to test if seedlings can recover from antibiotics. No significant recovery was observed.

First, the effectiveness of bleaching was tested by comparing plantings of wash-offs from the grains after bleaching and after mock treatment, showing the removal of the vast majority of microbiota. Three grains from each treatment were percolated in saline and dilutions were plated, showing no bacterial growth from treated samples, while mock-bleached samples showed significant bacterial growth. Since no tagged strain was introduced, all the counts are of native grain associated microbiota, that managed to colonize the plant.

On days 3 to 8, plants grown in an antibiotic free medium showed an increasing bacterial load (in absolute numbers per sample), which can be related to mass growth. Once the data has been standardized in regard to mass, it became clear that population remains stable (figure 12), meaning that the carrying capacity has most likely been reached. The colony counting was challenging due to the variety of types and sizes of colonies and because some plated dilutions showed changes not only in counts but in a morphological diversity of observed colonies. This could be due to a variety of synergistic and antagonistic interactions between microbes present in the samples. Therefore, as for wild-type communities, that this experiment should be considered more of a skewed snapshot than real absolute numbers of bacteria. Nonetheless, trend of growth of CFU in parallel to growth of root mass is still clearly visible. (Figure 12).

Both bleached treatments, except for single colonies in some Day 8 technical replicates, showed no bacterial growth.

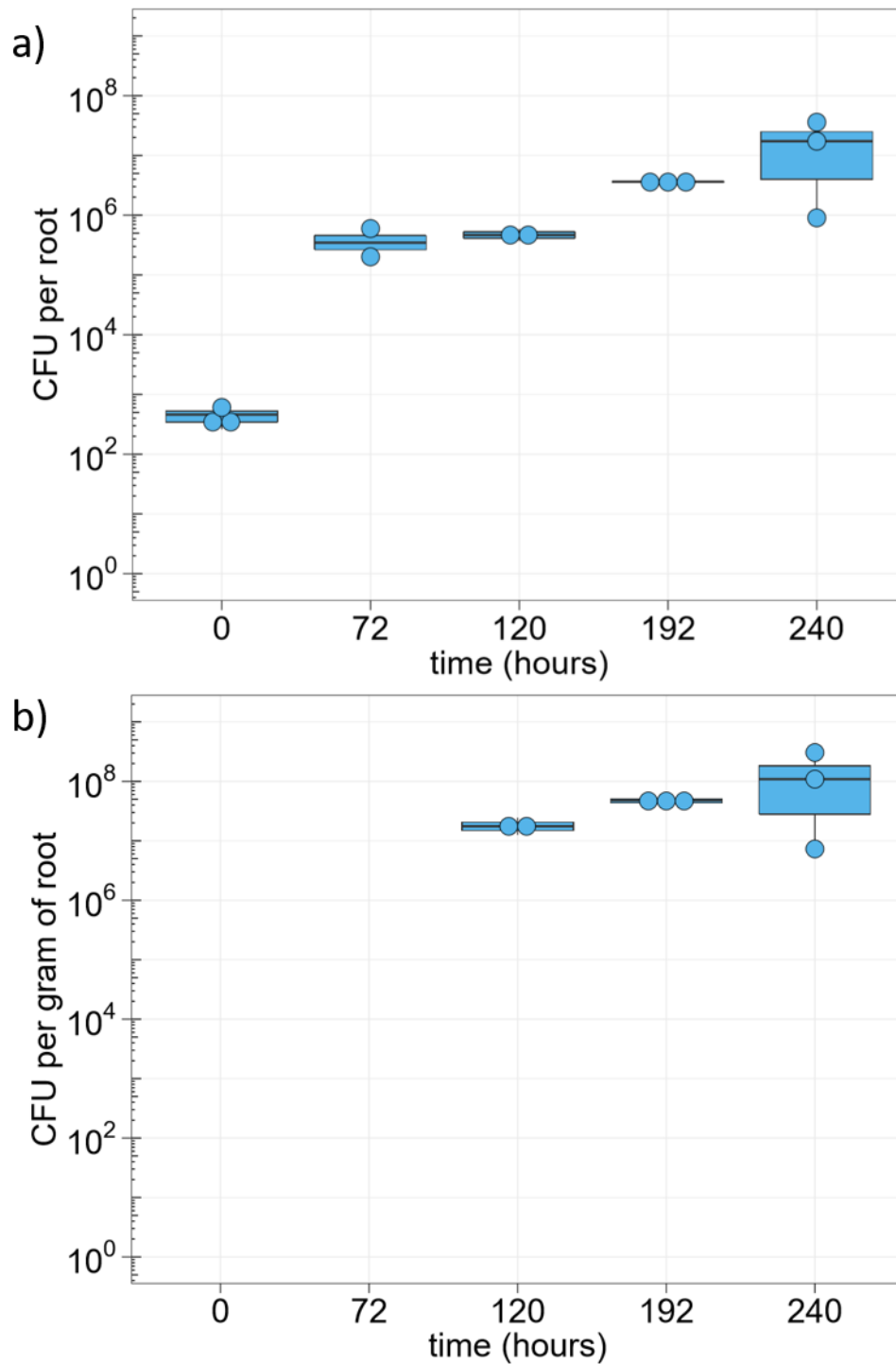


Figure 12. Total number of cultivable bacteria recovered from non-treated plants (native microbiota). Timepoint 0 is the grain percolation. a) per root, b) data standardized per gram of root mass; at the sampling time where root was too small to separate from the germ (up to 72-hour post germination), mass of the root was not recorded and the whole germ was homogenized. N=3; at 120 hours one of the samples showed no bacterial growth.

Another important observation was the difference of masses between the treated, treated with two days of recovery period in antibiotic free media and mock-treated plants (figure 13). Treated plants showed significantly slower growth and discoloration compared to

mock-treated plants. Two days of recovery had not improved the mass and morphology of the plants.

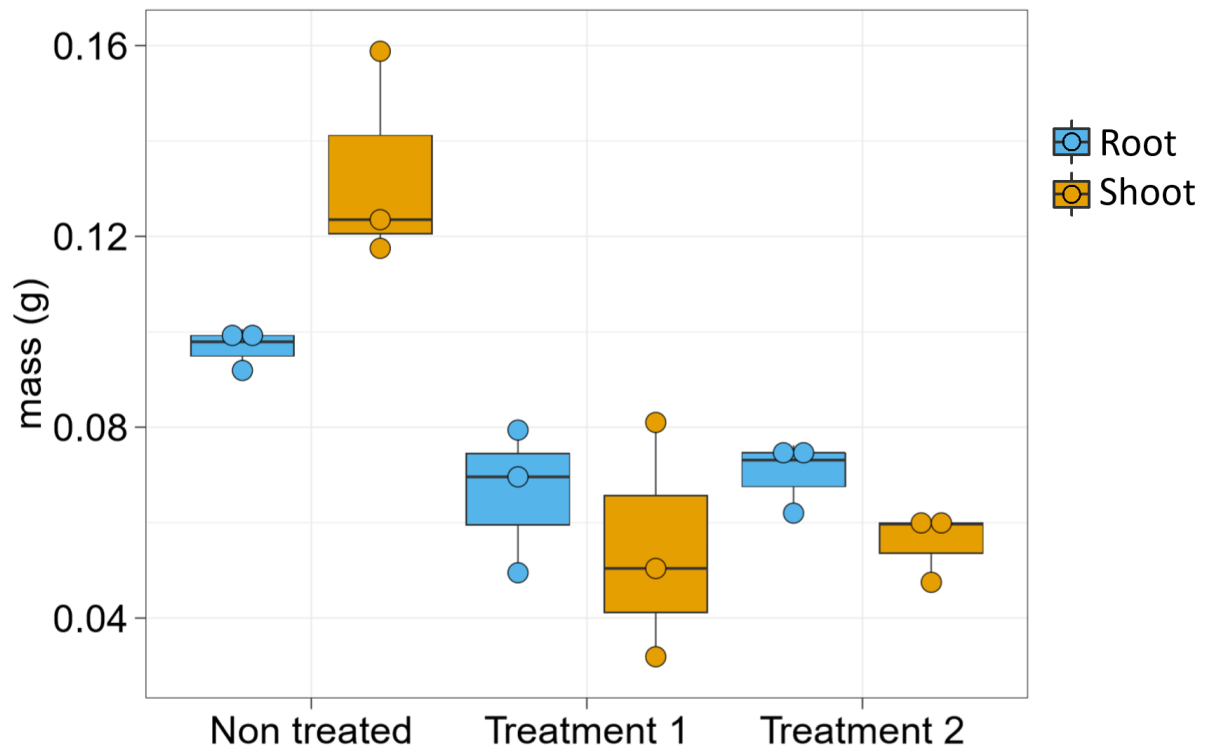


Figure 13: Impact of the bleaching and antibiotic treatment with and without recovery on plant mass (on day ten). Bleached plants, have lower mass in both shoot and root, compared to non-treated control plants. Plants do not recover within 3 days without antibiotics. N=3

Despite significant impairment of the growth, the cultivation methods I used enabled the maintenance of plants free of detectable bacteria. Two-day long recovery period (growth of treated plants on antibiotic free media) did not show a significant increase in the fresh weight of roots or shoots. On the other hand, rhizosphere and media remained free of cultivable bacteria. Insignificant growth of mold was observed on the grains, though plants remained unaffected in any other way. As a result of this experiment, kanamycin was substituted with neomycin, which shows reduced impact on plant morphology.

6.7 Colonization of germ-free plants

For this experiment, further minute modifications were introduced to cultivation methods. After mock or bleaching treatment, the grains were placed on agar solidified plant medium (with or without antibiotics and fungicide) in a deep-well plate. This setup was supposed to minimize the soaking of the grain, further reducing leakage of its contents to the media.

Unfortunately, such a germination method appeared to slow down the initial growth, requiring an additional 24 hours for germination. Moreover, roots grew through the surface of the agar disc, making it hard to remove the agar without damaging the plant (few plants had to be discarded due to damage). Overall, no significant benefit for this germination method was shown, therefore, the previously described germination method in the liquid plant medium had been used for further experiments.

Four samplings were performed during the experiment each from three biological replicates (four biological replicates for colonized plants on day thirteen) of each treatment and three technical replicates for each biological replicate, with an additional sampling of 24-hour old, antibiotic free media from to-be-inoculated plants. Samplings from the treated plants provided no significant bacterial growth except for one biological replicate (day 10).

On day nine, treated plants were moved to non-selective medium. On the day 10 medium was sampled (proved to be free of cultivable bacteria,) and the plants moved to tubes with fresh medium inoculated with ca. 1200 of *Gfp*-expressing *Pantoea agglomerans*. Final sampling was performed thirteen days after inculcation. See figure 14 for the detailed timeline.

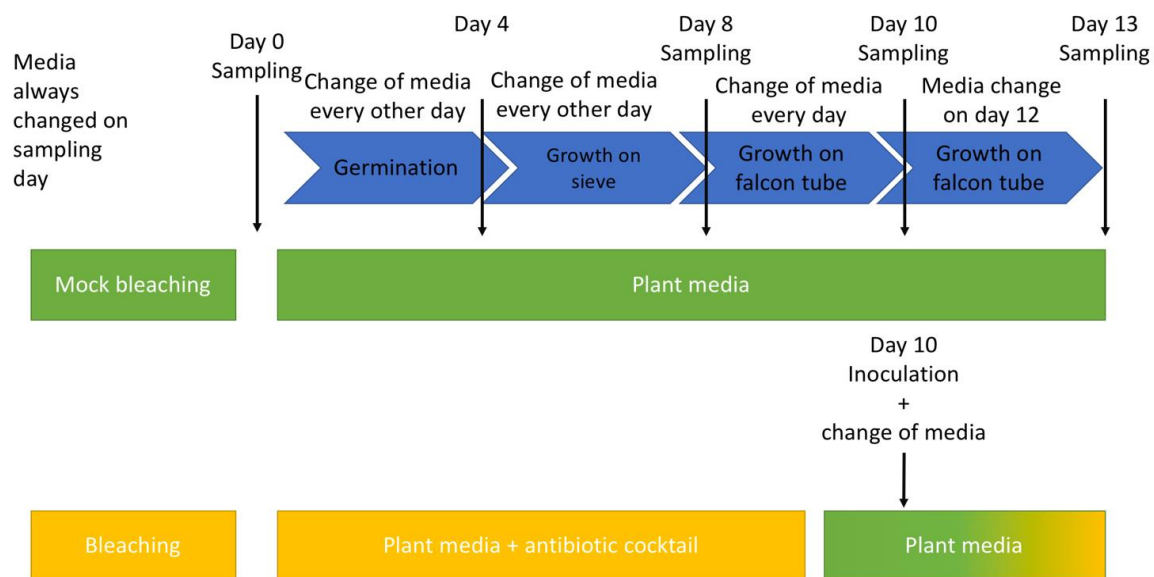


Figure 14: Timeline of the experiment.

Pantoea agglomerans established, within 3 days, a larger population than that in the previous grown root system inoculation experiment (6.5). Absolute number of colony forming units per root system was 4.00×10^5 with a minimum absolute deviation (MAD) of 1.30×10^5 and 7.3×10^6 with a MAD of 1.91×10^6 when standardizing per gram. The relatively high MAD highlights the diversity between the samples. However, the population density remained 10-100 times lower than what was achieved during colonization experiments, where the

bleached grains were inoculated directly. There are few plausible explanations to this observation; it is likely that the observed difference was either due to the shorter colonization period or the older age of the roots in combination with their smaller size due to use of the antibiotic treatment. It cannot be ruled out at this point that colonization with non-cultivable bacteria refrained *Panteoa agglomerans* cells from successfully colonizing. All the colonies observed on plates during the execution of this experiment expressed GFP and appeared green.

As plant media contained a significant number of tagged *Panteoa agglomerans* cells after 24 hours of cultivation, it is likely that the colonization process was still ongoing (a large number of freely swimming bacteria, ready to associate with the plant) or cells that proliferated on the root disassociated from its surface. This can be addressed by expanding post-inoculation time.

To address the age of roots and in an attempt to reduce new experiment needs to be designed and executed.

At the end of the experiment, I observed a significant difference in fresh weight between plants (see figure 15). Therefore, it can be hypothesized that the difference in plant fresh weight was caused either by antibiotic treatment or lack of growth-promoting bacteria at early growth stages.

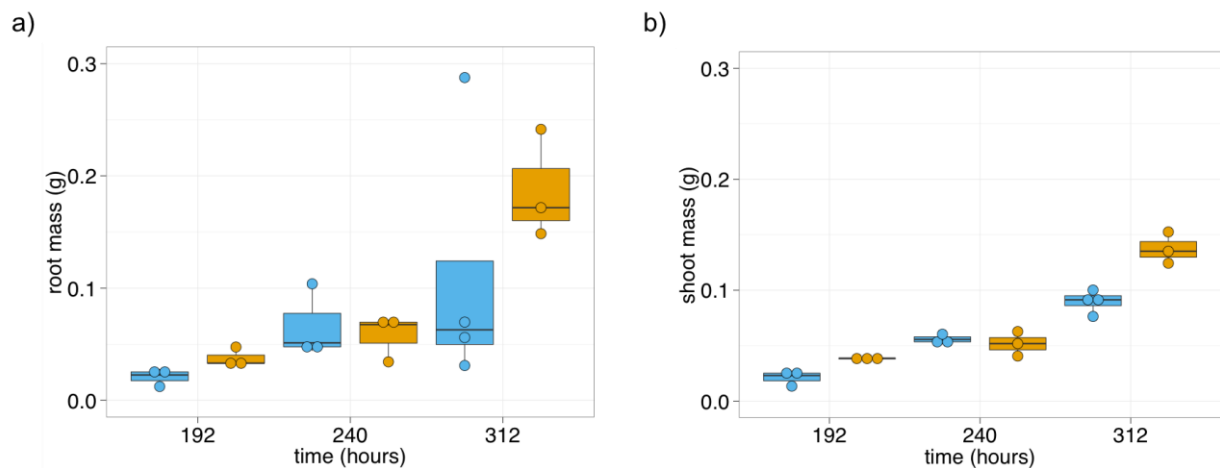


Figure 15: The mass (in grams) of treated (blue) and not treated (orange) plant. Root (a) and shoot (b) weighted separately.

Single colonizer experiment

A simple, single-colonizer experiment was performed in order to check all the changes introduced during the establishment of new gnotobiotic plant growth system and current experimental setup (figure 16 a). Post inoculation growth curve was observed before

reaching $9.6 \times 10^7 \pm 1.83 \times 10^7$ MAD (n=6) CFU per g saturation point (figure 16 b), which is in line with previous experiments.

This experiment validated the pipeline and established baseline for following experiments using the same set up. Number of colonizers CFU in media was measured as well (figure 16 d). Since media was replaced daily, meaning that registered CFU was shed from the root and/or proliferated in the media.

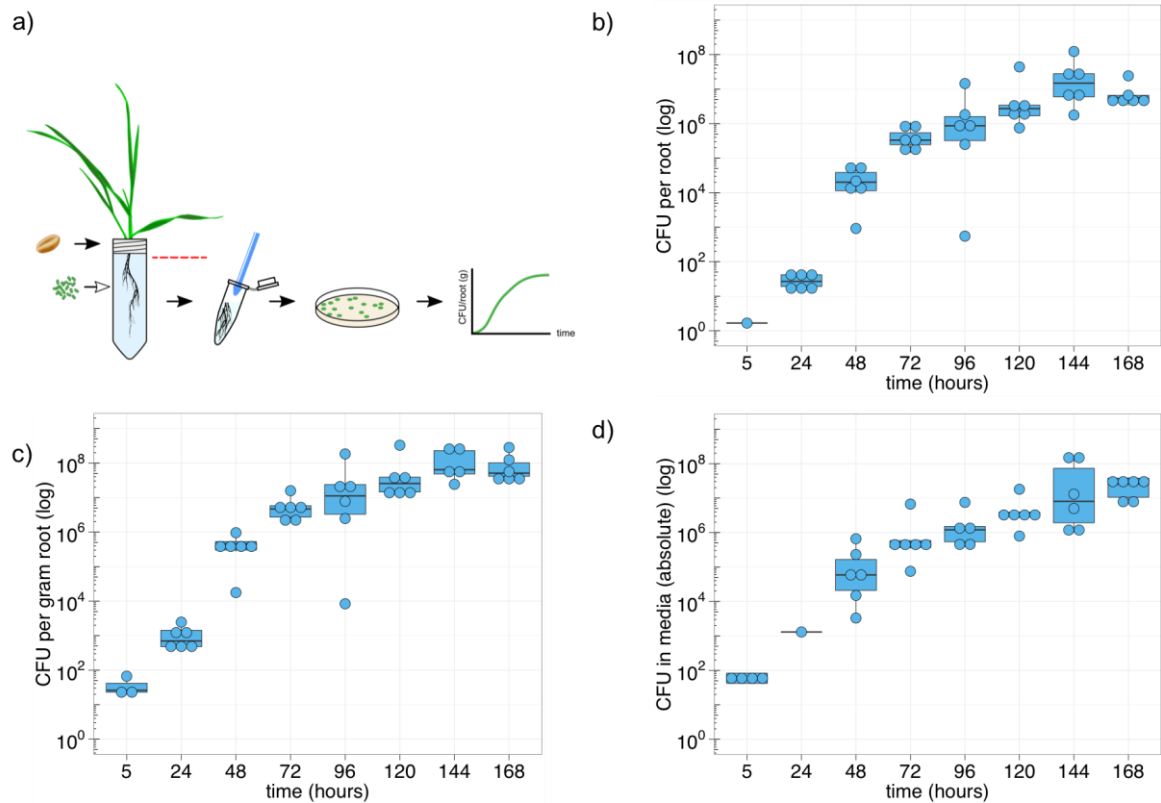


Figure 16. Single colonizer (GFP-tagged *Pantoea*) was introduced to the media. a) scheme of the experiment, b) CFU of *Pantoea* per root c) CFU of the colonizer per gram of root tissue, d) total number of colonizers in media. Population of the *Pantoea* on the root grows, until saturation point is reached. Same trend

Numerical priority effect experiments

The experimental design for numerical priority effect experiments is based on previous experiment. For the control experiment roots were inoculated with a 1:1 mixture of the GFP and mCherry tagged *Pantoea* (figure 17 a).

During the control experiment the first measurement (5 hours post inoculation) showed 91 with a MAD of 19 CFU/g of the first competitor (n=4; one sample excluded due to high contamination), and 40 ± 52 MAD; ratio of 1st:2nd colonizer being close to 2:1) The GFP-tagged strain reached a median population size of 1.2×10^8 with a MAD of 3.8×10^7 (n=5) while the mCherry-tagged strain population reached a similar median density $1.2 \times 10^8 \pm 5.2 \times 10^7$ MAD CFUs per gram; n=5). Consequently, I conclude that in this experimental system I do not observe difference in fitness between the colonizers. Notably, the total *Pantoea* population in the roots at the end of the experiment reached a median density of $2.2 \times 10^8 \pm 1.0 \times 10^8$ MAD CFUs per gram (n=5), which is within the same order of magnitude of the bacteria density observed in the single colonizer experiment (figure. 16 b). This result further supports our estimate of the root habitat carrying capacity.

To test for the effect of preemptive competition in *Pantoea* colonization of the wheat roots, I conducted colonization experiments where two competitor strains are inoculated sequentially with a delay of 24 or 48 hours between the first (mCherry-tagged) and second (GFP-tagged) incoming colonizers (Figure 17 b). The population size of both strains in the root sample was documented daily as described above. Performing the experiment with 24 h delay of the 2nd incoming colonizer shows an initial relative density of the 1st:2nd colonizers of ca. 12:1 (Figure 17 d; day 2) with 2nd colonizer population median density of $3.9 \times 10^3 \pm 2.0 \times 10^3$ MAD of CFUs per gram (n=5), while the 1st colonizer median density was $4.6 \times 10^4 \pm 4.2 \times 10^4$ MAD of CFUs per gram (n=5). Our results further show that the difference in population size between the two colonizers increases with time. Ten days post the 1st colonizer inoculation, the 1st colonizer reached a median density of 2.8×10^8 CFUs per gram (n=5), while the 2nd colonizer reached a median density of 5.9×10^5 CFUs per gram (n=5). Thus, the median of the total bacterial density at the end of the experiment was 2.8×10^8 CFUs per gram (n=5) with a relative density of the 1st:2nd colonizers being 475:1 (Figure 17 d).

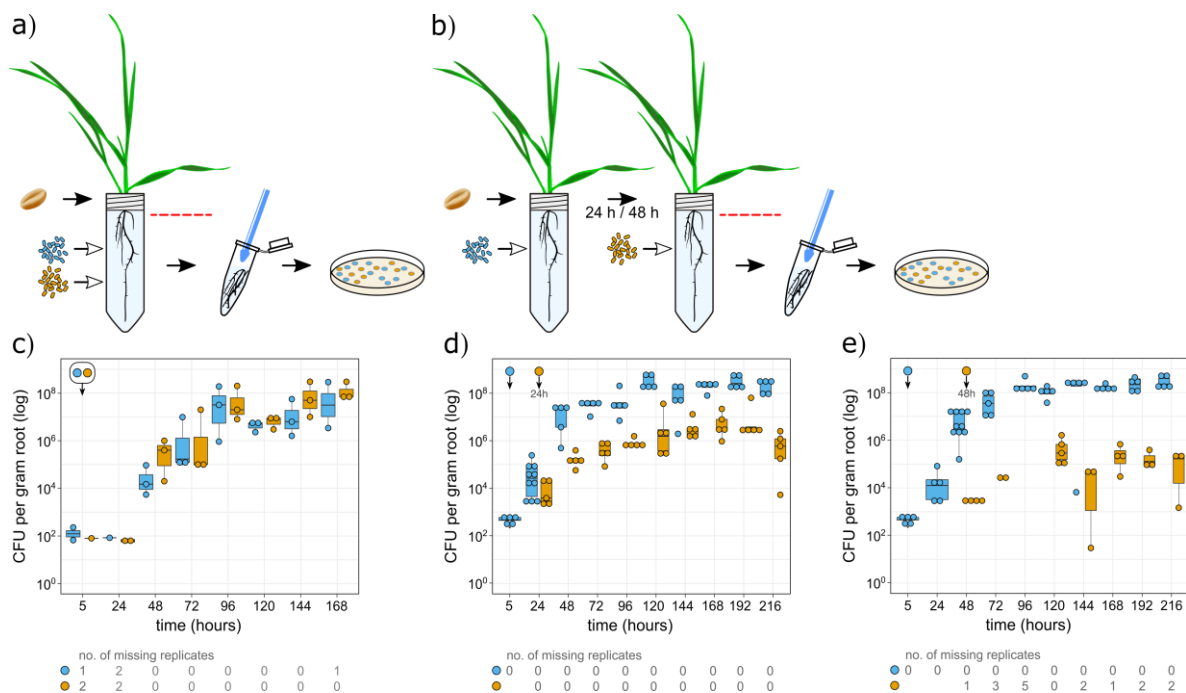


Figure: 17. Numerical priority effects. I inoculated wheat root with primary colonizer. Afterwards, I introduced secondary colonizer with 24 or 48 (a) hour delay. This resulted in secondary colonizer being less abundant compared to primary colonizer (100 fold less abundant for 24-hour delay experiment and 1,000 less abundant for 48-hour delay experiment). When I introduced both colonizers simultaneously (b), 1:1 ratio is maintained through the experiment.

Performing the numerical priority effect experiment with a 48h delay of the second colonizer reveals slightly different dynamics. Directly after the inoculation, the population size of the 2nd incoming colonizer was similar to that of the 2nd colonizer in the 24h experiment (median: $2.6 \times 10^3 \pm 1.4 \times 10^2$ MAD per gram ($n=5$); figure 17 e). Nonetheless, the difference from the 1st incoming colonizer was much larger with a 1st:2nd ratio of 1030:1. The population size of both colonizers increased over the experiment duration. Notably, the 2nd colonizer was not observed in several plants during the experiment, while the 1st colonizer was observed in all of the plants at all days. On the tenth day of the experiment the 1st colonizer reached a median density of $2.3 \times 10^8 \pm 8.4 \times 10^7$ MAD per gram ($n=5$), while the 2nd colonizer reached a median density of $1.5 \times 10^3 \pm 1.5 \times 10^3$ MAD per gram ($n=5$), with a total *Pantoea* population median density of $2.3 \times 10^8 \pm 8.4 \times 10^7$ MAD per gram root ($n=5$). The final ratio between the 1st:2nd colonizers was 155,762:1, hence the 1st colonizer had an even larger advantage over the 2nd incoming colonizer in the 48 hours delay experiment.

To further compare the impact of priority effects in the colonization of the root system, ratios of colonizers were calculated over the time of the experiment. In order to address high variability and skew of the data, log transformation was performed. Once common logarithm of these ratios in time was plotted (figure 18), regression models were calculated (table 6). Additionally, log transformation was used for *in vitro* bacteria relative fitness experiments, therefore using similar approach here improves cross-experiment data compatibility.

Medium from sampled plants were also plated. CFUs were calculated for total number of bacteria in whole media volume and plotted (figure 19 a-c). Log_{10} of competitors ratio was calculated and plotted (figure 19 d). Regression models are showed in table 7.

For the experiment with no delay, the first measure after five hours of inoculation shows population size of 2.9×10^3 vessel ($n=5$) for first competitor, and $8.0 \times 10^5 \pm 6.3 \times 10^3$ MAD vessel ($n=5$), with ratio of the 1st:2nd colonizer is: 1:275. Final *Pantoea* population, 120 hours after inoculation, was $2.3 \times 10^7 \pm 3.0 \times 10^6$ MAD vessel ($n=5$) for first competitor, and $1.2 \times 10^7 \pm 3.5 \times 10^6$ MAD per vessel ($n=5$), giving total of 3.5×10^7 CFU/g. Final ratio was (1st:2nd colonizer): 2:1.

In the experiment where the second colonizer was delayed by 24 hours, the first measure five hours post inoculation with second colonizer, shows $2.8 \times 10^5 \pm 4.5 \times 10^4$ MAD per vessel ($n=5$) for first competitor, and $1.3 \times 10^4 \pm 1.3 \times 10^5$ MAD vessel ($n=5$), with ratio of the 1st:2nd colonizer is: 21:1. Final measurement, 216 hours after inoculation was $6.8 \times 10^7 \pm 1.7 \times 10^7$ MAD vessel ($n=5$) for first competitor, and $6.0 \times 10^5 \pm 3.7 \times 10^7$ MAD per vessel ($n=5$), giving total of 6.9×10^7 CFU/g. Final ratio was (1st:2nd colonizer): 114:1.

In the samples where second colonizer was delayed by 48 hours, first measure five hours post inoculation with second colonizer, shows $2.7 \times 10^6 \pm 1.8 \times 10^6$ MAD per vessel ($n=5$) for first competitor, and $1.0 \times 10^4 \pm 2.1 \times 10^6$ MAD vessel ($n=5$), with ratio of the 1st:2nd colonizer is: 263:1. Final measurement, 216 hours after inoculation was $1.9 \times 10^8 \pm 2.0 \times 10^7$ MAD vessel ($n=5$) for first competitor, and $3.1 \times 10^4 \pm 2.5 \times 10^8$ MAD per vessel ($n=5$), giving total of 1.9×10^8 CFU/g. Final ratio was (1st:2nd colonizer): 6119:1.

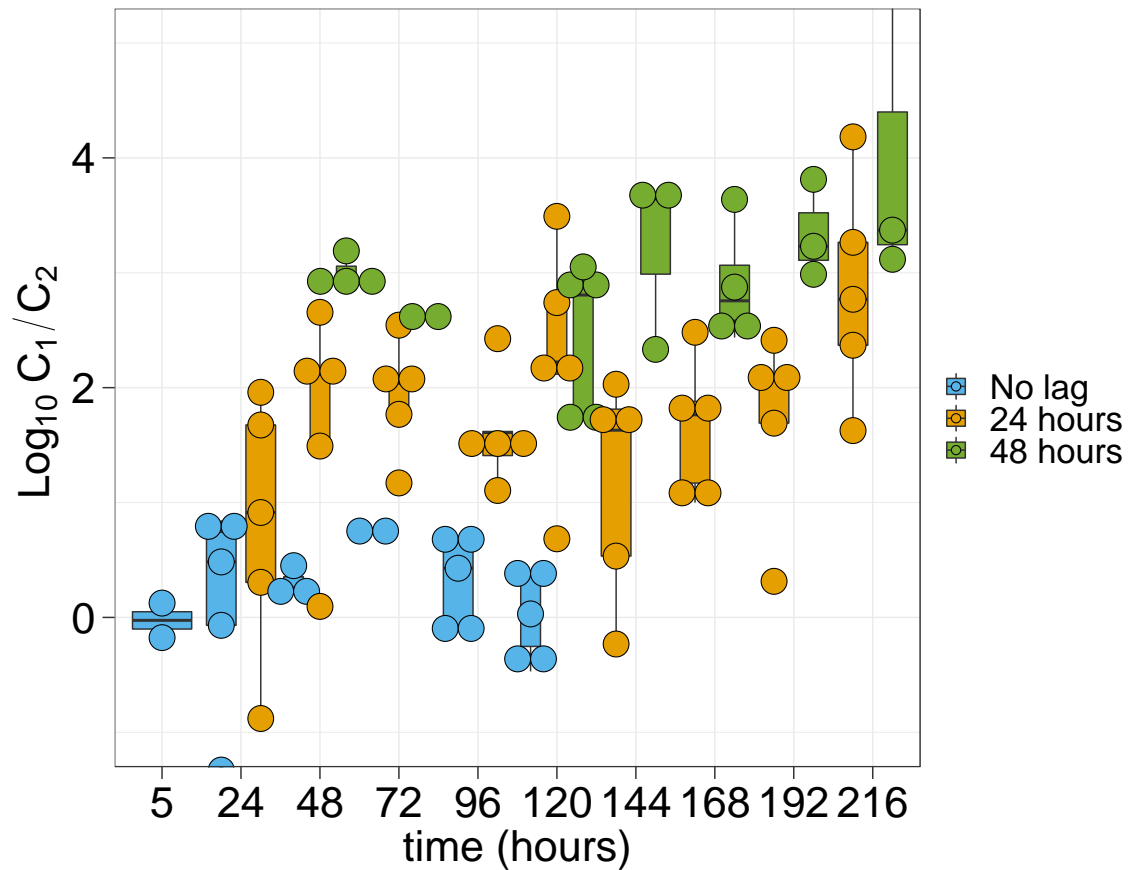


Figure 18. Plot of logarithmic ratios of colonizer over time for no delay experiment (1:1 ratio), 24-hour delay experiment and 48-hour delay experiment. While for no-delay experiments, ratio of competitors remains constant throughout the experiment, it changes for 24- and 48-hour lag experiments.

Table 6. Slope and p-value of the slope. Slope was calculated using common logarithm of the ratio of the competitors in plant roots.			
Experiment	Slope (b)	R-squared	P-value
No delay	0.0077	0.0007	0.9061
No delay - alternative experiment	-0.0723	0.0155	0.2730
24-hour delay	0.1203	0.0857	0.0287
48-hour delay	0.1255	0.1216	0.529

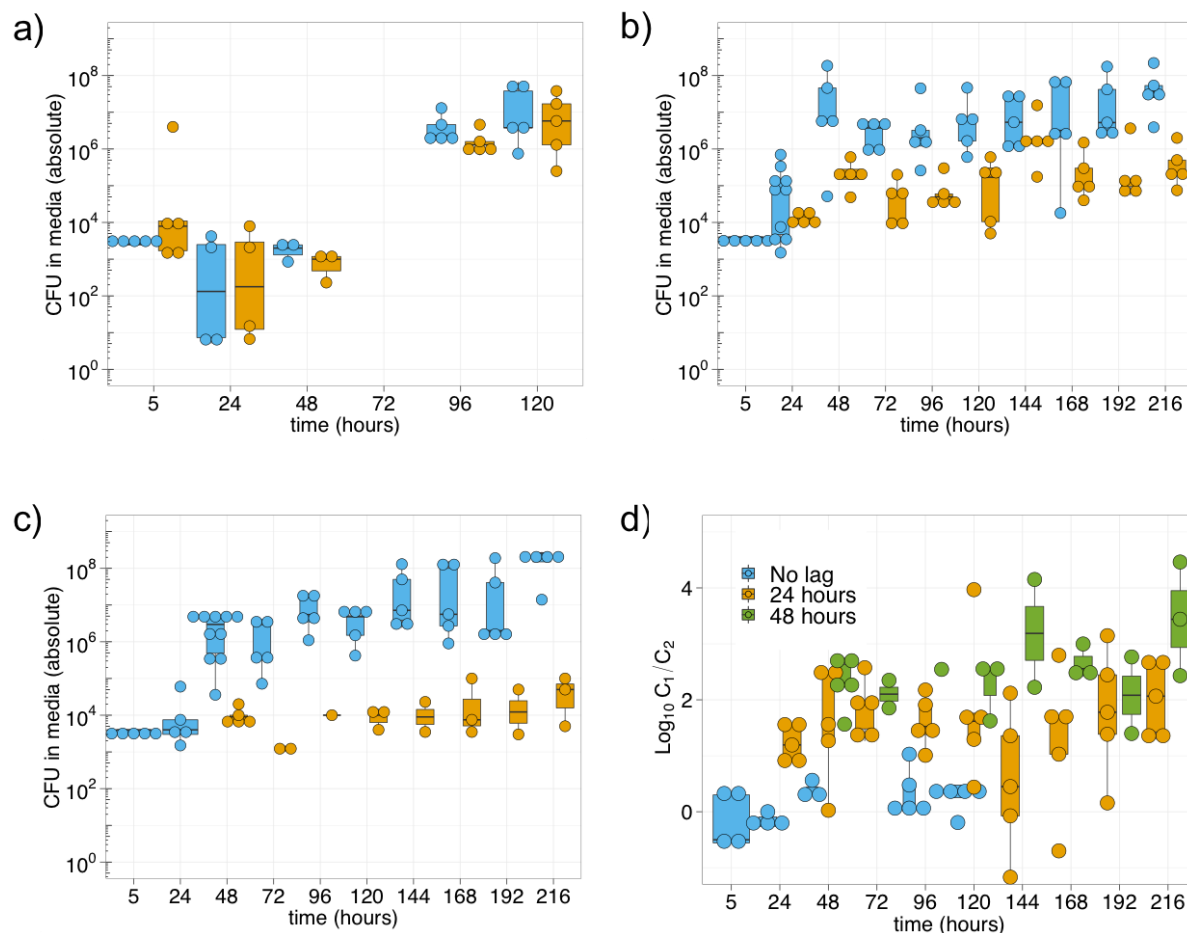


Figure 19. *Pantoea*'s abundance in media during a) no delay experiment, b) 24-hour delay and c) 48-hour delay. d) common logarithm of ratios of competitors in media.

Table 7. Slope and p-value of the slope. Slope was calculated using common logarithm of the ratio of the competitors in media.

Experiment	Slope (b)	R-squared	P-value
No delay	0.18306	0.2049	0.0344
24-hour delay	0.02826	0.005799	0.6191
48-hour delay	0.1159	0.1573	0.07504

6.8 No adaptive priority effects for early

In vitro fitness impact of priming

In vitro competitions were performed as described previously in chapter 6.3. This time I focused on assessing the impact of priming on fitness of *Pantoea* cultures. As visible by the

slope (figure 20), for both setups fitness difference between competitors is low (up to 1.5%). Statistical analysis shows that the slope was not statistically significant (table 8), further suggesting negligible fitness difference.

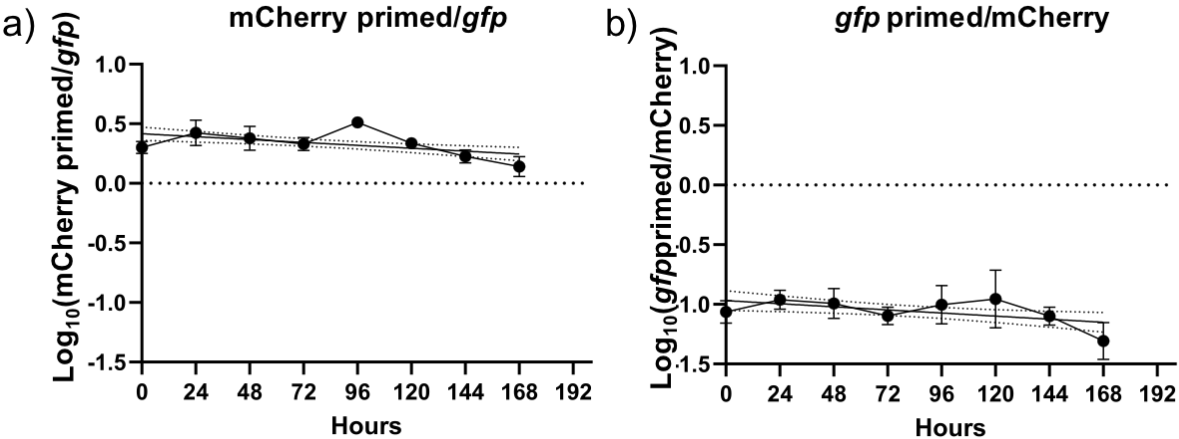


Figure 20. Competitions between *Pantoea* primed and non-primed strains presented as change in common logarithm value of the ratio of the competitors. N=8

Table 8. Relative fitness. Fitness cost calculated using mean of common logarithms of 1st competitor, against 2nd competitor. In all cases, priming causes fitness cost. N=8, 7 days each. *P-value of the regression.

a) R1 pTW1-mCherry Primed/R1 pRL153-GFP				
replicates	Slope (b)	P-value	FDR adjusted p-value	fitness (w) of strain A
1	-0.00056	0.964609	0.05	0.99972
2	-0.03889	0.214774	0.025	0.98056
3	-0.00677	0.714400	0.04375	0.99662
4	-0.02409	0.292576	0.03125	0.98795
5	-0.03363	0.119496	0.0125	0.98318
6	-0.04061	0.074156	0.00625	0.97970
7	-0.01673	0.417570	0.0375	0.99164
8	-0.03355	0.131233	0.01875	0.98323
Average				0.98782
Standard deviation				0.00751
Fitness cost				1.22%
Confidence Interval				0.00628
R1 pTW1-mCherry Primed is less fit than R1 pRL153-GFP				

b) R1 pRL153-GFP Primed/R1 pTW1-mCherry				
replicates	Slope (b)	P-value	FDR adjusted p-value	fitness (w) of strain A
1	-0.03249	0.249282	0.01875	0.98376
2	-0.06708	0.083599	0.0125	0.96646
3	-0.02609	0.260931	0.03125	0.98696
4	-0.01079	0.700655	0.04375	0.99460
5	-0.02904	0.254944	0.025	0.98548
6	-0.04140	0.036233	0.00625	0.97930
7	-0.01706	0.377136	0.0375	0.99147
8	-0.01847	0.766794	0.05	0.99076
Average				0.98485
Standard deviation				0.00886
Fitness cost				1.52%
Confidence Interval				0.00740
R1 pRL153-GFP Primed is less fit than R1 pTW1-mCherry				

Impact of priming on colonization dynamics

The stark differences were observed between the 1st and 2nd incoming colonizers in the lag experiments may be explained by the presence of an adaptive priority effect in *Pantoea* colonization of the root. To further test if early incoming colonizers have a fitness benefit over the late incoming colonizers, I performed an *in planta* competition, as shown in the figure 21, between *Pantoea* isolated from the root – i.e., primed – and *Pantoea* cultivated in liquid media (i.e., non-primed).

The priming of *Pantoea* was performed as in my standard inoculation procedure. Seven days post inoculation the primed *Pantoea* cells were isolated from the roots and dilutions were directly used (no *in vitro* cultivation step after isolation from plant) in a mixture with *Pantoea* grown in liquid media to inoculate germ-free roots (figure 21 a).

The documentation of colonizer population size directly after the inoculation shows that the initial ratio of primed to non-primed 1:7 (Fig. 4B. The population size of both colonizers increased over the experiment duration and stabilized at day 6 post inoculation. On day 9 of the experiment the primed colonizer reached a median density of $5.6 \times 10^6 \pm 2.3 \times 10^6$ MAD per gram (n=6), while the non-primed colonizer median density was $3.9 \times 10^7 \pm 1.1 \times 10^7$ MAD per gram (n=6), with a total population median density of $4.5 \times 10^7 \pm 1.0 \times 10^7$ MAD per gram root (n=6). The final ratio of the primed and non-primed competitors was thus 1:7.

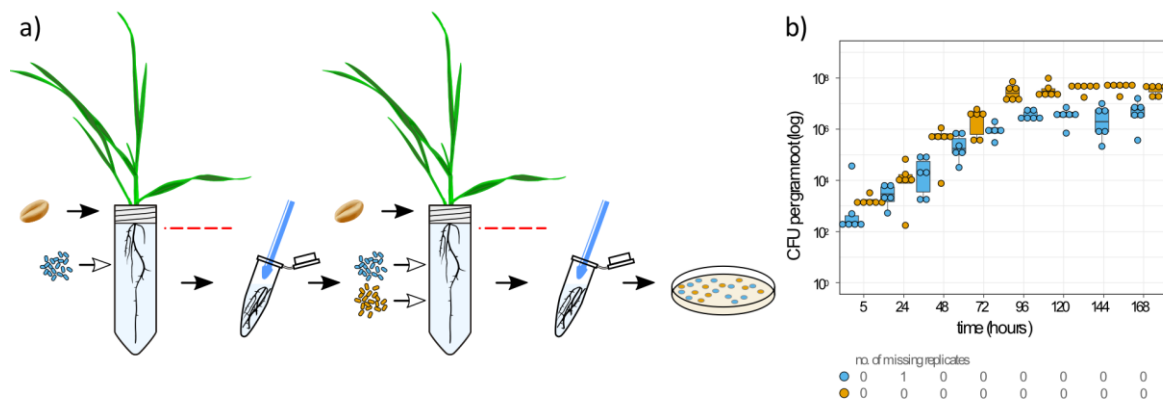


Figure 21 *In planta* competition for adaptive advantage. a) Two isogenic *Pantoea agglomerans* strains were prepared. First (in orange) was cultivated in R2A broth with transfer every 24 hours; second strain (blue) was inoculated into a plant. After seven days, both strains were used to inoculate fresh seedlings. Despite different pre-inoculation treatments, proportion between strains remained stable through whole experiment. b) population density of tagged colonizers throughout the experiment.

Ratios of CFUs of the competitors were calculated and log-transformed. As previously linear regression was applied (figure 22 a and b). Results of swap (switch of tags on primed/non-primed competitors) experiment are also reported. Low value of the slope confirms lack of priming effect. Counts obtained from media were processed in analogous manner (figure 22 c and d).

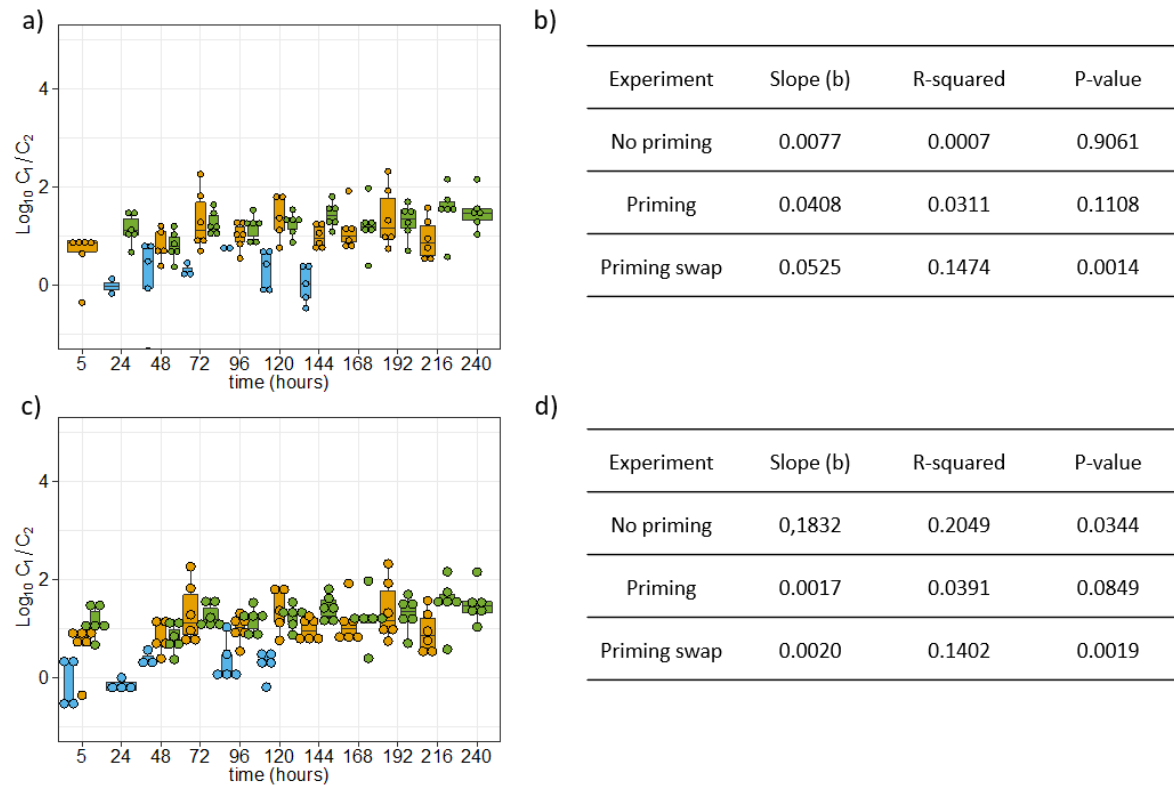
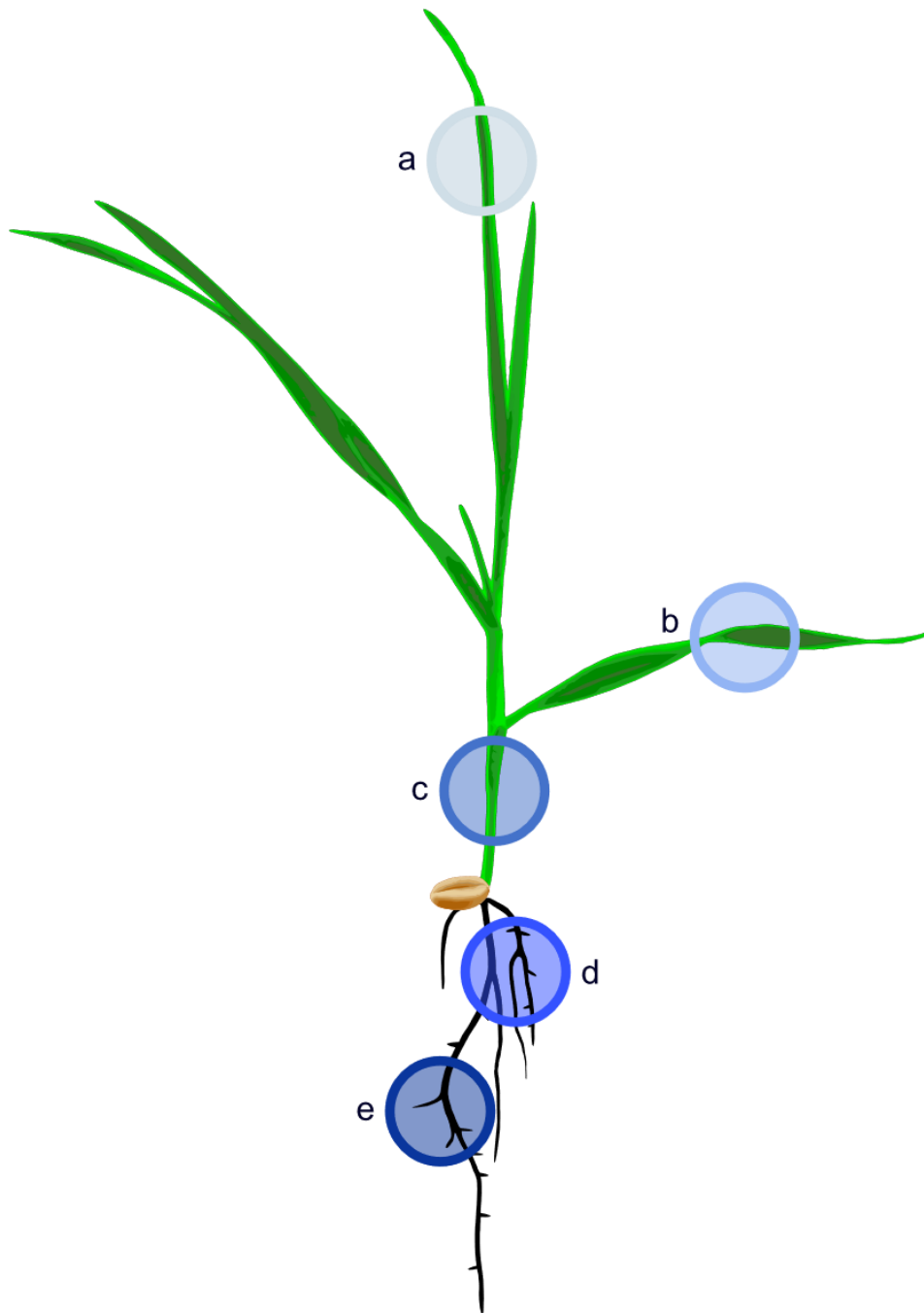


Figure 22. Results of priming experiment and swap experiment: a) common logarithm of ratio of competitors (C1 and C2) extracted from root throughout the experiment. Blue-no priming experiment (control); orange and green: priming experiment; b) Slope and statistical evaluation of the linear regression model; c) common logarithm of ratio of competitors (C1 and C2) in media throughout the experiment; d) slope and statistical evaluation of linear regression model

6.9 *Pantoea* persistence in the wheat tissue

To further follow the *Pantoea* presence in the wheat tissues, I sampled older plants that were inoculated during the colonization experiments. Our results show that within 32-40 days post inoculation *Pantoea* is present in the wheat plants, where the highest density was observed in the primary and crown root (figure 23). Additionally, *Pantoea* was highly abundant in the stem, while the population density was lowest in the leaves. Younger part of the root (crown root) harbors higher density of *Pantoea* than older primary root.



CFU per gram (fw)

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

Figure 23. Persistence of *Pantoea* in plants, 38 and 46 days post inoculation.

7 Discussion

In this thesis I performed experiments towards the aim to explore the dynamics of host colonization and to characterize the effect of priority effects. In comparison to previous work on host colonization dynamics performed with Hydra as the host (Wein et al. 2018), experimenting with wheat (i.e., my host) presents several differences and hence novel challenges. Firstly, through the experiment host forms ever growing habitat, secondly, different plants from the same cohort show significant variation in size (especially early during the experiment). To alleviate varied plant size, instead of using absolute values of bacterial populations, I used the population density that was calculated relative to gram of host tissue.

Challenges with obtaining and maintaining germ-free hosts were originating from two main reasons. Firstly, the removal of viable bacteria and fungi without damaging the germ was more demanding task than anticipated. Most available protocols are developed for seeds of *Arabidopsis* or other plants with smaller seeds, which can be easily penetrated by bacteriocidal agents. In wheat, while the embryo itself may be germ-free, the endosperm of seeds can still be inhabited by bacteria (Robinson et al. 2016b). Due to the structure of the seed, the embryo is not well protected from bleach (bran covers whole seed and it has to be penetrated to let bleach sterilize the endosperm). On top of that, it is known, that spores are, to a certain degree, resistant to bleaching (Young and Setlow 2003; Eissa et al. 2014).

Secondly, antibiotic resistance is widespread among soil-associated bacteria not only due to human intervention (Cycoń et al. 2019; Armalyté et al. 2019; Zhu et al. 2019) but can occur naturally as well (Allen et al. 2010). This means, that germinating the grains in an antibiotic cocktail may not be enough to assure removal of all bacteria, which was confirmed in preliminary experiments; only bleaching treatment and germination in antibiotic cocktail assured that most of the seedlings were free from cultivable bacteria.

Once the methodology for acquiring germ-free plants was established, reintroduction of *Pantoea* with phenotypic marker was possible. My findings show, that the wheat roots have a limited carrying capacity, which is well correlated with the root (i.e., habitat) size. Throughout the colonization experiments, the *Pantoea* population density increased and around 10^8 CFU/g of root fresh weight, independently of the tested individuals. Thus, further increase of the bacterial population was correlated with the plant growth. Notably, despite the bacterial population size increase, the density expressed as CFU per gram of plant tissue remained stable. The bacterial densities I observed in our experiments are in line with average bacterial density observed in the rhizosphere (10^6 - 10^9 CFU per gram) and the root endosphere (10^4 - 10^8 CFU per gram) (Bulgarelli et al. 2013). From this I conclude that our

liquid media cultivation approach provides a suitable environment for the wheat plants and supports growth of host associated bacteria and colonization experiments.

Following, data regarding *Pantoea* in spent plant media can be compared to observations of bacteria in soil, put together in beforementioned review by (Bulgarelli et al. 2013). Again, up to 2×10^6 CFUs per milliliter of spent media from *Pantoea* 'saturated' plants were observed, which is in line with 10^6 - 10^9 per gram of soil. Since the wild-type microbiota was assessed, higher population densities may be the result of trophic network present in soil and rhizosphere. Microbial interactions composing these networks include cross-feeding and other synergistic interactions (Wei et al. 2015). Trophic network may promote more efficient utilization of available nutrients, explaining plant's ability to maintain denser microbiota. This is not the case for my experimental system, where due to colonization with isogenic strains, no trophic networks can be formed: *Pantoea* can utilize only readily available nutrients.

During my colonization experiments, bacterial growth was observed not only on the plant itself, but in media as well, reaching values up to 2×10^6 CFU/ml. Since the plant media used in my experiments is lacking a significant carbon source, it is unable to promote bacterial growth by itself. Bacteria present in the media during the experiment were therefore shed from the root between the exchanges of media or were able to proliferate due to nutrients coming from the plant by means of rhizodeposition. It has been estimated that 10-16% of the nitrogen and 11-17% of the carbon fixed via photosynthesis is leaking into the rhizosphere (Nguyen 2003; Jones et al. 2009). Rhizodeposition supplies, among other, low-molecular-weight carbon substrates such as glucose, glycine, or citric acid (Bulgarelli et al. 2013) providing necessary carbon sources for bacteria. Thus, the plant provides enough nutrients not only for bacteria colonizing the root but also for free-swimming bacteria in media. Consequently, I conclude that nutrients deficit is not the reason for limited colonizer density on root tissue. Nonetheless, our results clearly show that the *Pantoea* density in the wheat root is stable during the late colonization stages. I hypothesize, that other factors may be involved in the regulation of the maximal bacteria population density. These include bacterial quorum sensing, and interactions with the plant immune system.

Regulation of bacterial population size via quorum sensing

Quorum sensing, includes various mechanisms with which bacteria coordinate their actions in orchestrated, multicellular-organism-like fashion (Fuqua et al. 1994). Bacteria release autoinducers (extracellular signaling molecules), which accumulate in the environment; where their accumulation in the environment supplies a signal that enables the bacteria to orchestrate the expression of dozens or even hundreds of genes, as a response to the

bacterial cell density (Papenfort and Bassler 2016). Examples of this phenomena are biofilm formation, production of antibiotic and virulence factors. Very vibrant and sophisticated example is symbiosis between bioluminescent *Vibrio fischeri* and its host *Euprymna scolopes* (Hawaiian bobtail squid). The bacteria 'light up' the squid at night when the bacterial population is densely packed in light organs. The bioluminescence stops in the morning, when light organs are cleansed from the majority of bacteria, reducing the population density (Verma and Miyashiro 2013).

Our observation of the root 'saturation' and the high abundance of *Pantoea* I observed in the plant media indicates bacterial shedding from the root. Bacterial shedding could be considered as a form of population density regulation in biofilm-like clusters observed on the root. A recent study of *Pseudomonas putida* isolated from tomato roots, showed that expression of putisolvin, a biosurfactant required for cell's escape from the biofilm, is regulated by the quorum sensing (Cárcamo-Oyarce et al. 2015). That research further described a stochastic auto induction in young colonies, where induced bacteria leave the population before inducing neighboring cells; only in aged biofilm, induction can be strong enough to cause biofilm collapse. *Pantoea stewartii* subspecies *stewartii* is has the ability to regulate biofilm structure depending on the cell density (Koutsoudis et al. 2006). Hence, our finding of *Pantoea* in the medium in high density points at aged biofilm at its peak density. This is in line with our observation of population density limit on the root.

Notably, host-associated bacteria may use exogenous metabolites sourced from the host for their quorum sensing molecules synthesis; in a way, including possible signals from the host in the quorum sensing signaling. For instance the photosynthetic bacterium *Rhodopseudomonas palustris* and at least one strain of stem-nodulating photosynthetic bradyrhizobia utilize p-coumarate originated from plant host to produce their quorum sensing molecule: fatty acyl-homoserine lactone (Schaefer et al. 2008; Ahlgren et al. 2011). Whether *Pantoea* density in the wheat root may be regulated by a similar mechanism can be tested experimentally and is subject for future investigations.

Regulation of bacterial population size by the plant immune system

Abovementioned density-dependent pathogenicity is caused by expression of *hrp/hrc* gene cluster; containing a Type 3 Secretion System (its name can be abbreviated to TTSS or T3SS). The T3SS is oftentimes related to avoidance of the plant host immune system using immune-suppressing effector proteins (Collmer et al. 2000; Block et al. 2008; Clarke et al. 2010; Waite et al. 2017). While direct immune suppression is not the only function of T3SS, it is the best known function for that system (Macho 2016). As shown in experimental work on *Pseudomonas syringae*, which may be associated with *Arabidopsis*, T3SS-defective mutants have reduced population density compared to the wildtype bacterium; the bacteria density can be increased if the host is deficient in *nahG*, which disrupts the salicylic acid mediated immunity pathway (Hauck et al. 2003). Salicylic acid mediated immunity pathway has many sophisticated regulatory mechanisms and the details of the mechanism of bacteria-mediated interference of the pathway remain unknown (Huang et al. 2018). In this study, plant immune system was not fully eradicating bacterial colonizers despite being activated. The plant immune system is not only able to eradicate pathogens but also to regulate the population size of resident bacteria. While simply damaging a plant can trigger a response of the immune system (Choi et al. 2016), at least some pathways of the plant immune system seem to be activated by microbes in a dose dependent manner (Denoux et al. 2008; Moroz et al. 2017). A similar mechanism might explain the observation of different *Pantoea* species and strains showing different host specificity and ability to reach different population densities depending on the host (maize, onion and fruit fly were tested) (Nadarasah and Stavrinides 2014). As shown in an immunodeficient *Arabidopsis* host, under favorable conditions of extremely high humidity, even the growth of commensal bacterial communities may turn into excessive proliferation, resulting in diseased plant phenotype (Xin et al. 2016). That observation clearly demonstrates that the plant immune system is at least partially responsible for regulation of saprophytic bacteria population size.

Priority effects in *Pantoea* colonization of the root.

In my experiments I looked for basic mechanism explaining vertical inheritance of seed associated bacteria in wheat. I hypothesized, that since embryo remains germ-free until germination, it has to be colonized anew, enabling microbes present in the environment to colonize the host. This opportunity for outsiders to colonize the host, suggests that there are mechanisms in place, that ensure stable vertical-like inheritance of *Pantoea*. Such mechanisms could be ecological priority effects, for which I tested.

In order to test for ecological priority effect, I used the simplified system, where isogenic bacteria tagged with different fluorophores colonize wheat root. Plants were cultivated in the presence of antibiotics, enabling us to exclude native microbiota and other contaminants from the environment. The colonization experiment with single colonizer, showed, that *Pantoea* isolated from Runal variety of *Triticum aestivum* can efficiently colonize Benchmark variety.

My attempt at colonizing plants with two isogenic strains of *Pantoea* harboring different fluorophores, were initiated by introducing both competing strains simultaneously, in ratio close to 1:1. This experiment was aimed at verifying relative fitness of the competitors. Since ratio of the competitors was maintained through the experiment, I concluded that the fluorophores used had similar impact on *Pantoea*'s fitness on plant host. This means that in further competitions, the fluorophores impact is negligible. Notably, the total population of competitors reached the expected host saturation (i.e., the root carrying capacity). In addition, *in vitro* competition experiments show low fitness difference between both pRL153-*gfp* harboring strains.

To test for numerical priority effect, a second colonizer was introduced into the plant media with 24- or 48-hour delay after the first colonizer. While final total population density remained within the expected carrying capacity, and at the time of its introduction roots were not saturated, the late arriving colonizer was not as successful as the first colonizer. One can hypothesize, that the greater the delay between the colonizers, the greater the benefit achieved through the priority effect, though I do not have enough data to verify that claim. This could be due to a) primary colonizer using up the nutrients, by that lowering their concentration and limiting the early growth of secondary colonizer, b) primary colonizer occupying colonization hotspots on the root c) population on the root is reaching saturation point and therefore colonization efficiency is reduced d) primary colonizer was already primed by the time second colonizer was introduced, making it more efficient at using/controlling the niche-

In order to test for adaptive priority effect, or priming, colonization competition experiment where one of the strains was pre-cultivated on a plant was performed. Though, I failed to achieve 1:1 ratio for the inoculation process, the ratio between colonizers was maintained through the experiment. This demonstrates that priming does not enhance *Pantoea*'s colonization capabilities.

How numerical priority effects may mediate vertical inheritance

Numerical effect could be one of mechanism that promotes transfer to another generation. Simply being present early on, before other competitor and/or being more abundant may be the mechanism that *Pantoea* uses to outcompete other colonizers that may arrive during germination and flowering. In my experiment, I showed that numerical/temporal advantage upon equally capable late-incoming colonizer is enough to enable successful colonization and dominance on the root. In this case, even though the colonization process may still be influenced by incoming bacteria, *Pantoea* will have chance to establish a stable colonization of the plant roots.

In context of hypothesized vertical transfer through the grain, this adaptation promotes rapid colonization of germinating embryo, without lag period. In the light of importance of numerical advantage, this means, that first colonizing *Pantoea* strain to establish population on the plant is more likely to remain main colonizer of next generation of plants. Existence of priority effect enables the exclusion of future colonists (Urban and De Meester 2009).

While (Ruppel et al. 1992) doesn't show CFU's directly (they used ELISA to comparatively quantify colonization), higher densities of *Pantoea* were found in root sap, compared to shoot sap; independent of the inoculation spot and density. This is analogous to what I observed in the older plants. While roots remained highly colonized, other parts of the plant showed signs of lower density of colonization. This way of bacterial distribution is common among other plants. For instance, in rice, fluorescently tagged rhizobia were recovered from surface sterilized roots (2.0×10^4 – 1.3×10^6 cfu/g fresh weight) and from leaf sheaths (1.0×10^3 – 2.5×10^5 cfu/g fresh weight) (Chi et al. 2005). Differences in population sizes were observed not only between parts of plant, but also between different colonizers (figure 24).

Furthermore, for pumpkins bacterial density varied between endorhiza, anthosphere and carposphere (1.6×10^4 , 1.0×10^7 , and 2.0×10^3 CFU/f on average, respectively) (Förnkrantz et al. 2012).

In the context of hypothesized vertical transfer through the grain, this adaptation promotes rapid colonization of germinating embryo, close proximity to the embryo means, that *Pantoea* can start proliferation and colonization without a lag period, as soon as conditions promote germination of the seed. According to the theoretical expectation, the existence of priority effect enables the exclusion of future colonizers (Urban and De Meester 2009). In the light of the importance of numerical advantage, this means, that first colonizing *Pantoea* strain to establish population on the plant is more likely to remain main colonizer of

next generation of plants, outcompeting soil-borne *Pantoea*, or even hypothetically other bacteria.

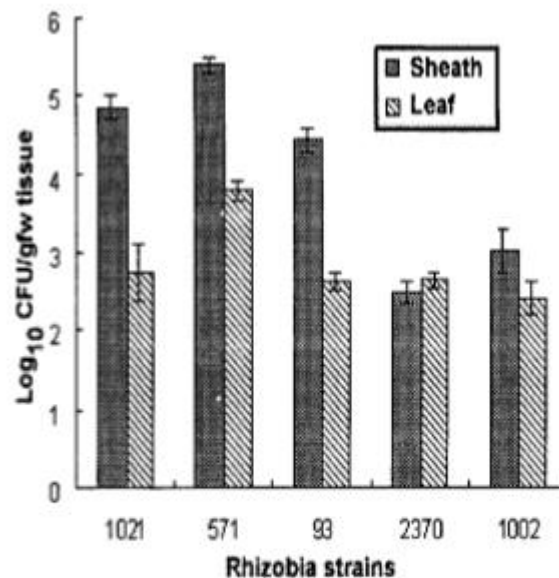


FIG. 4. Culturable population densities of *gfp*-tagged *S. meliloti* 1021, *A. caulinodans* ORS571, *M. huakuii* 93, *R. leguminosarum* USDA 2370, and *S. meliloti* 1002 within large tissue samples of leaf sheaths and leaves of rice plants grown in open potted soil plus vermiculite (1:1 ratio) and harvested at 125 dpi. The data reported are the means \pm the standard errors of the mean from three tissue sample replicates plated on TY media.

Figure 24. Population density of the colonizer depends on both: colonizer species and part of the plant. (Chi et al. 2005)

The final result of the thesis points towards the flowering and seed formation stages as the next critical wheat lifecycle stage for *Pantoea* colonization and maintenance among the generations of the host. Future work should likely focus on elucidating how *Pantoea* colonizes the wheat seeds.

8 References

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