Differences in root colonization by fluorescent *Pseudomonas* spp.

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Plant-Microbe Interactions

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Abstract

Pseudomonas fluorescens WCS374 and WCS417 are plant growth promoting rhizobacteria. *P. fluorescens* WCS417 is known to be able to induce systemic resistance in *Arabidopsis thaliana* and radish while *P. fluorescens* WCS374 can only do so in radish. On *A. thaliana* root colonization by *P. fluorescens* WCS417 is superior. The performed research shows that *P. fluorescens* WCS417 is a better root colonizer on *A. thaliana* accession Col-0 and the mutant *myb72-1* as well as on radish. The differences in root colonization are not caused by the capability of inducing systemic resistance in the plant neither by a difference in growth rates. The performed research does show a linkage with the capability of *P. fluorescens* WCS417 to use the carbon source D-sorbitol. This carbon source could not be used by *P. fluorescens* WCS374.

Introduction

Plants are dependent on beneficial microorganisms for their own health (Berendsen *et al.,* 2012). There are several ways in which beneficial microorganisms can help the plant to improve its health. One of these ways to improve plant health is the production of antibiotics like 2,4-diacetylphloroglucinol which is produced by *Pseudomonas fluorescens* CHA0 (Jousset *et al.,* 2010). With the production of this antibiotic the growth of other and potential harmful microbes is reduced. Another well studied way in which plant health is improved by beneficial microbes is known as induced systemic resistance. In the case of induced systemic resistance the plant reaches an enhanced defense capacity upon colonization by beneficial bacteria. When attacked by a pathogen the plant is able to respond faster and more effectively, this is called priming (Conrath *et al.,* 2002, 2006; Van Wees *et al.,* 2008). In contrast to systemic acquired resistance no pathogenesis-related genes are up regulated by enhanced levels of salicylic acid, though jasmonic acid and ethylene play a major role in inducing systemic resistance (Pieterse *et al., 1996*).

Vice versa, many beneficial microorganisms depend on the plant as well for their own well being. Most soils are low in carbon sources (Garbeva *et al.* 2011) and because plants secrete up to 40% of their photosynthates into the rhizosphere, a good environment originates for these microorganisms (Bais *et al.* 2006). Because of the higher concentration of nutrients in the rhizosphere microbial population densities are also higher. This is commonly known as the rhizosphere effect. For bacteria it is of great importance to be able to colonize the roots. To be able to colonize roots means an open access to nutrients needed for growth and survival.

To be a good root colonizer the microbe has to be able to detect nutrients and move towards these nutrients. They do so by means of flagella, and the ability to use different carbon sources, keep other microbes away for instance by producing antibiotics and to suppress the defense mechanism of the plant (Zamioudis and Pieterse, 2011). The defense mechanism of a plant is activated upon recognition of microbe-associated molecular patterns (MAMPs) like flagelin or lipopolysaccharides. To colonize the roots, beneficial microbes need to work with or around this activated defense mechanism in order to stay on the roots, for instance, by secreting effector molecules that suppress a MAMP-triggered immunity response (Zamioudis and Pieterse, 2011). When MAMPs are recognized by the plant an activation of the transcription factor gene *MYB72* in the roots also occurs (van Wees *et al.,* 2008). This gene is very important in the early steps of induced systemic resistance (van der Ent *et al.* 2008 and van Wees *et al.,* 2008). Furthermore, Doornbos *et al.* (2009) showed that *P. fluorescens* WCS417 has a reduced colonizing ability on the myb72 mutant. This gives room to the hypothesis that the *MYB72* gene doesn't only influence induced systemic resistance but also influences the chances for a plant to be colonized.

Research showed that not all beneficial microbes can induce systemic resistance in all plants (Bakker *et al.* 2007). The beneficial bacteria *Pseudomonas fluorescens* WCS417 is able to induce systemic resistance in Arabidopsis and radish while *Pseudomonas fluorescens* WCS374 is only able to induce systemic resistance in radish but not in Arabidopsis (Bakker *et al.* 2007). Furthermore, *P. fluorescens* WCS417 is a better colonizer of the Arabidopsis rhizosphere then *P. fluorescens* WCS374 which leads to the thought that root colonization and the ability of inducing systemic resistance are linked (Raaijmakers *et al.,* 1995).

The aim of this study was to investigate what determines the difference in root colonization between *P. fluorescens* WCS417 and WCS374 on Arabidopsis but not on Radish. We analyzed the root colonization on *Arabidopsis thaliana* accession Col-0, its mutant *myb72-1* and on *Raphanus satura* cultivar Saxanova. The mutant *myb72-1* is used because if the ability to induce systemic resistance plays a role in the ability to colonize the roots then on this mutant *P. fluorescens* WCS417 and WCS374 have to colonize the roots at equal rates. The same accounts for the use of radish on which both strains can induce systemic resistance and if the ability to induce systemic resistance plays a role in root colonization then root colonization on radish has to be equal between both strains as well. Furthermore we analyzed the root colonization on *Arabidopsis thaliana* accession Col-0 and its mutant *myb72-1* on plates, individual and in combination to see if one can outcompete the other. Also growth rates of both Pseudomonas strains were analyzed to see if this could be of influence on the root colonizing ability. And last we analyzed the use of different carbon sources between *P. fluorescens* WCS417 and WCS374.

Material and Methods

Cultivation of bacteria

For the performed experiments the following bacterial strains were used (table 1):

Table 1: Bacterial strains used and their characteristics.

Strain	**Characteristics**
Pseudomonas fluorescens WCS374r	High tolerance for the antibiotic rifampicine. This strain is capable of inducing systemic resistance in *Raphanus satura* cultivar Saxanova (radish).
Pseudomonas fluorescens WCS417r	High tolerance for the antibiotic rifampicine. This strain is capable of inducing systemic resistance in radish and in *Arabidopsis thaliana.*
Pseudomonas fluorescens WCS374k	High tolerance for the antibiotics kanamycin sulphate and streptomycin sulphate.

The bacterial strains *P. fluorescens* WCS374r and *P. fluorescens* WCS417r were grown on King's medium B agar (King *et al.* 1954) supplemented with the following antibiotics: 13 µg/ml chloramphenicol, 40 µg/ml ampicillin, 150 µg/ml rifampicine and 100µg/ml natamycin (Delvocid; DSM, Delft, Nl). Bacteria were incubated at 28 °C for 48 hours.

The bacterial strain *P. fluorescens* WCS374k was grown on plates with King's medium B agar (King *et al.* 1954) supplemented with 200 µg/ml kanamycin sulphate and 200 µ/ml streptomycin sulphate. Bacteria were incubated at 28 °C for 48 hours.

Determination of the growth rate

For analyzing the growth rates of P. fluorescens WCS374r and P. fluorescens WCS417r the strains were first grown on plates with King's medium B agar. Minimal medium (Penrose, D.M. 2002) was inoculated by pricking a sterile toothpick in a bacterial colony growing on a plate. Both strains incubated overnight at 24 °C. Cfu/ml were measured with a spectrophotometer at 660 nm. To an Erlenmeyer with 40 ml of minimal medium a volume of the overnight culture was added to get a total concentration of 10^7 cfu. Samples were shaking at 24 °C. Every hour the optical densities were measured until the samples reached an optical density op 1 or higher.

Sterilization of the seeds

For the performed experiment the following plants were used (table 2):

Table 2: plants used and their characteristics

Plant	**Characteristics**
Arabidopsis thaliana accession Col-0	
Arabidopsis thaliana mutant *myb72-1*	mutant form of *A. thaliana* with a DNA knockout of the gene *myb72*
Raphanus satura cultivar Saxanova (radish)	

Seeds are sterilized in a closed bell jar with a beaker containing 100 ml of bleach and 3,2 ml of a 37% HCl solution. *Arabidopsis thaliana* accession Col-0 and *myb72-1* seeds are placed around this beaker in open eppendorf tubes for 4 hours. Radish seeds are placed around the beaker in an open Petri dish for 8 hours. After sterilization the eppendorf tubes or Petri dishes are left open for another 15 minutes for ventilation.

Cultivation of plants

Seeds from *A. thaliana* accession Col-0 and its mutant *myb72-1* were sown in autoclaved sand mixed with half strength Hoagland solution (Hoagland and Arnon, 1938) and incubated at 4 °C for 48-62 hours in the dark and subsequently at 24 °C, 70% relative humidity and 200 µmol/m^2. After two weeks the seedlings are transferred either to 60 ml pots with a potting soil-sand mixture or to 60 ml pots with rockwool. Rockwool was autoclaved twice with a 24 hour interval. Radish seeds were sown directly in 60 ml pots with a mixture of potting soil and sand.

A. thaliana accession Col-0 and *myb72-1* are also sown directly on plates with MS (Murashige and Skoog, 1962) agar without sucrose. Before transferring the plates to the climate chambers (24 °C, 70% relative humidity and 200 µmol/m^2) plates were left at 4 °C for 48-62 hours in the dark.

Bacterial inoculation of plants

Bacteria were cultivated as described above. The bacteria were suspended in 10 ml of $MgSO_4$ 10 mM and spinned down at 4250 rpm for 5 minutes. After spinning down the bacteria, the supernatant was replaced with 10 ml of $MgSO_4$ 10 mM and herein the pellet was suspended. The process of spinning down and replacing the supernatant is repeated once more. Bacterial suspension were diluted to a starting concentration of 10^3 cfu/ml.

For inoculation of the plants growing in a potting soil-sand mixture 10^3 and 10^7 cfu/gram soil were added to the soil before sowing. Seedling on MS agar were inoculated by placing 10 µl of a bacterial suspension (10^5 cfu/ml) high on the root. For the experiment with rockwool two week-old seedlings were dipped in a bacterial suspension of 10^3 cfu/ml before transferring them.

Biolog ecoplate

To investigate which carbon sources can be used by *Pseudomonas fluorescens* WCS374r and *P. fluorescens* WCS417r the ecoplate pm1 (Biolog, Inc. Hayward CA, USA) was used. This plate contains 96 wells with different carbon sources. 100 µl was placed of a bacterial suspension (10^3 cfu/ml) in inoculation fluid IF-0 (Biolog, Inc. Hayward CA, USA) with 1 µl redox dye A (Biolog, Inc. Hayward CA, USA). The discoloration of each well is measured at 590 nm with the microplate reader (SPECTROstar, Ortenberg, Germany) for 24h. Every 15 minutes the absorbance was measured of each individual well. Before taking the measurements the plate was shaken for 5 minutes.

Results

Root colonization by *P. fluorescens* WCS374r and *P. fluorescens* WCS417r on *A. thaliana* accession Col-0, Mutant strain *myb72-1* and Radish.

Arabidopsis, *myb72-1* and radish were grown on a potting soil-sand mixture, non-autoclaved. The soil was inoculated with bacteria at 10^7 cfu and 10^3 cfu per gram. Root colonization was analyzed by the use of selective plating. The mutant *myb72-1* was used to exclude a possible role of the capability of inducing systemic resistance for root colonization. On radish both *P. fluorescens* WCS374 and WCS417r are capable of inducing systemic resistance while on Arabidopsis *P. fluorescens* WCS417 is only capable of doing so. Only for 10^7 cfu/gram t=0 was measured.

On radish (figure 1.A) root colonization by both *P. fluorescens* WCS374r and *P. fluorescens* WCS417r with an initial density of 10^7 cfu is equal at time point 0. After two weeks of incubation *P. fluorescens* WCS417r was detected at higher numbers of colony forming units (cfu) per gram root than *P. fluorescens* WCS374r (6.42 and 5.71 respectively). In the bulk soil *P. fluorescens* WCS417r also reach higher numbers of cfu per gram root than *P. fluorescens* WCS374r (5.28 and 4.48 respectively). Numbers of cfu are higher on radish than in bulk soil . After three weeks of incubation *P. fluorescens* WCS417r was detected at higher numbers of cfu per gram root than *P. fluorescens* WCS374r (6.28 and 5.81 respectively). The same applies for the bulk soil (5.11 and 4.31) though numbers of cfu per gram bulk soil are lower than numbers of cfu per gram root. The differences found in log cfu/gram root or log cfu/gram soil are all significant except for the bulk soil with *P. fluorescens* WCS417r versus radish with *P. fluorescens* WCS374r.

Two weeks after inoculation with *P. fluorescens* WCS417r, initial density of 10^3 cfu/gram root (figure 1.B), was detected at higher numbers of cfu per gram root than *P. fluorescens* WCS374r (5.68 and 2.96 respectively). Numbers of cfu per gram bulk soil with *P. fluorescens* WCS417r are lower than the numbers of cfu per gram root of radish with P fluorescens WCS374r (3.74 and 2.96 respectively). Numbers of cfu per gram bulk soil with WCS374r were below detection limit (see *).
After three weeks the same pattern applies. Numbers of cfu per gram root are highest for *P. fluorescens* WCS417r (5.10) and numbers of cfu per gram root are lowest for bulk soil with *P. fluorescens* WCS417r (3.47). *P. fluorescens* WCS417r on the roots of radish differ significantly from *P. fluorescens* WCS374r on the roots and *P. fluorescens* WCS417r on the bulk soil. Numbers of cfu per gram bulk soil with WCS374r were below detection limit (see *).

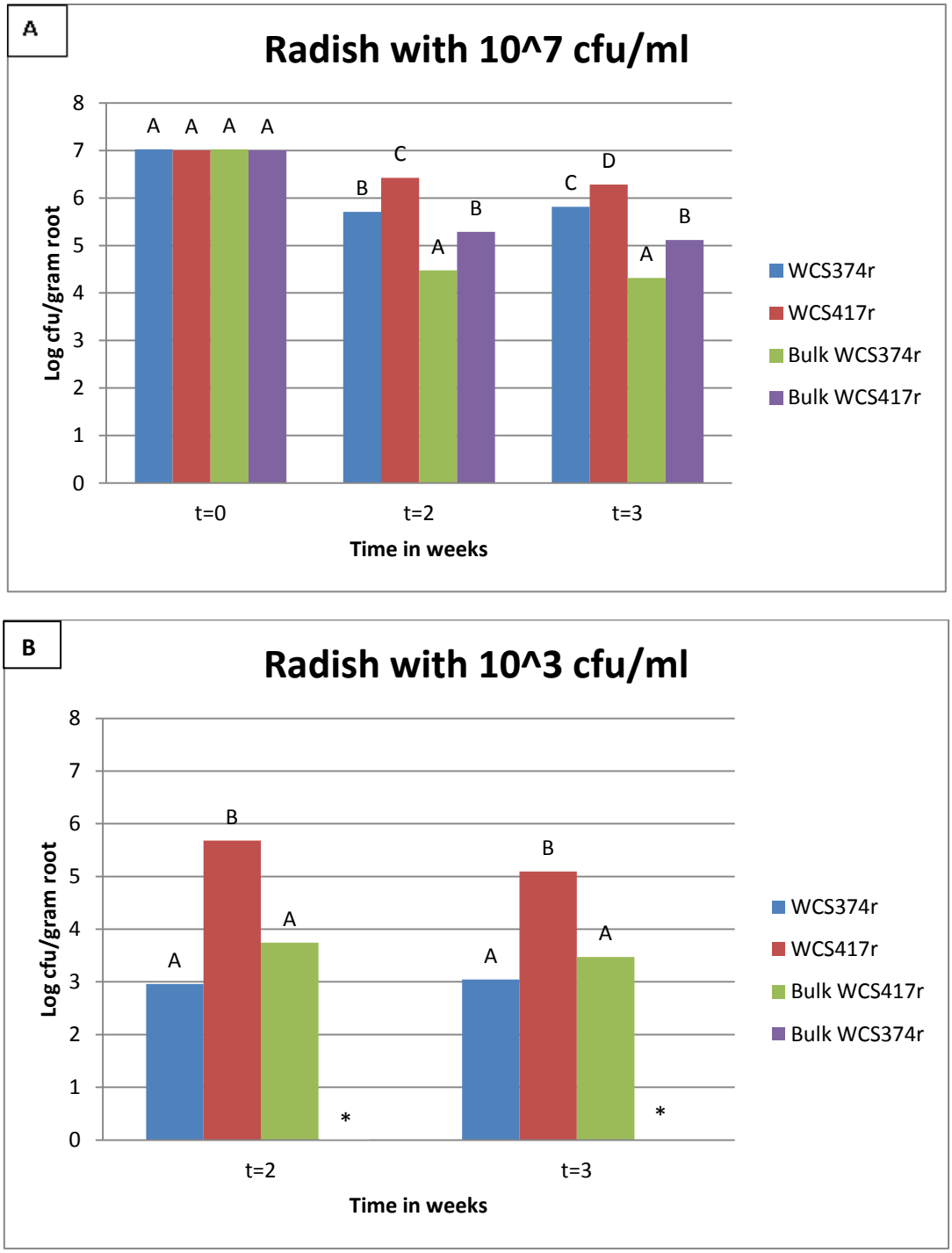


Figure 1. Colonization of radish roots by *P. fluorescens* WCS374r or *P. fluorescens* WCS417r two and three weeks after inoculation at 10^7 cfu per gram soil (A) or 10^3 cfu per gram soil (B) * indicates values below detection limits.

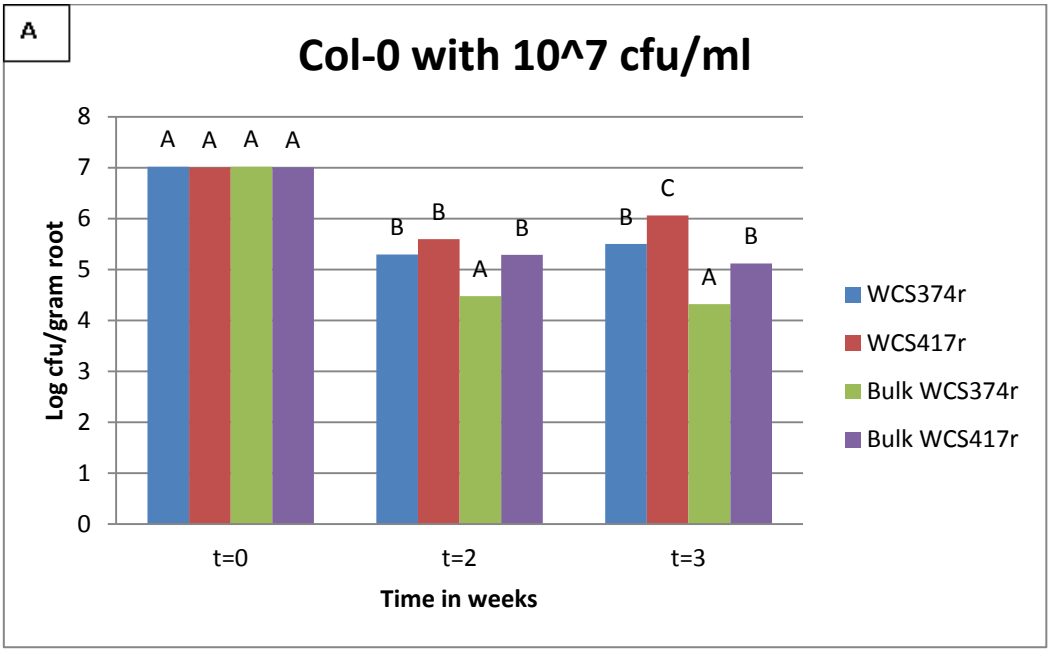
On *Arabidopsis thaliana* accession Col-0 (figure 2.A) root colonization by both *P. fluorescens* WCS374r and *P. fluorescens* WCS417r with an initial density of 10^7 cfu were equal at time point 0. Two weeks after inoculation numbers of cfu per gram root or bulk soil are lower when compared to cfu per gram root or bulk soil at the beginning of the experiment. Two weeks after inoculation numbers of cfu per gram root or bulk are significantly the same for *P. fluorescens* WCS417r on the roots and in the bulk soil and *P. fluorescens* WCS374r on the roots (5.59, 5.28, and 5.29 respectively) except for *P. fluorescens* WCS374r growing in bulk soil (4.48).

Three weeks after inoculation numbers of cfu per gram root is the highest for *P. fluorescens* WCS417r

grown on Arabidopsis (6.06). Numbers of cfu per gram root or bulk soil are significantly the same for *P. fluorescens* WCS374r on Arabidopsis and *P. fluorescens* WCS417r in bulk soil (5.50 and 5.12 respectively). Numbers of cfu per gram are the lowest for *P. fluorescens* WCS374r in bulk soil (4.32).

For Arabidopsis Col-0 growing on soil mixed with 10^3 cfu of *P. fluorescens* WCS374r or *P. fluorescens* WCS417r (figure 2.B) no initial densities are measured. Though two weeks after inoculation, numbers of cfu per gram are highest for *P. fluorescens* WCS417r on Arabidopsis (4.33) and lowest for *P. fluorescens* WCS417r in bulk soil (3.74). After three weeks the same pattern is visible. Numbers of cfu are highest for Arabidopsis wit *P. fluorescens* WCS417r (4.38) and lowest for *P. fluorescens* WCS417r in bulk soil (3.47). *P. fluorescens* WCS417r on the roots, in the bulk soil and *P. fluorescens* WCS374r on the roots all differ significantly.

Numbers of cfu per gram bulk soil mixed with *P. fluorescens* WCS374r are not shown because they were below detection limit (see *).



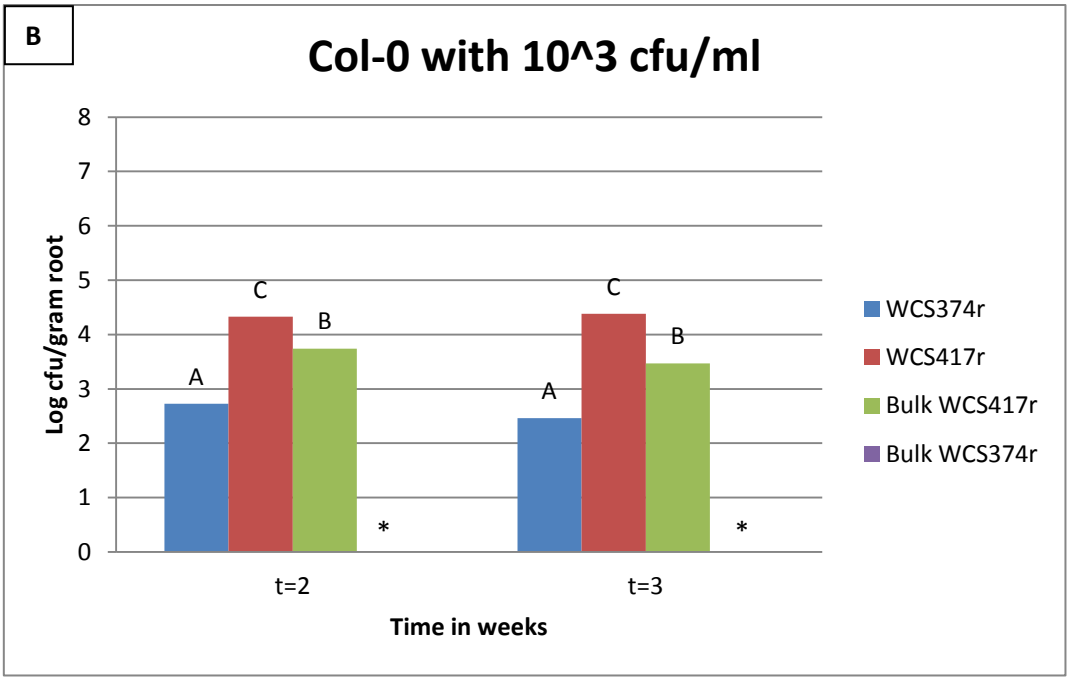


Figure 2. Colonization of *Arabidopsis thaliana* accession Col-0 roots by *P. fluorescens* WCS374r or *P. fluorescens* WCS417r two and three weeks after inoculation at 10^7 cfu per gram soil (A) or 10^3 cfu per gram soil (B) * indicates values below detection limits.

At the beginning of the experiment numbers of cfu were also equal at 10^7 cfu on Arabidopsis mutant *myb72-1* (figure 3.A). Two weeks after inoculation numbers of cfu were lower than at the onset of the experiment. *P. fluorescens* WCS417r is detected at higher numbers of cfu per gram root on *myb72-1* (5.76) though not significantly higher than *P. fluorescens* WCS374r (5.46). Compared to bulk soil numbers of cfu per gram are equal for *P. fluorescens* WCS417r grown in bulk (5.28) and *P. fluorescens* WCS374r grown on the roots of *myb72-1* (5.46). Numbers of cfu per gram bulk are lowest for *P. fluorescens* WCS374r (4.48). Three weeks after inoculation numbers of cfu per gram root are highest for *P. fluorescens* WCS417r (5.93). Numbers of cfu are not significantly different for *P. fluorescens* WCS374r grown on the roots of *Myb72-1* (5.31) and *P. fluorescens* WCS417r grown in bulk soil (5.12). Numbers of cfu per gram are lowest for *P. fluorescens* WCS374r grown in bulk soil (4.32).

At the beginning of the experiment no cfu are measured for the initial density of 10^3 cfu per gram soil (figure 3.B). Two weeks after inoculation *P. fluorescens* WCS417r reaches the highest numbers of cfu on the roots of *myb72-1* (4.35) and numbers of cfu are lowest for *P. fluorescens* WCS417r grown in bulk soil (3.74).
Three weeks after inoculation again numbers of cfu are highest for *P. fluorescens* WCS417r growing on the roots of *myb72-1* (3.93) and lowest for *P. fluorescens* WCS417r growing in bulk soil (3.47). Numbers of cfu per gram bulk soil mixed with *P. fluorescens* WCS374r are not shown because they were below detection limit (see *).

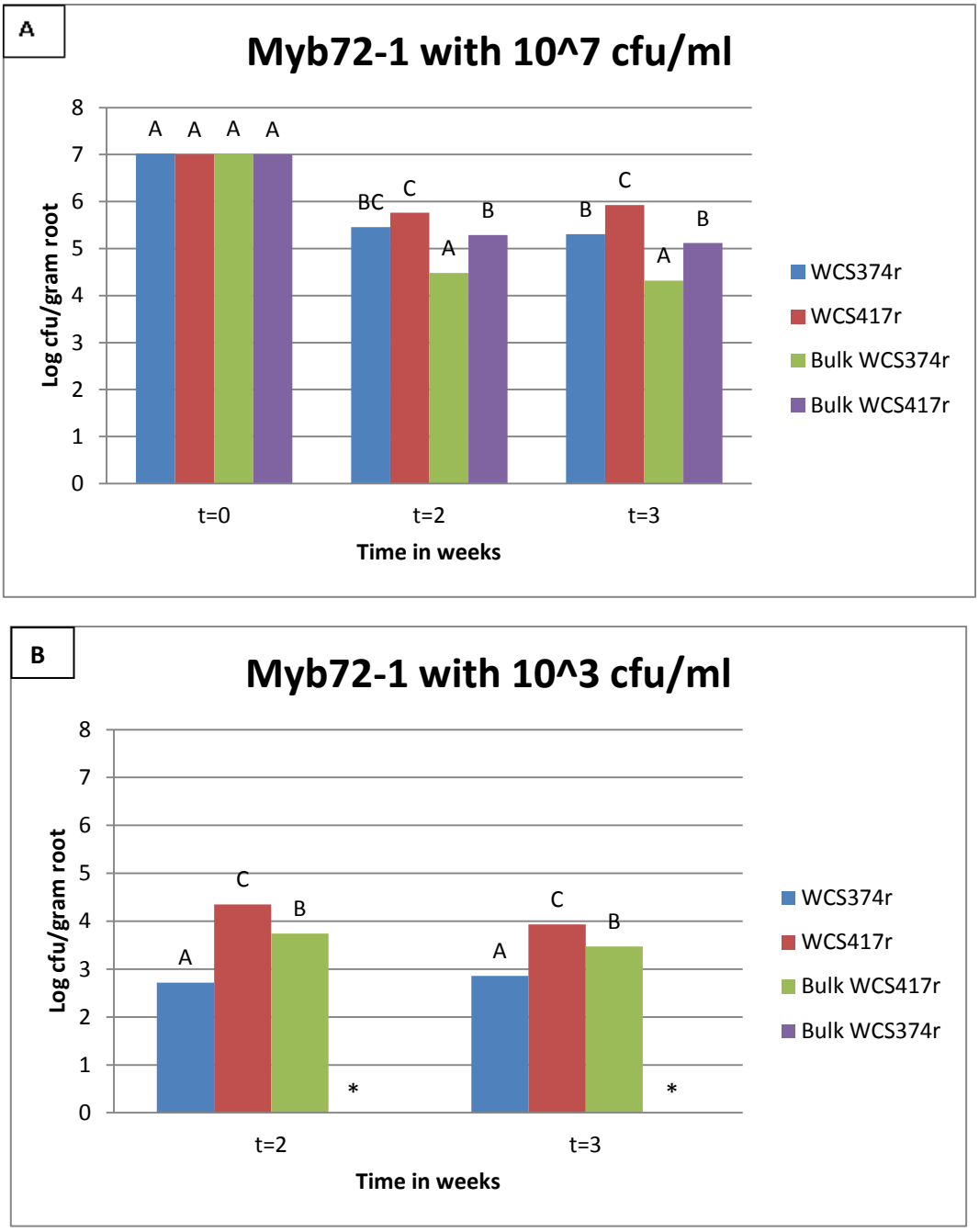


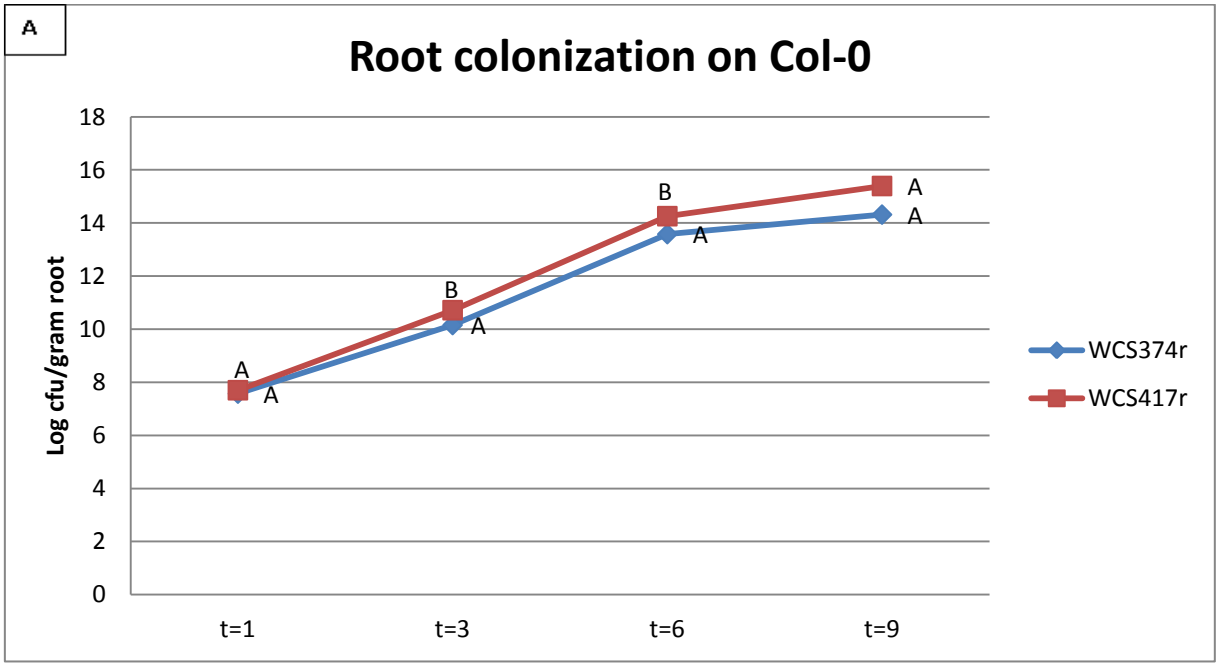
Figure 3. Colonization of *Arabidopsis thaliana* mutant *myb72-1* roots by *P. fluorescens* WCS374r or *P. fluorescens* WCS417r two and three weeks after inoculation at 10^7 cfu per gram soil (A) or 10^3 cfu per gram soil (B) * indicates values below detection limits.

Root colonization on *A. thaliana* accession Col-0 and *myb72-1* on MS-plates, bacterial strains without competition.

Roots of two week-old seedlings of *A. thaliana* accession Col-0 and *myb72-1* were inoculated with a drop of 10^3 cfu placed high on the root. Root colonization was determined after 1,3,6 and 9 days. On *Arabidopsis thaliana* accession Col-0 (figure 4.A), numbers of cfu are equal for *P. fluorescens* WCS374r and *P. fluorescens* WCS417r one day after inoculation (7.57 and 7.70 respectively). 3 and 6 days after inoculation *P. fluorescens* WCS417r reaches significantly higher numbers of cfu per gram root (10.71 and 14.26) than *P. fluorescens* WCS374r (10.16 and 13.58). After 9 days no difference in cfu could be measured between *P. fluorescens* WCS374r (14.32) and *P. fluorescens* WCS417r (15.39) on Col-0.

On the roots of *Arabidopsis thaliana myb72-1* (figure 4.B), numbers of cfu per gram root were not significantly different after 1 and 3 days. After 6 and 9 days *P. fluorescens* WCS417r reaches significantly higher numbers of cfu per gram root (14.09 and 14.49) than *P. fluorescens* WCS374r (13.38 and 13.99).

On day 1 no significant differences can be found in root colonization of *P. fluorescens* WCS374r and *P. fluorescens* WCS417r on Col-0 and *myb72-1*. After 3 days *P. fluorescens* WCS374r has the lowest numbers of cfu per gram root on Col-0. *P. fluorescens* WCS417r reaches the highest numbers of cfu per gram root with no significant differences between Col-0 and *myb72-1*. *P. fluorescens* WCS374r on *myb72-1* does not significantly differ from *P. fluorescens* 374r on Col-0 or *P. fluorescens* WCS417r on Col-0 and *myb72-1*.
On day 6 the density of *P. fluorescens* WCS374r does not significantly differ between Col-0 and *myb72-1* though they are significantly lower than cfu per gram root of *P. fluorescens* WCS417r. No differences in cfu of *P. fluorescens* WCS417r can be found between Col-0 and *myb72-1*.
After 9 days numbers of cfu are highest for *P. fluorescens* WCS417r on the roots of Col-0 and Lowest for *P. fluorescens* WCS374r on the roots of *myb72-1* (Data not shown).



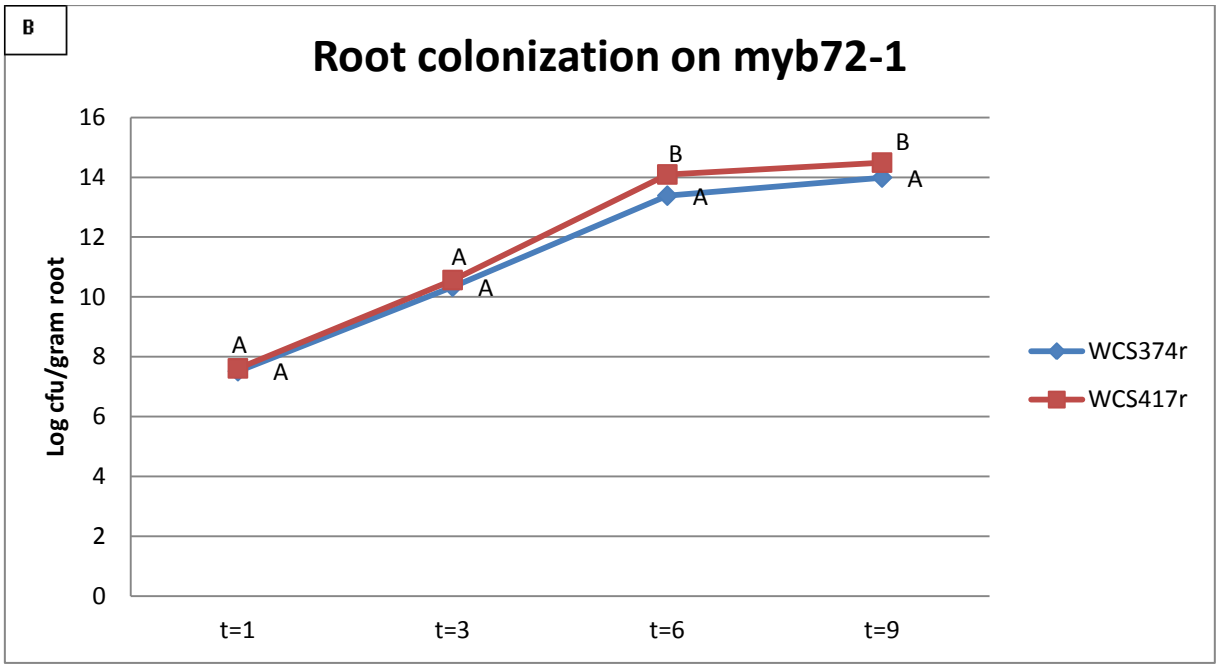


Figure 4. Log cfu per gram root is shown for *Arabidopsis thaliana* accession Col-0 (A) and its mutant *myb72-1* (B) grown on MS plates with a drop of 10^3 cfu of *P. fluorescens* WCS374r or *P. fluorescens* WCS417r placed high on the root.

Root colonization on *A. thaliana* accession Col-0 and *myb72-1* on MS-plates, bacterial strains in competition.

Roots of two week-old seedlings of *A. thaliana* accession Col-0 and *myb72-1* were inoculated with a drop of 10^3 cfu of a suspension with equal amounts of cfu of *P. fluorescens* WCS374k and *P. fluorescens* WCS417r. Root colonization was measured by means of selective plating on the same day as inoculation and after 3, 6, 8 and 15 days.

On *Arabidopsis thaliana* accession Col-0 (figure 5.A) the amount of cfu of *P. fluorescens* WCS374k and *P. fluorescens* WCS417r showed no significant differences at the time of inoculation (2.63 and 2.49 respectively). After 3, 6, 8 and 15 days *P. fluorescens* WCS417r (10.32, 10.36, 10.32, and 10.28 respectively)does significantly better in colonizing the roots than *P. fluorescens* WCS374k (6.59, 7.12, 7.69, and 7.38 respectively).

On the mutant *myb72-1* (figure 5.B), the amount of cfu per gram root by *P. fluorescens* WCS374k and *P. fluorescens* WCS417r showed no significant differences on the day of inoculation (2.63 versus 2.49). 3, 6, 8 and 15 days after inoculation however cfu per gram root are significantly higher for *P. fluorescens* WCS417r (10.16, 10.13, 9.90, and 10.23) when compared to *P. fluorescens* WCS374k (6.11, 8.02, 6.41, and 6.83).

After 3 days there is no significant difference in numbers of *P. fluorescens* WCS374k on Col-0 and *myb72-1* (6.59 and 6.11 respectively). The same is true for *P. fluorescens* WCS417r (on Col-0 10.32 and *myb72-1* 10.16). 6 days after inoculation there is no significant difference in root colonization by *P. fluorescens* WCS374k on Col-0 and *myb72-1* (7.12 and 8.02). *P. fluorescens* WCS417r reaches significant higher numbers of cfu per gram root on Col-0 (10.36) than on *myb72-1* (10.13). After 8 days *P. fluorescens* WCS374k and *P. fluorescens* WCS417r both reach higher numbers of cfu on Col-0. 15 days after inoculation no differences in root colonization by *P. fluorescens* WCS374k on Col-0 and *myb72-1* could be found. The same is true for *P. fluorescens* WCS417r (data not shown).

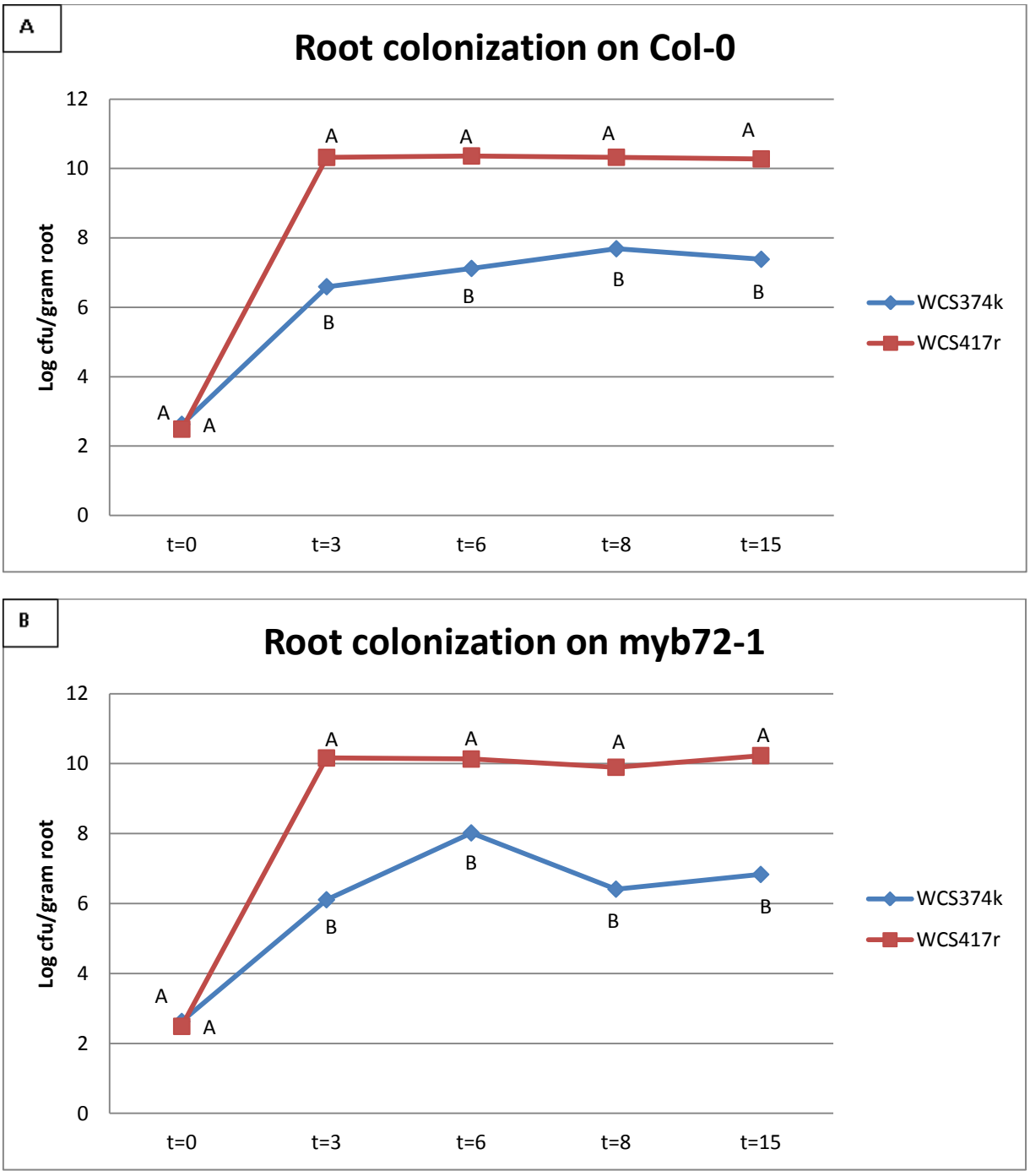


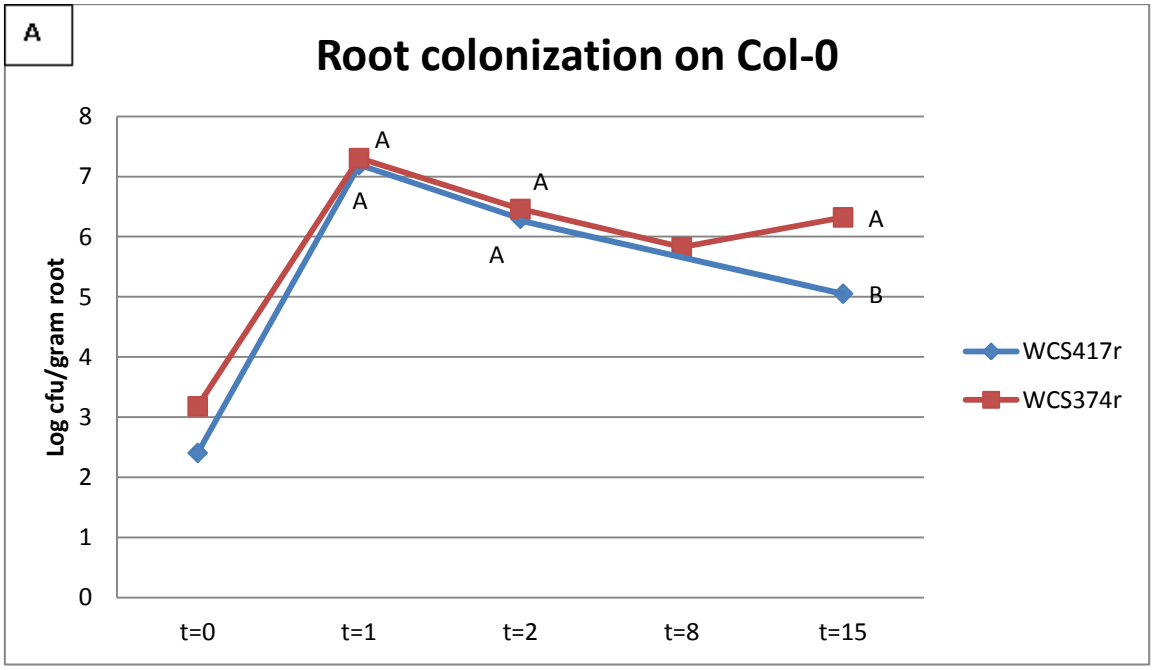
Figure 5. Log cfu per gram root is shown for *Arabidopsis thaliana* accession Col-0 (A) and its mutant *myb72-1* (B) grown on MS plates with a drop of 10^3 cfu of *P. fluorescens* WCS374k and *P. fluorescens* WCS417r together placed high on the root.

Root colonization on *A. thaliana* accession Col-0 and its mutant *myb72-1* when grown on rockwool.

Two week-old Col-0 and *myb72-1* seedlings were dipped in a bacterial suspension of 10^3 cfu/ml of either *P. fluorescens* WCS374r or *P. fluorescens* WCS417r before being transferred to 60ml pots with granulated rockwool. Root colonization was measured by means of selective plating. One day after inoculation (figure 6.A) cfu per gram root raised to a log cfu/g of more than 7 on Col-0 for both bacterial strains. The second day numbers of cfu per gram root declined. Numbers of cfu per gram root for *P. fluorescens* WCS417r kept declining while the numbers of cfu per gram root of *P. fluorescens* WCS374r inclined from day 8 till day 15. Numbers of cfu per gram root are higher for *P. fluorescens* WCS374r but differ only significantly from *P. fluorescens* WCS417r on the last day.

Also on *myb72-1* numbers of cfu per gram root increased from the moment of inoculation until day 1. After day one numbers of cfu per gram root declined. Numbers of cfu per gram root are higher for *P. fluorescens* WCS374r but differ only significant from *P. fluorescens* WCS417r on the second day and the last day (figure 6.B).

On the first day no differences were found between the root colonization between *P. fluorescens* WCS374r on Col-0 and on *myb72-1*. The same account for *P. fluorescens* WCS417r. On the second day numbers of cfu per gram root of *P. fluorescens* WCS374r are significantly lower on Col-0 than on *myb72-1*. No significant differences are found in cfu per gram root for *P. fluorescens* WCS417r on Col-0 or *myb72-1*. On the eight day no significant differences are found in the root colonization by *P. fluorescens* WCS374r on Col-0 or *myb72-1*. On the last day numbers of cfu per gram root of *P. fluorescens* WCS374r are significantly higher on Col-0 than on *myb72-1*. For *P. fluorescens* WCS417r no significant differences are found in numbers of cfu per gram root between Col-0 and *myb72-1* (Data not shown).



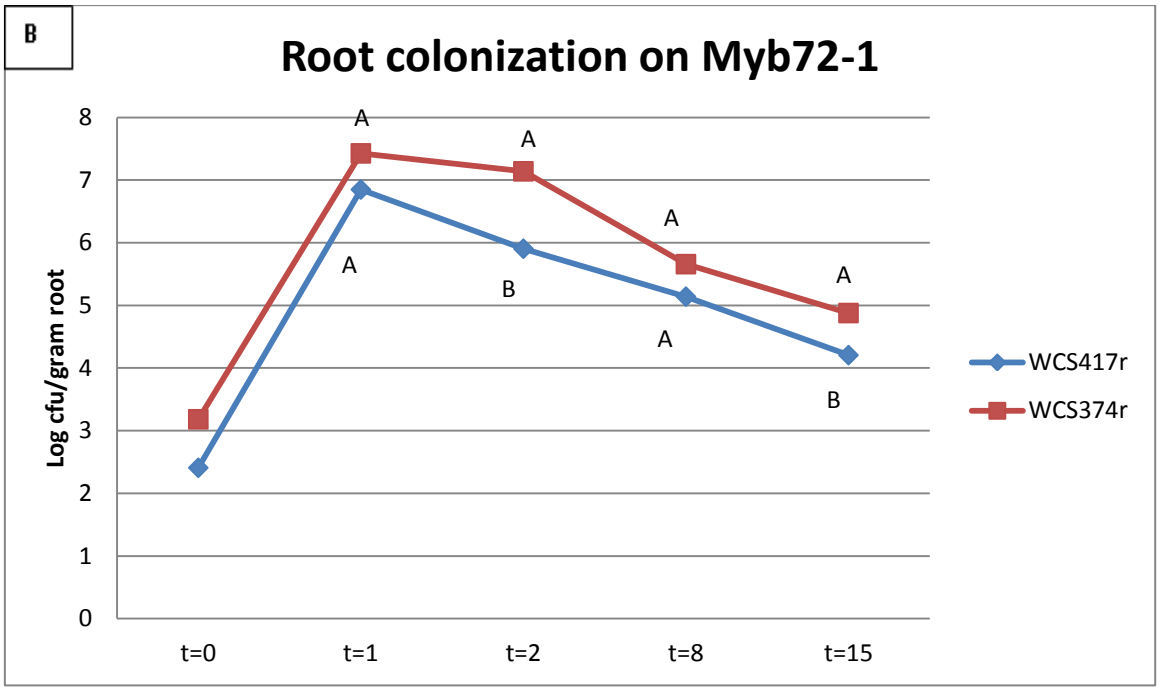


Figure 6. Log cfu per gram root is shown for *Arabidopsis thaliana* accession Col-0 (A) and its mutant *myb72-1* (B) grown on granulated rockwool after being dipped in a suspension of 10^3 cfu/ml of *P. fluorescens* WCS374r or *P. fluorescens* WCS417r.

Growth rate of *P. fluorescens* WCS374r and *P. fluorescens* WCS417r.

To see if the difference in root colonization between *P. fluorescens* WCS374r and *P. fluorescens* WCS417r are caused by the plant or the ability to colonize roots better than the other and not merely by a difference in the growth rate, the growth rates of both bacterial strains are measured in two different media.

In minimal medium *P. fluorescens* WCS374r and *P. fluorescens* WCS417r show no significant differences in their growth rates. In King's broth medium *P. fluorescens* WCS374r and *P. fluorescens* WCS417r also show no significant differences in their growth rates. However both bacterial strain have higher growth rates in King's broth medium than in minimal medium (figure 7).

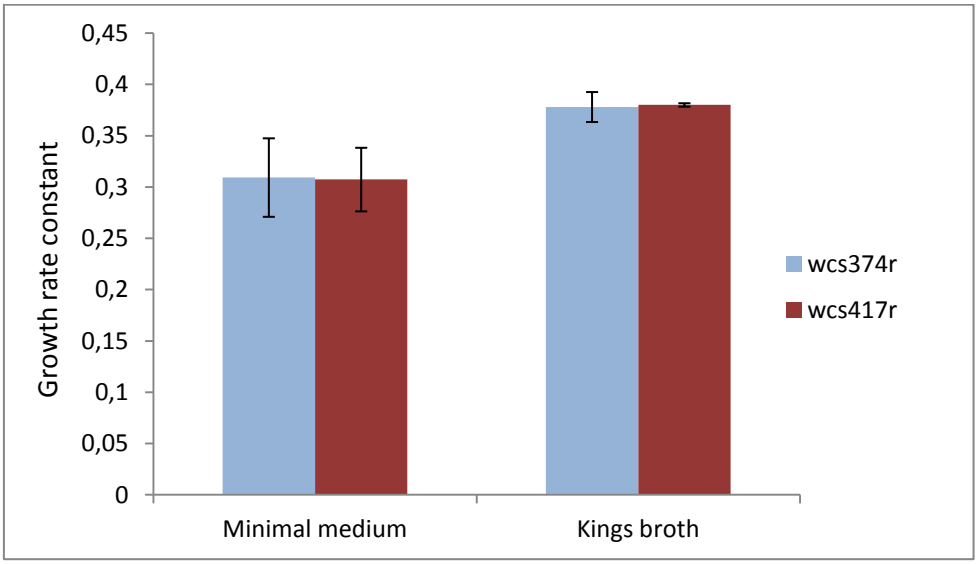


Figure 7. Growth rate constant for *P. fluorescens* WCS374r and *P. fluorescens* WCS417r in minimal medium and in King's broth medium.

Differences in the use of carbon sources by *P. fluorescens* WCS374r and *P. fluorescens* WCS417r.

The biolog ecoplate is a 96 wells plate with a different carbon source in each well. If the carbon source is used by the bacteria carbon dioxide is formed and the suspension turns purple. By measuring the absorption at 590 nm the rate of use can be measured.

The biolog ecoplate pm1 is used to see if there are any differences in which carbon source can be used by *P. fluorescens* WCS374r and *P. fluorescens* WCS417r.

When the wells of the ecoplate pm1 with *P. fluorescens* WCS374r (figure 8) are compared with the wells of the ecoplate pm1 with *P. fluorescens* WCS417r (figure 9) the wells A3 and B2 have turned purple in the biolog ecoplate with *P. fluorescens* WCS417r and not in biolog ecoplate with *P. fluorescens* WCS374r (see red circles in figures 8 and 9). Well A3 contains the carbon source N-acetyl-D-glucosamine and well B2 contains the carbon source D-sorbitol. Also the discoloration is heavier in the biolog ecoplate pm1 with *P. fluorescens* WCS417r than it is in biolog ecoplate pm1 with *P. fluorescens* WCS374r. The amount of discoloration is also made visible in table 3. The differences between *P. fluorescens* WCS374r and *P. fluorescens* WCS417r can be seen for well A3 and B2 (see red borders).

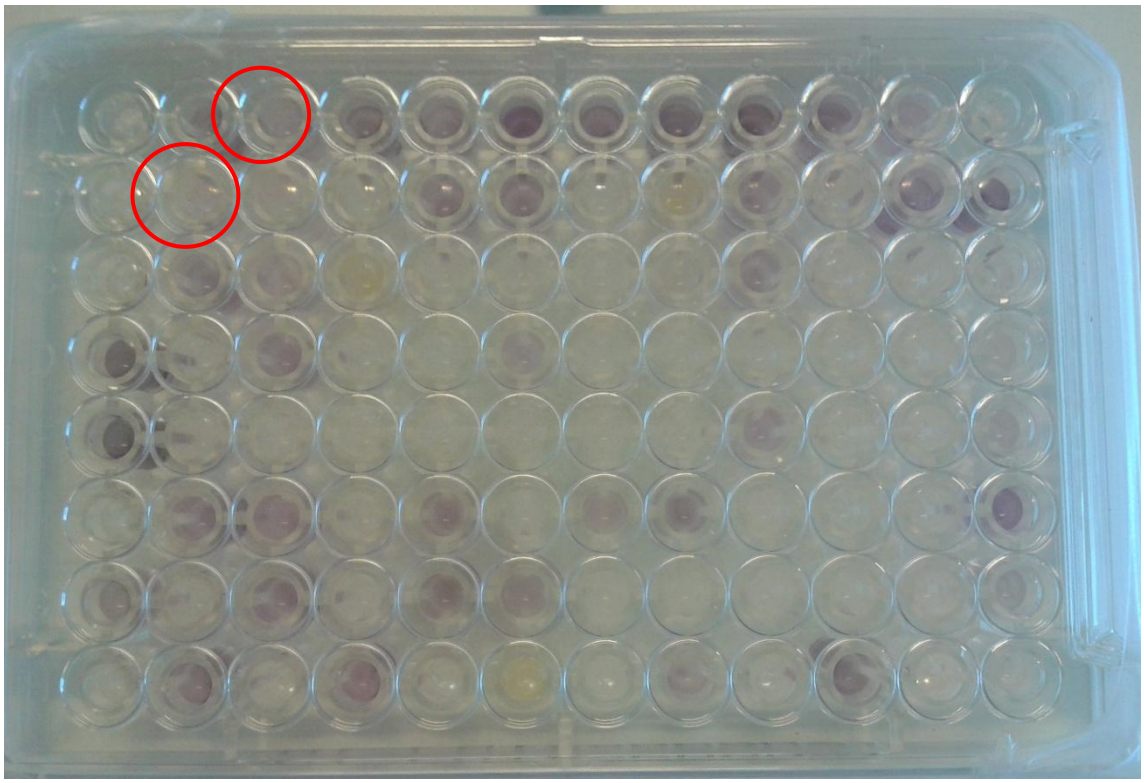


Figure 8. A picture of the biolog ecoplate pm1 with *P. fluorescens* WCS374r. Each well contains a different carbon source and if the well has turned purple this carbon source is used by the bacteria.

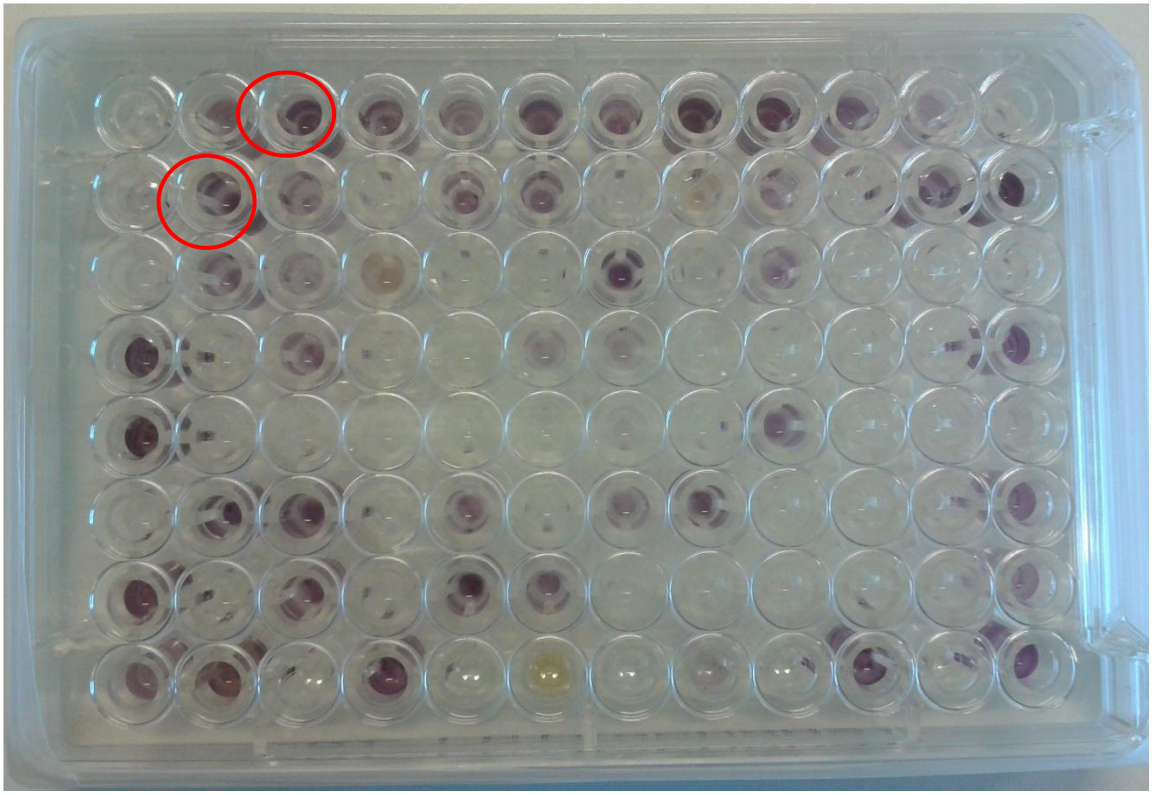


Figure 9. A picture of the biolog ecoplate pm1 with *P. fluorescens* WCS417r. Each well contains a different carbon source and if the well has turned purple this carbon source is used by the bacteria.

Table 3: Overview of the carbon sources in each well and to what extend the carbon source could be used by *P. fluorescens* WCS374r of *P. fluorescens* WCS417r. (-) means no discoloration, (+) means mild discoloration, (++) means strong discoloration. This table is based on a combination of visible discoloration and measured absorbance at 590 nm.

Carbon source	*P. fluorescens* WCS374r	*P. fluorescens* WCS417r
A1 Negative control	-	-
A2 L-Arabinose	+	+
A3 N-Acetyl-D-Glucosamine	-	++
A4 D-Saccaric acid	+	+
A5 Succinic Acid	-	+
A6 D-Galactose	++	++
A7 L-Aspartic Acid	+	+
A8 L-Proline	++	++
A9 D-Alanine	++	++
A10 D-Trehalose	+	+
A11 D-Mannose	+	-
A12 Dulcitol	-	-
B1 D-Serine	-	-
B2 D-Sorbitol	-	++
B3 Glycerol	-	+
B4 L-Fructose	-	-
B5 D-Glucuronic Acid	+	+
B6 D-Gluconic Acid	+	+
B7 D,L-a-Glycerol-Phosphate	-	-
B8 D-Xylose	-	-
B9 L-Lactic Acid	+	+
B10 Formic Acid	-	-
B11 D-Mannitol	+	+
B12 L-Glutamic Acid	+	++
C1 Glucose-6- Phosphate	-	-
C2 D-Galactonic Acid-g -Lactone	+	+
C3 D,L-Malic Acid	+	+
C4 D-Ribose	-	-
C5 Tween 20	-	-
C6 L-Rhamnose	-	-
C7 D-Fructose	-	++
C8 Acetic Acid	-	-
C9 a-D-Glucose	+	+
C10 Maltose	-	-
C11 D-Melibiose	-	-
C12 Thymidine	-	-

Carbon source	*P. fluorescens* WCS374r	*P. fluorescens* WCS417r
E1 L-Glutamine	++	++
E2 M-Tartaric Acid	-	-
E3 Glucose-1-Phosphate	-	-
E4 Fructose-6-Phosphate	-	-
E5 Tween 80	-	-
E6 a-Hydroxy Glutaric Acid-g -Lactone	-	-
E7 a-Hydroxy Butyric Acid	-	-
E8 b-Methyl-D glucoside	-	-
E9 Adonitol	+	+
E10 Maltotriose	-	-
E11 2-Deoxy Adenosine	-	-
E12 Adenosine	-	-
F1 Glycyl-L-Aspartic Acid	-	-
F2 Citric Acid	+	++
F3 M-Inositol	+	++
F4 D-Threonine	-	-
F5 Fumaric Acid	+	+
F6 Bromo Succinic Acid	-	-
F7 Propionic Acid	+	+
F8 Mucic Acid	+	+
F9 Glycolic Acid	-	-
F10 Glyoxylic Acid	-	-
F11 D-Cellobiose	-	-
F12 Inosine	+	+
G1 Glycyl-Lglutamic Acid	+	++
G2 Tricarballylic Acid	-	-
G3 L-Serine	+	++
G4 L-Threonine	-	-
G5 L-Alanine	+	++
G6 L-Alanyl-Glycine	+	+
G7 Acetoacetic Acid	-	-
G8 N-Acetyl-b-Dmannosamine	-	-
G9 Mono Methyl Succinate	-	-
G10 Methyl Pyruvate	-	-
G11 D-Malic Acid	-	-
G12 L-Malic Acid	+	+

D1 L-Asparagine	++	++		H1 Glycyl-L-Proline	-	+
D2 D-Aspartic Acid	-	-		H2 P-Hydroxy Phenyl Acetic Acid	+	++
D3 D-Glucosaminic Acid	+	+		H3 M-Hydroxy Phenyl Acetic Acid	-	-
D4 1,2-Propanediol	-	-		H4 Tyramine	+	++
D5 Tween 40	-	-		H5 D-Psicose	-	-
D6 a-Keto-Glutaric Acid	+	+		H6 L-Lyxose	-	-
D7 a-Keto-Butyric Acid	-	+		H7 Glucuronamide	-	-
D8 a-Methyl-D galactoside	-	-		H8 Pyruvic Acid	+	-
D9 a-D-Lactose	-	-		H9 L-Galactonic Acid-g - Lactone	-	-
D10 Lactulose	-	-		H10 D-Galacturonic Acid	+	++
D11 Sucrose	-	-		H11 Phenylethylamine	-	-
D12 Uridine	-	+		H12 2-Aminoethanol	-	+

Discussion

Two well-known plant-growth-promoting Rhizobacteria (PGPR) are *P. fluorescens* WCS374 and *P. fluorescens* WCS417. Together with the plant they can form a super organism with an enhanced fitness. It is of great importance for the bacteria to be a good colonizer on the roots because the rhizosphere is a nutrient rich environment in which they can grow well. Some PGPR are known to induce systemic resistance in the plant. In the case of induced systemic resistance the plant is primed for a possible attack by pathogens. In the case of an attack by such a pathogen the plant is able to respond faster an heavier upon the attack and therefore is more likely to survive. It was shown that the PGPR *P. fluorescens* WCS417r is capable of inducing systemic resistance in *Arabidopsis thaliana* and in other plants like radish (Bakker *et al.* 2007). The other PGPR *P. fluorescens* WCS374 is only capable of inducing systemic resistance in radish but not in *Arabidopsis thaliana*. Furthermore it is shown that *P. fluorescens* WCS417 is a better colonizer than *P. fluorescens* WCS374 of the Arabidopsis rhizosphere (Doornbos, 2009). Therefore we hypothesized that the ability to induce systemic resistance in the plant and good root colonization are linked.

Research shows that the *myb72* gene is very important in the early steps of induced systemic resistance (van der Ent *et al.* 2008) and is activated when the plant recognized MAMPs (van Wees *et al.*, 2008). MAMPs are among the first things a plant responds upon with a defense. Defense signaling is also enhanced upon colonization by beneficial microbes because they produce MAMPs, so for PGPR it is important to work around this defense signaling so they can colonize the roots. One of the hypotheses for the performed experiments here is that when the capability to induce systemic resistance is lost because of a mutation in the *myb72*-gene, root colonization by *P. fluorescens* WCS417 and *P. fluorescens* WCS374 is equal. Our second hypothesis is that when *P. fluorescens* WCS374 and *P. fluorescens* WCS417 colonize the roots of a plant in which they both are able to induce systemic resistance, root colonization is also equal but better than on the *Arabidopsis* mutant *myb72-1*.

Differences in root colonization

First root colonization was for both strains determined on radish, *Arabidopsis thaliana* accession Col-0 and its mutant *myb72-1* by means of selective plating. Plants were grown on a mixture of potting soil and sand which was mixed with *P. fluorescens* WCS374r or *P. fluorescens* WCS417r in densities of 10^3 cfu /gram soil or 10^7 cfu/gram soil. On radish *P. fluorescens* WCS417r is a better colonizer of radish roots after two and three weeks independent of the density of the bacteria (figure 1).
On the roots of *Arabidopsis thaliana* accession Col-0, no significant differences could be found in root colonization by *P. fluorescens* WCS374r or *P. fluorescens* WCS417r with the initial density of 10^7 cfu/gram soil after two weeks. However after three weeks *P. fluorescens* WCS417r showed to be a significant better root colonizer than *P. fluorescens* WCS374r. On Col-0 with initial densities of 10^3 cfu/gram soil of *P. fluorescens* WCS374r or *P. fluorescens* WCS417r results show that after two and three weeks *P. fluorescens* WCS417r is a significant better colonizer of the roots (figure 2).
On *myb72-1* with initial densities of 10^7 cfu/gram soil results show that after two weeks no significant difference could be found between *P. fluorescens* WCS374r and *P. fluorescens* WCS417r in root colonization but after three weeks *P. fluorescens* WCS417r did significantly better. On *myb72-1* with initial densities of 10^3 cfu/gram soil *P. fluorescens* WCS417r was significantly better than *P. fluorescens* WCS374r after two and three weeks (figure 3). Results are compared to cfu/gram of bulk soil. In the bulk

soil *P. fluorescens* WCS417r does also significantly better than *P. fluorescens* WCS374r. The results indicate that *P. fluorescens* WCS417r is indeed a better root colonizer.

Because the potting soil-sand mixture was not autoclaved the differences found cannot be directed with certainty to a better ability to colonize the roots. In the potting soil-sand mixture other microbes are present and are likely to have competed with one or both of the used strains. Therefore experiments are repeated on MS-plates so root colonization could be tested without any competition being of influence.

Before sowing Col-0 and *myb72-1* seeds were sterilized. Measurements were taken 1, 3, 6 and 9 days after inoculating the two week old seedlings with 10^3 cfu of *P. fluorescens* WCS374r or *P. fluorescens* WCS417r. One day after inoculation no significant differences could be found in root colonization on Col-0. 3, 6 and 9 days after inoculation *P. fluorescens* WCS417r showed to be a better root colonizer, though the difference in root colonization was not significant on the ninth day.
On *myb72-1* no differences in root colonization were found 1 and 3 days after inoculation. The difference was significant 6 and 9 days after inoculation in the advantage of *P. fluorescens* WCS417r. These results indicate that even in a sterile environment *P. fluorescens* WCS417r is a better root colonizer than *P. fluorescens* WCS374r (figure 4). The results also indicate that losing the ability to induce systemic resistance, by a loss of *myb72*-gene, is of no importance for root colonization. On the last day the numbers of cfu/gram root are in the same range which was not what we hypothesized.

In nature, however, there are always multiple microbes living in the rhizosphere of a plant therefore we examined root colonization of *P. fluorescens* WCS374k and *P. fluorescens* WCS417r when they are applied together on the root. The bacterial strains are selective for different plates so that root colonization could be measured by selective plating. Measurements were taken 3, 6 and 8 and 15 days after inoculating the two week old seedlings of Col-0 and *myb72-1* with 10^3 cfu of *P. fluorescens* WCS374k and *P. fluorescens* WCS417r. The suspension of the bacteria was also plated to see if both bacteria were there in equal amounts. The amount of cfu of *P. fluorescens* WCS374k and *P. fluorescens* WCS417r were equal in the suspension. Three days after inoculation numbers of cfu have risen for both bacterial strains on both plants. 3, 6, 8 and 15 days after inoculation *P. fluorescens* WCS417r does significantly better than *P. fluorescens* WCS374k on both Col-0 and *myb72-1*. These results show as well that *P. fluorescens* WCS417r is better at colonizing the roots than *P. fluorescens* WCS374k (figure 5). Again, the numbers of cfu/gram root are in the same range for Col-0 and *myb72-1* which indicates that the ability to colonize the root and the ability to induce systemic resistance are not linked.

Root colonization on Col-0 and *myb72-1* was also measured when the plants were placed in rockwool. Seedlings were transferred after two weeks from sand to rockwool and during the transfer the roots were dipped in a suspension of *P. fluorescens* WCS374r or *P. fluorescens* WCS417r at a density of 10^3 cfu/ml. Measurements were taken 1, 2, 8 and 15 days after being dipped. Compared to the suspension the numbers of cfu/gram root increase rapidly after one day for both Col-0 and *myb72-1*. On Col-0 *P. fluorescens* WCS374r was a better colonizer than *P. fluorescens* WCS417r though this difference is only significant on the last day. On *myb72-1 P. fluorescens* WCS374r was also a better root colonizer though only significant on days 2 and 15 after being dipped (figure 6).
This experiment has been performed before by Rogier Doornbos (Doorbos, R.F. *et al.* 2009, thesis) and his results show that *P. fluorescens* WCS417r is a significant better root colonizer than *P. fluorescens* WCS374r on Col-0. On *myb72-1* numbers of cfu were lower for both bacterial strains though *P.*

fluorescens WCS417r was also a better root colonizer on this plant. The difference he found on *myb72-1* was only significant though 2 days after dipping.

Overall, based on our results, we can say that *P. fluorescens* WCS417r is better root colonizer than *P. fluorescens* WCS374r except for the experiment performed with rockwool. The results show no direct link between the ability to induce systemic resistance and root colonization. Furthermore, we can say that there is a link between root colonization and the substrate used for growing the plants. *P. fluorescens* WCS417r is a better root colonizer except when the plants are grown on rockwool, than *P. fluorescens* WCS374r does better at colonizing the roots. Doornbos *et al.* (2010) also provides evidence that the substrate used for growing plants and root colonization are linked. Autoclaved clay shows higher cfu/gram root than on autoclaved potting soil-sand mixture, when the substrates are not autoclaved the results are even more diverse for the plants used.

Growth rate

Because *P. fluorescens* WCS417r proves to be a better root colonizer than *P. fluorescens* WCS374r, except in the case of the experiment on rockwool, the idea of *P. fluorescens* WCS417r growing faster arose. Results show that *P. fluorescens* WCS417r does not grow any faster than *P. fluorescens* WCS374r in both liquid minimal medium and liquid King's medium B (figure 7). Therefore one can conclude that growth rate is of no importance for being a good colonizer. These results also support the hypothesis of the substrate being of great importance for root colonization because there are no differences in growth rate though root colonization differs between *P. fluorescens* WCS374r and *P. fluorescens* WCS417r depending on the substrate used.

Use of different carbon sources

Another possibility for *P. fluorescens* WCS417r being a better root colonizer is that this bacterial strain can use more carbon sources than *P. fluorescens* WCS374r or that the carbon sources can be used more efficient. The biolog ecoplate pm1 is a 96 wells plate with a different carbon source in each well and is therefore suitable to see if there are any differences in the use of carbon sources between the two bacterial strains. The carbon sources used by the bacterial strains are the same except for N-acetyl-D-glucosamine and D-sorbitol which could only be used by *P. fluorescens* WCS417r (figures 8 and 9). N-acetyl-D-glucosamine is also known as chitin and can be found in fungi and in invertebrates (Carlström, D. 1957). It is not secreted by plants and therefore this carbon source is not the reason why *P. fluorescens* WCS417r is a better colonizer. D-sorbitol is produced in the leaves of plants (Li-song Chen and Lailiang Cheng, 2004). Photosynthates can leak into the rhizosphere and therefore D-sorbitol is a possible candidate for the difference in root colonization in favor of *P. fluorescens* WCS417r.
Overall, the purple discoloration is heavier in the plates with *P. fluorescens* WCS417r which might indicate that these carbon sources are also used more efficient, especially because the average initial density at time point 0 was higher for *P. fluorescens* WCS374r. A higher initial density means more bacteria that can use the carbon source which leads to the idea of a heavier discoloration. But this is not the case and therefore the conclusion can be drawn that *P. fluorescens* WCS417r uses the carbon sources more efficient.
Raw graphics of the absorbance curves also show an increase in absorbance for the biolog plate pm1 with *P. fluorescens* WCS374r in wells F10 and H9 in the beginning but this increase declines rapidly and the picture of the biolog ecoplate pm1 also shows no purple discoloration. Probably there was some

kind of filth in this well which caused the increase in the curves. These sugars could not be used cause then the wells would have turned to a purple color (see appendix A and figures 8 and 9).

Conclusion

Our results indicate that *P. fluorescens* WCS417r is a better root colonizer than *P. fluorescens* WCS374r when the plants used are grown on a potting soil-sand mixture or in a sterile environment on MS-plates (with or without competition). *P. fluorescens* WCS374r is a better root colonizer when the plants used are grown on rockwool. Because there are no differences in growth rates between the strains used we conclude that the substrate used for growing the plants determines which strain is the better root colonizer. Also, no big differences are found between root colonization on *A. thaliana* accession Col-0 and root colonization on its mutant *myb72-1,* we conclude that there is no link between the ability to induce systemic resistance and the ability to colonize the roots.

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Picture on the cover from:
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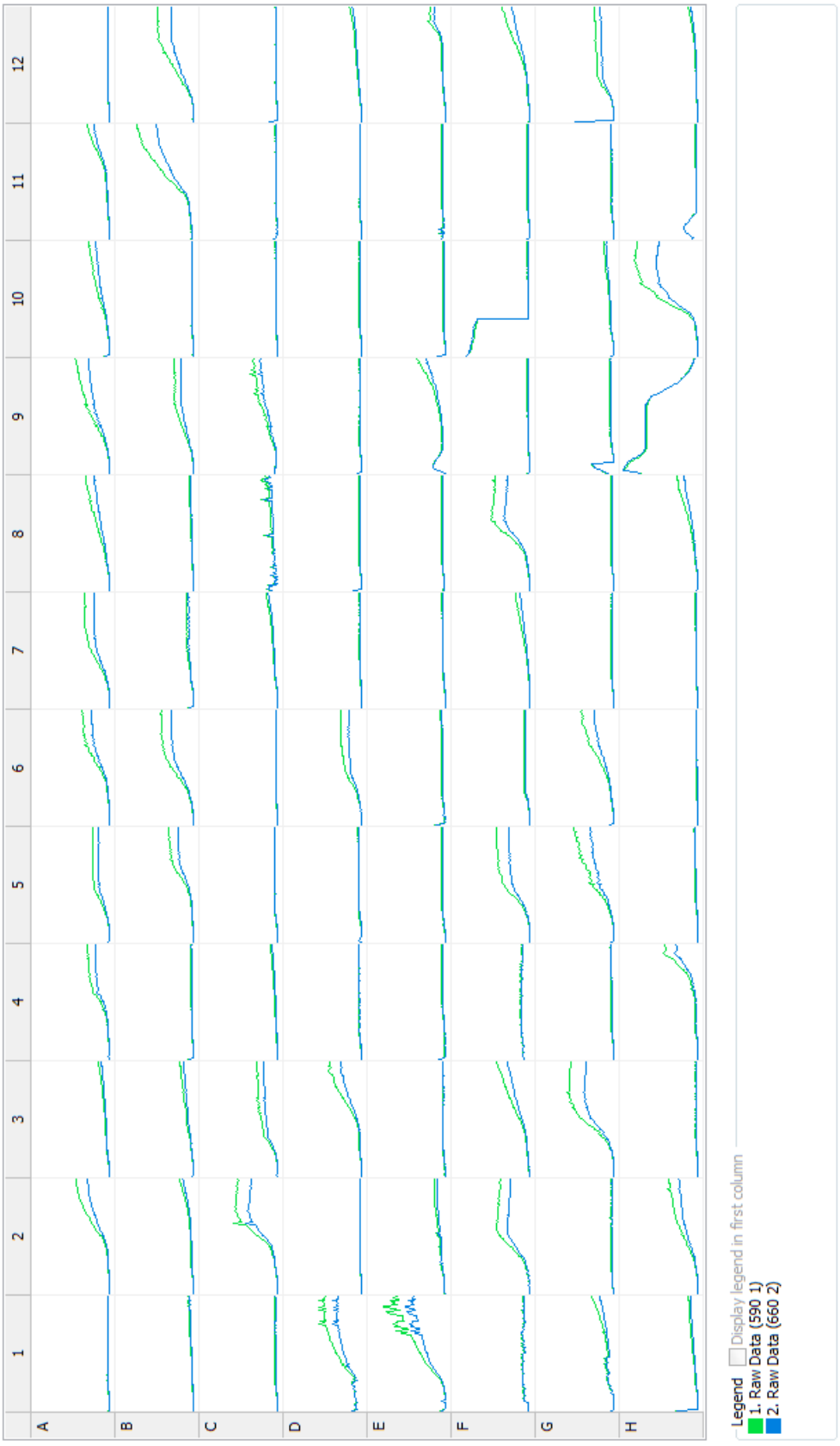
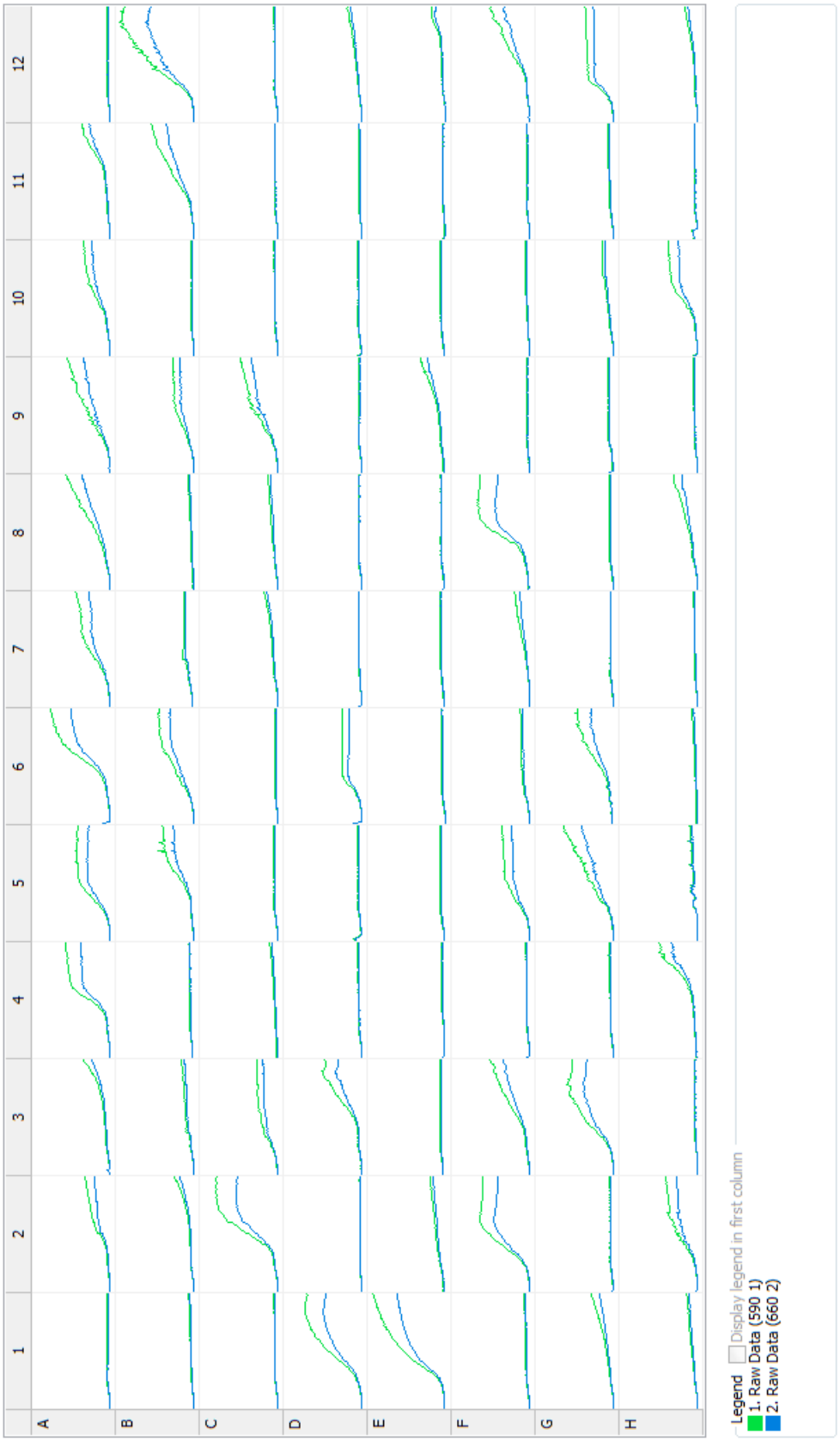
Appendix A – Curves of usage of carbon sources by *P. fluorescens* WCS374r



Appendix B- Curves of usage of carbon sources by *P. fluorescens* WCS417r



BiOLOG

Phenotype MicroArrays™

PM1 MicroPlate™

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 M-Tartaric Acid	E3 Glucose-1-Phosphate	E4 Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 M-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 P-Hydroxy Phenyl Acetic Acid	H3 M-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

FIGURE 1. Carbon Sources in PM1 MicroArray