Rhizoplane colonisation of peas by *Rhizobium leguminosarum* bv. *viceae* and a deleterious *Pseudomonas putida*

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Abstract

*Pseudomonas putida* strain Å313, a deleterious rhizosphere bacterium, reduced pea nitrogen content when inoculated alone or in combination with *Rhizobium leguminosarum* bv. *viceae* on plants in the presence of soil under greenhouse conditions. When plants were grown gnotobiotically in liquid media, mixed inocula of Å313 and rhizobia gave a higher proportion of small evenly distributed nodules when compared with a single rhizobial inoculation. In addition, the rhizobial root establishment was reduced by Å313 irrespective of inoculum density, indicating that Å313 has the capacity to interact with the early rhizobial infection process. When pea seedlings were simultaneously inoculated with Å313 and rhizobia, Å313 colonised the root hairs to the same extent as the rhizobia, according to analysis by immunofluorescence microscopy. This suggests that the root hair colonisation trait of *P. putida* interferes with the onset of the symbiotic process.

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1. Introduction

*Pseudomonas putida* strain Å313 is a non-fluorescent deleterious rhizosphere bacterium (DRB) that is phytotoxic to both monocotyledonous and dicotyledonous plant species, including peas [1–3]. It was originally isolated from roots of symptomless pea plants [1]. The bacterium is able to produce phytotoxins that are possibly hydrophobic, heat tolerant and stable at a pH range of 4–10. Strain Å313 invades the intercellular spaces of wheat root cortex [1]. In addition, the bacterium expresses an antimicrobial activity towards *Rhizobium leguminosarum* bv. *viceae* when studied in vitro [3]. The production of antimicrobial metabolites is likely to be an important mechanism by which rhizosphere bacteria are able to reduce or prevent colonisation of the roots by other rhizosphere bacteria.

The capacity for root colonisation by the DRB themselves, however, could be a critical trait for expression of the deleterious activity in the host plant [4–7]. For *Rhizobium*, the colonisation of the legume root hairs is of fundamental importance for establishing the legume-*Rhizobium* symbiosis [8]. Wiehe et al. [9] observed that *R. leguminosarum* bv. *viceae* colonised the roots of pea to a lower extent than a plant growth promoting...
rhizobacterial (PGPR) strain PsIA12 of *Pseudomonas fluorescens*. In monoxenic, nitrogen-free hydroponic cultures they observed an up to ten-fold lower root colonization in peas than in lupines. In agreement with their results in hydroponic cultures, electron microscopic observations confirmed poor colonization of the pea rhizoplane by single bacterial cells. Moreover, different strains of *Pseudomonas* spp. have been found to reduce nodule numbers and the capacity for nitrogen fixation [10,11].

The objective of this study was to examine how interactions with deleterious *P. putida* affect the development of a well-functioning symbiosis between peas and *R. leguminosarum* bv. *viceae*. In particular, the initial onset of the *Rhizobium*-pea symbiosis in the presence of *P. putida* was of interest.

2. Materials and methods

2.1. Bacterial strains and preparation of inocula

*P. putida* strain Å313, a non-fluorescent DRB isolated from pea roots by Aström [1] and further identified and characterised by Berggren et al. [2,3], was used in this study. In addition, three strains of *R. leguminosarum* bv. *viceae* were chosen from a Swedish collection [2] and have been used in previous studies as well [3]. Strains 3113 and 3114 were isolated from the nodules of *Vicia sativa* and strain 3162 was isolated from nodules of *Pisum sativum*. The choice of three strains was based on the expectation that the response induced by DRB is not *Rhizobium* strain-specific [3]. Results from other investigations indicate that DRB are not to be regarded as truly host specific with regard to plant species [1]. The *P. putida* strain was cultured on diluted TSA (tryptic soy broth, 3 g/l, technical agar, 12 g/l), suspended in sterilised distilled water (SDW) and stored at +4 °C pending cells from a 24 h-old culture in TSA in 10 mM phosphate buffer saline (PBS; 0.01 M, pH 7 + 0.1%)

2.2. Plant material and growth conditions

Seeds of a pea cultivar (*P. sativum* cv. Capella), that is known to produce tendrils to a higher extent than typical pea cultivars, were fractionated by size, surface-sterilised and pre-germinated for 3 days (the gnotobiotic experiments) before use [2].

The greenhouse experiments were carried out in field soil as previously described [2]. The soil originated from a field situated at Ultuna, Sweden (60° N, 17° E). The soil is a sandy loam of pH 6.5, with a total nitrogen content of 0.1%, an organic matter of 1.1%, slightly soluble phosphorus [13] of 6 mg/100 g soil and soluble phosphorus of 63 mg/100 g soil. This soil has a long history of continuous pea monoculture and seems to have a high frequency of deleterious rhizosphere bacteria, observed as poor establishment of peas under field conditions without any distinct deleterious symptoms on the plants. The *P. putida* used was previously isolated among other similar strains from pea roots in this soil [1].

The growth conditions in the greenhouse varied between 16 and 20 °C, referring to night and day temperature. Light was, when necessary, supplemented with Philips HPI-T 400 W mercury lamps to give 30 kLux at plant height to ensure a 16 h light period. The growth conditions in the chamber were 17/15 °C being the day/night temperature, respectively, a 16 h day length and a mean photosynthetic photon flux density of 45 kLux and 70% humidity.

The gnotobiotic experiments were carried out in test tubes (30 mm in diameter, 170 mm length), containing 20 g of oven-baked clay beads (diameter 3 mm, Leca™, Nystroms Cementvarufabrik AB, Rimbo, Sweden), soaked in 10 ml nitrogen-free Fähræus nutrient solution [14]. The tubes were closed with a cotton plug before autoclaving for 40 min at 121 °C. Thereafter, two surface-sterilized but pre-germinated apparently healthy seeds with a root length of about 5 mm were placed aseptically on the clay bead surface in each tube. After inoculation with 2 ml of bacterial suspension that was poured over each seedling, they were covered with a thin layer of moistened clay beads and the tubes were closed with cotton plugs to prevent evaporation and contamination. Tubes were incubated in a growth chamber and, when necessary, the plants were watered uniformly with SDW only.

2.3. Production of crude antisera for Immunofluorescence (IF) observations

This study concentrated on interactions of *P. putida* strain Å313 and *R. leguminosarum* bv. *viceae* strain 3162. For the preparation of antigens, *R. leguminosarum* bv. *viceae* strain 3162 and *P. putida* strain Å313 were cultured separately, harvested and washed twice with phosphate buffer saline (PBS; 0.01 M, pH 7 + 0.1%
 Tween 20), centrifuged and resuspended in PBS (10⁹ CFU ml⁻¹). The immunization procedure for the production of antisera was the same as previously described by Mårtensson et al. [15], except that intact cells of both *R. leguminosarum* bv. *viceae* and *P. putida* were used. The collected serum was purified by precipitation with saturated ammonium sulphate, centrifuged (10,000 g) for 30 min at 8 °C and dissolved in 0.02 M sodium phosphate buffer, pH 7.2 and stored frozen (−70 °C) prior to use.

### 2.4. Preparation of samples for in situ IF detection

Bacterial root colonisation of pea roots by *R. leguminosarum* bv. *viceae* and *P. putida* Å313 was observed on 1, 2, 3, 6 and 8 day-old root systems to be able to follow colonisation in time. On each sampling occasion, two pouches with single strain inoculation and four pouches with combined inoculation were harvested and the whole root system of one plant from each pouch was placed in a well (Multidish rectangular 4 well, NUNC™, 24 × 67 mm). The roots were then incubated with either of the two crude antisera (16 ml in each well, diluted 1:300 in PBS) for 24 h at 36 °C and rinsed twice in PBS. Thus, two root systems from the co-inoculated treatment were incubated with antisera against strain 3162 and the remaining two with antisera against strain Å313. A second incubation of the roots was performed with an additional antibody (fluorescein isothiocyanate (FITC)-conjugated pig-anti-rabbit immunoglobulins, DAKO, Glostrup, Denmark), diluted 1:200 in PBS, incubated and rinsed in PBS as described above. The cross-reactivity was checked in the antibody preparations by enzyme-linked immunosorbent assay (ELISA), and on the roots by IF. The respective antisera did not cross-react.

### 2.5. Interaction between DRB and Rhizobium in pea in field soil

The possible interference of DRB with the nitrogen-fixing process was investigated by inoculating *P. putida* and *R. leguminosarum* bv. *viceae* to pea seedlings in field soil and measuring the nitrogen content in the aerial plant parts as previously described [2]. A pregerminated seed was placed in each pot containing field soil and inoculated with 1 ml of a cell suspension of strain Å313 and/or strain 3162 per seed. In one of the treatments, the strain 3162 was applied six days later in order to magnify the effects of Å313. Controls were left without any inoculation. Following inoculation, the pots were placed in the greenhouse. After 10 days, eight plants showing healthy shoots and a uniform height were chosen from each treatment for observations. Nitrogen-free nutrient solution [16] was added on three occasions during the experiment. Forty-five days after the start of the experiment, flowering was recorded among the five treatments. Thereafter, the shoots and roots were dried at 60 °C over-night, weighed and analysed for their Kjeldahl nitrogen content.

### 2.6. Interactions between DRB and Rhizobium in pea under gnotobiotic conditions

The first gnotobiotic experiment was conducted in order to study the impact of Å313 on the nodule formation and the biological nitrogen fixation by rhizobia. Sterile tubes were co-inoculated with 2 ml of strain 3113 and/or strain Å313 (10⁶ CFU ml⁻¹). A delayed inoculation with 2 ml of strain 3113, one day and eight days after the start, was carried out for two of the treatments. The plants were harvested after 7 weeks of growth and nodules were counted and classified into either of two size groups, <5 or >5 mm in diameter. Shoots and roots were dried at 60 °C over night, weighed and analysed for Kjeldahl-nitrogen content. The experiment was set up in a random design with six replicates per treatment.

To determine whether the initial cell density influenced the bacterial colonisation rate of the pea roots, a second gnotobiotic experiment was carried out with three inoculum densities of strain Å313 and strain 3114. The inocula of strain Å313 and strain 3114 (10⁶ CFU ml⁻¹, respectively) were prepared and diluted in 10 mM MgSO₄, corresponding to 0.9 and 0.1 parts of the inoculum cell density. Tubes were inoculated with 2 ml of strain Å313 and strain 3114 in three different ratios; 1:1, 0.9:0.1 and 0.1:0.9 of Å313:3114, respectively. Tubes with Å313 or 3114 were used as controls. Nine replicates were used per combination, and three randomly chosen tubes per treatment were harvested after 3, 6 and 9 days of growth. Each of the two root systems per tube were washed and homogenised together in SDW. The homogenates were serially diluted and appropriate dilutions were spread on diluted TSA and YMA before incubation at 28 °C. The colonies appearing on TSA were counted after 2 days for strain Å313 and those on YMA after 3 days for strain 3114. The two strains could be separated from each other in vitro, based on differences in colour and shape.

### 2.7. Inoculation and growth of pea plants in growth pouches for in situ IF detection

The procedure of seed preparation and plant cultivation in the gnotobiotic growth pouch set-up has been described in detail by Berggren et al. [2]. Pea seeds of cv. Capella were surface-sterilised as before and pre-germinated in moistened, sterile vermiculite for four days at 22 °C. The pea-seedlings were then soaked for 15 min in bacterial cell suspensions (10⁷ CFU ml⁻¹) of strains 3162 and/or Å313, prepared from fresh cultures suspended in 0.01 M MgSO₄. The seedlings were
transferred aseptically to the growth pouches (two seedlings per pouch) and placed in a climate chamber. IF-stained roots were observed in a stereomicroscope equipped with fluorescent light (Leica) and with 6.3–50 times magnification.

2.8. Experimental design and statistical analyses

Data were analysed using one way ANOVA [17] and treatment means were compared using student’s t-test ($P < 0.05$). All the experiments were repeated once. However, since results followed a similar pattern, the data presented here are from single experiments only.

3. Results and discussion

3.1. Nitrogen fixation and dry matter production

In a previous study, we reported in vitro growth inhibition of *R. leguminosarum* bv. *viceae* by live cells of the deleterious *P. putida* strain Å313. This inhibition was also evident in the presence of the extracellular metabolites of *P. putida* [2]. The present study further confirms the deleterious nature of *P. putida*, since a negative effect of strain Å313 on the biological nitrogen fixation (BNF) process was observed in the pea rhizosphere. After inoculation of pea seedlings, planted in potted field soil, with Å313 alone or in combination with *R. leguminosarum* bv. *viceae* 3162, we obtained a significant ($P < 0.05$) reduction in the pea nitrogen content both in shoots, roots and nodules compared to that in non-treated peas or peas inoculated with strain 3162 alone (Table 1).

| Table 1 | Effects of deleterious *P. putida* strain Å313 and nitrogen-fixing *R. leguminosarum* bv. *viceae* strain 3162 on the growth and nitrogen content of pea (*P. sativum* cv. Capella) after 45 days of growth in field soil ($n = 8$) |
|---|---|---|---|
| Control | Inoculation day 0 &nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&n

* The values in the rows which are followed by the same letters are not significantly different according to the Duncan test ($P < 0.05$).
seedlings treated with a mixed inoculum also displayed a distribution of root nodules on the whole root system compared to the roots inoculated with *R. leguminosarum* bv. *viceae* strain 3113 alone, which developed nodules accumulating in clusters on the root parts close to the stem (data not shown).

When studying interactions between *Rhizobium* and *Azospirillum*, Plazinski and Rolfe [24] found an increase in nodule numbers to produce a non-nitrogen fixing (Fix/C0) response in clover, which suggest that *Azospirillum* probably acts via phytohormone production. Grimes and Mount [25] also reported an increased nodulation after co-inoculation with *Rhizobium* and *P. putida*. Åström [1] provided evidence in support of both diffusible and volatile metabolites as being some of the mechanisms of strain Å313, because its live cells caused reduced root hair development, while its culture filtrates induced dense root hair formation in wheat. Next to other metabolites, the isolate produces IAA in vitro. It is possible that the amounts of these metabolites produced by Å313 in the rhizosphere are sufficient to affect the root permeability and hence the root exudation and the root infection sites for *Rhizobium* in pea. We consider it likely that Å313 induces excessive root exudation.

### 3.3. Root colonization

We found that root colonization by *R. leguminosarum* bv. *viceae* strain 3114 was reduced when co-inoculated with *P. putida* strain A313, irrespective of the inoculum density (Fig. 1). This was also observed by Li and Alexander [28] and Bolton et al. [26]. A reduced root colonization could be explained by the ability of Å313 to produce inhibitory substances, shown to affect *Rhizobium* cell growth and chemotaxis in vitro [3]. Burla et al. [10] proposed a similar explanation for effects of *P. fluorescens* towards growth of *Rhizobium phaseoli*. In our study, no native microflora was present that interfered with establishment by 3114 and it was found in lower numbers on the root surface than Å313 in the same root environment. When Burla et al. [10] studied the in vitro effects of biocontrol bacteria, *P. fluorescens* strains 1 and 2 and *Serratia marcescens* during growth on *R. phaseoli*, they found that nodulation of bean roots was inhibited and suggested that the selection of microorganisms for biocontrol should involve studies on their influence on plant root mutualistic symbionts. Our study

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>3113 at day 0</th>
<th>Å313 and 3113 at day 0</th>
<th>Å313 and 3113 at day 1</th>
<th>Å313 and 3113 at day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry weight (mg) shoots</td>
<td>589.4A</td>
<td>608.4A</td>
<td>597.5A</td>
<td>549.8A</td>
</tr>
<tr>
<td>Dry weight (mg) roots and nodules</td>
<td>220.2A</td>
<td>236.6A</td>
<td>219.3A</td>
<td>212.4A</td>
</tr>
<tr>
<td>Nitrogen (mg g⁻¹) shoots</td>
<td>16.7A</td>
<td>17.3A</td>
<td>17.9A</td>
<td>15.8A</td>
</tr>
<tr>
<td>Nitrogen (mg g⁻¹) roots and nodules</td>
<td>7.5B</td>
<td>8.0B</td>
<td>7.6B</td>
<td>6.3A</td>
</tr>
</tbody>
</table>

Nodule number tube⁻¹ (containing two plants per tube)

| Diameter | 9.8A | 19.8B | nd | 17.3 AB |
| <5 mm in diameter | | | | |
| >5 mm in diameter | 5.8C | 0.1A | nd | 1.6B |

* The values in the rows followed by the same letters are not significantly different according to the Duncan test (*P* < 0.05).

* Not determined.

Fig. 1. Colonisation of the rhizosphere of pea (*P. sativum* cv. Capella) by seedling-inoculated *P. putida* strain Å313 (○) ((○) control Å313) and *R. leguminosarum* bv. *viceae* strain 3114 (□) ((□) control 3114) at three different inoculum cell densities (a) = 1 Å313:1 3114; (b) = 0.9 Å313:0.1 3114; (c) = 0.1 Å313:0.9 3114 (*n* = 9). Results are expressed per tube (containing two plants per tube). Error bars represent standard deviations for colony counts.
supports this view with regard to selection of PGPR-lacking biocontrol traits.

The above results as well as the antagonistic activity of Å313 towards R. leguminosarum bv. viceae reported in earlier studies [2,3] led us to expect that root colonisation by strain 3162 would be reduced in the presence of Å313. Instead, we found that the two bacterial types visibly colonised the root surface apparently without out-competing each other (Fig. 2). However, quantitative estimations of the cell numbers of the two bacterial strains were not established. Therefore, the possibility that the cell growth of strain 3162 was reduced due to the presence of strain Å313 could not be ruled out. It is, however, possible that the in situ activity of strain Å313 was not fully expressed at this early stage of root colonisation and that the results of such interactions may be detected at a later stage of the colonisation process.

Similar to the colonisation pattern of the root surface, we found that the root hairs were equally colonised by both strain 3162 and strain Å313 (Fig. 3). This is an interesting observation, as strain Å313 has been found to induce root hair deformations in vitro [2] similarly to the early stage of Rhizobium-legume infection process [30]. Hansen et al. [31] reported that barley root hairs were only weakly colonised by two P. fluorescens strains. Thus, root hair colonisation may not be a general trait among rhizosphere bacteria, but strain Å313 showed a high colonisation potential of the root hairs (Fig. 3). It seems that the high colonisation of the root hairs by Å313 may be partly responsible for the induced root hair deformation, which may account for the unfavourable nodule distribution by changing the number of infection sites available for Rhizobium infection (Table 2). We also found root hair development to be strongly reduced in the presence of strain Å313 as a single inoculant. Similarly, Begonia et al. [32] observed reduced and collapsed root hairs of birdsfoot trefoil in the presence of different DRB strains. Abnormal increases in root hair growth and decreases in the number of infected root hairs have been observed in clover [24], alfalfa [27] and soybean [33] inoculated with Rhizobium or Brady...
rhizobium in combination with Azospirillum spp. [34]. Bacterial production of sufficiently high concentrations of phytohormones may explain the observed root hair deformations in our study as well as the plant growth-promoting effects observed in studies with P. putida and Azospirillum [7,29,35,36].

4. Conclusions

From the findings presented here it can be concluded that selected rhizosphere bacteria, if present simultaneously with symbiotic nitrogen-fixing microorganisms, have a significant potential to affect root establishment and biological activity, besides having negative influence on plant productivity. Their effects on root hair formation indicates that the uptake of nutrients and water is disturbed, as also the distribution of root hair along the whole root system suggests that metabolites formed by DRB increase the release of root exudates and affect the integrity of plant cell membranes. Some substances produced by DRBs may be taken up by root cells and block metabolic processes. These are only some of the hypotheses worth further challenge.

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