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# Early modifications of *Brassica napus* root system architecture induced by a plant growth-promoting *Phyllobacterium* strain

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## Summary

• Plant growth-promoting bacteria (PGPB) have been reported to stimulate root morphogenesis. To improve our knowledge of the PGPB effect, the early modifications of *Brassica napus* root system architecture induced by the PGPB *Phyllobacterium* sp. (29-15) were analysed.

• Plants were grown in Petri dishes on a vertical medium supplemented with variable doses of *Phyllobacterium* sp. in gnotobiotic conditions. Root system elementary variables were measured in a nondestructive manner and the distribution of the bacteria throughout the primary root was quantified.

• *Phyllobacterium* sp. in doses from $3 \times 10^7$ to $3 \times 10^8$ colony-forming units $ml^{-1}$ significantly promoted *B. napus* total root length up to 50% by increasing both lateral root density throughout the primary root and growth rate of mature lateral roots. The primary root was progressively colonized by the bacteria from the tip to the base and the number of colonizing cells was positively correlated with the inoculum density.

• Relationships between inoculum density, root colonization and root system architecture emphasized the relevance of this approach to specify PGPB effects on plants.

**Key words:** plant growth-promoting bacteria (PGPB), root system architecture, oilseed rape (*Brassica napus*), *Phyllobacterium*, colonization.



## Introduction

Mutualistic bacteria used for crop inoculation are promising agents to aid sustainable agriculture since they contribute to a decrease mineral fertilizers and chemical pesticide treatment (Okon & Hadar, 1987; Bashan, 1998). They form loose associations with plants, living near, on, or even inside roots (Kloepper & Beauchamp, 1992). Among mutualistic bacteria, plant growth-promoting bacteria (PGPB), stimulate plant growth through direct mechanisms while biocontrol PGPB increase plant growth indirectly, through preventing deleterious effects of phytopathogenic microorganisms (Bashan & Holguin, 1998; Gamalero *et al.*, 2002).

Plant growth enhancement by PGPB involves diverse mechanisms not entirely elucidated. They may release key hormones for plant development such as indoleacetic acid (IAA) (Costacurta & Vanderleyden, 1995) or reduce the level of the growth-limiting hormone ethylene (Wang *et al.*, 2000). Altered mineral nutrition by way of a direct stimulation of the ionic transport system (Bertrand *et al.*, 2000) and mineral availability enhancement has also been suggested as a factor in plant growth promotion (Goldstein *et al.*, 1999). Whatever the mechanisms involved, the ultimate impact of PGPB is a quantitative change in root and/or shoot growth. Over the last two decades, the effects of PGPB on plant growth have been described and modifications in term of fresh and dry mass accumulation, root length and surface, lateral root number and density have been reported (Hadas & Okon, 1987; Frommel *et al.*, 1991; Sarig & Okon, 1992; Barbieri & Galli, 1993; Leinhos & Bergmann, 1995). However, the effects of PGPB

on root system architecture have rarely been studied (Gamalero *et al.*, 2002). Accurate analysis of morphometric data in a nondestructive way is required to obtain precise information on the mode of action of PGPB, in particular, on the root parameters that are modified, the way they are modified, and the starting point of the beneficial effects. That knowledge may be a crucial step for further investigation on mechanisms of PGPB–plant interaction.

Many studies have pointed out that plant growth might be promoted by various bacterial genera, including *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus* and *Pseudomonas* (Glick, 1995; Reinhold-Hurek & Hurek, 1997). We have previously identified an efficient plant growth-promoting *Phyllobacterium* strain (29-15) naturally associated with oilseed-rape (*Brassica napus*), a crop plant with both agronomic and environmental interests (Bertrand *et al.*, 2000). The genus *Phyllobacterium* was originally described by Knösel (1962) to include bacteria that develop within leaf nodules of tropical ornamental plants. The further isolation of *Phyllobacterium* strains from different plant root systems underscored that this genus is commonly associated with plants and also colonizes niches other than leaf nodules (Lambert *et al.*, 1990; Hallmann *et al.*, 1997; Sturz *et al.*, 1998; Rojas *et al.*, 2001). Nevertheless, knowledge about the *Phyllobacterium* genus, and particularly its PGPB properties, remains limited.

The aim of this study was to analyse the early modifications induced by the *Phyllobacterium* strain 29-15 on *B. napus* root system architecture. A dynamic approach was designed to point out elementary variables associated with the PGPB effect and the root colonization pattern when the plant was supplied with different bacterial doses.

## Materials and Methods

### Bacterium material and growth conditions

The *Phyllobacterium* sp. strain 29-15, isolated from *Brassica napus* roots and previously characterized as a PGPB was used (Bertrand *et al.*, 2000). It was cultivated in a rich liquid medium (E'; composition (per litre): 0.5 g $K_2HPO_4$, 0.2 g $MgSO_4(H_2O)_7$, 0.1 g NaCl, 0.8 g $KNO_3$, 0.05 g $CaCl_2(H_2O)_2$, 0.0047 g $FeCl_3$, 10 g mannitol and 3 g yeast extract, pH 6.8) at 28°C for 36 h. Culture of bacterial cells was pelleted by centrifugation (5000 *g*, 20 min), washed twice and resuspended in sterile physiological water (0.9% NaCl, w : v). The bacterial density of the suspension was determined using a calibration curve assessed by turbidity ($\lambda$ = 620 nm) and then adjusted in plant medium to a cell concentration 100 times higher than that required for inoculation. The precise bacterial concentration was checked through serial diluted and plated suspension on solid E' medium (1.5% agar, w : v) and then colony-forming units (cfu) were counted.

### Plant and Petri dish experiments

Oilseed rape (*B. napus* var. Navajo) seeds were surface-sterilized in 3% calcium hypochlorite (w : v) for 5 min and washed four times in sterile water. Seeds were placed for germination on the E' semisolid medium (0.8% agar, w : v), diluted 10 times, in Petri dishes for 48 h at 20°C in dark.

The bacterial suspension at the appropriate concentration was introduced in the plant medium (0.5 mM $CaSO_4$, 1 mM $KH_2PO_4$, 2 mM $KNO_3$, 0.5 mM $MgCl_2$, 50 μM NaFe-ethylenediaminetetraacetic acid (EDTA), 4 μM $H_3Bo_3$, 6 μM $MnSO_4(H_2O)$, 0.9 μM $ZnSO_4(H_2O)_7$, 1 μM $CuSO_4(H_2O)_5$, 0.1 μM $Na_2MoO_4(H_2O)_2$ and 2.5 mM 2-(*N*-Morpholino) ethanesulfonic acid (MES), pH 5.7) with 0.8% noble agar (w : v) added, at 50°C, just prior cooling in 22.8 × 22.8 cm Petri dishes (200 ml per box) (Polylabo, Strasbourg, France). In each Petri dish, four seedlings were placed (in line) onto the solidified medium at 4.5 cm from each other and from the box edges. The Petri dishes were installed vertically for 7 or 8 d in a growth chamber set for a 22°C/18°C light/dark thermoperiod and 16 h/8 h light/dark photoperiod at a photon flux density of 130 μmol $m^{-2}$ $s^{-1}$.

Each treatment (either the bacteria at each concentration mentioned below or the uninoculated control) consisted of three Petri dishes with four seedlings per dish. Over the 7-d experiment, the seven plants close to the average plant in each treatment were chosen using a quick visual screen based on uniformity among the 12 plants per treatment. Microbial sterility in the Petri dishes was also assessed: one seedling per Petri dish was aseptically crushed in sterile physiological water and then serially diluted and plated on solid E' medium.

Three independent experiments were then performed. The first experiment was designed to test a wide range of bacterial doses (i.e. $10^6$, $3 \times 10^7$ and $6 \times 10^8$ cfu $ml^{-1}$). In the two other experiments, we focused on a narrower range of bacterial doses (second experiment, $4 \times 10^5$, $4 \times 10^6$ and $4 \times 10^7$ cfu $ml^{-1}$; third experiment, $4 \times 10^7$, $1.5 \times 10^8$, $3 \times 10^8$ cfu $ml^{-1}$). Hence, the median concentration (i.e. $3–4 \times 10^7$) was assessed in triplicate.

### Root system architecture

Plant root development was estimated daily, using a flatbed scanner (Scanjet 5300C; Hewlett-Packard, Wilmington, DE, USA) at intermediate resolution (450 dpi) as a nondestructive method. Pictures of the growing root systems were obtained by digitizing Petri dishes from the bottom. Images were later analysed according to Freixes *et al.* (2002) using Optimas software (Optimas 6.5; Media Cybernetics, Silver Spring, MD, USA). On day 7, each visible secondary root was given a registration number and the coordinates of the insertion into the primary root of each root were recorded. The position of this insertion with respect to the base of the primary root was then calculated by summing the distances between each

individual older root (located closer to the base). The lengths of both primary and secondary roots were registered on that image and later on images corresponding to earlier days. The process was entirely repeated for the seven plants selected per treatment. Three main variables were calculated from the set of data: (1) mean elongation rate of the primary root, (2) elongation rate of each secondary root and (3) density of secondary roots. The two first parameters were calculated from successive (1-d interval) measurements of individual root length. Secondary root elongation rate was averaged in each plant on roots longer than 8 mm to avoid very young roots; it was then averaged on each treatment. The latter parameter was calculated as the mean of the root number in successive 4-cm sectors along the primary root. Because cessation of growth of branches is a common feature in dicots, this variable was also calculated. Among the previously cited root parameters, only those that well described the effect of *Phyllobacterium* sp. on *B. napus* root system were developed in the results.

### Root colonization pattern of *Phyllobacterium* sp.

To quantify root-colonizing bacteria, three segments of 1 cm were extracted in the oldest, intermediate and youngest zones of the primary root in three plants, 4 d and 8 d after transplanting in the third experiment. Segments were carefully removed from the medium surface, placed in sterile water and crushed. Bacterial cells were counted by indirect immunofluorescence test on glass slides using a polyclonal antiserum produced with whole cells of *Phyllobacterium* sp. (Schmidt *et al.*, 1968; Arsac & Cleyet-Marel, 1986).

### Statistical analysis

Data were analysed by Student's *t*-test (threshold $P = 0.05$) or by one-way analysis of variance and then multiple range Duncan test (threshold $P = 0.05$) using SUPERANOVA software (Abacus Concepts, Berkeley, CA, USA).

## Results

### The *Phyllobacterium* strain stimulated *B. napus* root growth

The *Phyllobacterium* strain increased *B. napus* total root length in a dose-dependent manner with an apparent maximum efficiency for bacterial concentrations around $10^8$ cfu ml$^{-1}$ (Fig. 1a). At two similar concentrations among those assessed in the three independent experiments ($3 \times 10^7$ cfu ml$^{-1}$ and $4 \times 10^7$ cfu ml$^{-1}$), this stimulation was statistically significant (from 20 to 40% more root length compared with control, Fig. 1a,b). At higher concentrations ($1.5 \times 10^8$ cfu ml$^{-1}$ and $3 \times 10^8$ cfu ml$^{-1}$), the stimulation was even larger (about 50% more root length compared with control plants). Promotion of total root length by the *Phyllobacterium* strain was apparent 3 d after transplanting at the high bacterial concentrations ($3 \times 10^7$ cfu ml$^{-1}$ and $6 \times 10^8$ cfu ml$^{-1}$) and 7 d after transplanting at a lower bacterial concentration ($10^6$ cfu ml$^{-1}$) (Fig. 1c).

### Stimulation of root growth was due to increase of lateral root length

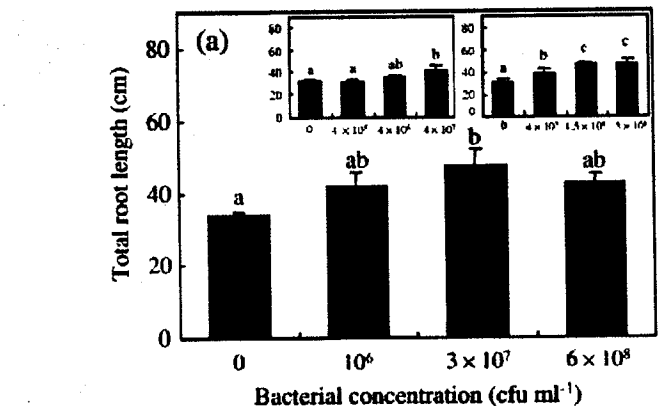
By day 7 after transplanting, the primary root length was unaffected by the bacterial concentration used in the three independent experiments. By contrast, at that time, there was a large increase in lateral root length (Fig. 2): this effect was significant for the bacterial concentrations of $3–4 \times 10^7$ cfu ml$^{-1}$ (from 55 to 60% increase in lateral root length) and was even larger for the bacterial concentrations of $1.5–3 \times 10^8$ cfu ml$^{-1}$ (about an 80% increase in lateral root length for both concentrations).

### The increase of lateral root length was due to an increased density of lateral roots on the branched zone of the primary root

Whatever the bacterial concentration tested in the three independent experiments performed, the branched zone of the primary root, i.e. the zone bearing lateral roots, was not modified (data not shown). On the other hand, lateral root density along the branched zone of the primary root was significantly increased by the *Phyllobacterium* strain; this result was statistically significant for all bacterial concentrations higher than $10^6$ cfu ml$^{-1}$ there was no evidence of an optimum (Fig. 3a). The increase was very similar in the three independent experiments performed and ranged from 20 to 45%. The impact of the bacteria on the branching density was obvious throughout the branched zone of the root (Fig. 3b); it was already clearly noticeable in the most apical region of the primary root (i.e. in the zone where the earliest lateral roots emerged) about 3 d after transplanting (Fig. 3b). By contrast, the emergence of lateral roots was not accelerated by the presence of bacteria at any concentrations, as shown by the lack of changes of either the branched zone length or the unbranched zone length at any time of the experiment.

### The increase of lateral root length was also due to an increase in both elongation rate of mature lateral roots and duration of elongation of lateral roots

An individual examination of each root allowed separate estimates of: (1) the mean elongation rate of mature lateral roots (roots emerged in the zone between 0 cm and 4 cm from the primary base) that were actively growing; and (2) the mean percentage of lateral roots that had ceased elongating. The mean elongation rate of mature laterals was increased from 20 to 44% by the bacteria from $3 \times 10^7$ to $3 \times$




(b)

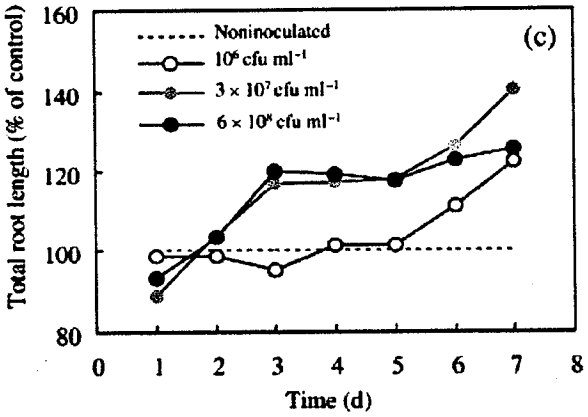



**Fig. 1** The effect of *Phyllobacterium* sp. on the total root system length of *Brassica napus* plants. (a) Seedlings were grown on initial bacterial concentrations of $10^6$, $3 \times 10^7$ and $6 \times 10^8$ cfu ml$^{-1}$ (main graph), $4 \times 10^5$, $4 \times 10^6$ and $4 \times 10^7$ cfu ml$^{-1}$ (inset, top left), $4 \times 10^7$, $1.5 \times 10^8$ and $3 \times 10^8$ cfu ml$^{-1}$ (inset, top right) on vertically orientated agar dishes. Data are means ± SE ($n = 7$) after 7 d of treatment; bars followed by the same letter are not significantly different (Duncan's test, $P = 0.05$). (b) Seven-day-old plants uninoculated (left) and inoculated with *Phyllobacterium* sp. at the initial concentration of $3 \times 10^7$ cfu ml$^{-1}$ (right). (c) Data are means ($n = 7$) compared with the uninoculated control (control = 100%) during 7 d of treatment; short-dashed line, uninoculated; open circles, $10^6$ cfu ml$^{-1}$; long-dashed line, $3 \times 10^7$; closed circles, $6 \times 10^8$ cfu ml$^{-1}$.

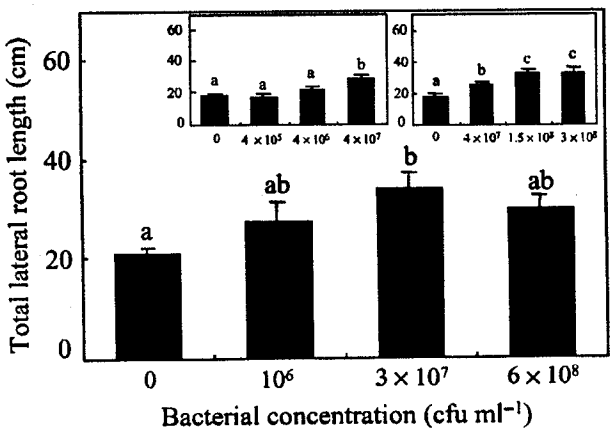



**Fig. 2** The effect of *Phyllobacterium* sp. on the total lateral root length of *Brassica napus* plants. Seedlings were grown on initial bacterial concentrations of $10^6$, $3 \times 10^7$ and $6 \times 10^8$ cfu ml$^{-1}$ (main graph), $4 \times 10^5$, $4 \times 10^6$ and $4 \times 10^7$ cfu ml$^{-1}$ (inset, top left), $4 \times 10^7$, $1.5 \times 10^8$ and $3 \times 10^8$ cfu ml$^{-1}$ (inset, top right). Data are means ± SE ($n = 7$) after 7 d of treatment; bars followed by the same letter are not significantly different (Duncan's test, $P = 0.05$).

$10^8$ cfu ml$^{-1}$ (Fig. 4a). At the same time, the presence of *Phyllobacterium* sp. in the root medium slightly decreased the percentage of lateral roots that had ceased growing over the 7-d experiment (Fig. 4b). Therefore, the presence of the bacteria slightly increased the duration of lateral root elongation.

## *Phyllobacterium* sp. colonization during root growth

Microscopic observations of cross-sections of roots carried out at the end of the experiment on different root zones revealed that *Phyllobacterium* sp. colonized all the root system and were located on root surface. However, bacterial cell number decreased from the base to the tip irrespective of bacterial inoculum concentrations assessed, (i.e. $4 \times 10^7$, $1.5 \times 10^8$ cfu ml$^{-1}$ and $3 \times 10^8$ cfu ml$^{-1}$; Table 1). Thus, a significant 10-fold gradient in bacterial concentrations was detected between the youngest and oldest segments of the primary root. The bacterial populations recovered in the root segments at the end of the experiment were not significantly different from those recovered 4 d after transplanting, except

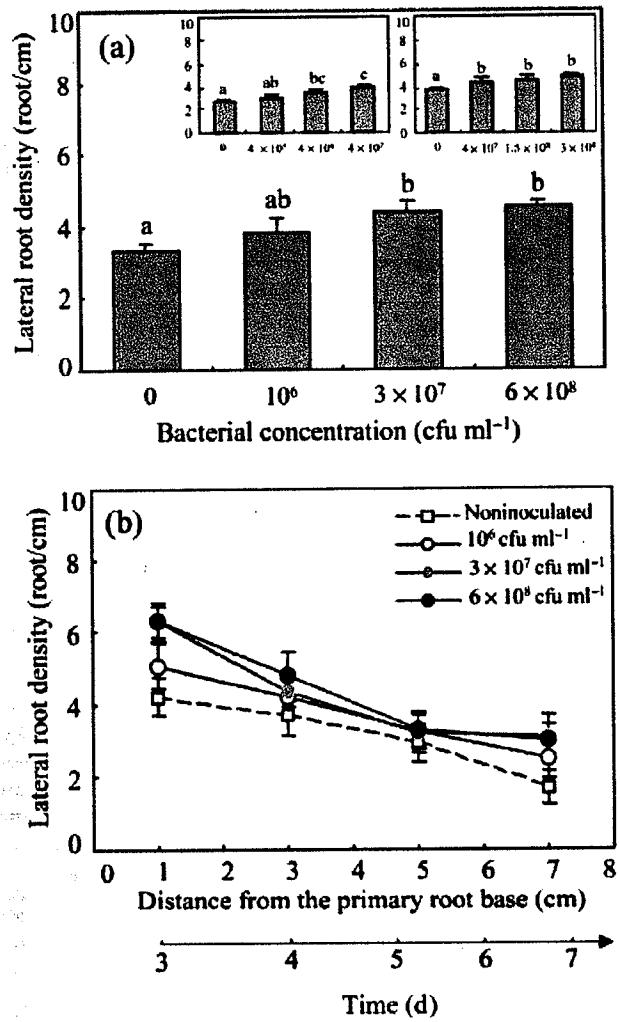



Fig. 3 The effect of *Phyllobacterium* sp. on the lateral root density of *Brassica napus* plants. (a) Seedlings were grown on initial bacterial concentrations of $10^6$, $3 \times 10^7$ and $6 \times 10^8$ cfu ml$^{-1}$ (main graph), $4 \times 10^5$, $4 \times 10^6$ and $4 \times 10^7$ cfu ml$^{-1}$ (inset, top left), $4 \times 10^7$, $1.5 \times 10^8$ and $3 \times 10^8$ cfu ml$^{-1}$ (inset, top right). Data are means ± SE ($n = 7$) after 7 d of treatment; bars followed by the same letter are not significantly different (Duncan's test, $P = 0.05$). (b) Data are means ($n = 7$) of the lateral root densities measured in each 2-cm segment of the primary root from the base. The second abscissa is an approximate correspondence between the branched zone length and time of growth; squares, uninoculated; open circles, $10^6$ cfu ml$^{-1}$; dashed line, $3 \times 10^7$; closed circles, $6 \times 10^8$ cfu ml$^{-1}$.

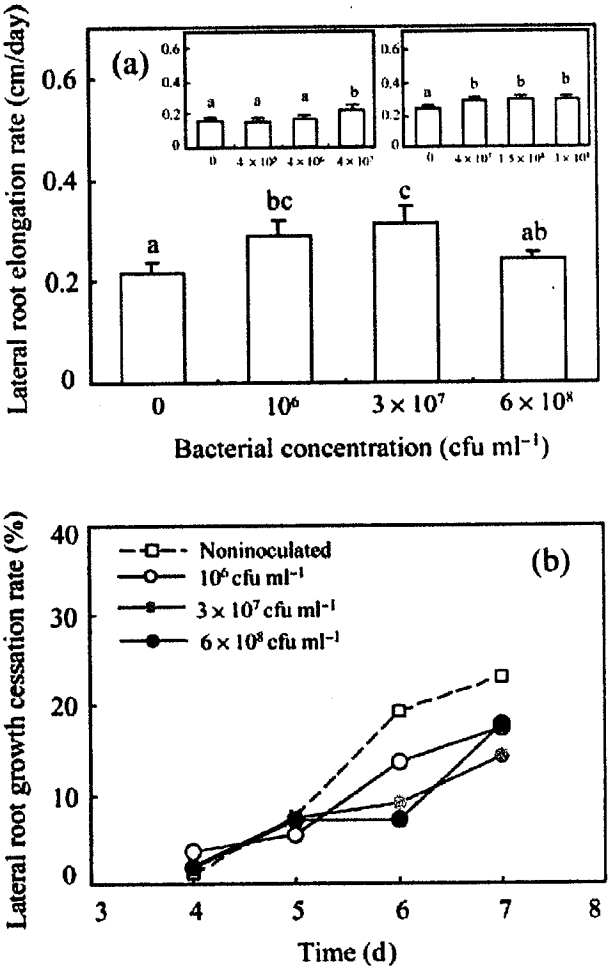



Fig. 4 The effect of *Phyllobacterium* sp. on the elongation rate of mature lateral roots (a) and lateral root growth cessation (b) of *Brassica napus* plants. (a) Seedlings were grown on initial bacterial concentrations of $10^6$, $3 \times 10^7$ and $6 \times 10^8$ cfu ml$^{-1}$ (main graph), $4 \times 10^5$, $4 \times 10^6$ and $4 \times 10^7$ cfu ml$^{-1}$ (inset, top left), $4 \times 10^7$, $1.5 \times 10^8$ and $3 \times 10^8$ cfu ml$^{-1}$ (inset, top right). Data are means ± SE ($n = 7$) after 7 d, calculated for the lateral roots that emerged in the zone between 0 and 4 cm from the primary root base. Bars followed by the same letter are not significantly different (Duncan's test, $P = 0.05$). (b) Data are means ± SE ($n = 7$) for 7 d of treatment; squares, uninoculated; open circles, $10^6$ cfu ml$^{-1}$; dashed line, $3 \times 10^7$; closed circles, $6 \times 10^8$ cfu ml$^{-1}$.

for the $1.5 \times 10^8$ cfu ml$^{-1}$ treatment in the oldest and intermediate primary root segments. There was a positive correlation between inoculum density and the concentration of bacteria recovered in the root segments, both 4 d and 8 d after transplanting (Table 1).

## Discussion

Several parameters of *B. napus* root system architecture were affected by the PGPB *Phyllobacterium* sp. The overall 40% promotion in total root length after 7 d at $3-4 \times 10^7$ cfu ml$^{-1}$ was due to an increase in the density and length of lateral roots. Similar effects have been reported for *Pseudomonas* strains on maize (Leinhos & Bergmann, 1995) and for *Azospirillum* on wheat (Barbieri & Galli, 1993). By contrast, the primary root length of *B. napus* plants was not modified by the *Phyllobacterium* strain at any concentrations after 7 d, which suggests that the effect on lateral roots was not the consequence of an earlier effect on the primary root.

Our kinetic approach revealed that the increase in total lateral root length induced by *Phyllobacterium* after 7 d was due

**Table 1** Concentration of *Phyllobacterium* sp. cells recovered from the basal, intermediate and tip zones of *Brassica napus* primary root

| Inoculum density introduced in the plant medium (cfu ml$^{-1}$) | Time after transplanting (d) | Bacteria recovered (log cell mg$^{-1}$ of fresh wt root) | | |
|---|---|---|---|---|
| | | Base | Intermediate zone | Tip |
| $3 \times 10^8$ | 4 | 5.64 a | 5.22 ab | 4.49 b |
| | 8 | 5.92 a | 5.52 b | 4.96 c |
| $1.5 \times 10^8$ | 4 | 5.06 a* | 4.79 ab* | 4.34 c |
| | 8 | 5.71 a* | 5.51 ab* | 4.15 c |
| $4 \times 10^7$ | 4 | 4.50 a | 4.38 a | 3.06 b |
| | 8 | 4.92 a | 4.44 ab | 3.92 c |

Seedlings were grown on initial bacterial concentrations of $4 \times 10^7$, $1.5 \times 10^8$ and $3 \times 10^8$ cfu ml$^{-1}$. Data are means ($n = 3$) after 4 d and 8 d. Within each bacterial treatment and for a given time (day 4 or 8), numbers followed by different letters are significantly different (Duncan's test, $P = 0.05$). Within each bacterial treatment and for a given root segment, numbers followed by an asterisk are significantly different (Student's $t$-test, $P = 0.05$).

to the stimulation of the elongation rate of mature lateral roots throughout the experiment (Fig. 4a). Furthermore, we observed that the stimulation of lateral root density occurred all along the zone of lateral root emergence and was particularly noticeable in the most basal region (Fig. 3b). In that region, lateral roots emerged about 3 d after transplanting. Because primordia initiation must precede lateral root emergence, this would suggest that the PGPB effect on primordia initiation started immediately upon transplanting. Because primordia development is essentially acropetal, the time needed for an initiated primordium to emerge from the primary root can be inferred from (1) the distance between the first emerged lateral root and the primary root tip, and (2) the rate of primary root growth (MacLeod, 1976). In *Phyllobacterium* sp.-treated plants, no effect on any of these variables was observed (data not shown) suggesting that the rate of primordia development was probably unaffected by the PGPB.

The early root modifications observed suggest that the build up of the interaction between the two partners is a rapid process. A spatio-temporal quantification of *Phyllobacterium* sp. on the primary root showed that root colonization occurred very early and progressed during root maturation, supporting an active bacterial involvement. Indeed, we found a 10-fold bacterial gradient between the youngest and oldest parts of the primary root. The experimental conditions used with an homogeneous dispersion of *Phyllobacterium* sp. in the agar medium undoubtedly facilitated root colonization since bacteria were continuously accessible to the growing root system.

The precise manipulation of the bacterial concentration in the root medium indicated that *Phyllobacterium* enhanced total root length in a dose-dependent manner, with an optimal concentration around $10^8$ cfu ml$^{-1}$. Such an optimal bacterial concentration on root modifications has often been observed in PGPB–plant interactions (Kapulnik *et al.*, 1985; Bashan, 1986; Harari *et al.*, 1988). However, the architectural analysis revealed that the optimal *Phyllobacterium* concentration differed according to the root parameter considered: the highest bacterial concentration ($6 \times 10^8$ cfu ml$^{-1}$) did not affect lateral root elongation but increased lateral root density when compared with control plants (Figs 3 and 4).

Quantification of *Phyllobacterium* root colonizing cells showed that the bacterial number retrieved on the primary root was positively correlated with the inoculum density in the medium. This correlation may explain the observed increased effect of the strain on root length until the optimal inoculum concentration around $10^8$ cfu ml$^{-1}$. Up to this concentration, there is also an increase in root colonization intensity but not in the promotive effects. These observations suggest that *Phyllobacterium* sp. express phytoregulators that induce a dose-dependent effect on the plant. It is possible that *Phyllobacterium* sp. stimulates *B. napus* growth through IAA production as this ability was found in pure culture (unpubl. data). This hormone is well known to have a dose effect on plant development (Patten & Glick, 1996). However, PGPB–plant interactions are now considered as a multifactorial process, and others factors not yet identified may therefore be involved in *B. napus* growth promotion by *Phyllobacterium* sp.

The elucidation of bacterial mechanisms responsible for plant growth promotion remains the major problem encountered in understanding PGPB. Identifying PGPB effects on plants may therefore bring new insights. Our investigation allowed us to identify a set of root parameters modified by the bacteria, the moment when the effect became significant and the most promotive bacterial doses. Data acquisition was possible since we used an accurate experimental design (i.e. a kinetic bioassay) that was proved to be microbiologically controlled, reliable and in which the interaction between both partners was facilitated. Such an approach could be helpful in the search for bacterial genes involved in the plant–PGPB interaction through comparison of wild bacterium type and mutant effects. Further investigations might be conducted to identify plant genes involved in the plant–PGPB interaction through comparison of plant mutants and wild-type responses.

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