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Letter to Editor

The Motility of Bacteria from Rhizosphere and Different Zones of Winter Wheat Roots

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Abstract

More than 800 rhizobacterial strains were isolated from winter wheat "rhizosphere" (the soil tightly adhering to the roots), "rhizoplane" (the root surface) and "endorhiza" (the interior of the roots) at different plant growth stages (two leaves, four leaves, flowering and full maturity). The data obtained clearly show that the proportion of motile strains gradually increased from "rhizosphere", through "rhizoplane", to "endorhiza". These results strongly suggest that flagellar motility is an important factor in the colonization of plant roots (especially the root interiors) by bacteria. However, high proportions of nonmotile bacteria among the bacterial isolates from the root surface at four leaves and flowering stages suggest that flagellar motility is not an absolutely necessary bacterial feature in colonization of plant roots. Pseudomonads and enterobacteria were the main motile bacteria, and *Cytophaga-Flavobacterium* the main nonmotile ones. The role of flagellar motility in plant root colonization is discussed in relation to other bacterial traits.

Keywords: bacterial motility, bacteria location, winter wheat, rhizosphere, rhizoplane, endorhiza

Introduction

Colonization of the root system is required for the beneficial effects of inoculant bacteria for applications such as biocontrol of soil-borne diseases, biofertilization and phytostimulation [1]. Flagellar motility may be an important trait for successful plant root colonization by bacteria. However, contradictory results on the significance of this feature are found in the literature [1, 2]. Howie *et al.* [3] with wheat, and Bowers and Parke [4] with peas, questioned the importance of bacterial motility in colonization of plant roots, because motile wild strains of *Pseudomonas fluorescens* and their non-motile mutants colonized the plant roots to the same degree. Also Scher *et al.* [5] found that root colonization rate and bacterial distribution on soybean roots by a motile wild strain and a non-flagellated mutant of *P. putida* did not differ significantly. Boelens *et al.* [6] obtained similar results. They found that motility of *P. fluorescens* appeared not to be an important property of the bacteria in their colonization of maize roots. The results of Germida *et al.* [7], who found that non-motile bacteria (mainly *Micrococcus* and *Flavobacterium)* constituted a large part of bacterial isolates from canola roots, suggest that the role of bacterial motility in root colonization by microorganisms is not great.

On the other hand, Bashan and Holguin [8] reported that motility of *Azospirillum brasilense* in the rhizosphere of wheat and soybean was essential for colonization of the root system. Although the nonmotile mutants proliferated similarly to their wild parents, they failed to colonize neighbouring roots. Similarly, the results of De Weger *et al.* [9], Sakai *et al.* [10] and Toyota and Ikeda [11] strongly suggest that motility of *P. fluorescens* and *P. putida* played an important role in the bacteria movement both towards and along roots of potato, spinach, tomato and melon. Also Catlow *et al.* [12] reported that flagellar motility appears to

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affect the distribution of *Rhizobium trifolii* on clover roots and Maplestone and Campbell [13] found that a non-motile strain of *Bacillus mycoides* spread down the wheat root system more slowly than a motile strain of *B. pumilus*.

We decided to evaluate the importance of the flagellar motility of rhizobacteria in colonization of the plant roots indirectly, by determining the proportions of motile strains in groups of bacterial isolates from winter wheat rhizosphere and from the surface and interior of winter wheat roots.

Experimental Procedures

Soils, Plants and Sampling Procedure

In Experiment 1, conducted with winter wheat (*Triticum aestivum* L.) cv. Gama on loess soil (pH(KCl) – 6.8; organic C content – 0.9%), the samples of the plant roots with adjacent soil were taken at four stages (two leaves – October 12, four leaves – April 25, flowering – June 5 and full maturity – July 25) of plant growth. In Experiment 2, conducted with winter wheat cv. Almari on alluvial soil (pH(KCl) – 6.2; organic C content – 1.6%), the samples of the plant roots with adjacent soil were taken only at the two-leaves stage (October 25) of plant growth. The winter wheat (in both cases sown in the second week of November) was grown in a field at Antopol and Kępa (both places are located near Puławy in the Lublin District in Poland) with loess and alluvial soil, respectively.

Preparations of Plant Roots with Adhering Soil for Isolation of Bacteria

Immediately after transporting the samples to the laboratory, bacteria were isolated (according to the methods described by Kobus *et al.* [14] – fractions 1, 2 and 4) from three zones: (1) "rhizosphere" – the soil tightly adhering to roots after shaking-off the excess of the soil, (2) "rhizoplane" – the root surface after scraping off the external layer of roots by shaking the soilless roots (roots previously washed of soil with tap water and then rinsed 10 times with sterile water by hand shaking) for 30 min in a suspension of coarse sand, and (3) "endorhiza" (some researchers have used the term "endorhizosphere) – the root interiors after homogenization of the roots, remaining after fraction 2, previously disinfected with 70% ethanol for 15 min and with 3% H_2O_2 for 15 min and rinsed 3 times with sterile water. The termes "rhizosphere," "rhizoplane" and "endorhiza" were adopted from Kloepper *et al.* [15].

Isolation of Bacteria

For the isolation of bacteria two different agar media were used: in Experiment 1 – King, Ward and Raney's medium "B" [16], and in Experiment 2 – the medium consisting of: Malt Agar (Difco) – 4.5 g; Tryptic-Soy Agar (Oxoid) – 4 g; Potato-Dextrose Agar (Difco) – 4 g; Corn Meal Agar (Difco) – 2 g; Antybiotic Medium nr 2 (Oxoid) – 2 g; Yeast Extract (Difco) – 0.5 g; aqueous extract of the alluvial soil (1:1 ww.) – 100 cm^3; K_2HO_4 – 1 g; KNO_3 – 0.5 g; $MgSO_4 \cdot 7\ H_2O$ – 0.4 g; $CaCl_2$ – 0.1 g; NaCl – 0.1 g; $FeCl_3$ – 10 mg; $ZnSO_4$ – 1 mg; thiamine – 100 µg; biotin – 5 µg; inositol – 5 µg; agar 7 g; H_2O dist. – 900 cm^3.

The rhizobacteria were isolated after 5 days of incubation at 27°C from all colonies growing on plates with the highest dilution of rhizosphere soil or root debris. The isolates were purified and then they were stored on agar slants with the same medium as was used for isolation of the bacteria.

Identification of the Isolated Bacteria

The isolated bacterial strains were identified on the basis of their phenotypic features according to Bergey's Manual of Systematic Bacteriology [17]. The methods – cell morphology; colony pigment production; Gram's staining; staining of bacterial flagella (Leifson method); high temperature resistance of spore formers; mode of utilization of glucose, lactose, arabinose and glycerol (Hugh and Leifson method); cytochrom oxidase production (Kovacs' method); catalase activity (3% H_2O_2); arginine dihydrolase test (Thornley's medium); fluorescent diffusible pigment production (King, Ward and Raney "B" medium); laevan formation from sucrose; growth on TTC, trehalose, adonitol and sorbitol; growth and alkali production on acetate and citrate (Simmon's agar); nitrate reduction (Griess-Ilosvay's reagent and Durham tubes); gelatin (Frazier's method), starch and cellulose hydrolysis (in tubes with colloidal suspensions of the polysaccharides); pectin degradation (on potato cubes); urease production (Christensen's agar); indole production (Kovacs' reagent); methyl red and Voges-proskauer tests (Barritt's modification); gliding motility (Hayes's medium) – for identification of bacteria are described in Laboratory Methods in Microbiology [16].

Bacterial Motility Test

The motility of the bacterial isolates was examined by "hanging drop" preparations of 24 hours old broth cultures [16].

Statistical Evaluations

For statistical evaluation of significant differences between the bacterial populations from different sites of bacterial location, confidence intervals of the motile strains proportions in groups of the isolated strains were calculated according to the equation [18]:

$$\frac{2Y + u_\alpha 2 - K}{2\left(n - u_\alpha{}^2\right)} \le p \le \frac{2Y + u_\alpha 2 + K}{2\left(n - u_\alpha{}^2\right)}$$

where: p is a confidence interval; $K = u_\alpha \sqrt{x}$ and $x = u_\alpha{}^2 + 4Y(1 - Y/n)$; Y is the number of motile strains; n is the number of all isolated strains; u_α is the Student's t value obtained from tables for an infinite number of degrees of freedom (1.645 for 90% confidence intervals, and 2.576 for 99% confidence intervals). The confidence limits are presented in Figs. 1 and 2 as percentages ($p \cdot 100$).

Results and Discussion

It is well known that the real rhizobacteria are copiotrophs in contrast to oligotrophic soil bacteria [19], so rich nutrient media were specially chosen in the present studies for isolation of rhizobacteria from winter wheat rhizosphere and roots. The well known (to detect bacterial fluorescent pigments) King, Ward and Raney's medium "B" [16], containing peptone, was used in Experiment 1, because a considerably larger proportion of rhizobacteria than of the soil bacteria required aminoacids [20, 21]. The other medium (used in Experiment 2) was a mixture of various media, containing many different nutrients. This medium was prepared to enable rhizobacteria with different nutrient requirements to grow.

The data from Fig. 1A clearly show that in the first three examined growth stages of winter wheat, the proportion of the motile strains gradually increased from "rhizosphere" (20-60%), through "rhizoplane" (43-76%), to "endorhiza" (65-87%). Only at the maturity stage, when the wheat roots were dead and the plant resistance forces against soil microorganisms disappeared did the differences between the zones become less clear. At that stage Gram positive bacteria constituted a majority (52-59%) of the bacterial isolates, contrary to the other plant growth stages (8-40%) (Fig. 1A and 1B). The data of Experiment 2 presented in Fig. 2 confirm the results obtained from Experiment 1. In this case, the proportion of motile strains in bacterial isolates increased from "rhizosphere" through "rhizoplane" to "endorhiza" to an even greater degree (a factor of 5.5 times). Our findings are in agreement with those of Pham Quang Hung and Annapurna [22] and Sato and Jiang [23], who found that more than 80% of endophytic bacteria in soybean tissues and more than 70% of isolates of Gram (-) bacteria from root surface of wheat were motile.

Pseudomonads have been found to be among the best bacterial root colonizers [23, 24, 25]. Also, pseudomonads were the main (22-98%) motile colonizers of wheat roots, particularly of endorhiza (except at the full maturity stage), in both experiments (Fig. 1A and Fig. 2). The majority of the isolates of this group (87% in Experiment 1 and 100% in Experiment 2) produced fluorescent pigments. All fluorescent pseudomonads in Experiment 2 and 64% in Experiment 1 were identified as *Pseudomonas fluorescens* (results not presented). Besides pseudomonads, numerous very motile bacteria belonging to the family *Enterobacteriaceae* were found on and (especially) in wheat roots at the two-leaves stage in both experiments (Fig. 1B and 2B). The isolates of this bacterial group were identified as *Erwinia carotovora* (70% of isolates in Experiment 1 and 100% in Experiment 2) and *E. herbicola* (*Pantoea agglomerans*) (results not presented). Furthermore, in Experiment 1 other motile bacteria (especially in the two last plant growth stages) were identified as belonging to the Gram-negative genera *Alcaligenes* and *Xanthomonas* as well as to Gram-positive *Bacillus* and coryneforms (Fig. 1A). In Experiment 2 Gram-positive coryneform bacteria and Gram-negative bacteria related to genera *Janthinobacterium* and *Chromobacterium* were found (Fig. 2). All wheat roots looked healthy and only 52% of *Erwinia* isolates showed an ability to degrade pectin. It is possible that they could be classified with other methods as members of the genus *Enterobacter*. These results are consistent with results of other studies. Bacteria belonging to genera *Pseudomonas*, *Erwinia* (and other enterobacteria), *Bacillus*, *Xanthomonas* and coryneforms were found on roots of different plants [15, 26].

The results presented in Figs. 1A and 2 strongly suggest that flagellar motility is an important feature in colonization of the plant roots by bacteria. But it should be emphasized that the ability of bacteria to colonize plant roots consists of many traits other than bacterial motility, *e.g.* chemotactic response toward root exudates, features increasing bacterial adherence to the root surface, ability to use many compounds from the plant root exudates, and various abilities to survive in the presence of competition of other rhizosphere microorganisms [1, 2, 27-31]. The bacterial growth rate can also be an important trait in the colonization of plant roots [28, 29]. De Leij *et al.* [32] reported that the bacterial population on wheat roots at the flowering stage was dominated by fast-growing bacteria on 0.1 strength TSA, in contrast to soil bacteria, but at the ripening stage, no differences in the growth rate between root and soil bacterial populations were observed. Similarly, in the present studies bacteria belonging to genera *Pseudomonas* and *Erwinia*, which were numerous on or in the younger wheat roots, were observed as faster growers in comparison to coryneforms, numerous at the maturity stage of wheat growth.

Presumably, it is not a coincidence that more motile bacteria are present in the vicinity of roots. It should be emphasized that the role of motility of bacteria in their colonization of plant roots is closely related to other bacterial traits important for colonization. Dekkers *et al.* [33] reported that the function of several colonization genes can be explained in relation to motility. For chemotaxis towards root exudates bacteria need to be motile. Several bacteria mutations (*e.g.* in genes encoding synthesis of the O-antigen of LPS, a site-specific recombinase and NADH dehydrogenases) that result in slower growth rate and decreased ability to colonize

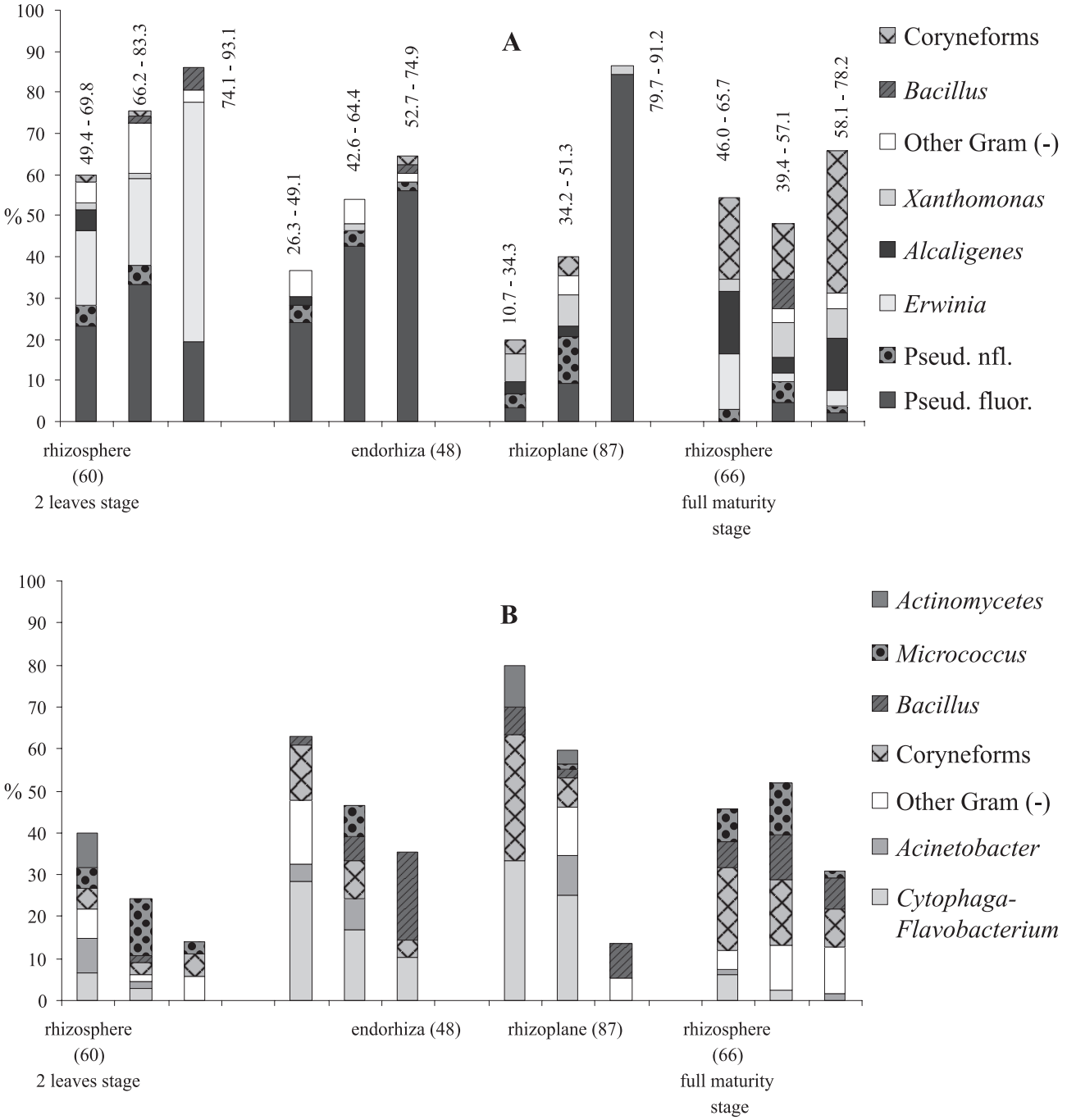

Fig. 1. Proportions and qualitative composition of the motile (A) and nonmotile (B) bacterial isolates from winter wheat rhizosphere, rhizoplane and endorhiza at different plant growth stages in Experiment 1. (The data in the brackets are the numbers of total isolates). The values above the bars in Fig. 1A represent 90% confidence intervals of the proportions of the motile strains. The proportions in the bars with the confidence intervals which do not overlap each other are significantly different at P = 0.1. "Pseud. nfl." means "nonfluorescent pseudomonads," and "Pseud. fluor." means "fluorescent pseudomonads."

rhizosphere are associated with a decrease in bacterial motility [33]. Also, in the opinion of Gottlieb [29], the high growth rate of rhizobacteria is probably correlated with the high rate of their translocation to new root surfaces. Moreover, the results of Turnbull *et al.* [34] showed that the motile *Pseudomonas putida* strain showed significantly greater attachment to wheat roots than the non-motile strains.

Flagellar motility presumably plays an important role in bacterial competition for nutrients through chemotaxis [1, 27, 28]. Capdevila *et al.* [24] found that bacterial mutants, both non-motile and with reduced motility properties, were completely displaced from the root tip by the motile wild-type pseudomonad strain, indicating that the motility was necessary for competitive root colonization. Similarly, tomato root colonization by the wild type of

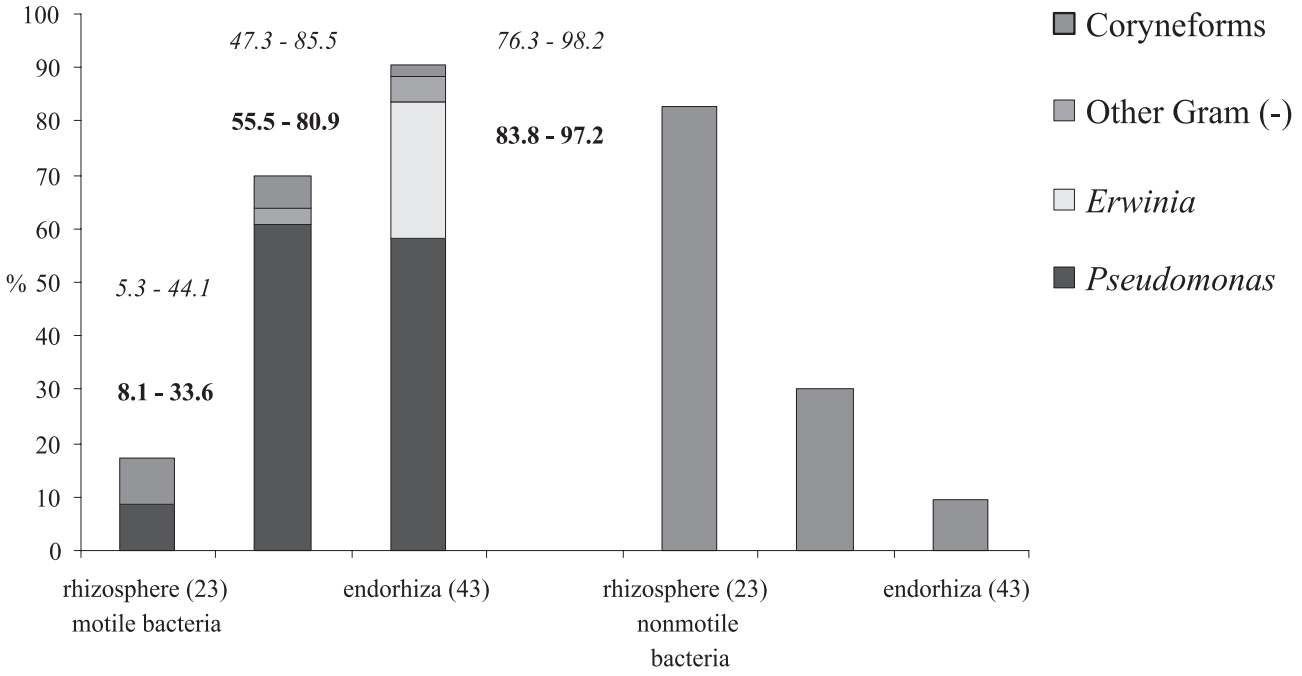

Fig. 2. Proportions and the qualitative composition of the motile (left side) and nonmotile (right side) bacterial isolates from winter wheat rhizosphere, rhizoplane and endorhiza at 2-leaves stage in Experiment 2. (The data in the brackets are the numbers of total isolates). The values above the bars of the motile isolates proportion (left side) represent 90% (the lower rows in bold face) and 99% (*the upper rows in italics*) confidence intervals. The proportions in the bars with the confidence intervals which do not overlap each other are significantly different at P = 0.1 (in bold face) or P = 0.01 (in italics).

Pseudomonas chlororaphis was at least 1000 times greater than that by the non-motile mutants in a competitive situation [35].

From the results presented in Figs. 1A and 2, the flagellar motility of rhizobacteria may be important in their internal root colonization. But it is only one of many various bacterial traits that help rhizobacteria in entering the root interior. For example, polysaccharide-degrading enzymes (cellulase, pectinase) are involved in the active entrance of bacteria into plant roots [36], and mutants lacking the O-antigen of LPS can be impaired in entering roots due to the higher hydrophobic character of their cell surfaces [29]. Van Peer *et al.* [37] reported that (motile) fluorescent *Pseudomonas* isolates obtained from endorhizosphere were distinct from (motile) fluorescent *Pseudomonas* isolates obtained from the tomato root surface. Isolates from the endorhizosphere especially were able to recolonize the endorhizosphere. This ability of the endorhizosphere strains was significantly correlated with their agglutination by tomato root agglutinin [37].

In general, bacteria invade roots through natural openings and wounds or through root hairs, and at epidermal cell junctions [26, 30]. The importance of areas of emerging lateral roots where the epidermis had been broken recently by expansion of the underlying cell layers is underlined by Cooley *et al.* [38], Quadt-Hallmann *et al.* [36] and Shishido *et al.* [39]. Possibly more nutrients are available in these places, so nonmotility would appear to be a disadvantageous property for bacteria colonizing the root interior, because it would impair their chemotactic response. Nonmotile mutants of some bacterial plant pathogens, belonging to genera *Pseudomonas*, *Erwinia*, *Ralstonia* and *Agrobacterium*, show decreased virulence, primarily due to their inability to enter plants [38]. Mutants of *Salmonella enterica* that were defective in flagellin synthesis or motility function, grew as quickly as the wild-type strain on the surface of *Arabidopsis thaliana* roots, but a microscopic investigation did not reveal any invasion at lateral root junctions when its inoculum was 10^4 CFU/ml [38].

Results presented in Fig. 1B also indicate that flagellar motility is not absolutely necessary for bacterial colonization of plant roots, because non-motile bacteria constituted a large part (and even a majority at the flowering and maturity stages in Experiment 1) of the bacterial isolates from the root surface. Relatively fast-growing bacteria belonging to the *Flavobacterium-Cytophaga* group were the most numerous (about 35-45%) among the non-motile isolates at four leaves and flowering stages (Fig. 1B). Also, *Cytophaga*-like bacteria constituted a dominant part of the bacterial communities in barley rhizosphere [40, 41], and *Flavobacterium* constituted a large part of bacterial isolates from canola and wheat roots [7, 23]. Moreover, nonmotile bacteria belonging to coryneforms and actinomycetes and to the genera *Bacillus* and *Micrococcus* were found on and in the wheat roots (Figs. 1B and 2). All of these groups of Gram-positive bacteria are known to be plant root endophytes [7, 15].

It should be emphasized that *Cytophaga-Flavobacterium* and other nonmotile bacteria were numerous mainly on older roots (Figs. 1A and 1B). These data are consistent with those obtained by Nihuis *et al.* [42]. They found that *Pseudomonas* colonized the rhizoplane of both young and old grass roots growing in natural soil, but *Flavobacterium* colonized mainly the rhizoplane of the old roots. Maybe lack of flagellar motility was one of the reasons for the impairment of the colonization of young roots by nonmotile bacteria, because, as was written previously, motility is very helpful for competitive root colonization.

The results of the present studies, based on the rhizobacteria isolation on the rich media and on classification of the isolates on the grounds only of their phenotypic (but not genetic), morphological and biochemical features, can be criticized. But it seems that the obtained image of population diversity of rhizobacteria colonizing the rhizosphere as well as the surface and interior of the wheat roots is real, because results of Marilley and Aragno [43], who studied amplified and cloned 16S rDNA obtained from bulk soil, the soil adhering to the roots and the washed roots (rhizoplane and endorhizosphere) of *Lolium perenne* and *Trifolium repens*, gave very similar patterns of bacterial distribution. The plant roots had a selective effect towards *γ-Proteobacteria* (mainly *Pseudomonas* and enterobacteria), to the detriment of the Gram-positive bacteria, leading to a dominance of *Pseudomonas* [43].

Modes of bacterial motility other than flagellar, *e.g.* swarming [44] and gliding [40] motilities (70% of the *Cytophaga-Flavobacterium* isolates in the present study exhibited the gliding motility), as well as passive movement on the root surface or interaction with other biota, are likely to be important additional mechanisms of bacterial movement in soil under field conditions [45]. For example, Mawdsley and Burns [46] have reported that a non-motile strain of *Flavobacterium* was able to migrate with the expanding root in the absence of downward water flow. But it should be mentioned that percolating water due to rainfall or irrigation can be a major transporting agent of both motile and non-motile bacteria in soil [45, 47-50].

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