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Role of Chemotaxis Genes in Wheat Root Colonization by Azospirillum brasilense

Mariam Wasim

ROLE OF CHEMOTAXIS GENES IN WHEAT ROOT COLONIZATION BY *AZOSPIRILLUM BRASILENSE*

by

MARIAM WASIM

Under the Direction of Gladys Alexandre-Jouline

ABSTRACT

Previous studies have shown that chemotaxis plays an important role in the colonization of the wheat roots surfaces by *Azospirillum brasilense* and a chemotaxis operon shown to control motility and chemotaxis in *A. brasilense* has been isolated. This study looked at the effects of mutations in individual genes coding for chemotaxis proteins from this operon on the ability of the cells to colonize the surface of sterile wheat roots. Using both quantitative and qualitative assays, the study shows differences in the colonization ability of the mutants relative to the wild type: the *cheB, cheR, cheBR*, and *cheOp* mutants were significantly impaired in wheat root colonization. Interestingly, the *cheA* mutant was not affected in its ability to colonize the wheat root surface relative to the wild type. Future studies will look for the factors that compensate for *cheA* impairment in the rhizosphere.

INDEX WORDS: *Azospirillum brasilense,* Chemotaxis, Wheat root colonization, c*heA, cheB, cheR, cheBR, cheOp,* Sp7.

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MARIAM WASIM

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MARIAM WASIM

Honors Thesis Director: Gladys Alexandre-Jouline
Honors Program Director: Timothy M. Renick

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Introduction

Azospirillum brasilense is a free-living soil bacterium, which colonizes the roots of many economically important crops including wheat, rice and corn. It is a member of the alpha subdivision of proteobacteria and it may enhance plant growth (Greer-Phillips *et al*., 2004). *A. brasilense* is an aerophilic bacterium, which has an oxidative type of metabolism and can use nitrate as an alterative electron acceptor (Alexandre *et al.*, 2000). *Azospirillum* spp. are diazotrophs that fix nitrogen as free-living organisms (Ramos *et al*., 2002). The bacterium obtains the nutrients necessary to replicate and survive from the plant and the plant benefits from the association with the bacterium because the bacteria produces hormones that increase the volume of the roots bulk thereby leading to improved plant nutrition. The beneficial effects of *Azospirillum* on plant growth have been applied in agricultural practices and inoculants containing *A. brasilense* have been used as biofertilizer for cereals all over the world.

The beneficial effects on the plant can only be seen once the bacteria have established significant population levels on the root surface. It is necessary for the bacteria to come in close contact with the root surface in order to establish colonies and proliferate on the plant surface. Chemotaxis may play an important role in bringing the bacteria in close proximity of the roots by sensing the chemical compounds released by the roots (Greer-Phillips *et al*., 2004). The mechanism for this chemoattraction has not been elucidated but there are a few plausible hypotheses. According to Katupitiya *et al*, when *A. brasilense* is inoculated to plant roots, the bacteria adsorb to the roots and proliferate on the surface (Katupitiya *et al*., 1994). Since most rhizobacteria possess

motility, either random or chemotactic, it can be assumed that it is likely to play a major role in the initial stages of interaction between the roots and the bacteria (Vanderbroek *et al*., 1995). Root exudates contain many substances with chemoattractant properties including simple sugars, organic acids, and amino acids (Alexandre *et al*., 2000). Once the bacteria are in proximity of the roots, they will detect the chemical compounds and attach to the root surface. A two-step attachment mechanism has been proposed for *A.Brasilense* through *in vitro* assays. In the first step, called the adsorption step, the bacteria weakly and quickly binds to the plant root surface. This step has been found to be mediated by the polar flagellum of the bacteria. The second step, or the anchoring step, occurs only in a medium with rich carbon and nitrogen content and is mediated via bacterial calcofluor-binding polysaccharides (Vanderbroek *et al*., 1998).

A. brasilense grows optimally in medium with sugars and organic acids such as fructose and malate, respectively (Alexandre, 2000). Consequently, further proliferation of the bacterium on the wheat root surface depends on the nitrogen and carbon content of the root exudates. Only the compounds that support bacterial growth serve as chemoattractants such as sugars and amino acids (Alexandre *et al*., 2000).

Since the active movement of *A. brasilense* is important for the primary colonization of wheat roots at the root hair zones, it can be hypothesized that chemotaxis play an important role in these interactions (Vanderbroek *et al*., 1998). According to Hauwaerts *et al*, “the successful colonization of the plant root surface by the bacterium is thought to be dependent on active motility and chemotaxis toward root exudates” (Hauwaerts *et al*., 2002).

Chemotaxis is the movement of cells along a chemical concentration gradient either towards or away from the chemical stimulus. Organisms use this mechanism to move toward favorable environments and to move away from unfavorable environments. It is essential for the bacteria to possess this ability to survive in its microenvironment. Bacteria use chemotaxis to move toward nutrients and away from toxic environments.

The chemotaxis mutants that were used in this study have been constructed and characterized in the Alexandre lab and there is no published data characterizing these mutants. The mutants were generated in the chemotaxis operon consisting of genes coding for the central excitation and adaptation pathway. Mutations in this operon have been shown to abolish taxis to all stimuli and also affect the pattern of motility (Hauwaerts *et al*., 2002).

According to Hauwaerts *et al.* (2002) the environmental information perceived by chemoreceptors is transduced to the CheA protein, which modulates the phosphorylation of CheY. When phosphorylated, the CheY protein binds to the flagellar motor and causes reversal of the default direction of the flagellar rotation. Covalent modification of chemoreceptors is carried out by the CheR methyltransferase and the CheB methylesterase, which is phosphorylated by CheA (Hauwaerts *et al.*, 2002). Figure 1 below shows the schematic of this chemotaxis operon in *A. brasilense*.

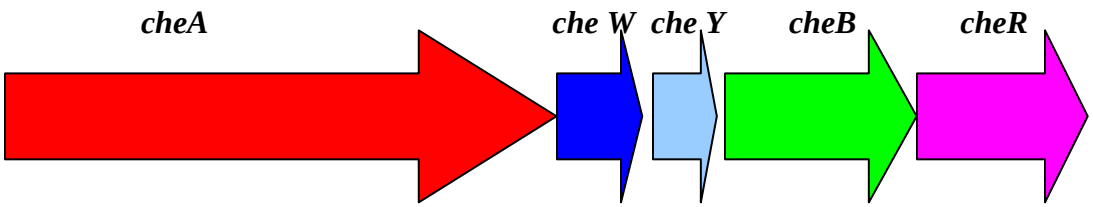

Figure 1. Chemotaxis Operon in *Azospirillum Brasilense*.

The mutant strains have been constructed by deleting certain genes from the chemotaxis operon. These genes are required to direct motility in *A. brasilense*. The mutants used for this study had been previously constructed by other members of the Alexandre lab. The *cheA*, *cheB*, and *cheR* mutants were constructed through knockout mutations, or mutations which essentially abolish the function of the entire gene. The *cheBR* was constructed by a knockout of both *cheB* and *cheR* genes. A polar mutation that affects all the genes in the operons in addition to *cheA* was made in *cheA* to construct the CheOp mutant. This mutation knocks out the entire operon.

The purpose of this study was to provide experimental evidence for the role of chemotaxis in the plant-root interaction. This was accomplished by determining the role of individual chemotaxis genes in wheat root colonization. The effects of mutating chemotaxis genes were directly studied through quantitative and qualitative assays. The study attempts to prove that the function of the entire chemotaxis operon is necessary for establishment of *A. brasilense* in the rhizosphere of wheat plants.

Methods

A. Bacterial strains and growth conditions

Six strains of *A.Brasilense* were used in this study. These strains include Sp7 (wildtype for chemotaxis), the *cheA*, *cheB*, *cheR*, *cheBR*, and *cheOp* mutants. All of the strains were grown in minimal media MMAB. The growth medium was supplemented with kanamycin (30μg/ml) for the CheOp mutant. The cells were incubated at 200 rpm and 28^{o}C in a rotary shaker overnight.

For mating, the donor *E. coli* Dh5-α (pJBA21Tc) was grown in LB with tetracycline (50μg/ml). The helper, *E. coli* HB101 (pRK2013) was grown in LB with kanamycin (25μg/ml). The recipient strains, Sp7, the *cheA*, *cheB*, *cheR*, *cheBR*, and *cheOp* mutants were inoculated as mentioned above. After mating, all strains were grown in minimal media with tetracycline (50μg/ml) and kanamycin (30μg/ml) for *cheOp* for selection of transconjugants carrying the pJBA21Tc plasmid. The cells were incubated at 200 rpm and 28^{o}C in a rotary shaker overnight.

B. Seeds sterilization

Wheat seeds (*Triticum aestivum* cv. Jagger) were used. Seeds were sterilized following the protocol outlined by Ramos *et al.* (2002). Seeds are immersed in a solution of 0.1% streptomycin and 0.002% tween and incubated in a rotary shaker at 100 rpm for 20 minutes. Then, 2 X 10 minute washes were done in sterile dH_2O. Seeds were then immersed in a solution of 0.1% $HgCl_2$ and incubated on a rotary shaker for 20 minutes. 8 X 10 minute washes were done in sterile dH_2O on rotary shaker. The seeds were then

aseptically transferred to 0.8% nutrient agar plates and allowed to germinate in the dark (aluminum foil) at 28^{o}C for 3 days (Ramos *et al*., 2002).

C. Seeds inoculation for quantitative assay

The cell cultures were grown overnight and washed twice in PBS (Phosphate-Buffered Saline). All strains were adjusted to an optical density (OD_{600}) of 0.44 and inoculated into 15 ml molten Fahraeus semi-soft (0.4% agar) medium. The medium was as described by Zamudia and Bastarrachea (1994) and modified with traces of sodium molybdate and contains 0.1 g of $CaCl_2.H_2O$, 0.12 g of $MgSO_4.7H_2O$, 0.1 g of KH_2PO_4, 0.15 g of Na_2HPO_4, and 0.005 g of ferric citrate per liter of dH_2O. After the medium had solidified, one germinated seed per tube was placed aseptically and 20 seeds were inoculated per strain. The seeds were placed in a plant chamber with 12 h light cycle and temperature maintained at 28^{o}C.

Colonization of the wheat root was measured at 6H, 24-28H and 48-50H. The plants were removed from the chamber and their roots were weighed in sterile plates. Five plants were used for every time point. The roots of the plant were cut and crushed in 1 ml of PBS. To measure the number of cells colonizing each root, serial dilutions of the root and buffer mixture were plated on MMAB and incubated for 2 days at 28^{o}C.

D. Triparental bacterial matings

To determine the pattern of colonization on wheat root surface, the pJBA21Tc plasmid was introduced in all strains through conjugation. This is a stable plasmid, which constitutively expresses the *gusA* gene encoding for the β-glucuronidase activity that can be effectively used to monitor wheat root interactions in *Azospirillum* because in presence

of a specific substrate the β-glucuronidase activity produces a blue precipitate (Ramos *et al*., 2002). The cell cultures of the donor, pJBA21Tc, helper, pRK2013, and recipients were inoculated as described above. 1.5 ml of donor, 2 ml of helper and 3 ml of recipient cultures were centrifuged at 3500 rpm for 10 minutes in sterile eppendorf tubes. The pellet was resuspended in 1 ml of 10 mM $MgSO_4$ and centrifuged at 3500 rpm for 5 minutes. This was repeated once with 1 ml and 500µl of $MgSO_4$. The final pellet was resuspended in 100µl of $MgSO_4$. 25µl of donor, helper and each recipient were mixed in sterile eppendorfs and dropped on D-medium plates. The plates were incubated at 28^{o}C for 16-20 hours. The drops were picked up with sterile inoculating loops and resuspended in 300µl of $MgSO_4$. 150µl of each strain with new plasmid were spread on MMAB (+C/-N) plates with tetracycline (50µg/ml). Kanamycin (25µg/ml) was added for the *cheOp* mutant. The plates were incubated at 28^{o}C for 2 days.

E. Seeds inoculation for qualitative assay

The protocol detailed in section c was followed for the qualitative assay as well. 400µl of each strain was inoculated to molten Fahraeus medium. The inoculum was plated on MMABTet[10] plates.

F. Root observation

To observe the bacterial colonization on the wheat roots, the plants were stained with X-Gluc (50µg/ml), substrate for *gusA.* Staining with X-Gluc will color the roots blue wherever the pJBA21Tc strains colonize. The plants were removed from the plant chamber after 24 hours of inoculation and rinsed with 50 mM Phosphate buffer (Ramos et al 2002). X-Gluc was added to phosphate buffer in beakers and the seeds were

immersed in this solution for 20 minutes in a vacuum. The beakers were then covered with parafilm and incubated at 37^{o}C for 12-16 hours. The root systems for 3 plants per strain were observed with the eye and brightfield microscopy.

Results

1. Quantitative assays

For each strain, approximately 10^6 cells were inoculated to the wheat seedlings. The number of cells inoculated for each strain is shown in Table 1. The results from the colonization counts at 6H, 24-28H, and 48-50H for five plants per strain are shown in Table 2. The number of colonies counted (CFU/ml) was divided by the weight of the root to measure the number of cells colonizing each root. Table 3 shows the average number of colonies per gram of root for the three time points. Figure 2 is a graphical representation of the data in Table 3.

Table 1. Number of Cells inoculated for each strain

Strain	(CFU/ml)
Sp7	6.67×10^6
CheA	3.33×10^6
CheB	1.10×10^6
CheR	5.67×10^5
CheBR	3.86×10^6
CheOp	5.39×10^6

Table 2. Number of colonies counted per gram of root for each strain

	6 H (cells/gm)	**24-28 H** (cells/gm)	**48-50 H** (cells/gm)
Sp7 – 1	3.62×10^5	3.23×10^6	3.87×10^6
Sp7 – 2	3.09×10^5	8.08×10^6	1.55×10^7
Sp7 – 3	1.09×10^5	5.68×10^6	2.25×10^6
Sp7 – 4	1.40×10^7	8.63×10^6	1.37×10^6
Sp7 – 5	2.72×10^7	3.46×10^6	7.41×10^6
Average	8.39×10^6	5.82×10^6	6.07×10^6
ST Dev*	1.21×10^7	2.52×10^6	5.73×10^6

CheA – 1	6.83×10^5	6.00×10^6	7.01×10^5
CheA – 2	6.78×10^5	6.45×10^6	9.38×10^6
CheA – 3	9.50×10^5	1.30×10^7	9.73×10^5
CheA – 4	6.30×10^5	2.85×10^7	2.74×10^6
CheA – 5	5.46×10^6	1.22×10^7	4.26×10^6
Average	1.68×10^6	1.32×10^7	3.61×10^6
ST Dev	2.11×10^6	9.10×10^6	2.88×10^6
CheB-1	2.72×10^3	3.80×10^4	1.98×10^4
CheB-2	2.17×10^3	1.18×10^4	1.99×10^4
CheB-3	1.43×10^3	4.12×10^4	1.08×10^4
CheB-4	NO Data	3.81×10^4	4.16×10^3
CheB-5	4.48×10^3	3.17×10^4	2.25×10^4
Average	2.70×10^3	3.22×10^4	1.54×10^4
ST Dev	1.30×10^3	1.19×10^4	7.71×10^3
CheR-1	6.69×10^3	5.01×10^4	4.39×10^4
CheR-2	1.27×10^3	2.37×10^4	1.77×10^4
CheR-3	2.06×10^3	7.12×10^4	1.00×10^4
CheR-4	2.24×10^3	3.28×10^4	3.24×10^4
CheR-5	3.62×10^3	4.73×10^4	1.61×10^4
Average	3.18×10^3	4.50×10^4	2.40×10^4
ST Dev	2.14×10^3	1.82×10^4	1.38×10^4
CheBR-1	1.62×10^3	4.72×10^4	4.03×10^3
CheBR-2	8.42×10^3	5.83×10^4	3.60×10^3
CheBR-3	8.55×10^3	1.24×10^5	2.54×10^4
CheBR-4	1.95×10^4	3.64×10^3	7.14×10^3
CheBR-5	3.38×10^3	2.66×10^4	1.99×10^4
Average	8.29×10^3	5.19×10^4	1.20×10^4
ST Dev	6.97×10^3	4.54×10^4	1.00×10^4
CheOp-1	3.39×10^4	1.20×10^5	4.44×10^5
CheOp-2	8.62×10^4	4.54×10^5	2.01×10^5
CheOp-3	2.06×10^4	3.00×10^5	1.71×10^5
CheOp-4	8.36×10^4	1.64×10^5	6.45×10^5
CheOp-5	4.14×10^4	2.22×10^5	2.13×10^4
Average	5.31×10^4	2.52×10^5	2.96×10^5
ST Dev	2.99×10^4	1.32×10^5	2.47×10^5

*ST Dev = Standard Deviation calculated from the average.

Table 3. Average Number of Cells per gram at 6H, 24-28H, 48-50H

	6 H (cells/gram)	**24-28 H (cells/gram)**	**48-50 H (cells/gram)**
Sp7	8.39×10^6	5.82×10^6	6.07×10^6
CheA	1.68×10^6	1.32×10^7	3.61×10^6
CheB	2.70×10^3	3.22×10^4	1.54×10^4
CheR	3.18×10^3	4.50×10^4	2.40×10^4
CheBR	8.29×10^3	5.19×10^4	1.20×10^4
CheOp	5.31×10^4	2.52×10^5	2.96×10^5

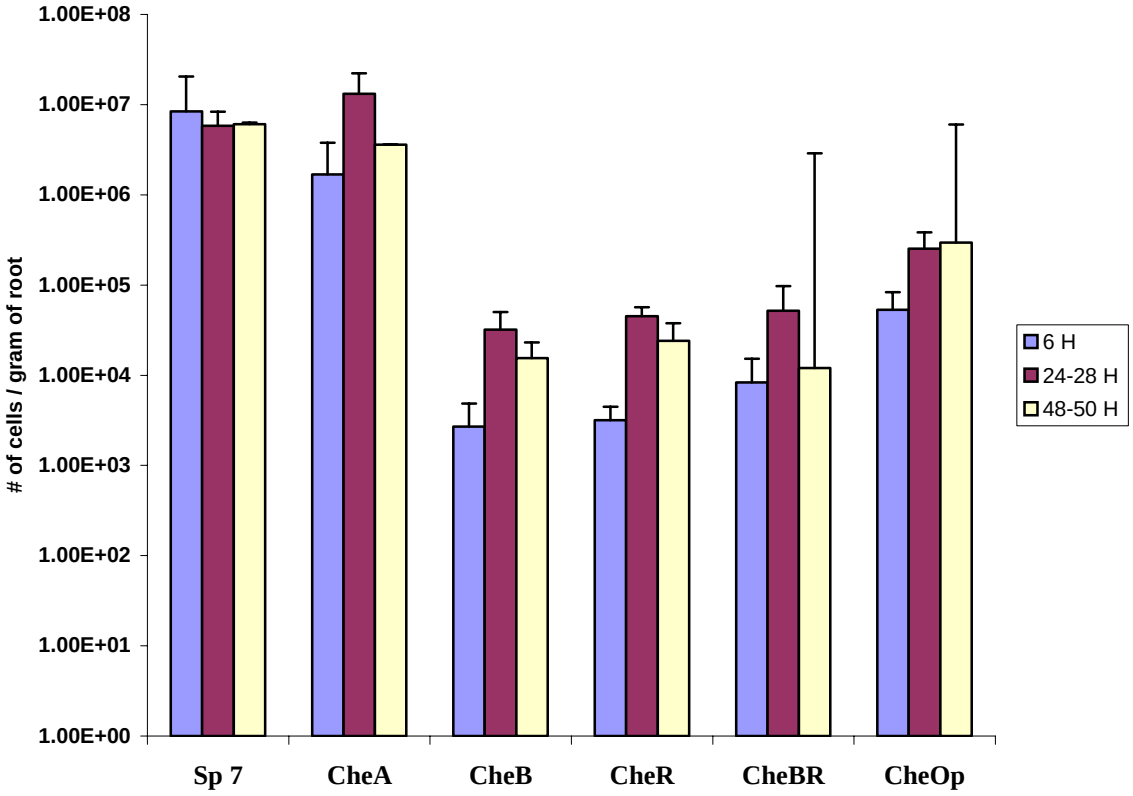

Figure 2. Plot of Table 3. Error bars indicate the standard deviation from the average of all plants.

Altogether, the data indicate that despite the fact that all strains were inoculated to the same levels to sterile wheat seedlings, they do not have the same ability to establish population on the roots after a short incubation time. The results also indicate that colonization of the wheat roots does not change beyond the 24-28H time point suggesting that population levels are established at their maximal levels at this time. Data shown in table 3 and in figure 2 clearly indicate that relative to the wild type strain, *A. brasilense* Sp7, the *cheB, cheR, cheBR,* and *cheOp* mutants were significantly impaired in wheat root colonization but the *cheA* mutant was not.

2. Qualitative assays

For all strains, approximately 10^5 cells were inoculated to the wheat seedling. Table 4 below shows the number of cells inoculated for each strain.

Table 4. Number of cells inoculated (CFU/ml) for qualitative assay

Strain	**(CFU/ml)**
Sp7	6.0 X 10^5
CheA	4.0 X 10^5
CheB	6.67 X 10^5
CheR	5.33 X 10^5
CheBR	4.33 X 10^5
CheOp	3.33 X 10^5

Visual Observations

-Root Morphology:

The roots for all plants were approximately the same in length. There were no significant differences in the root morphology. These observations were made after the plants had been incubated for 12 to 16 hours at 37^{o}C. The roots were then cut from the seedling and observed under a microscope.

-Root Colonization:

All of the plants were observed with the naked eye to locate root surface colonization of the *Azospirillum* strains. Sp7 had localized colonization on the roots and colonized mainly the root hairs and root tips; the color blue was very intense, suggesting high levels of colonization. The *cheA* mutant had a similar pattern of colonization than the wild type Sp7 strain but was more spread out than all the other strains. The *cheB* and *cheR* mutants colonized only the root hairs. The *cheBR* mutant colonized the root hairs and root tips; the color blue was less intense than with the wild type strain Sp7. No local colonization was observed for the *cheOp* mutant.

Microscopic Observations

The plants were observed using a brightfield microscope, the Nikon Eclipse E200 at 10X magnification.

-Root Colonization:

Sp7 colonized both the root hairs and the surface of the roots (Fig. 3). The *cheA* mutant colonized the roots with a pattern similar to the wild type (Fig. 4). Colonization by the *cheB* mutant was mainly localized to the root hairs with some surface colonization

(Fig. 5). The *cheR* mutant had more surface colonization than the *cheB* mutant but it is also localized on the root hairs (Fig. 6). The *cheBR* (Fig. 7) and the *cheOp* mutants both had colonization on root hair but very little surface colonization was observed. In Figure 8, individual colonies on the root hair and on the surface of the roots can be seen.

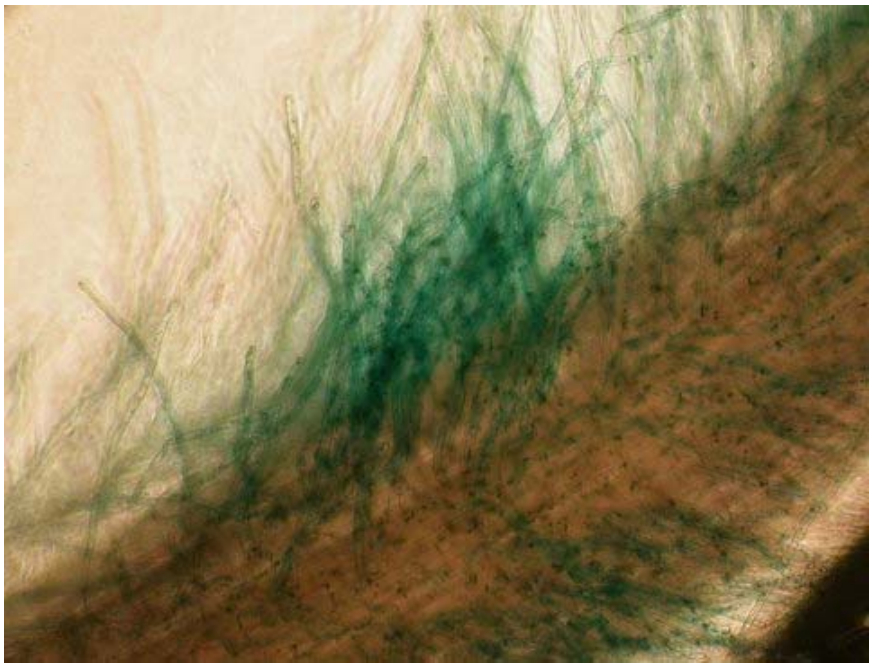

Figure 3. Root Colonization by Sp7. Observed with a brightfield microscope under 10X magnification. The blue color indicates the colonization on the root surface and root hairs.

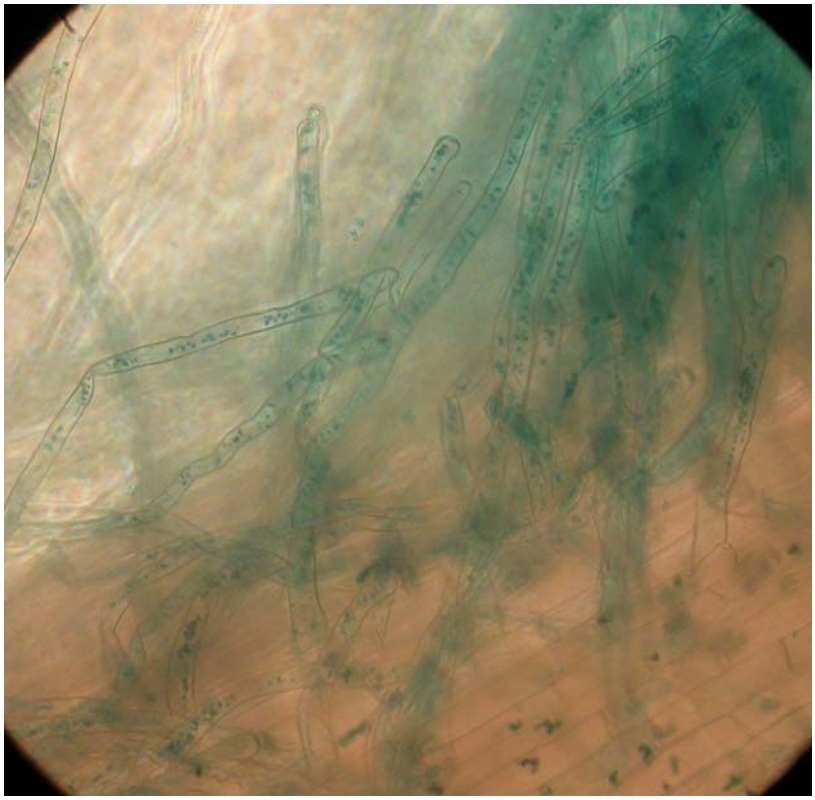

Figure 4. Root Colonization by *cheA*. Observed with a brightfield microscope under 10X magnification. The blue color indicates the colonization on the root surface and root hairs.

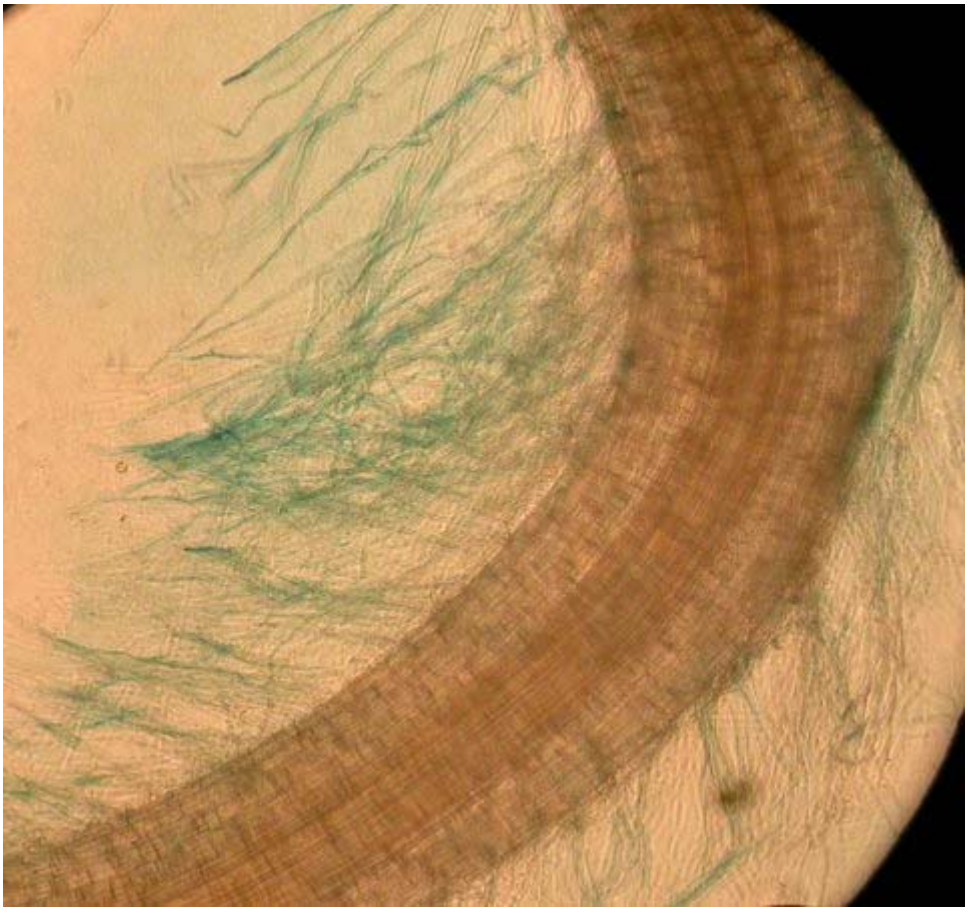

Figure 5. Root Colonization by *cheB*. Observed with a brightfield microscope under 10X magnification. The blue color indicates the colonization on the root surface and root hairs.

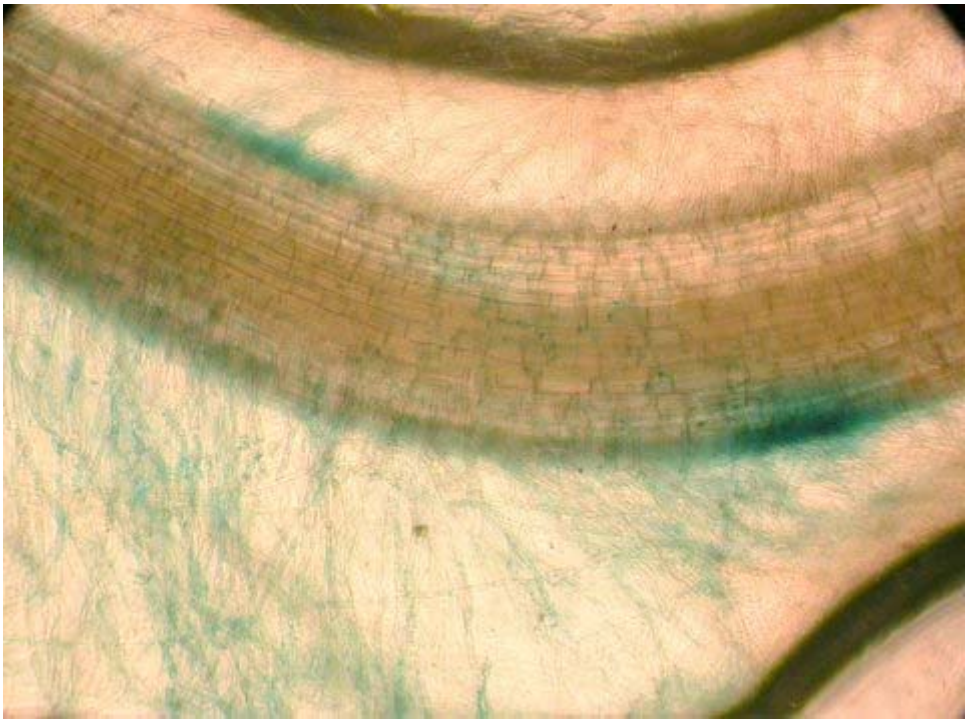

Figure 6. Root Colonization by *cheR*. Observed with a brightfield microscope under 10X magnification. The blue color indicates the colonization on the root surface and root hairs.

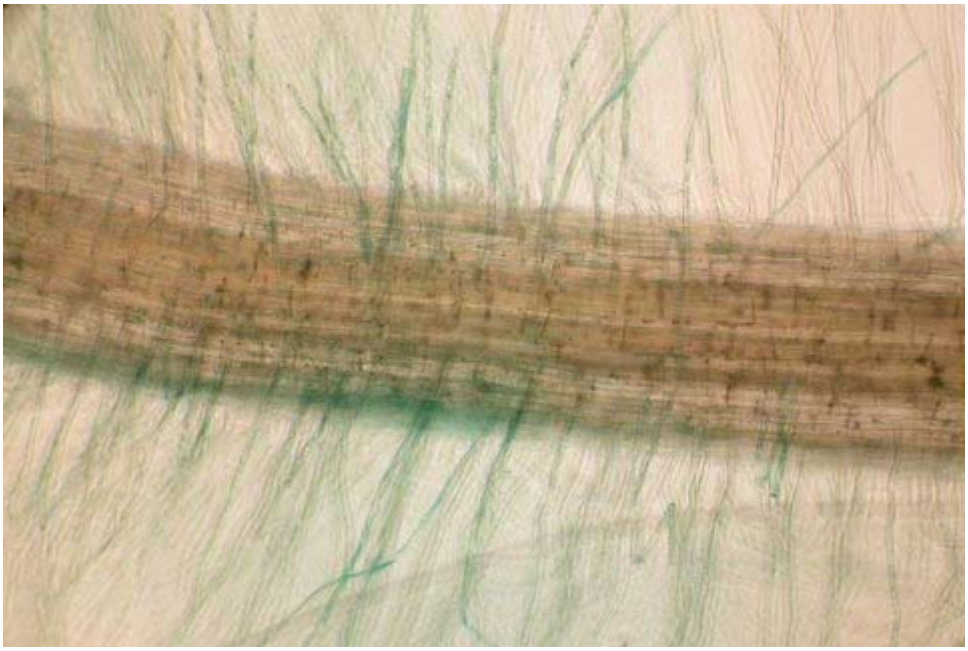

Figure 7. Root Colonization by *cheBR*. Observed with a brightfield microscope under 10X magnification. The blue color indicates the colonization on the root surface and root hairs.

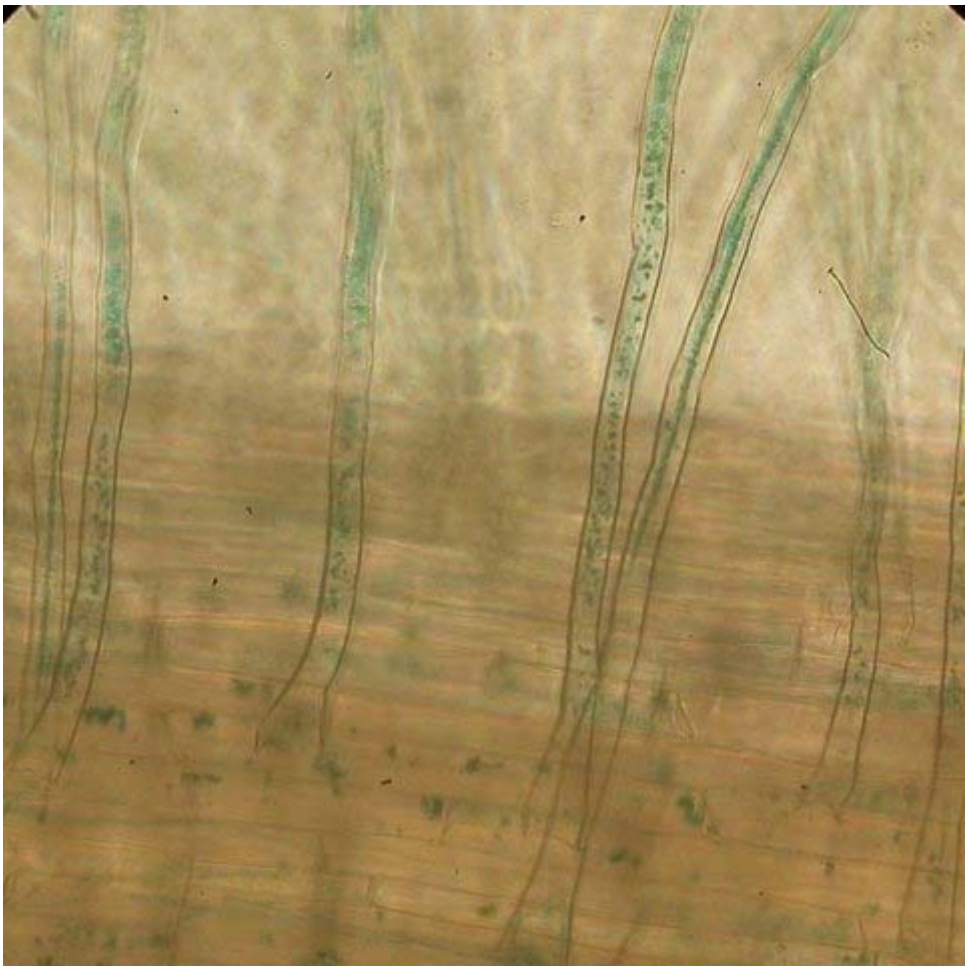

Figure 8. Root Colonization by c*heBR*. Observed with a brightfield microscope under 10X magnification. The blue color indicates the colonization on the root surface and root hairs. The blue dots indicate the individual colonies of the bacterium.

Discussion

The results of the study show that all mutants are impaired in wheat root colonization except the *cheA* mutant. In comparison with the wild type strain, Sp7, the *cheB, cheR, cheBR,* and *cheOp* mutants colonize the root in significantly lower numbers. At 6 hours, there was already a noticeable difference in the number of cells colonizing the roots. At 24 to 28 hours and 48 to 50 hours, this difference persists indicating not a delay but an impairment in the ability to colonize the wheat plant root. Interestingly, the *cheA* mutant was not impaired as the other mutants. There must be something else that compensates for *cheA. cheA* is involved in regulating motility and exopolysaccharide (EPS) production. EPS could be involved in root colonization giving CheA an advantage in colonizing the wheat root. All other mutants are impaired in EPS production.

The results indicate that chemotaxis may improve root colonization. Since all mutants except the *cheA* mutant are significantly impaired in motility, they colonize the roots to a lesser extent than the wild type *A. brasilense* Sp7. It is interesting to mention that the mutants are still able to colonize the roots. This may be because chemotaxis affects the competitiveness of the bacteria and not their intrinsic ability to form an association with the roots of the plants. The qualitative assay confirms the hypothesis that *A. brasilense* first colonizes the root hairs and then the root surface itself. Colonization for all mutants and wild type strain was observed at the root hairs, where there was significant growth for all strains. The colonization of all mutants, which reach lower population density on the root surfaces, was limited to the root hairs. The *cheA* mutant

and the wild type, which both reached high population number on the roots and colonized the root surface very efficiently, were found both on the root hairs and the root surface.

The results for this study show that the chemotaxis genes play an integral role in the wheat root colonization in *A. brasilense*. Further studies need to be done in order to conclusively understand the effects of these genes in the chemotaxis of *Azospirillum*.

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