Effects of plant root exudates on bacterial chemotaxis

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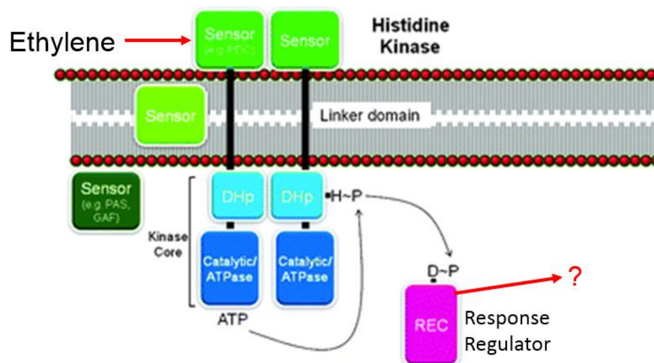
Introduction

Azospirillum brasilense is an alphaproteobacterium that is able to colonize the roots of various plants, including economically important crops such as wheat, corn and rice. In addition to being widespread in soils, these bacteria are able to enhance plant growth when inoculated to cereals (Fibach-Paldi et al., 2012; Puente et al., 1993). Evidence suggests that the ability of *A. brasilense* to promote plant growth depends on its production of phytohormone and its ability to fix nitrogen in the rhizosphere (Fibach-Paldi et al., 2012). The beneficial effects of *A. brasilense* on plant growth depend on the ability of the bacterium to colonize root surfaces. The ability to sense extracellular signals and migrate towards root surfaces, to attach and to grow are essential to successful colonization by *A. brasilense*. Plants excrete a variety of chemical compounds into the rhizosphere that directly impact microbial life. This project focuses on characterizing the role of chemotaxis toward compounds present in the exudates of preferred (wheat) or a non-preferred host plant (red clover) for colonization of the root surfaces by *A.brasilense*. Given the role of ethylene as a plant hormone produced in response to various stressors, the project also analyzed the pattern of expression of a gene coding for a putative ethylene-sensing protein in *A. brasilense*, named AzoETR1. AzoETR1 is predicted to be a membrane-anchored histidine kinase able to sense ethylene. Genome sequence analysis further suggests that AzoETR1 is part of a two-component signaling system. In this two-component signaling system, a stimulus, presumably ethylene, is predicted to interact with the sensory domain of AzoETR1 to stimulate autophosphorylation, which is subsequently transferred to a response regulator that modulates a signaling output (AzoETR1-RR). The phosphoryl group activates AzoETR1-RR, and a signal is sent downstream inducing a response of unknown nature at this point (Fig. 1). The observation that *A.brasilense* may detect and respond to ethylene is intriguing since this is a property limited to plants and cyanobacteria.



Perry, J., et al. 2011

Figure 1: Proposed AzoETR1 and AzoETR1-RR two-component system in *A. brasilence*. Ethylene is predicted to bind a conserved Cysteine residue in the active site of AzoETR1 to trigger autophosphorylation. The phosphoryl group should then be transferred from AzoETR1 to

the response regulator, AzoETR1-RR. AzoETR1 mediates the cellular response. The signaling output regulated by AzoETR1-RR is not yet known.

Methods

Expression of *azoETR1* and *azoETR1-RR*

Preparation of *A. brasilense* grown under nutrient-poor conditions

The experiment utilized two strains of *A. brasilense*, Sp7 and Sp245. Sp7 has been shown to be involved in plant root surface colonization while Sp245 can localize endophytically (Pereg-Gerk et al., 1998; Rothballer et al., 2003). Using a stock of *A. brasilense* cells stored at -80˚C, minimal medium (MMAB containing 3g K_2HPO_4, 1g NaH_2PO_4, 0.15g KCl, and 3g agar per liter) plates containing ampicillin (200 µg/mL) and supplemented with a carbon source were streaked with each strain. *A. brasilense* grew in a 28˚C incubator until isolated colonies formed. Each colony was inoculated into 5 ml minimal medium containing ampicillin (200 µg/ml). Cultures grew for 48 hours at 28˚C in a rotary shaker (220 rpm).

Preparation of *A. brasilense* grown under nutrient-rich conditions

Cultures grown in nutrient-rich media followed the same procedure as those grown under nutrient-poor conditions except that cells were inoculated into rich media (TY containing 10g tryptone and 5g yeast extract) at 28˚C, 220 rpm and grew for 12 hours.

Preparation of *A. brasilense* grown with differing amounts of exudates

Ten µLs of overnight cultures were inoculated into fresh TY and supplemented with ampicillin (200 µg/ml) and 0, 50, or 500 µl of exudates prepared from germinating wheat seeds as described in Table 2 using wheat late protocol. *A. brasilense* grew for 12 hours at 28˚C and 220 rpm under these conditions.

RT-PCR and Gel Electrophoresis

The cell concentration in each culture was determined using 600 nm (OD_{600}) measurements. This method estimates bacterial density as a function of turbidity. Next, two mLs of the 12 hour sample were washed three times in a solution of 0.8% (w/v) sterile KCl by centrifugation at 13,000 rpm, 4˚C and re-suspended in 0.8% KCl. The cell suspensions were sonicated in order to lyse the cells using a Fisher Scientific Sonic Dismembrator. Qiagen protocol was then followed to extract mRNA and synthesize cDNA via RT-PCR in a thermocycler. For gene specific PCR, the cDNA was used as a template with primers specific for azoETR1 (forward and reverse),

azoETR1-RR (forward and reverse), 16S rRNA (up and down) (Table 1), 10.5 µl nuclease free water, and 12.5 µl Master Mix (Promega) were added to separate PCR tubes. Gel electrophoresis was used to analyze the PCR products.

The experiment utilized several means to confirm the results. The mRNA for *azoETR1* and *azoETR1-RR* was amplified by PCR to check for DNA contamination. Sp7 and Sp245 gDNA were used to ensure that the primers maintained functionality, and 16S rRNA was amplified to verify that similar amounts of cells were utilized for each sample.

Table 1: Primers used in RT-PCR

Primer Designation	Primer Sequence
azoETR1 forward	5' – ATG TTC GGT GGC GTG GAA G – 3'
azoETR1 reverse	5' – TCA GGC ATG AGC GCG CTC C – 3'
azoETR1-RR forward	5' – TTG CAT GTC CTG GTG GCC – 3'
azoETR1-RR reverse	5' – TCA TGC GGG CCG CAG CTT CG – 3'
16S RNA up	5' – GGT CTG AGA GGA TGA TCA – 3'
16S RNA down	5' – TGC ACC CCA GCG TCT AGC – 3'

Preparation of exudates

Two types of seeds, Wheat and Red Clover, served as the sources of exudates. Wheat is a caryopsis while red clover is a legume. The seeds remained stored at -20˚C until use. The seeds were surface sterilized in a bleach – Triton X-100 solution for 5 minutes using a beaker and stir bar. This was followed by rinsing in deionized water, washing in 70% ethanol for 5 minutes, and rinsing in deionized water until ethanol was fully eliminated. Autoclaved filter paper was placed at the bottom of petri dishes. Cleaned seeds were individually placed in each of the 24 petri dishes. Of the 24 petri dishes, twelve contained wheat seeds while the remaining 12 contained red clover seeds. The seeds were covered with a second autoclaved filter paper. Two milliliters of pH 6.9 deionized water were added to each wheat seed plate while 3 milliliters of the same water were added to each red clover plate. The plates were then placed in the dark to allow the seeds to germinate. All seeds received one milliliter of water on the following Day 2 and were placed back in the dark to germinate.

A sample denoted as 'early' means that germination has occurred. The term 'late' describes post-germination in which the seeds have grown in the light and matured further. On Day 4, the red clover early (RCE) plates were combined into 3 new, separate plates with no filter paper (Fig.

2H). Exudates corresponding to red clover late (RCL) were treated similarly, but the bottom filter paper remained (Fig. 2G). Five milliliters of water were added to RCE, and they were left to incubate in the dark. Three milliliters of water were added to RCL, and the plate was incubated under light. All wheat plates remained in the dark. On Day 5, RCE remained incubating in the dark while RCL grew in the light. The wheat early (WE) plates were combined into 3 new, separate plates with no filter paper (Fig. 2F). The same was done with wheat late (WL) plates, but a bottom filter paper remained (Fig. 2E). Five milliliters of water were added to WE, and they were left to incubate in the dark. Three milliliters of water were added to WL, and it grew in the light. On Day 6, RCE exudates were extracted and stored in a -20˚C freezer. One milliliter of water was added to RCL and WL while WE remained incubating in the dark. On Day 7, RCL and WL remained growing in light, while WE exudates were extracted and stored at -20˚C. On Day 8, five milliliters of water were added to RCL and WL. They incubated for 48 hours and the exudates were extracted on Day 10 and stored in the freezer. All samples were lyophilized for two days. Two milliliters of the same pH 6.9 deionized water were used to suspend each lyophilized exudates powder. Table 2 displays the procedure.

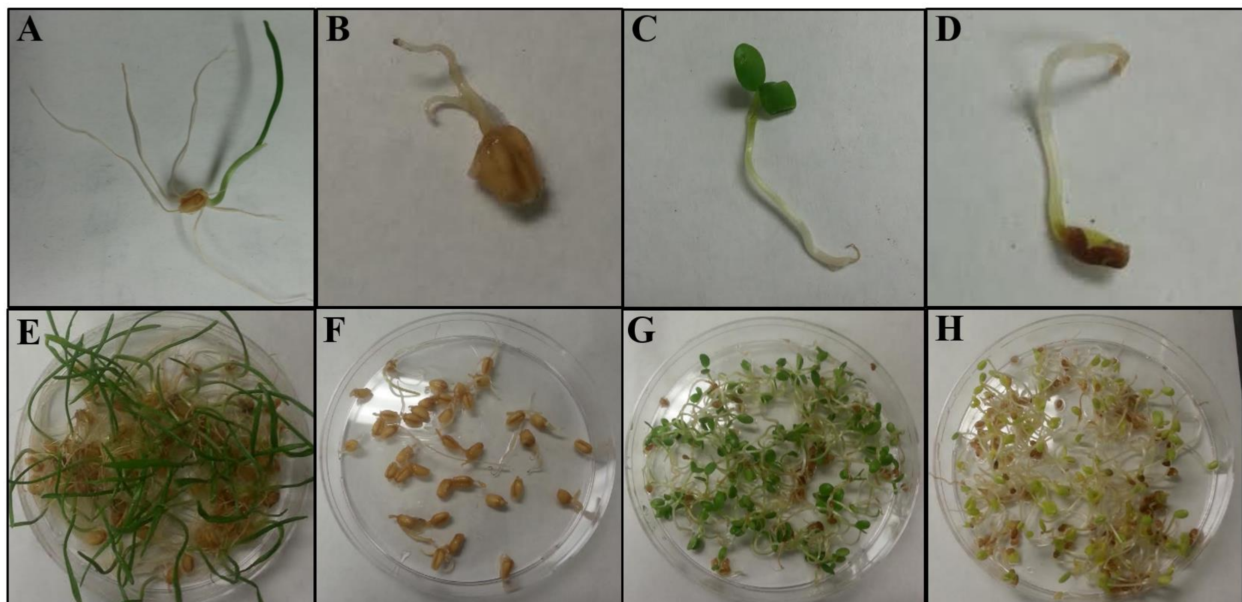


Figure 2: Early and late wheat and red clover seeds and plates. A) a single wheat late seed; B) a wheat early seed; C) a red clover late seed, and D) a red clover early seed. E) a plate of wheat late seeds; F) wheat early seeds; G) red clover late seeds, and H) red clover early seeds.

Table 2: Procedure for the preparation of the early and late exudates. Wheat Late is abbreviated as WL, Wheat Early as WE, Red Clover Late as RCL, and Red Clover Early as RCE.

<table>
<tr><th colspan="7">Procedure for exudates preparation</th></tr>
<tr><th>Exudates</th><th>Day 1</th><th>Day 2</th><th>Day 3</th><th colspan="3">Day 4</th></tr>
<tr><td>RCE</td><td rowspan="12">Added 3 milliliters of water</td><td rowspan="24">Germinating in the dark and received 1 milliliter of water</td><td rowspan="24">Germinating in the dark</td><td rowspan="24">Combined a set of 2 similar plates to a single new plate</td><td rowspan="2">RCE</td><td rowspan="6">Added 5 milliliters of water to incubate in the dark</td></tr>
<tr><td>RCE</td></tr>
<tr><td>RCE</td><td rowspan="2">RCE</td></tr>
<tr><td>RCE</td></tr>
<tr><td>RCE</td><td rowspan="2">RCE</td></tr>
<tr><td>RCE</td></tr>
<tr><td>RCL</td><td rowspan="2">RCL</td><td rowspan="6">Added 3 milliliters of water to grow in the light</td></tr>
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<tr><td>RCL</td><td rowspan="2">RCL</td></tr>
<tr><td>RCL</td></tr>
<tr><td>RCL</td><td rowspan="2">RCL</td></tr>
<tr><td>RCL</td></tr>
<tr><td>WE</td><td rowspan="12">Added 2 milliliters water</td><td rowspan="2">WE</td><td rowspan="12">Germinating in the dark and added 2 milliliters of water</td></tr>
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Procedure for exudates preparation continued 2				
Exudates	Day 5	Day 6	Day 7	Day 8
RCE RCE RCE	Incubating in dark	Extracted exudates and stored at -20^{0}C	Stored at -20^{0}C	Stored at -20^{0}C
RCL RCL RCL	Growing in light	Added one milliliter of water and growing in light	Growing in light	Added 5 milliliters of water to incubate in the dark
WE WE WE	Added 5 milliliters of water to incubate in the dark	Incubating in dark	Extracted exudates and stored in freezer	Stored at -20^{0}C
WL WL WL	Added 2 milliliters of water to grow in the light	Added one milliliter of water and growing in light	Growing in light	Added 5 milliliters of water to incubate in the dark

Procedure for exudates preparation continued 3				
Exudates	Day 9	Day 10	Day 11	Day 12
RCE RCE RCE	Stored at -20^{0}C	Stored at -20^{0}C	Lyophilized all samples	- removed samples from lyophilizer - added 2 milliliters of water to each sample - filter sterilized all samples - stored at -20^{0}C
RCL RCL RCL	Incubating in light	Extracted exudates and stored in freezer		
WE WE WE	Stored at -20^{0}C	Stored at -20^{0}C		
WL WL WL	Incubating in light	Extracted exudates and stored at -20^{0}C		

Preparation of *A. brasilense*

A. brasilense wild type strain Sp7 and its double knockout *ΔcheA1ΔcheA4* derivative were used. Sp7 exhibits root surface colonization utilizing chemotaxis while the double mutant serves as a chemotaxis null strain (Mukherjee et al., 2016). Each grew in nutrient rich media containing ampicillin (200 µg/mL) at 28˚C and 220 rpm. After growth, the culture's optical densities were estimated by measuring the absorbance at 600 nm (OD_{600}) and then standardized to OD600 = 0.4. The cultures were then washed three times in che buffer (containing 0.85g K_2HPO_4 and 0.68g KH_2PO per liter, pH 7.0) using 13,000 rpm centrifugation to remove any residual media or antibiotics.

Optimization of swarm plate assay

Each plate was filled with 25 ml of molten 0.3% minimal media (MMAB) that lacked a carbon and nitrogen source. A washed suspension of *A. brasilense* cells in che buffer was inoculated in the center. A sample of exudates were inoculated on the left and right of *A. brasilense* while che buffer was inoculated at the top and bottom of the plate (Fig. 3). This experiment tested differing amounts of bacteria, exudates and che buffer. *A. brasilense* cells were inoculated as volumes of 5 or 10 µl of suspension while the exudates and che buffer were tested as volumes of 1, 3, 5, 15, or 30 µl. The swarm plates did not contain antibiotics because there may be an unanticipated interaction between the antibiotics and the exudates. All swarm plates remained in an air or ethylene (air with 1 ppm ethylene) chamber for seven days.

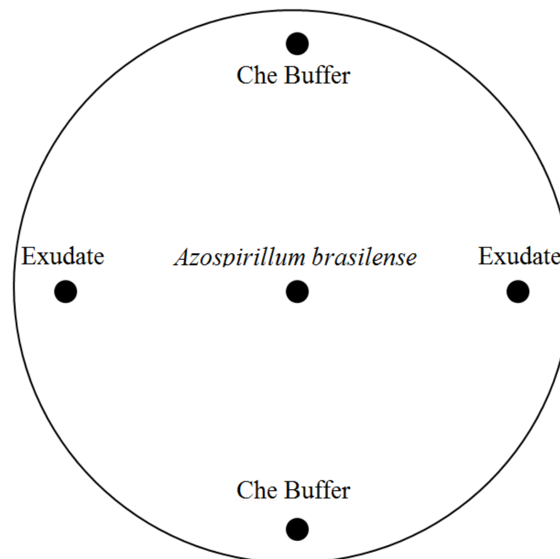

Figure 3: Swarm plate design. The minimal media (MMAB) plates lacked a carbon and nitrogen source. Each set of plates contained differing amounts of bacteria (5 or 10 µl), type of bacteria (Sp7 or *ΔcheA1ΔcheA4*), amounts of che buffer (1, 3, 5, 15, or 30 µl), and amounts of exudates (1, 3, 5, 15, or 30 µl).

Measurement Protocol

After seven days of growth, the movement of inoculated bacteria toward the buffer and the exudates were measured as shown in Fig. 4.

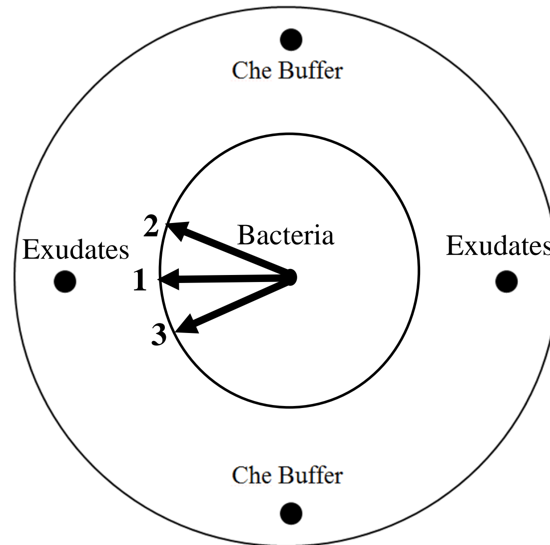

Figure 4: Measurement Protocol. The movement of the inoculated bacteria from the inoculation point in the direction of the exudates or buffer were measured at three locations for each direction, as displayed. Only measurements for the left most exudates are displayed.

Post optimization

These 0.3% minimal media (MMAB) agar plates contained 10 µl of bacteria (Sp7 or *ΔcheA1ΔcheA4*), 30 µl of exudates (wheat and red clover), and 30 µl che buffer. There were four biological replicates per type of exudates (WE, WL, RE, and RL), resulting in a total of sixteen plates. Another set of equivalent plates grew in ethylene (1ppm) for seven days with one caveat, only wheat early and late exudates were utilized. The same measuring protocol was employed when measuring these post optimization samples as were used in measuring the optimization samples.

Results and Discussion

Expression of *azoETR1* and *azoETR1-RR*

Since AzoETR1 was previously shown to bind ethylene (Binder lab- personal communication, data not shown), it is believed to mediate the interaction between *A. brasilense* and plants. Furthermore, AzoETR1is predicted to form a two-component signal transduction system with its cognate response regulator, AzoETR1-RR, implying that both genes must be expressed

concomitantly. This experiment utilized primers to target and anneal to the RNA of interest and the use of reverse transcriptase and PCR for synthesis and amplification. Due to the interaction of the translated proteins, both are expected to be synthesized under the same conditions.

Bands appear at 0.4, 1.5, and 0.4 kb and correspond to *azoETR1-RR*, *azoETR1*, and the 16S ribosome respectively (Fig. 5). The position of the bands for *azoETR1* cDNA and *azoETR1-RR* cDNA at 0.4 and 1.5 kb along with the lack of bands in the RNA samples suggests that the bands are of cDNA (Fig. 5A and D; Fig. 5B and E). No bands appeared for *azoETR1* RNA or *azoETR1-RR* RNA, indicating that the result was not caused by DNA contamination (Fig. 5B and E). The presence of bands for the gDNA of Sp7 and Sp245 implies that the primers functioned properly (Fig. 5C and F). Take note of samples 1-3 in Figure 5D. Samples 1 and 2 are not as bright or pronounced as Sample 3. This could be due to differential expression based on the amount of exudate or there could simply be less cells therefore causing a fainter band.

Testing under the conditions of high nutrient availability, low nutrient availability, and the presence of exudates, the concomitant expression of *azoETR1* and *azoETR1-RR* mRNA was maintained. This indicates constitutive expression at the transcriptional level. The presence of exudates and nutrients did not affect transcription, perhaps emphasizing the importance of this signaling interaction between *A. brasilense* and plants. Both strains of *A. brasilense*, Sp7 and Sp245, elicited constitutive expression, providing evidence that this result may be consistent within the species.

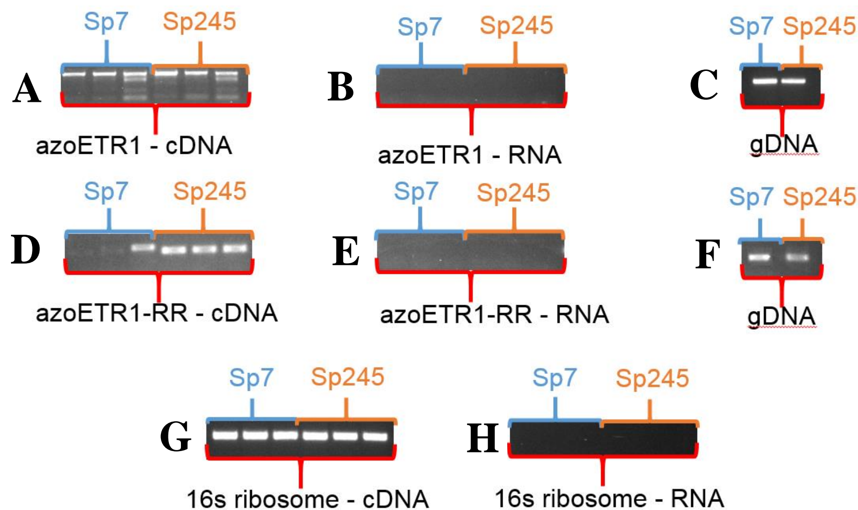

Figure 5: DNA gel electrophoresis of *azoETR1* and *azoETR1-RR*. The sizes of the amplicons corresponding to *azoETR1-RR*, *azoETR1*, and the 16S rRNA are 0.4kb, 1.5kb and 0.4 kb respectively. Sp7 and Sp245 were grown in nutrient rich media containing 0, 50, or 500µl of exudates for 12 hours.

Swarm Plate Assay

Optimization of swarm plate assay

Sample Sets 1 – 20 exhibited chemotactic rings with a circular shape, indicating no preference or inability to sense exudates (Table 3; Figs. 6A and B). The only conditions in which Sp7 did not elicit a circular shape was in Sample Sets 21 and 22 (Table 3; Figs. 6C and D). The chemotactic rings in these sets elicited an oval shape with Sp7 migrating strongly towards the wheat and red clover exudates (Figs. 6C and D). Note that Sample Sets 21 and 22 contained the highest amounts of bacteria and exudates - 10 µl of Sp7 and 30 µl of exudates. With the increased amount of exudates, *A. brasilense* sensed the exudates and migrated towards them utilizing chemotaxis.

Table 3: Swarm plates utilized differing bacteria, amounts of bacteria, amounts of exudates and the usage of ethylene.

Sample Set	Bacteria Used	Exudates used	Amount of bacteria used (µL)	Exudates amount (µL)	Grew in ethylene or air	Result: Chemotaxis Ring Shape
1	Sp7	Wheat	5	1	Ethylene	Circular
2	*ΔcheA1ΔcheA4*	Wheat	5	1	Ethylene	Circular
3	Sp7	Red Clover	5	1	Ethylene	Circular
4	*ΔcheA1ΔcheA4*	Red Clover	5	1	Ethylene	Circular
5	Sp7	Wheat	5	1	Air	Circular
6	*ΔcheA1ΔcheA4*	Wheat	5	1	Air	Circular
7	Sp7	Red Clover	5	1	Air	Circular
8	*ΔcheA1ΔcheA4*	Red Clover	5	1	Air	Circular
9	Sp7	Wheat	10	3	Ethylene	Circular
10	Sp7	Red Clover	10	3	Ethylene	Circular
11	Sp7	Wheat	10	3	Air	Circular
12	Sp7	Red Clover	10	3	Air	Circular
13	Sp7	Wheat	10	5	Ethylene	Circular
14	Sp7	Red Clover	10	5	Ethylene	Circular
15	Sp7	Wheat	10	5	Air	Circular
16	Sp7	Red Clover	10	5	Air	Circular
17	Sp7	Wheat	10	15	Air	Circular
18	Sp7	Red Clover	10	15	Air	Circular
19	Sp7	Wheat	10	30	Air	Circular
20	Sp7	Red Clover	10	30	Air	Circular
21	Sp7	Wheat	10	30	Air	Oval
22	Sp7	Red Clover	10	30	Air	Oval

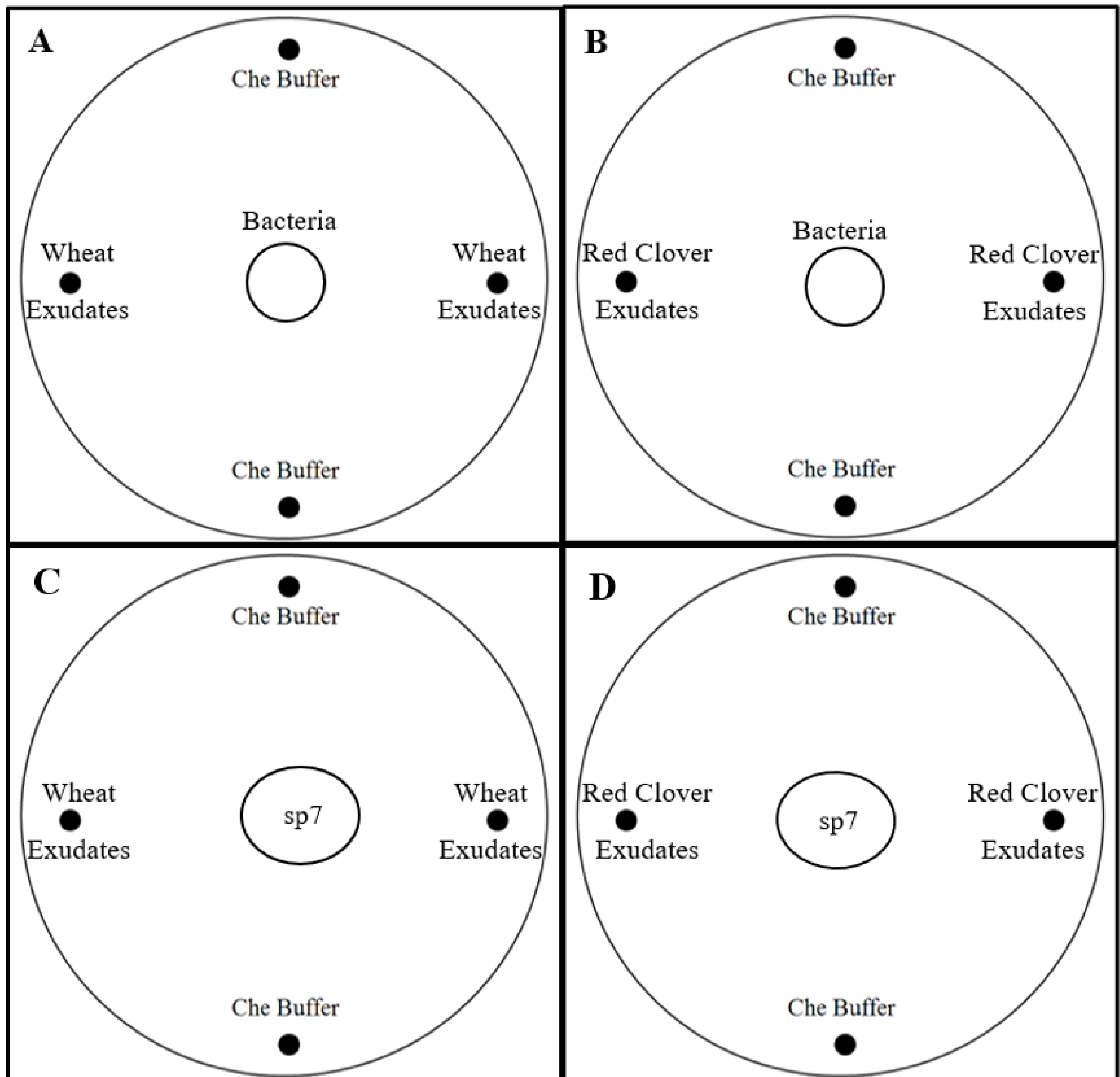

Figure 6: Chemotactic rings corresponding to different exudates. A) Sample Sets 1-20 with wheat exudates; B) Sample Sets 1 – 20 with red clover exudates; C) Sample Set 21 with Sp7 and wheat exudates, and D) Sample Set 22 with Sp7 and red clover exudates.

Post optimization samples in air

To further examine the association noted in the optimization samples, the 'post optimization' samples were constructed using the procedure and design outlined by Sample Sets 21 and 22 (Table 3; Figs 6C and D). There was a significant difference between colonization towards both types of exudates (both wheat and red clover) compared to che buffer (t-test = 2.83E-06). This is expected because *A. brasilense* localizes to the rhizosphere and should have no preference to the che buffer solution. Although *A. brasilense* chemotaxed strongly towards both exudates, it

elicited a stronger response towards wheat exudates (t-test wheat vs. che buffer = 0.0028; t-test red clover vs. che buffer = 0.0078). This observation is expected since *A. brasilense* is known to prefer to colonize cereals like wheat.

Comparing wheat to red clover exudates did not yield a significant difference (t-test = 0.79). Wheat late samples were compared to wheat early samples displaying no significant difference (t-test = 0.76). Comparing red clover late to red clover early resulted in a non-significant difference as well (t-test = 0.69). All samples with *ΔcheA1ΔcheA4* showed colonization at the inoculation sight with no evidence of chemotaxis (Fig. 7B). To visualize these results, Figure 8 was constructed. The average measurement for each set of exudates was divided by its associated che buffer measurement, yielding the chemotactic index. The error bars are representative of the standard error to the mean in which the value for the chemotactic index is divided by the square root of the number of samples – eight in this case. Figure 8 illustrates that late wheat exudates elicited a stronger attraction for Sp7 than early wheat exudates while early red clover exudates showed a stronger attraction for Sp7 than late red clover exudates.

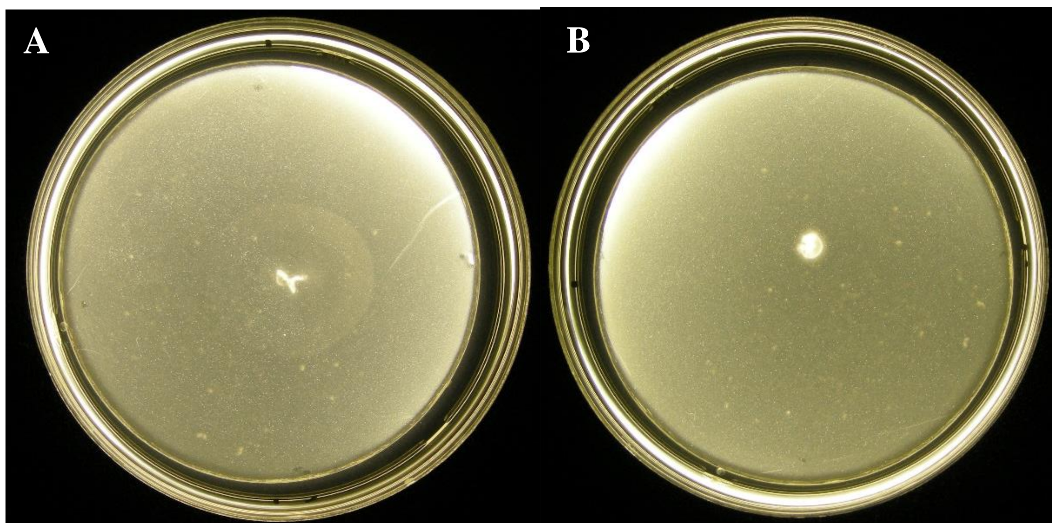

Figure 7: Pictures of *A. brasilense* on 0.3% minimal media (MMAB) agar plates grown in air for seven days. Each plate contained 10 µl *A. brasilense*, 30 µl exudates, and 30 µl che buffer. A) utilized strain Sp7 and B) utilized *ΔcheA1ΔcheA4*.

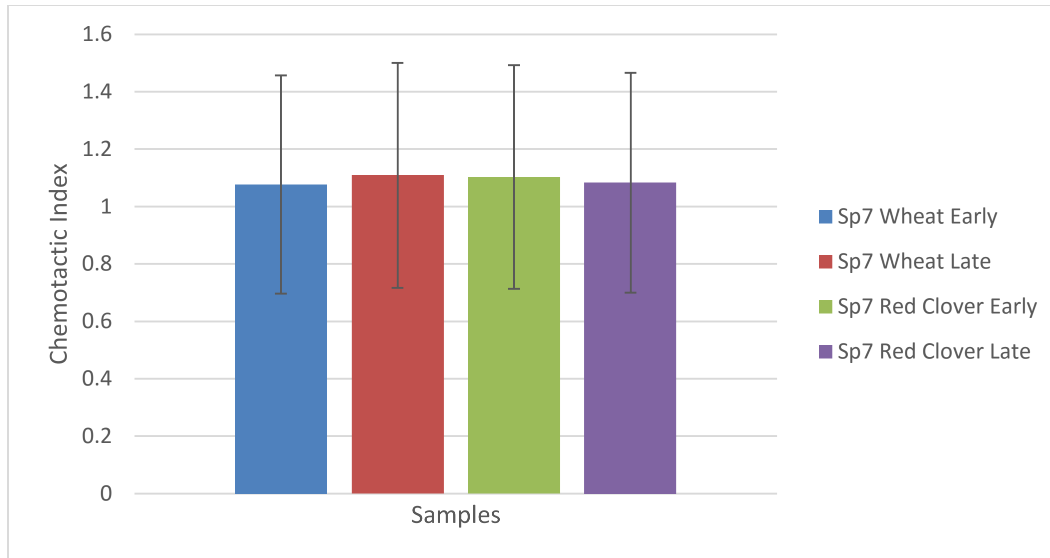

Figure 8: Swarm plate assay under air conditions. These 0.3% minimal media (MMAB) agar plates lacked a carbon and nitrogen source. Each agar plate utilized 10 µl Sp7, 30 µl exudates, and 30 µl che buffer arranged according to Figure 3. The cultures grew for seven days in air chambers.

Post optimization samples in ethylene

Sp7 grew very quickly under these conditions and by the time seven days had transpired, it had dispersed throughout the majority of the agar plate. However, Sp7 did not fully disperse towards the che buffer inoculation site (data not shown). Comparing wheat exudates (both early and late) to che buffer resulted in a marginally significant difference likely due to the timing of the experiment (t-test = 6.77E-01). If the measurements were taken before *A. brasilense* had dispersed throughout the majority of the plate, the result likely would have been significant like the air conditions. Analogous to the air results, *ΔcheA1ΔcheA4* displayed colonization only at the inoculation site with no evidence of chemotaxis, and there was not a significant difference between wheat early and late exudates (Fig. 9).

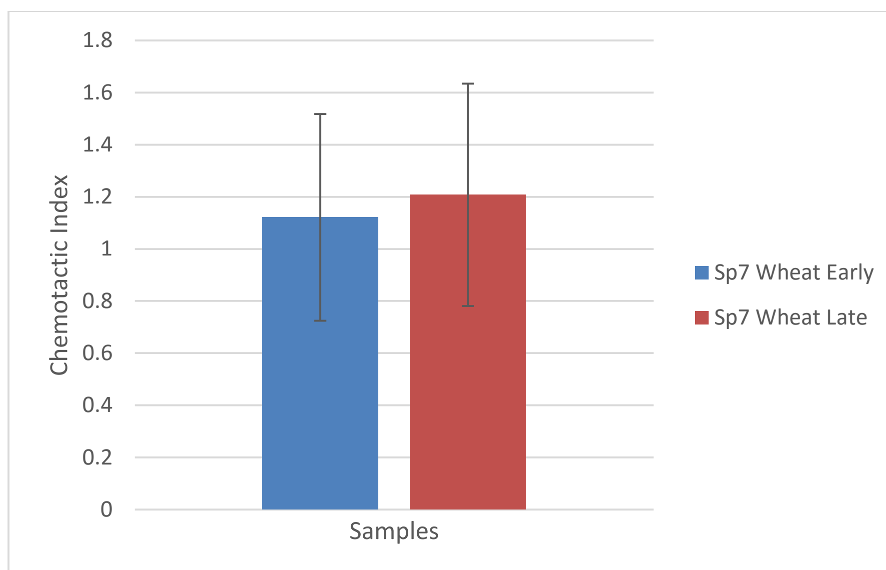

Figure 9: Swarm plate assay under ethylene conditions. The 0.3% minimal media (MMAB) agar plates lacked a nitrogen and carbon source. Each contained 10 µl Sp7, 30 µl wheat early or wheat late exudates, and 30 µl che buffer as outlined by Figure 3. The cultures grew for seven days in an ethylene chamber (1 ppm).

Implications of post optimization samples

These results assist in the identification of the molecules responsible for the attractive properties of the rhizosphere. Most notably, sugars and organic acids involved in the citric acid cycle have been implicated in this attraction (Alexandre et al., 2000). The interactions of other molecules found in the rhizosphere should be analyzed to gain further insight into these complex relations. The observation that late wheat exudates attracted *A. brasilense* more strongly than early exudates suggests that these attractant molecules are more concentrated in these samples post-germination. The opposite effect was noted when red clover exudates were tested: early exudates caused *A. brasilense* to be more significantly attracted. In this case, the attractant molecules may be more concentrated immediately after germination while they may be degraded or secreted at lower concentrations as germination progresses.

The rhizosphere represents an area of complex interactions between the resident microbes and the roots of plants. The colonization of the plant root surfaces by *A. brasilense* depends on secreted molecules that are attractant for this bacterium and seem to be produced at different levels at different times and in different plant species. Such differences could explain the preference of *A. brasilense* for colonization of cereal roots compared to legume roots.

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Acknowledgments

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