Communication

Corn Responsiveness to Azospirillum: Accessing the Effect of Root Exudates on the Bacterial Growth and Its Ability to Fix Nitrogen

Lucas Caiubi Pereira 1,*, Carolina Bertuzzi Pereira 1, Larissa Vinis Correia 1, Thaisa Cavalieri Matera 1, Rayssa Fernanda dos Santos 1, Cristiane de Carvalho 2, Elisete Aparecida Fernandes Osipi 3 and Alessandro Lucca Braccini 1

1 Department of Agronomy, Universidade Estadual de Maringá, Maringá-PR, CEP 87020-900, Brazil; carolbertuzzi14@gmail.com (C.B.P.); pg401097@uem.br (L.V.C.); pg53837@uem.br (T.C.M.); pg53835@uem.br (R.F.d.S.); albraccini@uem.br (A.L.B.)
2 Centro Estadual de Educação Tecnológica Paula Souza, Itú-SP, CEP 13306-220, Brazil; cristiane.carvalho10@etec.sp.gov.br
3 Center of Agrarian Science, Universidade Estadual do Norte do Paraná, Bandeirantes-PR, CEP 86360-000, Brazil; elisete@uenp.edu.br
* Correspondence: lucascaiubi@yahoo.com.br; Tel.: +55-44-30118963

Received: 11 June 2020; Accepted: 10 July 2020; Published: 21 July 2020

Abstract: Corn has shown different degrees of positive response to inoculation with the nitrogen-fixing bacteria of the genera Azospirillum. Part of it has been attributed to the plant genotypic variation, including the root exudates, that are used by the bacteria as energy source. In this study, we grew two corn hybrids that differ for their response to Azospirillum, to investigate the effect of different exudates profiles on the bacteria growth and nitrogenase activity. Employing high performance liquid chromatography, we identified nine amino acids (asparagine, aspartic acid, serine, glutamic acid, valine, phenylalanine, threonine, tryptophan and alanine), six sugars (glucose, sucrose, xylose, arabinose, fructose and galactose) and four organic acids (citrate, malate, succinate and fumarate). The less responsive corn genotype showed reduced plant growth (root volume, shoot dry mass and shoot N content), a lower concentration of Azospirillum cells within the root tissues, a higher content of asparagine and glucose and a reduced amount of metabolites that serve as bacterial energy source (all organic acids + five sugars, excluding glucose). The genotypes did not interfere in the ability of Azospirillum to colonize the substrate, but the metabolites released by the less responsive one reduced the nitrogenase activity.

Keywords: Azospirillum brasilense; Zea mays; nitrogenase activity; amino acids; sugars; organic acids

1. Introduction

Symbiosis is a biological phenomenon involving changes in the genome and metabolism of organisms from different species, usually with benefits to one or both [1]. In the plant–microbe relationships, two root symbiotic systems have been actively studied, the arbuscular mycorrhizae and root-associated bacteria [1]. While the first mentioned interaction is probably the most widespread in the ecosystem, the plant association with diazotrophic bacteria has been the more exploited symbiotic relationship in plant production [1,2].

Diazotrophic bacteria comprehend groups of free living, associated and nodule forming species capable of enzymatically reducing the atmospheric nitrogen (N) into plant bioavailable N compounds and, in the agriculture, have been shown capable of enhancing crop yield, while reducing the environmental impacts caused by mineral fertilizers [3,4], such as the production of phytohormones,
the N fixation mechanism of these bacteria directly benefit the plant growth; however, some of these bacteria can further provide indirect benefits to plants, including the inhibition of the host pathogenic organisms and the induction of systemic resistance to stresses [5]. Among them, *Azospirillum*, a free living diazotrophic bacterium of the Spirillaceae family, stands out as one of the best characterized and exploited plant-growth promoting microorganism in the agricultural production [3].

To date, twenty species of *Azospirillum* have been described [6], and some have reached commercialization in countries such as Brazil, India and France [3,4]. In the literature, most of the studies on *Azospirillum* focus on the specie *Azospirillum brasilense*, which, since its identification as promising N fixer when associated to grasses crops, has been investigated for its ability to replace nitrogenous fertilizers in sugarcane (*Saccharum* spp.), corn (*Zea mays*), wheat (*Triticum aestivum*), and rice (*Oryza sativa*) [3,6]. However, it is in the corn production that *A. brasilense* stands out as inoculant [6], not only because it allow for a 25% reduction in the need for nitrogenous fertilizer, but also for being capable of increasing the grain yield up to 30% [7].

Although largely prospected as N-fixing bacterium [8], nowadays, it is widely accepted that the benefits delivered by *A. brasilense* to cereal plants goes beyond those mechanisms, and includes the mitigation of abiotic stresses, the biological control of plant pathogens, and the production of phytohormones [6]. In the field, the application of *Azospirillum* is either carried out by coating the seeds with a bacterial suspension or by in-furrow soil applications, prior to sowing [9]. Irrespective of the method, a successful inoculation relies on the microorganism’s ability to survive and reproduce in the rhizosphere, a complex environment regulated by the host root-released compounds [10].

Over the cycle, plant roots continuously secrete soluble metabolites, that, based on their molecular weight, are classified into two clusters [11]: one formed by highly diversified group of low-molecular weight metabolites, such as amino acids, organic acids and sugars, and the other constituted of high-molecular weight and less diverse compounds, such as mucilage and proteins [11]. In the genera *Azospirillum*, root exudates play a bimodal role in the rhizosphere colonization, firstly as an energy source, and secondly, serving as chemoattractants that guide the bacterial migration toward the roots [10].

Despite the consistent benefits obtained with the corn inoculation with *Azospirillum*, different degrees of positive outcomes have been reported in the literature [4]. Part of it has been attributed to genotypic variation of the host genetic background, including the composition of the metabolites it releases toward the rhizosphere [12]. Crops genotypes, including corn, may differ in the amount and the composition of their exudates, but the implications of this for the rhizosphere colonization by *Azospirillum*, or for the benefits it provides to plants, remain a poorly-understood subject [13].

In this context, hypothesizing that root exudates may be involved in the corn responsiveness to *A. brasilense*, our objective was to investigate the effects of changes in the composition of these metabolites, linked to the crop genotype background on the ability of *Azospirillum* to colonize the rhizosphere, as well as on its N-fixing mechanism.

2. Results

We identified nine amino acids (asparagine, aspartic acid, serine, glutamic acid, valine, phenylalanine, threonine, tryptophan and alanine), six sugars (glucose, sucrose, xylose, arabinose, fructose and galactose) and four organic acids (citrate, malate, succinate and fumarate) in the exudates of the tested genotypes.

The genotypes showed comparable values for total content amino acids (Table 1), but the abundance of each class in the exudates differed with the corn lines (Appendix A). The more responsive one displayed lower content of aspartic acid upon full (100% N), lower alanine concentration under partial (75% N) and higher tryptophan abundance in both the partial N supply and inoculation (75% N + Azo) (Appendix A). In the inoculated plants, comparable results of alanine and threonine were found between the genotypes, while a higher release of asparagine and a lower concentration of
aspartic acid, serine, glutamic acid, valine, phenylalanine and tryptophan were identified in the less responsive genotypes (Appendix A).

In relation to sugars and organic acids, the genotypes differed for both, the total content (Table 1) and profile (Appendix B), with the less responsive ones showing a reduced concentration of both and, thus, of chemotactic compounds, i.e., metabolites that serve as chemoattracts and an energy source for *Azospirillum* (sum of all four organic acids + five sugars, excluding glucose). For the less responsive corn line, individual classes of sugar and organic acid were released in lower concentration, apart from glucose, whose concentration was higher upon only mineral N supply (full and partial) and statistical equivalent to the more responsive one under inoculation (Appendix B).

### Table 1. Means of total amino acids content (TAA), total sugar (TS), total organic acids (TOA), total of chemotaxis compounds (TCC), most probable number of diazotrophic bacteria (MPN), root volume (RV), shoot dry mass (SDM) and its N content (NCSDN) of two corn genotypes of high (HResp) and low (LResp) responsiveness to *A. brasilense* under full N mineral supply (100% N), N deprivation (75% N) as well as partial N supply coupled with inoculation (75% N + Azo).

| Treatments        | TAA (nmol g⁻¹) | TS (nmol g⁻¹) | TOA (nmol g⁻¹) | TCC * (nmol g⁻¹) |
|-------------------|----------------|---------------|----------------|-----------------|
|                   | HResp LResp    | HResp LResp   | HResp LResp    | HResp LResp     |
| 100% N            | 1633.15C       | 1062.3Ba      | 952.82Ab       | 394.88Ba        |
| 75% N             | 1040.15B       | 776.52Ca      | 750.60Ba       | 212.85Ca        |
| 75% N + Azo       | 1770.18A       | 1125.98Aa     | 928.2Ab        | 447.42Aa        |
| Means             | 1481.16        | 988.27        | 877.21         | 351.72          |
| CV (%)            | 2.67           | 2.00          | 3.25           | 2.19            |

According to the Tukey test, means followed by the same capital letter in the column belong to the same group at 5% probability. Means followed by the same lowercase letters in the row do not differ from each other by the F-test at a 5% probability level. * Sum of sugars (excluding glucose) + organic acids.

Regarding plant growth, the full supply of N in the form of mineral fertilizer provided plants showing equivalent results for root volume, shoot dry mass and shoot N content (Table 2). However, upon inoculation, the high responsive genotype showed comparable or higher growth parameters than the full N mineral supply (Table 2), while in the less responsive *Azospirillum*, it was unable to deliver growth parameter, at least, equivalent to mineral fertilization (Table 3).

### Table 2. Means of root volume (RV), shoot dry mass (SDM), shoot N content (NCSDN) and of the most probable number of *Azospirillum* (MPN) in the substrate and root tissue of two corn genotypes of high (HResp) and low (LResp) responsiveness to *A. brasilense* under full N mineral supply (100% N), N deprivation (75% N) as well as partial N supply coupled with inoculation (75% N + Azo).

| Treatments        | RV (mL) | SDM (g) | NCSDM (mg) | MPN Substrate | MPN Root Tissue |
|-------------------|---------|---------|------------|---------------|-----------------|
|                   | HResp   | LResp   | HResp      | LResp         | Substrate       | Root Tissue    |
| 100% N            | 22.34Ba | 22.56Aa | 9.31Ba     | 9.34Aa        | 10.54Aa         | 10.34Aa        |
| 75% N             | 10.54Ca | 11.01cb | 7.34Ca     | 7.45Ca        | 7.34Ba          | 6.65Cb         |
| 75% N + Azo       | 26.87Aa | 16.89Bb | 11.34Aa    | 8.56Bb        | 10.91Aa         | 7.56Bb         |
| Means             | 19.58   | 18.82   | 9.33       | 8.45          | 9.60            | 7.85           |
| CV (%)            | 10.23   | 8.95    | 4.34       | 5.45          | 8.45            |

According to the Tukey test, means followed by the same capital letter in the column belong to the same group at 5% probability. Means followed by the same lowercase letters in the row do not differ from each other by the F-test at a 5% probability level.

Regarding the colonization of the rhizosphere by the bacteria, the genotype background did not play a part in the *Azospirillum* establishment in the substrate, but significantly interfered in the bacteria ability to colonize internal tissues (Table 2), as observed in the reduced most probable number (MPN) of bacteria within the root tissues of the less responsive corn (Table 2). In artificial media...
(Table 3), the addition of synthetic compounds that mimic the profile of each genotype had no effect on *Azospirillum* count, but interfered in the N-fixing mechanism, with the less responsive genotype showing lower nitrogenase activity (Table 3).

| Growth Media | MPN (10^8 Cells g^-1) | Nitrogenase Activity (nmol C_2H_4 mg^-1 h^-1) |
|--------------|-----------------------|---------------------------------------------|
| Neat NFb     | 18.56A                | 387.21A                                    |
| NFb + HResp  | 20.54A                | 365.34A                                    |
| NFb + LResp  | 19.56A                | 267.23B                                    |
| Mean         | 19.55                 | 339.93                                     |
| CV (%)       | 9.34                  | 12.45                                      |

According to the Tukey test, means followed by the same capital letter in the column belong to the same group at 5% probability.

### 3. Discussion

Even though sugars are reported as the main class of root-released compounds by plants [13,14], in the present study, this trend has not been confirmed, as our root exudates contained 60% of amino acids, followed by 30% sugars and 10% organic acids. This contrasting result may be related to the high rates of amino acids released by plant roots at early growth stages, as we sampled the plants at seedling stages [15]. However, this higher amino acid content may also be attributed to the *Azospirillum*, which, as a diazotrophic microorganism, release high rates of N-derivate compounds [16].

Corroborating previous works, all N-depleted plants, as well as those of the less responsive upon inoculation (75% N + Azo), displayed lower amount of sugars, organic acids and chemotactic compounds [17]. All classes of sugars we identified have previously been reported in exudates of corn [18], rice [19] and of the model grass specie *Brachypodium* [20]. Regarding the organic acids, malate, citrate, succinate and fumarate are considered the most common classes released by corn roots [18], and they were all identified in our study.

In the case of *Azospirillum*, the plant’s root exudates are involved in the bacterial cells migration in the soil [21], in a process governed by energy taxis, i.e., the bacterial moves toward a higher concentration of a nutrient to seek a position at which the energy level is favorable [7,8]. Compared to sugars and organic acids, *Azospirillum* species have a limited reliance on amino acids to grow [4], which, thus, display null or weak attractiveness on the cells [11]. Concerning *A. brasilense*, the species used in this work, all classes of organic acids are considered to exert chemotaxis, while, among the sugars, glucose is not considered a chemoattractant [4] and galactose exerts induced taxis, i.e., only when the cells have previously been grown in media containing it [7,8].

However, chemotaxis should not be taken for granted in analyzing corn-*Azospirillum* symbiosis, as some metabolites that do not serve as energy source, or chemoattracts may interfere in the bacterial growth or metabolism, impacting, thus, the benefits delivered by it to plants [4,22,23]. In this context, asparagine that, such as in our work, has been reported as the most abundant amino acid released by corn [18], wheat [24] and the model grass *Brachypodium* [20] roots, has already been reported to impair the activity of the nitrogenase of *Azospirillum* [23]. In cereals, asparagine is involved in N storage and transportation, but its increase within root tissues has been documented as a metabolic response to stress, including nutritional deficiency [25,26].

This is consistent with other plant-growth promoting bacteria, such as *Bacillus*, that had a suppression of bacterial genes involved in the synthesis of protein after changes in the profile of corn exudates [18]. In this work, however, we only investigated the effects of the whole exudates on bacteria growth and nitrogenase activity, but as the less responsive genotype remained, showing a high amount
of asparagine, even under inoculation, we suggest that this amino acid may have contributed to reduce the enzyme activity.

Concerning the other amino acids, while aspartic acid have null effects on *Azospirillum* species, the tryptophan benefits the bacterial synthesis of plant growth regulators [23], as this latter compound is structurally and functionally related to the synthesis of auxin, the major phytohormone produced by *A. brasilense* [10,27]. In the literature, an increase in lateral roots due to inoculation has been well-documented and it is, often, ascribed to the bacterial auxin [1,2,5]. In this study, provided the higher concentration of tryptophan in the rhizosphere is of the high responsive genotype, it is plausible that *Azospirillum* may have had been capable of producing higher concentration of auxin [28], compared to the other genotype. This hypothesis is consistent with the results found for plant growth, as the less responsive genotype displayed reduced root volume data upon inoculation.

Despite the different chemical profiles, the substrate’s MPN values suggested that any genotype favored *Azospirillum* establishment in the substrate, a trend that was later confirmed by our data on bacterial count in supplemented media. This evidences the great degree of versatility of *Azospirillum* in using different carbon based compounds as energy source, a trait that enables the organism to adapt to a wide array of environments [3], including different agricultural practices and soil types, where cereal crops are grown [4].

*Azospirillum* colonizes all plant parts, but it exhibits a decreasing pattern concentration from roots to leaves [9]. For this reason, the bacteria are generally regarded as a root-surface microorganism, but the species colonizes the roots in a specie-specific way [10]. *A. brasilense*, the species used in this work, can further colonize the interior of root tissues [10], where the nitrogenase is more protected against the irreversible inactivation caused by the oxygen. Supporting Fukami et al. [9], our data on internal root colonization, as estimated by the MPN technique, suggest a genotype-dependency regarding tissues colonization, which, among other factors, depends on the bacterial ability to degrade the structural polysaccharide of cell walls pectin, whose content and structure may differ with the plant genetic background [29].

Overall, we showed that just as for their responsiveness, the genotypes differed for the composition of their exudates as well as for the ability of *Azospirillum* to establish within root tissues. This difference in profile, however, did not affect the ability of *A. brasilense* to establish in the substrate, or to grow in artificial medium, but it interfered in the nitrogenase activity. In this sense, it is likely that the weak growth performance of the less responsive genotype may be related to its exudates profile (lower relative amount of chemotaxis compounds, lower tryptophan concentration and high relative asparagine content), but also to its lower concentration of *Azospirillum* in the root tissues.

Our results corroborate that the benefits delivered by *Azospirillum* to corn are affected by the genotype background, and further confirm the involvement of root-released compounds in the degree of response. It is noteworthy that the plant-*Azospirillum* interaction is settled within a dynamic and complex ecosystem constituted by other compounds not identified in this study, but that may interfere in the behavior of microbial communities [14]. Therefore, further researches on the role of root exudates as mediators of the corn-*Azospirillum* symbiosis need to be carried out, including regarding the amino acids classes, as despite their null chemotaxis, they seemed to be determinant in the degree of the benefits provided by *A. brasilense*. Additionally, rather than being the cause of the responsiveness, root exudates are more likely to reflect the specificity of transcriprional signaling involved in the mutual recognitions between *Azospirillum* and its host plant [12,13].

4. Materials and Methods

We tested two inbred lines showing different N use efficiency under *Azospirillum* inoculation, as described by Zeffa et al. [30] and Vidotti [13]. The lines belong to the germplasm bank of the Universidade Estadual de Maringá (Brazil), and were obtained from successive self-pollinations of commercial hybrids with tropical background. Based on the work reported by Zeffa et al. [30],
we selected the line L7 as a high responsiveness hybrid to inoculation, while L16 was taken as the low one.

Seeds of both hybrids were sown in Leonard jars [31] containing sterilized substrate (sand and pulverized coal at 3:1 v/v) and sterile nutrient solution. Following Zezza et al. [30], the nutrient solution contained (L−1): 2.0 mmol Ca(NO3)2 (calcium nitrate tetrahydrate, Sigma-Aldrich, Saint-Louis, MO, USA); 0.75 mmol K2SO4 (potassium sulfate, Merck Millipore, Darmstadt, Germany); 0.65 mmol MgSO4 (magnesium sulfate heptahydrate, Sigma-Aldrich); 0.1 mmol KCl (potassium chloride BioXtra, Sigma-Aldrich); 0.25 mmol KH2PO4 (potassium phosphate monobasic, Supelco, Darmstadt, Germany); 1 × 10−3 mmol H3BO3 (boric acid BioXtra, Sigma-Aldrich); 1 × 10−3 mmol MnSO4 (sulfate monohydrate BioReagent, Sigma-Aldrich); 1 × 10−4 mmol CuSO4 (cupric sulfate pentahydrate, Sigma-Aldrich); 1 × 10−5 mmol ZnSO4 (zinc sulfate, J.T.Baker, Center Valley, PA, USA); 5 × 10−6 mmol (NH4)6Mo7O24 (ammonium molybdate tetrahydrateand, Sigma-Aldrich) and 0.1 mmol Fe-EDTA (Fe–EDTA, BioReagent, Sigma-Aldrich).

As bacterial source, a liquid formulation of Azospirillum brasilense containing a blend of the AbV5 and AbV6 strains was purchased at the local market (Masterfix Gramíneas, Stoller do Brasil, Campinas, Brazil). These strains derive from a Brazilian nationwide selection program for corn, and allow for a replacement of 25% of the total input of the needed nitrogenous fertilizer [3]. The crop inoculation was manually performed by mixing 100 mL of the commercial product with 1 kg of corn seeds in order to obtain 1.2 × 105 of viable cells seed−1. We tested three N management conditions: (i) full solution, i.e., supplying 2.0 mmol L−1 Ca(NO3)2 (100% N); (ii) partial N supply, i.e., only 75% of Ca(NO3)2 used in the full solution (75% N) and (iii) 75% of N + Azospirillum brasilense inoculation (75% N + Azo).

Under natural light and temperature conditions, the plants were grown in a greenhouse, where the recorded daily mean temperature ranged between 25 °C and 31 °C, and the daily mean air relative humidity ranged between 62% and 78%. After 25 days, we performed the following evaluations: root volume, shoot dry mass, shoot N content, rhizosphere bacterial count (soil and root tissues) and root exudate profiling.

4.1. Plant Growth Parameters

The root volume was determined as the difference between the water volume within a graduated cylinder before and after insertion of the two-times washed fresh roots [30]. The shoot parts were stored in paper bags and dried in a forced ventilation oven at 60 °C for 72 h for subsequent determination of the dry mass and its N content by the Kjeldahl digestion method [32]. Next, after weighting, the dried shoot was ground in a mill for 60 s at 17,000 rpm; then 0.2 g of the flour was placed in test tubes containing 2 g of a catalysis (copper sulfate and selenium powder) and 5 mL of concentrated sulfuric acid. Then, the tubes were gradually heated up to 350 °C on a block digestor for 2.5 h, up to the digestion phase for organic matter. Afterwards, the released ammonia distillation phase was started by a reaction to sodium hydroxide (50%), which was then collected in 4% boric acid solution. Finally, titration was carried out in standard chlorohydric acid solution (1 mol L−1) and the difference of the amount of nitrogen was calculated.

4.2. Rhizosphere Colonization

For bacterial count we used the most probable number technique (MPN) described by Dobereiner et al. [33]. To evaluate the substrate colonization, 10 g of the root surrounded substrate were collected and suspended in 45 mL saline solution (NaCl 0.85%), while, for assessing the internal colonization of internal tissues, roots tips were collected and five times washed with sterile distilled water and surface-disinfected by immersion in 70% ethanol (30 s), followed by an 8-min gentle agitation in 2% sodium hypochlorite. Then, 10 g of this root tissue were macerated and suspended in 45 mL saline solution (NaCl 0.85%).

For both the substrate and the root tissues, the saline solution was submitted to serial dilutions from 10−1 to 10−8. Then, 0.1 mL aliquot of each suspension was inoculated onto a semisolid nitrogen-free
medium [33], which was incubated in the dark at 30 °C for 72 h. After that period, the bacteria concentration was estimated, based on the biofilm formation employing the McCrady probability table [33]. We used the solid culture media NFb that favors the growth of the used strains as well as of other Azospirillum species [33], and its composition was as follow (L⁻¹): malic acid 5.00 g (D-malic acid, ReagentPlus, Sigma-Aldrich, Saint-Louis, MO, USA), KOH 4.00 g (potassium hydroxide BioXtra Sigma-Aldrich), K₂HPO₄ 0.50 g (potassium phosphate dibasic, Sigma-Aldrich), FeSO₄·7H₂O 0.05 g (iron(II) sulfate heptahydrate, ReagentPlus-Sigma-Aldrich), MnSO₄·7H₂O 0.01g (magnesium sulfate heptahydrate, ReagentPlus, Sigma-Aldrich), MgSO₄·7H₂O 0.10g (magnesium sulfate heptahydrate, ReagentPlus, Sigma-Aldrich), NaCl 0.02 g (sodium chloride, ACS, Sigma-Aldrich), CaCl₂ 0.01 g (calcium chloride, Sigma-Aldrich), Na₂MoO₄ 0.002 g (sodium molybdate, Sigma-Aldrich), bromothymol blue 0.5% in 95% methanol 2.00 mL (bromothymol blue, ACS reagent-Sigma-Aldrich), agar 1.8 g (agar powder, Sigma-Aldrich) and NH₄Cl 0.7 g (ammonium chloride, Sigma-Aldrich).

4.3. Exudates Collection and Analyses

The exudates were collected by immersing the whole root system in 100 mL of sterile ultra-pure water (Water Ultrapur, Supelco, Darmstadt, Germany), where it remained for 3 h, under stirring in an orbital shaker (60 rpm). Then, following Kawasaki et al. [20], the liquid solution was filtered (PHENEX RC syringe filter, Allcrom, São Paulo, Brazil) and submitted high performance liquid chromatography (HPLC) to quantify the amino acids, the sugars and the organic acids contents released by each genotype. The HPLC was performed as follow: HP 1100, chromatographic column: 4.0 × 125 mm C18, temperature of column: 40 °C, velocity of flow: 1.0 mL min⁻¹, wavelength: 338 nm, 262 nm (Pro), mobile phase A: 20 mmol sodium acetate solution (sodium acetate for HPLC, LiChropur, Darmstadt, Germany) phase B: 20 mmol (1:2:2 (v/v/v)) of sodium acetate solution: methanol (methanol for HPLC, LiChropur): acetonitrile (acetonitrile for HPC, LiChropur).

4.4. Nitrogenase Activity

To test the effects of each genotype root-released on the Azospirillum growth and the nitrogenase activity, 1 mL of the neat inoculant was inoculated on semisolid NFb medium, supplemented with compounds mimicking the exudates of each genotype under N deprivation (75%). The concentration of each compound used in the media preparation (nmol L⁻¹) was based on their abundance, according to the HPLC results (Appendix A). Neat NFb medium was used as control and, as amino acids source we used L-asparagine (BioReagent, Sigma-Aldrich), L-aspartic acid (BioReagent, Sigma-Aldrich), L-serine (Sigma-Aldrich), L-glutamic acid (ReagentPlus, Sigma-Aldrich), D-valine (BioReagent, Sigma-Aldrich), L-phenylalanine (non-animal source, Sigma-Aldrich), L-threonine (PharmaGrade, SAFC); L-tryptophan (non-animal source, Sigma-Aldrich) and L-Alanine (Sigma-Aldrich). As a source of sugars, we used D-glucose (Sigma-Aldrich), sucrose (BioReagent, Sigma-Aldrich), arabinose (Pharmaceutical Secondary Standard, Supelco), xylose (European Pharmacopoeia Reference Standard, Sigma-Aldrich), D-fructose (BioReagent, Sigma-Aldrich) and D-dalactose (BioReagent, Sigma-Aldrich). The organic acids (citrate, malate, succinate and fumarate) were purchased from Sigma-Aldrich, BioReagent line.

In accordance with Kim et al. [34], we used the acetylene-reduction assay to assess the nitrogenase activity, which is an indirect method that uses the enzyme’s ability to reduce acetylene gas to ethylene. Then, we incubated the NFb media for 48 h at 30 °C in the dark to then reduce their gas phase’s partial pressure of oxygen with a mixture of acetylene-air-N (10:10:80 v/v/v) (Bovine Serum Albumin, Sigma-Aldrich). Finally, after 24 h, the rate of ethylene production using gas chromatograph with a flame ionization detector HP-PLOT/AL203 column, and the MPN of Azospirillum were measured.

4.5. Experimental Design and Statistical Analyses

In the greenhouse, the experiment was conducted in the completely randomized design in a 2 × 3 factorial scheme, with four replications: two genotypes of different responsiveness × three
N managements. From individual HPLC results we further calculated the content of chemotactic compounds, i.e., the concentration of metabolites that serve as bacterial energy source (all organic acids + five six sugars, excluding glucose). When investigating the impact of genotype exudates on *Azospirillum* growth and N fixation, we tested three treatments in the completely randomized design: one neat NFb medium as a control and two supplemented NFb media, each, containing compounds representing the exudates upon 75% N. After checking the normality and homogeneity of variances, the means were compared using the Tukey test at a 5% probability level. To individually compare the content of each class of metabolites in the exudates, the F-test was conclusive.

**Author Contributions:** Conceptualization, L.C.P. and C.d.C.; methodology, L.C.P.; software, C.B.P.; investigation, L.C.P., L.V.C., T.C.M. and R.F.d.S.; resources, E.A.F.O. and A.L.B.; data curation, C.B.P.; writing—original draft preparation, L.C.P.; writing—review and editing, C.B.P., L.V.C. and R.F.d.S.; supervision, C.d.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was financed in part by the Coordenaçãode Aperfeiçoamento de Pessoal de Nível Superior–Brazil (CAPES)–Finance Code 001.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Appendix A**

**Table A1.** Means of asparagine, aspartic acid, serine, glutamic acid, valine, phenylalanine, threonine, tryptophan and alanine of two corn genotypes of high (HResp) and low (LResp) responsiveness to *A. brasilense* under full N mineral supply (100% N), partial N (75% N) as well as partial N supply coupled with inoculation (75% N + Azo).

| Amino Acids (nmol g⁻¹) | Classes       | N Management | HResp       | LResp       | Means    | CV (%) |
|------------------------|---------------|--------------|-------------|-------------|----------|--------|
|                        |               |              | 100% N      | 75% N       | 75% N + Azo |        |
| Asparagine             | 100% N        | 667.55a      | 687.75a     | 677.65      | 5.34     |
|                        | 75% N         | 897.15a      | 886.10a     | 891.63      | 6.35     |
|                        | 75% N + Azo   | 877.68b      | 1286.05a    | 1081.86     | 8.82     |
| Aspartic Acid          | 100% N        | 487.43b      | 557.00a     | 522.21      | 7.34     |
|                        | 75% N         | 115.48a      | 114.90a     | 115.19      | 8.36     |
|                        | 75% N + Azo   | 428.93a      | 290.75b     | 359.84      | 8.23     |
| Serine                 | 100% N        | 149.50a      | 140.18a     | 144.84      | 11.34    |
|                        | 75% N         | 10.30a       | 10.35a      | 10.32       | 10.94    |
|                        | 75% N + Azo   | 133.08a      | 83.05b      | 108.06      | 8.34     |
| Glutamic acid          | 100% N        | 155.75a      | 163.85a     | 159.80      | 6.74     |
|                        | 75% N         | 8.80a        | 9.08a       | 8.94        | 8.56     |
|                        | 75% N + Azo   | 142.60a      | 85.55b      | 114.08      | 9.31     |
| Valine                 | 100% N        | 47.20a       | 45.53a      | 46.36       | 6.45     |
|                        | 75% N         | 5.98a        | 5.70a       | 5.84        | 7.01     |
|                        | 75% N + Azo   | 55.43a       | 30.40b      | 42.91       | 5.64     |
| Phenylalanine          | 100% N        | 34.80a       | 34.83a      | 34.81       | 5.56     |
|                        | 75% N         | 4.05a        | 4.33a       | 4.19        | 6.31     |
|                        | 75% N + Azo   | 32.75a       | 19.48b      | 26.11       | 6.54     |
| Threonine              | 100% N        | 27.90a       | 25.78a      | 26.84       | 5.64     |
|                        | 75% N         | 3.00a        | 2.93a       | 2.96        | 5.67     |
|                        | 75% N + Azo   | 24.98a       | 13.75a      | 19.36       | 9.34     |
| Tryptophan             | 100% N        | 11.63a       | 12.45a      | 12.04       | 8.34     |
|                        | 75% N         | 0.93a        | 0.60b       | 0.76        | 6.45     |
|                        | 75% N + Azo   | 11.80a       | 9.43b       | 10.61       | 7.46     |
| Alanine                | 100% N        | 8.35a        | 8.85a       | 8.60        | 8.74     |
|                        | 75% N         | 0.20b        | 0.45a       | 0.33        | 9.56     |
|                        | 75% N + Azo   | 7.25a        | 7.43a       | 7.34        | 7.45     |

According to the F-test, means followed by the same letter in the row do not differ from each other at a 5% probability level.
Appendix B

Table A2. Means of sugars (glucose, sucrose, xylose, arabinose, fructose and galactose) and organic acids (citrate, malate, succinate and fumarate) of two corn genotypes of high (HResp) and low (LResp) responsiveness to *A. brasilense* under full N mineral supply (100% N), partial N (75% N) as well as partial N supply coupled with inoculation (75% N + Azo).

| N Management | Sugars (nmol g⁻¹) | Organic Acids (nmol g⁻¹) |
|--------------|-------------------|-------------------------|
|              | HResp | LResp | Means | CV (%) | HResp | LResp | Means | CV (%) |
| 100% N       | 771.05 | 858.10 | 814.58 | 6.34 | 198.8a | 107.6b | 153.09 | 6.67 |
| 75% N        | 650.30 | 697.10 | 673.70 | 6.56 | Citrate | 129.23a | 83.90b | 106.56 | 6.34 |
| 75% N + Azo  | 849.30 | 839.35 | 844.33 | 7.34 | 206.03a | 102.68b | 154.35 | 5.34 |
| 100% N       | 106.38 | 51.20  | 78.79  | 5.45 | 140.15a | 72.90b | 106.53 | 6.76 |
| 75% N        | 82.23  | 31.73  | 56.98  | 6.35 | 70.88a | 21.60b | 46.24  | 7.96 |
| 75% N + Azo  | 109.15 | 43.23  | 76.19  | 5.56 | 154.35a | 65.00b | 120.18 | 9.45 |
| 100% N       | 78.05  | 23.25  | 50.65  | 6.65 | 45.91a | 30.33b | 38.12  | 5.68 |
| 75% N        | 23.28  | 8.93   | 16.10  | 5.45 | 11.00a | 10.41b | 10.71  | 7.41 |
| 75% N + Azo  | 75.25  | 23.30  | 49.28  | 6.12 | 120.18 | 65.00b | 120.18 | 9.45 |
| 100% N       | 47.25  | 10.78  | 29.01  | 4.54 | 10.25a | 5.25b  | 7.75   | 4.46 |
| 75% N        | 11.63  | 5.13   | 8.38   | 5.34 | 1.75a  | 0.78b  | 1.26   | 4.89 |
| 75% N + Azo  | 44.40  | 12.55  | 28.48  | 5.67 | 54.48a | 31.25b | 42.86  | 5.01 |
| 100% N       | 54.73  | 8.90   | 31.81  | 6.87 | 10.25a | 5.25b  | 7.75   | 4.46 |
| 75% N        | 7.80   | 7.50   | 7.65   | 4.78 | Fumarate | 1.75a  | 0.78b  | 1.26  | 4.89 |
| 75% N + Azo  | 44.31  | 9.25   | 26.78  | 7.45 | 54.48a | 31.25b | 42.86  | 5.01 |
| 100% N       | 4.85a  | 0.60b  | 2.73   | 6.45 | 11.58a | 11.03b | 11.30  | 5.46 |
| 75% N        | 1.30a  | 0.23b  | 0.76   | 7.56 | 11.58a | 11.03b | 11.30  | 5.46 |
| 75% N + Azo  | 3.58a  | 0.53b  | 2.05   | 7.56 |

According to the F-test, means followed by the same letter in the row do not differ from each other at a 5% probability level.

References

1. Bonfante, P.; Genre, A. Mechanisms underlying beneficial plant—Fungus interactions in mycorrhizal symbiosis. *Nat. Commun.* 2010, 1, 1–11. [CrossRef] [PubMed]
2. Zahran, H.H. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev.* 1999, 63, 968–989. [CrossRef] [PubMed]
3. Bashan, Y.; Bashan, L.E. How the plant growth-promoting bacterium *Azospirillum* promotes plant growth—A critical assessment. *Adv. Agron.* 2010, 108, 77–136. [CrossRef]
4. Cassán, F.D.; Okon, Y.; Creus, C.M. *Handbook for Azospirillum: Technical Issues and Protocols*, 1st ed.; Springer International Publishing: New York, NY, USA, 2015; Volume 10, pp. 978–983.
5. Olanrewaju, O.S.; Glick, B.R.; Babalola, O.O. Mechanisms of action of plant growth promoting bacteria. *World J. Microb. Biot.* 2017, 33, 137–181. [CrossRef] [PubMed]
6. Fukami, J.; Cerezini, P.; Hungria, M. *Azospirillum*: Benefits that go far beyond biological nitrogen fixation. *AMB Express* 2018, 8, 73. [CrossRef]
7. Hungria, M.; Campo, R.J.; Souza, E.M.; Perosa, F.O. Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. *Plant Soil* 2010, 331, 413–421. [CrossRef]
8. Dobereiner, J.; Day, J.M. Associative symbioses in tropical grasses. In *Characterization of Microorganisms and Dinitrogen-Fixing Sites*, Proceedings of the First International Symposium on N₂-fixation; Newton, W.E., Nyman, C.J., Eds.; Washington University Press: Washington, DC, USA, 1976; Volume 2, pp. 518–538.
9. Fukami, J.; Nogueira, M.A.; Araujo, R.S.; Hungria, M. Accessing inoculation methods of maize and wheat with *Azospirillum brasilense*. *AMB Express* 2016, 6, 1–13. [CrossRef]
10. Steenhoudt, O.; Vanderleyden, J. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: Genetic, biochemical and ecological aspects. *FEMS Microbiol. Rev.* 2000, 24, 487–506. [CrossRef]
Plants 2020, 9, 923

11. Greer-Phillips, S.E.; Stephens, B.B.; Alexandre, G. An energy taxis transducer promotes root colonization by Azospirillum brasilense. J. Bacteriol. 2004, 186, 6595–6604. [CrossRef]

12. Vidotti, M.S.; Lyra, D.H.; Morosine, J.S.; Granato, I.S.C.; Quesine, M.C.; Azvedo, J.L.; Fritsche-Neto, R. Additive and heterozygous (dis)advantage GWAS models reveal candidate genes involved in the genotypic variation of maize hybrids to Azospirillum brasilense. PLoS ONE 2019, 14, e0222788. [CrossRef]

13. Vidotti, M.S.; Matias, F.I.; Alves, F.C.; Pérez-Rodrigues, P.; Beltran, G.A.; Burgueño, J.; Crossa, J.; Fritsche-Neto, R. Maize responsiveness to Azospirillum brasilense: Insights into genetic control, heterosis and genomic prediction. PLoS ONE 2019, 14, e0217571. [CrossRef] [PubMed]

14. Azazez, H.A.; Marschner, H.; Römheld, V.; Wittenmayer, L. Effects of a vesicular-arbuscular mycorrhizal fungus and other soil microorganisms on growth, mineral nutrient acquisition and root exudation of soil-grown maize plants. Mycorrhiza 1995, 5, 321–327. [CrossRef]

15. Jones, D.L.; Darrah, P.R. Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. Plant Soil 1994, 166, 247–257. [CrossRef]

16. Thuler, D.S.; Floh, E.I.S.; Handro, W.; Barbosa, H.R. Plant growth regulators and amino acids released by Azospirillum sp. in chemically defined media. Lett. Appl. Microbiol. 2003, 37, 174–178. [CrossRef]

17. Tawaraya, K.; Horie, R.; Shinano, T.; Wagatsuma, T.; Saito, K.; Oikawa, A. Metabolite profiling of soybean root exudates under phosphorus deficiency. Soil Sci. Plant Nutr. 2014, 60, 679–694. [CrossRef]

18. Carvalhais, L.C.; Dennis, P.G.; Fedoseyenko, D.; Hajirezaei, M.R.; Borris, R.; Wieren, N. Root exudation of sugars, amino acids, and organic acids by maize as affected by nitrogen, phosphorus, potassium, and iron deficiency. J. Plant Nutr. Soil Sci. 2011, 174, 3–11. [CrossRef]

19. Bacilo-Jiménez, M.; Aguilar-Flores, S.; Ventura-Zapata, E.; Pérez-Campos, E.; Bouquelet, S.; Zenteno, E. Chemical characterization of root exudates from rice (Oriza sativa) and their effects on the chemotactic response of endophytic bacteria. Plant Soil 2003, 249, 271–277. [CrossRef]

20. Kawasaki, A.; Donn, S.; Ryan, P.R.; Mathiesius, U.; Devilla, R.; Jones, A.; Watt, M. Microbiome and exudates of the root and rhizosphere of Brachypodium distachyon, a model for wheat. PLoS ONE 2016, 11, e0164533. [CrossRef] [PubMed]

21. Alexandre, G.; Greer, S.E.; Zhulin, I.B. Energy taxis is the dominant behavior in Azospirillum brasilense. J. Bacteriol. 2000, 182, 6042–6048. [CrossRef]

22. Van Bastelaere, E.; Lambrechts, M.; Vermeiren, H.; Van Dommelen, A.; Keijers, V.; Proost, P.; Vanderleyden, J. Characterization of a sugar-binding protein from Azospirillum brasilense mediating chemotaxis to and uptake of sugars. Mol. Microbiol. 1999, 32, 703–714. [CrossRef]

23. Sini, K.; Flouri, F.; Balis, C. Influence of Asparagine and Aspartic Acid on Growth of Azospirillum. In Azospirillum VI and Related Microorganisms, 1st ed.; Gallo, M.D., Vanderleyden, J., Zamoroczy, M., Eds.; Springer: Berlin/Heidelberg, Germany, 1995; Volume 37, pp. 331–334.

24. Warren, C.R. Wheat roots exudate a diverse array of organic N compounds and are highly proficient at their recapture. Plant Soil 2015, 397, 147–162. [CrossRef]

25. Lea, P.J.; Sodek, L.; Parry, M.A.J.; Shewry, P.R.; Halford, N.G. Asparagine in plants. Annu. Rev. Plant Biol. 2007, 58, 150–1–26. [CrossRef]

26. Bais, H.P.; Weir, T.L.; Perry, L.G.; Gilroy, S.; Vivanco, J.M. The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol. 2006, 57, 233–266. [CrossRef]

27. Ona, O.; Impe, J.; Prinsen, E.; Vanderleyden, J. Growth and indole-3-acetic acid biosynthesis of Azospirillum brasilense Sp245 is environmentally controlled. FEMS Microbiol. Lett. 2005, 246, 125–132. [CrossRef] [PubMed]

28. Jaeger, C.H.; Lindow, S.E.; Miller, W.; Clark, E.; Firestone, M.K. Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. Appl. Environ. Microbiol. 1999, 65, 2685–2690. [CrossRef] [PubMed]

29. Voragen, A.G.J.; Coenen, G.J.; Verhoeof, R.P.; Schols, H.A. Pectin, a versatile polysaccharide present in plant cell walls. Struct. Chem. 2009, 20, 263–275. [CrossRef]

30. Zeffa, D.M.; Perini, L.J.; Silva, M.B.; Sousa, N.V.; Scapim, C.A.; Oliveira, A.L.M.; Amaral-Júnior, A.T.; Gonçalvez, L.S.A. Azospirillum brasilense promotes increases in growth and nitrogen use efficiency of maize genotypes. PLoS ONE 2019, 14, e0215332. [CrossRef] [PubMed]

31. Vincent, J.M. A Manual for the Practical Study of the Root-Nodule Bacteria, 3rd ed.; IBP Handbook; Blackwell Scientific: Oxford, UK, 1970; pp. 1–164.
32. Lang, C.A. Simple microdetermination of Kjeldahl nitrogen in biological materials. *Anal. Chem.* **1958**, *30*, 1692–1694. [CrossRef]

33. Döbereiner, J.; Baldani, V.L.D.; Baldani, J.I. *Como Isolar e Identificar Bactérias Diazotróficas de Plantas Não-Leguminosas*; EMBRAPA: Brasília, DF, Brazil, 1995.

34. Deaker, R.; New, P.B.; Kennedy, I.R.; Sa, T. Wheat root colonization and nitrogenase activity by *Azospirillum* isolates from crop plants in Korea. *Can. J. Microbiol.* **2005**, *51*, 948–956. [CrossRef]