Comparison of N uptake of maize inoculated with two diazotrophic bacterial species grown under two N levels

Albiane Carvalho Dias, Gabriela Cavalcanti Alves, Thamires Ferreira Rodrigues Da Silva, Bruno José Rodrigues Alves, Leandro Azevedo Santos and Veronica Massena Reis

Department of Soils, Federal Rural University of Rio De Janeiro-UFRRJ, Seropédica, Brazil; Department of Research and Development, Embrapa Agrobiologia, Seropédica, Brazil

ABSTRACT
We investigated how plant inoculation with two diazotrophic bacteria would influence the modulation of root architecture and parameters associated with N uptake rate under high and low NO$_3$⁻ availability. The treatments were arranged in a 2 × 3 factorial design, the first factor being N dose (0.3 and 3.0 mM, or low and high N, respectively) and the second being inoculation with Herbaspirillum seropedicae strain ZAE94 (Hs-ZAE94), Azospirillum brasilense strain Sp245 (Ab-Sp245) and an uninoculated control. The parameters evaluated were: root architecture, mineral N in the growth solution, soluble metabolites, and activities of two N enzymes, dry mass, N accumulation, and the ratio of plant dry mass to N accumulation (NUE). Plant parameters improved with higher N availability. Only under high N, the inoculation with Ab-Sp245 brought about a change in root architecture with an improvement in root surface area, which was in agreement with an increased plant dry matter, N accumulation, and NUE, which was not always observed on either sampling date, 24 and 32 days after transfer to the hydroponic system. The shift in root architecture and the maintenance of N uptake per unit of root area by inoculation with Ab-Sp245 was likely the main effect.

Introduction

Maize is one of the most important staples in developing countries and a key animal feed component worldwide. This crop demands high soil fertility, and generally the supplementation of macro and micronutrients, especially nitrogen (N), are indispensable for high yields. However, efficient N fertilisation is challenging owing to application costs, which limit its use by low-income farmers, as well as the low efficiency of this practice, leading to negative environmental impacts (Lipper et al. 2014).

Biological N$_2$ fixation (BNF) is defined as a sustainable process supplying N to plants (Jensen et al. 2012), with inoculation with selected rhizobium strains successfully used in crops such as soybean and other legume species (Herridge et al. 2008). Yet, direct BNF contribution to cereals and grasses by associative diazotrophic bacteria remains limited, and N fertilisation cannot be dissociated from plant inoculation (Hungria et al. 2010). Some of these diazotrophs produce phytohormones and other substances that stimulate root growth and enhance water and nutrient uptake by plants (Bashan and Bashan 2010). For this reason, the inhibitory effect of mineral N on associative BNF is considered paradoxical (Carvalho et al. 2014). Regardless, the concomitant use of N fertilization and
inoculation of maize and wheat with diazotrophic bacteria has become a recommended practice for improving grain production (Fukami et al. 2016).

Traditionally, inoculation of grasses in South America most often use *Azospirillum brasilense* species, with different strains of the species recommended in different countries (Cassán et al. 2009; Cassán and Diaz-Zorita 2016). In Brazil, commercial inoculants contain the *A. brasilense* strains AbV5 and AbV6, recommended for wheat and maize by Hungria et al. (2010). However, the most studied *A. brasilense* strain is Sp245, which is considered a reference for the investigation of plant growth promotion mechanisms (Somers et al. 2005). It is a well-described diazotrophic strain/species isolated from wheat plants in Southern Brazil by Baldani et al. (1986a).

Another genus capable of promoting plant growth is *Herbaspirillum*, which was first isolated from different grasses (including maize) by Baldani et al. (1986b). It is described as an endophytic bacterium (Monteiro et al. 2012) and currently includes more than 19 described species (LPSN 2020). The ZAE94 strain of *Herbaspirillum seropedicae* was tested on maize under field conditions, producing a 34% increase in plant biomass over uninoculated plants (Alves et al. 2015).

The inoculation of maize plants with *A. brasilense* or *H. seropedicae* has shown positive results, but no single strain provides consistently better performance over the others (Dartora et al. 2016; Breda et al. 2020). Although *in vitro* studies have demonstrated its ability to fix N, root growth promotion has been considered the best explanation for the improved growth of inoculated plants. In this respect, higher use efficiency of N in fertilizer after inoculation of maize plants with these strains was the dominant effect demonstrated by Martins et al. (2017).

Inoculation with associative diazotrophs to improve N use efficiency is gaining increasing importance in crop management, with benefits from BNF possible even under conditions with low N availability (Oliveira et al. 2003). Interestingly, there is not much information from which to infer the possible interactions of the inoculated bacteria and mineral N availability with respect to plant response (Breda et al. 2020), as NO₃⁻ is also a modulator of root architecture and it might play a synergistic role in improving of soil exploration in plants (Forde 2014). In rainfed crop fields, NO₃⁻ is frequently the dominant form of N. Nitrate uptake by the plant occurs at the root level and is a substrate-inducible molecule (2 H⁺/NO₃⁻) requiring symport (Santi et al. 1995), with transport processes differing depending on the availability of NO₃⁻ in soil (Forde 2014; Krapp 2015).

Generally, NO₃⁻ concentrations below a threshold of 0.5 to 1 mM activate high-affinity transport systems (HATS), while low-affinity systems (LATS) take over at higher NO₃⁻ concentrations (Masclaux-Daubresse et al. 2010; Kant 2018). Attention is being drawn to the relatively broader action of transporters involved in low- and high-affinity NO₃⁻ systems, such as the NTR1 and NRT2 transporter families (Wang et al. 2012). Apart from the regulation of absorption, allocation, and translocation of N in the plant, they are involved in signalling hormonal processes that regulate root architecture, repress root elongation, induce lateral root production under high NO₃⁻ levels, or induce the elongation of primary roots while repressing lateral roots under low NO₃⁻ levels (Kant 2018). Hence, the extra doses of phytohormones that the inoculated bacteria could produce do not necessarily align with the internal signals of the plant. This could bring about metabolic changes that enhance or depress nutrient uptake rate, especially of N, consequently affecting plant growth. For this reason, more studies are required to understand how the incrementation of associative and endophytic diazotrophic associations with plants through inoculation would interfere with the process of N uptake and plant growth (Carvalho et al. 2014).

In light of the above, the present study was carried out to investigate how plant inoculation with diazotrophic bacteria described as plant growth-promoters influence the modulation of root architecture and parameters associated with the N uptake rate under high and low NO₃⁻ availability.
Material and methods

Experimental set up

The experiment was carried out in a hydroponic system set up in a greenhouse at Embrapa Agrobiologia (Rio de Janeiro, Brazil). Plastic pots 7.5 dm³ in volume were the experimental units, and were arranged in four complete blocks (repetitions) with a randomized design, to accommodate a 2 × 3 factorial scheme. The main factor comprised the N concentrations in growth solutions of 0.3 and 3.0 mM (low and high, respectively), using ammonium and nitrate in a ~ 1:6 ratio as the N source. The secondary factor was the bacterial inoculant: Herbaspirillum seropedicae strain ZAE94 (selected by Alves et al. 2015), Azospirillum brasilense strain Sp245, and an uninoculated treatment. Pots were filled with 5.5 dm³ of a nutrient solution and kept under intermittent aeration (45 min aeration, 15 min rest), and maize seedlings were grown in them for 36 days.

The triple-hybrid maize cultivar SHS5050 was used, and seeds were pre-germinated in a controlled environment plant growth chambers at 30°C with 12 h of light, followed with the application of the inoculation treatment. Before inoculation, maize seeds were superficially sterilized by immersion in a solution containing NaOCl (0.5%) and Tween 20 (0.01%) for 5 min at 65 rpm and 30°C, followed by 3 washes (5 min each) with phosphate buffer solution (50 mM; pH 7) under the same conditions. After the superficial disinfestation process (Johnston-Monje et al. 2016 – modified), seeds were pre-germinated in 2 cm³ phenolic foam cells in a growth chamber with circulated air at 30°C and a 12/12 h photoperiod cycle for 4 days. After this period, maize seedlings were selected by choosing those with root lengths of approximately 5 cm. The uniform plant stand was inoculated by immersing roots in a bacterial suspension 10⁹ cells mL⁻¹ for 1 h, according to the treatment assigned. Treated maize seedlings were transferred to the hydroponic system and left for 6 days to adapt to the growth medium. During the adaptation period, Hoagland’s modified nutrient solution (1/2 ionic force – supplementary material 1 – SM1) at an N concentration of 1.5 mM (Hoagland and Arnold 1950) was used. After this period, the two N levels (0.3 and 3.0 mM) were applied using the Hoagland’s solution at 1/2 ionic force. The pH of the nutrient solution was monitored daily and maintained at 5.8, by adding drops of 1 M NaOH solution or 1 M HCl as appropriate. Every 4 days, the nutrient solution was renewed (Table 1). A sample of the replaced solution was used for the quantification of ammonium and nitrate concentrations.

Inoculant production

The two bacterial strains were obtained from the Johanna Döbereiner Biological Resources Center (CRB-JD, Embrapa Agrobiologia, Seropédica, RJ, Brazil). The strains were initially grown on Petri dishes containing Nfb solid culture medium (Baldani et al. 2014) to obtain pure isolates. Pure colonies of the isolates were inoculated in test tubes with 5 mL of DYG malate medium pH 6.5 (Baldani et al. 2014) and incubated for 20 h at 30°C on a rotary shaker at 175 rpm. After this stage, 200 µL of the suspension was inoculated into vials containing 500 mL of the same medium and left to grow under the same conditions described above. After growth, bacterial numbers were equalised to 10⁹ cells mL⁻¹ with the aid of a Neubauer chamber, with cell numbers adjusted using a saline solution. After that, the seedlings were inoculated by immersing the maize roots (grown for 4 days as described above) for 1 h in the inoculating solutions in sterilised containers. The control treatment consisted only of growth medium diluted in saline solution.

Bacteria and mineral N monitoring

Two and 24 days after transfer (DAT) of the seedlings to the hydroponic solution (after inoculation), 1 g of roots was sampled for bacterial counting using the N-free semi-solid media JNFb and NFB for the strains ZAE94 and Sp245, respectively (Baldani et al. 2014). At 32 DAT, the same procedure was executed, but 10 g of roots was used. Bacterial counting was performed using the most probable
number technique with the application of McCrady’s Table using three replicates, as described by Baldani et al. (2014).

After gentle pot stirring, a 10 mL sample of the nutrient solution was collected daily, to monitor the N concentration. Analyses were performed to quantify NO$_3^−$ and NH$_4^+$ using UV spectrometry (Olsen 2008) and spectrophotometry of the salicylate complex (Kempers and Zweers 1986), respectively.

**Effect on plant growth and N acquisition**

At 24 and 32 DAT, leaf and root tissues were collected for analysis of soluble metabolites and enzymatic activity (Table 1). The first fully expanded leaf from the leaf apex, without the central vein (leaf+1), was chosen as a standard for aerial tissues. Roots were sampled whole. Leaf sampling was performed between 10 and 12 a.m. to reduce circadian effects on the enzymes analysed. The foliar and root tissues were stored in 80% alcohol for analysis of soluble fractions; 0.5 g was used for immediate determination of nitrate reductase activity (NRa) and 0.5 g was frozen in liquid nitrogen and stored at −80°C for glutamine synthetase enzyme activity (GSa).

**Root architecture**

Sampled roots immersed in 50% ethanol were scanned and characterised by image analyses using WinRHIZO Pro software (Regent Instruments, QC, Quebec, Canada) coupled to an Epson Expression 11000XL LA2400 image scanner, as described by Bauhus and Messier (1999) and Bouma et al. (2000). Roots were laid out in an acrylic container (30 × 40 cm), with water at an approximate depth of 1 cm, and placed onto the scanner. Total length (TL, m), surface area (SA, m$^2$), root volume (RV, m$^3$), and number of tips, forks, and crossings were recorded.

**Nitrate reductase activity (NRa – EC 1.7.1.1)**

Nitrate reductase activity (NRa) was assessed using the *in vivo* method described by Jaworski (1971) and adapted for maize according to Breda et al. (2020). Samples of 0.5 g of root and leaf tissue from the first completely expanded leaf were placed in an incubation solution composed of phosphate buffer, Tween 20, and deionised water. After the samples were subjected to vacuum treatment and subsequently immersed in a water bath, the middle reaction compound of sulphanilamide and N-(1-naphthyl) ethylenediamine was added to the samples for colour development. The amount of NO$_3^−$ produced was measured, to estimate NRa, and expressed as µmol NO$_3^−$ h$^{-1}$ g$^{-1}$ fresh mass. To avoid circadian effects, NRa was blocked during high incidence of daylight, as measured using a luxmeter.

---

**Table 1. Timetable of the hydroponic experiment.**

| DAP | DAT | Steps                  | Activities                                      | Variables analysed                                      |
|-----|-----|------------------------|-------------------------------------------------|--------------------------------------------------------|
| 1   |     | Germination            | Planting of trays                               | -                                                      |
| 4   | 0   | Plantlets development | Inoculation and transplanting to hydroponics    | Counting of the inoculant before and after application using roots |
| 6   | 2   |                        | IF and 1.5 N                                    | Bacterial counting on roots (BC)                       |
| 10  | 6   | N treatment            | N (3 mM and 0.3 mM)                             | BC and plant biometry including root measurement (WinRhizo Pro™). Enzymatic assay and soluble fractions |
| 28  | 24  | H2                     | Harvest 1                                       | -                                                      |
| 36  | 32  | H3                     | Harvest 2                                       | BC and plant biometry including root measurement. Enzymatic assay and soluble fractions |

*DAP- Days after planting. *DAT- Days after transplanting to hydroponics (n = 4). IF – ionic force of the Hoagland’s solution as described in SM1.
**Glutamine synthetase activity (EC 6.31.2)**

Samples of fresh leaf tissue and roots (0.5 g) were treated with liquid nitrogen, to produce a fine powder by manual grinding, to which 1.5 mL of an extraction buffer was added. The extraction buffer comprised 5 mL Tris-HCl 1 M at pH 8.0, 0.2 mL EDTA, 0.5 M pH 8.0, 1.5 g polyvinylpolypyrrolidone, 0.154 g dithiothreitol, 30 mL glycerol, 0.5 mL 200 mM phenylmethysulphonyl fluoride, and 100 mL deionised water. The homogenate was centrifuged at 14,000 rpm in a refrigerated centrifuge, model 5415 R (Eppendorf) at −4°C for 30 min. Total protein content was determined by measuring absorbance at 540 nm, with bovine serum albumin (Sigma) as the standard. Protein extracts containing 50 μg mL⁻¹ of protein were used to quantify GS activity according to the modified method described by Schiavon et al., 2008. For this purpose, an extraction solution was prepared using 2.5 mL of 1 M imidazole-HCl (pH 7.5), 2.5 mL of 0.1 M hydroxylamine-Tris pH 7.5, 0.203 g of MgCl₂ ⋅ 6H₂O, 0.184 g of L-glutamate, 0.138 g of ATP, and 17.5 μL of β-mercaptoethanol, then added to 100 mL of deionised water. An aliquot of 0.45 mL of this buffer was added to 0.5 mL of the protein extract. This mixture was then incubated at 30°C for 30 min. After that, the reaction was terminated by the addition of 0.35 mL of a ferric chloride reagent added to trichloroacetic acid and dissolved in 0.5 N HCl. Absorbance was measured at 540 nm using an Anthos Zenyth 200 ST microplate reader (Biochrom) with standard γ-glutamyl mmono-hydroxamate(Sigma). GSa was expressed in μmol of γ-glutamyl monohydroxamate produced per minute per mg of protein.

**N and sugar soluble fractions**

A 1 g sample of leaf or root tissue was added to 20 mL of ethanol/Milli-Q water™ (80% v/v), crushed for 3 min using a Turratec homogeniser (model TE-102 – Tecnal, Sao Paulo, Brazil) equipped with a 12 mm helix, and filtered through 4 layers of sterilised gauze and filter paper ø = 150 mm (Whatman™). The filtrate was then collected and transferred to a separating funnel with chloroform to form polar and nonpolar phases. The nonpolar phase was discarded and the polar phase was used for the analyses. The levels of nitrate (plus nitrite) were determined according to Miranda et al. (2001), free amino-N content by the ninhydrin method according to Yemm and Cocking (1955), ammonium levels by the colourimetric method (Mitchell 1972; Felker 1977), and soluble sugar contents according to Yemm and Willis (1954).

**Biometric evaluation**

For each harvest, the leaf area index was measured using a LI-3100 C metre (LI-COR™) using all existing leaves. Dry biomass of shoots and roots was quantified after 72 h in a forced air oven at 65°C.

**Total nitrogen (N)**

Root and shoot dry matter were ground in a Wiley mill to <2 mm. The ground material was analysed for N content following the Kjeldhal procedure, as described by Bremner and Mulvaney (1983). The N accumulated in the plant was quantified by the product of N content (g N kg⁻¹ of dry mass) and the corresponding plant dry mass.

The N use efficiency (NUE) was calculated using Eq. (1), as proposed by Xu et al. (2012):

\[
NUE(\text{g}^{-1}) = \frac{\text{Total plant dry mass}}{\text{Applied nitrogen}} \quad (1)
\]
**Statistical analysis**

After verification of homogeneity and error normality, the data were analysed using an ANOVA in R (R Core Team 2020). Significant differences between means were tested using the Fisher LSD test at a 5% probability level. Cell counting in the McCrady’s table was already a probability result produced by the method.

**Results**

**Bacterial counting**

Inoculants of *A. brasilense* Sp245 (Ab-Sp245) and *H. seropedicae* (Hs-ZAE94) containing \( >10^9 \) cells g\(^{-1}\) were used to prepare the respective immersion solutions, which were set up to a population of \( 4.5 \times 10^9 \) cells mL\(^{-1}\). Forty-eight hours after transferring inoculated maize plantlets to the high (3 mM N) and low N (0.3 mM N) growth solutions, the initial populations of both bacterial strains were similar irrespective of N level, at approximately \( 10^9 \) cells g\(^{-1}\) of root fresh weight on average (Suppl. Mat 2). At this time, uninoculated plants presented a natural population of \( 4.5 \times 10^4 \) cells g\(^{-1}\) root fresh weight. However, a decrease in the population of inoculated strains was observed at 24 and 32 DAT for plants growing in low N treatment. Only plants inoculated with Ab-Sp245 in high N treatment maintained slightly higher bacterial populations than the other treatment, \( 1.35 \times 10^7 \) cells g\(^{-1}\) root fresh weight at 32 DAT.

**Root system architecture**

Inoculation with Ab-Sp245 increased total root length (Figure 1(a)), root volume (Figure 1(b)), number of tips (Figure 1(c)), forks (Figure 1(d)), and crossings (Figure 1(e)) in high N treatment, principally at 32 DAT; total root surface area (Figure 1(f)) was also consistently significantly increased by inoculation, in high N treatment, but consistently when Ab-Sp245 was used.

In low N treatment, no significant differences owing to treatment were observed. Plants inoculated with Hs-ZAE94 presented a weak effect (detected at 24 DAT) for root length, volume, and tip number, but these were not significantly different to plants inoculated with Ab-Sp245 or that were uninoculated. In general, low N treatment limited the response of the root architecture to inoculation.

**Nitrogen acquisition by the plants**

Measurements of NH\(_4\)^+ and NO\(_3\)^− in plant growth solution were performed every day for 3 days after the solution was replaced. The daily average for the experimental period revealed fast utilization of NH\(_4\)^+ by plants, with concentrations in growth solution reducing to <0.2 mg N L\(^{-1}\) after 72 h for both N levels (Figure 2(a,b)). After 24 h under high N treatment, plants inoculated with Ab-Sp245 presented the lowest NH\(_4\)^+ concentrations, compared to those inoculated with Hs-ZAE94, but similar to the control (Figure 2(a)). No significant differences were observed for the remaining hours. In low N treatment, the effect of inoculation with Ab-Sp245 on the reduction of NH\(_4\)^+ concentration was observed after 48 h (Figure 2(b)).

The initial concentrations of NO\(_3\)^− were almost 10 times greater than that of NH\(_4\)^+, and utilisation by the plants was mostly steady up to 72 h, for both growth solutions (Figure 2(c,d)). However, in high N treatment, NO\(_3\)^− concentration was significantly lower in the treatment inoculated with Ab-Sp245 (Figure 2(c)) compared to plants inoculated with Hs-ZAE94, but without a significant difference to the control. The effect of inoculation was detected after 48 h in low N treatment with both strains, which reduced NO\(_3\)^− concentration by 57% compared to the control (Figure 2(d)).
**Enzymatic activity**

Nitrate reductase activity (NRa) in plant leaves was much more intense in high N treatment (Figure 3(a)), but no significant differences were observed in roots at each sampling date (Figure 3(b)). Nonetheless, an increase in NRa was evident from 24 to 32 DAT for both plant organs. Plant inoculation with Ab-Sp245 or Hs-ZAE94 did not induce differences in NRa, compared with uninoculated plants.

In high N treatment, the activity of glutamine synthetase (GSa) in leaves was lower when inoculated with either strain at 24 DAT, but this difference was no longer observed at 32 DAT (Figure 3(c)). Unlike the effect on leaves, in low N treatment, inoculation with both strains increased GSa in roots at 32 DAT, even though roots in plants inoculated with Hs-ZAE94 presented GSa similar to uninoculated plants (Figure 3(d)). The sampling date and the N level of the growth solution exerted only discrete effects on GSa in leaves, with even more erratic effects in roots.

**Soluble fractions**

In low N treatment, the differences in the nitrate content of leaves were marginal (<0.5 mmol NO$_3^-$ g$^{-1}$ fresh weight (FW)), without any effect of treatment at 24 or 32 DAT (Figure 4(a)). In high N treatment, plant leaves presented approximately 20 mmol NO$_3^-$ g$^{-1}$ FW for the control and the treatment inoculated with Hs-ZAE94, but for plants inoculated with Ab-Sp245, the concentration decreased to about half that, at 24 DAT. At 32 DAT, nitrate content in leaves decreased to below 5 mmol NO$_3^-$ g$^{-1}$ FW, and no significant difference between treatment was observed. In addition, nitrate in roots increased from nominal levels at 24 DAT to just below 4 mmol NO$_3^-$ g$^{-1}$ FW at 32 DAT at low N, but there was no effect of treatment (Figure 4(b)). However, in high N treatment, nitrate content in roots of the control treatment was approximately 15 mmol NO$_3^-$ g$^{-1}$ FW, and for both of the inoculation treatment, it significantly decreased to 7 mmol NO$_3^-$ g$^{-1}$ FW at 24 DAT. At 32 DAT, all treatment presented nitrate content concentrations of approximately 7 mmol NO$_3^-$ g$^{-1}$ FW.

In contrast to what was observed for NO$_3^-$ in maize tissues, NH$_4^+$ concentration in leaves and roots were less indicative of N levels in the growth solution, with the effects of inoculation only observed in high N treatment (Figure 4(c,d)). In high N treatment, the concentration of NH$_4^+$ in leaves was lower when inoculated with either strain at 24 DAT, although this effect disappeared at 32 DAT. Additionally, NH$_4^+$ concentration in roots did not follow the same behaviour, and was reduced only in inoculations with Ab-Sp245 at 32 DAT.

Soluble sugars in leaves at 24 DAT were almost two times higher in high N than at low N treatment, irrespective of inoculation treatment (Figure 4(e)), but this difference was reduced at 32 DAT. No differences were also observed among treatment for roots at 24 or 32 DAT (Figure 4(f)).

Amino-N concentration in leaves followed a pattern similar to that of the other soluble components presented herein. At 24 DAT concentrations were higher than at 32 DAT in high N treatment, and inoculation resulted in values approximately 50% lower than that of the control (Figure 4(g)). For roots, amino-N concentrations tended to increase from 24 to 32 DAT, but no effect of treatment was observed irrespective of N level in the growth solution (Figure 4(h)).

**Plant growth and N accumulation**

The dry mass accumulation by maize plants growing in high N was more than double that growing in low N treatment, with a significant effect from inoculation with Ab-Sp245, which became more evident at 32 DAT (Figure 5(a)). However, when shoots and roots were analysed separately, the inoculation effect was significant for both sampling dates. At 24 DAT, shoot dry matter of plants inoculated with Ab-Sp245 were higher than that observed for the uninoculated control, but similar to that of plants inoculated with Hs-ZAE94, regardless of the N level in the growth solution (Figure 5(a, b)). At this date, treatment had no effect on root dry mass. Eight days later (32 DAT), shoots of plants inoculated with Ab-Sp245 reached an average of 16.5 g dry matter in high N treatment, 25% higher
than that observed for the uninoculated control or when Hs-ZAE94 was used as an inoculant (Figure 5(a)). Therefore, shoot dry mass accumulation was 57% lower than in high N treatment when plants were inoculated with Ab-Sp245 and growing in low N treatment, but this improved shoot dry mass by 36% compared to the uninoculated control (Figure 5(b)). At 32 DAT, root dry matter also increased in plants inoculated with Ab-Sp245, being 29 and 57% higher than the control in high and low N treatment, respectively (Figure 5(a,b)).

Leaf area and the shoot-to-root ratio did not change from 24 to 32 DAT but increased with the level of N and was unaffected by inoculation (Figure 5(d,e)). Under the stressed condition of low
N treatment, roots represented approximately 30% of plant dry matter, a percentage that was halved in high N treatment.

At 24 DAT, N accumulation by the plants in low N treatment was not modified by the inoculation, contrary to what was observed in high N treatment, in which plants inoculated with Ab-Sp245 accumulated approximately 30% more N than the non-inoculated control. Plants inoculated with Hs-ZAE94 showed an intermediate response (Figure 6(a)). Eight days later, these values did not change, and there were no statistically significant differences between treatment (Figure 6(b)).

With regard to NUE, no statistical differences between treatment were observed at 24 DAT under either growth N levels, but plants growing in low N treatment presented an NUE above 30, whereas approximately half of that figure was estimated for the plants growing in high N treatment (Figure 6(c)). At 32 DAT, the trend was the same, but NUE was close to 50 for non-inoculated plants and 30% more for plants inoculated with Ab-Sp245, suggesting possible BNF contributions. In high N treatment, NUE was approximately 15, without any statistical difference among inoculation treatments (Figure 6(d)).

Figure 2. Analysis of ammonium and nitrate depletion on the hydroponic solution during 72 h (n = 4), pH of the solution was correct every day to 5.8.
Discussion

For cereal crops, the commercial utilisation of inoculants based on diazotrophic bacteria has been focussed on favouring growth promotion by transferring fixed-$N_2$ to the plant and the enhancement of the radicular system through hormonal effects; with the latter also providing aiding exploitation of nutrients and water in soil. To provide such services to the plant, the inoculated bacteria must be persistently associated with the root system (Carvalho et al. 2014). In our study, the inoculation resulted in increased numbers of diazotrophic bacteria after 48 h, approximately four orders of magnitude above the control. Superficial seed sterilisation did not prevent the establishment of a population of $10^4$ cells in the uninoculated treatment, as some diazotrophs (among other bacterial groups) are capable of dwelling in seed tissues (Kandel et al. 2017). For this reason, the possibility of competition between seed-carried populations and inoculated strains cannot be disregarded. The convergence of both bacterial populations to a common level of approximately $10^6$ to $10^7$ cells g FW$^{-1}$ at 32 DAT is a result of the limited microbial population the root environment can sustain. Even under this homeostatic constraint, the root population associated with Ab-Sp245 appeared to be consistently higher in high N, but not low N treatment, confirming that $NO_3^-$ availability is a key factor for the competence of this strain in the root system (Vanbleu and Vanderleyden 2007). In contrast to $A. brasilense$, which is capable of colonising the plant rhizosphere and roots, $H. seropedicae$ is considered to be an obligate or facultative endophyte with a very low survival.
Figure 4. Soluble fraction of maize plants inoculated or not with Ab-Sp245 and Hs-ZAE94 at 24 and 32 DIH under two N levels (0.3 and 3.0 mM N) after 72 h of renewing the nutrient solution. Columns represent mean value and bars represent the standard error of 4 replicates. Letters above bars indicate significant differences at the p < 0.05.
rates outside the plant (James and Olivares 1998). This could indicate that persistence of the latter is dependent on plant genotype; which would explain the better performance of Hs-ZAE94 in a similar experiment but with a different maize genotype, reported by Breda et al. (2020), or the contrasting response of different maize genotypes to inoculation with the same strain under field conditions (Alves et al. 2015). In fact, we could not confirm the proportion of the quantified diazotrophic population that was Hs-ZAE94 or Ab-Sp245, owing to methodological limitations.

As expected, plants growing in high N treatment presented greater roots and shoot dry matter than in low N treatment, which was also the case for the leaf area index. The decrease in shoot-to-root ratio in low N treatment is the result of the plant redirecting photosynthates from branching to...
the elongation of primary roots, in the search for nutrients (Mi et al. 2010; Forde 2014). Even when the shoot-to-root ratio was not altered, inoculation with Ab-Sp245 significantly increased shoot and root dry mass under both N levels, establishing a relationship between bacterial population and plant development.

Biological N₂ fixation is the main characteristic process of diazotrophic bacteria, but in many situations the shift in root architecture by phytohormones produced by the bacteria is the major mechanism responsible for plant growth (Carvalho et al. 2014). In addition to this, our data revealed the influence of N level on root parameters, which is much more potent in high N, and related to the modulation of root architecture by NH₄⁺ and NO₃⁻. At uniform concentrations in the growth medium, similar to the high N concentration in the present study (2.6 mM NO₃⁻), NO₃⁻ functions as a gene signal for auxin production, inducing the formation and elongation of lateral roots. In contrast, under low NO₃⁻ levels, the signalling for hormonal induction of root ramification is repressed in favour of primary root elongation (Jia and von Wirén 2020). Concentrations of NH₄⁺ implemented in this experiment were relatively low for much of the time (<0.4 mM). Hence, if an effect was present, it was on root elongation and branching, but possibly overshadowed by the influence of a six-fold higher NO₃⁻ concentration (Jia and von Wirén 2020). Despite the effect of mineral N on root modulation, consistent changes in root architecture were observed when plants were inoculated with Ab-Sp245, indicating that this practice has the potential to influence the plant even with concurrent management of N fertilisation.

The increased number of root tips, forks, and crossings captured by WinRhizo® provides a reasonable reference for a shift in root branching or lateral root emergence (Krell et al. 2018),
which was observed with inoculations with Ab-Sp245 in high N, in our study. Similar trends were also observed in low N treatment, but without significant differences between treatment.

Root branching and lateral root elongation are dependent on the flux of auxin to lateral root tips, which can be induced when NO$_3^-$ is present, or repressed, depending on the concentration of organic N formed from nitrate reduction and assimilation (Forde 2014). On the other hand, under N starved conditions, the plant may inhibit root branching, which could be the underlying situation in low N treatment. The level of mineral N in growth solutions decreased significantly between solution repositions, especially NH$_4^+$, which seemed preferentially taken up by the plant, leading to N starvation conditions (<100 mM) that could have limited root growth (Jia and von Wirén 2020).

The observed changes in root morphology provided a possible explanation for the highest N accumulation by the plant inoculated with Ab-Sp245 and the enhanced NH$_4^+$ and NO$_3^-$ uptake in high N treatment. Assuming that the difference in total N between 24 and 32 DAT for each treatment represents the total N taken up by maize in the 8-day interval, we estimated the mean N-influx rate by relating the daily N uptake with the respective root surface area. The N-influx rate was greater in high N than low N treatment, as expected. However, it was similar among inoculation treatment (Fisher’s LSD Test, p > 0.05) with a mean of 25.1 ± 1.2 mg N cm$^{-2}$ day$^{-1}$ (data not shown), without interaction between N level and inoculation. Therefore, the influence of Ab-Sp245 on the increased N uptake by the plant is probably related to its effect of increasing root surface area. Under such conditions, a significant contribution to the plant by BNF was unlikely, as the remaining concentration of mineral N after 72 h was relatively high (>1 mM), indicating N surplus. It is well known that high N availability as NH$_4^+$ or NO$_3^-$ is detrimental to N$_2$ fixation, especially in the case of *A. brasilense* and *H. seropedicae* that downregulate nitrogenase through specific mechanisms, such as an increase in glutamine levels (Smercina et al. 2019).

In low N treatment, plant uptake of N through BNF would be more likely to occur. In this study, the levels of GSa in roots were increased by inoculation, principally when Hs-ZAE94 was used, suggesting BNF activity, even though the lower N accumulation by plants and the absence of significant differences between treatment suggest that this process is of relatively minor importance. Following the model proposed by Pii et al. (2019), plant inoculation with *Azospirillum* downregulates HATS, reducing NO$_3^-$ uptake, but compensation by BNF would guarantee the accumulation of N by the plant. Nevertheless, although we believe that BNF could complement N accumulation by the plant, our data indicated a greater NO$_3^-$ uptake from low NO$_3^-$ solution as a result of inoculation, suggesting an upregulation of HATS by Ab-Sp245 and Hb-ZAE94. Although this is in partial disagreement with Pii et al. (2019), we do not have a clear explanation for the increased N use efficiency. Perhaps this combination of processes improved the allocation of absorbed N into plant structures differentially than the uninoculated maize. A hypothesis of a metabolic change driven by association with associative diazotrophic bacteria might be that changes in root exposure to auxin levels (as an effect of inoculation) provoked a feedback response in the NRT family of transporters; with a focus on internal N mobilisation and allocation and sugar metabolism (Wang et al. 2012).

Another effect of inoculation with diazotrophic bacteria would be the improvement of N assimilation by the plant. In high N treatment, reduced levels of NO$_3^-$ in shoot and root tissues in inoculated plants, principally with Ab-Sp245, hypothetically due to increased NRa, owing to greater plant biomass and N accumulation in this treatment. However, our results showed that NRa in plant leaves was only affected by the N level in the growth solution and roots were practically unresponsive to differences in external N levels, without any effect of inoculation treatment. The almost equal availability of soluble sugars in leaves and roots refutes any energy or C-skeleton limitations for all treatment conditions. Hence, there is a possibility that the NRa of Ab-Sp245 may contribute to N acquisition by the plant, as proposed by Ferreira et al. (1987). The lower NH$_4^+$ and amino-N concentrations, together with reduced GSa activity in leaves of inoculated plants in N treatment, seem to indicate that the N absorption process was more dominant in roots when plants were inoculated; as a result of direct NH$_4^+$ supply through the reduction of NO$_3^-$ by the bacteria.
Conclusion

An understanding of how maize reacts to inoculation under varying mineral N availability, especially during the first weeks of growth, is strategic to verifying if inoculants with diazotrophic bacteria can help with N acquisition by the plant. A genotypic effect is recognised (Zeffa et al. 2019) and plant elements with positive responses to N fertilisation are important for the development of cereals with enhanced N uptake efficiency, as millions of tons of N-fertilizer are applied worldwide and less than 50% of these applications can be efficiently used by crops.

Our results demonstrated that a single inoculation with suitable bacteria can improve root architecture and, consequently, an increase in plant biomass and N content. The weaker performance of Hs-ZAE94 can be attributed to a reduced competence of the strain, considering the maize genotype used (Alves et al. 2015). Regardless of the number of processes involved in N acquisition when plants are inoculated with diazotrophic bacteria, the increased root surface area due to a shift in root architecture was the main effect of inoculation with Ab-Sp245. Plant physiological responses which potentially interfered with N uptake rates either complimented bacterial effects or did not significantly affect them.

Acknowledgements

We are greatful the National Council of Scientific and Technological Development (CNPq), the Coordination of Improvement of Higher Education Personnel (CAPES), the Carlos Chagas Foundation for Supporting Research in the Rio de Janeiro State (FAPERJ), the Newton Fund, Biotechnology and Biological Sciences Research Council (BBSRC) and the Brazilian National Council for State Funding Agencies (CONCAP).

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Coordination of Improvement of Higher Education Personnel - CAPES [financing code 001]; National Council of Scientific and Technological Development - CNPq Brazil, for the research fellowships provided to VMR [grant number INCT 456133/2014-2], ACD [grant number 165649/2017-8] and TFRS [grant number 130847/2019-4]; Newton Fund ‘Understanding and Exploiting Biological Nitrogen Fixation for Improvement of Brazilian Agriculture’ [grant number BB/N013476/1], co-funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and the Brazilian National Council for State Funding Agencies (CONCAP).

References

Alves GC, Videira SS, Urquiaga S, Reis VM. 2015. Differential plant growth promotion and nitrogen fixation in two genotypes of maize by several Herbaspirillum inoculants. Plant Soil. 387(1–2):307–321. doi:10.1007/s11104-014-2295-2.

Baldani JI, Baldani VLD, Seldin L, Döbereiner J. 1986b. Characterization of Herbaspirillum seropedicae gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. Int J Syst Bacteriol. 36(1):86–93. doi:10.1099/00207713-36-1-86.

Baldani JI, Reis VM, Videira SS, Boddey LH, Baldani VLD. 2014. The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. Plant Soil. 384(1–2):413–431. doi:10.1007/s11104-014-2186-6.

Baldani V, Alvarez M, Baldani J, Dobereiner J. 1986a. Establishment of inoculated Azospirillum spp. in the Rhizosphere and in Roots of Field Grown Wheat and Sorghum. Plant Soil. 90:35–46.

Bashan Y, Bashan LE. 2010. How the plant growth-promoting bacterium Azospirillum promotes plant growth - a critical assessment. Adv Agron. 108:77–136.

Bauhus J, Messier C. 1999. Evaluation of fine root length and diameter measurements obtained using RHIZO image analysis. Agron J. 91(1):142–147. doi:10.2134/agronj1999.00021962009100010022x.

Bouma TJ, Nielsen KL, Koutstaal B. 2000. Sample preparation and scanning protocol for computerized analysis of root length and diameter. Plant Soil. 218/2(1/2):185–196. doi:10.1023/A:1014905040177.
Breda FADF, Alves GC, Lopez BDO, Aragão AR, Araujo AP, Reis VM. 2020. Inoculation of diazotrophic bacteria modifies the growth rate and grain yield of maize at different levels of nitrogen supply. Arch Agron Soil Sci. 66(14):1948–1962. doi:10.1080/03650340.2019.1702164.

Brencher JM, Mulvaney CS. 1983. Nitrogen-Total. In: Page AL, Miller RH, Keeney DR, editors. Methods of soil analysis. Part 2. Chemical and microbiological properties. Madison (Wisconsin): American Society of Agronomy, Soil Science Society of America; p. 595–624. doi:10.2134/agronmonogr92.2.ed.c31

Carvalho TLG, Balsemão-Pires E, Saraiva RM, Ferreira PCG, Hemery AS. 2014. Nitrogen signalling in plant interactions with associative and endophytic diazotrophic bacteria. J Exp Bot. 65(19):5631–5642. doi:10.1093/jxbерe319.

Cassán F, Díaz-Zorita M. 2016. Azospirillum sp In Current Agriculture: From the Laboratory to the Field Soil. Biol Biochem. 103:117–130.

Cassán F, Perrig D, Sgroy V, Mascriarelli O, Penna C, Luna V. 2009. Azospirillum brasilense Az39 and Bradyrhizobium japonicum E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (Zea mays L.) and soybean (Glycine max L.). Eur J Soil Biol. 45(1):28–35. doi:10.1016/j.ejsoobi.2008.08.005.

Dartora J, Marini D, Goncalves EDV, Guimarães VF. 2016. Co-inoculation of Azospirillum brasilense and Herbaspirillum seropedicae in maize. Revista Brasileira De Engenharia Agricola E Ambiental. 20(6):545–550. doi:10.1590/1807-1929/agriambi.v20n6p545-550.

Felker P. 1977. Microdetermination of nitrogen in seed protein extracts. with the Salicylate-dichloroisocyanurate Color Reaction. Analytical Chemistry. 49(7):1080–1081. doi:10.1021/ac50015a053.

Ferreira MCB, Fernandes MS, Dobereiner J. 1987. Role of Azospirillum brasilense nitrate reductase in nitrate assimilation by wheat plants. Biol Fert Soils. 4:47–53.

Forde BG. 2014. Nitrogen signalling pathways shaping root system architecture: an update. Current Opinion in Plant Biology. 21:30–36. doi:10.1016/j.pbi.2014.06.004.

Fukami J, Nogueira MA, Araujo RS, Hungria M. 2016. Accessing inoculation methods of maize and wheat with Azospirillum brasilense. AMB Express. 3(6):1–13.

Herridge DF, Peoples MB, Boddey RM. 2008. Marschner Review: global inputs of biological nitrogen fixation in agricultural systems. Plant Soil. 311(1–2):1–18. doi:10.1007/s11104-008-9668-3.

Hoagland DR, Arnold DL. 1950. The water-culture method for growing plants without soil. Circular Calif Agric. 347:32–39.

Hungria M, Campo RJ, Souza EM, Pedrosa FO. 2010. Inoculation with selected strains of Azospirillum brasilense and A. lipoferum improves yields of maize and wheat in Brazil. Plant Soil. 331(1–2):413–425. doi:10.1007/s11104-009-0262-0.

James EK, Oliivares FL. 1998. Infection and colonization of sugar cane and other gramineaceous plants by endophytic diazotrophs. Critical Reviews in Plant Sciences. 17(1):77–119. doi:10.1080/0735268991304195.

Jaworski EG. 1971. Nitrate reductase assay in intact plant tissues. Biochemical and Biophysical Research Communications. 43(6):1274–1279. doi:10.1016/S0006-291X(71)80010-4.

Jensen ES, Peoples MB, Boddey RM, Greshoff PM, Hauggaard-Nielsen H, Alves B, Morrison MJ. 2012. Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. Agron Sustain Dev. 32(2):329–364. doi:10.1007/s13593-011-0056-7.

Jia Z, Von Wirén N. 2020. Signaling pathways underlying nitrogen-dependent changes in root system architecture: from model to crop species. J Exp Bot. 71(15):4393–4404. doi:10.1093/jxb/eraa033.

Johnston-Monje D, Lundberg DS, Lazarovits G, Reis VM, Raizada MN. 2016. Bacterial populations in juvenile maize rhizospheres originate from both seed and soil. Plant Soil. 405(1–2):337–355. doi:10.1007/s11104-016-2826-0.

Kandel SL, Joubert PM, Doty SL. 2017. Bacterial endophyte colonization and distribution within plants. Microorganisms. 5(4):77. doi:10.3390/microorganisms5040077.

Kant S. 2018. Understanding nitrate uptake, signaling and remobilisation for improving plant nitrogen use efficiency. Seminars in Cell & Developmental Biology. 74:89–96. doi:10.1016/j.semcdb.2017.08.034.

Kempers AJ, Zweers A. 1986. Ammonium determination in soil extracts by the salicylate method. Communications in Soil Sci Plant Anal. 17(7):715–723. doi:10.1080/00103628609367745.

Krapp A. 2015. Plant nitrogen assimilation and its regulation: a complex puzzle with missing pieces. Curr Opin Plant Biol. 25:115–122. doi:10.1016/j.pbi.2015.05.010.

Krell V, Unger S, Jakobs-Schoenwandt D, Patel AV. 2018. Importance of phosphorus supply through endophytic Metarhizium brunneum for root:shoot allocation and root architecture in potato plants. Plant Soil. 430(1–2):87–97. doi:10.1007/s11104-018-3718-2.

Lipper L, Thornton P, Campbell BM, Baedeker T, Braimoh A, Bwalya M, Caron P, Cattaneo A, Garrity D, Henry K, et al. 2014. Climate-smart agriculture for food security. Nature Climate Change. 4(12):1068–1072. doi:10.1038/nclimate2437.

LPSN – list of prokaryotic names with standing in nomenclature. http://www.bacterio.net/-allnamesdl.html. Accessed 2020 Jan 14.

Martins MR, Jantalia CP, Reis VM, Döwisch I, Polidoro JC, Alves BJR, Boddey RM, Urquiaga S. 2017. Impact of plant growth-promoting bacteria on grain yield, protein content, and urea-15N recovery by maize in a Cerrado Oxisol. Plant Soil. 422(1–2):239–250.
Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gau fichon L, Suzuki A. 2010. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann Bot. 105 (7):1141–1157.

Mi G, Chen F, Zhang F. 2010. Physiological and genetic mechanisms for nitrogen-use efficiency in maize. J Crop Sci Biotech. 10(2):57–63.

Miranda KM, Espey MG, Wink DA. 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide. 5(1):67–71.

Mitchell HL. 1972. Micro determination of nitrogen in plant & tissues. J Aoac. 55(1):01–03.

Monteiro RA, Balsanelli E, Wassem R, Marin AM, Lcc B-S, Schmidt MA, Tadra-Sfeir MZ, Pankievicz VCS, Cruz LM, Chubatsu LS, et al. 2012. Herbaspirillum-plant interactions: microscopical, histological and molecular aspects. Plant Soil. 356:175–196.

Oliveira ALM, Canuto EL, Reis VM, Baldani JI. 2003. Response of micropropagated sugarcane varieties to inoculation with endophytic diazotrophic bacteria. Braz J Microbiol. 34(Suppl.1):59–61.

Olsen KK. 2008. Multiple wavelength ultraviolet determinations of nitrate concentration, method comparisons from the preakness brook monitoring project, October 2005 to October 2006. Water Air Soil Pollut. 187:195–202.

Pii Y, Aldrighetti A, Valentinuzzi F, Mimmo T, Cesco S. 2019. Azospirillum brasilense inoculation counteracts the induction of nitrate uptake in maize plants. J Exp Bot. 70(4):1313–1324.

R Core Team. 2020. R: a language and environment for statistical computing disposable https://www.R-project.org Accessed Jan 29.

Santi S, Locci G, Pinton R, Cesco S, Varanini Z. 1995. Plasma membrane H+-ATPase in maize roots induced for NO₃⁻ uptake. Plant Physiol. 109:1277–1283.

Schiavon M, Ertani A, Nardi S. 2008. Effects of an Alfalfa protein hydrolysate on the gene expression and activity of enzymes of the tricarboxylic acid (TCA) cycle and nitrogen metabolism in Zea mays L. J Agric Food Chem. 56:11800–11808.

Smercina DN, Evans SE, Friesen ML, Tiemann LK. 2019. To fix or not to fix: controls on free-living nitrogen fixation in the rhizosphere. Appl Environ Microbiol. 85(6):e02546–e02518.

Somers E, Ptacek D, Gysegom P, Srinivasan M, Vanderleyden J. 2005. Azospirillum brasilense produces the auxin-like phenylacetic acid by using the key enzyme for indole-3- acetic acid biosynthesis. Appl Environ Microbiol. 71 (4):1803–1810.

Vanbleu E, Vanderleyden J. 2007. Molecular genetics of rhizosphere and plant-root colonization. In: Elmerich C, Newton WE, editors. Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations. Nitrogen fixation: origins, applications and research progress. Vol. 5. Dordrecht: Springer. p. 85-112.

Wang YY, Hsu PK, Tsay YF. 2012. Uptake, allocation and signaling of nitrate. Trends Plant Sci. 17(8):458–467.

Xu G, Fan X, Miller AJ. 2012. Plant nitrogen assimilation and use efficiency. Annu Rev Plant Biol. 63:153–182.

Yemm EW, Cocking EC. 1955. The determination of aminoacid with ninhydrin. Analyst. 80(948):209–213.

Yemm EW, Willis AJ. 1954. The estimation of carbohydrate in plants extracts by anthrone. Biochim J. 57(3):508–514.

Zeffa DM, Perini LJ, Silva MB, Sousa NV, Scapim CA, Oliveira ALM, Amaral-Júnior AT, Gonçalvez LSA. 2019. Azospirillum brasilense promotes increases in growth and nitrogen use efficiency of maize genotypes. PLoS ONE. 14:e0215332.