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Isotopic evidence of significant assimilation of atmospheric-derived nitrogen fixed by *Azospirillum brasilense* co-inoculated with phosphate-solubilising *Pantoea dispersa* in pepper seedling

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ABSTRACT

The contribution of plant growth-promoting bacteria (PGPB) of the genus *Azospirillum* to the plant N budget through biological nitrogen fixation (BNF) is still controversial. The aim of this study was to determine the contribution of BNF by *Azospirillum brasilense* on pepper grown at different N levels, attained using the ^{15}N natural abundance method. To this end, pepper plants were grown in a growth chamber and treated with *A. brasilense* combined with *Pantoea dispersa* and then irrigated at four different N levels (0, 1, 3 and 7 mM NO_3^-). The assimilation of fixed N was clear from the lower $\delta^{15}N$ values observed in bacteria-treated plants compared with those of non-bacteria treated plants. The percentage of BNF-derived N decreased with decreasing NO_3^- levels in the growth medium. BNF contribution to the total nitrogen content of plants was found to be as high as 46%. The results suggest that the bacteria have a potential to supply a considerable amount of N to pepper seedlings, as well as to stimulate plant growth and N uptake when *Azospirillum–Pantoea* treatment is combined with low NO_3^- levels.

1. Introduction

Bacteria of the genus *Azospirillum* are free-living aerobic heterotrophs that fix nitrogen under microaerobic conditions. They belong to the group of plant growth-promoting bacteria (PGPB) and, under certain environmental conditions have a positive effect on plant growth and crop yield (Bashan et al., 1989). The stimulatory effect of *Azospirillum* on plant development has been attributed to diverse mechanisms, including the transfer of fixed nitrogen to the plant as well as the production of phytohormones, mainly indole acetic acid (IAA) and gibberellins, which modify the plant metabolism and morphology and lead to better mineral and water uptake (Bashan et al., 2004). The benefit of nitrogen fixation by *Azospirillum* in agriculture was first established by Döbereiner et al. (1976). However, although N_2 fixation was the first mechanism suggested for PGPB to promote plant growth, this contribution it is still controversial. Some studies in sugar cane (Mirza et al., 2001; Oliveira et al., 2002) and rice (Rodrigues et al., 2008) have pointed to the considerable contribution of N_2 fixation to the plant N budget. On the other hand, low but critical contribution of biological nitrogen fixation (BNF) to wheat plants have been reported (Malik et al., 2002). Finally, several studies have failed to find any significant transfer of nitrogen from BNF to the plant by *Azospirillum* (Mantelin and Touraine, 2004). N_2 fixation by *Azospirillum* is modified by several factors, including the presence of nitrogen in the growth substrate, so that, in general, the bacteria are able to fix N_2 under N-limiting conditions, whereas ammonium, glutamine, nitrate and nitrite have been shown to repress N_2 fixation (Steenhoudt and Vanderleyden, 2000). However, certain diazotrophic bacterial strains of *Azospirillum* spp and *Pantoea agglomerans* were able to fix N_2 in association with wheat, when additional inorganic nitrogen (ammonia or nitrate) was supplied to plants (Ruppel and Merbach, 1997). On the other hand, the transfer of fixed N_2 to the plant is determined by plant/soil-bacteria interactions, which are highly dependent on plant genotype and environmental conditions (Bashan, 1999; Boddey et al., 1991). According to Wood et al. (2001), the inability of the host plant to release carbon to the rhizosphere is a significant constraint in the development of associative N_2-fixing systems since this limits the transfer of newly fixed N_2 to the plants. Van Dommelen et al. (2009) showed that inoculation of wheat plants with the *A. brasilense* mutant 7029, with its drastically reduced glutamine synthetase activity and therefore enhanced capacity to excrete ammonium, increased the plant growth promotion capacity compared with the wild-type strain at suboptimal N fertilization levels.

Since multiple factors affect N_2 fixation and therefore N transfer to plants, quantification of N transfer is necessary to assess whether the ammonium released by the bacteria is used as N

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source. The most suitable methods for determining the extent of fixed N_2 transfer to plants include ^{15}N isotope dilution (ID) and ^{15}N natural abundance (NA) techniques (James, 2000). Oberson et al. (2007) tested both methods and showed comparable results for both techniques. The principal advantage of the NA method over ID is that it does not involve the addition of N and so it is applicable to experiments where no N fertilization is carried out. For example, NA has been extensively used to estimate the contribution of N_2 fixation by bacteria in symbiotic association with leguminous and non-leguminous plants (Chalk and Ladha, 1999; Houngnandan et al., 2008; Malarvizhi and Ladha, 1999). In such a case, non-N_2 fixing plants are needed as reference plants in which ^{15}N natural abundance is assumed to be identical to that of soil-derived N in the N_2-fixing plant (Shearer and Kohl, 1993). This assumption constitutes the main limitation for using this method since the selection of a suitable reference plant that does not fix N_2 and with similar phenology, rooting patterns and growth form to the plants of interest is not always reliable. In non-symbiotic associations of N_2-fixing bacteria with non-fixing plants, such as the *Azospirillum*–pepper plant association studied in this research work, this limitation does not apply since the plant material studied with no added bacteria can be used as a 'perfect' reference plants.

This study focuses on the use of *Azospirillum brasilense* associated with *Pantoea dispersa* in sweet pepper, an important greenhouse crop in the Mediterranean area. Pepper is a long duration crop, with high yields which removes large quantities of plant nutrients, especially nitrogen. In this crop, the vegetative and reproductive stages overlap and, so that a continuous supply of nutrients is needed throughout their life span. The use of N_2-fixing bacteria can therefore be considered as an environmentally friendly practice that helps supply nutrients and minimize losses.

Azospirillum has been shown to be more successful when it is co-inoculated with other microorganims such as phosphate-solubilising bacteria (Bashan et al., 2004). To this end, *A. brasilense* was co-applied with *P. dispersa* whose beneficial effect on plant development arises from its capacity to solubilise phosphorus compounds and help control pathogenic organism (Son et al., 2006). The aim of this study was to determine the contribution of BNF by *A. brasilense* on pepper grown with different NO_3^- supplies using the NA method to evaluate the use of these bacteria as a partial substitute to N chemical fertilization.

2. Materials and methods

Investigation of the contribution of BNF by *A. brasilense* was carried out in two pot experiments with pepper seedlings cultivated in growth chamber. The plant-growth promoting bacteria were applied as Biopron® PMC-3 (Spanish Patent number 2234417, Probelte S.A.), containing bacterial population densities higher than 10^9 CFU g^{-1} of *A. brasilense* M_3 and C_3 immobilized in natural inert support to allow bacterial survival for long periods of time. These strains are deposited in the Spanish Type Culture Collection (CECT) with the numbers CECT-5801 (*P. dispersa*) and CECT-5802 (*A. brasilense*). The population of bacteria present in the inoculant was checked by an external approved laboratory (Biolab S.A., Madrid, Spain), following principles of Good Laboratory Practices (Directive 87/18/EEC) Bacterial population densities were 3.5×10^9 and 4.1×10^9 CFU g^{-1} of *A. brasilense* M_3 and *P. dispersa* C_3, respectively.

2.1. Plant material and growth conditions

Pepper (*Capsicum annuum* L.) seeds of the commercial hybrid 'Quito' (Syngenta Seeds S.A., Barcelona, Spain) were germinated on wet filter paper in a dark chamber at 25 °C for 3 days before being transferred to 1 L pots (1 plant per pot) filled with unfertilized peat ($pH_{H_2O} = 5.65$, conductivity 0.86 dS/m, nutrients (mg/L): 53 K, 164 Ca, 48 Mg and 292 total P, in a 1:10 soil:solution ratio) previously sterilized in an autoclave by fractional sterilizing (tyndelization). The pots were placed in a growth chamber programmed at 25/15 °C for a 16/8 h light/dark cycle, relative humidity of 60/80% and a photon flux density of 500 μmol m^{-2} s^{-1}. The experimental design consisted of controls (non-bacteria treated plants) and plants treated with bacteria (15 g of Biopron® per plant). Both controls and treated plants were irrigated at four different N levels (0, 1, 3 and 7 mM NO_3^-) during the experiment, resulting in a total amount of N applied of 0, 11, 33 and 77 mg N $plant^{-1}$. Nitrate was applied as KNO_3 with a value $\delta^{15}N$ of 10.57‰. In order to assess the possible nitrogen added with the inoculant, the total dissolved N content of Biopron and its $\delta^{15}N$ were determined after extraction with water. In addition, two set of plants were grown under control conditions (with no addition of Biopron) and with 15 g of sterilized-treated BioPron (to inactivate the bacteria), respectively. The later was used to account for the N added with the inoculum and its respective contribution to the plant $\delta^{15}N$. Four replicates per treatment (constituted by three plants per replicate) were placed in a randomized design. Forty-seven days after sowing, the chlorophyll index was measured in the uppermost fully expanded leaves using a portable chlorophyll meter SPAD-502 (Konica Minolta, Osaka, Japan). This tool determines the relative amount of chlorophyll by measuring the absorbance of the leaf in the red and near-infrared regions. Then, the shoot and root were separated, carefully washed, and afterwards weighed. Plant and aqueous extract samples were freeze-dried, weighed and ground into very fine powders for subsequent determination of $\delta^{15}N$ and total N. Nitrogen uptake was calculated as N accumulated in the plant (mg N) per root mass to provide information concerning the N uptake capacity of the root.

2.2. ^{15}N natural abundance method

$\delta^{15}N$ in shoots, roots and freeze-dried aqueous extracts was determined using a ThermoFinnigan FlashEA 1112 Elemental Analyzer (Thermo Fisher Scientific Inc., Milan, Italy), connected to a Finnigan MAT DELTA^{plus} Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific Inc., Bremen, Germany) with a Finnigan MAT ConFlo II connection interphase. Dried samples were weighed (3–4 mg) in tin capsules with a Mettler Toledo MX5 microbalance. The values of the isotope ratio were expressed in δ‰ according to the formula:

$$\delta = \left(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right) \times 1000 \quad (\text{‰})$$

where R is $^{15}N/^{14}N$. Following international convention, the standards used were the nitrogen isotope ratio in air. Samples were referenced against the following materials certified by the International Atomic Energy Agency: IAEA-N-1 ($(NH_4)_2SO_4$), IAEA-N-2 ($(NH_4)_2SO_4$), and IAEA-NO3 (KNO_3).

$\delta^{15}N$ of the whole plants were calculated as indicated by Robinson (2001) by a isotope mass balance according to Eq. (1).

$$\partial^{15}N_{plant} = \frac{\partial^{15}N_{shoot} m_{shoot} + \partial^{15}N_{root} m_{root}}{m_{shoot} + m_{root}} \quad (1)$$

where $\delta^{15}N_{shoot}$ and $\delta^{15}N_{root}$ are $\delta^{15}N$ values of shoot (leaves and stem) and root, respectively, and m_{shoot} and m_{root} the shoot and root dry weights.

The theoretical $\delta^{15}N$ values of non-bacteria treated plants were calculated by considering that the difference in N content between plants irrigated with 0 mM NO_3^- and nitrate-treatedplants resulted