Applied Soil Ecology 107 (2016) 313–319

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Nitrogen metabolism and growth of wheat plant under diazotrophic endophytic bacteria inoculation

Adriana Parada Dias da Silveira[a,*], Valéria Marino Rodrigues Sala[a], Elke Jurandy Bran Nogueira Cardoso[b], Elaine Godoy Labanca[a], Matheus Aparecido Pereira Cipriano[a]

[a] Agronomic Institute/IAC, Center of Soil and Environmental Resources, P.O. Box-28, 13010-970, Campinas, SP, Brazil
[b] University of São Paulo/ESALQ, Department of Soil Science, Pádua Dias Avenue, P.O. Box 11, 13418-900, Piracicaba, SP, Brazil

ARTICLE INFO

Article history:
Received 15 December 2015
Received in revised form 8 July 2016
Accepted 11 July 2016
Available online xxx

Keywords:
Nitrate reductase
Glutamine synthetase
Phytohormones
Plant growth-promoting bacteria

ABSTRACT

The plant growth-promoting bacteria (PGPB) improve nutrient uptake, crop yield, reduce fertilization cost and minimize the environmental pollution by decreasing nitrogen leaching. This study reports the impact of three diazotrophic endophytic bacteria on the metabolism, physiology and growth of wheat (*Triticum aestivum hard* L.) plants. A greenhouse experiment was carried out on an unsterilized soil with three levels of nitrogen fertilizer: no-fertilizer, half-recommended N-fertilizer rate and full recommended N-fertilizer rate; and with the inoculation of spontaneous antiobiotic resistant mutants strains, IAC-AT-8 (*Azospirillum brasilense*), IAC-HT-11 (*Achromobacter insolitus*) and IAC-HT-12 (*Zoogloea ramigera*). All the three bacteria were able to modify, in a specific way, the nitrate reductase and the glutamine synthetase activities and improve the chlorophyll content and promote root and shoot weight. All the strains produced IAA in pure cells culture and showed the presence of gene *nif*H. The N content and the N-use efficiency index were dependent on the endophytic bacteria strain and on the plant tissue assessed. The performance of the above parameters was affected by the nitrogen rate. The different responses suggest that the successful colonization and the growth promotion were achieved by distinct mechanisms. In general, the N metabolism and plant growth were positively impacted by the strains inoculation, mainly *A. insolitus*, strain IAC-HT-11. Moreover, up to now this is the first study showing *Achromobacter insolitus* as an endophyte bacterium able to promote wheat plant growth related to the plant N metabolism. The results confirm that the endophyte strains act as PGPB and have potential to be developed as inoculants to integrate crop management.

1. Introduction

Due to the great importance of nitrogen, many studies have been carried out aiming to maximize plant N-use efficiency, through biological nitrogen fixation (BNF) or soil nitrogen uptake. The BNF is closely related to mechanisms of N_2 assimilation by diazotrophic bacteria freely or in simbiosis with plants (Bhattacharjee and Mukhopadhway, 2008). These bacteria bear the *nif* genes making them able to fix N_2 and play an important role as plant growth-promoting (Zahid et al., 2015). Those plant growth-promoting bacteria (PGPB) can increase the N uptake and assimilation capacity, due to the production of phytohormones (increasing root growth), or by stimulating the NH_4^+ enzymatic production and assimilation (James and Olivares, 1997; Oliveira et al., 2003; Marcos et al., 2016) and the N transport system in plants (Mantelin and Touraine, 2004). Many PGPB, such as *Azospirillum*, induce beneficial changes in the plants by physiological, structural and molecular modifications (Guerrero-Molina et al., 2015).

Inorganic fertilization allows the plant benefits simultaneously from the endophytic activity and from the assimilation of mineral N (Shaharoona et al., 2008). Therefore, a research conducted to study the interaction mechanisms between diazotrophic endophytic bacteria and enzymes of plant metabolism help to select bacteria strains for plant growth- promotion purpose. Strains of the genus *Azospirillum* can synthesize hormones such auxins (Bashan et al., 2004), being the most commonly, indole acetic acid (IAA). A fine-tuned cross talk between all the hormones produced by endophyte and by the host leads to a successful interaction.

* Corresponding author.
E-mail address: apdsil@iac.sp.gov.br (A.P.D.d. Silveira).

http://dx.doi.org/10.1016/j.apsoil.2016.07.005
0929-1393/

The activity of several metabolic routes depends on the way these hormones modulate the expression of several genes (Pieterse et al., 2012). The endophytic bacteria *Azospirillum brasiliense* strain IAC-AT-8, *Achromobacter insolitus* strain IAC-HT-11 and *Zoogloea ramigera* strain IAC-HT-12 (Sala et al., 2008b), obtained from wheat roots, were selected due to their positive performance under gnotobiotic, greenhouse with sterilized substrate and field conditions (Sala et al., 2005, 2007a,b, 2008a). The research reported here was carried out in order to evaluate the possible benefits of these endophytic bacteria, the role they play in the nitrogen metabolism of wheat plant, and how the soil N level affects these processes.

2. Material and methods

2.1. Bacterial strains and culturing conditions

The strains were grown on a liquid culture medium: NFb medium for IAC-AT-8 (*Azospirillum brasilense*, Genbank accession no. DQ386149) and JNFb medium for IAC-HT-11 (*Achromobacter insolitus*, Genbank accession no. DQ386150) and IAC-HT-12 (*Zoogloea ramigera*, Genbank accession no. DQ389143), at 28 °C on a shaker at 150 rpm for 24 h, until reaching the exponential growth phase with 10^9 CFU mL^{-1} (Dobereiner et al., 1995). These strains were marked with antibiotic resistance in order to ensure the appropriated colonization of wheat plants, as follow: IAC-AT-8, 100 μg mL^{-1} neomycine and 50 μg mL^{-1} streptomycin; IAC-HT-11, streptomycin, chloramphenicol and neomycine at 100 μg mL^{-1}; IAC-HT-12, streptomycin and chloramphenicol at 100 μg mL^{-1}.

2.2. Plant cultivation and strains inoculation

Wheat (*Triticum aestivum hard* L.) plants were grown in pots (5 L), containing non-sterilized soil, eutrophic red oxisol, with the following fertility characteristics (0–20 cm deep): NH_4 12.37 and NO_3 3.93 mg kg^{-1}, Mo 41 g dm^3, pH at $CaCl_2$ 5.4, P 76 mg dm^3, K 5.3, Ca 44, Mg 16, H + Al 31, S.B. 65.3, cationic exchange capacity (C.E.C) 96 mmolc dm^{-3}, Base saturation (%) 68, B 0.29, Cu 6, Fe 14, Mn 57 and Zn 4.3 mg dm^3. Nine wheat seeds of the genotype IAC-370 (*Triticum aestivum hard* L.) were sown in each pot, and after germination plants were thinned out to 3 per pot.

Bacterial inoculation was performed after plants were thinned out by adding 5 mL of each bacteria culture medium (strain IAC-AT-8 in Nfb culture medium, and strains IAC-HT-11 and IAC-HT- 12, in JNFb medium) at 10^8 cfu ml^{-1}, to each pot. Control plants were inoculated with the same amount of autoclaved medium. We applied three levels of N fertilizer using urea: unfertilized control (N0), 600 mg N pot^{-1} corresponding to the half-recommended rate (N1) and 1200 mg N pot^{-1} corresponding to the full recommended rate (N2), according to Raij et al. (1996). The N fertilization was divided into 3 applications, 20% at planting, 40% on day 15, and 40% on day 30. The experiment was completely randomized, with 10 replicates per treatment, and it was carried out in a greenhouse for 70 days with a 12-h photoperiod. Plants were watered as needed with autoclaved water.

2.3. Enzymatic analysis and chlorophyll content

After 40 days of the inoculation, the youngest fully-expanded leaves were collected, between 9 and 10 am, to determine the activity of the nitrate reductase, glutamine synthetase and chlorophyll content, with five replicates of each treatment. To determine nitrate reductase activity, 200 mg of tissue-plant discs were placed in 5 mL of buffered substrate (200 mM KNO_3 in 50 mM phosphate buffer, pH 7.5) containing 0.5% Tween-20, according to Reed et al. (1980). The activity was measured in a spectrophotometer at 540 nm. The enzyme glutamine synthetase activity was assessed by the formation of y-glutamilhidroximate from glutamate and hydroxylamine, which replace ammonia (Rhodes et al., 1975). We used 1 g of previously macerated plant tissue in liquid nitrogen, and added 2 mL of extraction buffer (0.05 M Tris-HCl, 1 mM EDTA-NO_2, and 0.02 mL/L mercaptoethanol, pH 7.5) and centrifuged at 15000 rpm at 4° C for 20 min. We added to 300 μL of supernatant sample 0.5 mL of 500 mM sodium glutamate, 0.3 mL 100 mM hydroxylamine, 0.2 mL of 50 mM ATP and incubated at 30° C for 30 min. The reaction was stopped by adding 2 mM ferric chloride (10% $FeCl_3$,24% trichloroacetic acid and 6 N HCl in 1:1:1). The precipitated protein was removed by centrifugation (10 min, 3000 rpm). Absorbance was read at 540 nm. To analyze the chlorophyll content, dimethyl sulfoxide (DMSO) was used to extract the pigments; 7 mL of dimethyl sulfoxide were added to 0.05 g of leaf discs, and incubated at 65 °C for 30 min in sealed tubes and kept in the dark. Absorbance was read at 663 nm and 645 nm. To calculate the chlorophyll content (Chl a + Chl b), the following equations, proposed by Arnon (1949), were used: Chla = [0.0127 × (663 nm) − 0.00269 × (645 nm)] and Chlb = [0.0229 × (645 nm) − 0.00468 × (663 nm)]. Results were expressed in μg mL^{-1} of extract.

2.4. IAA production

We performed the quantification of IAA production according to Kojima (1996) adapted for microbial cultures. The strains were grown in 250 mL of Dygs medium supplemented with 100 μg mL^{-1} of L-tryptophan and incubated for 72 h at 30 °C on orbital shaker in the dark. The samples were centrifuged at 14000 x *g* for 15 min at 4 °C and the supernatant was filtered through Millipore filter at 0.22 μm. An aliquot of 5 mL plus 100 μL [^{3}H] IAA solution used as internal standard was acidified with 1 N HCl at pH 2.5. Each sample was portioned 3 times against 6 mL of diethyl-ether. After the last partition, diethyl-ether was evaporated to dryness and re-suspended in 300 μL of methanol. The samples were analyzed on high performance liquid chromatography (HPLC), using a reverse phase C18 column. Pure IAA was used as standard. A solution of methanol: acetic acid: water (10%: 0.5%: 89%) was used as a mobile phase at the rate of 1 mL min^{-1}.

2.5. Amplification of nifgene

The primers Polf (TGC-GAY-CCS-AAR-GCB-GAC-TC) and Polr (ATS-GCC-ATC-ATY-TCP-CCG-GA) described for *Azotobacter vinelandii* (Poly et al., 2001) and the primers PPf (GCA-AGT-CCA- CCA-CCT-CC) and PPr (TCG-CGT-GGA-CCT-TGT-TG) described for *Azospirillum brasilense* (Reinhardt et al., 2008) were used for the amplification of *nif*H gene. The genomic DNA extraction was done with the Wizard Genomic DNA Purification Kit (Promega). The reaction mixture with 50 μL of final volume contained: 25 μL of PCR Master Mix (Promega), 20 μL of DNAse Free Water, 2 μM of each forward and reverse primer and 1 μL of template DNA (10 ng). The step-up PCR procedure included denaturation at 94 °C for 3 min, follow by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 7 min. Amplification products were then analyzed by electrophoresis in 1.5% agarose gel in 1xTAE buffer.

The data were processed by analysis of variance and Duncan's multiple range test at 5% for means comparisons. The enzymatic activity and plant growth parameters related to N-dose were analyzed by polynomial regression. For these statistical analyses, the Sisvar software was used.