Chapter 27
Protocol for the Quality Control of *Azospirillum* spp. Inoculants

Fabricio Cassán, Claudio Penna, Cecilia Creus, Débora Radovancich, Emilia Monteleone, Inés García de Salamone, Luciana Di Salvo, Isabel Mentel, Julia García, María del Carmen Pasarello Mayans, Lina Lett, Mariana Puente, Olga Correa, Karina Punschke Valerio, Rosana Massa, Melina Catafesta, Alejandro Rossi, Marisa Díaz, Silvia Righes, Susana Carletti, and Enrique Rodríguez Cáceres

Abstract *Azospirillum* has been one of the most studied genera of plant growth promoting rhizobacteria (PGPR) worldwide over the past 50 years. The use of these microorganisms in agriculture practices has been adopted due to their ability to associate in rhizospheric, epiphytic, or endophytic ways with roots and promote whole plant growth or crop productivity. The biological treatment of seeds (inoculation) in more

F. Cassán (✉)
Laboratorio de Fisiología Vegetal y de la Interacción Planta-Microorganismo, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina
e-mail: fcassan@exa.unrc.edu.ar

C. Penna • R. Massa
Stoller Biociencias SRL, CABA, Argentina

C. Creus
Laboratorio de Bioquímica Vegetal y Microbiana. Unidad Integrada Balcarce: Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata-EEA Balcarce, INTA, Balcarce, Buenos Aires, Argentina

D. Radovancich
Laboratorio de Especialidades Medicinales Formosa SA (LAFORMED SA), Formosa, Argentina

E. Monteleone
Nitrasoil Argentina S.A., Quilmes, Buenos Aires, Argentina

I.G. de Salamone • L. Di Salvo
Cátedra de Microbiología Agrícola, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina

I. Mentel
Planta Piloto de Procesos Industriales Microbiológicos (PROIMI-CONICET), San Miguel de Tucumán, Argentina

J. García • M. Puente
Laboratorio de Bacterias Promotoras del Crecimiento Vegetal, IMYZA, INTA Castelar, Castelar, Buenos Aires, Argentina

F.D. Cassan et al. (eds.), *Handbook for Azospirillum*,
DOI 10.1007/978-3-319-06542-7_27

than a hundred species of economic or ecological interest has become a common practice in many countries. In Argentina, the Az39 strain of *Azospirillum brasilense*, belonging to the Culture Collection of the Instituto de Microbiología y Zoología Agrícola (IMYZA) of INTA Castelar, was selected in the 1980s after an intensive program to isolate and identify microorganisms for agriculture, according to their agronomic behavior. Since then, its ability to cover the premise for which it was selected has determined that Az39 is largely adopted by inoculant companies in Argentina with the aim of producing biological products for the treatment of several crops. In this chapter, those methods developed and standardized by the network Red de Control de Calidad de Inoculantes (REDCAI) of the Asociación Argentina de Microbiología (AAM) have been adapted as a guide for the quantification of *Azospirillum* spp. and the detection of contaminating microorganisms in biological products, as two of the most basic and important quality control parameters of inoculants.

27.1 Theoretical Framework

In the past 25 years, plant growth promoting diazotrophic rhizobacteria (PGPR) have been widely used in studies of agricultural microbiology worldwide, due to their ability to improve the yield of crops, preserve agro-ecosystems, and reduce the

M. del Carmen Pasarello Mayans • K. Punschke Valerio
Dirección General de Servicios Agrícolas, Ministerio de Ganadería Agricultura y Pesca, Montevideo, Uruguay

L. Lett
Laboratorio Integrado de Microbiología Agrícola y de Alimentos, Facultad de Agronomía, Universidad Nacional del Centro de la Provincia de Buenos Aires, Azul, Buenos Aires, Argentina

O. Correa
Cátedra de Microbiología Agrícola, Facultad de Agronomía, Instituto de Biociencias Agrícolas y Ambientales, Universidad de Buenos Aires, CONICET, Buenos Aires, Argentina

M. Catafesta
NOVA S.A. Cañada de Gómez, Santa Fe, Argentina

A. Rossi • M. Díaz
Rizobacter Argentina S.A., Pergamino, Buenos Aires, Argentina

S. Righes
Marketing Agrícola S.R.L. Córdoba Capital, Córdoba, Argentina

S. Carletti
Microbiología, Departamento de Ciencias Básicas, Universidad Nacional de Luján, Luján, Buenos Aires, Argentina

E.R. Cáceres
Plantec Laboratorio de Investigaciones Agronómicas, Castelar, Buenos Aires, Argentina

environmental impact of the use of mineral fertilizers. Numerous publications have reported the identification and possible manipulation of PGPR, as well as the association of PGPR with higher plants. The most successful associations include those established between nonlegumes or grasses and rhizobacteria of the genera *Pseudomonas*, *Bacillus*, *Azotobacter*, and *Azospirillum*. Among these, *Azospirillum* is one of the most studied due to its ability to significantly improve the growth, development, and yield of numerous species of agricultural interest worldwide (Bashan et al. 2004). The organisms belonging to this genus colonize mainly the elongation zone and root hairs. Some strains of *Azospirillum* sp. can be found inside plants and are thus called facultative endophytes (Figueiredo et al. 2008). Despite the different forms of interaction, when these diazotrophs are associated with grasses, they lead to yield increases of between 5 and 30 % (Baldani et al. 1983; Okon and Labandera 1994). The ability of these organisms to promote plant growth was initially attributed to the process of biological nitrogen fixation, in both rhizospheric and endophytic conditions, but this model had lower agricultural significance than initially expected. Today, one of the main mechanisms proposed to explain the ability of these organisms to promote plant growth is related to their ability to produce or metabolize plant hormone-like compounds, such as indole acetic acid, cytokinins (Tien et al. 1979), gibberellins (Bottini et al. 1989), and ethylene (Strzelczyk et al. 1994), as well as other molecules that regulate plant growth. Many studies have detailed the beneficial effects of the inoculation with PGPR and the important morphophysiological changes that occur in inoculated plants. However, in many cases, the mechanisms or compounds responsible for generating this response have not been identified.

27.1.1 Inoculants of Azospirillum *spp. in Argentina*

When studies of the genus *Azospirillum* began in Argentina with the aim to introduce them as rhizobacteria of agricultural use, one of the main drawbacks was that there were no local isolates of this microorganism. Following the guidelines proposed by Dr. Johanna Döbereiner from the laboratory of Agrobiology of EMBRAPA, Brazil (Döbereiner and Day 1976), and Dr. Yaacov Okon from the Hebrew University of Jerusalem, Israel (Okon et al. 1977), a considerable number of presumptive isolates of this genus were obtained, but due to the lack of a simple and accurate descriptive method or confirmatory molecular techniques they were only considered as presumptive. With this premise, subsequent studies aimed to isolate bacterial strains of *Azospirillum* from the main crops of Argentina, mainly maize and wheat.

After extensive research on the physiological properties of the presumptive isolates, such as the use of different carbon and nitrogen sources, a defined culture medium, highly useful for recognition and isolation of strains obtained in natural conditions, was developed. The main features to identify the microorganisms in this medium were scarlet red staining of the colonies and rough and flattened structure on their surface. These allowed easy identification and selection even in the presence of other rhizosphere microorganisms. Thus, this medium, proposed by Enrique Rodríguez Cáceres in 1982, was named RC (Fig. 27.2). After that, this new tool allowed isolating a large number of strains from different crops and obtaining a collection of 64 strains, which were lyophilized for conservation at the Instituto de Microbiología y Zoología Agrícola (IMYZA) of the Instituto Nacional de Tecnología Agropecuaria (INTA) Castelar (Buenos Aires, Argentina).

27.1.2 Azospirillum brasilense *Az39*

An intense program was then held at the IMYZA from 1981 until 1995, to select and identify strains of *Azospirillum* sp. potentially applicable in agriculture and to assess their ability to promote growth in cultivable species such as wheat and corn in experimental fields of the province of Buenos Aires (Rodríguez Cáceres et al. 1995). The information obtained allowed confirming the positive effect of inoculation with *A. brasilense* in both crops and selecting the Az39 strain (obtained from the roots of wheat grown in soils of Marcos Juárez, Córdoba, Argentina) and the Cd strain (collection strain) as the ones with best performance within the group, due to their ability to increase yields of both crops from 13 to 33 %. Based on the information generated with this program, and in agreement with the inoculant companies of our country, the Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA) postulated the *A. brasilense* Az39 native strain as that recommended for the production of inoculants initially intended for crops of maize and wheat in Argentina.

27.2 Protocol for the Evaluation of Inoculants Containing *Azospirillum* spp.

27.2.1 Conservation of Samples

In the case that the samples are transported to the laboratory in their original container, during transportation and storage, these must be kept under the conditions recommended by the manufacturer. Rapid temperature changes can cause changes in moisture in the solid products by condensation or induce physiological changes in the microorganisms. Control samples must be kept at room temperature and away from direct sunlight.

27.2.2 Sampling Procedures

Depending on the availability and sample sizes, these can be divided according to the following recommendations:

27.2.2.1 Aqueous Liquid Inoculants

- Sanitize containers with alcohol 70°.
- Homogenize by manual agitation.
- Remove 10 mL of the product under flame or laminar flow, with syringe and needle or sterile pipette.

27.2.3 Enumeration of Viable Cells of Azospirillum spp. Using the Plate Count Technique by Spreading on the Surface in Red Congo (RC) Medium

27.2.3.1 Preparation of the Homogenate

- Shake the container vigorously for 30 s.
- Remove 10 mL of the product under flame or laminar flow, with syringe and needle or sterile pipette.
- Dilute with 90 mL of physiological solution, with the addition of Tween 80 to a final concentration of 0.01 % (0.360 mL of Tween 80 stock solution. Methodological Annex 3) per 90 mL of physiological solution. This is considered the 10^{-1} dilution.
- Shake for 15–20 min in any of the following conditions:
  - Linear stirrer with a 250-mL Erlenmeyer flask (point 3 or similar).
  - Orbital shaker with a 500-mL Erlenmeyer flask at 150 rpm (2.5 of eccentricity).
  - Magnetic stirrer with 250-mL Erlenmeyer flask at 300–350 rpm with a magnetic bar (40 × 7 mm).

27.2.3.2 Preparation of Working Dilutions

- Remove the Erlenmeyer flask from the agitator (the 10^{-1} dilution must be homogeneous, avoiding precipitation or phase separation).
- Remove 1 mL of the 10^{-1} dilution, preferably with automatic pipette, and place it in a test tube containing 9.0 mL of sterile saline.
- This dilution is called 10^{-2}. Vortex for 20 s to mix thoroughly.
- Repeat the previous step until dilution 10^{-7}, as shown in Fig. 27.1.

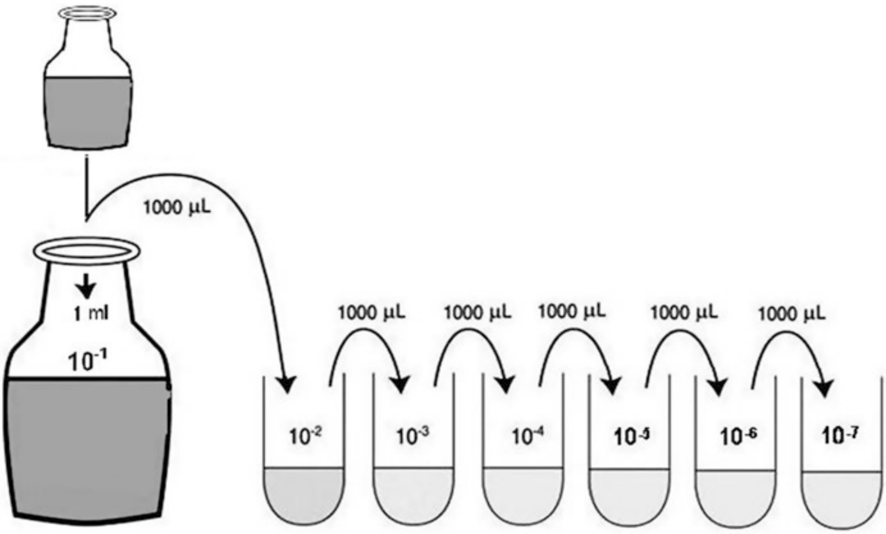

Fig. 27.1 Scheme of the tenfold dilution procedure to estimate viable cell enumeration

27.2.3.3 Dilution Plating

- Perform plate dilutions 10^{-4}, 10^{-5}, and 10^{-6} in triplicate, by spreading of 0.1 mL of the adequate dilution onto the surface of Petri dishes containing RC medium (15–20 mL) according to Rodríguez Cáceres (1982), with a Drigalsky spatula. The spatula to be used in spreading can be built with a glass rod (preferably 4 mm in diameter), a conditioned platinum spatula, a welding electrode, etc.
- Dry the plates in an incubator prior to their use.
- Begin with the highest dilution. Place 0.1 mL in the middle of the plate and, with the Drigalsky spatula previously sterilized, spread the liquid on the surface.
- Leave for 15 min with the agar side down until the liquid is completely absorbed.

27.2.3.4 Incubation

- Incubate the plates upside down in an incubation chamber between 28 and 30 °C. If space is limited and it is necessary to stack plates, take care not to exceed six plates.

27.2.3.5 Reading

- Reading is carried out 4 days after seeding and verified 2 days later.
- Plates showing between 30 and 300 colonies are counted to verify the proportionality between dilutions. If two successive dilutions present plates within these values, the corresponding calculation for each dilution is made according

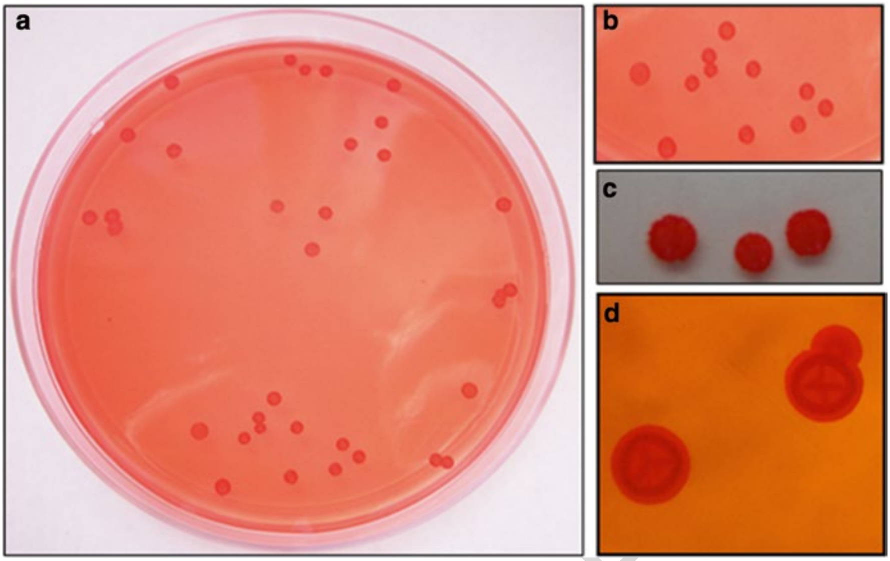

Fig. 27.2 Scarlet red colonies of *Azospirillum* spp. in Petri dishes containing RC culture medium, according to Rodríguez Cáceres (1982). Photographic credit: (**a**, **b**) Mariana Puente, (**c**) Luciana Di Salvo, and (**d**) Lina Lett

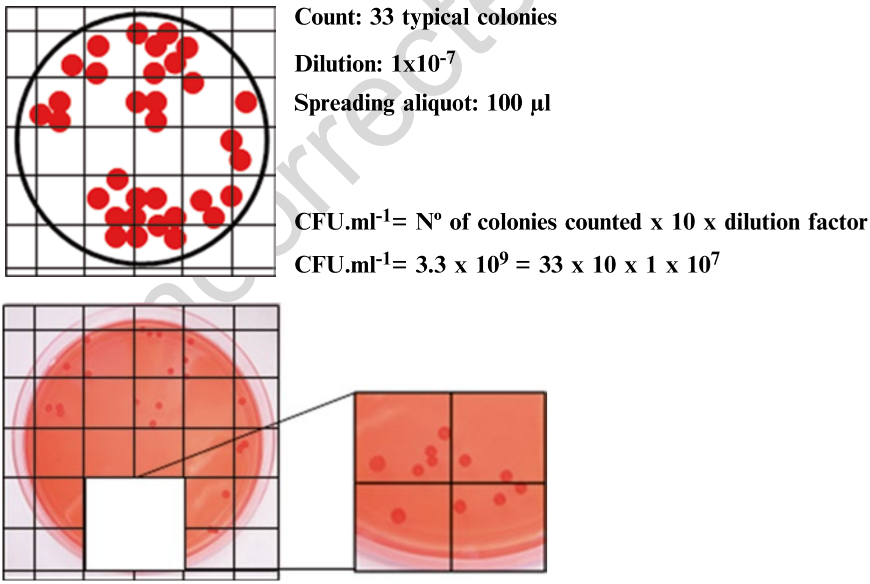

Fig. 27.3 Schematic counting of the colonies in plate. Photographic credit: Mariana Puente

to the formula described below and then the result is averaged. Typical colonies of *Azospirillum* spp. are usually scarlet red, circular, convex, 1–3 mm in diameter, and raised edges, as shown in Fig. 27.2. Count example is shown in Fig. 27.3.

Formula

The result is expressed as colony forming units per milliliter ($CFU \cdot mL^{-1}$) and is calculated as follows:

$$CFU \cdot mL^{-1} = \text{no of colonies counted} \times 10 \times \text{dilution factor}$$

where number of colonies is the average number of colonies present in the three reading plates, aliquot factor is 10 if 0.1 mL is used for spreading on the plates, and dilution factor is the inverse of the dilution in which the colonies are counted to obtain the result.

27.2.4 Reference Procedure for the Detection of Contaminating Microorganisms

To detect contaminating microorganisms, we suggest direct spreading from the container with spatula and by depletion in plates with trypticase soy agar (TSA) for bacteria in general or with Sabouraud agar for saprophytic fungi. In addition, we suggest carrying out Gram staining and observing a direct sample under a microscope.

27.2.4.1 Spreading in Culture Medium for the Detection or Quantification of Contaminants

- Spread on the surface a loaded spatula obtained directly from the sample without burning.
- Incubate the TSA plates in an incubation chamber at 28–30 °C for 48–72 h and the Sabouraud agar plates at 24 °C for 72 h.

27.2.4.2 Observation of Slides Stained with Gram Stain Modified by Hucker

Preparation of Smears

- On a clean slide, place a drop of the material to be analyzed.
- Spread with a spatula and fix by cutting several times the oxidant flame of the burner.
- Proceed to staining.

Staining Technique

(a) Reagents
 Preparation of reagents and solutions. See Methodological Annex 4. Follow the procedure of the manufacturer if you work with a commercial kit.

(b) Smear staining

- Wash with the crystal violet working solution for 1 min.
- Rinse and wash with water.
- Wash with Lugol's iodine solution and leave for 1 min. Rinse and wash with water.
- Discolor with alcohol until total removal of the stain.
- Rinse and wash with water.
- Wash with safranin solution for 2–3 min. Rinse and wash with water.
- Dry and observe under a microscope.

Recording

Seek for the presence of microorganisms with staining and morphological features not compatible with *Azospirillum* sp., i.e., Gram (+) or Gram (−) rods of great size, cocci, and vibrios. Additionally, the cell morphology of *Azospirillum* sp. should be determined due to its cell dimorphism.

27.2.4.3 Observation of a Direct Slide (Fresh)

- On a clean slide, place an aliquot of the material to be analyzed (a drop of the formula if the inoculant is liquid or a drop of dilution 10^{-1} if the inoculant is solid, allowing the particulate to sediment).
- Cover with a coverslip.
- Observe under the microscope.

Recording

Bacteria of the genus *Azospirillum* sp. are mobile Gram (−) rods, but their shape and mobility depend on the physiological state in which the sample is preserved. You should seek for the presence of microorganisms with morphological features compatible to those mentioned and high mobility in spiral form.

27.2.5 Evaluation of pH

Aqueous Liquid Inoculant

pH is measured directly from the product.

Fig. 27.4 Growth of *Azospirillum brasilense* Az39 in NFb semisolid medium and evaluation of the diazotrophic ability in microaerophilic conditions, according to Döbereiner et al. (1995). Photographic credit: Carolina Castaño

27.2.6 Procedure to Evaluate the Microaerophilic and Diazotrophic Capacity of Presumptive Bacterial Colonies

Since it is common to observe dimorphism of colonies in RC medium plates inoculated with *Azospirillum* sp., our strategy to assume the presence of the microorganism is based on the evaluation of the bacterial ability to fix atmospheric nitrogen under microaerophilic conditions, by means of a procedure modified from Döbereiner et al. (1995).

27.2.6.1 Preparation of the Medium

- Prepare semisolid NFb medium as mentioned in point 2 of the Methodological Annex and as it is presented in Fig. 27.4.
- Load 10 mL sterile flasks with 5 mL of freshly sterilized medium.

27.2.6.2 Inoculation of NFb Medium

- Inoculation is carried out from the presumptive bacterial colonies obtained in the RC medium.
- Select a colony with an inoculating loop and seed at depth on the semisolid medium.
- Incubate between 4 and 6 days in an oven at 28–30 °C.
- Presumptive growth is considered in those cases in which the presence of a cloud or halo of growth is observed below the surface of the culture medium (Fig. 27.4).

Methodological Appendix

Agar-RC Medium (Agar–Congo Red)

Component	Amount
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
$FeCl_3 \cdot 6H_2O$	0.015 g
DL-malic acid	5.0 g
KOH	4.8 g
Congo red solution[a]	15.0 mL
Agar	20.0 g
H_2O	1,000 mL

Adjust to pH 7 with 0.1 N of KOH

[a]Congo red solution

Component	Amount
Congo red	2.5 g
H_2O	1,000 mL

NFb Semisolid Medium to Evaluate the Microaerophilic Diazotrophic Activity

Component	Amount
D-malic acid	5.0 g
K_2PO_4H	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.1 g
$Ca_2Cl \cdot 2H_2O$	0.02 g
Bromothymol blue solution[b]	2.0 mL
Micronutrient solution[c]	2.0 mL
$FeCl_3 \cdot 6H_2O$	0.015 g
KOH	4.5 g
Agar	1.8 g
Distilled water	1,000 mL

Adjust to pH 6.8 with 0.1 N of KOH

[b]Bromothymol blue basic solution

Component	Amount
Bromothymol blue	0.5 g
KOH 0.2 N	100 mL

Component	Amount
H_3BO_3	0.286 g
$MnSO_4{\cdot}H_2O$	0.235 g
$ZnSO_4{\cdot}7H_2O$	0.024 g
$CuSO_4{\cdot}5H_2O$	0.008 g
$Na_2MoO_4{\cdot}2H_2O$	0.2 g
H_2O	200.0 mL

[c] Micronutrient solution

Tween 80 Stock Solution at 2.5 % (w/v)

Component	Amount
Tween 80	5 g
Distilled water	200 mL

Gram Staining Technique Modified by Hucker

Crystal Violet Reaction

Crystal violet mother solution (*A*)	
Crystal violet	5 g
Alcohol 95°	25 mL
Ammonium oxalate mother solution (*B*)	
Ammonium oxalate	2 g
Distilled water	200 mL

Prepare solutions A and B separately and use a *working solution* prepared with 4 mL of solution A, 36 mL of distilled water, and 160 mL of solution B.

Lugol's Iodine Solution

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 mL

Safranin Reagent

Safranin	2.5 g
Alcohol 95°	100 mL

References

Baldani VLD, Baldani JI, Döbereiner J (1983) Effects of *Azospirillum* inoculation on root infection and nitrogen in wheat. Can J Microbiol 29(8):869–881

Bashan Y, Holguin G, de-Bashan L (2004) *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). Can J Microbiol 50:521–577

Bottini R, Fulchieri M, Pearce D, Pharis R (1989) Identification of gibberellins A_1, A_3, and Iso-A_3 in cultures of *A. Lipoferum*. Plant Physiol 90:45–47

Döbereiner J, Day JM (1976) Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites. In: Newton WE, Nyman CJ (eds) Proceedings of the 1st international symposium on nitrogen fixation. Washington State University Press, Pullman, pp 518–538

Döbereiner J, Baldani V, Baldani I (1995) Como isolar e identificar bacterias diazotróficas de plantas nao-leguminosas. MAARA-EMBRAPA-CNPAB, Brasilia, p 60

Figueiredo M, Burity H, Stamford N, Santos C (2008) Microrganismos e agrobiodiversidade: o novo desafio para a agricultura. Agrolivros, Guaiba, p 100

Okon Y, Labandera C (1994) Agronomic application of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biol Biochem 26(12):1591–1601

Okon Y, Albrecht S, Burris R (1977) Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Appl Environ Microbiol 33:85–88

Rodríguez Cáceres E (1982) Improved medium for isolation of *Azospirillum* spp. Appl Environ Microbiol 44(4):990–991

Rodríguez Cáceres E, González Anta G, López JR, Pacheco Basurco JC, Parada JL (1995) Response of field grown wheat to inoculation with *Azospirillum brasilense* and *Bacillus polymyxa* in semiarid region of Argentina. Arid Soil Res Rehabil 10:13–20

Strzelczyk E, Kamper M, Li C (1994) Cytocinin-like-substances and ethylene production by *Azospirillum* in media with different carbon sources. Microbiol Res 149:55–60

Tien T, Gaskin M, Hubbell D (1979) Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl Environ Microbiol 37:1016–1024