academicJournals

Vol. 8(31), pp. 2898-2903, 30 July, 2014
DOI: 10.5897/AJMR2014.6974
Article Number: 6A71A2E46414
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Biological nitrogen fixation by diazotrophic bacteria in association with grasses of economic importance

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Received 19 June, 2014; Accepted 14 July, 2014

The capacity to transform atmospheric N_2 into ammonia, which initially enhanced the research, has become just one of the potentialities verified by diazotrophic bacteria, and today, it is known that these microorganisms produce various plant-growth-promoting substances. Due to the enormous data obtained so far, diazotrophic bacteria have the potential to be used as biofertilizers in different crops or grasses, it is expected that the application of this alternative fertilizer will reduce the need for N application in these crops, since an efficient nitrogen use is an essential factor for a sustainable agriculture and the world agribusiness. Investment in research and diffusion of biological nitrogen fixation (BNF), through multidisciplinary and integrated studies can bring great benefit to the planet, increasing food production, reducing the use of fossil fuels and the contamination of water resources, by decreasing the use of external sources of nitrogenous fertilizers. Therefore, this review will show the characteristics of diazotrophic bacteria (nitrogen-fixing), their way of acting with microorganism endophytes and their contribution when in association with grasses of economic importance.

Key words: Endophytic diazotrophic bacteria, inoculant, biological nitrogen fixation (BNF), grass.

INTRODUCTION

Among the various biological processes occurring in nature, the only one that obtains nitrogen (N) that can benefit plants is the biological nitrogen fixation (BNF) (Alves, 2007). The BNF is performed by a limited group of organisms known as diazotrophic, especially bacteria living in association with plants. These bacteria have the nitrogenase enzymatic complex and are able to break the triple bond connecting two atoms of atmospheric nitrogen and to reduce N_2 to ammonia (NH_3^+) (Nunes et al., 2003). The efficient use of atmospheric nitrogen is an essential factor for a sustainable agriculture and the world agribusiness (Alves, 2011).

Associative diazotrophic bacteria, from all sorts of genera and species, have been reported to be in

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association with a great number of non-leguminous plants, in both tropical and temperate climates (Reis Junior et al., 2008). Unlike symbiotic bacteria, they excrete only part of the fixed N directly to the plant with which they are associated, and it is important to point out that the BNF process performed by these bacteria in association with Poaceae plants (grasses) is able to meet only part of the plant demand for N (Hungria, 2011).

The increasing interest in the use of inoculants containing these bacteria that promote the growth and increase plant productivity is due to the high cost of chemical fertilizers and the awareness towards a sustainable, less polluting agriculture (Hungria, 2011). The projections are that in the next years, there will be a substantial increase in the use of fertilizers in Brazil in order to meet the intensification in agriculture. Therefore, it is essential to find alternatives for the more efficient use of fertilizers and, in this context, atmospheric nitrogen-fixing bacteria can play an important and strategic role to guarantee high productivities and low costs, with less dependence on agricultural inputs. It is estimated that the savings resulting from the inoculation with these bacteria in grasses can be of about 2 billion dollars a year (Hungria, 2011).

The possibility of significant increases in nitrogen availability through BNF in grasses, such as rice, wheat, corn, sorghum and sugarcane, has been reported by many authors (Hungria et al., 2010; Dalla Santa et al., 2008; Sala et al., 2005; Reis Junior et al., 2008; Xavier, 2006; Lana et al., 2012; Ferreira et al., 2011).

Based on the aforementioned issues, the use and knowledge on these bacteria that supply N through biological fixation and increase fertilizer use efficiency, as an alternative for nitrogen nutrition in grasses, represent a viable economic strategy and one of the most important tactics in the world nowadays. Thus, this review aims to address the characteristics of diazotrophic bacteria (nitrogen-fixing), their form of action as endophytic microorganisms and their contribution when associated with grasses of economic importance.

DIAZOTROPHIC BACTERIA

Diazotrophic bacteria can be found either distributed in the environment (free-living bacteria) or associated with plants (endophytic bacteria). These microorganisms have nitrogenase enzymatic complex and thus are known as nitrogen fixers.

Unlike rhizobium in symbiosis with leguminous plants, diazotrophic bacteria do not form nodules and colonize from roots to leaves, from the rhizosphere to plant tissue. In this second case, bacteria are called endophytic and it is believed that these are responsible for the gain in N through BNF observed in many crops. The division of the term into facultative and obligate endophytes was proposed to distinguish, respectively, strains able to colonize both the surface and the interior of the root, which colonize the interior and the aerial part of plant tissues without issuing any symptom of pathogenicity (Baldani et al., 1997). These microorganisms can readily deliver to plants the fixed N and other growth-promoting molecules (BaldaniI et al., 1997).

It is known that the associations involving diazotrophic bacteria occur in different interaction degrees and, in many cases, are related to the specificity of interaction between microbe and host plant genetic characteristics (Olivares et al., 1997). Low-specificity diazotrophic bacteria usually colonize superficial areas of the plant, and some strains of *Azospirillum* are found inside plants, thus being referred to as facultative endophytes. These bacteria preferably colonize plant rhizoplane and rhizosphere, due to the accumulation of a variety of organic compounds released by roots through exudation, secretion and deposition (Dobbelaere et al., 2003). Bacteria that preferably colonize internal plant tissue are called obligate endophytes, like *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp., *Azoarcus* spp. and *Burkholderia* spp., and generally have a limited spectrum of host plants (Baldani et al., 1997).

The capacity of colonizing internal plant tissue can confer on these obligate endophytic bacteria ecological advantages over the others. Internal plant tissues provide a more uniform and protected environment for microorganisms than the surface does, where they are exposed to the extreme environmental conditions of temperature, osmotic potential, ultraviolet radiation and microbial competition, which are the most limiting factors to the survival of bacteria along time (Cocking, 2003).

In addition to the capacity for BNF, diazotrophic bacteria associated with grasses stimulate plant growth directly by: a) producing growth hormones like auxins, gibberellins and cytokinins (Dobbelaere et al., 2003; Khaliq et al., 2004; Donate-Correa et al., 2004; Radwan et al., 2004; Creus et al., 2004), which particularly stimulate root growth, increasing root hair density and the emergence rate of secondary roots, which leads to better water and nutrient absorption, increasing plant production and its capacity to tolerate environmental stress (Dobbelaere et al., 1999); b) acting in the solubilization of zinc phosphates and oxides (Rodriguez et al., 2004; Baldotto et al., 2010), excreting organic acids and their associated protons, which directly dissolve the phosphate material and zinc oxides, which later can be made available to plants, since these ions are soluble in acid environments (Vassilev and Vassileva, 2003; Vessey, 2003), and c) increasing nitrogen reductase activity when occurring endophytically in plants (Cássan et al., 2008).

Furthemore, diazotrophic bacteria stimulate plant growth indirectly by: a) acting in the biological control of pathogens (Mariano et al., 2004; Correa et al., 2008) through various mechanisms like production of chitinases, glucanases and antibiosis, and b) synthesis of siderophores, which are molecules secreted by micro-organisms that sequestrate low molecular weight Fe and make it available to plants in the form of a siderophore-Fe^{3+} complex, acting in plant growth and immobilizing the Fe that would be available for phytopathogen proliferation (Vessey, 2003).

In general, it is believed that diazotrophic bacteria promote plant growth through a combination of all of these mechanisms (Dobbelaere et al., 2003).

BIOLOGICAL NITROGEN FIXATION

Although gaseous nitrogen (N_2) constitutes 78% of the atmosphere, no animal or plant can use it as nutrient, because of the triple bond that exists between the two atoms of the N_2, which are one of the strongest bonds that can be found in nature (Hungria, 2011).

For BNF to occur, the N triple bond must be broken and then three atoms of H are bound to each N, forming $2NH_3$ (ammonia). For this, the host plant gives carbohydrates to the microorganism, which in turn breaks N_2 triple bond through a biochemical system (nitrogenase complex), delivering ammonia (NH_3) to the plant in return (Nunes et al., 2003).

It is believed that N_2 biological reduction happens in three steps: 1) reduction of Fe-protein I by ferredoxins; 2) transfer of electrons of Fe-protein I to Fe-Mo protein II; and 3) the actual reduction of the substrate in the active site of the Fe-Mo protein (to which N_2 binds in the presence of the electrons that will be used to break the triple bond) (Nunes et al., 2003).

In the cases of obligate (e.g. *Herbaspirillum* spp.) or facultative (e.g. *Azospirillum* spp.) endophytic bacteria, the same nitrogenase complex converts atmospheric N_2 into ammonia. Nevertheless, unlike symbiotic bacteria, associative bacteria excrete only part of the fixed nitrogen directly to the plant they are associated with; later, bacteria mineralization can contribute to additional N supply to plants (Hungria, 2011).

CONTRIBUTION OF BIOLOGICAL NITROGEN FIXATION TO GRASSES OF ECONOMIC IMPORTANCE

The studies with diazotrophic bacteria related to grasses in Brazil began in early 1950, with the isolation of *Azobacte*r in acid soils from the Baixada Fluminense (Dobereiner, 1953). However, it was only from 1970s on that these bacteria gained worldwide prominence, when the Embrapa researcher Dr. Joana Dobereiner (1924-2000) discovered the biological nitrogen fixation capacity of the genus *Azospirillum* when associated with grasses. Since then, the world scientific community, aiming to identify new bacteria (Govindarajan et al., 2008), has done many studies. Currently, it is known that various species of bacteria are able to establish associations with many grasses (Bhattacharjee et al., 2008).

However, in this review we highlight only the contributions from the biological nitrogen fixation and (or) from the growth-promoting effect, by the synthesis of phytohormones in the main grasses of economic importance.

In studies conducted in India, Govindarajan et al. (2008) observed increases of 40 and 42% in the contribution of BNF in rice plants inoculated with *Burkholderia vietnamiensis* in pots and field, respectively. In 2006, Govindarajam and co-workers verified an increase of 20% in sugarcane dry mass production when inoculated with *B. vietnamiensis* in field conditions, but BNF was not quantified.

In Argentina, García de Salamone and Dobereiner (1996) tested four corn genotypes inoculated with *Azospirillum* regarding BNF contribution, using soil marked with ^{15}N, and observed significant BNF contributions of 58.3 and 48% for the genotypes Dekalb 4D70 and CMS 22, respectively.

In Uruguay, Montanez et al. (2009) tested nineteen corn genotypes under greenhouse conditions, using the ^{15}N-isotope dilution technique, and verified that the percentage of N from the air ranged from 12 to 33%, showing that corn can obtain significant amounts of N through the BNF.

In Egypt, El-Komy et al. (2003) quantified BNF in wheat plants inoculated with the Z-78 strain of *Herbaspirillum seropedicae* in greenhouse conditions, through the ^{15}N-isotope dilution technique, and found accumulations of 24.6 and 26.5% of N from the BNF in leaves and grains, respectively.

In Brazil, Alves (2007), through the ^{15}N natural abundance technique, observed that the inoculation of *H. seropedicae* increases the contribution of N from BNF in 67 and 44% in experiment with corn in the first and second cropping seasons, respectively. In 2011, Alves found that the inoculation with the BR11417 strain of *H. seropedicae* in corn contributed in average with 26% of the N required for crop development.

Xavier (2006) verified that the average contribution of BNF ranged from 7 to 19% using the ^{15}N-isotope dilution technique and from 24 to 53% using the ^{15}N natural abundance technique in ten varieties of sugarcane. Oliveira et al. (2006) evaluated the effect of the inoculation with a bacteria mixture in micropropagated material of the varieties SP 70-1143 and SP 81-3250 using the ^{15}N natural abundance technique in different soil types, and verified that the BNF contributed with up to 38% of the nitrogen. However, there were cases in which the BNF was much lower or null as a function of bacteria mixture inoculation, soil type and fertilization (Oliveira et al., 2006).

Using the strategy of combining inoculation and the application of nitrogen fertilizers, Dalla Santa et al. (2004a) found the possibility of substitution of up to 40% of the recommended N dose for corn in experiments using the RAM-7 and RAM-5 strains of *Azospirillum* sp. For barley, the presence of the inoculant based on the RAM-7 strain of *Azospirillum* sp. substituted 20% of the recommended N fertilization (Dalla Santa et al., 2004b). For wheat, this same inoculation associated with 48 kg ha^{-1} of N promoted productivity similar to that obtained in the treatment with 60 kg ha^{-1} of N, with or without inoculation, suggesting the possibility of partial substitution of inorganic nitrogen fertilization (Dalla Santa et al., 2008). Ferreira et al. (2011) verified that the inoculation with the ZAE94 strain of *H. seropedicae* was able to supply up to 50 kg ha^{-1} of N, depending on the rice cultivar used. Working with corn and wheat in greenhouse conditions, Riggs et al. (2001) verified that the inoculation with *H. seropedicae* promoted increases in dry mass production from 49 to 82%, when applied along with the nitrogen fertilizer. Dobbelaere et al. (2002) verified that the effect of the inoculation with the Sp 245 strain of *Azospirillum brasilense* and the KBC1 strain of *Azospirillum irakense* in corn plants was higher when associated with nitrogen doses. Sabino and co-workers (2012) also observed that the inoculation with the M130 strain of *H. seropedicae* combined with nitrogen fertilization led to better results regarding development and grain production of the rice cultivars IR42 and IAC4440.

Although it is not a consolidated agricultural practice, especially in Brazil, commercial inoculants containing mixture of diazotrophic bacteria were launched in the global market. In the United States, a product named Azo-Green™ was produced and recommended to increase seed vigor, root system establishment, resistance to frost and improvement in plant health (Reis, 2007). In Italy, Germany and Belgium a product named Zea Nit™ containing a mixture of *A. brasilense* (CD strain) and *Azospirillum lipoferum* (BR17 strain), in liquid and peat formulations, was developed by the company Heligenetics and recommended to reduce in 30 and 40% nitrogen application required by the crop. Another product based on *Azospirillum*, strain CRT1, was launched in France.

In Mexico, an inoculant based on *Azospirillum* was developed by the University of Puebla and has been successfully used for corn, wheat and barley. Still in Mexico, a company named Asia commercializes a product for corn and sorghum and another one for wheat and barley, containing a mixture of strains of *A. brasilense* (Reis, 2007). In Argentina, a product named Graminante™, was launched based on calcium carbonate powder, containing a mixture of strains of *Azospirillum*, that is able to increase grain production in nearly 20% (Reis, 2007). In India, many industries produced biofertilizers containing *Azospirillum* for various crops and even *Gluconacetobacter* for sugarcane (Reis, 2007). In Brazil, Embrapa Soybean in partnership with the private company Total Biotecnologias launched a liquid inoculant containing the strains Ab-V5 and Ab-V6 of *Azospirillum brasilense* and many other companies have been developing formulations and performing agronomic efficiency tests with these same strains (Hungria, 2011).

However, various studies have shown both interesting and promising results. In the study of Hungria et al. (2010), the inoculation with the V5 and V6 strains of *A. brasilense* promoted an increase of about 30 and 18% in the average grain yield of corn and wheat, respectively, as compared to the control treatment, without inoculation. Dotto et al. (2010) verified that the inoculation with *H. seropedicae* in corn promoted an increase of 8.6% in the productivity of the hybrid AS 1540. Zilli et al. (2008) showed that the inoculation of corn seeds with *H. seropedicae* contributed significantly to the increase in grain yield for the hybrid BRS1010, but not for the variety BRS 4157. Pedraza et al. (2008), working with rice, also reported that the inoculation with *Azospirillum* spp. increases grain yield as compared to the control (without either N or inoculant).

There are countless positive effects of the inoculation with diazotrophic bacteria in association with grasses. Lana et al. (2012) showed that the inoculation with *A. brasiliense* promoted increase in grain productivity and dry mass of corn plants of 26 and 7.2%, respectively. Reis Junior et al. (2008) observed that the inoculation with *Azospirillum amazonense* led to significant increase in dry mass production and root nitrogen content in corn plants cultivated in greenhouse and harvested 25 days after planting. Braccini et al. (2012) found relative increase in dry mass production with the inoculation of *A. brasilense* in corn seeds. Guimarães et al. (2007), working with rice in greenhouse conditions, observed increases of up to 34% in total shoot nitrogen of plants inoculated with the ZAE 94 strain of *H. seropedicae*, as compared to the absolute control. Guimarães et al. (2010) observed that the inoculation with *H. seropedicae* and *Burkholderia* sp. contributes to increases in dry mass, nitrogen accumulation and grain production of the rice cultivars IR42 and IAC4440. Dobbelaere et al. (2001) verified increases in the contents of N, P and K of corn leaves when working with bacteria of the genus *Azospirillum*; while Francisco et al. (2012) found increase in Zn concentrations of corn leaves when inoculated with *A. brasilense* + 30 kg ha^{-1} of N. Guimarães (2006) observed increases of 64% in grain nitrogen accumulation of rice plants (variety IR 42) inoculated with the ZAE 94 strain of *Herbaspirillum seropedicae* and fertilized with 50 kg ha^{-1} of N, as compared to the control, without either inoculation or fertilization.

Increases in dry mass production and productivity in response to the inoculation can be attributed to the stimulus that diazotrophic bacteria give to root system development, with the increase in root hair density, length, volume and number of lateral roots, resulting in higher capacity to absorb and use water and nutrients, as reported by Huergo et al. (2008).

Despite the encouraging results, the use of inoculants containing these bacteria as a common practice in agriculture requires a careful critical analysis due to the high variability usually observed in the response of different plant genotypes under different soil and climate conditions (Oliveira et al., 2006). Indeed, the reasons for the variability in the responses of grasses to BNF have not been fully elucidated yet. It has been suggested that the plant genotype and environment interaction exercises a decisive role on the efficiency of diazotrófico (Gyaneshwar et al., 2002).

CONCLUSIONS

BNF behaves as an important source of N to the plant system and, with adequate management and the use of BNF-efficient genotypes, it is possible to reduce nitrogen fertilization in grasses.

Investment in research and diffusion of BNF, through multidisciplinary and integrated studies can bring great benefit to the planet, increasing food production, reducing the use of fossil fuels and the contamination of water resources, by decreasing the use of external sources of nitrogenous fertilizers.

Therefore, the exploration and use of BNF in agricultural systems aiming to complement the N from industrial fertilizers is an essential, environmentally friendly and economically viable strategy.

Conflict of Interests

The authors have not declared any conflict of interests.

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