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Azospirillum: Diversity, Distribution, and Biotechnology Applications

Biswajit Batabyal*

Consultant Microbiologist, Serum Analysis Center Pvt. Ltd., Kolkata, (W.B) - India

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Abstract

The genus Azospirillum comprises plant-growth-promoting bacteria (PGPB), which have been broadly studied. The benefits to plants by inoculation with Azospirillum have been primarily attributed to its capacity to fix atmospheric nitrogen, but also to its capacity to synthesize phytohormones, in particular indole-3-acetic acid. Recently, an increasing number of studies has attributed an important role of Azospirillum in conferring to plants tolerance of abiotic and biotic stresses, which may be mediated by phytohormones acting as signaling molecules. Tolerance of biotic stresses is controlled by mechanisms of induced systemic resistance, mediated by increased levels of phytohormones in the jasmonic acid/ethylene pathway, independent of salicylic acid (SA), whereas in the systemic acquired resistance—a mechanism previously studied with phytopathogens—it is controlled by intermediate levels of SA. Both mechanisms are related to the NPR1 protein, acting as a co-activator in the induction of defense genes. Azospirillum can also promote plant growth by mechanisms of tolerance of abiotic stresses, named as induced systemic tolerance, mediated by antioxidants, osmotic adjustment, production of phytohormones, and defense strategies such as the expression of pathogenesis-related genes. The mechanisms triggered by Azospirillum in plants can help in the search for more-sustainable agricultural practices and possibly reveal the use of PGPB as a major strategy to mitigate the effects of biotic and abiotic stresses on agricultural productivity. The development of cultivars with improved nitrogen use efficiency (NUE) together with the application of plant growth-promoting bacteria is considered one of the main strategies for reduction of fertilizers use. Although Azospirillum strains used in commercial inoculants formulations presents diastrophic activity, it has been reported that their ability to produce phytohormones plays a pivotal role in plant growth-promotion, leading to a general recommendation of its use in association with regular N-fertilizer doses. In addition, a high variability in the effectiveness of Azospirillum inoculants is still reported under field conditions, contributing to the adoption of the inoculation technology as an additional management practice rather than its use as an alternative practice to the use of chemical inputs in agriculture.

Keywords: Azospirillum; Diversity; Distribution; Applications

Introduction

Azospirillum is a Gram-negative, microaerophilic, non-fermentative and nitrogen-fixing bacterial genus from the family of Rhodospirillaceae. [1, 2] Azospirillum bacteria can promote plant growth [3]. Azospirillum brasilense is a well studied, nitrogen-fixing (diazotroph), genetically tractable, Gram-negative, alpha-proteobacterium bacterium [4]. A. brasilense is able to fix nitrogen in the presence of low oxygen levels, making it a microaerobic diazotroph. Originally isolated from nitrogen poor soils in the Netherlands in 1925,

***Corresponding Author**
E.Mail: biswajit.batabyal@gmail.com

It is widely found in the rhizospheres of grasses around the world where it confers plant growth promotion [5, 6].
Whether growth promotion occurs through direct nitrogen flux from the bacteria to the plant or through hormone regulation is debated [7, 8].The two most commonly studied strains are Sp7 (ATCC 29145) and Sp245.
The genome of A. brasilense Sp245 has been sequenced and is 7Mbp in size and spread across 7 chromosomes. The high GC content (70%) makes it challenging to engineer. [9] Sp245 can be transformed with OriV origin of replication plasmids through conjugation and elect roporation. The strain is natively resistant to both spectinomycin and ampicillin antibiotics. Ka namycin resistance is used as a selectable marker. A. brasilense has a high evolutionary adaptation rate driven by codon mutation and transposon hopping.
A strain originally classified as Roseomonas fauriae was reclassified as A. brasilense. It was first isolated from a hand wound of a woman in Hawaii in 1971, and was named for Yvonne Faur "for her contributions to public health bacteriology and, specifically, for her contribution to the recognition of pink-pigmented bacteria [10, 11].
The genus Azospirillum comprises free-living, nitrogen-fixing bacteria that are known as plant growth-promoting rhizobacteria (PGPR), which can colonize, by adhesion, the root surface or the intercellular spaces of the host plant roots. The potential role of the PGPR in association with economically important cereals and other grasses is to promote plant growth by several mechanisms including nitrogen fixation and phytohormone production [12]. Several species of Azospirillum are able to secrete phytohormones such as auxins, gibberellins, cytokinins, and nitric oxide as signals of plant growth promotion [13, 14]. Azospirillum genomes, as previously suggested for various strains, are larger and are comprised of multiple replicons indicating a potential for genome plasticity [15]. Genomic rearrangements can occur spontaneously where replicons can be lost upon the formation of new megaplasmids [16, 17]. Moreover, genome sequencing of some Azospirillum species revealed that significant part of the genome has been horizontally acquired [17]. Up until now, 16 Azospirillum species have been characterized; however complete genomic sequences of only Azospirillum brasilense, Azospirillum lipoferum, Azospirillum sp. B510, and a draft of Azospirillum amazonense genome have been published [18].
Azospirillum amazonense was found to be associated with the roots and rhizosphere of several grasses including sugar-cane, maize, sorghum, and rice revealing a broad ecological distribution in Brazil. Studies revealed that A. amazonense is phylogenetically closer to Rhodospirillum centenum and Azospirillum irakense than to A. brasilense. Unlike other Azospirillum strains, A. amazonense can grow in the presence of sucrose as sole carbon source and is also better adapted to soil acidity, which offers the bacterium additional advantages for colonization of plant root tissue in acid environments [19, 20]. Moreover, A. amazonense genomic analyses revealed the presence of genes not commonly distributed in other Azospirillum species such as those responsible for the utilization of salicin as carbon source (similar to A. irakense) and a gene cluster (RubisCO) implicated in carbon fixation. However, our understanding of phytohormone production in A. amazonense is still incomplete.
The genomic plasticity of A. amazonense is probably related to the versatile gene repertoire present in the genome of this bacterium suggesting that horizontal gene transfer may have an impact on the adaptation and evolution of this species. Gene organization and phylogenetic analysis demonstrated that genes coding for proteins responsible for the nitrogen fixation process, carbon fixation (RubisCOs), and molecular hydrogen oxidation (hydrogenases) is more closely related to Rhizobiales members than to related species.
The importance of A. amazonense genetic variability, an in silico comparative genomic analysis using subtractive hybridization was performed using total coding sequences (CDS) from A. amazonense to compare with genomes of closely related bacteria. The analysis of conserved and specific A. amazonense coding sequences indicated features that distinguished A. amazonense from other Azospirillum species.

Furthermore, the specific interesting features related to phytohormone production may provide several cues to establish A. amazonense pathways for auxin biosynthesis.

Inoculation technology with plant growth-promoting bacteria (PGPB) has been presented worldwide as an important tool for reaching sustainability in agriculture due to its low environmental and production costs compared with industrial inputs. However, different from symbiotic relationships, where plant-bacteria interactions have been widely exploited and are relatively well understood, the associative interactions driven by PGPB are discreet and elicited by factors that have only recently started to be clarified [21,22]. It is not difficult to realize that the broad adoption of PGPB inoculation as regular agricultural practice is somehow impaired by the lack of scientific knowledge regarding the ecology, physiology and biochemistry of associative plant-bacteria interactions. Although feasible, the replacement of chemical inputs in commercial agriculture with bioproducts developed from and based on the rational exploitation of plant-microbe natural relationships, such as nutrient-provider bacteria in substitution for soluble fertilizers, or biocontrol agents in substitution for pesticides, remains a major challenge [23]. In this sense, efforts to strengthen inoculation technology in non-leguminous crops with PGPB need to incorporate a broader understanding of the determinants of bacterial rhizocompetence and competitiveness necessary to successfully achieve a plant-PGPB interaction. In the same way, one must consider the physiological status of the inoculated microorganisms such that high viability is maintained under adverse conditions found in soil and/or during storage. Such challenges are magnified if one considers the identification of PGPB with high biotechnological potential in distinct phylogenetic clusters, and the low probability of finding conditions to produce high-quality inoculants that can be universally applied for any PGPB [24].

Commercial inoculants carrying PGPB are generally available as dry or liquid formulations of different organic and/or inorganic materials, which may be prepared with cells grown in a liquid culture medium or via the direct use of bacterial broth for producing liquid inoculants, or obtaining dehydrated cells that may be incorporated in a solid or liquid carrier [25, 26, and 27]. Liquid inoculants simplify both the industrial production and the field application, although compared with solid formulations, such as peat- or polymer-based inoculants, bacteria in liquid inoculants appear to be more sensitive to stressful conditions and can exhibit decreased viability when used on seeds or soil [28]. Effectiveness of PGPB inoculants has been improved by immobilization of inoculant cells in polymeric carriers, such as alginate and starch foam [29, 30]. Thus, the actual demand is for improved liquid inoculants formulations, which are replacing peat-based inoculants and currently comprise ~80% of doses sold for soybean crops in Brazil [31]. While the technical criteria required to produce high-performance peat-based inoculants are well defined, these same criteria are treated as industrial secrets or proprietary information for liquid inoculants. Even considering the presence of high-performance Azospirillum-based liquid inoculants on the market, the information regarding the composition of these inoculants is mostly limited to that presented on the product label, which increases the difficultly of conducting a thorough scientific analysis of the role of each ingredient in the formulation and its effects on the final quality of the product applied in the field [24]. This may be best exemplified by inoculants formulations using the PGPB Azospirillum brasilense, which are prepared from different bacterial strains and are available in a wide variety of commercial products worldwide; variations in their performance under field conditions are still reported.

The diazotroph A. brasilense is considered a model PGPB, and a great amount of information regarding the physiology of its growth and development has been published [32]. However, it is unclear when this available knowledge is in fact applied in the inoculants industry. Quality control assessments of agricultural inoculants are commonly defined by the presence of contaminants and the population density of viable cells in the final product throughout its shelf-life, and determining any information about the physiological state of the inoculants PGPB strain in a commercial product is not required. To consider inoculants production solely in terms of the yield of microbial biomass from a specific strain has the potential to produce poor-quality inoculants, which plays against the broad adoption of PGPB inoculation as an alternative to conventional agronomical practices. To implement this new paradigm in modern agriculture, commercial inoculants formulations should be produced with bacterial cells at a high population density and in high physiological state to enable them to face adverse conditions that occur both during storage (shelf life) and at the time of its use (on seeds and in soil) [24,28]. It is not difficult to realize that the broad adoption by the industrial and regulatory agencies of the available PGPB physiology knowledge, as well as the implementation of research studies aimed to better understand the physiological characteristics for which no such knowledge is yet available, should result in better inoculants with higher field performance.

At least in Brazil, the inoculation of cereals with diazotrophic PGPB, such as maize inoculation with A. brasilense, is considered an additional practice and its adoption occurs under the application of regular amounts of N fertilizer that can exceed 200 kg ha^{-1}. Concerns leading to the distrust of the natural nitrogen inputs provided by diazotrophic PGPB substituting at least part of the nitrogen demand in commercial crops reflect the lack of scientific information available for the commercial inoculants formulations. In addition, there is a huge variability in inoculation efficiency as a result of the use of low-quality inoculants [25, 28]. Maize inoculants are mainly applied over seeds before sowing, which is an additional practice that presents risks of lowering the germination rate if the seeds are mechanically damaged during this process. Furthermore, inoculating seeds places the bacteria in contact with the pesticides and agrochemicals commonly found covering commercial seeds, which are then sown close to the fertilizers applied in the soil. Intending to determine whether conditioning the PGPB A. brasilense Ab-V5 to accumulate high amounts of polyhydroxybutyrate (PHB) and exopolysaccharides (EPS) during its growth leads to an improved inoculants performance in the field, a culture medium was developed, and growth conditions were defined to lead this bacterium to increase its biopolymer contents. The importance of the cellular content of PHB and EPS on improving the viability of A. brasilense was evaluated by a short-period assay carried out under greenhouse conditions. Bacterial biomass produced under the conditions defined so on was used to produce liquid inoculants, which were applied on seeds or topdressing in maize plants; the results of these treatments were compared with the performance of a peat inoculants applied on seeds.

Projections of population increases, especially in developing countries, as well as of life expectancy worldwide, imply greater needs for food and feed. To achieve higher productivity, agriculture is being intensified, mainly with monocultures highly dependent on increased chemical inputs, including pesticides and fertilizers [33, 34]. However, to ensure long-term food production, we must develop sustainable agricultural practices, based on conservationist practices, to achieve economic returns for farmers, but with stability in long-term production and minimal adverse impact on the environment [35]. In this context, the use of microbial inoculants plays a key role, and we may say that we are starting a “microgreen revolution.”

The nomenclature “plant-growth-promoting bacteria (PGPBs)” has been increasingly used for bacteria able to promote plant growth by a variety of individual or combined mechanisms. By this definition, rhizobia—studied and used in commercial inoculants for more than a century—are also PGPBs. Undoubtedly, besides rhizobia, the most studied and used PGPB is *Azospirillum*, encompassing bacteria with a remarkable capacity to benefit a range of plant species [36,37,38, 39, and 40]

The genus *Spirillum* was first reported by Beijerinck (1925), [41] and decades later reclassified as *Azospirillum*, because of its ability to fix atmospheric nitrogen (N_2), discovered and reported by the group of Dr. Johanna Döbereiner in Brazil, in the 1970s [4]. After the discovery that *Azospirillum* was diazotrophic, several studies evaluated its capacity to fix N_2 and to replace N-fertilizers when associated with grasses [42], including sugarcane (*Saccharum* spp.), grain crops such as maize (*Zea mays* L.), wheat

(*Triticum aestivum* L.), and rice (*Oryza sativa* L.), pastures such as *Brachiaria* (= *Uruchloa*), among others [32, 39, 40, 43, 44, 45]. Twenty species of *Azospirillum* (DSMZ 2018) have been described so far, but *A. brasilense* and *A. lipoferum* have been the subjects of the highest numbers of physiological and genetic studies [46, 47].

Beneficial results have been obtained consistently with *Azospirillum* applied to a variety of crops [40, 48, 49] in dozens of commercial inoculants worldwide [50]. Intriguingly, although the Brazilian research group headed by Dr. Döbereiner contributed to dozens of studies with *Azospirillum* [46, 51 and 52], it was only in 2009 that the first commercial inoculant containing *A. brasilense* started to be commercialized in the country [37, 38]; however, more than 3 million doses of inoculants are now applied annually by farmers, for inoculation both of non-legumes and for co-inoculation of legumes.

Although the most prevalent reported benefit of *Azospirillum* has been its capacity of fixing N2, an increasing number of studies describe other properties that imply growth-promotion. One main property of *Azospirillum* relies on the synthesis of phytohormones and other compounds, including auxins [53], cytokinins [6], gibberellins [54], abscisic acid [55], ethylene [56], and salicylic acid [57]. Phytohormones greatly affect root growth, resulting in improvements in uptake of moisture and nutrients [58]. Some *Azospirillum* strains can solubilize inorganic phosphorus, making it more readily available to the plants and resulting in higher yields [59]. There are also reports of *Azospirillum* helping in the mitigation of abiotic stresses, such as salinity and drought [60, 61, 62], by triggering induced systemic tolerance (IST). *Azospirillum* has also been reported to help in the mitigation of excessive compost and heavy metals [36, 63]. Another important feature of *Azospirillum* is related to biological control of plant pathogens [64,65, 66 and 67], enabled by the synthesis of siderophores, and limiting the availability of iron (Fe) to phytopathogens [67], or causing alterations in the metabolism of the host plant, including the synthesis of a variety of secondary metabolites that increase plant resistance to infection by pathogens, a mechanism known as induction of systemic resistance (ISR) [68]. Due to the several mechanisms reported to promote plant growth, Bashan and De-Bashan (2010) [36] proposed the "theory of multiple mechanisms" in which the bacterium acts in a cumulative or sequential pattern of effects, resulting from mechanisms occurring simultaneously or consecutively. In this review we will give emphasis to the mechanisms of *Azospirillum* that can improve plant tolerance of biotic and abiotic stresses.

Conclusion

Azospirillum is currently one of the most broadly studied and commercially employed PGPB. Previous studies with *Azospirillum* emphasize its capacity of fixing atmospheric N2, followed by benefits in promoting plant growth via synthesis of phytohormones. More recently, it has been shown that the benefits should be extended to the capacity of some *Azospirillum* strains to protect plants from biotic stresses, triggering ISR defense mechanisms, and from abiotic stresses, through IST. The mechanisms discussed in this review of tolerance of abiotic and biotic stresses promoted by inoculation of *Azospirillum* in plants, encompassing detoxification of oxidative stress, ISR and IST. The mechanisms that PGPB use to cope with biotic and abiotic stresses vary with the plant species and cultivar and with the bacterial species and strains, and also depend on the phytopathogen and the intensity of the abiotic stress. Further studies to elucidate the mechanisms of action of PGPB—as well as of the response of plants to stresses—are of fundamental importance for understanding the potential and increasing the use of PGPB as an important and sustainable strategy to mitigate the effects of biotic and abiotic stresses in agriculture.

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