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REVIEW ARTICLE

Agronomic and environmental aspects of diazotrophic bacteria in rice fields

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Abstract This article provides an overview of the free-living and plant-associated nitrogen (N)-fixing bacterial communities in wet rice fields, with a focus on describing the elements affecting community assemblages in this waterlogged soil–plant system. Nitrogen is a crucial nutrient for rice yield and growth. Characteristics of the rice paddy ecosystem promote N-fertilizer losses, resulting in negative impacts on the environment. Public concerns on sustainable rice crop production and food security have accentuated interest in exploring biological supplementary nitrogen sources. Biological N-fixation is a significant source of the nitrogen in agroecosystems. The nitrogen requirement of rice crops can be partly remedied by managing and promoting the activities of N-fixing microorganisms. These changes are leading towards a cleaner approach that maintains sustainability while simultaneously improving crop production targets. The use of N-fixing microorganisms as biofertilizers and the factors driving the success of this technology in wet rice paddies are also discussed.

Keywords Microbial community · Biofertilizer · Paddies · Nitrogen fixation

Introduction

Rice (*Oriza sativa* L.) is one of the world's three leading cereal crops and an important dietary component for more than 3.5 billion people. As such, it significantly impacts individual and family-level food security in Asia (Tonini and Cabrera 2011). Rice cropping systems can be upland, lowland, rainfed, wet or deepwater and range from one to three crops per year [Global Rice Science Partnership (GRiSP 2013]. Intensively cropped wet rice systems account for the majority (75 %) of global rice production, and the majority (93 million ha) of land used to grow rice (GRiSP 2013). Some wet rice-growing regions have been in production for centuries, with steady yields using traditional rice varieties. These wet rice fields, which are commonly flooded for part of the growing season in order to control weeds and pests, are more fertile than non-wet systems, allowing for greater planting density and greater per hectare yields (Ghosh and Bhat 1998). Part of this increased fertility is attributed to the presence of nitrogen (N)-fixing bacteria, called diazotrophs, that metabolize atmospheric nitrogen (N_2) and release it in a form which is available for rice uptake (Watanabe and Liu 1992).

New rice varieties were introduced in the second half of the 20th century that provided increased yields, and these yield gains were almost doubled when synthetic fertilizers were used (Khush 2001). Nitrogen is one of the primary macronutrients needed for rice growth and one of the main factors to be considered for achieving a high-yielding rice crop, particularly when modern rice varieties are used (Chen and Wang 2014). The most common mechanism used to provide sufficient N to rice plants grown in modern intensive wet cropping systems is through the addition of synthetic N-fertilizers (Cai et al. 2007).

While the use of synthetic fertilizers cannot be eliminated without influencing food security, the use of N-fertilizers is not without challenges. Only a portion of the applied N-

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fertilizer is used by the rice plant, with up to 70 % of the applied synthetic N lost from the system (Ghosh and Bhat 1998; Trân Van et al. 2000; Fageria and Baligar 2007). The unused fertilizer leaches into surrounding water bodies and contributes to eutrophication. Synthetic fertilizer losses can also adversely affect the soil (e.g. concentration of heavy metals, soil acidification) and increase the emission of greenhouse gases (Zhang et al. 2013). As the use of chemicals in rice culture causes environment hazards and the rate of rice yield growth slows, there is renewed interest in exploring the possibility of "biofertilization", an area of research which seeks to understand and harness naturally occurring biological processes to enhance rice yields (Prakamhang et al. 2009).

The primary biological mechanism by which N is available to plants is referred to as biological nitrogen fixation (BNF), which is performed by prokaryotic diazotrophs (N-fixing), which are extensively distributed among the Bacteria and Archaea domains (Xie et al. 2014). Plants also can obtain nitrogen by N-mineralization of organic matter and from native NH_4 and NO_3 in the soil (Ghosh and Bhat 1998).

A large body of evidence suggests that diazotrophic bacteria can be used and managed in rice production systems to improve the quantity and/or quality of rice while using the same or decreased synthetic fertilizer inputs (Biswas et al. 2000a, b; Das and Saha 2007; García de Salamone et al. 2010; Yanni and Dazzo 2010; Javaid 2011; Jha et al. 2013; Souza et al. 2013). Although it is common to advocate for the use of diazotrophic bacteria as biofertilizers, and as an alternative to synthetic fertilizer use, a more applied and immediate use of this technology for intensively cropped wet rice systems is only one component of multi-faceted management strategy. An action plan has been proposed that acknowledges both the need of individual farmers to maintain production and profitability and the need of society to protect the environment and provide for long-term continued yields. In order to harness the N-fixing capabilities of diazotrophic bacteria it is important to understand the underlying ecology of these organisms throughout the rice production cycle, identify agronomically beneficial interactions between microbes, fertilizer and plants (Rodrigues et al. 2008; Orr et al. 2012) and identify native plant-associated and free-living bacteria that fix N_2 at high numbers in these specific production systems. In this review we explore the agronomic and ecological aspects of BNF in wet rice systems and discuss experiments pertaining to other rice cropping systems.

Ecology of diazotrofic bacteria in wet rice fields

Nitrogen is one of the primary structural elements of all living organisms and an essential component for the growth of plants, including rice (Tabuchi et al. 2007). Atmospheric nitrogen (N_2) consists of two N atoms bound together with a triple bond which renders it. This structure renders the N_2 unavailable to plants (Wagner 2011) and, consequently, plants and animals cannot directly obtain nitrogen from the atmosphere (Fields 2004). The nitrogenase enzyme is needed to catalyze the breaking of the N_2 triple bond. After this bond is broken, three hydrogen atoms are inserted onto to each N atom, and the hydrogen atoms combine to form ammonia (NH_3) (Wagner 2011). In this way, N_2 is removed from air and converted into the un-ionized species of water-soluble NH_3. The ionized species NH_4 (ammonium) can be acquired by plants (Fig. 1).

The most well-known diazotroph—plant interactions consist of those involving bacteria, such as rhizobia that are involved in the formation of root nodules on leguminous dicotyledonous herbaceous plants (e.g. peas and beans) and *Frankia* sp. that form root nodules on woody plants (non-legumes) (Hurst et al. 2014). It has been suggested the supply of nitrogen from the symbiotic association between bacteria (e.g. *Rhizobia* or *Frankia*) and plants occurs at higher rates than that in non-symbiotic systems (Unkovich and Baldock 2008). However, numerous biomes do not have the benefit of nitrogen inputs from symbiotic N-fixers, and thus the demand for nitrogen can be filled by non-symbiotic nitrogen fixation (Reed et al. 2011). Rice is a monocotyledonous non-legume cereal grain, and the diazotrophs associated with rice do not form root nodules. Biological nitrogen fixation has been estimated to contribute about 30 kg N ha^{-1} per year to rice systems (Herridge et al. 2008).

The nitrogenase enzyme of N-fixing microorganisms can be broken down by oxygen (Lery et al. 2010). Prevailing N-fixing bacteria fix nitrogen in microaerophilic situations (Barraquio et al. 1982). In paddy fields, flooding changes the plant root zone conditions from aerobic to anaerobic

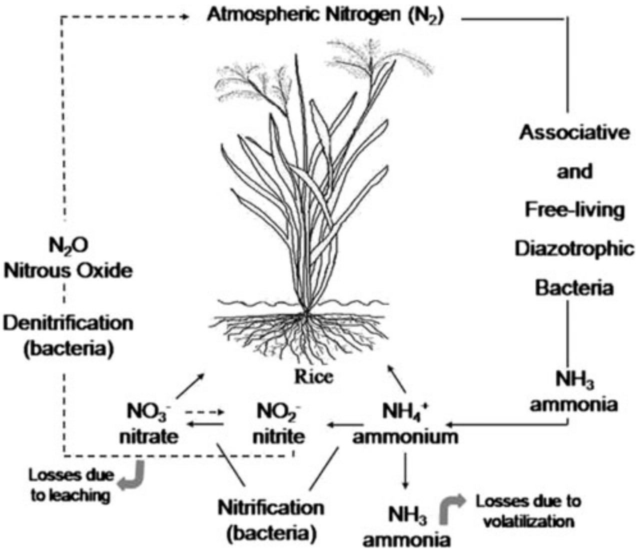

Fig. 1 Summary diagram of nitrogen (N) transformations in flooded rice field with an overview of diazotrophic bacteria action and fertilizer losses through volatilization and leaching. *Dotted line* shows the denitrification process from NO_2^- to N_2O

(Ghosh and Bhat 1998), and rice roots release oxygen (O_2) and thereby oxygenate the rhizosphere zone. The interfaces of the oxic and anoxic layers that are present in the flooded rice field favors the colonization of microorganisms belonging to a variety of respiration strategies, including strict anaerobes, facultative anaerobes, microaerophiles and aerobic organisms (Newton 2007; Chen et al. 2009). For example, the microaerobic genera *Azospirillum* and *Azoarcus* show a trend to move toward locations where the presence of O_2 is ideal for its metabolism (low O_2 concentration) (Reis et al. 2011). The obligate anaerobe *Clostridium* fixes N_2 in the absence of air. The facultative anaerobe *Klebsiella pneumoniae* is able to develop in the presence of O_2 but to fix N_2 only in its absence (Roger 1996). *Azotobacter* and *Rhizobium* need aerobic environments to obtain resources for N_2-fixing. The secretion of polysaccharides and the formation of heterocysts by cyanobacteria are also mechanisms used to form microarobic conditions for N-fixation (Pratte et al. 2006; Lery et al. 2010). Carbon replenishment in standing water, the rhizosphere and the soil layer also promote an ideal niche for autotrophic and heterotrophic rice-associated diazotrophs (Ladha and Reddy 2003).

Associative diazotrophs live in “close association” with the rice plant; they can be divided into endophytes that live within the plant tissues (Prayitno and Rolfe 2010), rhizospheric bacteria that live outside of (but in close association with) the rice root (Prasanna et al. 2009) and epiphytic bacteria that live outside of (but in close association with) other rice plant parts (Gnanamanickam and Immanuel 2007). Free-living diazotrophs are an important font of nitrogen in many ecosystems, including wet rice fields. In rice systems, the free-living diazotrophs are mostly cyanobacteria and photosynthetic bacteria found in the topsoil and floodwater (Ladha and Reddy 2003).

There is no formal definition of “closely associated”, and some diazotrophic bacteria can be both free-living and associative. The general ecological relationship between the associative diazotrophs and the rice plant is assumed to be protocooperative, with the rice plant providing carbon and phytohormones and the diazotrophs providing bioavailable nitrogen (Lu et al. 2006; Dalton and Kramer 2007). However, significant gaps remain in our knowledge of the precise relationship(s) between individual rice cultivars and specific diazotrophic species or community assemblages in applied agronomic settings (Knauth et al. 2005).

The complex relationships between diazotrophs and other biotic and abiotic components of rice ecosystems are impacted by environmental (natural) and agronomic (anthropogenic) conditions. As with all bacteria, the primary drivers affecting which diazotrophs are present in any particular location relate to physical and chemical parameters, such as pH, oxygen availability, temperature and phosphorus (P) and nitrogen levels (Quesada et al. 1997; Nayak and Prasanna 2007; Orr et al. 2012). In addition to these broad environmental influences, diazotroph community structure in wet rice cropping systems is influenced by plant-specific and management-specific inputs (Pedraza et al. 2009).

The numbers and types of bacteria that are present in a crop system are believed to impact yields, system health and supports of specific production systems over time (Van der Heijden et al. 2008). It is generally assumed that greater diversity of bacterial communities is correlated with the desired outcomes, with the implication that effects on diversity influence the stability and function of a community in a given environment and consequently the sustainability of crop fields (Jones and Bradford 2001; Hsu and Buckley 2009; Eisenhauer et al. 2012).

The current working hypothesis underlying diazotrophic community studies is that by identifying the drivers which affect the number, types and evenness of diazotrophs in intensively cropped wet rice systems, these factors can be managed to maximize profit for individual farmers while minimizing environmental impact on the broader community (Berthrong et al. 2014). The agronomic drivers, namely, plant cultivar/genotype, fertilizer and agrochemical input, should be considered when screening bacterial strains for use in an inoculation program (Table 1).

Plant cultivar/genotype

Rice plant cultivar and genotype appear to influence which types of diazotrophs colonize and associate with rice plants (Hardoim et al. 2011), even when all other parameters are identical. This has been attributed to the expression of genetically encoded features that influence plant metabolism and the constitution of root exudates (Aira et al. 2010), such as sugar that accumulates in the root exudate and varies with rice genotype (Hertenberger et al. 2002; Naher et al. 2008). Chamam et al. (2013) evaluated under gnotobiotic conditions the effects of two diazotrophs strains used as inoculum in rice seeds; one isolated from the Cigalon rice cultivar and the other isolated from the Nipponbare rice cultivar. These authors observed that each rice cultivar performed best when inoculated with its original diazotroph strain, demonstrating that specific bacterial agronomic benefits can be plant cultivar-dependent.

Root exudates are the leading factor for the approximation of the bacteria that live near the plant roots (Naher et al. 2011). Although the precise compound(s) in most root exudes, as well as their impacts in the rhizosphere are as yet undetermined, it has been proposed that the community structure of the N-fixing organisms associating with roots varies according to differences in plant exudates (Ladha and Reddy 2003; Tawaraya et al. 2009). For example, plant exudates can affect the bacterial genes and subsequent release of indole-3- acetic acid (IAA) by *Azospirillum* and are therefore directly involved in *Azospirillum*-–plant communications (Puyvelde et al.

Table 1 Agronomic drivers related to diazotrophic communities in rice fields

Agronomic drivers	Microbial group	Effect	Source of sample	Experimental conditions	Reference
Plant genotype	Microbial communities (phospholipid fatty acid analysis)	Differences in soil Gram-positive and Gram-negative bacterial were observed in transgenic rice rhizosphere when compared with nontransgenic	Clay soil from plow layer rice field	Pot	Wu et al. 2009
	Populations of endophytic diazotrophs (cultured bacteria characterized by 16S rDNA techniques)	Diversity contrast between wild and modern rice cultivars	Roots of wild and modern rice cultivars	Field	Engelhard et al. 2000
	Bacterial communities (16S rRNA—DGGE)	48.8 % of the bacterial community variability were explained by plant genotype	Roots from rice cultivars	Field	Hardoim et al. 2011
	Diazotrophic communities (mRNA-based profiling of *nifH* gene)	Differences between rice cultivars	Roots from rice cultivars	Pot	Knauth et al. 2005
	Herbaspirillum sp. (cultured bacteria)	Greater occurrence of bacterial populations inside wild rice than in cultivated rice	Stems of wild and cultivated rice	Pot	Elbeltagy et al. 2001
Fertilizer input	Diazotrophic bacteria (cultured bacteria)	Nitrogen fertilizer application promoted bacterial abundance	Rhizosphere soil, roots and basal stem samples	Field	Muthukumarasamy et al. 2007
	Bacterial community (16S rRNA—qPCR, RFLP)	Inorganic fertilizer input did not influence bacterial abundance	Soil	Field	Wu et al. 2011
	Stenotrophomonas maltophilia (cultured bacteria)	Nitrogen fertilizer input promoted bacterial population growth	Soil	Pot	Othman et al. 2013
	Endophytic diazotrophs communities (*nifH*—DGGE)	Bacterial diversity decrease with nitrogen fertilizer input	Roots, stems and leaves	Pot	Prakamhang et al. 2009
	Diazotrophic bacteria (*nifH*—T-RFLP)	Combined nitrogen affected the *nifH* gene pools	Roots	Pot	Tan et al. 2003
Agrochemicals	*Anabaena* and *Nostoc* (cultured bacteria)	Propanil and butachlor impacting cyanobacterial growth and nitrogenase activity	Water-soil, algal cultures	Field, pot	Irisarri et al. 2001; He et al. 2013
	Cyanobacterial (cultured bacteria)	Monosulfuron, bispyribac-sodium inhibit cyanobacterial photosynthesis and nitrogenase activities	Water-soil, algal cultures	Field, pot	Okmen and Ugur 2011; Shen and Luo 2011
	Microbial community (*nifH*—DGGE)	Butachlor application altered microbial community	Soil from the upper layer	Field	Chen et al. 2009
	Anabaena doliolum (cultured bacteria)	Carbofuran treatment caused growth inhibition (more than 50 %), reduction of nitrogenase activity (38 %)	Algal cultures	Pot	Hammouda 1999

DGGE Denaturing gradient gel electrophoresis; *nifH* nitrogenase reductase subunit of the nitrogenase enzyme; *qPCR* quantitative PCR; *T-RFLP* Terminal restriction fragment length polymorphism

2011). Rice plant roots provide a variety of amino acids, carbohydrates, sugars and secondary elements that supply nutrition to colonizing microorganisms in the rhizosphere (Aulakh et al. 2001; Bacilio-Jiménez et al. 2003). In early stages of rice development, these exudates comprise compounds of simple sugars that selectively enhance the chemotactic responses of some bacteria (Bacilio-Jiménez et al. 2003). In this way, the plant can exert an important effect on the mixed strain inoculum, with positive or negative actions on rhizosphere microorganisms that will have an effect on their ability to establish advantageous relations with the plant (Hartmann et al. 2009).

Fertilizer input

Ingredients commonly found in both organic and synthetic fertilizers, such as nitrogen, phosphorus, potassium, sulfur and calcium, are essential components of microorganisms and, therefore, tend to modify the growth and activity of the microbial community, including diazotrophs (Cruz et al. 2009; Wu et al. 2011; Costa et al. 2013; Othman et al. 2013). Depending on the level, form and combination of other components, the effects of N-fertilization on the diazotrophic bacterial community can be favorable (Kanungo et al. 1997; Muthukumarasamy et al. 2007; Othman et al. 2013) or harmful (Wu et al. 2009; Omar and Ismail 1999). A pH of 5 has been shown to affect soil microbiota (Fierer and Jackson 2006). Consequently, the application of N-fertilizers, which tend to reduce soil pH, has a potentially negative effect on soil microorganisms (Guo et al. 2010).

The changes caused to the diazotrophic assemblages according to the levels and nature of carbon and nitrogen are less consistent (Hsu and Buckley 2009). N-fertilization usually generates lower N-fixation rates since diazotrophic bacteria do not fix nitrogen when it is abundant (Reed et al. 2011). Thus, plant nitrogen absorption from the roots and a decrease in fertilizer loads provide a friendly microenvironment in terms of the establishment of diazotrophic bacteria (Kanungo et al. 1997). Moreover, a high nitrogen concentration may lead to osmotic stress and the ultimate death of the microorganisms (Maheshwari et al. 2012), which eventually results in soil infertility (Duarah et al. 2011).

Pedraza et al. (2009) verified significant improvement in grain yield in a rice rainfed field following inoculation with *Azospirillum brasilense*, both with and without additional N-fertilization. The action of phosphate-solubilizing bacteria (PSB) on the availability and uptake of rice nutrients was the subject of investigation in a pot experiment study carried out by Duarah et al. (2011). The results demonstrated that with or without the use of nitrogen, phosphorus, and potassium (NPK) fertilizers, the addition of PSB increased NPK (when applied) uptake, germination index, root length and biomass as compared to the control. The point highlighted by these authors was that excessive utilization of NPK fertilizers resulted in low bacterial enzyme activity. It has also been shown that the utilization of NPK fertilizers can influence the diversity of diazotrophic bacteria (Prakamhang et al. 2009) which in turn can impact N-fixation rates (Hsu and Buckley 2009). In a rice field study, Wu and co-workers (2011) applied the 16S rRNA approach and found no statistical significant influence of inorganic fertilizers on bacterial abundance. However, application of the fertilizer together with resulted in a significant increase in bacterial abundance which was not related to the soil pH values. Similar results were found in a model paddy microcosm study with the use of urea and rice straw in relation to the effects on N-fixing activity (Tanaka et al. 2006). Soil nitrogen-fixing activity was reduced in microcosmos treated with urea; however, in the rice straw treatment N-fixation increased by 40 % in relation to the control. In another study, Sarkar et al. (2014) verified that the diversity of the diazotroph assemblage was higher in rice fields treated with an organic fertilizer than in those subjected to long-term N-fertilizer inputs.

In summary, based on the studies mentioned above, it can be concluded that in agricultural systems, irrespective of the (long-term) use of synthetic fertilizer, the application of biofertilizers in a field context can reduce the amount of synthetic fertilizer required to increase rice crop development and yield when biofertilizers are used in combination with their synthetic counterparts.

Agrochemicals

The answer to the question of whether or not herbicide use influences diazotrophs remains disputed. Long-term utilization of agrochemicals in cropping systems has been shown to modify soil diazotrophic bacterial communities (Orr et al. 2012). Pesticide use in non-rice crops has also been shown to impact diazotrophs in soil (Das and Mukherjee 2000). However, it has also been shown that when the correct amount is applied, agrochemicals do not notably impact diazotrophic communities and that in modest concentrations they can actually stimulate nitrogenase activity (Okmen and Ugur 2011; Shen and Luo 2011).

Pseudomonas aeruginosa (De Vleesschauwer et al. 2006), *Pseudomonas* sp. (Chaiharn et al. 2009) and *Azospirillum* sp. B510 (Yasuda et al. 2009) have been reported to benefit rice plants with increases in the levels of defense enzyme activity in response to pest attack, reduction of disease due to the iron sequestration by siderophores and/or the production of compounds harmful to competing organisms. Ji and coworkers (2014) identified diazotrophic bacteria from rice plants using a *nifH* gene technique. The bioassay demonstrated an antagonistic effect between all of the diazotrophic bacteria isolated in the study and the phytopathogenic fungi *Fusarium oxysporum* and *Rhizoctonia solani*. The antagonist potential of *Azospirillum* sp., *Burkholderia brasilensis* and *Herbaspirillum seropedicae* against *Fusarium* sp. was also verified in a rice seed inoculation experiment by Araújo et al. (2010) which showed that these bacteria promoted the rate of seed germination and less fungal infection.

By influencing bacterial activity, the application of herbicides can provide a competitive advantage to a specific bacterial group (e.g. heterotrophs), causing an imbalance in microbial processes, such as the conversion of organic matter and degradation of extraneous components (Zabaloy et al. 2008). In this context, herbicide application may have the potential to affect microbial abundance (Moorman and Dowler 1991; Maurhofer et al. 1994), such as the densities of pathogenic and beneficial organisms, respectively, and thereby the response of microbiota to herbicides could impact crop health. Gimsing et al. (2004) reported that the mineralization of the herbicide Glyphosate in rice farms was highly correlated with *Pseudomonas* spp. number. Zabaloy and Gómez (2008) verified the impact of Glyphosate on the soil microbial community and observed a stimulatory effect on heterotrophic bacteria density and a transient increase in the proportion of carbon sources utilized by the bacterial community.

All of the studies reported in this section describe agronomic factors that can interfere with the plant–microorganism interaction in the rice field. These factors can be used either to select effective bacterial strains (e.g. cultivar/genotype) or to ensure the most suitable conditions for biofertilizer application and management (fertilizer and agrochemical input).

In flooded rice fields the aerobic and anaerobic conditions which develop, with the formation of microhabitats in the rhizosphere and in plant tissues, changes in organic carbon availablity and the lower mineral nitrogen concentration in the soil, creates an optimal habitat for N-fixing bacteria, influencing both the number and diversity of these microorganisms (Kanungo et al. 1997). The most frequent bacterial taxa associated to rice fields across all of the studies cited herein are presented in Table 2.

Advances in Gramineae development using diazotrophic bacteria

Biofertilizers

Biofertilizers are bioinoculants consisting of living or latent cells of microorganisms which when inoculated onto seeds, plant surfaces or the soil re-locate to inside plant tissue or onto the root surface, resulting in improved plant mineral nutrient availability and input (Vessey 2003). According to Okon and Labandera-Gonzalez (1994), the term biofertilizer is not suitable because biofertilizers do not replace fertilizers, rather they enhance plant nutrient uptake.

An example of a natural association that can result in crop gain is the positive effect on plant growth due to the development of a relationship between the microorganism and either the rice plant, or other plants in the field, such as the aquatic fern *Azolla*, which can be co-grown with the crop. There is a natural symbiosis among *Azolla* and diazotrophic bacteria, and farmers in regions where *Azolla* is endemic were quick to notice that rice production increased in the presence of these small aquatic ferns. The rice and *Azolla* plants provide a physical habitat for the bacteria, and the products of the bacterial metabolism serve as growth promoters for the rice plants (Bocchi and Malgioglio 2010). The biofertilizer industry originated over 1000 years ago in Vietnam, where a few families had monopolies on *Azolla* propagation (Lumpkin and Plueknett 1981). While the use of *Azolla* continues today, this system alone cannot adequately provide for the needs of modern rice production (Bocchi and Malgioglio 2010).

As with any agronomic input, the goal of biofertilizer use is to increase grain yield and improve grain quality. Both experimental and commercial field studies have demonstrated that rice plants inoculated with either individual diazotrophic strains or specific groups of strains have increased grain yields and/or improved grain quality (Govindarajan et al. 2008; García de Salamone et al. 2010; Isawa et al. 2010; Yanni and Dazzo 2010; Bhattacharjee et al. 2012) (Table 3). Various studies have examined the benefits of biofertilizer use on other rice growth parameters, with the implied assumption that improvement in any of these parameters would likely result in improved plant health and ultimately improved grain yield and quality. The parameters studied include plant biomass (Bhattacharjee et al. 2012), tiller numbers (Govindarajan et al. 2008), plant height (Saadatnia and Riahi 2009) and increased root length (Jha et al. 2009). A third set of measurements that are often taken when the goal is to evaluate the efficacy of biofertilizers is to assess the uptake and distribution of specific nutrients by rice plants, most commonly nitrogen and phosphorus (Cong et al. 2009).

A number of barriers need to be overcome in order to expand the small-scale localized use of naturally occurring bacteria in food production to large-scale food production systems. The dairy and wine industries are good examples of how to harness the desired qualities of naturally occurring microbial interactions and then systematically apply them in their respective production/processing systems (Hutkins 2006). The average size of a typical operation in many food production industries/systems has increased over the years, as has the throughput, or in the case of rice, yield per hectare. While small-scale and traditionally managed operations still exist, the dairy and wine industry is dominated by producers with large production capacities. In the fermented food industry, these scale-ups came with fundamental changes in the production system, namely, a change from using undefined natural cultures with large variations in quality from batch to batch to

Table 2 The most frequent diazotrophic bacteria associated to rice fields

Bacteria	Source of sample	Technique	Experimental conditions	Reference
Anabaena spp., *Nostoc* spp.	Water, soil, rhizosphere of rice fields	Counting of cyanobacteria	Samples collected from diverse rice agroecological regions and soil types	Irisarri et al. 2001; Prasanna and Nayak 2007
Azoarcus spp.	Rice soil	DNA/RNA—DGGE fingerprinting; *nifH*—-qPCR	Wet rice field	Mårtensson et al. 2009
Azospirillum spp.	Rice shoots and roots	16S rDNA, morphological and physiological features	Greenhouse, field	Elbeltagy et al. 2001; Jha et al. 2009; Prayitno and Rolfe 2010
Azotobacter sp.	Rice rhizosphere	Biochemical characteristics	Field	Torres-Rubio et al. 2000
Bacillus spp., *Stenotrophomonas* sp., *Xanthomonas* sp.	Surface of rice seeds	16S rRNA sequences	Wet rice field	Mano et al. 2006
Burkholderia spp., *Gluconacetobacter* spp., *Herbaspirillum* spp.	Rice roots	Phylogenetic probes and fluorescent in situ hybridization	Field	Jha et al. 2009
Enterobacter sp., *Klebsiella* sp.	Rice soils	Fatty acid methyl ester characterization	Field	Islam et al. 2012b
Ochrobactrum spp., *Stenotrophomonas* spp.	Rice seeds, roots, shoots and soils	16S rRNA PCR–DGGE	Pot	Hardoim et al. 2012 Othman et al. 2013
Paenibacillus spp.	Rice leaves, stems and roots	*nifH* sequence analysis	Wet rice field	Ji et al. 2014
Pantoea spp., *Pseudomonas* spp.	Rice root and rhizospheric soil	16S rRNA sequencing and fatty acid methyl ester analysis	Field	Spence et al. 2014
Rhizobium spp.	Rice roots and rhizospheric soil	Most probable number—16S rRNA analysis	Field	Yanni et al. 1997
Serratia spp.	Rice roots and stems	Fragment length polymorphism—16S rRNA sequencing	Wet rice field	Gyaneshwar et al. 2001
Sphingomonas spp.	Endophytically in roots and rhizospheric soil of rice	Morphological and physiological features	Field	Prayitno and Rolfe 2010 Chung et al. 2011

Table 3 Improvements in rice development with the biological nitrogen fixation and nutrient uptake mechanisms and commercial products using diazotrophic bacteria

Bacterial species	Mechanisms of plant benefit	Experimental conditions	Improvements	Reference	Bacteria strain, product name/Company
Anabaena spp., *Aulosira* spp., *Gleotrichia* spp., *Nostoc* spp., *Tolypothrix* spp.	Nutrient uptake	Pot	Increase of 103.5 and 110.3 % in grain and straw yield, respectively	Bhuvaneshwari et al. 2012	
Anabaena iyengarii var. tenuis, *Nostoc* sp., *Nostoc commune*, *Nostoc linckia*	BNF	Field	Increase of 0.16 (t ha^{-1}) in grain yield and 0.2 % in entire grain quality	Pereira et al. 2009	
Anabaena sphaerica, *Aulosira fertilissima*, *Azospirillum* sp., *Cylindrospermum majus*, *Nostoc hatei*, *Pseudomonas* spp., *Westiellopsis prolifica*	Fertilizer use efficiency	Field	Increase of 10–32 % in rice yield in traditonal varieties	Jha et al. 2013	
Anabaena variabilis, *Azotobacter* sp., *Chlorella vulgaris*	BNF	Laboratory	Increased seed germination and plant height	Zayadan et al. 2014	*Azotobacter* sp., Agri Life/Gujarat State Fertilizers & Chemical Ltd., Omega Ecotech Products Ltd.
Azospirillum sp.	BNF, growth-promoting substances release and nutrient uptake	Field	Increased grain yield, plant height, panicle and root length, root dry weight and nutrient uptake	Islam et al. 2012a	
Azospirillum brasilense	Nitrogen uptake	Field	Increased nitrogen content by 16 and 50 kg ha^{-1} and increased rice biomass	García de Salamone et al. 2010	
Azospirillum amazonense	BNF	Greenhouse	Increased grain dry matter accumulation (7–11.6 %), panicle numbers (3–18.6 %) and nitrogen content (3.5–18.5 %)	Rodrigues et al. 2008	
Bacillus sp.	BNF, IAA, siderophore secretion	Greenhouse	Promoted rice root and shoot growth	Beneduzi et al. 2008	*Bacillus* sp., Biopromoter-Phosphobacteria/ ManiDharma Biotech Pvt.
Burkholderia vietnamiensis	Nutrient uptake	Pot/Field	Increased grain yield by 13–22 %	Trân Van et al. 2000	
Burkholderia vietnamiensis, *Herbaspirillum* sp.	Phosphate solubilization and/or nitrogen use efficiency	Greenhouse	Increase rice grain yield from 33 to 47 % in relation to uninoculated/ tricalcium phosphate fertilized plants	Estrada et al. 2013	
Enterobacter sp.	BNF and IAA secretion	Greenhouse	Increased plant dry weight, chlorophyll and protein content and root elongation	Keyeo et al. 2011	
Pseudomonas spp., *Bacillus subtilis*, *Bacillus amyloliquefaciens*	Nitrogen uptake improvemnent	Field	Saved 43 kg of N fertilizer ha^{-1} together with increasing rice yield by 270 kg ha^{-1}	Cong et al. 2009	*Pseudomonas* sp., Proradix/ Sourcon Padena
Rhizobium sp., *Corynebacterium* sp.	Root improvement and efficient nutrient uptake	Laboratory	Increase in rice biomass of between 32 and 36 % vs. the uninoculated control	Naher et al. 2009	*Rhizobium* sp., Biopromoter/ ManiDharma Biotech Pvt..; *Rhizobium*, *Azospirillum*, *Acetobacter* and *Azotobacter*, Symbion-N/T.Stanes Company
Rhizobium leguminosarum bv. *trifolii*	Nitrogen uptake improvement	Field	Rice grain yield increased an average of 19.5 %	Yanni and Dazzo 2010	

BNF Biological nitrogen fixation; *IAA* Indole-3- acetic acid using defined cultures that provide a product of consistent quality. Many biofertilizer proponents are currently focusing on isolating and characterizing diazotrophic bacteria from rice systems in an effort to identify those strains that would best serve as defined cultures for intensively cropped wet rice (Ashrafuzzaman et al. 2009; Araújo et al. 2013).

The first step in manufacturing inoculants is to obtain diazotrophic strains that are efficient at fixing nitrogen under the conditions used for a specific rice system (Mia and Shamsuddin 2010). Endophytic beneficial diazotrophic strains can be isolated from surface-sterilized rice plants in high-yield rice fields (Gyaneshwar et al. 2001). A second consideration when screening strains for their use as a biofertilizer is to evaluate their interaction with the host plant. At the present time, this generally means determining if the selected diazotrophic strain or mix of strains is able to colonize rice plant seeds or roots (Chi et al. 2005; Etesami et al. 2013). This interaction is governed not only by the bacteria but also depends on the specific variety of rice that is being grown. In a mixed inoculum, plant genotype is an important factor to be considered (Hardoim et al. 2011; Araújo et al. 2013). Biofertilizer candidates need to be able to survive in a wide range of environments and to tolerate pesticides and herbicides, as well as be able to effectively compete with the native flora (Kavadia et al. 2011). In addition to their qualities in the field, potential biofertilizer strains need to be amenable to large-scale production and distribution, i.e. easy to make ready for use, with an economical carrier and proper transport and storage. This includes being able to both grow the strain efficiently in broth culture while maintaining the desired field qualities and process the stock cultures so that they survive during storage (Pindi and Satyanarayana 2012).

Other practical considerations include appropriate handling, application and adhesion of the inoculant to the rice seed and persistence of the inoculant on the seed and soil environment. The bacterial inoculum can be applied by seed treatment, seedling dip, root dip, nursery inoculation and rhizosphere, soil or planting pit application (Muraleedharan et al. 2010; Earanna and Muruli 2011). The planting technique, which involves growing rice seedlings in nurseries and then transplanting these seedlings to pre-prepared fields, is an approach which has been shown to improve the establishment and survival of the living organism inoculum, with better survival results in the field environment compared to non-inoculated seedlings (Solaiman and Hirata 1997).

A important factor underlying the economic feasibility of any large-scale use of biofertilizer is the establishment of production parameters that are reliable, quality-controlled and cost-effective. Consequently, in terms of efficacy and commercial application, beneficial bacteria that are able to colonize the intercellular or intracellular spaces of rice tissues (endophytes) (Baldani et al. 2000) may be the better choice as inoculum candidates than rhizosphere and free-living bacteria (Bhattacharjee et al. 2008). Endophytes have a direct supply of nutrients inside the host and a low oxygen habitat, both conditions favoring the nitrogenase enzyme (Bhattacharjee et al. 2008). In return, microorganisms occupying this niche provide benefits in the form of fixed nitrogen (Prakamhang et al. 2009). Additionally, the anti-plant pathogen actions of these strains are more efficient than those of other plant-associated bacteria because endophytes colonize a niche similar to that of the phytopathogens where they specifically can exert their competitive activities for space and nutrients (Etesami et al. 2013).

Beneficial mechanisms other than N-fixation shared by diazotrophs and plants

In addition to N-fixation, biofertilizers can also promote the ability of diazotrophs to solubilize phosphorus so that it is available to the rice plant, provide specific iron chelators and synthesize plant growth-promoting hormones [such as cytokinins, gibberellins, indole-3-acetic acid (IAA)]. Some bacteria are also able to metabolize xenobiotic elements that can be used to control plant pathogens are useful; the potential of these strains in biofertilizers have also been explored (Al-Taweil et al. 2009) (Table 4).

However, some bacterial groups contain strains which both promote plant growth and cause animal and plant disease, such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Pantoea ananatis*. Consequently, these strains should not be used as biofertilizers (García-Fraile et al. 2012).

Phosphate solubilization and mineralization Phosphate-solubilizing bacteria convert insoluble forms of phosphorus (organic and inorganic) to forms that the plant is able to take up (available P) (Leelahawonge and Pongsilp 2009). The activities of phosphatase enzymes promote mineralization of organic phosphorus (Rodríguez et al. 2006). The mechanism by which microorganisms solubilize inorganic phosphorus requires the presence of organic acids in chelation and exchange reactions (Asea et al. 1988). Panhwar et al. (2014) investigated rice seed development in a high alluminum and low pH environment after PSB inoculation. These authors observed improvements in root size, seedling dry weight—probably due to the solubilization of phosphorous—and the production of polysaccharides by bacterial strains that increased the pH and decreased the aluminum concentration in the suspension.

Sequestering iron Siderophores are low-molecular weight high-affinity iron chelating compounds synthesized by bacteria and fungi which bind ferric (Fe^{3+}) and other heavy metals (such as Al, Cd, Cu, Pb and Zn). As such, they provide conditions conducive to plant development by promoting iron availability and alleviating the heavy metal concentration in contaminated soil (Indiragandhi et al. 2008). For, the availability of soluble ferrous (Fe^{2+}) is relatively higher in wet rice

Table 4 Plant-promoting mechanisms and commercial products using nitrogen-fixing bacteria

Bacteria	Mechanisms of plant benefit	Experiment conditions	Improvements	Reference	Bacteria strain, product name/company
Azospirillum sp.	Growth-promoting effect	Greenhouse	Increased seed production by 17 % and biomass by 6-12 %	Isawa et al. 2010	Paddy *Azospirillum* sp./ManiDharma Biotech Pvt. *Azospirillum* sp., Sardar/Gujarat State Fertilizers & Chemical Ltd. *Azospirillum* sp. Azogreen/Omega Ecotech Products Ltd.
Bacillus spp.	Decreases phytopathogen growth	Greenhouse	Promoted plant growth and induced resistance to bacterial leaf blight disease	Chithrashree et al. 2011	*Azospirillum brasilense* and *Azospirillum lipoferum*, Bio-N/ BioTech UPLB
Burkholderia cepacia, *Pseudomonas* sp.	No conclusive data on mechanisms	Pot/Field	Increased shoot length by up to 60 %, shoot dry weight by up to 33 % and grain yield by up to 26 %	Jha et al. 2009	
Combination of *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Azospirillum lipoferum*, and *Burkholderia vietnamiensis*	IAA secretion	Pot/field	All bacteria together increased rice yield by between 9.5 and 23.6 %; *Burkholderia vietnamiensis* alone enhanced yield by 5.6–12.16 %	Govindarajan et al. 2008	*Azospirillum lipoferum*, Nitrofix™/ AgriLife
Rhizobium leguminosarum bv. *trifolii*, *Rhizobium* sp., *Bradyrhizobium* sp.	Changes growth physiology or root morphology	Pot	Increased rice grain and straw yields by 4–22 %; *Rhizobium* inoculation increased NPK intake	Biswas et al. 2000a	
Rhizobium leguminosarum bv. *trifolii*	IAA and 1-aminocyclopropane-1-carboxylic acid (ACC) release	Laboratory	Increased root branching by 64-82 % and shoot nitrogen content of the rice plants by 28 %	Bhattacharjee et al. 2012	

fields, and its compounds may cause physiological stress to the rice plant. In this context, bacteria capable of sequestering iron can promote the health of rice plants (Etesami et al. 2013). Siderophores play an additional role as biocontrols, decreasing the accessibility of ferric ions to the phytopathogens (Beneduzi et al. 2012). Chaiharn and co-workers (2009) screened bacterial siderophore producers in rice rhizospheric soil. Of the 216 bacterial isolates tested, 48 showed siderophore production, with isolated strains of *Streptomyces* sp., *Pseudomonas* sp., *Bacillus firmus* and *Kocuria rhizophila* effectively inhibiting the mycelial development of the rice pathogenic fungi. In another study, Naureen et al. (2015) verified a bacterial antagonistic effect against *Rhizoctonia solani*, the causative pathogen of rice sheath blight disease and correlated the antagonistic property to the quantity of siderophores produced by bacterial strains isolated from rice fields.

Phytohormone production Phytohormones are organic compounds that influence growth and development of plants at very low levels (Dobbelaere et al. 2003). Plant-associated microorganisms, such as bacteria, algae, and fungi, have the ability to release phytohormones (Mwajita et al. 2013). In gramineous plants, such as rice, IAA produced by *Azospirillum* sp. (Prakamhang et al. 2009) has been cited to be the principal factor in the plant growth process through its action on root tissue development (Cassán et al. 2014) which in turn enhances soil mineral and water intake by the plant (Matsuda et al. 2005). Manickavelu et al. (2006) reported that the extracts of the cyanobacteria *Plectonima* sp. showed an effect on root induction in rice callus that was probably related to the production of growth regulators such as IAA.

These multiple mechanisms combined or separately may be responsible for the promotion of plant growth and yield increase, thereby resulting in crop improvement.

Bacterial taxa associated to rice which have been shown to be promising candidates for future use as biofertilizers in rice fields are *Azospirillum*, *Rhizobium* and Cyanobacteria.

The genus *Azospirillum*

Azospirillum sp. are Gram-negative members of Class Alphaproteobacteria that live in association with plant roots and is able to fix nitrogen under microaerophilic conditions (Reis et al. 2011). In rice, *Azospirillum* has been show to create colonies around the surface of the roots, fix nitrogen and produce plant growth regulators that increase development and the panicle and harvest indices (Rodrigues et al. 2008; Khorshidi et al. 2011). The most frequent phytohorme released by *Azospirillum* is an auxin (IAA) (Moghaddam et al. 2012), and this auxin is responsible for the plant growth-promoting action of *Azospirillum* (Dobbelaere et al. 2003). However the release of phytohormone by bacteria not always beneficial to the plant, and in some situations it can result in an inhibition of plant growth (Rodrigues et al. 2008). Plant response to the action of IAA will depend on the concentration of the phytohormone in the rhizosphere of the plant (Lambrecht et al. 2000). *Azospirillum* has been shown to be one of the most dynamic organisms incorporating plant-derived carbon in the rice rhizosphere (Lu et al. 2006). Isawa and co-workers (2010) assessed *Azospirillum* sp. strain B510 as an inoculum in rice and observed that it promoted the number of tillers at the beginning of rice growth and increased seed production by increasing panicle quantity. This strain has been found to adapt well to various stress conditions (Drogue et al. 2014) and to increase the mass and length of root hairs, as well as the frequency of emergence of lateral roots (Okon and Labandera-Gonzales 1994), which results in higher nutrient and water uptake by the plant (Henry et al. 2012). *Azospirillum* has been used in association with synthetic nitrogen to achieve greater rice yields than fertilizer used alone (Islam et al. 2012a).

Evaluations of *Azospirillum* as a biofertlizer in rice have focused mainly on *A. lipoferum* and *A. brasilense* (García de Salamone et al. 2010; Reis et al. 2011). In another study, Rodrigues et al. (2008) evaluated the auxin production and nitrogenase activity of *A. amazonense* in association with rice plant yield. These authors reported that this bacterial species promotes rice growth by fixing N_2.

The Genus *Rhizobium*

Rhizobium is also a Gram-negative alphaproteobacterial diazotroph. Members of this genus are commonly associated with root nodules in leguminous plants; however, *Rhizobium* has been found to be naturally associated with cereal grains (Dazzo et al. 2000). Some *Rhizobium* species have been evaluated as biofertilizer strains that are associated with the rice rhizosphere (Chi et al. 2005). Bhattacharjee and colleagues (2012) documented the ability of *Rhizobium* to interact naturally with rice and its beneficial effect on rice plant performance. There is also interest in its use as an endophyte inside rice tissues (Perrine-Walker et al. 2007). Yanni and Dazzo (2010) analyzed the effect of inoculating one or many *Rhizobium leguminosarum* bv. *trifolii* strains into five rice varieties over five culture periods. They also rated the inoculation with different N-fertilizer inputs. Their results revealed that middle doses of N-fertilizer was associated with an increase in rice grain yield and that inoculation with associations of *Rhizobium* strains achieved better results as a biofertilizer than inoculation with only one strain.

Cyanobacteria group

Cyanobacteria are free-living Gram-negative photoautotrophs. In rice production systems, *Anabaena* and *Nostoc* are the most common genera of diazotrophic cyanobacteria

(Irisarri et al. 2001; Prasanna et al. 2009), but members of genera *Calothrix*, *Cylindrospermum*, *Nodularia*, *Scytonema* and *Tolypotrix* can be present at low abundance (Irisarri et al. 2001).

There are numerous reports on the beneficial use of cyanobacteria alone and as part of a microbial consortium-based biofertilizer in terms of increasing the growth and yield of rice (Saadatnia and Riahi 2009; Pereira et al. 2009; Singh et al. 2011; Bhuvaneshwari et al. 2012; Prasanna et al. 2012; Jha et al. 2013). In rice fields, nitrogen fixed by cyanobacteria can be made accessible to the plants via exudation, the destruction of microbial cells and/or degradation (Singh et al. 2013). Nayak and Prasanna (2007) inoculated cyanobacteria into rice plants and observed that the amount of N fixed and thus promptly accessible by the rice plants increased by 2–10 %, with the remaining nitrogen becoming available upon decomposition of dead algae.

Cyanobacteria are the major elements in rice fields that can be used as an inoculum. However, the use of cyanobacterial inoculants has faced challenges, including the inability of laboratory strains (1) to compete with native flora and be maintained in culture for a prolonged time or (2) to fix nitrogen efficiently in the presence of N-fertilizer (Akoijam et al. 2012). Cyanobacteria release a broad range of extracellular compounds, including plant growth-promoting substances, which impact plant crop growth and productivity (Mazhar and Hasnain 2011). Prasanna et al. (2012) investigated the results from a combination of plant growth-promoting bacteria and cyanobacteria for effective nutrient management of rice. They found an interactive action between the bacterial (*Providencia* sp. and *Brevundimonas* sp.) and cyanobacterial (*Anabaena* sp.) species, resulting in an improvement of N-fixing capacity. In waterlogged rice fields, *Azolla* (fern)–*Anabaena* symbiosis has an economically and agronomically important impact value (Unkovich and Baldock 2008). *Anabaena*, a member of the cyanobacterial order Nostocales, has been identified as a symbiont of *Azolla*. The *Azolla*–*Anabaena* complex can promote rice production due its potential to fix nitrogen in significant amounts (Pabby et al. 2003). Moreover, the use of this water fern as green manure can improve the ability of the soil to retain water and nutrients (Nayak et al. 2004), as well as help control weed emergence (Biswas et al. 2005).

Conclusions

More rice must be produced using less land, while minimizing costly and environmentally unfavorable inputs. Taking into account the current food security issues, researchers are looking to harness the functionality of diazotrophic bacteria to improve intensively cropped wet rice production yields. There are also economic and environmental demands related to the increasing costs of synthetic fertilizer, based on both the negative effects of fossil energy use and on demands from society to diminish or at least not increase the use of synthetic fertilizer. Recent advances in biological N-fixation have been promising, and applied field studies are demonstrating the potential of biofertilizers and green manures containing diazotrophic bacteria to decrease the use of N-fertilizers while maintaining yields. In addition, the diazotrophs commonly used as rice inoculums do not seem to have adverse effects on humans. The results of environmental screening studies of the natural environments of these microorganisms and of greenhouse and field studies demonstrate that the positive effects of long-term biofertilizer use outweigh the negative ones. Although hurdles associated with the large-scale commercialization of biofertilizers remain, a roadmap to the industrialization of natural biological processes is available from the fermented foods industry. In the short term, it will be possible to reduce N-fertilization through the use of diazotrophic inoculum in combination with green manures, but also by adding legumes in crop rotations, incorporating rice straw and applying fertilizer more precisely. Further studies are needed to clarify the ecology of natural diazotrophic groups (phylotypes) in different management conditions and the effects of their application in long-term wet rice cropping experiments. In addition, it is imperative that research be translated into practical applications that can be implemented by farmers in a cost-efficient manner and that diazotrophs biofertilizer be included in rice production recommendations and breeding programs with the aim of establishing efficient and beneficial associations of crop varieties and microflora.

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