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Review

Contribution of forage grasses to biological nitrogen fixation and their response to diazotroph inoculation. Review

Dania Fonseca López [a]

Nelson Vivas Quila [b]

Raúl Cuervo Mulet [c]

Carlos Eduardo Rodríguez Molano [d]*

[a] Universidad Santo Tomás. Centro de Investigación en Ciencia y Tecnología para el Desarrollo Sostenible. Tunja, Colombia.

[b] Universidad del Cauca. Facultad de Ciencias Agrarias. Grupo de Investigación NUTRIFACA. Popayán, Colombia.

[c] Universidad de San Buenaventura. Facultad de Ingeniería. Cali, Colombia.

[d] Universidad Pedagógica y Tecnológica de Colombia UPTC. Facultad de Ciencias Agropecuarias. Tunja, Colombia.

* Corresponding author: carlos.rodriguez@uptc.edu.co

Abstract:

The use of chemical inputs has led to the loss of microbial diversity involved in the N cycle, such as diazotrophic bacteria, which are inhibited by saturation of the receptors responsible for activating nitrogenase. Biological nitrogen fixation (BNF) in forage grasses can be used as an ecosystem service. The aim of this review was to analyze the contribution of forage grasses to BNF and their response to inoculation of non-symbiotic diazotrophs in order to find study opportunities. The analysis of the information was carried out using the prisma methodology of systematic reviews and meta-analyses. It should be noted that the main forage species that contribute to BNF are *Brachiaria* sp. and *Pennisetum* sp. The inoculation of *Azospirillum* sp. has generated a growth-promoting effect in grasses, but the response of the inoculated forage depends mainly on the synergy between plant and bacteria, showing neutral, antagonistic, and positive effects.

Keywords: Fertilization, Nitrogen fixation, Forage, Nitrogenase, Pastures.

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Introduction

In livestock systems, animal feed is economically viable when the ration is mainly made up of forage. Nonetheless, it is necessary to produce grass in an eco-efficient scenario to compensate for the environmental footprint caused by livestock farming, considering that in Colombia it occupies 80 % of agricultural land(1). The proposed strategies include the use of improved forage species, diversification of the system(1) and the utilization of natural phenomena such as biological nitrogen fixation (BNF)(2). This is a process in which diazotrophs transform atmospheric nitrogen (N) into ammonium from the nitrogenase enzyme complex, and contributes about 62 %, which is equivalent to 11.29 million tonnes (Mt) of nitrogen per year, which enters the Latin American agricultural ecosystem, while chemical fertilization contributes approximately 6.81 Mt N per year(3). BNF is a resource that can be used as a technological tool to reduce the application of nitrogen fertilizers of synthetic origin that have low efficiency (approximately 40-50 %) and contribute to the emission of greenhouse gases (ammonium, ammonia, and nitrous oxide)(4) and soil salinization(5). Nevertheless, little is known about the contribution of forage grasses to BNF and the bacterial species with the best productive effect. Therefore, this paper aimed to analyze the contribution of forage grasses to BNF and their response to the application of biofertilizers constituted by non-symbiotic diazotrophic bacteria, pure and in consortium, based on a systematic review of literature to find study opportunities.

The prisma methodology of systematic reviews(6) was used; the databases consulted were Scopus and Web of Science; for the search for information, the following criteria were established: a) specificity, based on the use of Boolean operators, b) sensitivity, with CAB descriptors; c) comprehensiveness, through the verification of descriptors of interest. The search strategy was based on the following routes: TITLE-ABS-KEY (“Biofertilizer”) and TITLE-ABS-KEY (“Biofertilizer and Grass”). With the general search, a total of 6,813 records were found between the Scopus (n= 4,621) and Web of Science (n= 2,192) databases. The search was limited to the Boolean connectors “Biofertilizer and Grass” from which 128 records were found (Scopus: 84 records and Web of Science: 44 records), which were imported into the Mendeley software and grouped by years; the analysis was limited to the period 2012-2022 (n= 80 records), then duplicate documents were removed (n= 2 records). Articles evaluating the effect of the application of biofertilizers on forages or the contribution of nitrogen fixed by these plants were included in the analysis. Publications with a title outside the search of interest (n= 5) and with only descriptive information that did not meet the inclusion criteria (n= 13 records) were excluded. Each record was independently reviewed by all authors for a total of 50 studies included within the review. The results of the analysis were defined as: a) Nitrogen fixed by forage grasses, b) Biofertilizers applied and their effect on forage grasses. The data of interest in the study (fixed nitrogen and plant effect) were tabulated and grouped by topic to measure their effect. A nonlinear regression analysis was performed with the number of records obtained from the sigmoidal models 3,4, Gompertz 3, and Hill 3. The models with the highest fit were selected based on the significance value and fit of the coefficient of determination to establish the overall trend of the area of interest.

Biological nitrogen fixation in forage grasses

In this review, it was identified that the test of choice for determining nitrogen fixed by forage grasses is natural abundance of ^{15}N(7). In the main studies reporting N fixed by forage, it is highlighted that the rate of N fixation differs between species (Table 1). This has a direct relationship with the populations of diazotrophic bacteria that interact with each type of forage, in *Brachiaria* sp., approximately 10^2 to 10^8 CFU g^{-1} soil are estimated(8). On the other hand, in *Pennisetum* sp., the diazotrophic bacterial population is reported to be 10^2 to 10^6 CFU g^{-1} soil(9).

Table 1: Some reports of forage species contributing to biological nitrogen fixation according to the review analysis

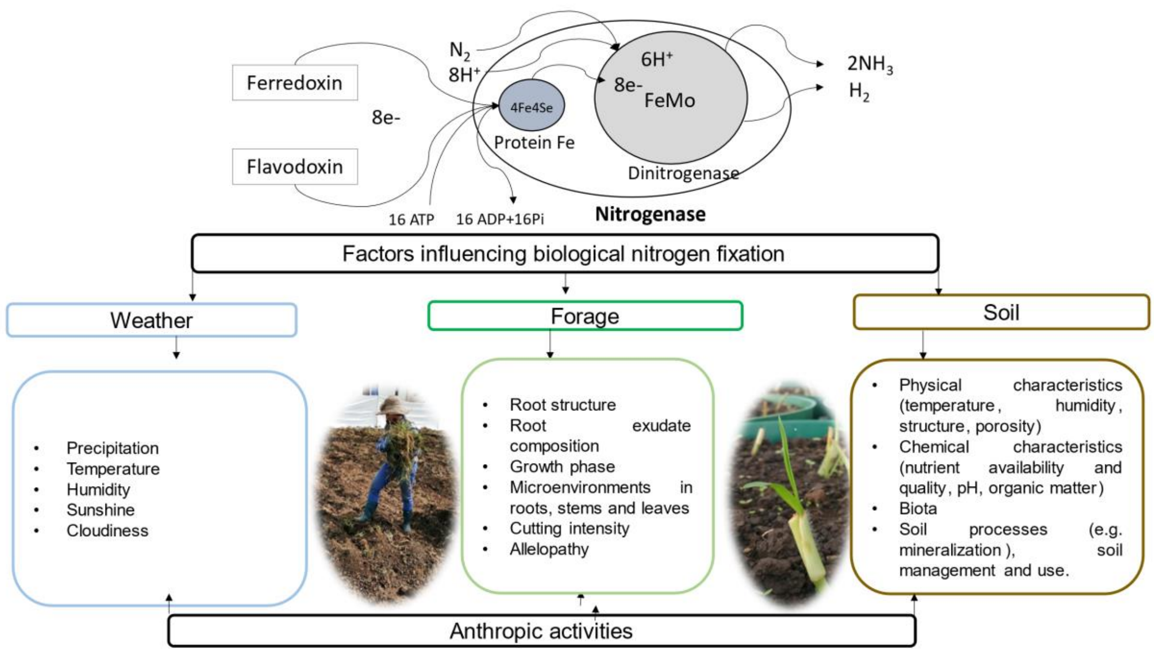
Crop	N fixed (%)	Source
Aristida laevis	36	Marques AC, *et al*(2)
Pennisetum purpureum	18-70	De-Morais RF, *et al*(10)
Megathyrsus maximus sp.	16 - 39	De-Carvalho EX, *et al*(11)
Brachiaria sp.	5.1 – 45	Leite RDC, *et al*(12)
Miscanthus giganteus	16	Leite RC, *et al*(13)

Source: prepared based on the indicated citations.

It was found that the main bacterial genera that persist in the rhizosphere and plant tissue of *Brachiaria* sp., *Pennisetum* sp., *Megathyrsus* sp., and *Panicum* sp., correspond to *Enterobacter* sp. (6 %)(10), *Azospirillum* sp. (25 %)(12,13,14), *Azotobacter* sp., *Bacillus* sp. (14 %)(2,15), *Herbaspirillum* sp. (11 %), *Burkholderia* sp. (8 %)(14), *Bradyrhizobium* sp. (6 %), *Klebsiella* sp. (5 %)(11,16), *Sphingomonas* sp. (4 %)(17), other (2 %). However, their distribution in roots, leaves and stems varies by forage species, locality, and soil type(18).

These microorganisms do not cause structural modifications in the plant and are encoded by the *nifH*[4] gene. The BNF process is carried out in sites with lower oxygen saturation to avoid nitrogenase inactivation, such as in clays, or through a reduction in intracellular oxygen concentration through an increase in cellular respiration[19]. During the reaction, eight electrons are pumped at high speed from a donor agent (ferredoxin or flavodoxin) to the nitrogenase enzyme complex consisting of the metalloenzymes dinitrogenase reductase or protein Fe encoded by the *nifH* gene and the dinitrogenase metalloenzyme encoded by the *nifD* and *nifK* genes[20]. Dinitrogenase reductase transfers each electron to dinitrogenase and they are stored in the FeMo cofactor, the binding site of N until it is reduced to NH_3, thus consuming 16 ATP, and producing 2 mol of ammonium and 1 mol of H_2 for each fixed N molecule[21]. As a result of the review, it was found that the differences in fixed nitrogen ranges between forages and species of the same genus are mainly determined by the factors: plant, soil, anthropogenic activities, and climate (Figure 1).

Figure 1: Factors influencing biological N fixation in forage grasses



Source: prepared based on citations[2,5,11,18,21,22].

Effect of climate on forage BNF

Although there are few studies analyzing the effect of climate on the BNF process, it is highlighted that cloudiness has a negative influence on this process due to the lower availability of photoassimilates that are produced in the leaves and distributed to the roots for the formation of rhizo-exudates[23]. The increased production of photoassimilates seems to have a direct relationship with the persistence of inoculated diazotrophs, which favors their effect; for example, with the application of *Azospirillum brasilense* in *Urochloa brizantha*, it has been observed that at the beginning of the dry season in which solar radiation increases, the mass of the roots of inoculated plants was 27 % higher than in non-inoculated plants, and although during the transition period the production of grass decreased, in inoculated plants, it decreased by only 7 % and its height increased by 16 % compared to non-inoculated plants, due to the greater absorption of nutrients[(12)]. Similar responses are reported with the application of *Bacillus* sp. on *Megathyrsus maximus*[(24)].

Ensuring the persistence of diazotrophic communities can reduce dependence on nitrogen fertilization[(13)]; however, in the rainy season, N fixation due to bacterial effect may decrease perhaps due to the entrainment of microorganisms[(1,9)]. This may explain why it is reported that at the end of the wet season, root biomass decreases by 15 % in inoculated plants and there is a lower N content in the leaves compared to plants fertilized with N[(12)].

In N-deficient environments, BNF increases as a control response when there are low mineralization rates[(4,25)]. Thus, higher accumulated N is reported in autumn than in spring due to the effect of a lower temperature in the forages *Axonopus affinis* (37.6 kg N ha^{-1}.), *Paspalum notatum* (27.7 kg N ha^{-1}.) and *Andropogon lateralis* (1.6 kg N ha^{-1}), estimating that on average, the percentage of N from BNF is 33 %, 22 %, and 25 % respectively[(2)].

Soil effect on forage BNF

Soil characteristics also influence BNF[(22)], a greater diversity of diazotrophic populations in soils with high organic matter is highlighted. The persistence of these microorganisms is modulated by the type and quality of nutrients in the soil[(22)], it is explained that diazotrophs increase their activity with the presence of iron (Fe), molybdenum (Mo), and vanadium (V) because these elements can be exchanged to be part of the nitrogenase structure. This enzyme, when inactivated by oxygen, requires anaerobic microsites to catabolize nitrogen fixation, which is why it seems that in clay soils there is greater chemical and mineral mobilization, and eventually greater BNF[(21)].

Effect of anthropogenic activities on forage BNF

Soil is a system that is naturally self-regulating, but abrupt changes in its characteristics due to anthropogenic management activities (tillage, fertilization) and use (permanent pastures with and without intervention, livestock) cause imbalance in bacterial communities since they alter the structure of the pores, the availability of elements, the content of organic carbon and the pH, factors that determine the richness, uniformity, and diversity of microorganisms[(2,22)].

Excessive application of Ca, nitrate and N during fertilization has a negative effect on diazotrophic populations[17]. The main cause is related to soil pH[12,22]; variations of 1.5 in the soil pH value can reduce the growth of microorganisms by up to 50 % in soils with a pH between 5 and 7[12,22]. There are reports of the inhibition of the growth of some microbial populations, such as *Azotobacter*, *Azospirillum*, *Herbaspirillum* and *Gluconacetobacter diazotrophicus*, with high fertilization doses of N[2,10,26], for example, with the application of 430 kg N ha^{-1} in *B. brizantha* and *B. ruziziensis*[27]. Nevertheless, the type and amount of fertilizer applied influences the abundance and diversity of microbial populations; an increase in methanotrophs with inputs greater than 200 μg N g^{-1} of ammonia has been observed when the active site of ammonia monooxygenase is exceeded[28]. In general, the structural modification of the bacterial community is a natural mechanism for controlling the nitrogen status in the soil[2].

Effect of the plant factor on forage BNF

The morphophysiological characteristics of grasses generate dissimilar microenvironments in leaves, stems, and roots, which promote the selective growth of members of the bacterial population during the growth phase[4]. In the early phase, the activity of rhizospheric diazotrophic populations is greater due to an increase in rhizo-depositions as a mechanism for plant recovery after grazing[13]. The interaction between diazotrophic bacteria and plants occurs through rhizo-depositions that include several molecules such as sugars, polysaccharides, inorganic organic acids, amino acids, vitamins, flavonoids, siderophores, peptides, proteins, and fatty acids[29]. These chemical signals control the interactions that take place in the soil and are responsible for promoting the selective growth of members of the rhizospheric community and allow the movement of bacteria to the plant root and root hairs[2,4]. The diverse functional capacity of diazotrophic bacteria allows them to modulate the growth response of forage and generate positive, negative, or neutral interactions. The main findings in relation to forage response with diazotroph inoculation are discussed below.

Biofertilizers made up of diazotrophs that have been used in grasses

From 1985 onwards, the first scientific studies in the area of biofertilizers applied to forage were reported, although historically it is a practice that dates back to 500 B.C., originating in India, a country that continues to lead scientific advances with a 30 % global share, followed by Brazil (10 %) and China (8.8 %). In the area of biofertilizers applied to forages, authors such as Gupta *et al*[4], Li H *et al*[15] and De Sousa *et al*[30] stand out. Rapid growth is estimated in the area with an inflection point by 2034 (Table 2), a projection that shows the existence of study opportunities that are linked to the phenomenon of climate change and the challenge of using sustainable fertilization strategies that reduce the application of chemicals obtained by burning fossil fuels such as urea.

Table 2: Nonlinear regression models obtained for the searches "Biofertilizer" and "Biofertilizer and grass"

Boolean code	Model	Inflection year	Durbin Watson	a	b	R^2	*P*-value
Biofertilizer	Sigmoidal 3 Parameter	2034	1.07	10988	5.7	0.99	0.01
	Sigmoidal, 3 Parameter	2029	1.87	21.42	4.34	0.96	0.01
	Sigmoidal, 4 Parameter	2018	2.89	26.96	5.4	0.90	0.01
Biofertilizer and grass	Gompertz, 3 Parameter	2018	2.93	33.68	10.42	0.90	0.01
	Hill, 3 Parameter	2016	0.82	21.34	92.85	0.58	0.01

Source: Authors' own preparation.

The trend of biofertilizer use in forages is sigmoidal with an inflection point towards the year 2029, as observed in the logistic model with the highest fit that obtained a Durbin Watson value close to 2[7], although the prediction by the Gompertz and Hill models is earlier, they have a lower fit (R^2), therefore, they do not predict reliable behavior (Table 2). The inflection point is associated with the rapid growth phase of the technology and corresponds to the maximum value of the curve from which biofertilizer-related publications are expected to begin to decline. These predictions with high variation are related to areas of application in increasing development, and organic fertilization is beginning to gain importance in the livestock sector due to the rise in the cost of chemical fertilizers.

From the review analysis, it was found that biofertilizers used in pastures have been applied by seed inoculation in the product for 30 min to 24 h, followed by a drying time prior to sowing[2,31] or by spraying in dosages ranging from 200 – 500 ml of inoculant ha^{-1} diluted in water at 0.1 - 1.3 % in a minimum concentration of 10^6 CFU ml^{-1} or 10^6 CFU g^{-1}[32-36].

The inoculation of microorganisms can modify the development of forage with high variability between genera and strains applied or even cause no effect or generate a negative response[35] (Table 3). When biofertilizers have been applied together with a synthetic N source, responses greater than or equivalent to the application of 100 % of the N requirement have been achieved due to more efficient absorption, reducing N losses caused by leaching by up to 95 %[36]. The best results in terms of production and economy have been observed with the combined application of the inoculant and N[36-40].

Table 3: Some studies of the effect of diazotroph application on forage grasses

Forage	**Inoculant**	**Percentage increase in biological parameters compared to non-inoculated plants**	**Source**
Brachiaria decumbens	*Herbaspirillum rubrisubalbicans* and *H. seropedicae*	12 % in crude protein	(1)
Megathyrsus maximus	*Bacillus* sp. and *Bacillus megaterium*	7.32 %, 25.3 % , 3.32 %, 20.3 %, 2.43 % in height, root biomass, digestibility, protein and neutral detergent fiber, respectively	(15)
Avena saliva L.	*Klebsiella* sp.	20 % in biomass	(16)
Panicum virgatum L.	*Burkholderia phytofirmans*	27 % in height	(19)
Brachiaria ruziziensis	*A. brasilense*	31.49 % in the relative content of water in leaves	(27)
Lolium multiflorum	*Pseudomonas fluorescens* and *Bacillus subtilis*	63 and 51 % in the production of dry mass of plants and biomass, respectively	(32)
Brachiaria brizantha	*Burkholderia pyrrocinia* and *Pseudomonas fluorescens*	770 %, 300 %, 17 % in root biomass, dry matter and chlorophyll, respectively	(33)
Panicum virgatum L.	*Azospirillum brasilense*	23 % in biomass	(34)
Avena saliva L.	*Sinorhizobium meliloti*, *Bacillus megaterium*, *Enterobacter* sp., *A. chroococcum*, *Pseudomonas* sp.	10.34 and 28.92 % in height and root length (28.92 %)	(37)
Pennisetum clandestinum	*Klebsiella* sp., *Beijerinckia* sp., *Achromobacter* sp.	52 %, 170 %, 134 % in shoot length, shoot dry weight and root length, respectively	(41)
Megathyrsus maximus	*Bacillus* sp.	30.8 % and 12.7 % in biomass production and height, respectively	(42)
Avena saliva L.	*Providencia rettgeri*, *Advenella incenata*, *Acinetobacter calcoaceticus*, *Serratia plymuthica*,	81.19 %, 26.89 %, 10.94 % in height, root length and chlorophyll, respectively.	(43)

Avena saliva L.	*Acinetobacter calcoaceticus* *Bacillus thuringiensis* and *B. thuringiensis*	92 % in germinated seeds	(44)
Phleum pratense L.	*Bacillus subtilis*	26.6 % and 63.8 % in shoots and roots, respectively	(45)
Pennisetum purpureum Schumach	*Sphingomonas, Pantoea, Bacillus* and *Enterobacter*	116.01 % increase in shoot dry weight	(46)
Sorghum bicolor L.	*Azotobacter* sp. and *Burkholderia* sp.	21.5 % and 16.8 % in crude protein and dry matter digestibility, respectively	(47)

Source: prepared based on the indicated quotations.

The positive response of the plant with the inoculation of diazotrophs is mainly due to two main conditions; first, because it favors the availability of nitrogen in the soil, which is an element that is part of proteins, amino acids, DNA, RNA, cytochromes, nucleic acids, and chlorophyll(2,21); and second, because of the production of secondary metabolites of bacterial origin such as: a) auxins that are involved in cell growth, differentiation, and division(16), b) gibberellins, which are hormones involved in the regulation of cell division and elongation, seed germination, bud appearance and stem growth(48), c) cytokines, which are related to the regulation of cell growth(48), d) siderophores, which are compounds that can bind to iron, making it available for use in metabolic processes(26) and e) biosurfactants, which are chemical agents that form micelles and allow better interaction between the membrane of microorganisms and nutrients dissolved in the soil and in rhizo-depositions(49).

Of these biomolecules, auxins are the most studied; indole-acetic acid stands out, which is synthesized from tryptophan, which can be derived from the following pathways: indole-3-acetonitrile, indole-3-acetamide, indole-3-pyruvic acid or tryptamine(48,50). This hormone is produced by some diazotrophs, for example: *Stenotrophomonas* spp., *Pseudomonas* spp.(49), *Azospirillum* spp.(51), *Azotobacter* spp., and *Pseudomonas* spp.(26). Its main effect is related to the modification of the structure, elongation and increase of forage root biomass(37), which favors the absorption of nutrients.

The hormonal stimulus that can be indirectly caused by the application of diazotrophs to the plant can favor its phenotypic plasticity in shady environments(23), in drought conditions(15) or saline soils(46). Physiologically, tolerance to stress conditions is related to an increase in the activity of the superoxide dismutase and catalase enzymes that eliminate H from free radicals generated under stressful conditions(32). An increase in the contents of proline, glutathione reductase(42), and ACC-deaminase(46) has also been reported.

On the other hand, greater availability of N in the soil due to bacterial effect allows the plant to increase the production of chlorophyll as it is part of its chemical structure, which leads to an increase in the photosynthetic rate of the plant and consequently in the production of biomass(32). Compositionally, it can promote the crude protein content of forage(1) and the production of unsaturated fatty acids(14).

Despite the aforementioned synergisms, antagonistic responses are reported with the inoculation of diazotrophs(2), due to the effect of nitrogenase inactivation due to exposure to high doses of N. Nevertheless, the lack of response may also be due to a low dose of inoculant applied(23), which can be inhibited by allelopathic control of the plant, which generates low survival, adaptation, and persistence of the inoculated microorganisms. In fact, the variability among the ecosystem can limit the response of bacteria because the BNF process occurs only in favorable environments that allow the persistence of the alpha-proteobacterial taxonomic group(9,51).

Conclusions

BNF is the main source of N in perennial meadows where synthetic N is not applied and in areas of severe drought where the plant manages to maintain its growth thanks to structural adaptations such as the reduction of aerial material to increase root length. The specific signaling mechanisms that allow the expression of proteins for the production of hormones and enzymes that make these modifications possible and potentiate microbial communities specialized in BNF to favor plant survival under extreme conditions are unknown. However, it has been identified that the species of *Brachiaria* spp. and *Pennisetum* spp. have high potential to contribute to the BNF process due to the persistence of alpha proteobacteria in the rhizosphere and in the tissue of roots, stems, and leaves.

Azospirillum spp. and *Azotobacter* spp. are highlighted, but of these, *Azospirillum brasilense* has the greatest potential to fix N due to the ability to infect forage tissue, which eventually facilitates its survival. Nonetheless, it is unknown whether the colonization of this isolate along with other endophytic microorganisms resists the plant's defense system during prolonged exposure times, and perhaps this is related to the lack of productive response with the application of some inoculants. This is why the biotechnological development of these products aims at the study of native microorganisms to avoid a negative allelopathic response by the plant.

Increased dry matter with the application of biofertilizers is the main response observed according to the review analysis, this effect may eventually allow shorter grazing intervals and the intensification of rotations in livestock systems. It has also been observed that the application of diazotrophs can stimulate the phenotypic plasticity of the plant in shaded conditions, which is why the use of biofertilizers can be a cost-effective option in silvopastoral systems.

There are still challenges such as ensuring positive interactions between applied microorganisms and native strains, developing biofertilizers combined with chemical fertilizers and biostimulants, reducing the technical costs of isolation, massification and obtaining the final product, formulating products by crop and according to the stage of growth, using monitoring methods for the detection and quantification of persistent bacterial populations that allow adjusting the dosage and frequency of use of biofertilizers according to management, crop, environmental conditions and soil type, and encouraging their application in farm systems as an ecosystem service.

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