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A Malian native *Azospirillum* sp. Az6-based biofertilizer improves growth and yield of both rice (*Oryza sativa* L.) and maize (*Zea mays* L.)

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The objective of this study was to improve rice and maize yields using native *Azospirillum*-based biofertilizer. To reach this objective, samples of rhizosphere soil, non-rhizosphere soil and roots of maize plants were collected from the particular locations of Samanko and Bamako of the south Mali. Thirty-three different colonies of bacteria were isolated from the different samples. Based on their better growth in nitrogen free semi-solid medium, their morphological, biochemical and plant growth promotion characteristic, ten bacterial isolates were identified as *Azospirillum* isolates following the Bergey's Manual of Determinative Bacteriology. Ten isolates were selected: Az1, Az2, Az3, Az4, Az5, Az6, Az7, Az8, Az9 and Az10. Strain Az6 showed great potential on both rice and maize production. Therefore, this strain is suggested for large scale rice and maize fields' application. While the *Azospirillum* sp. Az5, Az6 and Az10 strains are suggested for large scale application in maize field, which may reduce production cost. Top dressing with 25% of the recommended nitrogen-fertilizer was found to decrease maize grain yield.

Key words: *Azospirillum*, Nitrogen-fixing bacteria, plant growth-promoting rhizobacteria (PGPR), biopesticide, maize, rice, Mali.

INTRODUCTION

In Mali, in recent years, a significant drop in cereal yields, mainly maize (*Zea mays* L.) which occupies an important place in agricultural production systems in agroecological zones has been registered. Maize is one of the main cereals used to feed people in Africa (Macauley and Ramadjita, 2015; Ranum et al., 2014). Although a food crop, maize represents also a cash crop for many small farmers. It has a high yield potential, and it is easy to prepare and digest. All parts of the plant (stem, leaves, tops and seeds) have economic value. They can all be used to produce a wide variety of food (for Human and animals) and non-food products (Ranum et al., 2014).

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Maize is one of the most crucial and strategic cereals for achieving food self-sufficiency in Mali. Several factors explain its reduced yields such as soil mineral deficiencies, soil salinity, pH, temperature, heavy metals, phytopathogens, poor farming practices and harmful insects. Among these factors, soil nitrogen deficiencies, soil salinity and phytopathogens constitute the main constraints in Mali.

In recent years, much work in the world has been devoted to the use of soil bacteria to improve plant production (by improving its growth and protection), while reducing the use of chemical compounds which are more are expensive and harmful to Human and the environment. These plant growth-promoting rhizobacteria (PGPR) belong to different genera such as *Pseudomonas*, *Azospirillum*, *Azotobacter* and *Bacillus* (Sharma et al., 2017). These plant growth-promoting rhizobacteria (PGPR) improve plant growth and nutrition through phyto-stimulatory activities, hormonal mechanisms and phytoprotective functions (antagonism, competition or induction of systemic resistance) (Kannojia et al., 2019). The use of phytosanitary products can impact the soil microbial biodiversity (Annapurna et al., 2013). To do this, a possible strategy is based on the use of microorganisms whose phytobenefic effects would limit the use of chemical inputs (Abiala et al., 2015; Biessy et al., 2019).

There is evidence that inoculating maize seeds with effective rhizobacteria can improve maize production (Amogou et al., 2019; Kuan et al., 2016). Among the rhizobacteria most often used in organic fertilization, *Azospirillum* have shown a significant effect on maize, wheat, rice, sorghum and sugar cane production (Santa et al., 2004; Galindo et al., 2019) and have phytoprotective activities (Pieterse et al., 2003; Moënne-Loccoz et al., 2015).

Although Mali is among the biggest rice and maize producing countries in Africa, and nitrogen fertilization is more than necessary to increase the production of these cereals, very few studies have been undertaken so far on the use of native free and endophyte *Azospirillum* as organic fertilizer to improve rice and maize yields. This study aims to isolate effective *Azospirillum* isolates and formulate efficient as well as easy to use *Azospirillum*-based biofertilizer to improve maize and rice growth and yields.

MATERIAL AND METHODS

Collection of samples: Maize fields of particular locations of two different sites of the districts of Bamako were selected for sample collection. The locations were Samanko agricultural fields and LaboREM experimental site. Rhizosphere soils were collected from the rhizosphere regions of the maize plant at the depth of 2-3 cm and non-rhizosphere soil sample was collected from 1.80 m away from each plant. The plants were uprooted for root sample and the soils attached to the roots were removed. All samples were taken in different polythene bags and brought to the laboratory. The samples were preserved in refrigerator.

Preparation of samples: By placing the roots under a gentle stream of water soils attached to the roots were removed. When the roots were free of adhering soil, these were thoroughly washed for several times with sterile distilled water. Using sterile scissors, the roots were cut into small pieces. One gram of each root samples was used for the purpose. The roots were rinsed successively in disinfectant 2% Chloramine T and subsequently sterile 0.5 M PO_4 buffer (pH-7) five times. Afterwards, these have been washed with sterile distilled water three times; the root pieces were macerated in sterile mortars and serially diluted.

Isolation of *Azospirillum* bacteria

Media used in Azospirillum isolation

The media used in this study were those recommended by Baldani et al. (2014) and Caceres (1982). N-free semisolid malate medium (NFb-medium): (Gramme / liter of Distilled water (g/l D.W.)) DL-malic acid, 5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; KOH, 4; NaCl, 0.1; CaCl , 0.02; agar, 1.75; trace 2 element solution, 2 ml; alcoholic solution of Bromothymol Blue (5%), 2 ml; Fe EDTA, 4 ml; vitamin solution, 1 ml; NaOH to adjust the pH to 6.8. The trace element solution contained: 200 mg Na_2MoO_4 .$2H_2O$; 235 mg $MnSO_4.H_2O$; 280 mg H_3BO_3; 8 mg $CuSO_4.5H_2O$; 24 mg $ZnSO_4.7H_2O$; 200 ml D.W. The vitamin solution contained: 10 mg biotin; 20 mg pyridoxine; 100 ml D.W. Congo Red Agar (CRA) medium: (g/L D.W.) DL-malic acid, 5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; KOH, 4.5; NaCl, 0.1; agar, 15-20; yeast extract, 0.5; $FeCl_3.6H_2O$, 0.015; 15 ml Congo red solution (0.25%); NaOH to adjust the pH to 7.0.

Azospirillum isolation

The isolation of *Azospirillum* was done according to the technique of Hossain et al. (2015). Briefly, Nfb semi-solid medium in screw-capped tubes was inoculated with 0.1 ml of each sample suspension, using a sterile pipette and was incubated at 37°C for 72 h. After incubation, *Azospirillum* appeared in the tubes forming characteristic thin dense, white pellicle few mm below the surface at the medium (Dobereiner, 1980). The pellicles were examined microscopically for the presence of gram negative, fibroid and actively motile cells. According to Krieg (1981), a loopful of the pellicle developed in tubes was transferred to fresh semi-solid Nfb-medium in screw-capped tubes and the tubes were incubated at 37°C. The white sub-surface pellicle formed after 72 h in the fresh medium was checked by microscopic examination for the presence of gram negative, curved, motile cells and transferred into the fresh semi-solid Nfb-medium thrice, each transfer being made at 72 h intervals. Then, a loopful of the pellicle was streaked on the plates of Nfb-medium, containing 20 mg yeast extract per liter and solidified with 1.5% agar. The plates were incubated at 37°C for one week. Small, dry, slightly convex and rugose colonies were transferred to the slants of solid malate medium containing 0.1% ammonium chloride. Cultures in the slants were streaked on the Congo red medium to get pure colony. The pure colonies were transferred to the slants of same medium and preserved.

Characterization of the isolates

Morphological characteristics of the isolates

From the morphological view point, the isolates have been observed macroscopically and their specific morphology and cultural characteristics have been appreciated by culturing them on potato dextrose agar (PDA) plate media (Rosemary et al., 2013).

Biochemical characteristics of the isolates

The catalase (Venkateshwarran et al., 1999), oxydase (Caridis et al., 1991), cellulase (Gupta et al., 2012), and urease (Varenyam et al., 2010) tests were done according to methods described by the cited authors. Indole, hydrogen sulfide, nitrate reduction, carbohydrate fermentation, Voges and methyl red tests were done using Hossain et al. (2015). In each case, growth of the isolates was recorded by visual observation (Hossain et al., 2015). Bergey's Manual of Determinative Bacteriology (1994) was used to identify the tested isolates.

Plant growth promoting characteristics

To determine the plant growth promoting characteristics, the followings tests have been performed**:** promoting plant growth; the production of chitinase (Han et al., 2009), Indole Acetic Acid (Bric et al., 1991), siderophores (Schwyn and Neilands, 1987; Milagres et al., 1999), Cyanuric acid (Babana, 2003), and phosphate solubilization (Babana and Antoun, 2006; Dicko et al., 2018; Kassogué et al., 2015).

Greenhouse experiments

Experiment 1: Determination of the effect of Azospirillum isolates on the growth of rice

In this experiment, a complete randomized design (CRD) with 11 treatments was used. The *Azospirillum* isolates and a non-inoculated control represented the treatments. Rice cultivar (Adny11), the most appreciated by farmers and consumers in Mali, was used as plant test in this experiment. Each treatment was replicated 3 times. Five seeds of the tested rice variety were seeded directly in a plastic pot (100 mm w x 75mm d x 85mm h) filled with 2.50 kg of air-dried soil from LaboREM-Biotech test site. Rice seeds treated with sterile distilled water alone were considered as control. The pots were held on racks and grown under greenhouse conditions and watered regularly. After one week, the seedling was thinned to two plants per pot. Growth parameters such as shoot length and root length were recorded 45 days after planting. Collected data were subjected to analysis of variance and comparison of means using protected LSD test (P≤0.05). The statistical package SAS (Version 9 – SAS Institute) was used for all analysis (SAS, 1990).

Experiment 2: Determination of the effect of Azospirillum isolates on the growth of maize

Efficiency of *Azospirillum* isolates on plant growth and nutrient uptake in maize was evaluated under greenhouse conditions by seed bacterization. Bacterization of surface sterilized seeds was performed by imbibing the seeds in each *Azospirillum* isolate cell suspension (A600=0.5) for 6 h at 60 rpm. Maize and rice seeds treated with sterile distilled water alone were considered as control. Seeds either inoculated with bacteria or untreated were sown in plastic pots (100 mm w × 75 mm d × 85 mm h) filled with approximately 2.50 kg of air-dried soil from LaboREM-Biotech test site. The pots were held on racks in a complete randomized block design (CRD) with the dose of N applied as blocks and the *Azospirillum* isolates as treatments. Each treatment was replicated two times. The pots were maintained under greenhouse conditions and watered regularly. Growth parameters such as shoot length and root length were recorded 45 days after planting. Grain production (number and weight of the grains harvested per treatment) was recorded after harvesting.

RESULTS AND DISCUSSION

Nitrogen-fixing bacteria isolated

Sixty-six nitrogen-fixing bacteria were isolated from soils and different parts (Rhizoplane, xylem and endosphere) of maize plants, sampled in the two sites (Samanko and LaboREM, Bamako, Mali) investigated in this study. Based on their ability to grow better and faster in Nfb semi-solid medium in screw capped test tubes but not in Nfb agar medium in plates, 10 isolates were selected out for further studies. No isolate was obtained from non-rhizosphere soil, contrary to the rhizosphere soil and the rhizoplane where the maximum number of isolates was obtained. All the bacterial isolates from LaboREM-Biotech location were from the rhizoplane.

Azospirillum bacteria identified

Colony aspects of isolate Az10 on Nitrogen free medium (Figure 1A) and Congo red medium (Figure 1B) are presented in Figure 1, while Gram and vegetative cells of *Azospirillum* sp. Az6 are presented in Figure 2.

Data collected on morphological and biochemical characteristics of the ten nitrogen-fixing bacterial isolates showed that: all the ten selected isolates presented brownish and flat colonies on *Azospirillum* medium. They were curved, Gram negative, mobile, catalase and oxidase positive. They produced hydrogen sulfide, reduced nitrate and utilized lactose, mannitol and galactose as carbon source. The ten selected bacteria isolate can utilize biotin, but cannot hydrolyze the gelatin nor utilize xylose, glucose and sucrose, as carbon source. Considering all the identified characteristics, Az1, Az2, Az5, Az6 and Az10 were identified as *Azospirillum brasinense*.

The analysis of data in Table 1, shows that only the *Azospirillum* isolate Az5 produced all the compounds assessed, followed by the isolates Az3 and AZ8 who were only cellulase negative. Contrary to Az3 and Az8, the isolate AZ9 produced all the compounds but did not solubilize phosphates. Siderophores and cyanhydric acid have been produced by all tested isolates, but the isolates Az2, Az4, Az7 and Az10 were not able to produce the indole acetic acid. All selected *Azospirillum* isolates can grow normally at a temperature between 22 to 45°C, at pH range of 4.5 to 9.8, and in media containing 40 to 500 mM of NaCl.

Effect of the *Azospirillum* isolates on rice growth

Inoculation with the isolated *Azospirillum* sp. strains significantly increased seed germination, plant height, root and shoot fresh and dry weights of rice *(Oryza sativa L.* cv. Adny11) (Table 2). No significant effect was observed between the different repetitions. This indicates,

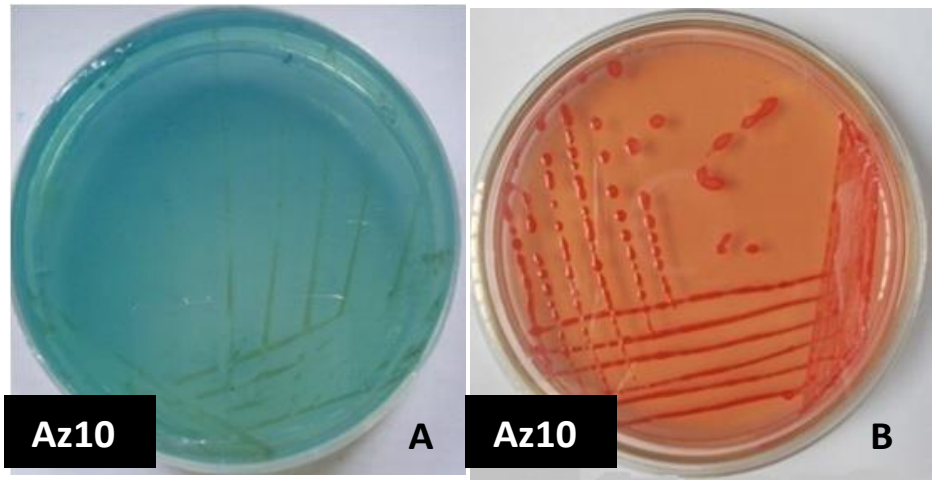

Figure 1. Aspect of *Azospirillum* sp. (Az10) colonies after 7 days.

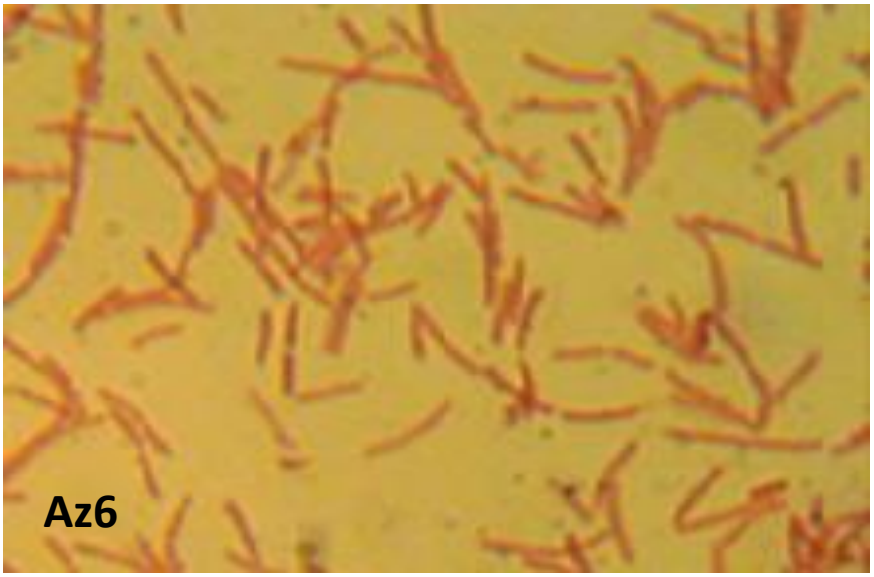

Figure 2. Gram and vegetative cells of *Azospirillium sp.* AZ6 incubated at 37°C on NFB-medium.

Table 1. Phosphate solubilization; production of cellulase, chitinase, cyanhydrique acid (CNA), indole acetic acid (IAA), and siderophores by the ten selected *Azospirillum* isolates.

Characteristics	*Azospirillum* isolates									
	Az1	Az2	Az3	Az4	Az5	Az6	Az7	Az8	Az9	Az10
Cellulase	+	+	-	-	+	-	+	-	+	-
Chitinase	-	-	+	-	+	-	+	+	+	-
CNA	+	+	+	+	+	+	+	+	+	+
IAA	+	-	+	-	+	+	-	+	+	-
Siderophores production	+	+	+	+	+	+	+	+	+	+
Phosphates solubilization	-	-	+	-	+	-	-	+	-	+

- Do not produce the enzyme/compound assessed; + Produce the enzyme/assessed compounds.

Table 2. Analyze of variance for the germination rate, plant height, root length, fresh and dry shoot biomass, fresh and dry root biomass and stem diameter of rice *(Oryza sativa L.* cv. Adny11).

Sources of variation	DDL	Mean square							
		Germination percentage (%)	Height (cm)	Root length (cm)	Shoot biomass (g)	Root biomass (g)	Dry shoot biomass (g)	Dry root biomass (g)	Stem diameter (cm)
Répétition	2	0.54SN	0.43SN	0.41SN	0.31SN	0.10SN	0.63SN	0.14SN	0.32SN
Traitement	10	11.94***	8.04**	67.94***	30.90***	12.39***	13.79***	26.69***	10.14***

*. **. ***, significant at p<0.05, p<0.01 and p<0.001 respectively, NS: not significant, DOF; degree of freedom.

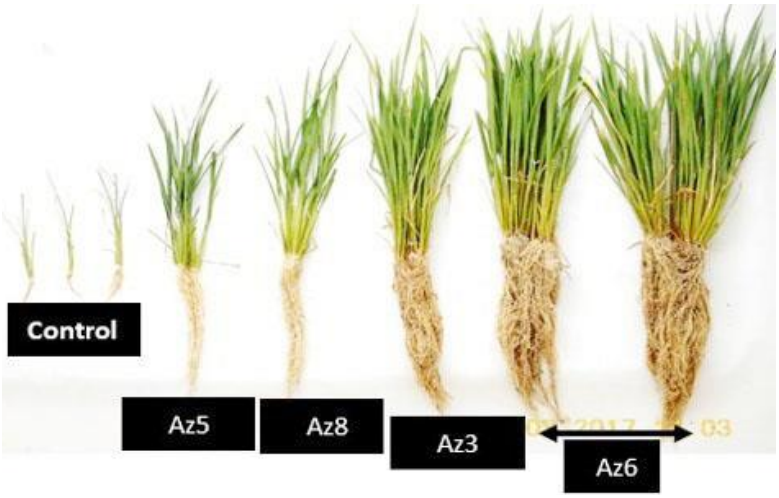

Figure 3. Effect of inoculation with of *Azospirillum* isolates (Az3, Az5, Az6 and Az8) on rice growth compared to non-inoculated maize plants.

for example, that the percentage germination of seed treated or not by *Azospirillum sp.* will not be affected differently by the different repetitions.

The effects of *Azospirillum* sp. isolates on germination rate, plant height, root length, fresh shoot and root biomass and stem diameter of rice (*O. sativa* L. cv. Adny11); showed that seed inoculation significantly enhanced rice seed germination. However, the rate of enhancement varied with bacterial strains. All the *Azospirillum* sp. strains tested, significantly increased seed germination over the non-treated control. The highest percentage of seed germination was recorded with Az4 (85%) and highest plant height, root and shoot biomass were recorded with Az6, followed by Az3, Az8 and Az5 (Figure 3).

Effect of the *Azospirillum* isolates on maize growth and production

Inoculation with *Azospirillum* strains significantly affected leaves number, stem length, grain number and grain weight (Table 3). Grain number and grain weight were also significantly affected by nitrogen level, while no significant effect of nitrogen level on the efficacy of the isolates was observed.

Maize seed inoculation with the *Azospirillum* isolates significantly enhanced all the analyzed parameters. In average, compared to non-inoculated maize plants, an increase in the number of leaves by 9.83%, the stem length by 32.33%, the number of grains by 90.75% and total grain weight by 70.83% were obtained (Table 4). No significant difference was observed between the *Azospirillum* isolates tested (Table 4). However, they showed high quality and well-filled ears compared to the control (Figure 4). The analysis of data in Table 5 showed that the application of 25% of the recommended dose of nitrogen, after inoculation with the *Azospirillum* isolates, decreased the number of maize grains by 45.34% and the total grains weight by 31.40%.

DISCUSSION

In this study, no bacterial isolate was obtained from bulk soil with few nutrients to support high quality growth of

Table 3. Analyze of variance for the leave number, the stem length, the number of ears, the number of grains/pots and the weight of grains produced (g)/pots of maize *(Zea mays.* cv. Dembagnuman).

Sources of variation	DDL	Means square				
		Leave number	Stem length	Number of ears	Number of grains/pots	Weight of grains produced (g)/pot
Nitrogen level	1	0.25^{NS}	0.0005^{NS}	0.02^{NS}	84826**	2251.02*
Repetitions	1	1^{NS}	0.014^{NS}	0.39^{NS}	583.02^{NS}	46.10^{NS}
Nitrogen*Repetitions	1	1^{NS}	0.004^{NS}	0.016^{NS}	2835.56^{NS}	119.52^{NS}
Isolates	3	2.42*	0.42*	0.56^{NS}	41965.44*	3349.33*
Isolates*repetitions	3	1^{NS}	0.08^{NS}	0.057^{NS}	470.69^{NS}	34.56^{NS}
Isolates*Nitrogen	3	1.25^{NS}	0.025^{NS}	0.266^{NS}	4232.52^{NS}	205.13^{NS}

Table 4. Effect of *Azospirillum* isolates on maize leaves number, stem length, number of grains/pot and Weight of grains produced (g)/pot.

Azospirillum isolates	Leaves number	Stem length	Number of grains/pots	Weight of grains produced (g)/pot
Az5	16.50^{a}	2.69^{a}	295.25^{a}	86.35^{a}
Az6	17.00^{a}	2.62^{a}	324.50^{a}	81.28^{a}
Az10	16.75^{a}	2.67^{a}	275.88^{a}	64.60^{a}
Control	15.25^{b}	2.01^{b}	156.51^{b}	45.31^{b}

Figure 4. Effect of *Azospirillum* sp. isolates Az6, Az5 and Az10 on the ears, the filling of the ears and the quality of maize grains produced.

Table 5. Effect of application of 25% of the recommended dose of nitrogen after inoculation with the *Azospirillum* isolates on the number of grains/pots and the weight of grains produced(g)/pots.

Nitrogen doses	Number of grains/pots	Weight of grains produced (g)/pot
0	321.13^{a}	75.55^{a}
25	175.50^{b}	51.83^{b}

microorganisms. In contrary, 10 bacteria were isolated from the rhizosphere and endosphere of maize grown at Samanko and the experimental plot of LaboREM-Biotech. These results are in conformity with those previously reported by Kabir et al. (1996) in Mali. According to New and Kennedy (1989), wheat rhizosphere provides an

ecological niche protected against soil acidity. These results suggest that plant rhizosphere offer a high quantity and diversity of nutrient for *Azospirillum* and a protective environment for a high-quality growth and activities. In fact, Wang et al. (2017) working on the effects of plant root exudates on the composition of the belowground microbiome, demonstrated that plant species and plant genotype were key factors driving the changes in the belowground bacterial community composition in agro ecosystems. Likewise, Brusamarello-Santos et al. (2017) suggesting to exploit the highly diverse maize genetic resources in terms of beneficial plant-bacterial interactions for optimizing maize growth, with reduced N fertilization inputs. In fact, Rilling et al. (2018) showed a compartmentalization between rhizosphere and root endosphere for both the abundance and diversity of total (16S rRNA) and putative N_2-fixing bacterial communities on wheat plants, and Johnston-Monje et al. (2016) studying bacterial populations in juvenile maize rhizospheres originate from both seed and soil, concluded that the most common bacterial cells in juvenile maize rhizospheres are seed transmitted.

All the *Azospirillum* sp. strains isolated in this study produce siderophores and some of them produce indole acetic acid. Inoculation of these isolates significantly enhanced rice and maize seed germination as well as allowed healthy plants. In fact, Araújo et al. (2010) reported that faster germination reduces the period of heterotrophism and reduces the chances of attack by soil pathogens. Besides the production of phytohormones, all the isolated *Azospirillum* sp. strains produce siderophores, and some of them can produce cyanhydric acid and solubilize phosphorus as well as increase the plant height, the number of leaves, the stem diameter of rice and maize plants; and the number of grains and the total weight of maize grains. The results were also supported by the results of Isawa et al. (2010) and Bao et al. (2013), who reported a significant enhancement of rice growth in terms of tiller numbers, shoot length and shoot biomass consecutive to rice seed inoculation with *Azospirillum* B510, under greenhouse and field conditions. Pandiaranja and Govindara (2012) reported that the strains of *Azospirillum* help the plants for better growing by means of utilization of various parameters from the soil to the plants.

In this study, the application of 25% of the recommended dose of nitrogen after inoculation with the selected *Azospirillum* sp. strains tested decreased the number of maize grains by 45.34% and the total grains weight by 31.40%. In fact, Zeffa et al. (2018) reported no significant increase in grain yield when inoculation with bacteria from the *Azospirillum* genus was realized together with nitrogen fertilization, but did not observe a significant decrease in production. According to research results of Steenhoudt and Vanderleyden (2006), Nif gene is inactive in excess N_2. These results indicate that addition of nitrogen after inoculation with *Azospirillum* strains is non-additive.

Conclusion

The present studies specify that the isolated strain *Azospirillum* sp. Az6 was suitable for inoculating on paddy (*O. sativa* L. cv. Adny 11), while *Azospirillum* sp. Az6, Az5 and Az10 strains were suitable for inoculating on maize (*Zea mays* cv. Dembagnuman). *Azospirillum* sp. Az5, Az6, and Az10 strains produced the highest number of leaves, stem length and yield (number of grains and total grains weight) when compared to the non-inoculated control other isolates. However, only *Azospirillum* sp. Az6 strain showed great potential on both rice and maize production. Therefore, this strain is suggested for large scale rice and maize fields' application, while the *Azospirillum* sp. Az5 Az6 and Az10 strains are suggested for large scale application in maize field, which may reduce production cost.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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