EFFECTIVENESS OF ISOLATED NATIVE *AZOSPIRILLUM SPP* ON GERMINATION AND GROWTH PROPERTIES OF RICE

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Abstract

The present study deals with three *Azospirillum* strains (Azo-1, 2 and 3) were isolated from the root of *Saccharum spontaneum* L. (Kaing), *Saccharum officinarum* L. (Kyan), *Dichanthium caricosum* (L.) A. Camus. (Padaw ni) of Poaceae family during 2019. The growth responses of rice inoculation with indigenous isolated strains were studied. These experiments were carried out with 3 isolated strains from the roots of some grasses to know the germination percentage, shoot and root length. The inoculation of isolated strains increased in germination up to 4.12%, 16.25% in shoot length and 12.97% in root length over the control by isolated strain Azo-3. In pot culture, the inoculation of isolated strains increased in plant height (21.87%), leaf area (18.78%), tillering number (27.75%), panicle length (8.88%) and fertile seed per panicle (10.94%), and 1000 grain weight (5.28%) over the control by Azo-3. These results indicated that certain diazotrophs can promote vegetative growth and yield characters of rice.

Keywords: *Azospirillum*, isolate, inoculation, indigenous

Introduction

Plant nutrients, which come primarily from chemical fertilizer, are essential for crop production. In agriculture, nitrogen is an essential element for crop growth and development. Nitrogen is a basic constituent of chlorophyll, proteins and all enzymes are involved in photosynthesis, especially Rubisco which alone accounts for more than 75 % of the total leaf nitrogen (Hak *et al*., 1993).

Soil microorganisms, like *Azospirillum* spp., *Azotobacter* sp. and *Enterobacter* sp. have shown to encourage plant growth, by promoting the outbreak of secondary roots. Inoculation with indigenous *Azospirillum* is an important procedure when studying their inherent capacity to benefit crops. In some cases, indigenous strains can perform better than introduced strains in promoting the growth of crops due to their superior adaptability to the environment (Kanimozhi and Panneerselvam, 2011).

Azospirillum species are commonly found in soils and in association with roots of plants namely rice, maize, wheat and legumes. Rhizosphere colonization by *Azospirillum* species has been shown to stimulate the growth of a variety of plant species (Lopez-de-victoria. 1989).

Bio-fertilizers are substances which comprise of living microorganisms that stimulate the plant growth by increasing the supply or availability of primary nutrients to the plant and the synthesis of growth promoting substances. Hence, bio-fertilizers can be expected to reduce the use of chemical fertilizers (Keyeo *et al*., 2011).

Biofertilizer, an alternate low cost resource have gained prime importance in recent decades and play a vital role in maintaining long term soil fertility and sustainability. They are cost effective, eco-friendly and renewable sources of plant nutrients to supplement chemical fertilizers. Nitrogen fixing and P- solubilizing inoculants are important biofertilizers used in rice (Singh *et al*., 2015).

In Myanmar, a few works have been done on the effects of *Azospirillum* inoculation on growth of rice. The aims of the present study were to isolate *Azospirillum* spp. from native grasses,

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to obtain more knowledge on the useful microorganisms isolated from local area, to know the effects of *Azospirillum* spp. on germination and vegetative growth of rice plant under natural soil-climatic conditions and to develop the effective *Azospirillum* spp. inoculation and useful as biofertilizer.

Material and Methods

Plant Samples Collection

Plants and root samples of *Saccharum spontaneum* L. (Kaing), *Saccharum officinarum* L. (Kyan), *Dichanthium caricosum* (L.) A. Camus. (Padaw ni), were collected from Monywa University Campus, Monywa Township, Sagaing Region during 2019.

Culture Media and Solution

Azospirillum strains were cultured in the following nitrogen free semi-solid malate medium (NFb) and Congo Red Agar Medium (CRA) according to Dobereiner and Baldani (1980).

Isolation of *Azospirillum* strains

In the present, following procedure were used for the isolation *Azospirillum* strains from the roots of three grasses of Poaceae family. Fresh roots samples were washed in rapidly running tap water for 5 minutes to remove the soil particles adhering to the root surface. The roots were rinsed in sterile water and then these roots were cut into small pieces (3-5 mm) and placed into the test tube. Small amount of sterile water was added into the test tube. These small pieces were macerated with glass rod and 0.5 ml introduced into 10 ml semisolid NFb medium. These test tubes were incubated at 33˚C. After 72 hours, white halo pellicle formed 3-6 mm below the media surface. It was a sign of nitrogenase activity. When the cultures medium exhibited a positive nitrogenase activity, they streaked out on Congo Red Agar (CRA) plates. Typical pink, often wrinkled colonies were picked out and transferred into semi-solid NFb medium and transfer into CRA medium for purification. The isolated strains were designated as **Azo-(*Azospirillum*-)** only just for this research work.

Germination Test

The test crop rice seeds (Sin Ekari – 2) were obtained from the Myanmar Agriculture Service, Zalote Research Center, Monywa District in Sagaing Region. The seeds were surface sterilized by immersion in hydrogen peroxide (H_2O_2) for 30s. The seeds were rinsed for five times of distilled water and floating seeds were discarded. Then, the seeds were air dried over a clean filter paper. Sterilized seeds were placed into the bacterial culture medium for 1 hour and control are inoculated with only nutrient medium as shown in Figure 2 and 3. And then the seeds are placed on agar plate and germinated in dark at 30 °C for 120 hours. The percentage germination of rice on each plate was counted and then shoot and root lengths were measured and recorded. Average germination percentage, shoot and root length were calculated for each plate.

Pot culture experiment

The second set of experiments was designed to evaluate the carryout effects of seedling vigor on yield parameters at maturity during May to August, 2019, in pot culture. The pot experiment was structured following a randomized complete plot design. The isolated *Azospirillum* strains (Azo-1, Azo-2 and Azo-3) were used to treat tested crop. Potted soil was watered and puddle before planting. Representative, 22-days old similar sized seedlings of rice plants were transplanted in to pots (35 cm by 20 cm by 15 cm and two plants per pot) containing 7 kg of soil. At transplanting, immerse rice roots into liquid innoculant for 10 - 15 min before transplanting and 25 ml of innoculant spread into each pot at regreening and flowering stages. Tap water was used to irrigate the potted plant. After harvesting, randomly 20 panicles were selected and measure the length of panicle and counted for total seed per panicle. Data were analysed by analysis of variance and the treatment mean were compared relative to control following the F-test on IRRISTAT.

Climatological Data

Climatological data (monthly mean maximum and minimum temperature, rainfall and humidity) were taken from Meteorology and Hydrology Department, Monywa Township in Sagaing Region during 2019.

Table 1 Monthly annual Temperature, rainfall and humidity of Monywa District during 2019

2019	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Mean
Maxi Temp: (°C)	29.5	33.4	35.9	40.2	41.9	37.3	36	34.9	34	34.3	33	29.3	34.98
Mini Temp: (°C)	14.1	16.7	19.3	24.2	26.1	26.4	25.6	25.7	25	23.4	21.3	15.5	21.94
Rainfall (mm)	3	Tr	Tr	15	39	169	35	114	99	16	26	Tr	516.00
Humidity (%)	70	66	54	52	53	70	70	78	80	80	75	71	68.25

Source: Department of Meteorology and Hydrology, Monywa District

Results

Isolated *Azospirillum* strains

All isolated strains (Azo-1, 2 and 3) were isolated from the root of *Saccharum spontaneum* L. (Kaing), *Saccharum officinarium* L. (Kyan) and *Dichanthium caricosum* (L.) A. Camus. (Padawni) of Poaceae family as shown Figure 1. They were isolated and culture on NFb and CRA nutrient medium and then sub-culture to obtain the pure culture. All strains in NFb medium showed white colour pellicle formation about 2 – 5 mm below the surface of the medium and colour changed from pale yellow to dark blue colour after 72 hours incubation. And then, the isolated stains were transferred to CRA medium. In this medium, the colonies of isolated strains were dark pink or red colour, 1.0 – 2.5 mm in diameter after 96 hours incubation as shown in Figure 2.

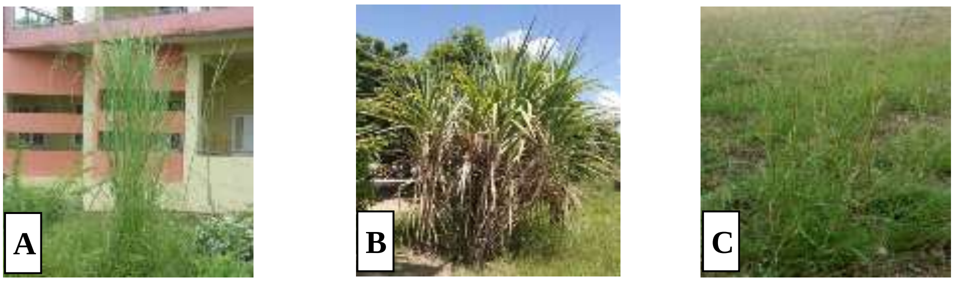

Figure 1 (A) Plant Habit of *Saccharum spontaneum* L.
(B) Plant Habit of *Saccharum officinarium* L.
(C) Plant Habit of *Dichanthium caricosum* (L.) A. Camus.

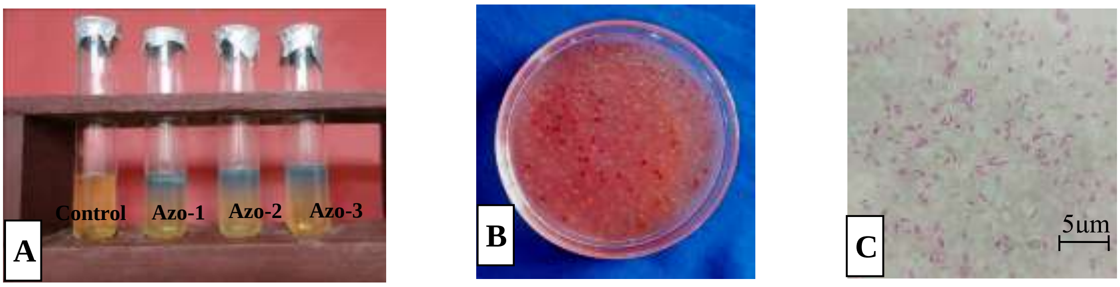

Figure 2 Isolated *Azospirillum* strain culture in semisolid NFb medium
(A) Isolated *Azospirillum* strains in NFb medium
(B) Isolated *Azospirillum* strain spread on CRA medium
(C) Photomicrograph of isolated *Azospirillum* strain

Effect of isolated strains on germination on rice

The results of the germination tests has indicated that among the three isolated strains, Azo-1, Azo-2 and Azo-3 were found to be the most effective strains which provided 96%, 94% and 97% while the control is 93% in germination percentage. Moreover, the isolated strain also gave the highest length of shoot and root length such as 6.78 cm, 6.32 cm and 7.14 cm and control has 5.94 cm and 6.64 cm, 6.18cm and 6.94 cm in root length while the control has 6.04 cm respectively as shown in Table 1 and Figure 3.

Table 1 Effect of inoculation on germination percentage, root and shoot length of rice

Treatment	Germination %	Shoot length (cm)	Root length (cm)
Control	93	5.94	6.04
Azo-1	96	6.78	6.64
Azo -2	94	6.32	6.18
Azo-3	97	7.14	6.94

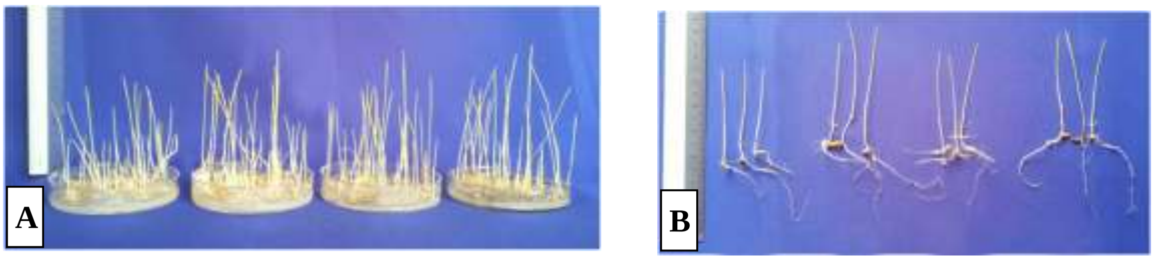

Figure 3 A. Effect of rice germination inoculated with Azo-1, 2, 3 and control
B. Shoot and root formation of rice inoculated with Azo-1, 2, 3 and control

Pot culture experiment

In the pot culture, the growth responses to inoculation varied during vegetative growth stages for rice seedling. The inoculation of rice with Azo-1, 2 and 3 were higher significant in plant height, leaf area and tillering numbers of rice at 20, 40 and 60 days after sowing when compared to control Figure 4 - 6.

Figure 4 Effect of isolated strain and control on plant height of rice after 20 days after sowing

Figure 5 Effect of rice inoculation with isolated strains and control after 40 days after sowing

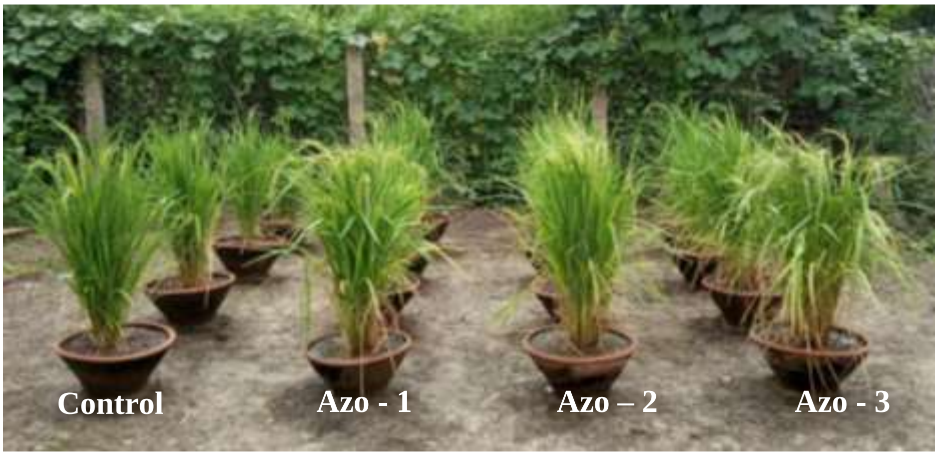

Figure 6 Effect of rice inoculation with isolated strains and control after 60 days after sowing

Effect of isolated *Azospirillum* strains on plant height

In the present study, the mean of plant height ranged from 36.33 to 46.50 cm. The highest length was found in treated plant with Azo-3 (46.50 cm) where as the lowest length was observed in control (36.33 cm) at 60 days after sowing. All treated plants possessed higher plant height than the control ones. The plant height of treated plants with Azo-3 has significantly higher than Azo-1, 2 and control (Table 2).

Table 2 Effects of isolated strains on plant height of rice after 20, 40 and 60 days after sowing

Factor	Plant Height (cm) Growing Period (DAS)			Mean(cm)
	20DAS	40DAS	60DAS	
T_1 (Azo - 1)	11.88	24.43	41.50	25.94
T_2 (Azo - 2)	11.13	23.93	38.25	24.44
T_3 (Azo - 3)	12.33	24.75	46.50	27.86
Control	11.13	23.08	36.33	23.51
F-test	*	**	**	
5%LSD	0.80	0.65	1.13	
CV%	4.5	1.8	1.8	

** = 1% level of significant, *= 5% level of significant, ns = non-significant, DAS = day after sowing

Effect of isolated *Azospirillum* strains on leaf area

The effects of isolated strains on leaf area of rice at 20, 40 and 60 days are showed in Table 3. Leaves area increased with the age in all treatments. The highest leaf area was found in treatment with Azo-3 (53.25 cm^2) and followed by Azo-1 (45.86 cm^2), Azo-2 (44.86 cm^2) and the lowest leaf area was observed in the control at 60 days after sowing.

Table 3 Effect of inoculation on leaf area of rice after 20, 40 and 60 days after sowing

Factor	Leaf Area (cm^2) Growing Period (DAS)			Mean(cm^2)
	20DAS	40DAS	60DAS	
T_1 (Azo - 1)	28.20	35.38	45.86	36.48
T_2 (Azo - 2)	26.88	33.86	44.86	35.20
T_3 (Azo - 3)	30.38	37.50	53.25	40.38
Control	25.63	32.25	43.25	33.71
F-test	**	**	**	
5%LSD	0.86	1.42	1.83	
CV%	2.0	2.6	2.5	

** = 1% level of significant, *= 5% level of significant, ns = non-significant, DAS = day after sowing

Effect of isolated *Azospirillum* strains on tillering number

The result indicated that the number of tiller per plant ranged from 47.75 with inoculation while the control has 34.50. All of the treatments were increased in number of tiller with the age in all accounting times (20, 40 and 60 days). At 60 days, comparison on the number of tillers per plant, Azo-3 showed significantly difference from the other treatments and control as shown in Table 4.

Table 4 Effect of isolated *Azospirillum* strains on tillering number of rice

Factor	Tiller Number Growing Period (DAS)			Mean
	20DAS	40DAS	60DAS	
T_1 (Azo - 1)	13.33	36.50	39.25	29.69
T_2 (Azo - 2)	10.75	34.63	37.50	27.63
T_3 (Azo - 3)	14.50	40.75	47.75	34.33
Control	10.38	30.63	34.50	25.17
F-test	**	**	**	
5%LSD	1.05	1.01	0.83	
CV%	5.6	1.8	1.4	

** = 1% level of significant, *= 5% level of significant, ns = non-significant, DAS = day after sowing

Table 5 Effect of isolated *Azospirillum* strains on panicle length, fertile seeds per panicle and 1000 grains weight

Factor	Panicle length (cm)	Fertile seed/ panicle	1000 grains weight (g)
T_1 (Azo - 1)	25.95	189.82	20.50
T_2 (Azo - 2)	25.83	185.63	20.33
T_3 (Azo - 3)	27.63	200.15	21.20
Control	25.18	178.25	20.08
F-test	**	**	ns
5%LSD	0.62	1.94	0.88
CV%	1.5	0.7	2.8

** = 1% level of significant, *= 5% level of significant, ns = non-significant, DAS = day after sowing

Effect of isolated *Azospirillum* strains on panicle length

The mean of panicle length was measured from 25.83-27.63 cm in the treatment while the control has 25.18 cm. The highest panicle length was found in treated plant with Azo-3 (27.63 cm) but the lowest length was observed in control (25.18 cm). The panicle lengths of treated plants with Azo-3 have significantly more than Azo-1 and Azo-2 over the control as shown in Table 5.

Effect of isolated *Azospirillum* strains on fertile seeds per panicle

The mean values of fertile seeds per panicle were accounted from 178.25 in non inoculated plant to 200.15 for inoculated plant. In contrast, the inoculation of plant with isolated strains had positive significant effect on fertile seeds per panicle. When the plants inoculated with isolated strain were 189.82, 185.63, and 200.15. (Table 5).

Effect of isolated *Azospirillum* strains on 1000 grains weight of rice

In the present study, the inoculation of isolated *Azospirillum* strains on 1000 grains weight arranged from 20.33-21.20 g in the treatment while the control has 20.08 g. One thousand grains weight of rice was significantly improved by the strain Azo-3 expect Azo-1 and 2. The most significant strain Azo-3 which increased up to 21.20 g (5.28%) over the control (Table 5).

Discussion and Conclusion

In the present study, all isolated strains (Azo-1, 2 and 3) were isolated from the root of *Saccharum spontaneum* L. (Kaing), *Saccharum officinarium* L. (Kyan) and *Dichanthium caricosum* (L.) A. Camus. (Padawni) of Poaceae family.

The isolates made from roots of grasses forming the subsurface pellicle in N-free semisolid malate medium is often taken to be an absolute proof of the presence of *Azospirillum* spp. (Okon *et al.* 1977). Appearance of pellicle formation on Nfb semi-solid medium indicated successful isolation of *Azospirillum* (Kanimozhi and Panneerselvam, 2011).

In this study, for the isolation of *Azospirillum* spp., Nfb semisolid medium was used. After 72 h incubation, the Nfb semi-solid medium showed pale yellow to dark blue colour, white colour pellicle formed 2 - 5 mm below the surface of the medium. Then the isolated strains were culture on CRA medium. In this medium, the colonies of the isolated strain were pale to dark pink or scarlet colour, irregular form and convex, 1.0 – 2.5 mm in diameter. Therefore, the result characters are in agreement with the above mentioned literature.

Good seed germination behavior is important for horticulture and agriculture. Uneven or poor germination and subsequently inhomogeneous seedling growth can lead to great financial losses (Ghiyasi *et al*., 2008 b).

Sanzida *et al*. (2008) stated that inoculation effect of *Azospirillum* spp. on growth of wheat at 30 days up to 43.24% over the control in germination percentage. Win Naing (2009) stated that the germination percentage of isolated *Azospirillum* strain promote 18.37% over the control in the treatment of rice. Similarly, Zaw Lwin Oo (2010) and Htar Htar (2013) stated that inoculation of endophytic bacteria strains on germination increased up to 7.29% and 20.0% in rice over the control.

The inoculations of isolated *Azospirillum* strains (Azo-1, 2 and 3) were 96%, 94% and 97% while the control has 93% in germination percentage, 6.78 cm, 6.32 cm and 7.14 cm and control has 5.94 cm in shoot length and 6.64 cm, 6.18 cm and 6.94 cm and 6.04 cm in control in root length respectively. Therefore, the inoculation of isolated strains increased in germination up to 4.12%, 16.25% in shoot length and 12.97% in root length over the control by the isolated strain Azo-3.

Inoculation with indigenous *Azospirillum* is an important procedure when studying their inherent capacity to benefit crops. In some cases, indigenous strains can perform better than introduced strains in promoting the growth of crops due to their superior adaptability to the environment (Kanimozhi and Panneerselvam, 2011).

The effect of *Azospirillum* inoculation on the total yield increase of field grown plants generally ranged from 10 to 30% (Watanabe and Lin, 1984). Okon (1985) evaluated the worldwide success of *Azospirillum* inoculation and concluded that positive effects on yield were obtained in approximately 65% of all field experiments. Yield increases due to inoculation were reported in 75 % of all experiments using summer cereals and only in 50% of the experiments using spring wheat (Smith *et al*., 1984*b*). Recently, about 70-75% of all pot experiments in cotton and several vegetables resulted in yield increase (Bashan *et al*., 1989).

Zaw Lwin Oo (2010) state that the inoculation of rice with isolated *Azospirillum* strains increased plant height upto 17.69%, 33.30% in tillering number, 14.28% in leaf area and 10.50% in total seed per panicle. Similary, Htet Htet Aung (2014) has been shown that the inoculation of wheat with the isolated *Azospirillum* strains increased upto12.64% in plant height, 30.26% in tillering number and 30.10% in leaf area over the control.

Tiller yield in percent over control was ranged from 8 to 27.75% at maturity in case of Azo-1, 2 and 3. Biswas *et al*. (2000) who have reported 16% increase in number of panicle per plant of rice and suggested that the improvement was due to increased availability of nutrients and phytohormones like indole acetic acid and ethylene.

The bacterias of the *Azospirillum* genera presents application potential in agricultural systems, around 70% of the experiments up to 30% in productivity (Dalla *et al*. 2004). In the same way, Win Naing (2009) has been shown that inoculation with endophytic microorganisms (*Azospirillum* sp.) in Ma-naw-thu-kha rice significantly increased in grain yield up to 43.15% over the control. Similarly, Kleopper *et al.* (1992) has been reported that wheat yield increased up to 30% with *Azotobacter* inoculation and up to 43 % with *Baccilus* inoculation. In this study, it was observed that grain yield increased between 3.98% and 10.94%. Among the selected strains, strain Azo-3 was effective and significant in grain yield up to 10.94 % over the control.

In the present study inoculation with selected strains of *Azospirillum* sp. caused significant increase in length of panicle with ranges of 2.52 – 8.88 % respectively. The best strain contributing significant increase in panicle length was Azo-3 (8.88 %) over the control. This is an agreement with the findings of Wedad *et al.* (1988).

In the present study, the total seed per panicle increase up to 10.94% over the control. This result was agreed with Hussain *et al.* (2009) who have reported 22.90% increased in number of grain per panicle. Also, an increase of 30-40% in grain yield of rice due to nitrogen fixing bacteria inoculation over control was recorded by Su San and Yan (1998). Bashan *et al*. (1989) reported that rhizobacteria and AM fungi increase plant growth but controverts results were observing by many authors where occasionally microorganisms inoculated decreasing plant growth. But, in this result work, the inoculation of *Azospirillum* sp. increase plant growth and yield of rice.

Hassamzadeh *et al.* (2007) also showed that seed priming with PGPR has caused an increase in 1000 grain weight of barley. Gholami *et al.* (2009) have been reported that seed priming with *Azotobacter brasilense* DSM 1690 increased 44% 1000 grain weight more than no priming. In the present, the inoculation of isolated *Azospirillum* strains increased 1000 grain weight up to 5.28 % by Azo-3 over the compared to control.

In the present study, comparison of the inoculated *Azospirillu*m strains among each other demonstrated that the strain of Azo-3 performed better than the other isolated strains and control. The higher plant height, number of tillers, total seed per panicle, 1,000 grain weight and grain yield respond to all inoculants compare to control clearly showed the beneficial role of these rhizobacteria. These findings can be concluded that in future valued inoculants could be developed with these organisms to use as biofertilizer for cereal crops like rice, wheat and maize etc. Therefore, further research in this area will be able to develop a sustainable biofertilizer technology for greater and environment friendly cereal production system.

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