The Bioscan 9(3): 1191-1196, 2014 (Supplement on Plant Pathology)
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

ENDOPHYTIC BACTERIA AS BIOFERTILIZERS FOR MAIZE (*ZEA MAYS L.*)

Y. K. JHALA*, H. N. SHELAT, R. V. VYAS AND D. G. PANPATTE
Department of Microbiology and Biofertilizer project,
Anand Agricultural University, Gujarat - 388 001 (INDIA)
e-mail: yogeshvari.jhala@gmail.com

KEYWORDS
Endophytic
Acetobacter
Azospirillum
Bacterization
Recommended dose
Pikovskaya

Received on :
10.05.2014

Accepted on :
18.07.2014

***Corresponding author**

ABSTRACT
Total ten isolates of endophytic bacteria mainly belonging to genera *Acetobacter* and *Azospirillum* having higher nitrogen fixing efficiency were isolated from surface sterilized plant parts of different plant species using LGIP and NFB media selective for growth of *Acetobacter* and *Azospirillum* respectively. All the isolates can colonize maize endophytically under laboratory conditions. *In vitro* nitrogen fixation rates of all the isolates ranged from 4.0 to 36.3 mg N fixed / gm of sugar consumed among which isolate A 10 showed the highest N fixing capacity *i.e.* 36.3 mg /g of sugar. All the isolates were also capable of solubilizing tri calcium phosphate in Pikovskaya's broth, the soluble phosphate content was found in the range of 1.19 - 21.0 µg/ml and 4.44-23.81 µg/ml at 3 and 5 DAI, respectively. Seed inoculation of all the isolates significantly influenced growth of maize *cv*. Narmada moti in lab and field conditions. The highest grain yield (5694 Kg/ha) was obtained when seeds were inoculated with isolate A 10 was applied alongwith 75% recommended dose (RD) of N fertilizer which was significantly superior as compared to 100 % RD of chemical N fertilizer sowing savings of 25% of Nitrogenous fertilizer through bacterization.

INTRODUCTION

Zea mays L. popularly known as 'corn' is one of the most important cereal of the world, ranking third amongst the food crops next to rice and wheat both in respect of area and production. It is well known that "corn" crop is considered among the most important cereal crops either all over the world that consumes huge quantities of chemical nitrogenous fertilizers (16 million tones/year). Many attempts have been tried to replace a part of those harmful chemical fertilizers by biofertilizers to get yield of a good quality without loss in its quantity. Bacteria belonging to genus *Azospirillum* and *Acetobacter* are considered among the best endophytic N fixers who can be used as biofertilizer for maize (Hungria *et al.,* 2010; Mellado *et al.,* 1998) Microorganisms on the roots and in the rhizosphere get benefits from root exudates. Some of the microorganisms are capable of entering plant as endophytes that do not cause harm and can ascertain a mutualistic association (Azovedo *et al.,* 2000; Hallmann *et al.*, 1987; Perotti, 1926). Kado (1992) defined endophytic bacteria that reside within plant tissue without doing substantial harm or gaining benefit other than securing residency. So in present investigation an attempt was made to evaluate *Azospirillum* and *Acetobacter* species for their potential to replace a part of these chemicals and thereby reducing the cost of maize cultivation for betterment of farming community. To achieve the goal current study was undertaken with objective to isolate the endophytic plant growth promoting agents and their *in vitro* and *in vivo* efficacy testing on corn crop.

MATERIALS AND METHODS

In present study, isolation of endophytic bacteria mainly belonging to genera *Acetobacter* and *Azospirillum* was attempted from various plant parts (root, stem and leaves) of species *viz*. *Cynodon dactylon* (Durva), *Pothos scandens* (Money plant), *Ipomea batata* (Sweet potato), *Saccharum officinarum* (Sugarcane) *cv*. CO.LK-8001 and CO.-84135, *Musa paradica* (Banana) and *Zea mays* (maize) *cv*. GM-6 as per method suggested by Dalal and Kulkarni, 2012. Standard strains of *Azospirillum lipoferum* (ASA-1) and *Acetobacter diazotrophicus* (ACG-1) were collected from Department of Agriculture Microbiology, B.A.C.A., A.A.U. Anand and used as positive check during entire study. Single colony with typical morphology of *Azospirillum and Acetobacter* as described by Dobereiner, (1995) were picked from these plates to subculture by re-streaking onto NFB and LGIP plates and used for further study. By comparing characteristics of isolates with standard check the isolates showing similarity with them were further studied for their plant growth promoting activity.

Confirmation of endophytic colonization of maize by endophytic bacterial isolates in laboratory conditions

As this study was aimed to detect efficacy of endophytic bacteria on maize, it was essential to confirm weather the

Table 1: Endophytic bacterial isolates of different plant parts and species

Sr no.	Name of isolate	Source of organism Scientific name	Common name	Plant part
1.	A-1	*Pothos scandens*	Money plant	Leaf
2.	A-2	*Cynodon dactylon*	Durva	Leaf
3.	A--3	*Ipomea batata*	Sweet potato	Root
4.	A-4	*Saccharum officinarum cv.* CO.-84135	Sugarcane	Stem
5.	A-5	*Zea mays cv.* GM-6	Maize	Stem
6.	ACG-1	*Acetobacter diazotrophicus* from *Saccharum officinarum*	Sugarcane	Stem
7.	A-6	*Pothos scandens*	Money plant	Leaf
8.	A-7	*Cynodon dactylon*	Durva	Leaf
9.	A-8	*Musa paradica*	Banana	Root
10.	A-9	*Saccharum officinarum cv.* CO.LK-8001	Sugarcane	Root
11.	A-10	*Zea mays cv.*GM-6	Maize	Stem
12.	ASA-1	*Azospirillum lipoferum* from *Pennisetum glucam*	Bajara	Root

Table 2: Bacterial counts from *in vitro* colonized maize roots

Isolate	*Acetobacter* counts (cfu gm^{-1} fresh root weight)	*Azospirillum* counts
A-1	1.1×10^5	-
A-2	1.0×10^5	-
A-3	6.8×10^5	-
A-4	7.8×10^5	-
A-5	7.9×10^5	-
ACG-1	1.1×10^5	-
A-6	-	7.9×10^5
A-7	-	8.4×10^5
A-8	-	6.2×10^5
A-9	-	6.8×10^5
A -10	-	9.0×10^5
ASA-1	-	7.7×10^5

Table 3: *In vitro* nitrogen fixation capacity of isolate

Isolate	mg nitrogen /g of sugarconsumed
A-1	20.0
A-2	8.1
A-3	23.9
A-4	29.9
A-5	24.3
ACG-1	10.4
A-6	23.3
A-7	4.0
A-8	21.3
A-9	20.8
A-10	36.3
ASA-1	17.4

isolates can colonize internal plant tissues of maize. For this study, surface sterilized maize seeds were inoculated with 0.01mL. of previously grown starter cultures of isolates. The treated seeds were then allowed to grow on sterilized 1% water agar tubes under dark conditions. Control seeds without treatment were also used as check. After 14 days of growing period, the plantlets were carefully removed from the water agar medium with intact root system and fresh weight of roots was noted as well as microbial population within the plants was also monitored (Cohen *et al.,* 1980).

Efficiency of endophytic bacterial isolates

All isolates were tested for their nitrogen fixing capacity in culture media after one week of inoculation through Micro-Kjeldahl method (A.O.A.C., 1965) and sugar utilization was estimated by Fehling's method. The rate of nitrogen fixation was expressed as mg nitrogen fixed/gram of sucrose consumed.

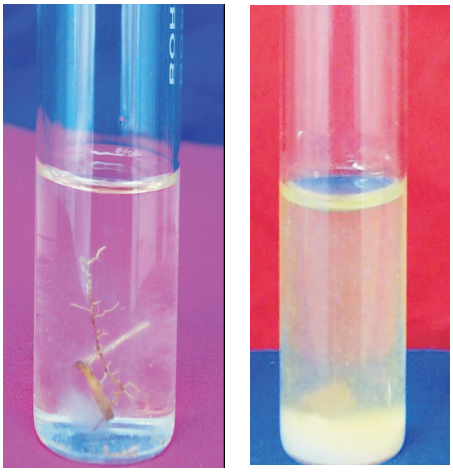
All isolates were tested for their phosphate solubilizing capacity in Pikovskaya's Medium. Here, Pikovskaya's broth was inoculated with 100μL bacterial culture and soluble phosphate content was estimated as per the method given by APHA, (1995) at 3 and 5 days after inoculation.

In vitro efficacy of isolates on maize seeds was tested on solid water agar in petri plates following the similar method as mentioned in confirmation of endophytic colonization following Completely Randomized Design.

The efficient isolates obtained were further evaluated under field conditions for their potential of replacing the chemical fertilizers without affecting the yield attributes of the crop following Randomized Block Design.

RESULTS AND DISCUSSION

Isolation of microorganisms, screening for desirable characters and selection of efficient strains are important steps to optimize high crop yields and improve the sustainability of the



Sugarcane root Sweet potato root

Figure 1: Oozing of *Acetobacter* sp. from different plant parts by using LGIP broth

ecosystem. In all, total 24 strains were isolated from different plant parts and species. Out of these, total 10 isolates were selected on the basis of their appearance and vigor to grow on NFB (5-isolates) and LGIP (5-isolates) medium (Table 1). As plant is the preliminary source of nutrition when microorganisms colonize the interior of plant roots or other organs of plant. Endophytic bacteria occurs at the extent of 10^3-10^6 gm^{-1} fresh weight (Hallman *et al.,* 1997). So, during enrichment in artificial medium, after 2-3 days of the inoculation due to limitation of nutrients inside plant part, microorganisms oozes outside the plant part and grow luxariuosly in the NFB (Fig. III) and LGIP medium. (Fig. 1). After transferring on to the semisolid LGIP medium isolates A-1 to A-5 were forming yellowish colored surface pellicle which showed micro aerobic nature of the organism. (Cavalcante and Dobereiner, 1988). Yellowish color of pellicle was found due to strong acid production and assimilation of Bromothymol blue (Boddey *et al.,* 1991) which results in rapid decrease in the pH of the isolation medium below 3.0 eliminating the chances of growth of contaminants. (Li and Macrae, 1992).

Table 4: *In vitro* phosphate solubilization efficiency of isolates

Isolates	P ìg /ml3 DAI	P ìg /ml5 DAI
A-1	3.22	2.78
A-2	11.04	18.96
A-3	1.89	7.44
A-4	1.19	4.44
A-5	10.22	12.30
ACG-1	12.70	9.15
A-6	11.22	6.33
A-7	14.96	23.81
A-8	21.00	20.19
A-9	2.93	10.04
A-10	3.89	11.11
ASA-1	4.00	10.15

Table 5: *In vitro* efficacy testing of isolates on maize *cv.* Narmada moti

Treatment	Root length (cm)	Shoot length (cm)	Fresh biomass weight(gm)	Dry biomass weight(gm)
Control	6.50^{d}	5.67^{b}	0.49^{g}	0.22^{g}
A-1	16.50^{ab}	16.00^{a}	0.97^{ab}	0.50^{ab}
A2	13.67^{bc}	13.00^{abc}	0.74^{de}	0.34^{e}
A-3	14.00^{abc}	14.67^{ab}	0.56^{fg}	0.27^{f}
A-4	15.33^{ab}	9.33^{cd}	0.71^{e}	0.35^{e}
A-5	10.50^{c}	12.33^{abc}	0.87^{bcd}	0.43^{cd}
ACG 1	15.33^{ab}	9.00^{cd}	0.68^{ef}	0.46^{bc}
A-6	16.67^{ab}	11.33^{abc}	0.77^{cde}	0.40^{d}
A-7	18.17^{a}	12.67^{abc}	0.89^{abc}	0.50^{ab}
A-8	15.67^{ab}	9.33^{cd}	0.95^{ab}	0.50^{ab}
A-9	15.33^{ab}	15.00^{ab}	0.87^{bcd}	0.48^{ab}
A-10	18.33^{a}	16.33^{a}	1.02^{a}	0.51^{a}
ASA-1	16.00^{ab}	10.67^{bc}	0.89^{abc}	0.35^{e}
S.Em.	1.27	1.47	0.043	0.013
CD at 5 %	3.68	4.27	0.13	0.037
CV %	14.48	20.73	9.16	5.25

Note: Treatment means with the letter/letters in common are not significant by Duncan's New Multiple Range Test at 5% level of significance

Sugarcane root Sweet potato root

Enriched cultures of isolates A-6 to A-10 formed a very thin vein like subsurface pellicles after 2-3 days of incubation on semisolid NFB medium due to its micro aerophilic nature (Fernando, 2007)

Durva leaves moneyplant leaves

Moreover, all the growth patterns were confirmed keeping standard strains ACG-1 and ASA-1 as check.

Confirmation of endophytic colonization of maize by *Acetobacter* and *Azospirillum* in laboratory condition.

Density of *Acetobacter* isolates was found in the range of 1.0 x 10^5 - 9.0 x 10^5cfu gm^{-1}fresh root weight after 14 days of inoculation, (Table II) markable difference in root fresh weight and number of bacteria was found as compared to control. The plants showing higher bacterial counts was reflected in higher root mass suggesting plant growth promoting effect was due to bacterial inoculation. Until recently, intracellular colonization of maize plants with *Acetobacter diazotrophicus* was found to be quite difficult to establish but Cocking *et al.,* 2005 succeeded in achieving intracellular colonization of maize by *Acetobacter diazotrophicus* where it persists inside membrane bounded vesicles in the cytoplasm of root cells and also demonstrated that conditions within intracellularly colonised root cells of maize were suitable for nitrogenase gene expression. *G. diazotrophicus* colonize lateral root junctions in high number (James and Olivares, 1997) suggesting that they may find their way through cracks formed at the emergence of lateral roots or at the zone of differentiation

Table VI: Seed inoculation efficacy of bacterial isolates on growth and yield of maize *cv.* Narmada moti

Treatment	Plant population at 10 DAS	PlantheightAt 30 DAS (cm)	Cob weight (kg/ha)	Grain yield (kg /ha)	Fodder yield(kg/ha)	1000 grain weight (gm)
T_1:Absolute control	107^{c}	108.3^{b}	4074^{c}	2700^{d}	2784^{c}	181.0^{e}
T_2:100kg/ha N	121^{bc}	123.3^{b}	5463^{abc}	4167^{abcd}	3189^{abc}	208.5^{d}
T_3:75 kg/ha N	116^{c}	110.3^{b}	4630^{bc}	3561^{cd}	3292^{abc}	193.8^{de}
T_4: T_3 + A-3	126^{abc}	160.7^{a}	5185^{bc}	3876^{bcd}	3498^{ab}	220.2^{bcd}
T_5: T_3 + A-6	124^{bc}	160.7^{a}	5741^{abc}	5169^{ab}	3644^{a}	232.9^{abc}
T_6: T_3 + ACG-1	115^{c}	175.0^{a}	4815^{bc}	3602^{bcd}	3086^{bc}	211.2^{bcd}
T_7: T_3 + A-9	153^{ab}	169.3^{a}	5556^{abc}	5030^{abc}	3395^{ab}	215.6^{bcd}
T_8: T_3 + A-10	158^{a}	176.0^{a}	6944^{a}	5694^{a}	3704^{a}	245.3^{a}
T_9: T_3 + ASA-1	122^{c}	152.7^{a}	5926^{ab}	4496^{abc}	3189^{abc}	239.5^{ab}
S.Em.	9.59	6.92	495.03	464.72	169.78	8.37
CD at 5%	28.48	20.56	1470.81	1380.75;	504.45	24.87
CV %	13.07	8.07	16.0	20.35	8.87	6.70

Table 7: Soil inoculation efficacy of endophytic bacterial isolates on growth and yield of maize *cv.* Narmada moti

Treatment	Plantpopulationat 10 DAS	PlantHeightAt 30 DAS(cm)	Cobweight (kg/ha)	Grainyield (kg /ha)	Fodderyield (kg/ha)	1000 grain weight(gm)
T_1:Absolute control	97^{d}	97.33^{d}	3333^{abc}	2533^{d}	2984^{b}	188.6^{b}
T_2:100kg/ha N	124^{bcd}	121.67^{bc}	4630^{abc}	3846^{bc}	3086^{ab}	252.2^{a}
T_3:75 kg/ha N	104^{d}	111.67^{c}	3981^{abc}	3069^{cd}	3241^{b}	233.3^{a}
T_4: T_3 + A-3	114^{cd}	157.67^{a}	4444^{abc}	3543^{bcd}	3241^{b}	263.3^{a}
T_5: T_3 + A-6	154^{ab}	146.69^{ab}	5741^{ab}	3982^{abc}	3447^{b}	246.9^{a}
T_6: T_3 + ACG-1	125^{bcd}	156.62^{a}	4074^{abc}	3270^{bcd}	3447^{b}	239.3^{a}
T_7: T_3 + A-9	148^{abc}	160.00^{a}	5370^{ab}	3792^{bc}	3704^{b}	259.1^{a}
T_8: T_3 + A-10	167^{a}	163.03^{a}	6481^{a}	5070^{a}	4321^{a}	275.1^{a}
T_9: T_3 + ASA-1	143^{abc}	164.35^{a}	5000^{abc}	4334^{ab}	3498^{b}	243.9^{a}
S.Em.	10.11	7.98	521.39	332.39	221.42	12.31
CD at 5%	30.05	23.9	1549.13	987.57	663.81	36.58
CV %	13.41	9.78	18.88	15.49	11.15	8.72

Note: Treatment means with the letter/letters in common are not significant by Duncan's New Multiple Range Test at 5% level of significance.

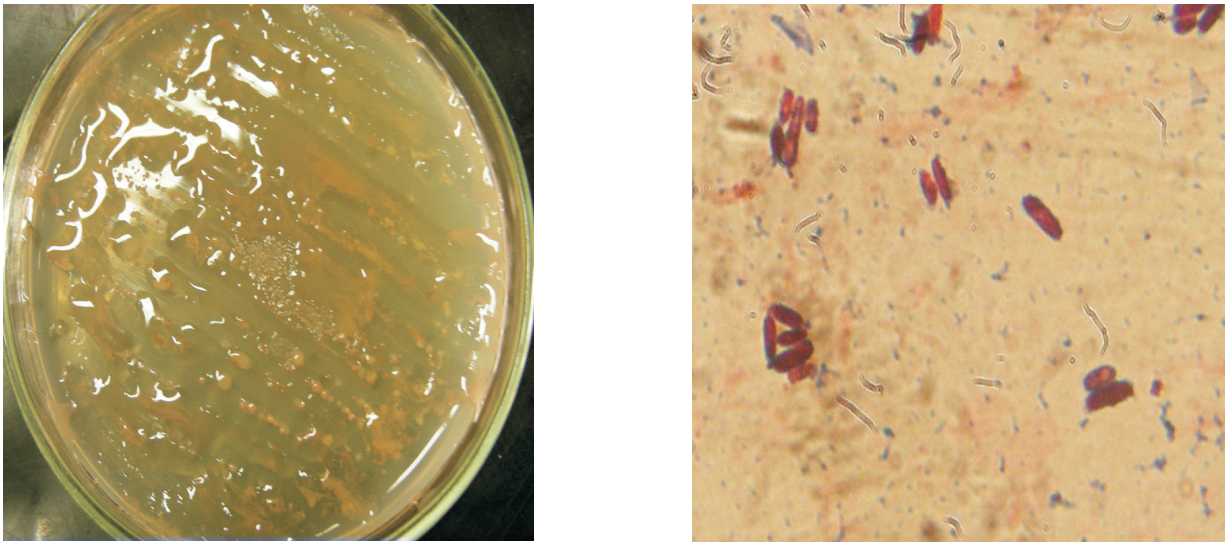


Figure 2: morphological and microscopic appearance of *Acetobacter* isolates

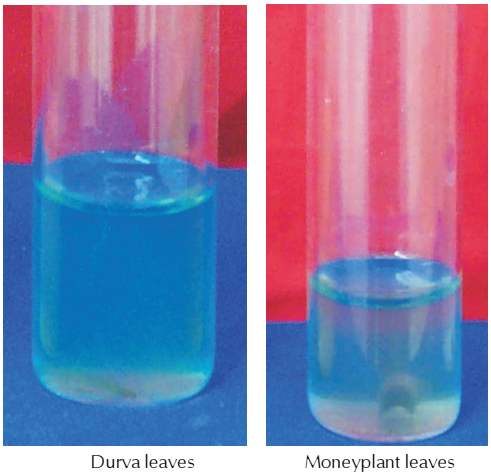


Figure 3: Oozing of *Azospirillum* sp. from plants by using NFB medium

of roots. Similar colonization pattern was observed by *Azospirillum* spp.

Nitrogen fixation efficiency of isolates

Comparison of endophytic diazotrophic bacteria with free living nitrogen fixers showed that internalized bacteria are much more likely to contribute significantly to nitrogen economy of the plant (Quispel, 1991, Cocking, 2003). *In vitro* nitrogen fixation efficiency of isolates was assessed before going for field trials. The results of this experiment are mentioned in Table III. All the isolates were confirmed to have ability of fixing atmospheric nitrogen. It was revealed from the result that nitrogen fixing potentiality of these isolates were ranged from 4.0-36.3 mg N / g of sugar consumed and isolate A-10 was showing highest nitrogen fixation capacity among all the isolates (36.3 mg N/g of sugar consumed). Previously N fixing ability of *Aceobacter diazotrophicus* ranging from 102-385 mg/g of sucrose consumed (Bhowmik and Konde (1997) and of *Azospirillum* strains ranged from10-19.5 mg N/gm of malate used (Saxena, 1983).

***In vitro* phosphate solubilizing activity of isolates**

Data regarding phosphate solubilization activity of isolates are presented in Table 4. Estimation of P in the medium revealed that all the strains released P from tri calcium phosphate (TCP). Isolate A-8 recorded maximum soluble

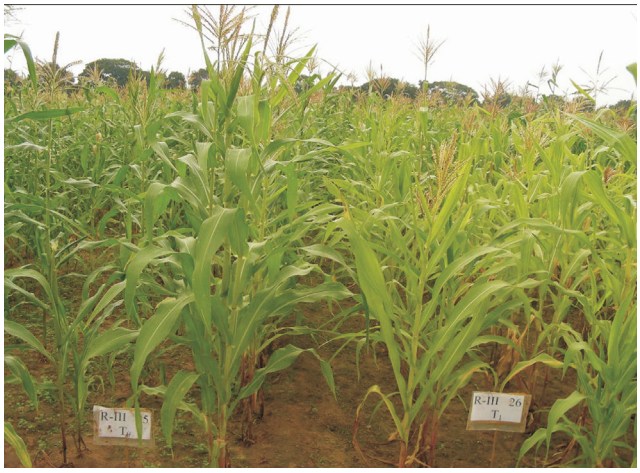


Figure 4: Seed inoculation efficacy of endophytic bacterial isolates (T_9) as compared to control

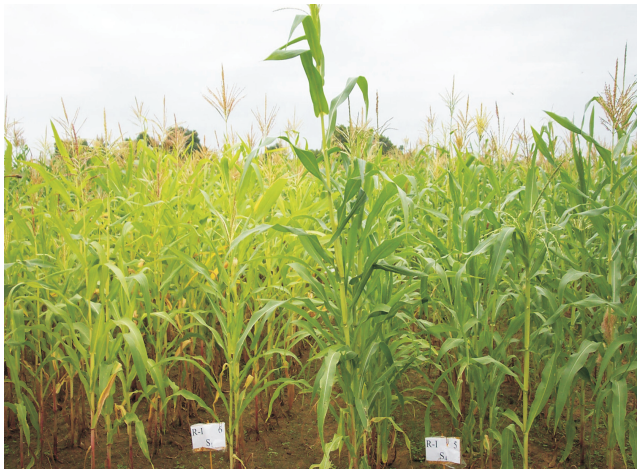


Figure 5: Soil inoculation efficacy of endophytic bacterial isolates (T_9) in comparison to control

phosphate (21.00 P μg/mL) at 3 DAI, closely followed by A-7 (14.96 P μg/mL) and A-5 (11.22 P μg/mL) and with isolates A-1, ACG-1, A-6 and A-8 decreased after 5 DAI because of utilization of solubilized phosphate by microorganisms. The present findings established the phosphate solubilization as an additional benefit of endophytic bacterial isolate and thereby, apart from fixing atmospheric nitrogen all the isolates can also improve the availability of phosphorous in crop's rhizosphere.

In vitro efficacy testing of endophytic bacterial isolates on maize *cv.* Narmada moti

Data regarding *in vitro* efficacy testing of endophytic bacterial isolates on maize *cv*. Narmada moti are presented in table V. Data revealed that all the bacterial inoculants showed significant increase in root and shoot length and biomass weight. Isolate A-10 reported significant increase in root length (18.33 cm), shoot length (16.33 cm), fresh biomass (1.02 gm) and dry biomass (0.51 gm) of maize *cv*. Narmada moti as compared to uninoculated control.

In comparison to control, the treated plants were noticeably taller and had more adventitious roots. Dry weight determination confirmed that, regardless of maize genotypes, bacterized plants produced higher shoot and root growth than non inoculated controls. Similar results were reported by Pathak and Chakraboti, 2014 showing maize seed treatment with *Azospirillum* exhibited significant increase in root and shoot fresh weight.

In vivo efficacy of endophytic bacterial isolates on maize *cv.* Narmada moti (Field trial)

The results revealed that T_8 and T_7 recorded highest plant population 158 and 153 plants/plot, respectively which was significantly higher over T_1 (107 plants/plot) and T_2 (121 plants/plot) at 10 DAS (Table 4). Plant height at 30 DAS was significantly influenced due to treatments of all the bacterial strains. Treatments T_4 to T_9 were at par with each other for plant height and among all the treatments T_8 recorded significantly highest plant height (176.00 cm) at 30 DAS, which was significantly higher over T_1 (108.00 cm) and T_2 (123.33 cm). Here, the results also revealed that bacterial inoculation along with 75 % R.D. of N gave significantly higher grain and fodder yield (Fig. IV) over R.D. of N suggesting 25 % chemical fertilizer saving in maize crop.

Treatments T_8 and T_5 recorded significant increase in fodder yield 3704 kg/ha and 3644 kg/ha, respectively over T_1 (2784 kg/ha) and T_2 (3189 kg/ha). Among all the treatments, T_8 and T_7 recorded significant increase in grain yield 5694 kg/ha and 5030 kg/ha, respectively, which were significantly higher over T_1 (2700 kg/ha) and T_2 (4167 kg/ha). Data also revealed that among all the treatments, T_8 recorded the highest 1000 grain weight (245.3 gm) closely followed by T_9 (239.5 gm) and T_5 (232.9 gm) which were significantly higher over T_1 (181.0 gm) and T_2 (208.5 gm). Similarly, Swêdrzyñska *et al.* (2001) reported that maize (*Zea mays sp. Saccharata* L.) inoculated with *Azospirillum brasilense* showed 27% increase in yield and higher cob mass than uninoculated control under different cultivation conditions.

Soil inoculation efficacy of isolates on maize cv. Narmada moti

Effect of bacterial inoculation on growth attributes and yield are presented in Table VII. Treatment T_8 recorded significant increase in plant population (167 plant/plot), closely followed by T_5 (154 plants/plot) and T_7 (148 plants/plot) which were significantly superior over T_1 (97 plants/plot) and T_2 (124 plants/plot). The results indicated that all the bacterial inoculants (T_4 to T_9) were found to be at par with each other for plant height at 30 DAS among which T_9 recorded maximum plant height (164.35 cm), closely followed by T_8 (163.03 cm) which were significantly higher over T_1 (97.33 cm) and T_2 (121.67 cm) (Fig. V). Treatment T_8 recorded statistically higher cob weight (6481 kg/ha)) which was significantly higher over T_1 (3333 kg/ha) and T_2 (4630 kg/ha) Moreover, treatments T_5 (5741 kg/ha), T_7 (5370 kg/ha), and T_9 (5000 kg/ha) were at par for cob weight.

It is ascertained from the results that treatment T_8 recorded increase in grain yield (5070 kg/ha) and fodder yield (4321 kg/ha) which were significantly higher over T_1 (2533 kg/ha and 2984 kg/ha) and T_2 (3846 kg/ha and 3086 kg/ha).

The positive effect caused by the bacterial inoculation on grain

and fodder yield was observed by comparing the treatment that received the ideal quantity of recommended nitrogen fertilizer for the experiment. The grain and fodder yield of the treatment in which the inoculation is associated with 75% of nitrogen were superior over treatment of 100% nitrogen without inoculation demonstrating that the presence of the inoculants substituted 25% of recommended nitrogenous fertilizer dose in this assay. T_8 recorded highest 1000 grain weight (275.1 gm) closely followed by T_4 (263.3 gm) and T_7 (259.1 gm) which were significantly higher over absolute control (188.6 gm) and statistically at par with T_2 (252.2 gm). Similar results were obtained by Hnamte *et al.* (2013), wherein they showed that combined inoculation of biofertilizers (N, P and K) along with vermicompost and inorganic fertilizers was most effective in producing highest seed yield of coriander.

Riggs *et al.* (2001) conducted greenhouse experiment in maize without N fertilizer. Inoculation of *G. diazotrophicus* Pal-5 significantly increased dry weight of maize genotypes Mo17, B14 and B84 by 42.6, 25.2 and 15.6 percentage, respectively. In field trail where N @ 224 kg/ha was applied, maize genotypes B73xMo17, 36H36 and 3905 showed increase in yield by 25.3, 23.4 and 14.4 percentage, respectively.

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