

Original Research Article

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Influence of Diazotrophic Bacteria on Growth and Biomass Production of Sugarcane *invitro*

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ABSTRACT

An *in vitro* experiment was carried out at Microbiology Laboratory, Department of soil Science of Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh to determine the effect of diazotrophic bacteria inoculation on growth and biomass production of sugarcane. Two indigenous diazotrophic bacterial strain isolated from sugarcane rhizosphere i.e. *Bacillus cereus* (BUSo 13) and *Acinetobacter calcoaceticus* (BUSo 9) and one reference strain, *Azospirillum barsilense* (SP 7) collected from ATCC, USA were used to conduct the experiment. Three levels of nitrogen viz., no nitrogen, 50% nitrogen and 100 % nitrogen were provided for the crop. The results of the experiment revealed that diazotrophic bacterial inoculation in sugarcane increased growth parameters and biomass yield significantly over control. The highest leaf greenness (38.67), number of leaves per plant (9.67), plant height (76.00 cm), root length (31.67 cm), dry matter yield (3.17 g), N content in plant (1.52%) and N-uptake (48.12 mg g⁻¹) were obtained in treatment T₆ receiving 50% N along with *Bacillus cereus* (BUSo 13) inoculation.

Keywords

Influence,
Diazotrophic
Bacteria, Growth,
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Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the world's most important sugar crops, providing over 76% of the sugar for human consumption. Sugarcane is an exhaustive crop that can uptake great amount of soil nutrients for its biomass production. The use efficiency of applied N fertilizers in sugarcane applied with recommended dose of N in the range of

250 to 400 kg ha⁻¹ is only 20-30% and hence at every harvest of the crop, soil suffers a net loss of 50-100 kg N/ha. One avenue to remediate the problem associated with synthetic N fertilizers is the use of microbes capable of biological N₂ fixation (BNF) (de Carvalho *et al.*, 2011). Free-living N₂-fixing bacteria belonging to the genera *Beijerinckia*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Derrxia*, *Enterobacter*, and *Erwinia* appear to be

frequent colonizers of sugarcane (Dobereiner and Ruschel 1958; Arias *et al.* 1978; Hegazi *et al.* 1979; Purchase 1980; Rennie *et al.* 1982; Gracioli *et al.* 1983; Seldin *et al.* 1984). A nitrogen balance study performed by Lima *et al.* (1987) provided direct evidence of a significant contribution of plant associated nitrogen fixation in sugarcane, ranging from 60 to 80% of total plant N, equivalent to over 200 kg N ha⁻¹year⁻¹. Various classes of bacteria have been reported to improve plant growth and metabolism, known as plant growth promoting rhizobacteria (PGPR). Various strains of *Bacillus*, *Enterobacter*, *Burkholderia*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Erwinia*, *Flavobacterium*, *Rhizobium* and *Serratia* (Rodriguez and Fraga, 1999; Sturz and Nowak, 2000; Sahin *et al.*, 2004) have been identified as PGPR. Serna-cock *et al.* (2011) observed that *Azospirillum brasilense* and *Trichoderma lignorum* exercised the greatest effect on stem diameter, root systems, and plant foliation in sugarcane. In a greenhouse study on sugarbeet, three different *Bacillus* isolates fixed nitrogen and increased growth (Çakmakçi *et al.*, 2006). Similarly, inoculation with a strain of *Bacillus* sp. also increased growth of roots and shoot parts of rice plants (Beneduzi *et al.*, 2008). In another study, Hafeez *et al.* (2006) noted that selected *Bacillus* sp. used as bio-inoculants on wheat resulted in increases in plant biomass, root length, and plant nitrogen and phosphorous content. Dadook *et al.* (2013) reported *Acinetobacter calcoaceticus* as a nitrogen fixing diazotrophs which isolated from asparagus rhizosphere. Previously research studies on *Acinetobacter* focused on growth potential, indole-3-acetic acid (IAA) production, inorganic phosphate solubilization, and nitrogen fixation (Huddedar *et al.*, 2002; Indiragandhi *et al.*, 2008; Khan *et al.*, 2011b). *Acinetobacter calcoaceticus* was recently reported as

gibberellins producing bacterium (Kang *et al.*, 2009). Two indigenous bacterial strain i.e. *Bacillus cereus* (BUSo 13) and *Acinetobacter calcoaceticus* (BUSo 9) have been isolated from sugarcane rhizosphere soil. These two bacteria found to have high potentiality of nitrogen fixation and IAA production. Though *Bacillus* sp. and *Acinetobacter* sp. inoculation has beneficial effect on different crops but no works has been done with these bacteria on sugarcane. Therefore, to identify preliminarily their effects on sugarcane growth and biomass yield an *in vitro* experiment was conducted to observe the influence of diazotrophic bacterial inoculation on growth, biomass yield and nitrogen uptake of sugarcane plant.

Materials and Methods

An *in vitro* experiment was carried out to determine the effect of diazotrophic bacteria inoculation on growth and biomass production of sugarcane. The experiment was conducted for eight weeks starting from 05 November, 2011 to 05 January 2012. Two diazotrophic bacterial strain isolated from sugarcane rhizosphere i.e. *Bacillus cereus* (BUSo 13) and *Acinetobacter calcoaceticus* (BUSo 9) and one reference strain, *Azospirillum brasilense* (SP 7), collected from ATCC, USA were used to conduct the experiment. Seedlings from sugarcane bud chips were used in this experiment. Hoagland solution was used to grow sugarcane seedling in test tube. The composition of Hoagland's solution used in this experiment was (mL L⁻¹): 2M KNO₃(2.5), 2 M Ca(NO₃)₂.4H₂O (2.5), 1.5% FeEDTA solution (1.5), 2M MgSO₄.7H₂O (1), 1 M NH₄NO₃ (1), 1M KH₂PO₄ (pH to 6.0 with 3M KOH) (0.5) and Minors (H₃BO₃-2.86 gL⁻¹, MnCl₂. 2H₂O-1.81 gL⁻¹, ZnSO₄.7H₂O-0.22 gL⁻¹, CuSO₄-0.051 gL⁻¹, Na₂MoO₄.2H₂O-0.12 gL⁻¹) (Hoagland *et al.* 1950). Three levels of nitrogen viz., no nitrogen, 50% nitrogen and 100 % nitrogen

were provided for the crop. The experiment was laid out in a Completely Randomized Design (CRD) with three replications having 11 treatment combinations. The experimental test tubes were arranged into 3 blocks representing replications to reduce heterogeneous effects. Each block consisted of 11 test tubes and the size of each test tube was 20 cm x 3.5 cm (125 ml). All test tubes were placed on a rack made with stainless steel wire (Picture 1).



Picture.1 Test-tube placed on rack made with stainless steel wire.



Eleven treatments were considered for the experiment as follows: T₁: Control (no nitrogen), T₂: 50% N, T₃: 100 % N, T₄: 50% N + *Acinetobacter calcoaceticus* (BUSo 9), T₅: 50% N + *Azospirillum barsilense* (SP 7), T₆: 50% N + *Bacillus cereus* (BUSo 13), T₇: 100 % N + *Acinetobacter calcoaceticus* (BUSo 9), T₈: 100 % N+ *Azospirillum barsilense* (SP 7), T₉: 100 % N + *Bacillus cereus* (BUSo 13), T₁₀: 50% N + mixed inoculums, T₁₁: 100 % N + mixed inoculums. Bud chips from sugarcane variety Isd 37 were collected for growing seedlings for the

experiment. Bud chips were surface sterilized by 70% ethanol for 30 second and in 3% H₂O₂ for 5 minutes. After that it was washed with sterile water for five times. Then bud chips were placed in sterilized soil in polyethylene bags for germination and maintained in a growth chamber. After germination 30 days old seedlings were used for the experiment (Picture 2).



Picture.2 Bud chip seedling

Preparation of inoculums

Bacterial strains were grown in respective broth for two days. Then the broth was centrifuged at 6000 rpm for 5 minutes. After that supernatants were discarded and cell pellets were washed two times with sterile water and collected in a 30 mL bottle. These solutions were centrifuged by vortex mixture to homogenize the strains in sterile water. Bacterial concentration was adjusted using a spectrophotometer at 540 nm and 0.1 ml of suspension containing 10⁸ cells were inoculated.

Root soaking with inoculum

Before inoculation roots of germinated seedlings were surface sterilized with 70 percent ethanol for 30 second and washed several times with sterile distilled water. Roots of germinated seedlings were soaked in the respective bacterial cells for 30 min for the bacteria to adhere to the roots.

Inoculum application rate

In each test tube (20 cm x 3.5 cm) 100 mL

Hoagland solution containing different nitrogen levels and same amount of other nutrient were added according to respective treatments. Seedlings of sugarcane was placed on a wire ring fitted to the neck of the test tubes. Thus, the rings were placed bellow 3 cm from the neck of the test tubes. The open end of test tubes was covered with sterilized cotton for avoid contamination. Plantation of bud chip seedlings was done on 05 November, 2011. After two days of planting, 0.5 mL of inoculums was added into test tubes according to respective treatments. For supplying nutrients, sterilized Hogland solution with respective N content were regularly added to the test tube according to respective treatments to maintain the initial level of solution. During the study, the rack was regularly placed in a natural condition for giving sunlight to the plants. After four weeks of growth, the same amount of inoculum was added according to respective treatments.

Harvesting was done on 05 January 2012. Mother stalks were selected from each test tube and tagged for collecting data. The entire plants including the shoot and roots were harvested very carefully and data on number of leaves, leaf greenness (SPAD Value), plant height, Root length and biomass yield were recorded soon after harvesting. Leaf samples of sugarcane were dried in an oven at 65-70⁰C for 72 hours and then ground by a grinding machine to pass through a 20-mesh sieve and stored in small paper bags and kept into a desiccator for chemical analysis. The samples were analyzed for nitrogen contents. N contents in the digest were determined following the Micro-Kjeldahl method (Jackson,1973). Nitrogen uptake was calculated by using following formula:

$$\text{Nutrient uptake (mg g}^{-1}\text{)} = \frac{\% \text{ Nitrogen} \times Y \text{ (g plant}^{-1}\text{)}}{100}$$

Here,

% Nitrogen = Nitrogen content (%) of plant
Y (g plant⁻¹) = Total dry matter production of plant

The collected data were compiled and tabulated in proper form and were subjected to statistical analysis by using the computer package Statistix 10 program for Windows Version. Computation and preparation of graphs were done by the use of Microsoft Excel 2003 program.

Results and Discussion

The influence of diazotrophic bacterial inoculation along with nitrogen on yield contributing characters, biomass yield, nitrogen content and nitrogen uptake by sugarcane plant are presented and discussed bellow.

Number of leaves per plant

Results presented in Table 1 revealed that number of leaves per plant of sugarcane varied significantly due to different treatments. The highest number of leaves was obtained in treatment T₆ (50% N + *Bacillus cereus* (BUSo 13) and T₁₀ (50% N + mixed inoculum) (both 9.67) which was statistically identical with treatments T₃ (100% N) (9.33) but superior to the rest of the treatments. The effect of treatment T₃ was, however, statistically similar to treatment T₅ but this treatment statistically ranked second. The lowest number of leaves per plant was found in control. This result corroborates with that of Serna-Cock *et al* (2011) who reported higher number of leaves in sugarcane with *Azospirillum* inoculation. This result was also in harmony with that of Ai'shah *et al* (2009) who showed higher leaf formation due to diazotrophic bacterial inoculation in oil palm.

Leaf greenness (SPAD value)

Inoculation of diazotrophic bacteria along with different levels of N showed a positive effect on leaf greenness (SPAD value) of sugarcane (Fig. 1). The leaf greenness (SPAD value) ranged from 32.33 to 38.67. The highest leaf greenness was found in T₆ (38.67) which was statistically at par with treatment T₁₀ (36.67) and T₃ (36.33) but superior to the rest of the treatments. The effect of treatment T₁₀ and T₃ were statistically similar to all other treatments except control. The lowest leaf greenness was obtained in control (32.33). This result was in accordance with that of Morais *et al* (2011) who found significant increase in leaf chlorophyll content with inoculation of mixture of five diazotroph strains in sugarcane. Keyeo *et al* (2011) reported higher chlorophyll content in rice leaf with inoculation of diazotrophic bacteria.

Plant height

Plant height of sugarcane responded significantly due to different treatment combinations (Table 1, Plate 3). The highest plant height (76.0 cm) was obtained in treatment T₆ receiving 50% N along with *Bacillus cereus* (BUSo 13) inoculation whose effect was statistically similar to treatments T₁₀ (73.0 cm) where 50% N along with mixture of strains was inoculated and T₃ (71.0 cm) (100% N) but superior to the rest of the treatments. The effect of treatment T₁₀ and T₃ were, however, statistically similar to treatment T₅ and ranked second. The lowest plant height (23.0 cm) was obtained in control (no nitrogen). This result corroborates with that of Chakraborty *et al* (2013) who reported higher cane height with inoculation of diazotroph biofertilizer *Azotobacter vinelandii* followed by *Paenibacillus polymyxa*. Khan *et al* (2010) found higher plant height with inoculation of *Azospirillum* in mustard.

Solaiman *et al* (2011) found highest plant height in rice with inoculation of *Rhizobium sp* isolated from grasspea.

Root length

Different treatments exerted significant variations in root length of sugarcane plant (Table 1, Picture 3). Root length of sugarcane ranged from 17.00 cm to 31.67 cm. The highest root length was recorded in treatment T₆ (31.67 cm) which was statistically similar to T₁₀ (30.33 cm), T₃ (30.00 cm), T₅ (29.67 cm), T₉ (28.33 cm) and T₈ (27.33 cm) but superior to the rest of the treatments. The effect of treatments T₁₀, T₃, T₅, T₉ and T₈ were, however statistically similar to T₄ and T₁₁ and ranked second. This result corroborates with that of Paungfoo-Lonhienne *et al* (2014) who reported stimulated growth of sugarcane plants with diazotrophs *Burkholderia australis sp. nov.* inoculation. This result was also at par with that of Mia *et al* (2010) who found higher root length with inoculation of diazotrophs in banana. Solaiman *et al* (2011) who found highest root length in rice with inoculation of *Rhizobium sp* isolated from grasspea.

Biomass yield

The influence of different treatments were found significant in recording biomass yield of sugarcane (Table 1). The biomass yield of sugarcane ranged from 1.42 to 3.17 g plant⁻¹. The maximum biomass yield (3.17 g plant⁻¹) was obtained in treatment T₆ receiving 50% N along with *Bacillus cereus* (BUSo 13) inoculation, which was followed by T₁₀ (50% N + mixture of inoculum), T₃ (100 % N) and T₅ (50% N+ *Azospirillum barsilense*). All other treatments except control produced statistically similar biomass yield with that of T₅. The lowest biomass yield (1.42 g plant⁻¹) was obtained in control (no nitrogen). This result corroborates with the findings of

Muthukumarasamy *et al* (2006) who reported higher sugarcane biomass yield with half of the recommended dose of nitrogen plus inoculation of *Gluconacetobacter diazotrophicus* and co inoculation of *Gluconacetobacter diazotrophicus* and *Herbaspirillum sp.* Govindarajan *et al* (2007) found higher biomass yield in sugarcane with inoculation of *G. diazotrophicus* and *Klebsiella sp.* GR9 along with half recommended dose of nitrogen. Increased biomass yield in sugarcane with inoculation of *Azospirillum* was also reported by Umrit *et al* (2005). Solaiman *et al* (2011) found highest dry matter in rice with inoculation of *Rhizobium sp* isolated from grasspea.

Nitrogen content in sugarcane plant (%)

A significant difference was observed on N content in sugarcane plant due to inoculation of diazotrophs along with different nitrogen levels (Fig. 4.2). Nitrogen content in sugarcane plant varied from 1.29 to 1.52%. The maximum nitrogen content (1.52 %) was

found in treatment T₆ where 50% N along with *Bacillus cereus* (BUSo13) was inoculated. Whose effect was statistically identical with T₁₀, T₃, T₅ and T₉ but superior to rest of the treatments. The effect of T₁₀, T₃, T₅ and T₉ were, however, statistically similar to treatment T₂ and ranked second. The lowest nitrogen content (1.29%) was obtained in control (no nitrogen). This result was at par with the findings of Hari and Srinivasan (2005) who reported that biofertilizer application along with lower nitrogen level significantly improved nitrogen content of sugarcane stem.

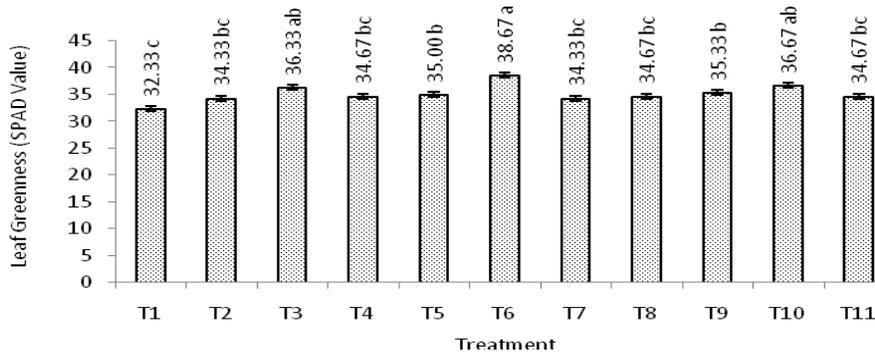
Kim *et al* (2010) also reported significantly higher nitrogen content in red pepper and rice with inoculation of diazotrophic bacteria *Azospirillum brasilense*. Govindarajan *et al* (2007) found higher leaf nitrogen content in sugarcane with inoculation of diazotrophs *G. diazotrophicus* and *Klebsiella sp.* GR9 along with half recommended dose of nitrogen. Askary *et al* (2009) found higher N content in wheat with *Azospirillum* inoculation.

Table.1 Influence of diazotrophic bacteria on growth and biomass yield of sugarcane

Treatment	No. of leaves plant ⁻¹	Plant height (cm)	Root length (cm)	Biomass (g plant ⁻¹)
T ₁	6.00 e	23.00 e	17.00 e	1.42 d
T ₂	7.00 de	56.00 d	22.67 d	2.17 c
T ₃	9.33 ab	71.00 abc	30.00 ab	2.77 b
T ₄	7.67 cd	59.67 d	25.67 bcd	2.26 c
T ₅	8.33 bc	65.67 bcd	29.67 ab	2.47 bc
T ₆	9.67 a	76.00 a	31.67 a	3.17 a
T ₇	7.33 cd	57.67 d	24.33 cd	2.24 c
T ₈	7.67 cd	61.00 d	27.33 abcd	2.28 c
T ₉	8.00 cd	62.33 cd	28.33 abc	2.35 c
T ₁₀	9.67 a	73.00 ab	30.33 ab	2.79 b
T ₁₁	7.00 de	63.00 cd	26.67 bcd	2.41 c
CV (%)	8.74	9.45	10.53	8.05

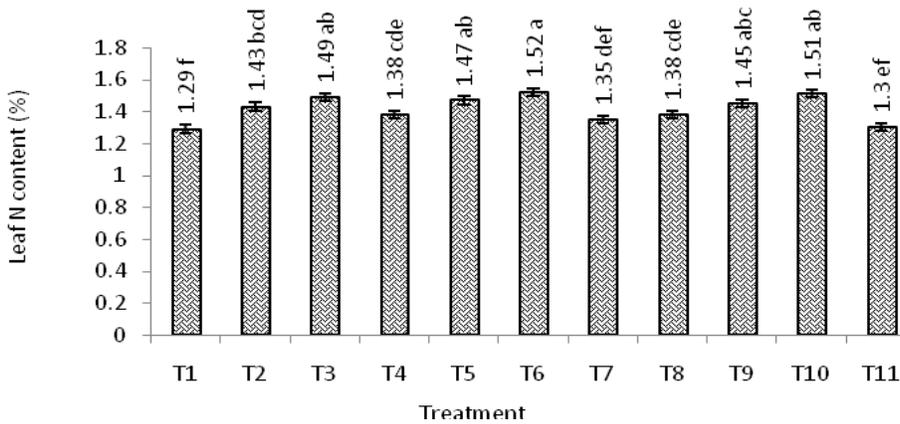
Means followed by uncommon letters are statistically different from each other at 5% level of provability by DMRT

Figure.1 Influence of diazotrophic bacterial inoculation on leaf greenness of sugarcane



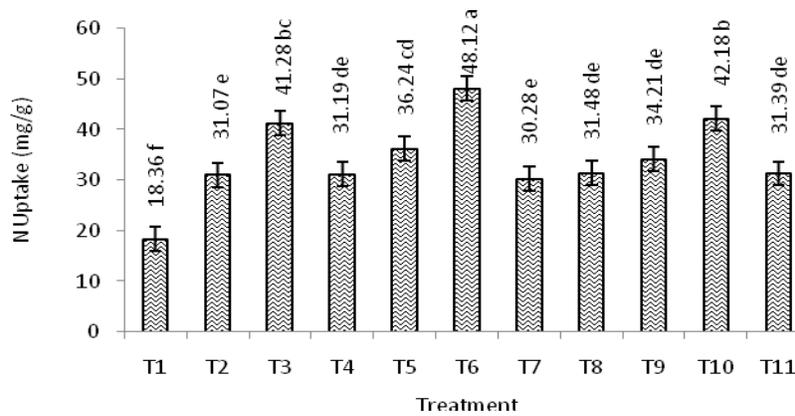
Vertical bars represent standard error of treatment means
Means followed by uncommon letters are statistically different from each other at 5% level of provability by DMRT

Figure.2 Influence of diazotrophic bacterial inoculation on nitrogen content in sugarcane plant.



Vertical bars represent standard error of treatment means

Figure.3 Influence of diazotrophic bacterial inoculation on nitrogen uptake in sugarcane plant.



Vertical bars represent standard error of treatment means
Means followed by uncommon letters are statistically different from each other at 5% level of provability by DMRT

Picture.3 Root and shoot growth in sugarcane due to diazotroph inoculation



Acinetobacter sp. & Control

Azospirillum sp & Control

Bacillus sp.& Control

Nitrogen uptake

A marked difference was observed in N uptake by sugarcane plant due to inoculation of diazotrophs along with different levels of nitrogen (Fig. 4.3). Nitrogen uptake by sugarcane plant varied from 18.36 to 48.12 mg g⁻¹. The maximum N uptake by sugarcane plant was recorded in treatment T₆ (48.12 mg g⁻¹) receiving 50% N + *Bacillus cereus* (BUSo 13) inoculation which was followed by T₁₀ (50% N + mixed inoculum) and T₃ (100% N). The effect of treatment T₃ was, however, statistically similar to treatment T₅ and ranked 3rd.

The lowest N uptake (18.36 mg g⁻¹) was obtained in control (no nitrogen). This result was in harmony with that of Suman *et al* (2005) who found increased nitrogen uptake with *Gluconacetobacter diazotrophicus* inoculation in sugarcane.

The results of the experiment revealed that diazotrophic bacterial inoculation in sugarcane increased growth parameters and biomass yield significantly. The highest leaf

greenness (38.67), number of leaves per plant (9.67), plant height (76.00 cm), root length (31.67 cm), dry matter yield (3.17 g), N content in plant (1.52%) and N-uptake (48.12 mg g⁻¹) were obtained in treatment T₆ receiving 50% N along with *Bacillus cereus* (BUSo 13) inoculation. The highest values as observed on these parameters were might be due to production of different growth promoting substances i.e. IAA, hormones and vitamins which influenced photosynthesis, plant height, root elongation and leaf formation which might led to more uptake of nutrients and increased plant growth and biomass yield. Inoculation of *Acinetobacter calcoaceticus* (BuSo 9) and *Azospirillum brasilense*(SP 7) in sugarcane was inferior compared to *Bacillus cereus* (BUSo 13) regarding growth parameter and biomass yield. This might be due to less affinity of *Acinetobacter calcoaceticus* sp., and non-compatibility of *Azospirillum brasilense* (SP 7) with local sugarcane variety. *Azospirillum brasilense* (SP7) strain was collected from USA. Treatment T₁₀ receiving 50% N along with mixture of inoculum also showed statistically similar performance as that of T₆

(50% N + *Bacillus cereus*) regarding growth parameter, biomass yield and nitrogen uptake. Biomass yield increase over control (no nitrogen) and 100% N were found 123.24% and 14.44%, respectively in treatment receiving 50%N + *Bacillus cereus* (BUSo 13) inoculation and 96.48 % and 0.72%, respectively in treatment receiving 50% N + mixture of diazotrophs inoculums. The findings of the study corroborate with the work of many researcher. *Bacillus* is also found to have potential to increase the yield, growth and nutrition of raspberry plant under organic growing conditions (Orhan *et al.*, 2006). Bacterial strain *Bacillus cereus* when tested in vitro are found to solubilize phosphate and thus helps in growth of plant (Husen, 2003). Garcia *et al* (2004) reported positive effect of *Bacillus licheniformis* on the yield of tomato and pepper. In a greenhouse study on sugarbeet, three different *Bacillus* isolates fixed nitrogen and increased growth (Çakmakçi *et al.* 2006). Similarly, inoculation with a strain of *Bacillus* sp. also increased growth of roots and shoot parts of rice plants (Beneduzi *et al.* 2008). In another study, Hafeez *et al* (2006) noted that selected *Bacillus* sp. used as bio-inoculants on wheat resulted in increases in plant biomass, root length, and plant nitrogen and phosphorous content.

From the above discussion it may be concluded that *Bacillus cereus* (BUSo 13) or mixture inoculum of *Bacillus cereus* (BUSo 13), *Acinetobacter calcoaceticus* (BUSo 9) and *Azospirillum brasilense* (SP7) inoculation along with 50% N showed better performance compared to other diazotroph treatment combinations regarding growth parameters, biomass yield and nitrogen uptake in sugarcane. Treatment T₆ receiving 50% N + *Bacillus cereus* and treatment T₁₀ receiving 50% N + mixture inoculum performed best in respect of growth parameters, biomass yield and nitrogen uptake in sugarcane.

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