Genetics and Plant Physiology – 2011, Volume 1 (3–4), pp. 130–138
©2011 Published by the Institute of Plant Physiology and Genetics – Bulgarian Academy of Sciences
Available online at http://www.ifrg-bg.com

GROWTH PERFORMANCE OF *SORGHUM BICOLOR* (L.) MOENCH IN RESPONSE TO INOCULATION WITH *GLUCONACETOBACTER DIAZOTROPHICUS*

Kumarasamy V. and K. Santhaguru*

Centre for Research and P.G. Department of Botany, Thiagarajar College (Madurai Kamaraj University), Madurai-625 009, India

Received: *11 May 2011* Accepted: *18 November 2011*

Summary. Diazotrophic endophytes associated with gramineous plants received considerable attention because of their ability to fix and transfer fixed nitrogen to the host plants. *Sorghum bicolor*, a multipurpose fodder crop rich in sugar is well suited for dry-land farming. The present study was focused on the effect of *Gluconacetobacter diazotrophicus* on the growth performance of *Sorghum bicolor* (L.) Monech. *Sorghum bicolor* plants were grown in a greenhouse and the impact of inoculation with 20 isolates of *G. diazotrophicus* and the reference strain PAL5 on dry weight (DW), total N, soluble sugars, and chlorophyll content (Chl) was studied. The stem isolate of *G. diazotrophicus* SoS2 induced a significant increase in plant biomass, total N, soluble sugars and Chl content in *S. bicolor* over the other stem isolates (SoS1, SoS3, SoS4, SoS5, SoS12, SoS13, SoS14, SoS15, SoS16 and EcS1) as well as root and rhizosphere isolates. Within the stem isolates, the lowest performer was SoS13. Among the 5 root isolates (SoR1, SoR2, SoR3, EcR1 and EcR2), SoR1 and EcR1 were more efficient in inducing dry weight, total N, soluble sugars, and Chl content in the plant. Within the 4 rhizosphere isolates (SoRS2, CdRS1, CdRS2 and ZmRS1), SoRS2 performed better on the basis of induction of DW, total N, soluble sugars, and Chl in the plant, in contrast to the poorer performance shown by ZmRS1. The isolates of *Saccharum officinarum* origin were more efficient than the isolates of *Eleusine coracana* origin. Further, the isolates originating from stem were more efficient than the ones originating from root and rhizosphere. However, the reference strain PAL5 expressed its superiority over the 20 isolates of *G. diazotrophicus*.

Key words: *Gluconacetobacter diazotrophicus*; growth; *Sorghum bicolor*; total chlorophyll; total nitrogen; total soluble sugars.

***Abbreviations*:** ANOVA – Analysis of variance; Chl – Chlorophyll; Dry weight – DW; Fresh weight – FW; IAA – Indole-3-acetic acid; N – Nitrogen; P – Phosphorus.

*Corresponding author: guruks02@yahoo.co.in

INTRODUCTION

Search for endophytic diazotrophs in non-leguminous plant species is gaining momentum, since attempts to transfer *nif* genes to cereals have not been successful. Several endophytic diazotrophic bacteria viz. *Gluconacetobacter diazotrophicus* (Cavalcante and Dobereiner, 1988), *Azoarcus* spp. (Reinhold-Hurek et al., 1993), and *Herbaspirillum seropedicae* (Baldani, et al., 1986) have been isolated from the interior of plant tissues. Since these bacteria live within plant tissues, they fix nitrogen more efficiently than the diazotrophs that remain in the rhizosphere or on the rhizoplane (Dobereiner et al., 1995). This may be due to the fact that the plant interior provides low oxygen environment and the required photosynthates directly for nitrogen fixation. In return, the endophytic diazotrophs provide fixed nitrogen and/or plant growth promoting substances to the host (Sevilla and Kennedy, 2000).

Although nitrogen-fixing potential of endophytic diazotrophs is established, little is known about the inoculation effects of these bacteria on host plants. In an earlier study, Sevilla et al. (1998) reported that *Gluconacetobacter diazotrophicus* inoculation enhanced the growth of sugarcane plants in sand supplied with N-deficient medium. Oliveira et al. (2002) evaluated the inoculation effect of endophytic nitrogen-fixing bacteria on the development of micropropagated sugarcane plants and concluded that a complex endophytic community was necessary to promote high transference of fixed nitrogen and growth promoting effects. The present work aimed at evaluating the effects of 20 isolates of *Gluconacetobacter diazotrophicus* along with the reference strain of *G. diazotrophicus* PAL5 on the growth performance of *Sorghum bicolor*.

MATERIALS AND METHODS

Gluconacetobacter diazotrophicus PAL5 obtained from Empraba, Brazil and 20 isolates of *G. diazotrophicus* obtained from the Department of Botany, Thiagarajar College, Madurai, were used in the present study. The names of the isolates and their origin with respect to the location of collection of the plant species and rhizosphere soil and plant parts used for isolation are given in Table 1. The cultures of *G. diazorophicus* were maintained on LGI agar slants (Cavalcante and Dobereiner, 1988) containing K_2HPO_4, 0.2 g; KH_2PO_4, 0.6 g; $MgSO_4$ $7H_2O$, 0.2 g; $CaCl_2$ $2H_2O$, 0.02 g; Na_2MoO_4 $2H_2O$, 0.002 g; $FeCl_3$ $6H_2O$, 0.01 g; 0.5% bromothymol blue in 0.2 N NaOH, 5 ml; cane sugar, 100 g; yeast extract, 0.02 g; agar, 15 g and 1000 ml water.

Seeds of *Sorghum bicolor* (variety Co27) obtained from Tamil Nadu Seed Testing Centre, Madurai, were surface sterilized with 0.1% $HgCl_2$ for 5 min and sown at the rate of 10 per pot (12.5 cm height and 15 cm in diameter) containing sterile sand-soil mixture (2:1 w/w). Four days after emergence, the seedlings were thinned out to 4 in each pot. *G. diazotrophicus* culture was grown in LGI broth (Cavalcante and Dobereiner, 1988) for 3 days in a 'Schigenics' rotary shaker with a speed of 120 strokes min^{-1}. Seven-day-old seedlings were inoculated with 2 ml of *G. diazotrophicus* culture ($1x10^9$ cells/ml) by introducing the cell

Table 1: *Gluconacetobacter diazotrophicus* isolates used in the present study.

Location	Host plant	Origin	Isolates of *G. diazotrophicus*
Rajapalayam Virudhunagar district	*Saccharum officinarum*	Rhizosphere	SoRS2
Rajapalayam Virudhunagar district	*Saccharum officinarum*	Root	SoR1
Rajapalayam Virudhunagar district	*Saccharum officinarum*	Stem	SoS1
Keezhadi Sivagangai district	*Saccharum officinarum*	Stem	SoS2, SoS3
Kondhagai Sivagangai district	*Saccharum officinarum*	Stem	SoS4
Ambathurai Dindigul district	*Saccharum officinarum*	Stem	SoS5, SoS12, SoS13
Samiyarpatti Dindigul district	*Saccharum officinarum*	Stem	SoS14, SoS15, SoS16
Samiyarpatti Dindigul district	*Saccharum officinarum*	Root	SoR2, SoR3
Samiyarpatti Dindigul district	*Eleusine coracana*	Stem	EcS1
Ambathurai Dindigul district	*Eleusine coracana*	Root	EcR2
Thirunagar Madurai district	*Eleusine coracana*	Root	EcR1
Kannivadi Dindigul district	*Cynodon dactylon*	Rhizosphere	CdRS1, CdRS2
Kannivadi Dindigul district	*Zea mays*	Rhizosphere	ZmRS1

suspension in soil around the base of each seedling. The plants were grown in a greenhouse under conditions of broad daylight and a temperature range of 28 to 30°C. Sterile tap water and plant nutrient solution (Wilson and Reisenauer, 1963) were used on alternate days to water the plants. Each treatment was repeated 4 times for harvest at 35 days after inoculation with *G. diazotrophicus*. On harvest root, stem, and leaves were cut into bits and dried separately at 90°C for 72 h for determination of DW.

The dried and powdered plant materials (root, stem and leaves) were mixed together and total N and soluble sugars in these samples were estimated. Total N was determined by the modified micro-Kjeldahl method (Umbreit et al., 1972). Ten milligrams of powdered plant material in a micro-Kjeldahl flask were digested after adding 0.5 ml of concentrated H_2SO_4 and 50 mg catalyst (preparation of catalyst: 1 g of $CuSO_4$, 8 g of K_2SO_4 and 1 g of selenium dioxide were ground separately and mixed together) in

a 'Tempo' digestion rack. When the digest turned to apple green colour, an aliquot of 0.1 ml was mixed with 2 ml of water, 2 ml of colour reagent (preparation of colour reagent: 4 g of KI and 4 g of HgI_2 were dissolved in 25 ml of distilled water. 1.75 g of gum arabic were dissolved in 750 ml of boiling distilled water and cooled. To this gum arabic solution, the potassium iodide – mercuric iodide solution was mixed and made up to 1000 ml with distilled water) and 3 ml of 2N NaOH. The absorbance of the resultant coloured solution was read at 490 nm with a 'Bausch and Lomb' Spectronic – 20 colorimeter. The quantity of total N was calculated from a standard curve prepared using NH_4Cl as standard.

Total soluble sugars were estimated by the Anthrone method (Mooris, 1948). The dried and powdered plant material (50 mg) was mixed with 10 ml of 80% ethyl alcohol in a test tube and kept in a boiling water bath for 10 min after covering the mouth of the tube with marble. After cooling, the alcohol extract was centrifuged at 3000 x *g* for 10 minutes. To 0.1 ml of the alcohol extract, 4 ml of freshly prepared anthrone reagent (0.2 g of anthrone in 100 ml of concentrated sulphuric acid) was added and kept in a boiling water bath for 10 min. After cooling, the absorbance of the coloured solution was read at 624 nm against reagent blank. Authentic glucose was used as standard.

The chlorophyll content in leaf tissue was estimated according to the method of Arnon (1949). Fresh leaf tissue (100 mg) was homogenized with 80% chilled acetone in diffused light with a mortar and pestle and centrifuged at 3000 x *g* for 10 min. The supernatant fraction was saved and the pellet was re-extracted with 80% acetone till the extract turned non-green. The supernatant fractions were pooled and made up to a known volume with 80% acetone. The absorbance was measured at 645 and 663 nm. The chlorophyll content was calculated on a FW basis using the formula:

$$\text{Total chlorophyll (mg Chl g FW}^{-1}) = = \frac{20.2 \times A_{645} + 8.02 \times A_{663}}{1 \times 1000 \times w} \times v$$

where:
l = path length in cm (1 cm)
v = volume of the extract in ml
and
w = fresh weight of the sample in g.

The data were subjected to statistical analysis by using Costat package for one way ANOVA and Student Newman Keuls test.

RESULTS AND DISCUSSION

Improvement of *S. bicolor* growth using 20 isolates of *G. diazotrophicus* was assessed in a greenhouse using sterilized sand-soil mixture (2:1 w/w) as potting mix. Inoculation with different isolates of *G. diazotrophicus* increased the DW (5-128%), total nitrogen (4-148%), total soluble sugars (7-46%), and total Chl (75-419%) of *S. bicolor* (Table 2). Among the three sources of isolates, stem, root and rhizosphere, the stem isolates performed better, followed by root and rhizosphere isolates. However, the effect of strain type PAL5 was found to be superior to all the 20 isolates. The isolate SoS2 was the best amongst the stem isolates in improving DW, total nitrogen, soluble sugars and Chl content (128, 148, 46 and 418%, respectively) compared with the control plants, in contrast to the poor performance shown by the isolate SoS14

Table 2: Effect of inoculation with *Gluconacetobacter diazotrophicus* on dry weight, total nitrogen, soluble sugars and chlorophyll content of *Sorghum bicolor* at 35 DAI.

Treatments	Dry weight [g plant^{-1}]	Total N [mg g DW^{-1}]	Total soluble sugars [mg g DW^{-1}]	Total chlorophyll [mg g FW^{-1}]
Control	0.443±0.039^{g}	19.690±1.480^{j}	12.669±0.93^{b}	1.220±0.147^{f}
PAL5	1.261±0.002^{a}	55.166±1.101^{a}	20.838±1.90^{a}	6.407±0.608^{a}
SoS1	0.758±0.020^{cd}	36.313±0.251^{cde}	16.437±1.65^{ab}	2.311±0.363^{ef}
SoS2	1.012±0.194^{b}	48.913±2.025^{b}	18.531±2.24^{ab}	6.340±0.505^{a}
SoS3	0.771±0.013^{cd}	36.853±0.933^{cde}	16.707±1.06^{ab}	2.969±0.385^{de}
SoS4	0.954±0.064^{b}	41.717±0.446^{c}	17.436±1.08^{ab}	5.940±0.305^{ab}
SoS5	0.726±0.105^{cde}	31.661±3.377^{efg}	15.522±2.42^{ab}	2.806±0.467^{e}
SoS12	0.769±0.020^{cd}	32.044±0.700^{efg}	16.665±2.19^{ab}	3.289±0.211^{de}
SoS13	0.605±0.038^{defg}	29.493±3.440^{fgh}	14.802±2.59^{ab}	3.009±0.094^{de}
SoS14	0.647±0.037^{def}	27.685±0.736^{fgh}	15.510±3.89^{ab}	2.965±0.127^{de}
SoS15	0.774±0.037^{cd}	37.811±2.008^{cd}	17.172±2.48^{ab}	3.106±0.470^{de}
SoS16	0.637±0.035^{def}	30.623±3.789^{fgh}	14.427±2.55^{b}	3.069±0.136^{de}
SoR1	0.762±0.025^{cd}	33.400±4.003^{def}	16.428±2.76^{ab}	3.570±0.393^{de}
SoR2	0.564±0.024^{efg}	25.384±1.940^{hi}	13.731±2.74^{b}	2.204±0.548^{ef}
SoR3	0.601±0.019^{defg}	29.129±1.040^{fgh}	15.627±2.26^{ab}	2.330±0.216^{ef}
SoRS2	0.690±0.138^{cde}	31.043±0.461^{efgh}	15.135±0.72^{ab}	4.188±0.306^{cd}
EcS1	0.779±0.013^{cd}	36.616±2.241^{cde}	14.877±2.25^{ab}	3.197±1.220^{de}
EcR1	0.875±0.050^{bc}	40.770±1.380^{c}	14.499±2.67^{b}	5.140±0.120^{bc}
EcR2	0.564±0.024^{efg}	38.775±2.768^{cd}	15.318±1.06^{ab}	4.738±1.081^{c}
CdRS1	0.478±0.090^{fg}	26.958±1.080^{ghi}	14.208±1.26^{b}	3.260±0.298^{de}
CdRS2	0.548±0.128^{efg}	23.220±3.681^{ij}	15.600±0.62^{ab}	3.522±0.463^{de}
ZmRS1	0.462±0.051^{fg}	20.417±3.894^{j}	13.629±0.65^{b}	2.145±0.408^{ef}
LSD (<0.05)	0.117***	3.792***	3.439*	0.806***

± Standard deviation; Values suffixed with different letters in the same column indicate significant differences; *, **, *** = level of significance.

(Fig. 1). Within the root isolates, SoR1 and EcR1 expressed their superiority over the other two – SoR3 and EcR2, in their ability to increase all tested parameters (Fig. 2). Apart from the stem and the root isolates, rhizosphere isolates also influenced *S. bicolor* positively. The isolate SoRS2 contributed significantly to biomass production and accumulation of total nitrogen, soluble sugars and Chl (56,

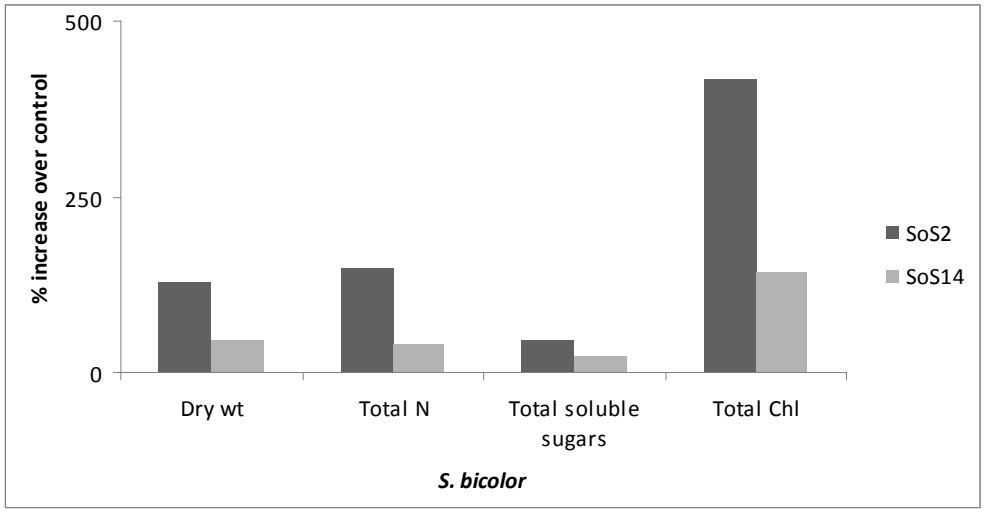

Fig. 1: Effect of inoculation with stem isolates of *G. diazotrophicus* on dry weight, total nitrogen, soluble sugars, and chlorophyll content of *S. bicolor* at 35 DAI.

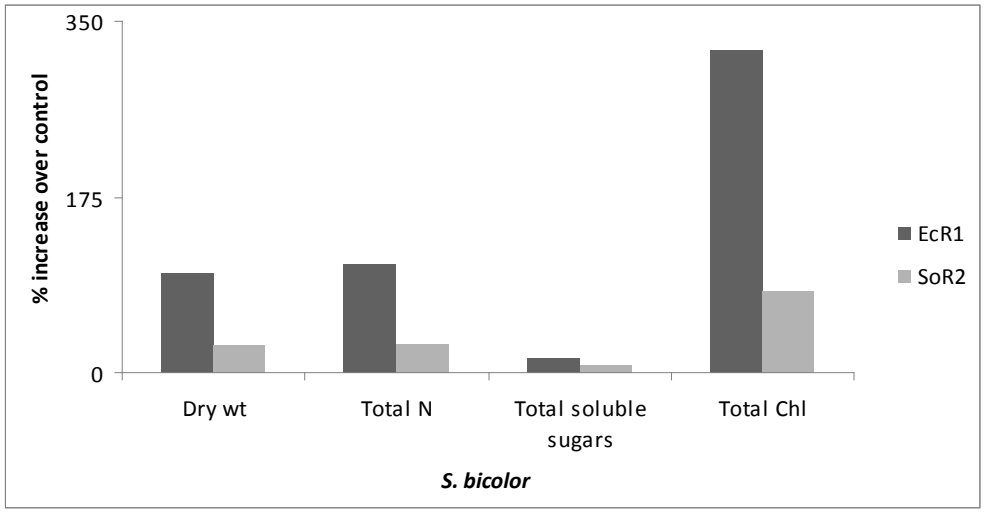

Fig. 2: Effect of inoculation with root isolates of *G. diazotrophicus* on dry weight, total nitrogen, soluble sugars, and chlorophyll content of *S. bicolor* at 35 DAI.

58, 19 and 243%, respectively) compared with the control plants (Fig. 3). The isolate ZmRS1 from *Zea mays* rhizosphere performed poorly among the stem, root and rhizosphere isolates.

Although *G. diazotrophicus* is found to have association with several plants like *Saccharum officinarum*, *Pennisetum purpureum*, *Ipomea batatas, Coffea arabica*, *Eleusine coracana* and *Ananas cosmosus*, only a few reports are available on its effects of inoculation in plants. Endophytic diazotrophs have been shown to increase biomass and nitrogen fixation in micropropagated sugarcane plants (Oliveira et al., 2002; Muthukumarasamy et al., 2006). Further, Sevilla et al. (2001) reported that *G. diazotrophicus* inoculation

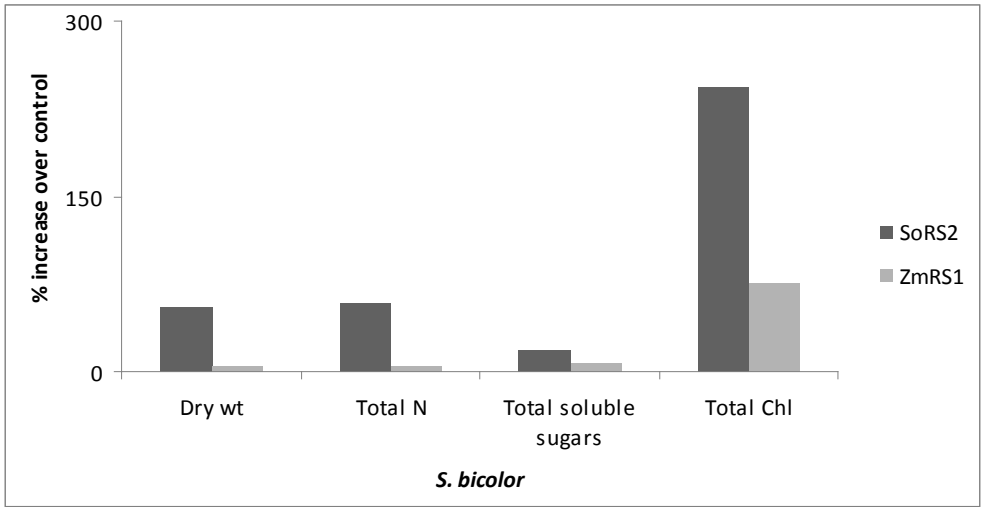

Fig. 3: Effect of inoculation with rhizosphere isolates of *G. diazotrophicus* on dry weight, total nitrogen, soluble sugars, and chlorophyll content of *S. bicolor* at 35 DAI.

enhanced the growth and nitrogen content in sugarcane under N-deficient conditions. Production of plant growth hormones by *G. diazotrophicus*, in addition to nitrogen fixation, is responsible for plant growth (Sevilla et al., 1998). Mehnaz and Lazarovits (2006) reported that *G. diazotrophicus* DS1 inoculation improved the growth of corn as indicated by root/shoot weight. The increased plant growth as indicated by plant biomass shown in the present study could as well be the result of nitrogen fixation and growth hormone production by the isolates since all the isolates fixed nitrogen and produced IAA in the culture medium (data not shown).

Mineral phosphate solubilization is considered to be a plant growth promoting characteristic of rhizosphere bacteria and this property has been found in several strains of *G. diazorophicus* isolated from sugarcane (Maheshkumar et al., 1999). The improved growth performance of *S. bicolor* upon *G. diazotrophicus* inoculation could be the result of improved nutrient uptake, especially of P by the inoculated plants, as all 20 isolates solubilized mineral phosphate (Kumarasamy, 2002). The nitrogen-fixing system demands energy for nitrogenase to function. This demand must be met by photosynthate supply from the host plant. An increase in total carbohydrate content in *S. bicolor* and rice upon inoculation with *G. diazotrophicus* and *Herbaspirillum seropedicae* has been reported (Bastian et al., 1999; Gyneshwar et al., 2002). James et al. (1994) have stated that the efficiency of endophytic diazotrophs may be due to the plant providing photosynthate and also a low oxygen environment created which is required for the function of nitrogenase. Thus, the higher total soluble sugars found in inoculated plants could satisfy the energy needs of *G. diazotrophicus*. The significant effect of *G. diazotrophicus* inoculation on plant growth differed depending on the origin of the isolates. The present study could help in the selection of the stem isolate SoS2 from *S. officinarum* as a potential isolate stimulating growth promotion in *S. bicolor*.

ACKNOWLEDGEMENT

The authors thank Empraba, Brazil for providing *Gluconacetobacter diazotrophicus* PAL5 and Dr. N. Sivaraman, Associate Professor of English (Retd), Thiagarajar College, Madurai, for reading the manuscript.

REFERENCE

Arnon DI, 1949. Copper enzymes in isolated chloroplasts. 1. Polyphenol oxidases in *Beta vulgaris*. Plant Physiol, 24: 1–15.

Baldani JI, VLD Baldani, L Seldin, J Dobereiner, 1986. Characterization of *Herbaspirillum seropedicae* gen. nov. sp. nov., a root associated nitrogen-fixing bacterium. Int J Sys Bacterial, 36: 86–93.

Bastian F, F Rapparini, R Baraldi, P Piccoli, R Bottini, 1999. Inoculation with *Acetobacter diazotrophicus* increases glucose and fructose content in shoots of *Sorghum bicolor* (L.) Monech. Symbiosis, 2: 147–156.

Cavalcante VA, J Dobereiner, 1988. A new acid-tolerant nitrogen fixing bacterium associated with sugarcane. Plant Soil, 108: 23–31.

Dobereiner J, VLD Baldani, VM Reis, 1995. Endophytic occurrence of diazotrophic bacteria in non-leguminous crops. In: *Azospirillum* VI and related microorganisms, Eds. I. Fendrik, M. del Gallo, J. Vanderleyden, M. Zamroczy, Springer, Berlin, 3–14.

Gyaneshwar P, EK James, PM Reddy, JK Ladha 2002. *Herbaspirillum* colonization increases growth and nitrogen accumulation in aluminium tolerant rice varieties. New Phytol, 154: 131–145.

James EK, VM Reis, FL Olivares, JI Baldani, J Dobereiner, 1994. Infection of sugarcane by the nitrogen – fixing bacterium *Acetobacter diazotrophicus*. J Exp Bot, 45: 757–766.

Kumarasamy V, 2002. Studies on diazotrophs associated with grasses and their rhizosphere in Sothern Tamil Nadu. Ph.D. Thesis, Madurai Kamaraj University, Madurai.

Maheshkumar KS, PU Krishnaraj, AR Alagawadi, 1999. Mineral phosphorus solubilizing activity of *Acetobacter diazotrophicus*: a bacterium associated with sugarcane. Curr Sci, 76: 874–875.

Mehnaz S, G Lazarovits, 2006. Inoculation effects of *Gluconacetobacter azoto-captans* and *Azospirillum lipoferum* on corn plant growth under greenhouse conditions. Microbial Ecol, 51: 326–335.

Mooris L, 1948. Quantitative determination of carbohydrates with dray wood's anthrone reagent. Science, 107: 254–255.

Muthukumarasamy R, M Govindarajan, M Vadivelu, G Revathy, 2006. N – fertilizer saving by the inoculation of *Gluconacetobacter diazotrophicus a*nd *Herbaspirillum* sp. in micro-propagated sugarcane plants. Microbial Res, 161: 238–245.

Oliveira ALM, S Urquiaga, J Dobereiner, JJ Baldani, 2002. The effect of inoculating endophytic N_2 fixing bacteria on micropropagated sugarcane plants. Plant Soil, 242: 205–215.

Reinhold-Hurek B, T Hurek, M Gills, B Hoste, M Vancanneyt, K Kersters, J DeLey, 1993. *Azoarcus* gen. nov.

Nitrogen-fixing proteobacteria associated with roots of kallar grass (*Leptochloa fusca* (L.) Kunth) and description of two species, *Azoarcus indicus* sp. nov and *Azoarcus communis* sp. nov. Int J Sys Bacteriol, 43: 574–584.

Sevilla M, C Kennedy, 2000. Genetic analysis of nitrogen fixation and plant growth stimulating properties of *Acetobacter diazotrophicus*, an endophyte of sugarcane. In: Prokaryotic nitrogen fixation: a model system for analysis of a biological process, Ed. E.W. Triplett, Horizon Scientific Press, UK, 737–760.

Sevilla M, ALM Oliveira, I Baldani, C Kennedy, 1998. Contributions of the bacterial endophyte *Acetobacter diazotrophicus* to sugarcane nutrition: a preliminary study. Symbiosis, 25: 181–192.

Sevilla M, RH Burris, N Gunapala, C Kennedy, 2001. Comparison of benefit to sugarcane plant growth and ^{15}N incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* Wild-type and Nif mutant strains. Molecular Plant Microbe Interactions, 14: 358–366.

Umbreit WW, RH Burris, J Stauffer, 1972. Method for nitrogen. In: Manometric and biochemical techniques (5th edition), Burgers Publishing Company, Minnesota, 259–260.

Wilson DO, HM Reisenauer, 1963. Cobalt requirement of symbiotically grown alfalfa. Plant Soil, 19: 364–373