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Endophytic Diazotroph Gluconacetobacter Diazotrophicus On Am Fungal Spores

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Abstract: Gluconacetobacter diazotrophicus is an endophytic plant growth promoting multi beneficial bacteria known to occur large numbers in sugar rich plants, in the present investigation attempts are made to isolate Gluconacetobacter diazotrophicus from rhizosphere soil and sugarcane parts, in addition a special interest were made for the isolation of Gluconacetobacter diazotrophicus from AM fungal spores. The findings of the present research clearly showed the endophytic occurrence of Gluconacetobacter diazotrophicus in different parts of sugarcane, i.e., Gluconacetobacter diazotrophicus successfully isolated from the extracts of different parts namely root bud and leaves whereas its isolation from rhizosphere soil samples ends with 50% success i.e., two samples fails to show any growth on semisolid LGI medium, very interestingly the extracts of AM fungal spores positively showed growth on semisolid LGI medium. In biochemical character studies the isolates were identified as Gluconacetobacter diazotrophicus and the present finds again proved its endophytic nature in sugarcane as well as in AM fungal spores. The field experiments showed enhanced growth and yield of sugarcane in the treatment having combination of G.diazotrophicus and AM fungi with 50 and 75% graded levels of N, P and K fertilizers

Key words: Gluconacetobacter diazotrophicus, AM fungal spores, Sugarcane, endophyte, rhizosphere and diazotroph.

INTRODUCTION :

Sugar is the universal sweetening agent used throughout the world for the taste sweet and all the countries in the world producing sugar from sugar rich plants. Among, sugar rich plants about 80% of sugar was obtained from very familiar sugar rich plant namely sugarcane (Saccharum officinarum L.).

India is one of the developing country in the world and having all the required sources for the cultivation of sugarcane and also having neck to neck race with Brazil for the first position in terms of production, whereas already India having the world maximum area under the cultivation of sugarcane. In the cultivation of sugarcane much important has given to nutrients which are supplied through chemical fertilizers, and in connection with nutrient inputs sugarcane removes huge quantum of nutrients from soil due to its long standing period in the fields. As a developing country India in the recent years facing so much of problematic issues about price hike for the petroleum products, many of these chemical fertilizers are synthesized from petroleum products and their price hike indirectly influences prices hike of chemical fertilizers as well as other commodities.

The natural availability of petroleum reservoir gets depleted year by year and in the future world should face lots of difficulties for all types of petroleum products. Hence it's our urgent need for the world to find an alternative method to improve the availability of nutrients. In the present world we needs to utilize biological sources or methods for the betterment of living things particularly for human beings and animals and by these ways we can able to minimize the chemical pollution in the soundings as well as in the living systems, even though different biological sources are available to improve the nutrients status in soil, in which one feasible way is to improve the availability of nutrients though biofertilizer.

Biofertilizers are nothing but preparations containing selected effective microorganisms intended to crop plants to supply nutrients like N, P, K and growth promoting substances to obtain maximum yield. Gluconacetobacter diazotrophicus is an entophytic bacterium supplies N and P nutrients in sustainable amount and also provide growth promoting substances to almost all types of sugar rich plants, regarding its entophytic nature different controversies are raised by scientist and they have isolated Gluconacetobacter diazotrophicus successfully from soil. Many scientific studies clearly indicate symbiotic relationship between Gluconacetobacter diazotrophicus and AM fungi. In many cases particularly in the absence of sugar rich plants the endophytic bacterium Gluconaceto bacter diazotrophicus become a fungal endophyte only on the spores of AM fungi.

G. diazotrophicus and AM fungal inoculation enhances the growth and development of sugarcane by fixing

nitrogen in various parts of sugarcane via roots, stem, bud and leaves along with producing growth promoting hormones and by solubilizing, mobilizing phosphorus, potash and zinc compounds and protecting plants from stresses and pathogens.

Based on above view the present research was undertaken with the following research aspects:

1. Isolation of Gluconacetobacter diazotrophicus from rhizosphere soil and different parts of sugarcane.
2. Isolation of Gluconacetobacter diazotrophicus from AM fungal spores.
3. Inoculation effect of Gluconacetobacter diazotrophicus and AM fungi on the growth and yield of sugarcane.

REVIEW OF LITERATURE:

Discovery of Gluconacetobacter diazotrophicus

Cavalcante and Dobereiner (1988) isolated and identified a new diazotroph from stem, leaf and roots of sugarcane and named it as Saccharobacter nitrocaptans. Later, Gillis et al. (1989) showed that this bacterium belonged to the genus Acetobacter by taxonomic studies and confirmed it to be a new species under this genus by DNA/DNA binding experiments, and the organism was renamed as G. diazotrophicus.

The outstanding specific beneficial bacterial association of G. diazotrophicus with sugarcane was elucidated in Brazil (Cavalcante and Dobereiner, 1988), Australia (Li and MacRae, 1991), Mexico (Fuentes – Ramirez et al., 1993), Uruguay (Ureta et al., 1995), Canada and Cuba (Dong et al., 1995) and India (Jayakumar and Thangaraju, 1996; Muthukumarasamy, 1996).

The unusual physiological properties of this diazotroph including growth and nitrogen fixation at high sucrose concentration (30% sucrose) and acidic pH even below 3 and rDNA analysis led to its identification as Acetobacter diazotrophicus. This is only N2 fixing species of acetobacter so far identified (Gillis et al., (1989). A. diazotrophicus is recently renamed as Gluconacetobacter diazotrophicus (Yamada et al., 1997; 1998; Franke et al., 1999) by the analysis of partial sequences of 16s ribosomal RNA. The characterization of different species of Gluconacetobacter was clearly explained by Muthukumarasamy et al. (2005). In contrast to the classic rhizosphere association which contributed to BNF in non-leguminous crops a similar relationship between the endophytic G. diazotrophicus and sugarcane has been well documented (Li and MacRae, 1992; Reis et al., 1994; Loganathan and Nair, 1999, 2003, 2004).

This organism is a proteobacterium that is an excellent model system for bacterial endophytes of plants. It basically belongs to phylum proteobacteria comprising gram negative bacteria in the section α-proteobacteria, order Rhodospirillales (Garrity, 2004). A bacterial endophyte is defined here as a bacterium that resides within plant tissues without causing any disease symptoms and without inducing any organized symbiotic structure such as a root nodule. G. diazotrophicus is known to survive only within plant tissues and cannot survive in soil. It has a number of very interesting phenotypes such as the ability to produce significant amounts of plant growth hormones in cultures (Fuentes-ramirez et al., 1993; Bastian et al., 1998), enhance growth of sugarcane in the presence of comparatively higher levels of combined nitrogen (Sevilla et al., 1998), fix N2 inside sugarcane (Sevilla et al., 1998; Sevilla and Kennedy, 2001a) and solubilize phosphate (Maheshkumar et al., 1999)

Entrance of G. diazotrophicus in sugarcane through AM fungi and mealy bugs:

The use of reporter genes such as uid A gene (Christiansen Weniger, 1997; Vande Broek et al., 1993; Sevilla and Kennedy, 2001b), lac Z (Gopalaswamy et al., 2000) and green fluorescent protein (GFP) (Chalfie et al., 1994; Galbraith et al., 1995) have facilitated investigation of plant microbe interactions including those involving grass associated diazotrophs.

Although G. diazotrophicus has been isolated from all parts of the sugarcane plant, their exact location is still to be established (James and Olivares, 1998). James et al. (1994) reported that G. diazotrophicus first colonized the root and lower stem epidermal surface of sugarcane during the first 15 days after planting and then entered throughout the plant via the translocation stream. Since that study was published, further micrographs showing xylem vessels colonized by G. diazotrophicus have been presented (Dobereiner et al., 1994; James and Olivares, 1998; Reis et al., 1999; Fuentes – Ramirez et al., 1999). However, results have presented in contrast with these studies (Dong et al., 1994). These authors suggested that the intercellular spaces in the sucrose storage parenchyma of the stems of mature sugarcane stalks were the most likely location.

Recently James et al. (2001) have again confirmed their previous findings through immunogold labeling and suggested that the xylem is the principal location of G. diazotrophicus is fully expanded up to leaves of inoculated green house grown plants and this may be more representative of the field situation.

Endophytic nature of G. diazotrophicus was reported by Dobereiner et al. (1988). These organisms infected sugarcane through damaged tissue (Boddey et al., 1991). Many authors described that G. diazotrophicus is spread among cane cultivars by the mealy bugs associated with sugarcane (Ashbold and Inkerman, 1990). Consistent with this notation are the observation that the honey dew excreted by this insect is rich in carbon sources and is very acidic.

Caballero – Mellado and Martinez – Romero (1994) reported that dispersal of G. diazotrophicus by sugarcane seeds may not occur and unable to isolate bacteria from seeds. The possibility of infecting sugarcane seedlings with endophytic diazotroph via AM fungi represents a unique possibility of introducing selected bacteria into plants which then became further propagated within stem cuttings (Dobereiner, 1992). Paula et al. (1991) reported that AM fungal hyphae may have promoted the penetration and spread of the G. diazotrophicus in cortical root regions in mycorrhizal infection zone. With this initial spread in roots the bacteria may be able to reach root structure and later translocated to the stems.

Paula et al., (1991) found the presence of several

diazotrophic bacteria in spores of Glomus clarum including G. diazotrophicus and Klebsiella spp. They obtained sterile spores of AM fungi and coinoculated sweet potato, sweet sorghum and sugarcane with these spores with G. diazotrophicus, Klebsiella spp and others. In those treatments inoculated with both microbes the number of diazotrophic cells within surface sterilized roots and tops of sweet potato and sugarcane was significantly higher than in the uninoculated treatment or in the treatments inoculated with diazotrophs only.

Isopi et al. (1995) have co-inoculated sorghum plants with AM fungi and G. diazotrophicus. The fungi increased bacterial infection on roots, stem and leaves. The bacterium also increased N and P levels in the plants especially when co-inoculated with the fungus. Prabudoss and Stella (2010) observed increased cane height and cane yield by the inoculation of G. diazotrophicus + AM fungi in sugarcane plants. There was considerable reduction in these parameters for those plants received AM fungi + G. diazotrophicus (heat killed cells). AM fungal colonization was also reduced in this treatment (Panneerselvam, 1997).

Occurrence and distribution of AM fungi in the rhizosphere soil:

Arbuscular mycorrhizae (AM fungi) are ubiquitous in nature and their occurrence is obvious in association with plants grown in cultivated soils, sand dunes, coal mines and aquatic environments (Hayman, 1980), the environments like marine habitates (Kannan and Lakshminarayanan, 1989), saline patches (Thapar and Uniyal, 1990), mangrove vegetation (Lingan, 1994), different types of soils (Srinivas et al., 1993), agroforestry trees in alfisol. They were known to enhance plant growth and biomass through better uptake of nutrients with water, resistance to drought and increased tolerance to invading plant pathogens (Tarafdar and Rao, 1990).

A study was conducted in Kerala, India in which the roots of 46 crop species, harbours the infection of mycorrhizae but it shows in various degrees (Girija and Nair, 1985). Nalini et al. (1987) made a preliminary survey of the AM spore types present in the root zone of two-year-old field grown Leucaena leucocephala plants, and found spores of AM fungi belongs to the genus Glomus and varied in their intensity of occurrence as G. versiforme,G. geosporum, Glomus mosseae and G. clariodeum.

Mohankumar and Mahadevan (1987) studied the effect of certain ecological factors such as soil pH, temperature, moisture, organic matter and macro and micro elements of the soil on AM fungal distribution in Kalakkad reserve forest, a tropical forest in India. Changes in soil conditions modify the dominance of particular fungi during mycorrhizal formation in field soil. Natural or intentional distribution is a common feature of the habitat of all AM fungi, and an understanding of the effects and distribution at the population level complement existing studies of the effects on individual fungi (Jasper et al., 1993).

Tarafdar and Rao (1990) surveyed for the occurrence of AM fungi infection in twelve tree species at different sites of Indian arid zone. The per cent as well as density of mycorrhizal colonization varied with the plant species and the locations. Even the plants of same family differed in the intensity of mycorrhizal association in a particular soil. The infection rate in general was independent of plant age. The AM fungal genera found in tree species were Gigaspora, Glomus, Acaulospora and Endogone.

Thapar and Uniyal (1990) reported that the frequency and level of cortical infection in nursery seedlings belonging to 19 Genera and 14 families of angiosperms at New forest Dehradun, India. The frequency of AM infection and percentage of infection in roots among species within the family and among different host Genera were found to be variable. Glomus macrocarpum and Sclerocystis coremioides were found in the rhizosphere of infected roots. Highest level of root colonization (92%) was recorded in Michelia champaca and Toona ciliate, whereas very low (1 to 6%) levels were recorded in Albizia lebbek, Grewia robusta and Madhace longifolia. The percentage of colonization was almost same in Terminalia Arjuna, Dendeocalamus strictus, Dalbergia sissoo, Acacia auriculiformis, Emblica officinalis, Olia glandulifera, Acacia nilotica, Serraca asoca.

Santhaguru et al. (1995) surveyed for the occurrence of AM spores in the rhizosphere soils of 20 species of tree legumes in the reserved forest of Alagar hills of Tamilnadu, India. Nearly 75 per cent of plant species showed AM fungal infection, but with varying frequency altogether 21 species of AM fungi belonging to five genera (Acaulospora, Gigaspora, Glomus, Sclerocystis and Scutellospora) were recorded.

Soil and root samples collected from fields cropped to spring wheat and lentil at 11 sites across four zones of Saskatchewan in Canada were analyzed for spore numbers, level of AM fungal colonization, and AM fungal species. The number of AM fungal spore detected in field soil ranged from 78 to 272 100 g-1 soil. AM fungal colonization of wheat and lentil at all the field study sites, but levels of colonization in the two crops varied from site to site and the differences were more pronounced in wheat than in lentil (Tarafdar and Marschner, 1994).

Five common species of AM fungi were recorded from rhizosphere soils of the salt marsh plants. Population of spores of AM fungi and effective inoculums potential of these fungi in rhizosphere soils as determined by the MPN-method were low (Sengupta and Chaudhuri, 1990).

Isoenzyme techniques have featured in taxonomic and population genetic studies of AM fungi. The methodology was also further extended to enable the identification of different fungal symbionts in endo and ectomycorrhizas (Secilia and Bagyaraj, 1994).

Fluorescent antibody techniques have been successfully used to trace the hyphae of Gigaspore margarita in the field after inoculation (Fries and Allen, 1991). The application of molecular techniques to AM fungi has the potential to allow precise identification and quantification of mycorrhizal fungi within roots of field grown plants. Nucleotide sequences of a highly conserved region of the 18 S ribosomal genes have been compared in isolates of selected Glomus species (Simon et al., 1992; Koide and Mosse, 2004; Rilling, 2004; Cho et al., 2009).

Interaction between soil bacterial population and AM fungi in rhizosphere soil:

Mycorrhizal fungi interact with a wide range of other soil organisms, in the root, in the rhizosphere and in the bulk soil. The positive influence of the soil microorganisms in interaction with AM fungi on plant growth was reported by many workers (Bagyaraj, 1984; Boby et al., 2008; Hu et al., 2009).

Secilia and Bagyaraj (1987) estimated the population of total bacteria, nitrogen fixing bacteria and actinomycetes in the root zones of different AM fungi pot culture viz., G. fasciculatum, G. margarita and Sclerocystis dussii. They observed that the total bacteria, nitrogen fixing bacteria and actinomycetes were significantly greater in mycorrhizal pot cultures.

The most numerous and active populations of Pseudomonas fluorescens were found in the exorhizosphere, while the greatest development of A. brasilense occurred in the rhizoplane and in the endorhizosphere. AM fungi rapidly stimulated the development of the P. fluorescens population on the surface of the extra radicular mycelium. The endorhizosphere had the most favourable effect on A. brasilense in the endorhizosphere (Klyuchinkova and Kozhevin, 1990).

Krishnaraj and Sreenivasa (1990) isolated the oligotrophic bacteria associated with AM fungi cultures viz., G. fasciculatum, G. mosseae and A. laevis in Rhodes grass (Chloris gayana Kunth). They observed higher population of oligotrophic bacteria in association with AM fungal inoculum, which also varied in the number of associated bacteria. G. fasciculatum harboured the highest number of oligotrophic bacteria while A. laevis had the least counts of oligotrophic bacteria.

Rhizosphere micro flora favoured AM fungal association and vice-versa in cassava root region. AM fungi in cassava plants with no phosphorus enhanced fungal, bacterial and actinomycetes population than the uninoculated plants. Maximum microbial populations were obtained at fourth month stage of the crop under partially sterile condition (Peretto et al., 1995).

Increase in grain yield of various genotypes of wheat was higher when G. fasciculatum and A. brasilense were co-inoculated (Singh et al., 1990b). The role of AM fungal spores on the infection of sweet potato plants by G. diazotrophicus was studied and interactions between these two indicated that AM fungi increased the translocation of G. diazotrophicus to aerial parts of the plant and G. diazotrophicus enhanced the sporulation of G. clarum in soil and within the roots (Paula et al., 1991; Sathyan and Thangaraju, 2003).

Micro propagated sugarcane seedling inoculated with the AM fungal spores containing the diazotrophs contained much higher number of G. diazotrophicus in aerial parts than seedlings inoculated in vitro with the bacteria alone. When grown in non-sterile soil, the sugarcane seedlings again showed the greatest colonization percentage of 65 per cent in the aerial parts after inoculation with AM fungal spores containing the diaztrophs (Paula et al., 1991).

Krishnaraj and Sreenivasa (1990) observed that the Capsicum annum inoculated with AM fungi harboured more number of bacteria in the endorhizosphere than the uninoculated plants.

Jones and Sreenivasa (1993a) studied the effect of inoculation of G. fasciculatum and or phosphate solubilizing bacterium, Pseudomonas striata at four levels of two forms of P on rhizosphere micro flora viz., total bacteria, fungi and actinomycetes, Azotobacter and P solubilizer populations in sunflower (Helianthus annuus L.) at 30, 60 and 90 days after sowing. The population of these organisms increased significantly with the increase in the level of P and age of the host plant inoculated with both the organisms up to 60 days as compared to plants inoculated with either of them and later the microbial population decreased.

MATERIALS AND METHODS:

Isolation of Gluconacetobater diazotrophicus from soil and AM fungal spores of sugarcane rhizosphere:

The enrichment culture technique as described by cavalcante and dobereiner (1988) was followed for the isolation of G.diazotrophicus from AM fungal spores, sugarcane samples, (Root,Bud,Leaves) and sugarcane rhizosphere soil samples collected from the below mentioned different locations like Annamalainagar, sivapuri, vilagam and vallampadugai cuddalore district, tamilnadu, india.

Isolation of G. diazotrophius:

In the present research an attempt was made to isolate G. diazotrophius from rhizosphere soil samples of sugarcane. About 1g of sugarcane rhizosphere soil samples from different locations of cuddalore district namely, Annamalai nagar, sivapuri, vilagam and vallampadugai were taken and are serially diluted upto 10-4,about 1ml of aliquots were transferred from 10-3and 10-4 to the test tubes containing enriched semisolid LGI medium and are incubated for about 4-7 days for pellicle formation. From the positive tubes, subsurface pellicles were transferred into LGI plates to study the colony characters and also for characterization

Isolation and characterization of AM fungi:

Soil samples from Annamalainagar, Sivapuri, Vilagam and Vallampadugai were collected and are used for the isolation of AM fungal spores by wet sieving (1000 – 45 μm) and decanting method as described by Gerdemann and Nicolson (1963). These spores were cleaned of soil particles by sucrose density gradient centrifugation method and washed with distilled water (Mertz et al., 1979). This spore's suspension was counted with stereo zoom microscope (× 47). During counting, morphologically similar spores were separated into groups, mounted and identified. The soil texture and the types also analyzed.

Based on the taxonomic key of Gerdemann and Trappe (1974) the spores of G. fasciculatum, Glomus mosseae, Glomus versiforme, Aculospora laevis and Gigaspora margarita were identified.

Isolation of Gluconacetobacter diazotrophicus from Am fungal spores:

The identified spores were collected, surface sterilized with 1% chloramines T and crushed with the help of pestle and mortar by using few ml of sterile water. From the crushed spores samples about one ml were transferred in to different test tubes containing sterilized semisolid LGI medium and incubated for 4 to 7 days for pellicle formation. The tubes shows orange coloured sub surface pellicle were taken with the help of sterile inoculation needle and streaked on plates containing solid LGI and acetic LGI medium and are incubated for about 4 to 7days, colonies were observed and orange coloured colonies after 4 days were taken and used for further studies.

Selection of efficient Gluconacetobacter diazotro phicus isolate for field studies

The nitrogen fixing capacity of the isolates was evaluated by using Acetylene Reduction Activity (ARA) following the standard procedure (Bergensen, 1980). Twenty five ml of semisolid LGI medium was prepared in 100 ml vials. The vials were inoculated with 25 µl of G. diazotrophicus isolates and incubated under static condition in an incubator 28 ± 1°C. After 5 days of growth the cotton plugs were replaced by suba-seal septa and tightened with aluminium cap. The air in the vial was replaced with nitrogen gas. Ten per cent (v/v) of the inert gas was removed and ten per cent pure acetylene gas was injected. The vials were incubated for 24 h at room temperature. After incubation, 1 ml of gas sample was withdrawn and injected into the gas chromatograph (Systronics 4010, India) fitted with porapak Q column (6″ 1/8″) and FID detector. The column tempera ture was maintained at 80°C. Nitrogen gas was used as carrier gas at the flow rate of 20ml min-1.

The acetylene reduction activity of the strains was calculated using the formula:

Sample peak length of ethylene (mm) Attenuation Volume of gas phase of flask 0.0006

Incubation time (h) Volume of gas simple injected into gas chromatograph (ml)

The acetylene reduction activity of the sample was expressed as n moles of ethylene formed mg of protein h-1. At the end of experimental period the cell protein content of the cultures were determined following the method described by Lowry et al. (1951).

RESULTS AND DICUSSION

Table 1: Isolation of G.diazotrophicus from rhizosphere soil, AM fungal spores and sugarcane Samples:

S.No	Places	Pellicle formation				
		Sugarcane rhizosphere soil	Sugarcane parts			
			Root	Bud	Leaves	VAM Spores
1	Annamalainagar	-	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days
2	Sivapuri	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days
3	Vallampadugai	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days
4	Vilagam	-	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days	Orange coloured subsurface pellicle formed within 7days

(Fails to form orange coloured subsurface pellicles within 7days)

Isolation of Gluconacetobacter diazotrophicus from rhizosphere soil and different parts of sugarcane

G. diazotrophicus were isolated from rhizosphere soil and sugarcane samples collected from four different locations (Annamalainagar, sivapuri, vallampadugai and Vilagam, Cuddalore District, Tamilnadu) and three different parts viz., root, bud and leaves. The G. diazotrophicus were isolated only from two rhizosphere soil samples namely sivapuri and vallampadugai and other two rhizosphere soils fails to show any positive character like formation of sub surface pellicle for the presence of G.diazotrophicus where as all the parts of sugarcane like root, bud and leaves positively responded for the presence of G.diazotrophicus and formed yellow light to orange coloured sub surface pellicle on colourless semisolid LGI medium and typical heavy orange yellow coloured subsurface pellicle on acetic LGI semisolid medium were observed (Table 1).The present study clearly showed the endophytic nature of G.diazotrophicus and poor survivability in the soil, only two rhizosphere soil samples showed pellicle formation and others fails to show pellicles on semisolid LGI tubes G. diazotrophicus was isolated from all the parts of sugarcane. If the absence of this diazotroph in certain varieties may be due to high N and P fertilization of these varieties with the report made by Li and Mac Rae (1991), Fuentes – Ramirez et al. (1993) and Baldani et al. (1997). The isolates were isolated from all parts of sugarcane viz., root, stem, bud and leaf (Cavalcante and Dobereiner, 1988). More isolates were

obtained when the macerates of surface sterilized root, stem, bud and leaves of sugarcane were used than the plant bits as such. Isolation of G. diazotrophicus from sugarcane rhizosphere soil ends with 50 per cent success and in the case of isolation from AM fungal spores positively ends and about three isolates were also isolated this observation is substantiated by Dong et al. (1994) and James et al. (1994) and these observation illustrated the endophytic nature of the bacterium G. diazotrophicus (Reis et al. 1994; dos Santos et al., 2010). It is the only nitrogen fixing bacterial endophyte and leading to designated as a model organism to evaluate the plant bacterial interactions in non-legumes (Dobbelaere et al., 2003).

Table 2: Characterization of G. diazotrophicus isolates from different parts of sugarcane

S.No.	Name of the isolates	Gram reaction	Shape	Nitrate reductase activity	Motility	Catalase activity	Oxidase activity	H_2S formation
•	GdASR	Negative	Rod shaped	–	+	+	+	+
•	GdASB	Negative	Rod shaped	–	+	+	+	+
•	GdASL	Negative	Rod shaped	–	+	+	+	+
•	GdAVS	Negative	Rod shaped	–	+	+	+	+
•	GdSSRS	Negative	Rod shaped	–	+	+	+	+
•	GdSSR	Negative	Rod shaped	–	+	+	+	+
•	GdSSB	Negative	Rod shaped	–	+	+	+	+
•	GdSSL	Negative	Rod shaped	–	+	+	+	+
•	GdSVS	Negative	Rod shaped	–	+	+	+	+
•	GdSVRS	Negative	Rod shaped	–	+	+	+	+
•	GdVSR	Negative	Rod shaped	–	+	+	+	+
•	GdVSB	Negative	Rod shaped	–	+	+	+	+
•	GdVSL	Negative	Rod shaped	–	+	+	+	+
•	GdVVS	Negative	Rod shaped	–	+	+	+	+
•	GdVSR*	Negative	Rod shaped	–	+	+	+	+
•	GdVSB*	Negative	Rod shaped	–	+	+	+	+
•	GdVSL*	Negative	Rod shaped	–	+	+	+	+
•	GdVVS*	Negative	Rod shaped	–	+	+	+	+
•	PAL5 (Reference Strain)	Negative	Rod shaped	–	+	+	+	+

GdA- G. diazotrophicus isolates from Annamalainagar
GdS- G. diazotrophicus isolates from Sivapuri
GdV- G. diazotrophicus isolates from Vallampadugai
GdV*- G. diazotrophicus isolates from Vilagam
GdVS- G. diazotrophicus isolates from VAM Spores

Characterization of G. diazotrophicus isolates

The eighteen isolates and the reference strain PAL5 of G. diazotrophicus were confirmed by performing characterization tests viz., gram reaction, motility, catalase activity, oxidase activity, nitrate reductase activity, hydrogen sulphide formation and growth under different conditions. Comparison of the isolates with type strain PAL5 was done. The results confirmed that the isolates were G. diazotrophicus.

All the twenty isolates produced yellow to dark yellowish orange coloured sub surface pellicles on semisolid LGI and acetic LGI medium. They developed brown pigmented colonies on potato infusion agar medium. This was in accordance with the findings of Cavalcante and Dobereiner (1988). No nitrate reductase activity was observed in the isolates (Boddey et al., 1995). The isolates were gram negative and rod shaped as described by Cavalcante and Dobereiner (1988). The tested isolates were motile, catalase and oxidase positive activities exhibiting the reactions similar to the type strain PAL5. All the isolates produced hydrogen sulphide. The isolates formed smooth, initially small white colonies, which became yellow, orange and finally dark orange on LGI and acetic LGI plates. The diameter was around 2-3 mm, but in potato infusion agar plates diameter was 4-5mm and colonies were chocolate brown with light coloured margins (Cavalcante and Dobereiner, 1988; Rojas and Mellado, 2003).

The isolates developed yellow coloured sub surface pellicles on semisolid LGI and developed yellowish orange coloured sub surface pellicle on acetic LGI semisolid medium, which showed the microaerobic nature of the organism (Cavalcante and Dobereiner, 1988). The yellow colour of the isolate is due to assimilation of bromothymol blue in the medium and strong acid production (Boddey et al., 1991; Sathyan and Thangaraju, 2003). The natural occurrence and colony characters were extensively studied in India by Muthukumarasamy et al. (2005).

Table 3: Nitrogen fixing efficiency of G. diazotrophicus isolates from different parts of sugarcane

S.No.	Name of the isolates	Nitrogenase activity (n moles C_2H_4/ h / mg of cell protein)
1.	GdASR	325.90
2.	GdASB	380.96
3.	GdASL	208.10
4.	GdAVS	384.10
5.	GdSSRS	212.00
6.	GdSSR	340.10
7.	GdSSB	384.00
8.	GdSSL	210.10
9.	GdSVS	384.25
10.	GdVSRS	214.10
11.	GdVSR	340.97
12.	GdVSB	384.80
13.	GdVSL	215.00
14.	GdVVS	389.10
15.	GdVSR*	338.20
16.	GdVSB*	380.00
17.	GdVSL*	210.10
18.	GdVVS*	384.10
19.	PAL5	360.20
SE		2.30
CD (p = 0.05)		4.80

All the isolate recorded appreciable amount of nitrogen, among 18 isolate and reference stain, the isolate from vallampudugai VAM spores showed maximum nitrogenase activity in the form of nitrogen fixation (389.10 n moles C2H4/hr/mg cell protein). Based on N fixing efficiency it was noticed that all isolates from AM fungal spores recorded significant values compared with other isolate hence the isolate from vallampudugai VAM spores was selected and used for field studies. The nitrogenase activity of G. diazotrophicus strains was first proved by Cavalcante and Dobereiner (1988) in a report indicating 240 n moles C2H4 h-1 mg cell protein-1 and later confirmed by Gillis et al. (1989). In the present study, all the isolates showed appreciable amounts of nitrogenase activity ranging from 208.10 to 389.10 n moles C2H4h-1 mg cell protein-1. The variation may be due to collection of sugarcane samples from different locations and different varieties. The same was also reported by Caballero-Mellado and Martinez-Romera (1994); Oliveira et al. (2009).

Reis et al. (1994) reported an increase in nitrogenase activity (350 and 420 n moles C2H4h-1 mg cell protein-1 respectively) of two strains viz., PAL5 and PPE4 due to supplementation with cane juice. In the present study, the G. diazotrophicus GdVVS and GdSVS strains recorded above 380 n moles of C2H4 h-1 mg cell protein-1 in acetic LGI semisolid medium without any supplementation. In general, the nitrogenase activity was more than type strain PAL5 indicating the superiority of the local isolates.

Table 4: Characterization of different AM fungal isolates from sugarcane rhizosphere soil

S.No.	Characters	*Glomus mosseae*	*Glomus fasciculatum*	*Glomus versiforme*	*Acaulospora laevis*	*Gigaspora margarita*
1	Size of spore	120 µm	100 – 120 µm	125 – 150 µm	400 µm	200 – 300 µm
2	Spore shape	Globose	Globose hypogeous	Globose	Globose	Ectocarpic
3	Colour of spore	Yellow to brown	Yellow to reddish brown	Yellow to brown	Outer wall – brown Inner wall – Hyaline Ellipsoid	White when young and slightly yellowish at maturity
4	Sporocarp	Present	Present	Present	Present	Absent
5	Thickness of spore wall	3 – 4 µm	4 – 14 µm	3 – 4 µm	4 – 8 µm	> 20µm
6	Subtending hyphae	Cylindric flared	Absent	Cylindric or flared	Not observable	Bulbous (30 – 50 µm)

The isolated spores were characterized under stereozoom microscope according to Gerdemann and Trappe (1974) and identified as G. mosseae, G. fasciculatum, G. versiforme, A. laevis and Gi. margarita, they were found in all the soil types (Table3).

The results of the present survey in three different locations in Cuddalore District of Tamil Nadu State of India, where sugarcane is grown as a commercial crop revealed the ubiquitous nature of AM fungi in sugarcane rhizosphere soil and the occurrence of AM fungi in soils has been reported in various kinds of environments (Koide and Mosse, 2004; Rilling, 2004, 2006; Bouwmeester et al., 2007; Cho et al., 2009). The rhizosphere soil and root samples collected from wheat and lentil at 11 sites across four zones of Saskatchewan in Canada were analysed for spore number, level of AM fungal colonization and AM fungal species. The number of spores ranged from 78 to 272 100 g-1 soil. The level of colonization varied from site to site and the difference were more pronounced in wheat than lentil (Talukdar and Germida, 1993; Begum and Priya, 2004). The root colonization percentage in sweet potato ranged from 30 to 70 per cent (Sathiyavathi, 1996) and in cotton ranged from 40-80 (Jayanthi, 2008).

Table 5: Effect of G. diazotrophicus and AM fungi with graded levels of N P K fertilizers on the growth and yield of sugarcane under field condition

Treatment		Milleable canes (000 ha^{-1})	Cane girth (mm)	Individual cane weight (kg)	Cane yield (t ha^{-1})
T_0	Absolute control	66.00	69.70	0.760	56.00
T_1	100%NPK (control)	102.50	86. 60	1.000	108.00
T_2	75% NPK(control)	96.00	80.60	0.930	90.00
T_3	75% NPK+ *G. diazotrophicus*	97.60	86.00	1.100	103.00
T_4	75% NPK+ AM fungi	97.0 0	82.90	0.980	97.00
T_5	75% NPK+ *G. diazotrophicus* + AM fungi	105.00	89.00	1.150	117.00
T_6	50% NPK (control)	89.70	76.30	0.840	76.00
T_7	50% NPK+ *G. diazotrophicus*	97.00	86.20	1.090	103.00
T_8	50% NPK+ AM fungi	94.00	83.10	0.930	88.00
T_9	50% NPK+ *G. diazotrophicus* + AM fungi	106.00	89.10	1.160	119.00
	SE	0.41	0.09	0.03	0.89
	CD (p = 0.05)	0.97	0.18	0.06	1.80

Observation at harvest

Milleable canes

There was significant difference in the milleable can count among the treatments, 50 per cent NPK + G. diazotrophicus + AM fungi recorded increased number of milleable canes (106.00 '000 ha-1) followed by 75 per cent NPK + G. diazotrophicus + AM fungi (105.00 '000 ha-1) over absolute control (66.00 '000 ha-1) and the values recorded in 50 and 75 per cent NPK + G. diazotrophicus were on par with one another (Table 4).

Individual cane weight

The treatment receiving both 50 per cent NPK + G. diazotrophicus + AM fungi (1.160kg) and 75 per cent NPK+ G. diazotrophicus + AM fungi (1.150 kg) showed an increase over 100 per cent NPK control (1.000 kg) and appreciable weight of cane noticed in 50 and 75 per cent NPK + individual inoculation of G. diazotrophicus treatments.

Cane yield

A significant increase was observed in the treatments receiving 50 and 75 per cent NPK+ G. diazotrophicus + AM fungi than other treatments and control. Maximum cane yield was noticed in 50and 75 per cent NPK+ G. diazotrophicus + AM fungi treatment (119.00 t ha-1and 117.00 t ha-1) followed by 100 per cent N P K treatments (117.00 t ha-1).

Regarding the growth parameters like milleable cane, cane girth and cane yield the G. diazotrophicus + AM fungi and 50 and 75 per cent NPK treated plants performed better than the absolute and other controls. Thangaraju and Jayakumar (2002) and Muthukumarasamy et al. (1999) have recorded can height and cane yield of 50 per cent N and P + G. diazotrophicus on par with 100 per cent N and P treatment. In the present study, G. diazotrophicus treatment in combination of AM fungi with 50 per cent inorganic NPK fertilizers registered more number of milleable cane, cane girth and cane yield than 75 and 100 per cent NPK treatment. Inoculation of G. diazotrophicus + AM fungi could enhance the cane height, cane girth and cane yield. Hence possibility of saving 50 per cent of fertilizer NPK through G. diazotrophicus + AM fungi inoculation was reported (Thangaraju and Govindarajan, 2001). The present results were in accordance with the above findings.

SUMMARY AND CONCLUSION

In the present research about 18 isolates of G. diazotrophicus were isolated from rhizosphere soil, different parts of sugarcane and AM fungal spores, all the isolates were characterized and tested for its efficiency on nitrogen fixation. Among the isolates, the best isolate from vallampadugai AM fungal spores were selected and used for field studies, likewise the best AM fungal isolate for host preference was selected(Glomus fasciculatum) and used in field studies with graded levels of NPK fertilizers. The best growth and yield parameters were noticed in the treatment-T9 which constitutes 50% NPK + G. diazotrophicus + AM fungi and also T9 showed significant values for all the parameters compared with control and other treatment except T5 which recorded on par values with best treatment. Hence by using G. diazotrophicus and AM fungi in sugarcane cultivation by farmers could save 50 – 75 per cent usage of chemical fertilizers.

FUTURE STUDY

The present research showed the endophytic nature of G. diazotrophicus in AM fungal spores, Hence further advanced studies are needed to investigate the inoculation of AM fungi along with G. diazotrophicus as endophyte with in the spores

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