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Original article

# Influence of organic fertilization on the number of culturable diazotrophic endophytic bacteria isolated from sugarcane

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ABSTRACT

The numbers of culturable diazotrophic endophytic bacteria (CDEB) from roots, stems and leaves of sugarcane submitted to organic, inorganic or no fertilization were compared. In order to determine the size of the $N_2$ fixing populations, the Most Probable Number technique (MPN) was used. The quantification of diazotrophic bacteria by using the acetylene reduction assay (ARA) was more accurate than observing the bacterial growth in the vials; to confirm $N_2$ fixing capability, the detection of gene *nifH* was performed on a sample of 105 isolated bacteria. The production of extracellular enzymes involved in the penetration of the plants by the bacteria was also studied. The results showed that organic fertilization enhances the number of CDEB when compared with conventional fertilization used throughout the growing season. The maximum number of bacteria was detected in the roots. Roots and stems presented the greatest number of CDEB in the middle of the cropping season and in leaves numbers varied according to the treatment. Using two pairs of primers and two different methods, the *nifH* gene was found in 104 of the 105 tested isolates. Larger amounts of pectinase were released by isolates from sugarcane treated with conventional fertilizers (66%), whereas larger amounts of cellulase were released by strains isolated from sugarcane treated with organic fertilizers (80%).



## 1. Introduction

Human activity is causing major increases in the amount of nitrogen cycling between living organisms and the environment. The consequences of such activity have already caused the rate of nitrogen entering the land-based nitrogen-cycle to double, and that rate is continuing to climb. Excessive nitrogen additions can pollute ecosystems and alter both their ecological functioning and the living communities they support [58]. Organic agricultural methods are believed to be more environmentally sound than conventional agriculture, which is dependent on the routine use of herbicides, pesticides and inorganic nutrient application. Organic agriculture results in less leaching of nutrients and higher carbon storage [14], less erosion [41] and lower levels of pesticides in water systems [24,27]. Organic manures are not only sources of major nutrients, but they also provide other micronutrients and plant growth-promoting molecules, which together lead to increased crop yields [27].

In natural ecosystems, plants represent a huge ecological niche, in which a great diversity of microorganisms is found. The microbial population is spread all over the host, continually interacting with the plant: on the surface of roots, stems and leaves or colonizing its inner tissues [46]. Some of these microbes are deleterious and others are beneficial to plants. Among the latter are plant growth promoting bacteria, able to synthesize substances that enhance plant growth, stimulate plant defenses or promote biocontrol [38].

Sugarcane (*Saccharum officinarum* L.) is grown in more than 120 countries, mainly in Brazil and India [3]. For centuries, Brazilian sugarcane has been cultivated with low N inputs, suggesting a possible interaction between the plant and diazotrophic bacteria [11]. Several genera of diazotrophic, endophytic bacteria were isolated from roots, stems and leaves of sugarcane: *Enterobacter*, *Pantoea*, *Klebsiella*, *Pseudomonas*, *Herbaspirillum*, *Gluconacetobacter*, *Azospirillum* [4,5,28,53]. Biological Nitrogen Fixation (BNF), the reduction of $N_2$ to ammonium, causes great transformations in the Nitrogen cycle. BNF is carried out by prokaryotes only, bacteria and archaea, which include most of the bacterial phylogenetic groups [9]. BNF is extremely important because it plays an effective role in the natural interactions between organisms and is a powerful

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agricultural weapon. Cereals grown with N supplies much below their need, associated with diazotrophic bacteria can obtain up to 30% of their N from BNF when fertilized with ample PK and minor elements. The largest effect in this group was obtained with sugarcane, which can obtain up to 150 kg N $ha^{-1}$ from BNF [11]. A considerable fraction of such exchanges involves endophytic $N_2$-fixing bacteria [50], which interact with the host plant cells and tissues according to different degrees of dependence [22]. Due to its characteristics, the use of organic manures together with BNF may enhance the benefits to the environment.

To obtain information about the bacteria-host-environment interaction, it is important to consider the number of microorganisms involved in the interaction and to try to learn which mechanisms promote such interaction. Within certain limits, the larger the size of the bacterial population, the greater its contribution to the interaction. Persello-Cartieaux and co-workers [37] demonstrated for *Pseudomonas thivervalensis* that an inoculum of ideal number ($10^5$ CFU $ml^{-1}$) produced favorable morphological changes to colonized plantlets whereas inocula above $10^6$ CFU $ml^{-1}$ caused irreversible damage to plants. Continuous environmental changes have increased the interest in studies about the consequences of such changes for living organisms. Microorganisms that play an important role in nature can be used to evaluate these consequences. The size of a given bacterial population may indicate if certain human practices which impact the environment have positive or negative consequences. One way to estimate viable microbial population sizes in different environments, such as water, plant and animal tissues [21,47] is the Most Probable Number (MPN) [36,39,48,51]. In order to function, endophytic bacteria need to colonize the host plant and their penetration depends on appropriate entry mechanisms. One such mechanism allowing for the active penetration of endophytic bacteria into plant tissues involves the use of hydrolytic enzymes such as pectinase and cellulase [43]. Pectinolytic and cellulolytic enzymes are produced by a number of endophytic bacteria such as *Azoarcus* sp. [19] and *Klebsiella oxytoca* [26].

The aim of this study was to quantitatively compare the culturable endophytic nitrogen-fixing bacteria populations in sugarcane submitted to organic, conventional or no fertilization, to detect the presence of the *nifH* gene in those bacteria and to asses their production of the enzymes used to penetrate the plant.

## 2. Material and methods

### 2.1. Sugarcane

*Saccharum sp* (variety SP801816) grown under the following conditions: organic fertilization (ORG-F), conventional fertilization (CONV-F) and no fertilization, or control (NO-F), hereinafter designated by the letters indicated in parentheses. Sugarcane was grown in Fazenda São Francisco, in Sertãozinho, São Paulo State (latitude 21°10′; longitude 48°07′). Three adjacent areas, one for each type of fertilization, measuring five square kilometers of purple latossoil were chosen as the collection site. Soil pH was close to neutral, between 6.0 and 7.0. The plants were 10 months old and 2.5 m high at the beginning of the experiment. Three individual plants, having been submitted to each type of fertilization, were collected randomly from each experiment area every month, from March to October, 2006.

Sugarcane was fertilized once, immediately after cutting the preceding crop. For organic fertilization (ORG-F) 100 $m^3$ $ha^{-1}$ of vinasse containing 80 kg of N e 300 kg of K were used. For conventional fertilization (CONV-F) 465 kg $ha^{-1}$ of NPK fertilizer 20-0-20 (93 kg $ha^{-1}$ of N and K). Organic fertilizer is certified by international agencies as: Farm Verified Organic, Inc, North Dakota, USA, accredited for IFOAM (1997); International Federation of Organic Agriculture Movements – by ECOCERT International, certify agency Franc-German, accredited by European Community (1999); and by the International Certification Services of Japan (2000). Sugarcane organic treatment consists of: the filter cake (residues from sugar), vinasse (residues from the alcohol distillery), phosphate rock and basaltic rock.

### 2.2. Microorganisms

Culturable Diazotrophic Endophytic Bacteria (CDEB) from sugarcane (*Saccharum* sp.). Root, stem and leaves of sugarcane plants were analyzed in order to determine the number of diazotrophic endophytic bacteria.

### 2.3. Culture media

Two synthetic semisolid media, deprived of combined nitrogen source, were tested to grow diazotrophic endophytic bacteria: LGI-P [44] and NFb [10].

### 2.4. Plant disinfection and CDEB enumeration

Samples of roots, stems and leaves weighing 3 g were washed in tap water. Using a sterilized punch, the inner section of the stem was withdrawn to avoid contamination. Roots and leaves were disinfected externally [2]. To perform the control of disinfection, samples of leaves and roots with cut tips were closed with paraffin to isolate the internal organ content, sealing the endophytic bacteria inside the organ. These materials were incubated for 24 h at 30 °C, in a nutrient broth. In case of microbial growth, all the study material was discarded. After the disinfection, leaves and roots were floated in distilled sterile water (1:10 p/v) and processed for 1 min in a Warring blender. Samples of stem removed from the plant were macerated in distilled sterile water (dilution 1:10) with sterile mortar and pestle. The extracts of organs were submitted to decimal dilutions to obtain the suspensions with decreased concentrations of bacteria. Preliminary tests were performed to determine the most adequate incubation period and to determine if MPN should be obtained through culture growth (turbidity) or through ARA. The cultures were incubated during 2, 3, 4, 5, 7, 14 and 21 days. In the 21 days incubation test, fresh medium was added to the cultures, according to the Villemin and co-workers method [57].

### 2.5. Most probable number technique (MPN)

Thirty five 10 ml vials, containing four milliliters of LGI-P and NFb media, were inoculated with 250 μl of sequenced dilutions ($10^{-1}$ to $10^{-7}$) of triturated organs. The vials were incubated for 5 days at 30 °C. The MPN was estimated by consulting the McCrady table [29]. The MPN of CDEB was estimated during eight months in different plant organs submitted to organic, conventional and no fertilization, during the growing season.

### 2.6. Acetylene reduction assay (ARA)

Nitrogenase activity by ARA was performed following Anderson and co-workers [1]. The bacterial cultures with acetylene 10% in the air phase were incubated without shaking at 30 °C, for 72 h.

Samples of vials presenting positive ARA were spread on LGI-P solid medium. Colonies with different characteristics (e.g. color, size, mucosity, rough or smooth edge) were isolated and again tested for their ability to reduce acetylene and grown in medium LGI-P liquid for DNA extraction in order to look for the presence of the *nifH* gene and to test for pectinase and one type of cellulase, the endoglucanase.