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Differential niche occupation and the biotechnological potential of *Methylobacterium* species associated with sugarcane plants

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This work highlighted a putative link between the physiological activity and genetic diversity of *Methylobacterium* species and the association with sugarcane roots and rhizoplane. In total, 40 isolates previously described as pink-pigmented facultative methylotrophic bacteria (*PPFMs*), were evaluated for their ability to fix nitrogen and solubilize inorganic phosphate, amylase and pectinase activity. This *in vitro* potential was positively correlated with the community isolated from the root tissues than those from the rhizoplane. Regarding the genomic fingerprinting, the (BOX-PCR) approach revealed a low similarity among the isolates, occurring sole 7 haplotypes harboring more than 70% of similarity among band patterns. These results revealed that the genomic fingerprinting of the isolates recovery from roots is different from the rhizoplane. Besides that, these haplotypes occurred on both sugarcane varieties. Using a phylogenetic sequencing approach based on the 16S rRNA gene, we observed a high abundance of sequences similar to *Methylobacterium radiotolerans* colonizing both plant tissue and sugarcane varieties were observed. Hence, it was suggested that the plant should select those *Methylobacterium* spp. with a high biotechnological potential to promote plant growth. Therefore, the bioprospection of specific endophytic bacterial groups comprise an important source of biotechnological potential to improve sugarcane growth and production.

Key words: Pink-pigmented facultative methylotrophic bacteria, plant growth promotion, BOX-PCR, 16S rDNA, *Methylobacterium radiotolerans*.

INTRODUCTION

Sugarcane is one of the main agricultural products in the Brazilian market, principally related to the production of sugar and ethanol (Unica, 2017). The intense agricultural practices in soil planted with sugarcane have raised

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public concerns over the dynamic of chemical, physical and biological factors on soil and its consequent impacts in plant development and production (Galdos et al., 2009; Stirling et al., 2016; Bordonal et al., 2018). In order to overcome this problem, some researchers have suggested the use of microorganisms associated with plant as a sustainable alternative that could reduce the environmental impact on the ecosystem caused by agrochemicals and then improve plant growth promotion (Ambrosini et al., 2015; Majumder et al., 2016; Oliveira et al., 2017; Leite et al., 2018).

The plants are colonized by a myriad diversity of microorganisms inhabiting the inner tissues of the plants and the plant surface such as rizoplane and phylloplane. They can be characterized as beneficial when these microbes harbor important functions related to plant growth and development. During the last decades, the evolution of the *in vitro* cultivation drove many authors to isolate a high diversity of beneficial microorganisms directly from the environment and associated to host (Rodrigues et al., 2018; Batista et al., 2018). The necessity to understand the mechanisms involved in the interaction between plant and bacteria and the biotechnological potentials driving the sustainable crop cultivation, leads the bioprospection of specific microbial groups that develop a close interaction with the plant tissues (Dourado et al., 2012; Batista et al., 2016).

One of the most important microorganisms is the one that belongs to the genus *Methylobacterium*. These microbes are classified in the α-Proteobacteria sub-class, a group of bacteria known as pink-pigmented facultative methylotrophs (PPFMs), which can grow on single compounds such as formaldehyde, methylamine and methanol compounds. Some authors have shown that this ability is an evolutionary advantage for survival of this genus, in order to avoid competition in the soil and rapidly colonize the plant (Ardanov et al., 2015). The PPFMs were reported to distribute ubiquitously in association with many plant species either epiphytically or endophytically (Dourado et al., 2012). In addition, some authors demonstrated that members of this group harbor a strong symbiotic interaction with the plant, showing an ability to promote its growth (Chistoserdova et al., 2003), by direct ways related to nutrient availability or phytohormones production or by indirect ways inducing systemic resistance to plants and controlling pathogens (Dourado et al., 2015). For example, *Methylobacterium nodulans* (Sy et al., 2001; Jourand et al., 2005) and *Methylobacterium* sp. suggested as a new species (Raja et al., 2006) have been reported to have the ability to form nodules and fix atmospheric nitrogen. In addition, another species such as *Methylobacterium radiotolerans* (Madhaiyan et al., 2015) have been reported to have the ability to fix nitrogen when associated with plants. Ardanov et al. (2012) demonstrated that when strains of *Methylobacterium* spp. were inoculated in potato plants at high density and then, they observed the biocontrol of the pathogen *Pectobacterium atrosepticum*. Madhaiyan et al. (2005) described the ability of *Methylobacterium extorquens* strains to promote plant growth when associated with the leaves of *Saccharum officinarum* L, through atmospheric nitrogen fixation. Marx et al. (2012) depicted the complete genome of six strains of *Methylobacterium* spp. and showed that those strains harbor some key gene cluster related to atmospheric nitrogen fixation, plant nodulation, radio resistance, endophyte colonization, and chlorometane degradation.

Besides all this knowledge regarding the association of this specific bacterial group and plants, Dourado et al. (2012) cited that there is a less diversity of the genus *Methylobacterium* associated with sugarcane when compared with five other plant host. In addition to this, these authors performed a crossed study of the 16S rRNA and *mxaF* genes and observed that all the strains associated with sugarcane were similar to uncultured methylotrophic bacterium or *Methylobacterium* spp. suggesting a possible outcome of the reduced number of microorganisms sequenced and deposited in the database such as GenBank. Hence, these results rose perspectives to bioprospecting *Methylobacterium* strains associated with sugarcane and its high biotechnological potential and ability to rapidly colonize the plant tissues (Hardoim et al., 2008).

Therefore, bioprospecting microorganisms belonging to the genus *Methylobacterium* associated with sugarcane, might be an important mechanism for plant growth promotion and might comprise an opportunity of sustainable agriculture decreasing the environment pollution, principally in Brazilian regions were the use of chemical fertilization become critical expensive.

This lack of knowledge, leads the authors of this work, to isolate bacteria of the genus *Methylobacterium* from two different varieties and niche in plant. In addition, analyze its *in vitro* biotechnological potential and diversity within the genus when associated with the rhizoplane and roots of two sugarcane varieties.

Then, it was hypothesized that the biotechnological potential characteristics of plant growth promotion, the genomic profile and the taxonomical properties, might be crucial clues to raise insights in the recruitment and association of sugarcane plants and bacteria within the genus *Methylobacterium*.

MATERIALS AND METHODS

Plant samples

The sugarcane plants were obtained from the Sugarcane Experimental Station Carpina (EECAC) (latitude 7° 50' 51.87'' S and longitude 35° 14' 19.17'' W) at the Federal Rural University of Pernambuco (UFRPE), cultivated in a dystrocohesive Yellow Argisol, according to Santos et al. (2013), corresponding to Ultisol (Soil Survey Staff, 1998). The two sugarcane varieties RB 92579 (medium maturation) and RB 867515 (late maturation), were cultivated in distinct plots however under the same soil type and climatic conditions. To avoid any bias of the border effect, healthy

plants were sampled at the middle of the plots. The distance between the three replicates was 5 m. Six plants (10 months of growth) were sampled and taken to the Laboratory of Genetics and Microbial Biotechnology (LGBM), Academic Unit of Garanhuns (UAG/UFRPE) to perform further analyses. In total, twelve samples comprising two distinct varieties, two niche in the plant (rhizoplane and root) and three plants per sample were obtained.

Bacterial assessment and culturing conditions

The roots of each plant were separated, washed and cut into small fragments of 1 cm, approximately. Thus, 3 g of these small roots fragments were mashed in 500 ml of phosphate buffered saline (PBS) solution. Then, 25 g of glass pearls (0.1 cm diameter) were added and the solutions maintained under agitation for 60 min (28°C). The solutions were inoculated following serial dilutions in MMS solid medium supplemented with Cercobyn 700 (50 gL^{-1}). The methylotrophic bacteria communities were cultured, utilizing a specific medium (methanol and minerals salts, MMS) according to Jayashree et al. (2011).

Endophytic methylotrophic bacteria was obtained using serially washing approach; 1 min in 70% ethanol, 3 min in sodium hypochlorite solution (2% available Cl^-), 30 s in 70% ethanol and two rinses in sterilized distilled water. The disinfection process was checked by plating aliquots of sterile water used in the final rinse, onto 10% trypticase soy agar (TSA) supplemented with Cercobyn 700 (50 gL^{-1}) and incubating the plates at 28°C for 2 to 15 days. Then, the tissues were cut aseptically into small fragments of about 2 cm and macerated in 10 ml of PBS using crucibles and pestles. The material was transferred to 15 ml tubes and incubated under agitation (120 rpm) at 28°C for 1 h. Serial dilutions in PBS were inoculated on dish plates containing solid MMS medium supplemented with Cercobyn 700 (50 gL^{-1}). The plates were incubated at 28°C and evaluated after 15 days. The PPFM bacteria population density was quantified by counting the pink colonies forming units per gram of fresh weight of each plant tissue ($CFU.g^{-1}$ fresh weight). In total, four plates (replicates) by each sample was used for statistical analyses (Azevedo et al., 2000).

After incubation and colonies counting, 40 pink pigmented colonies were picked off the plates by random and inoculated on a new 10% TSA agar culture medium, incubated at 28°C for 2 days, and were stored at 4°C. In addition, these colonies were also cultivated on 10% TSA, incubated at 28°C for 18 h, suspended in 20% glycerol solution and stored at -80°C.

Screening for PPFM bacteria able to fix nitrogen *in vitro*

The ability to *in vitro* fix nitrogen was tested where each strain was seeded in semi-solid medium (BNF) and incubated at 28°C for 10 days. The experiments were performed independently and in triplicate. The positive results were characterized by the presence of pellicles within the culture medium. In addition, the strains were re-inoculated in the BNF medium to avoid any residual growth or false positives (data not shown). The test included a positive control, the bacterial strain EN303, and *Pseudomonas oryzihabitans* (Kuklinsky et al., 2004). This test is still a widely-used approach to perform bacteria screening for *in vitro* physiological potential, as assumed by many authors in recent works (Quecine et al., 2012; Oliveira et al., 2017; Batista et al., 2018; Leite et al., 2018; Rodriguez et al., 2018).

Screening for inorganic phosphate-solubilizing endophytic and epiphytic bacteria

The assessment of the potential to solubilize inorganic phosphate by PPFM bacteria was carried out in solid medium supplemented with $CaHPO_4$ (Verma et al., 2001). Further, the test included a positive control, the bacterial strain EN303, and *P. oryzihabitans*. Then, the plates were incubated at 28°C for 20 days and evaluated every two days. The experiment was conducted in three rounds. The potential to solubilize $CaHPO_4$ *in vitro* was measured according to Berraquero et al. (1976).

Screening the potential to produce extracellular enzymes *in vitro*

The amylase activity was analyzed according to Stamford et al. (2001). Briefly, the strains were inoculated in a solid medium containing starch 1% (w/w), pH 7.3. The plates were incubated for 72 h at 28°C. Then, the plates were flooded with an iodine solution (1%) for 10 min. The plate was washed with a saline solution to visualize the degradation of halo beyond the colonies.

The pectinolytic activity was determined by inoculating isolated strains in a culture medium containing the following: $(NH_4)_2SO_4$ (2.0 g/L), K_2HPO_4 (4.0 g/L), Na_2HPO_4 (60 g/L), $FeSO_4.7H_2O$ (0.2 g/L), $CaCl_2$ (1.0 mg/L), H_3BO_3 (10 µg/L), yeast extract (1.0 g/L), citric pectin (5.0 g/L) and agar (15 g/L). This medium was adjusted to a specific pH condition (8.0) in order to observe the activity of pectin methylesterase. The dish plates were incubated for 72 h at 28°C. After the bacterial growth, the plates were flooded with a Lugol solution and maintained for 10 min to observe the halo around the colonies. The potential to produce extracellular enzymes in the solid medium was evaluated through an enzymatic index (Ceska et al., 1971; Alves et al., 2002; Carrim et al., 2006).

DNA isolation

The bacterial strains were cultured from isolated colonies in 5 ml of the liquid medium TSA for 48 h under 120 rpm at 28°C. After the period of culture growth and the multiplication of PPMF bacteria, 4 ml were centrifuged at 12,000 rpm for 5 min. The precipitate was re-suspended in 500 µL of extraction buffer. Then, a commercial kit was used for the bacterial genomic DNA extraction (Genomic DNA Purification Kit, Fermentas) according to the manufacturer's instructions.

Amplification with BOX-PCR primers and sequencing of the 16S rRNA gene from PPFM strains

The isolates were submitted to the genome profiling technique BOX-PCR (Rademaker and de Brujin, 1997). The BOX-PCR was performed using approximately 5 ng of genomic DNA from each isolate added to a PCR reaction containing the primer BOX-A1R (5'-CTACGGCAAGGCGACGCTGACG-3'). The resulting amplicons were separated on a 2% agarose gel. Afterwards, the gel was stained with ethidium bromide and observed under UV light. The clustering of isolates was performed based on the BOX-PCR band's profile matrix obtained using an ImageQuant TL Unidimensional software (Amersham Biosciences, UK, v2003). This matrix was used to compare and cluster the samples by unweighted pair group method using arithmetic averages (UPGMA) based on the "Jaccard" algorithm.

A sub-sample of 15 strains was selected from the grouping patterns of the BOX-PCR cluster and submitted to a polymerase chain reaction (PCR). The reaction was performed in 25 µl final volume containing 1 µl (0.5 to 10.0 ng) of total DNA, 0.2 mM of P27F primer (5'- GAGAGTTTGATCCTGGCTCAG-3'), 0.2 mM of 1492R primer (5'-TACGGYTACCTTGTTACGACT -3') (Lane, 1991), 0.2 mM of each dNTP, 0.02 $mg.ml^{-1}$ BSA, 3.75 mM $MgCl_2$ and 0.05 U of Taq DNA polymerase (Fermentas). The reaction was

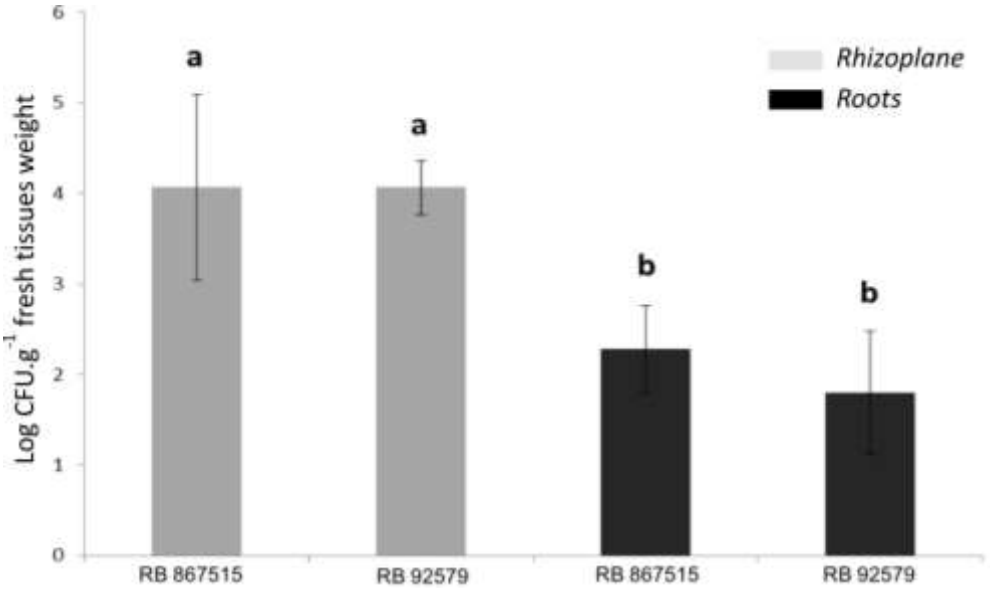

Figure 1. Abundance of facultative methylotrophic pink-pigmented bacteria over the experiments variables, such as cultivar (RB 86-7515 and RB 92-579). Light gray represent rhizoplane count of CFU fw plant tissues^{-1} and dark gray represent roots count of CFU fw plant tissues^{-1}.

subjected to a temperature-controlled thermal cycler performing an initial denaturation at 94°C for 4 min, 35 additional cycles of denaturation at 94°C for 30 s each, annealing at 63°C for 1 min and primer extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. After amplification, the PCR products were visualized by agarose gel electrophoresis (1.5% w/v) in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA).

The PCR products were purified using a Super Charger Switch Kit and Sanger sequenced using the 1387R primer (Heuer et al., 1997). Analyses of sequences were performed with the basic sequence alignment BLAST program, which was run against the database on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST). The reference sequences were aligned using MEGA 7 and based on UPGMA and neighbor-joining algorithm (Saitou and Nei, 1987), the results were plotted in a phylogenetic tree. The nucleotide sequences of each strain obtained in this study have been submitted to the GenBank and were assigned accession numbers as listed from KX830817 to KX830831.

Statistical analyses

The plating counting results were evaluated by an analysis of variance under a significance of 95%, followed by a Tukey test. A semi-quantitative analysis was performed to show the relative frequency of isolates able to fix atmospheric nitrogen, solubilize phosphate and produce extracellular activity in the culture medium. Afterward, through an exploratory approach, a principal component analyses biplot (PCAs) provided an overview of the correlation between the *in vitro* biotechnological potential of the isolates and the environmental variables of plant tissues (root or rhizoplane) and sugarcane varieties (RB-86 7515 or RB-92 579). The significance of these treatments (Niche and Varieties) on the distribution of the samples was tested using a PERMANOVA. These tests were performed using the software PAST (Hammer et al., 2001) under 9999 permutations of a Monte Carlo test. Additionally, the quantitative index values from the biotechnological tests were submitted for an analysis of variances, followed by the Tukey test with a significance of 95%. Those tests were performed using the R Statistics Software Package (R team development).

RESULTS AND DISCUSSION

Endophytic and epiphytic PPFMs associated with sugarcane

The methodology described allowed the isolation of PPMF bacteria endophytically and epiphytically associated with sugarcane. Colonies were morphologically differentiated with respect to the texture and morphology as well as the pink pigmentation and the ability to grow in MMS with methanol as the sole source of nutrients (data not shown). The population density of the PPFM bacteria ranged from 10^2 to 10^5 CFU/g (fresh weight) of plant tissues specifically, 10^1 to 10^2 CFU/g (fresh weight) isolated from roots and 10^4 to 10^5 CFU/g (fresh weight) isolated from rhizoplane (Figure 1). No significant difference was observed between the two varieties tested. This result showed a higher abundance of cultivable PPMFs strains colonizing the surface of the sugarcane's roots.

Hardoim et al. (2008) described a pyramidal pathway of plant colonization, suggesting decreased microbial diversity throughout the plant tissues, depending on the specificity or level of the interaction (Toyama et al., 1998; Zhang et al., 2003; Dourado et al., 2012). Therefore, it its suggested that higher amount of methanol released from

the cell disruption from the plant surface such as roots or leaves might attract a higher abundance of PPMFs bacteria, then corroborating the results found in this study (Ardanov et al., 2012).

It has been assumed that the colonization of plant tissues might be a two-way path, where the plant needs to recruit "*bacterial helpers*" to improve its growth and, in return, the bacterial strains can obtain shelter while helping the plant to develop (Ardanov et al., 2012). The association between plant and bacteria has a long evolutionary history and is mediated by many biotic factors such as plant physiological genetics and phenotypes characteristics such as plant species, tissues, physiological state and genetic factors. In addition some authors assume that this interaction depends also on the bacterial characteristics such as the genomic fingerprinting and its biotechnological potentials (Kuklinsky et al., 2004; Costa et al., 2014). The congruence among those features may determine which specific groups of microorganisms inhabit the inner tissues of the plant (Rosenblueth and Martines-Romero, 2006; Cerqueira et al., 2012). These assumptions corroborated the results obtained in this present study. First, a higher abundance in the number of PPMF strains colonizing the surface of the plants as compared to the abundance of strains that were able to colonize the inner tissues of the plant were observed, suggesting that even inside a specific bacterial group there is a high selective pressure of the plant selecting the microbial community colonizing the surface of the plant (Walitang et al., 2017).

In addition, Dourado et al. (2015) found that the community of *Methylobacterium* spp. that was associated endophytically to 6 plant hosts was able to fix atmospheric nitrogen, solubilize phosphate, produce phytohormones, extracellular enzymes and promote the biocontrol of pathogens and induce systemic resistance.

Screening for the *in vitro* potential of PPFMs

Kuklinsky et al. (2004) results also corroborate the results of the present study where they found a higher abundance of bacteria in the rhizoplane as compared to the inner tissues, describing that the rizoplane is characterized as an open-source of the higher amount of carbon compounds released by plants. On the other hand, they showed that when associated endophytically with plants, these microorganisms have a higher capability for biotechnological potentials or beneficial functions such as the solubilization of inorganic phosphate (BNF) and phytohormones production such as indol-acetic-acid (IAA) (Assumpção et al., 2009).

In the present study, the strains were first evaluated for their ability to fix nitrogen in a semi-solid medium (BNF). Overall, the results demonstrated that 83% of the strains were capable of fixing biological nitrogen, showing a horizontal halo within the semi-solid BNF culture medium (data not shown). It was also observed that 17 were isolated from roots and 16 from rhizoplane. All the strains isolated from roots variety RB 92-579 fix atmospheric nitrogen, while 9 were isolated from rhizoplane. Regarding the variety RB 86-7515, 7 isolates from roots were positive for BNF and all the strains from rhizoplane were positive.

The same bacterial strains were evaluated for their ability to solubilize inorganic phosphate *in vitro* by forming a clear halo around the colony growth. It was observed that from 40 isolates, 33% were positive for phosphate solubilization. In this context the majority of the positive (10 strains), were isolated from inside the roots tissues, from both varieties. Further, a semi-quantitative analysis was performed over the index of solubilization. The solubilization indexes of inorganic $CaHPO_4$ ranged from 0.33 to 2.23. The statistically higher indexes were produced by the strains UAGM2 = 1.50, UAGM3 = 1.48, UAGM7 = 1.42, UAGM54 = 1.40, UAGM62 = 1.45, UAGM69 = 2.23, UAGM91 = 2.22 and UAGM92 = 1.50 (Table 1). According to the mean comparison (Tukey test, $p < 0.05$). These strains solubilize as much phosphate (IS = 2.33) as the positive control EN 303 (*P. oryzihabitans*), which was included in the test (data not shown).

The screening for the ability to produce extracellular enzymes such as amylase demonstrated that 5 strains were able to produce amylase, 3 of which were isolated from root tissues and 2 from rhizoplane. In addition, the enzymatic index of amylase production ranged from 1.43 to 3.01. The strain UAGM 59 (endophytic isolate) was significantly more efficient ($p < 0.05$) than the others strains tested, producing an index of 3.01 (Table 1).

In general, 70% of the strains were able to produce pectin methylesterase (pH 8.0) (Table 1). Interestingly, it was observed that all the strains isolated from the roots tissues were able to produce pectinase, in contrast to the 6 strains from rhizoplane. The enzymatic index ranged from 2.81 to 16.73. The strain UAGM 2 (endophytic isolate) showed the highest enzymatic index ($p < 0.05$; Table 1).

This feature has been described as a crucial mechanism driving the association of plants and microbes (Shameer and Prasad, 2018). Specifically, it can also be described as one advantageous mechanism for some methylotrophic strains. The main source of methanol in plants is the demethylation of the cell-wall pectin by pectin methylesterase (Trotsenko et al., 2001). Jourand et al. (2005) demonstrated that the use of methanol as a substrate for the microbial community might be the key to promote an association between plant and PPFM bacteria. In this context, Omer et al. (2004) showed that the group of strains similar with the genus *Methylobacterium* has an advantage with regard to the colonization of the plant's tissues.

Recent studies have selected those microbes with high biotechnological potential (even greater than the control) and abilities to rapid colonize the plants and multiplicate,

Table 1. Description of the pink pigmented facultative methylotrophic bacterial source of isolation. Semi-quantitative and qualitative analyses of growth promotion potential of pink-pigmented facultative methylotrophic bacteria, over enzymatic production (Amylase-*Amil*; Pectinase in pH 8.0-*Pec-pH 8*; Biological Nitrogen Fixation-*BNF*. Solubilization of inorganic phosphate-*Sol. CaHPO₄*.

Strain	Source of Isolation		Biotecnological potential			
	Niche	Cultivar	Amil	Pec-pH 8	BNF	Sol. $CaPO_4$
UAGM2	Root	RB 86-7515	-	16.73^{a}	+	1.50^{a}
UAGM3	Root	RB 86-7515	-	3.20^{jkl}	-	1.48^{a}
UAGM7	Root	RB 86-7515	-	5.63^{cdefgh}	-	1.42^{a}
UAGM8	Root	RB 86-7515	2.03^{b}	7.10^{bc}	+	-
UAGM11	Root	RB 86-7515	-	5.80^{cdefg}	+	0.96^{b}
UAGM12	Root	RB 86-7515	-	5.04^{dfghi}	+	-
UAGM14	Root	RB 86-7515	-	5.96^{cdef}	-	1.12^{b}
UAGM15	Root	RB 86-7515	-	4.32^{fghijk}	+	-
UAGM16	Root	RB 86-7515	-	5.13^{defghi}	+	-
UAGM20	Root	RB 86-7515	-	4.60^{fghijk}	+	-
UAGM54	Root	RB 92-579	-	6.37^{cde}	+	1.40^{a}
UAGM56	Root	RB 92-579	-	6.49^{cd}	+	0.74^{b}
UAGM57	Root	RB 92-579	-	5.89^{cdefg}	+	-
UAGM59	Root	RB 92-579	3.105^{a}	4.19^{ghijk}	+	-
UAGM62	Root	RB 92-579	-	8.44^{b}	+	1.45^{a}
UAGM64	Root	RB 92-579	-	5.01^{defghi}	+	0.88^{b}
UAGM65	Root	RB 92-579	-	7.34^{bc}	+	-
UAGM66	Root	RB 92-579	1.431^{b}	5.36^{defgh}	+	-
UAGM68	Root	RB 92-579	-	7.16^{bc}	+	1.07^{b}
UAGM99	Root	RB 92-579	-	4.76^{efghij}	+	-
UAGM22	Rizoplane	RB 92-579	-	-	+	-
UAGM23	Rizoplane	RB 92-579	-	-	+	-
UAGM24	Rizoplane	RB 92-579	-	-	+	-
UAGM25	Rizoplane	RB 92-579	-	-	+	-
UAGM26	Rizoplane	RB 92-579	-	-	+	-
UAGM27	Rizoplane	RB 92-579	-	-	-	0.33^{b}
UAGM28	Rizoplane	RB 92-579	-	-	+	-
UAGM33	Rizoplane	RB 92-579	-	3.95^{hijkl}	+	-
UAGM69	Rizoplane	RB 92-579	-		-	2.23^{a}
UAGM71	Rizoplane	RB 86-7515	-	4.60^{fghijk}	+	-
UAGM73	Rizoplane	RB 86-7515	1.82^{b}	3.59^{ijkl}	+	0.61^{b}
UAGM77	Rizoplane	RB 86-7515	-	-	+	0.58^{b}
UAGM80	Rizoplane	RB 92-579	-	3.97^{hijkl}	+	0.79^{b}
UAGM82	Rizoplane	RB 86-7515	-	2.86^{kl}	+	-
UAGM83	Rizoplane	RB 92-579	-	2.81^{kl}	+	-
UAGM86	Rizoplane	RB 92-579	-	-	+	-
UAGM87	Rizoplane	RB 86-7515	-	-	+	-
UAGM91	Rizoplane	RB 92-579	1.95^{b}	-	-	2.22^{a}
UAGM92	Rizoplane	RB 92-579	-	-	-	1.50^{a}
UAGM98	Rizoplane	RB 92-579	-	-	+	0.61^{b}

*To each physiological test a Tukey analysis was perfomed. The letters mean the significance of the statistical test.

to be applied as bioinoculantes in green house and filed conditions as plant growth promoters (Ibort et al., 2018).

The demand for chemical fertilization in agriculture has historically increased as the economy and population growth (Sruthilaxmi and Babu, 2017). At the same stand, the interest for sustainable source of nutrients and factors improving plant growth has increased.

In this context, the bioprospection of microorganisms

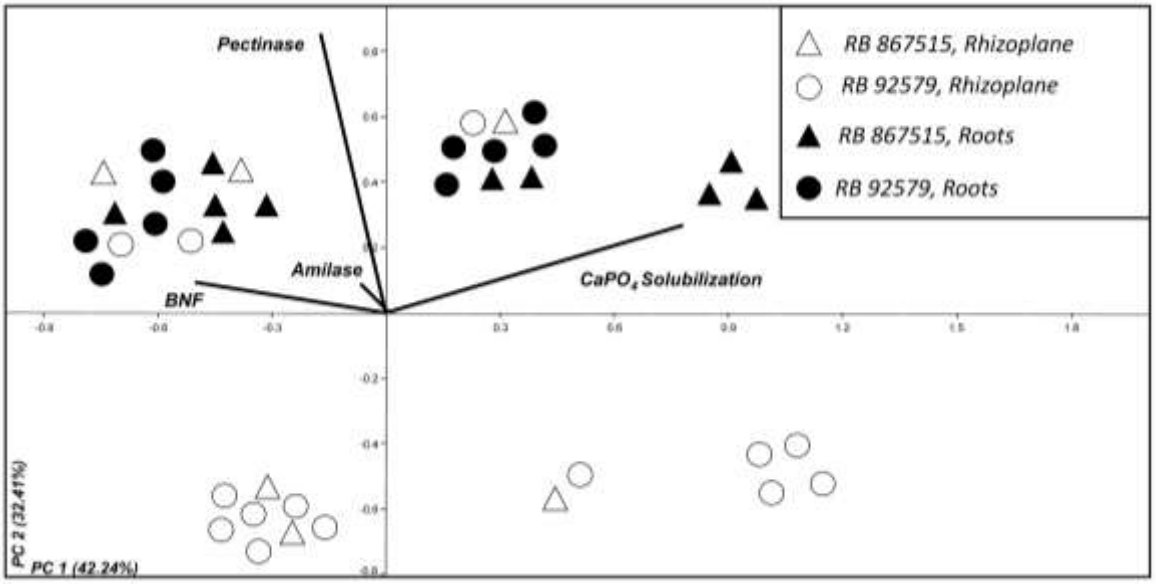

Figure 2. Principal components analyses (PCA), biplot describing the PPMFs strains isolated from roots and rhizoplane of sugarcane plants correlated with the principal plant growth promotion *in vitro* potentials. Bl: The open circles represent the strains isolated form rhizoplane, variety RB 92579. Black circles represent the strains isolated from roots, variety RB 92579; the black triangles represent the strains isolated from the roots, variety RB 867515.

associated with plant has been frequently cited. According to Bashan et al. (2014), a better bio-inoculant is characterized principally by the microbe that has some advantageous mechanisms to colonize the plant and harbor some plant growth promotion characteristics. In this context, it is suggested that the genus *Methylobacterium* associated endophytically to sugarcane might be an important group of microorganisms source for bio-inoculants processes (Senthilkumar and Krishnamoorthy, 2017).

In this context, a principal component analysis (PCA) through an exploratory analysis of these biotechnological potentials was demonstrated and the abilities to promote plant growth (nitrogen fixation, phosphate solubilization and enzymatic production, amylase and pectin methylesterase) were observed to be more correlated with the endophytic strains (Figure 2). Corroborating these last results, the PERMANOVA showed a significant and higher influence on the treatments tested such as niche (Pseudo-F = 22.837; p-value = 0.0001) and varieties (Pseudo-F = 5.741; p-value = 0.0037). In addition, no significant interaction was observed between these factors. The first axis of the PCA explained 42.24% of the variation, indicating a distinction on the type of isolates that is able to fix nitrogen *in vitro*, solubilize phosphate and produce extracellular enzymes. The second axis explained 32.41% of the PCA variation. The biplot explained the distribution of the potential for plant growth promotion of the isolates according to its source of isolation (plant tissues and varieties). Therefore, these results corroborated the present suggestions mentioned.

Genomic fingerprinting and phylogenetic analysis of the PPFM bacteria

In order to get an overview of the genomic diversity among the isolates, the BOX-PCR band profiles demonstrated patterns of genomic fingerprinting among the strains. Firstly, a high genomic diversity was observed consequently at a low similarity (Figure 3).
Fifteen (15) isolates were selected to represent the 30% clusters of the BOX-PCR (Figure 3). Those isolates were subjected to a 16S rRNA gene partial sequencing. The identification of the 15 isolates demonstrated that the PPFMs tested for their *in vitro* potential belong to the genus *Methylobacterium* (Figure 3). In addition, the BLAST alignment results showed a low diversity beyond the sugarcane tissues and surface. This last assumption comes from the results that indicated 10 isolates similar to *M. radiotolerans*, 2 isolates to *Methylobacterium fujisawaense,* 2 isolates to *Methylobacterium indicum* and 1 isolate to *Methylobacterium komagatae* (Table 2); these results comprise the best hits in the NCBI database, with the highest similarity and coverage, then encompassing the lowest e-value, thus, those taxonomical identification came from type strains of already published works. In this context, the phylogenetic tree demonstrated the occurrence of a specific group comprising the strains similar to *M. radiotolerans* (Figure 4).

Finally, clustering the results of BOX-PCR and the sequencing approach, it was observed that there is no genomic similarity among the strains colonizing the rhizoplane and those isolated from inside the roots

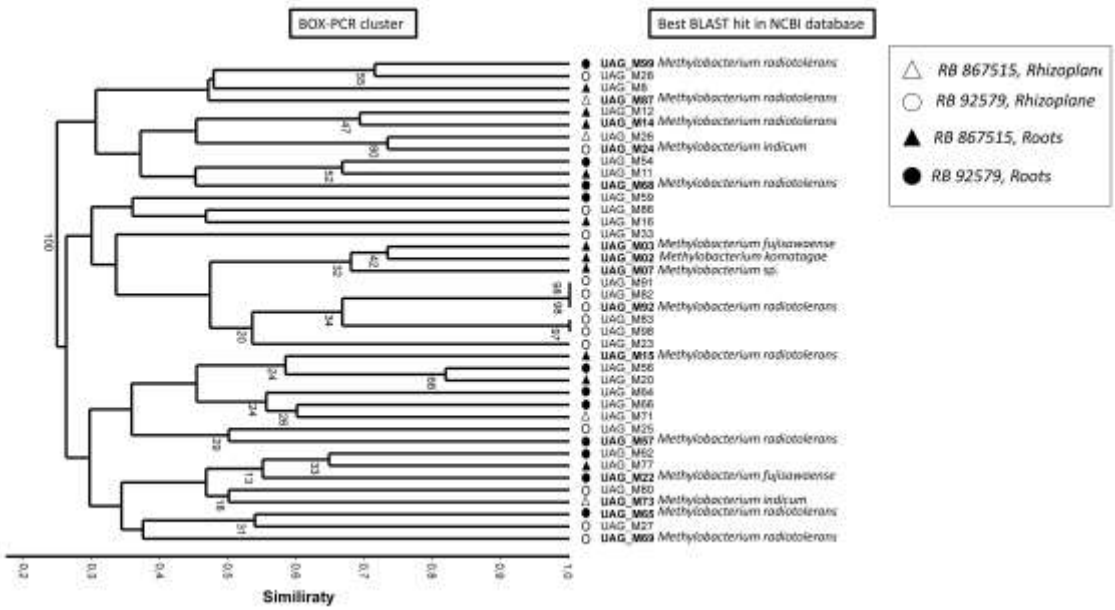

Figure 3. BOX-PCR technique describing the genomic profiling of the PPMFs isolates. The open triangle represents the strains isolated from Rhizoplane, variety RB 867515. The open circles represent the strains isolated form Rhizoplane, variety RB 92579. Black circles represent the strains isolated from Roots, variety RB 92579; the black triangles represent the strains isolated from the Roots, variety RB 867515. Those strains bold marked were selected to a partial sequencing of the 16S rRNA gene. The strains identification comprises the best hit of BLAST against the NCBI database.

Table 2. Genetic characteristics of the pink pigmented methylotrophics bacteria isolated from sugarcane and sequenced of the 16S rRNA gene[a].

Strain	16S rRNA gene			e-value
	Accession N°	Best Hit in NCBI	% Similarity	
UAGM2	AB986547.1	*Methylobacterium komatagae*	99	5×10^{-6}
UAGM3	KT720195	*Methylobacterium fujisawaense*	100	9×10^{-7}
UAGM7	KX022837.1	*Methylobacterium* spp.	100	3.1×10^{-7}
UAGM14	KT336732	*Methylobacterium radiotolerans*	99	4.2×10^{-8}
UAGM15	KT336727	*Methylobacterium radiotolerans*	100	1×10^{-6}
UAGM22	KT720188	*Methylobacterium fujisawaense*	99	1.7×10^{-7}
UAGM24	KP272101	*Methylobacterium indicum*	100	3.1×10^{-5}
UAGM57	KT390763	*Methylobacterium radiotolerans*	99	2.5×10^{-6}
UAGM65	KT336727	*Methylobacterium radiotolerans*	99	9×10^{-8}
UAGM68	KX022837.1	*Methylobacterium* spp.	100	1×10^{-7}
UAGM69	KT336732	*Methylobacterium radiotolerans*	100	5.3×10^{-8}
UAGM73	KP272101	*Methylobacterium indicum*	100	4×10^{-8}
UAGM87	KT336732	*Methylobacterium radiotolerans*	99	5.2×10^{-7}
UAGM92	KT336727	*Methylobacterium radiotolerans*	100	1×10^{-6}
UAGM99	KT390763	*Methylobacterium radiotolerans*	99	4.2×10^{-8}

tissues. However, the phylogenetic analysis revealed that the strains similar to *M. radiotolerans* colonize both plant tissues (roots and rhizoplane) and varieties (RB 867515 and RB 92579).

Previously, Jourand et al. (2005) described a close relationship between strains of *M. nodulans* and *Crotalaria podocarpa*, suggesting that methylotrophic bacteria might have different levels of symbiosis. The

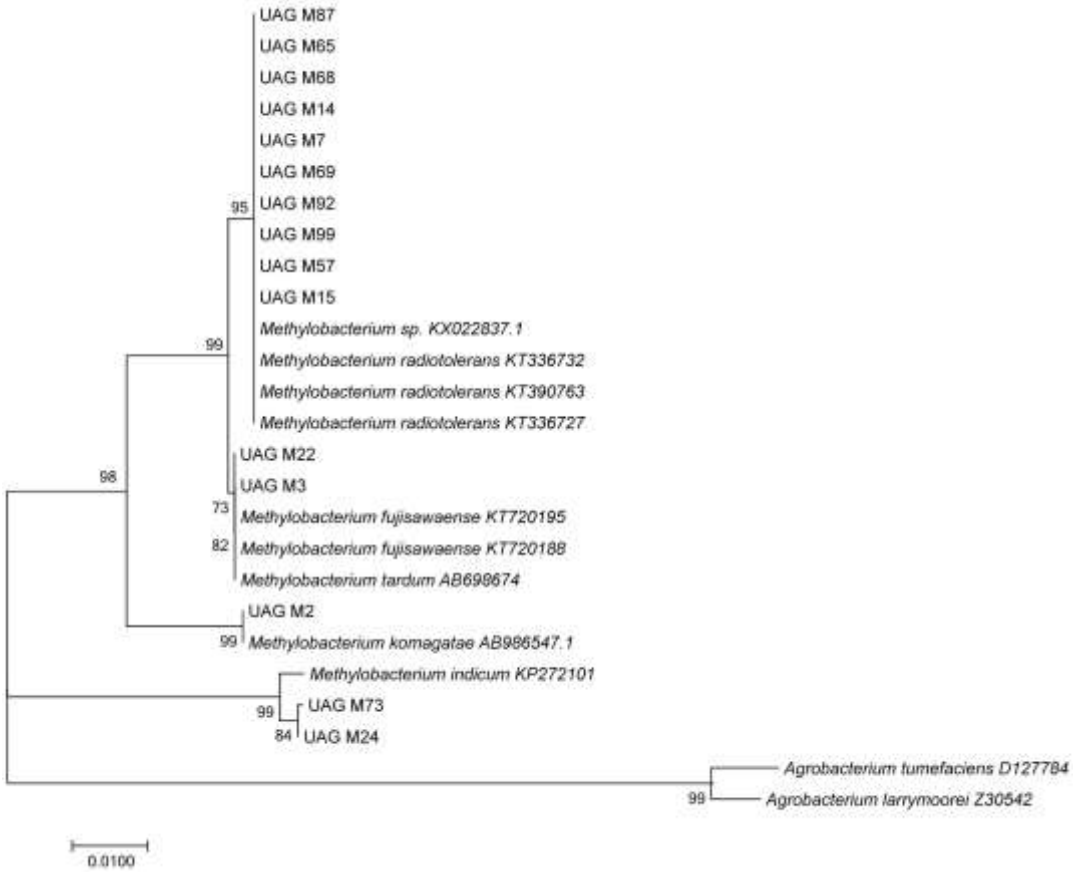

Figure 4. Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method. The percentage taxa clustered together in the bootstrap test (1000 replicates). The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. There were 720 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

authors described that the association must be a very specific and strong plant microbe interaction, considering that the plant exudes specific toxic methylated compounds such as pyrrolizidine alkaloids (Wink and Mohamed, 2003) to select specific and high specialized microbes, in order to prompt the plant development (Sánchez-López et al., 2018). Therefore, the plant's selection of crucial symbiotic microorganisms is based on the methylotrophic abilities of the *Methylobacterium* spp., which might detoxify these compounds and use them as a source of nutrients (Sy et al., 2001). This close relationship has been reported for Rhizobium strains that are resistant to mimosine on the rhizoplane of *Leucaena leucocephala* (Soedarjo et al., 1994). This was also described for *R. etli* in maize (Stamford et al., 2001). Then, relying on an agricultural plant such as sugarcane (Rosenblueth et al., 2004), it was suggested that plant might select a specific diversity of *Methylobacterium* spp. and those strain colonizing the plant tissues are determined by its functionality, particularly in relation to key *in vitro* biotechnological potentials. This provides insight into the mechanisms that might drive the bioprospecting process that leads to the promotion of plant growth.

Conclusion

The main goal of this study was to show that the association between sugarcane and *Methylobacterium* spp. are based on many specific traits; the *in vitro* potentials to promote plant development such as atmospheric nitrogen fixation, phosphate solubilization and extracellular enzyme activity, and specific genomic fingerprints, and not in relation to its taxonomical identity or phylogenetic distance. Hence, those are only some steps in the complete understanding of the interaction between *Methylobacterium* genus and sugarcane. The complete mechanisms that might determine this relationship are yet to be elucidated. For this reason, further genomic studies must be conducted in order to understand these interaction mechanisms between *M. radiotolerans* and sugarcane, and even more, their interaction under greenhouse conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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