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Bacterial biofilm production and water stress resistance by rhizobacteria associated to sugarcane (*Saccharum officinarum*) Linnaeus (POACEAE)

Produção de biofilme bacteriano e resistência à estresse hídrico por rizobactérias associadas à cana-de-açúcar (*Saccharum officinarum*) Linnaeus (POACEAE)

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ABSTRACT: The water restriction is a limiting factor in agricultural production. Some rhizobacteria, to live in symbiosis with the plant, can benefit plant growth. Among the skills of micro-organisms is the ability to survive in extreme environments, such as water stress, as well as having the biofilm production ability. The objective of this study was to evaluate the potential of four bacterial isolates from sugarcane rhizosphere as resistance to water restriction and biofilm production. A qualitative and other quantitative assay to produce biofilm was conducted. The qualitative assay was based on colorimetry with crystal violet. The qualitative test was developed by the violet colorimetric ratio of technical and optical density at 540 nm. Bacterial growth testing in low water activity was developed by inoculating the bacterial isolates in the TSA culture medium (10%) supplemented with sorbitol (405 gL^{-1}) at 28 °C, yielding the corresponding Aw value 0.919. All strains were able to produce biofilms in both tests however isolated *Bacillus subtilis* was most effective, with the greatest biofilm production ratio. All isolates were also skilled in the growing culture medium with low water activity.
KEYWORDS: plant protection, rhizobacteria, plant gowth promotion, symbiosis.

RESUMO: A restrição hídrica é um fator limitante na produção agrícola. Algumas rizobactérias, que vivem em simbiose com a planta, são capazes de beneficiar o crescimento da planta. Entre as habilidades dos micro-organismos está a capacidade de sobreviver em ambientes extremos, como o estresse hídrico, além de possuir a capacidade de produção de biofilme. O objetivo deste estudo foi avaliar o potencial de quatro isolados bacterianos da rizosfera da cana-de-açúcar de resistência à restrição hídrica e produção de biofilme. Foi realizado um ensaio qualitativo e outro quantitativo para a produção de biofilme. O ensaio qualitativo foi baseado em colorimetria com cristal violeta. O teste qualitativo foi desenvolvido pela razão colorimétrica datécnica do cristal violeta na densidade óptica a 540 nm. O teste de crescimento bacteriano em baixa atividade de água foi desenvolvido inoculando os isolados bacterianos no meio de cultura TSA (10%) suplementado com sorbitol (405 gL^{-1}) a 28 °C, produzindo o valor Aw correspondente de 0,919. Todas as cepas foram capazes de produzir biofilmes em ambos os testes, porém o isolado *Bacillus subtilis* foi mais eficaz, com a maior taxa de produção de biofilme. Todos os isolados também foram qualificados no meio de cultura em crescimento com baixa atividade de água.
PALAVRAS-CHAVE: proteção de plantas, rrizobactéria, promoção de crescimento em plantas, simbiose.

INTRODUCTION

On the soil there are many bacteria that are in the rhizosphere, and about 7 to about 15% total surface area of the roots is occupied by these microbial cells (GRAY; SMITH, 2005). Among the micro-organisms that inhabit the rhizosphere, many are beneficial and have functions that benefit plants also soil structure.

Among these microorganisms are the rhizobacteria which can colonize the roots, stimulating them directly or benefiting from the growth and development of various plants. These bacteria are called "Plant Growth-promoting Rhizobacteria" (PGPR) (GYANESHWAR et al., 2001; GRAY; SMITH, 2005; BARRIUSO et al., 2005; KOKALIS-BURELLE; KLOEPPER; REDDY, 2006; SILVA et al., 2019).

A significant factor that should be highlighted since the influence of crop yields is the availability of water and nutrients. Regarding water, the rains do not always meet the actual water requirement of the plants; hence the importance of irrigation which, when well-planned has undoubted economic return.

Water stress is a major cause of crop losses and understands how plants respond to this environmental change is important to strategize and avoid losses. Thus, the use of micro-organisms tolerant to drought and which can protect plants and promoting their growth under drought stresses may be an alternative to this problem (KAVAMURA et al., 2012).

In treated plants with bacteria under stress, Mayak, Tirosh and Glick (2004) found growth stimulus. Liddycoat, Greenberg and Wolyn (2009) observed differential growth of asparagus depending on genotype for one growth was favored by the presence of bacteria only under ideal conditions irrigation, while for other genotypes occurred in favoring water stress situation. The authors demonstrated so a differential response of the interaction between bacteria and plant associated with the water condition.

Bacterial biofilms are multicellular aggregates adhered to a substrate biotic or not embedded in polymer matrices (DANHORN; FUQUA, 2007). They may consist of a single microbial species (e.g., some biofilms associated to infection and biofilm growing on medical implants) or, more often, by various species forming mold consortium, algae, bacteria, and other microorganisms (WIMPENNY; MANZ; SZEWZYK, 2000).

The secondary metabolites production and biofilm formation can be adjusted by population monitoring mechanism or "quorum sensing" (QS) (CHOWDHURY et al., 2009). Through this system unicellular produce signaling substances, known as self-inducers, that freely diffuse across cell membrane to environment. Above a certain concentration, these substances trigger changes in the expression of specific genes. Thus, QS allows a coordinated response of the organism as a function of population density (SILVA et al., 2019).

The QS system is involved in the rhizospheric competence to regulate mobility, tolerance to stresses, horizontal gene transfer, surfactants, antibiotics, extracellular enzymes, and biofilm formation (DECHO et al., 2010).

The biofilm protects the formation when inoculated bacterial population in the soil and seeds and favors the maintenance of the density for beginning beneficial or deleterious interactions between plant and bacteria (DANHORN; FUQUA, 2007).

In the face of what was discussed, the objective of this study was to evaluate the potential formation of bacterial bilfilm on abiotic surface and resistance to fluid restriction to promote plant growth and use of inoculants production programs.

MATERIAL AND METHODS

Bacterial species

Bacterial species used in this study are identified and all sequences are deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/): *Bacillus subitilis* (accession number KT998653.1), *B. megaterium* (accession number KT998652.1), *B. pumilus* (accession number KT998656.1), and *Enterobacter cloaceae* (accession number KT998648.1).

Biofilm formation on abiotic surface

Was inoculated 100 µL grown bacterial isolates (10^6 UFC.mL^{-1}) in TSB (Trypticase Soy Broth) culture medium in microtubes with 900 µL of TSB culture medium with 10% of sucrose. All innocuous were calibrated by the scale of Mac Farland. After incubation at 40 °C for 96 hours, planktonic cells were removed and each microtube was washed twice with distilled water, followed by addition of violet crystal 25 μL 0.1% in each microtube. After fifteen minutes of incubation at room temperature, the microtubes were washed twice with distilled water, one more time (O'TOOLE; KOLTER, 1998).

Biofilm quantification in abiotic surface

Biofilm formation was quantified by the addition of 400 μL of ethyl alcohol (95%) plus 600μL of distilled water, forming a volume of 1000μL in each microtube to solubilize the crystal violet incorporated into the wall. The optical density (OD) of the solubilized dye was determined spectrophotometrically (Biospectro SP-22) at 540 nm.

Growth in medium with low water activity

Bacterial isolates were plated in Petri dishes of 150 mm diameter containing TSA culture medium (10%) supplemented with sorbitol (405 gL^{-1}) at 28 ° C, yielding A_w value corresponding to 0.919 (point which is a minimum water activity for most bacteria).

Statistical analysis

The obtained results were subject to descriptive analysis and percentage of biofilm formation by F test by ASSISTAT (SILVA; AZEVEDO, 2002)

RESULTS AND DISCUSSION

Biofilm formation on abiotic surface

The presence of biofilm on abiotic surface was evidenced by the colorimetric reaction in formation of a violet color (Figure 1).

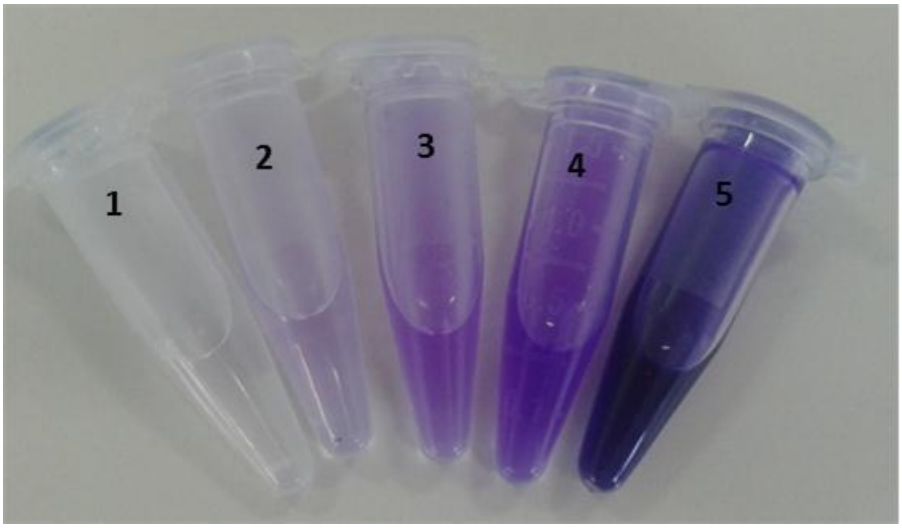

Figure 1. Biofilm formation by bacteria isolated in TSB culture medium containing sucrose, based on absorbance values obtained, DO 540 nm. 1) negative control; 2) DO540nm <0.1 (-) (no formation); 3) DO540nm 0.1-0.2 (+) (low formation); 4) DO540nm 0.2-1.0 (++) (average formation) 5) DO540nm> 1.0 (+++) (high formation) biofilm.

Biofilm quantification in abiotic surface

By quantitative biofilm assay was detected that all bacterial isolates can produce biofilm in abiotic surface, with highest production was detected by the isolate *B. subtilis* (Figure 2).

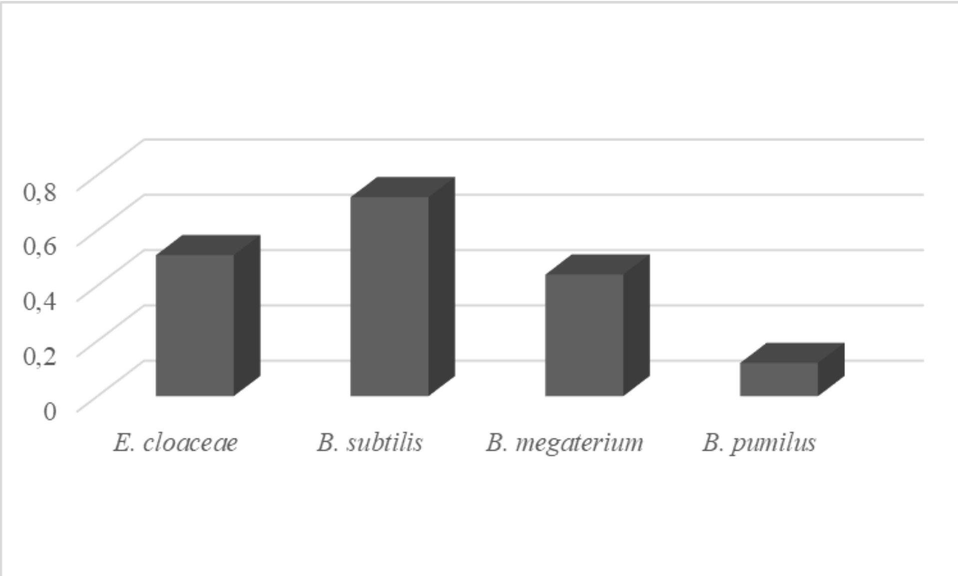

Figure 2. Biofilm formation on abiotic surface by bacterial strains in TSB medium supplemented with 10% sucrose.

Water is a critical component in the metabolism of all living beings and facilitates many vital biological reactions to be solvent, transportation and cooling agent (MUNDREE et al., 2002). The water deficit, cellular dehydration or osmotic stress are terms used in the literature to define the lack of water causes changes in the cell turgor (HMIDA-SAYARI et al., 2005).

The improvement of crops under stress conditions may occur with the use of technologies such as the application of bacteria capable of promoting plant growth and physiological changes beneficial to plants.

The presence of bacteria can lead to production of osmoregulatory substances by the plant and act synergistically, working on drought tolerance. Further, can induce or be mediating tolerance to abiotic stresses such as salinity, drought, floods, very high or low temperatures, nutrient deficiency, and toxic metals (DIMKPA et al., 2009).

The Bacteria can also produce osmotolerant substances such as, for example, glycine betaine which can act synergistically with other plant compounds in reduced water potential of the cells helping in drought tolerance (DIMKPA et al., 2009). Still, osmotolerant bacteria are capable of synthesizing or osmolytes solutes which increase osmotic cytoplasmic osmolarity and give stability to the cells, they are used as inoculants for plants, and appear to positively influence the plants under water stress conditions (YUWONO; HANDAYANI; SOEDARSONO, 2005).

These bacteria can promote growth in dry matter of root and shoot of rice plants under water stress with more significant effects between treated plants and control in most severe water conditions (YUWONO; HANDAYANI; SOEDARSONO, 2005).

Studies with inoculation of bacteria showed that treated plants had higher water potential than the control plants under water stress (CREUS; SUEUDO; BARASSI, 2004), thus guaranteeing advantages in regulating the fluid balance. The inoculation of these bacteria in plants can also alleviate the negative effects caused by stress, ensuring greater photosynthesis and stomatal conductance (HAN; LEE, 2005).

Growth in medium with low water activity

From the bacterial isolates tested in this experiment, they were all capable of growth in medium with low water activity (Aw 0.919, 30 ° C) (Table 1).

Table 1. Growth of bacterial isolates into five days of incubation on medium in TSA (10%) supplemented with sorbitol, producing 0,919Aw value at 28 °C.

Isolate	Growth
E. cloaceae	Positive
B. subitilis	Positive
B. megaterium	Positive
B. pumilus	Positive

Creus, Sueudo and Barassi (2004), studying with wheat plants inoculated with *Azospirillum*, reported that treated plants have a higher moisture, water potential and water fraction apoplastic larger than control plants under stress conditions, occurring ABA also related to anti-stresses signs, especially water stress, stimulates stomatal closure (HARTUNG; SLOVIK 1991).

The proline production can be stimulated in plants in the presence of bacteria in response to biotic and abiotic stresses can mediate the osmotic adjustment and protect membranes and proteins against adverse effects of increasing the concentration of inorganic ions (GROVER et al., 2011).

For Dimkpa et al. (2009), bacteria can mediate changes in the elasticity of the walls of root cells, which would be one of the first steps to improve tolerance to drought stress. Bacteria can also aid in recovery after drought stress, while not preventing water loss during stress, may cause the water levels in the cell as soon return to normal (MAYAK; TIROSH; GLICK, 2004).

According to Morris and Monier (2003) bacteria associated with plants are often viewed gathered in communities called aggregates, microcolonies and biofilm. Bacterial biofilms are a structured community of cells adhered to a substrate biotic or not embedded in a polymeric matrix produced by the bacteria themselves.

The process of formation of biofilms involves adherence of bacteria to the substrate and the aggregation of a cell to the other. Adherence there is participation of fimbriae, adhesins (DAVEY; O'TOOLE, 2000), and DNA molecules that are secreted into the environment (WHITCHURCH et al, 2002). The aggregation and maturation of the biofilm involve increasing the number of cells and deposition of large amounts of extracellular matrix (EPS, proteins, and other substances) to form the skeleton of biofilm (DAVEY, O'TOOLE, 2000). However, the maintenance of normal architecture of these biofilms has proved entirely dependent on the presence of these molecules (DENNY, 1995).

According Xavier and Foster (2007), the biofilm formation occurs within the plant through vertical colonization, in which there is a progressive increase of bacteria occurring growth and dispersion of microbial cells with subsequent increase in the production of biofilm by 10 numerous bacterial species.

Biofilm formation appears to be a widespread feature between rhizobacteria, as all isolated was positive. Biofilm can assist in root colonization during plant growth (RAMEY et al., 2004) and can also protect cells from nutrient deprivation, changes in pH, oxygen free radicals, antibiotics, phagocytosis (JEFFERSON, 2004) and limiting conditions Water (CHANG et al., 2007). Some exopolysaccharides are highly hydrated due to incorporation of water in its structure by hydrogen bonds which could prevent desiccation in some biofilms (FLEMMING; WINGENDER; MAYES, 2000).

Bacterial biofilms are found in many plants as colonization strategy phyllosphere and rhizosphere (KNEE et al., 2001). Most studies on rhizosphere Pseudomonas fluorescens biofilms has approached, used as a biological control agent and *Azospirillum* *brasilense*, bacteria promoting plant growth (FUJISHIGE et al., 2006). These bacteria have intrinsic characteristics that favor the ability to act as promoters of growth, such as competitiveness, saprophytic efficiency, production of antibiotic substances, answer like "quorum sensing" and formation of biofilms (ANDERSEN et al. 2003; SILVA et al., 2019).

Water restriction is a limiting factor in agricultural yield. Some rhizobacteria, to live in symbiosis with plant, can benefit plant growth. All isolates in this study were able to produce biofilm but also to grow in culture medium with low water activity, being promising enforcement officers in agriculture, as an increase in agricultural production. Once the rhizobacteria have affinity with the root system of the plants, they will be drawn through the root exudates flags. Thus, they are capable of conferring protection to plants as water stress, as other biotic and abiotic factors.

CONCLUSION

The isolate *B. subtilis* was the most efficient in biofilm production, however, all isolates showed in this study was able to growth in culture medium with low water activity, which is promising as a growth promoter in cultivated plants.

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