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Application of biopolymers to preserve *Pseudomonas fluorescens* cells and their efficiency in the biological control of *Macrophomina* sp.

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Abstract

Agricultural crops have been increasingly attacked by microorganisms that cause low productivity and severe losses to producers. In this context, chemical products are no longer sufficient to control pathogens, leading to the introduction of biological inoculants that can inhibit unwanted microorganisms. However, these products must be formulated properly to have their useful life guaranteed. In this sense, this study aimed to assess the use of carboxymethylcellulose and xanthan gum biopolymers in the conservation of cells of *Pseudomonas fluorencens*, an effective rhizobacteria in the biological control of the *Macrophomina* sp. fungus, responsible for black rot in different cultures. The inoculant produced from the bacteria fermentation was stored and assessed at 7, 15, 30, 45, 60, 90, 120, 150, 180, and 210 days after adding the biopolymers. The cell culture was then fermented and added with biopolymers, followed by pH measure and CFU mL^{-1} count. Antagonist activity against *Macrophomina* sp. Was assessed through the methods of fungal culture method in the antagonist culture, paired culture, and central risk pairing. Assessments were performed at 24, 48, and 72 hours after incubation in a growth chamber. Percentage of growth inhibition was then calculated and subjected to Tukey test at 5% significance level. According to the results, the biopolymers were able to preserve the *Pseudomonas fluorescens* cells for 210 days and ensure better antagonist potential against the pathogen, corroborating that the market should provide products with longer shelf life.

Keywords: Inoculant; Preservative; Biocontrol; Microorganism.
Abbreviations: BDA_potato dextrose-agar; BOD_biochemical oxygen demand; CCY_casein-casein-yeast; CFU_ colony forming units; CMC_carboxymethylcellulose; COVs_volatile organic compounds; GX_xanthan gum; MGI_percentage of mycelial growth inhibition pH_hydrogen potential; µL_ microliter.

Introduction

Major agricultural crops are often affected by various pests and diseases that cause severe economic damage and losses. Their origin can be bacterial, fungal, viral, or caused by nematodes. In turn, diseases triggered by fungus attack crops from start to finish and represent a major concern to producers, such as *Macrophomina phaseolina*, responsible for causing diseases in more than 500 different cultures, also known as stem or root rot (Marroni and Germani, 2011).

Macrophomina phaseolina fungus also caused a disease known as black rot, whose effect is to reduce stand of plants in the field and quality of seeds. It also induced early maturation and death of plants, generating symptoms such as wilt and necrosis due to blockage of vascular bundles in their structures, secretion of enzymes, and pathogenic toxins (Bellé and Fontana, 2018).

Chemical products are the most widely used pathogen control method, but intense application has generated microbial resistance and environmental contamination, leading to the introduction of biological control using antagonistic microorganisms, marketed as inoculants (Santos and Silva, 2014). The formulation of such inoculants must ensure microbial growth and continuous viability of their cells over the storage period, providing that releasing the number of microorganisms in the product is suitable for symbiosis with the plants and exert their function (Bashan et al., 2014).

Macrophomina phaseolina is a fungus found in the soil and is difficult to control due to their resistance structures, called microsclerotia. The bacterium *Pseudomonas fluorescens* is one of the microorganisms able to control this pathogen due to its capacity to produce several antibiotic compounds (Santos et al., 2010). Antibiosis is the relationship where one or more metabolites produced by a microorganism cause a harmful effect on another, inhibiting germination and growth or inactivating the cell by chemical toxicity (Mariano et al., 2005). Inoculants produced with *P. fluorescens* have

been used as a biocontrol agent due to their versatile metabolism, being found in soil, plants, and water, in addition to its ability to form biofilm (Raza et al., 2016).

Furthermore, the use of products formulated with microorganisms requires additives that protect cells against abiotic stresses. The example is polymers, which are able to limit heat transfer and their good rheological properties, increasing the inoculant useful life (Praveen Biradar and Santhosh, 2018a).

Some polymer-based vehicles have been tested in rhizobacteria, such as xanthan gum and carboxymethylcellulose as adjuvants in *P. fluorescens* (Praveen Biradar and Santhosh, 2018b). Carboxymethylcellulose is used to produce inoculants since it forms an adhesive film that promotes better fixation on leguminous seeds, defending against harmful microorganisms during storage, in addition to providing cell encapsulation and avoiding environmental stresses (Reis and Alves, 2015). In contrast, since it is a biodegradable and accessible biopolymer, which also provides cell encapsulation, xanthan gum can be used to ensure the release of microorganisms into the environment after a longer period (Silva et al., 2009).

Therefore, this study aimed to assess the efficiency of using carboxymethylcellulose polymers and xanthan gum on cell viability and antagonist activity of *Pseudomonas fluorescens* against the pathogen *Macrophomina* sp. at different periods during 210 storage days.

Results and Discussion

UFC mL^{-1} Maintenance during storage

The fermentation of *P. fluorescens* showed a successful multiplication of the microorganism, reaching 6.0x10^{9} CFU mL^{-1} after 96 hours. The culture medium was designed to meet the nutritional needs of the microorganism, usually cultivated in King's B medium, but it does not guarantee long shelf life. According to Biniarz et al. (2018), other possible formulations have been tested to optimize this medium, requiring to develop an alternative medium to supplement the composition of soy molasses, a nutrient-rich component effective for the metabolism of some microorganisms.

Table 1 indicated the efficiency of adding biopolymers to this medium after fermentation at maintaining cell viability, where a the treatments show difference only 7 days after the shelf-life start.

The assessments revealed that the use of xanthan gum ensured good CFU mL^{-1} at all times. Only between 45 and 90 days that the use of carboxymethylcellulose was statistically equal to the treatments without preservation and with xanthan gum. However, after 90 days, it presented higher values of CFU mL^{-1} in relation to the witness.

According to Biniarz et al. (2018) and Scales et al. (2014), the ideal pH for *Pseudomonas* to grow is between 4 and 8; however, it was found that at very low pH, the microorganism is inhibited. Figure 1 shows that pH between 7.3 and 7.5 generated better results in preserving the number of cells.

Gonçalves et al. (2017) observed that the growth of *P. fluorescens* had better performance under pH conditions closer to neutral, which may corroborate the good result from the tested fermentation, as it reached 10^{9} CFU mL^{-1} at pH equal to 7.3.

Trivedi et al. (2005) demonstrated that using alginate in the inoculant formulation with *P. corrugata* ensured superior results in relation to liquid or charcoal-based inoculants for corn plant inoculation. In turn, Taurian et al. (2010) found that adding glycerol to the culture medium preserved the viability of *P. fluorescens* for six months. Such a result can be justified by the findings of Manikandan et al. (2010), who observed that glycerol helps to maintain cells hydrated by retaining more water.

Praveen Biradar and Santhosh (2018b) carried out several tests with different types of polymeric additives in *P. fluorescens* formulation and found that using xanthan gum as an adjuvant generated better results in combination with cell protectors in relation to carboxymethylcellulose.

Antagonist potential analysis

The assessment of the antagonism between *P. fluorescens* and *Macrophomina* sp. showed that the results from using biopolymers to preserve *P. fluorescens* cells were significant in relation to the use of the inoculant without biopolymer addition when analyzing the efficiency in *Macrophomina* sp. biocontrol (Table 2).

Aimed at assessing the biocontrol potential of the bacteria on the fungus, the tests using the fungal culture method on antagonist culture (method 1) showed that all treatments were significantly equal until the 150th day. However, after 210 days, a difference between treatments regarding the witness started to appear by adding the biopolymer. This reinforces the efficiency of using biopolymers to preserve cells and ensure the inhibiting potential of pathogens, since the witness showed an inhibition percentage of the pathogen of only 80.4%, while the treatments with carboxymethylcellulose and xanthan gum continued to ensure 100% inhibition even after 210 days (Figure 2).

In turn, the test using the paired culture (method 2) and pairing with central risk (method 3) methods revealed the antagonist activity of *P. fluorescens* with the biopolymer-based treatments also superior to the witness (Figures 3 and 4).

Table 2 and figures 2, 3 and 4 show that after 210 days, the efficiency of *P. fluorescens* in exerting the antagonist potential decreased, but the use of biopolymers generated significant results. According to Praveen Biradar and Santhosh (2018a), the use of polymeric additives such as cell protectors, adjuvants, surfactants, and preservatives has a significant effect on the viability of *P. fluorescens*, much like the use of xanthan gum and carboxymethylcellulose as adjuvants in this study.

According to Bashan et al. (2014), several potentially beneficial strains found in the literature are not available in the market for not being properly formulated. In this sense, our results reinforce such a scenario, as well as the need to invest in research on biopolymers as to maintain microorganisms viable in various formulations.

Some studies have identified the production of volatile organic compounds (VOCs) in microorganisms. Raza et al. (2016) analyzed the production of VOCs by *P. fluorescens* for biocontrol potential of growth restriction and virulence of *Ralstonia solanacearum*, a pathogenic tomato bacterium, as a safer and cheaper alternative for disease control than agrochemicals. Other studies have reported the production of antimicrobial VOCs by *P. fluorescens*, inhibiting the growth of the phytopathogen *Botrytis cinerea* (Hernández-León et al., 2015).

Wallace et al. (2017) also found the antagonist potential of *P. fluorescens* detected against *Penicillium expansum*.

Table1. Influence of carboxymethylcellulose (CMC) and xanthan gum (XG) use on the *Pseudomonas fluorescens* cells preservation during 210 days of storage at 5 °C [(1)].

Colony Forming Units ($CFUmL^{-1}$)			
Time (days)	witness	CMC	XG
0	$6.0x10^9$ a	$6.0x10^9$ a	$6.0x10^9$ a
7	$5.0x10^8$ c	$1.3x10^9$ b	$6.3x10^9$ a
15	$2.5x10^8$ b	$7.5x10^8$ a	$5.0x10^8$ a
30	$2.0x10^8$ a	$3.6x10^8$ a	$3.9x10^8$ a
45	$1.7x10^8$ b	$2.1x10^8$ ab	$3.7x10^8$ a
60	$1.2x10^8$ b	$2.1x10^8$ ab	$3.7x10^8$ a
90	$1.0x10^8$ b	$1.9x10^8$ ab	$3.4x10^8$ a
120	$9.0x10^7$ b	$1.8x10^8$ a	$2.5x10^8$ a
150	$7.5x10^7$ b	$1.8x10^8$ a	$1.6x10^8$ a
180	$6.5x10^7$ b	$1.7x10^8$ a	$1.3x10^8$ a
210	$5.0x10^7$ b	$1.5x10^8$ a	$1.0x10^8$ a
CV (%)[(2)]		1.75	

[(1)] Means followed by the same lowercase letter, in the lines, do not differ by the Tukey test at 5%. [(2)] CV: Coefficient of Variation.

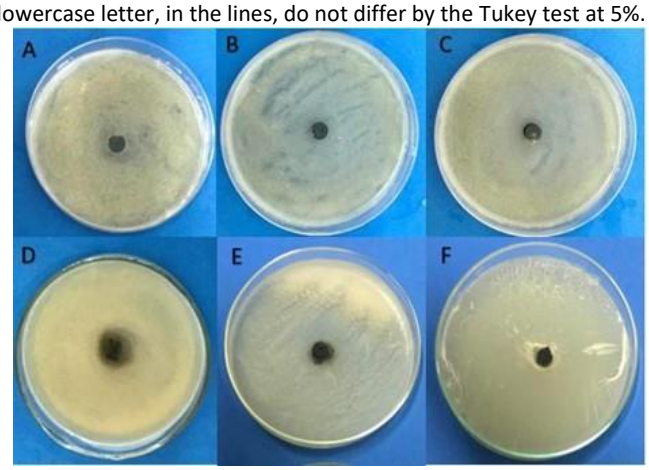

Fig 2. Biocontrol of *Macrophomina* sp. by *Pseudomonas fluorescens* through method 1 with and without addition of biopolymer in 7 and 210 days. Legend: A (7-day witness), B (7-day carboxymethylcellulose), C (7-day xanthan gum), D (210-day witness), E (210-day carboxymethylcellulose), and F (210-day xanthan gum).

Table 2. Efficiency of the use of biopolymers in inhibiting the mycelial growth of *Macrophomina* sp. by *Pseudomonas fluorescens* [(1)].

Inhibition percentage (MGI %)			
Treatment	Method 1	Method 2	Method 3
Time: 7 days			
Witness	100 a	41.12 c	70.33 c
CMC	100 a	52.30 a	77.47 b
XG	100 a	47.66 b	78.35 a
Time: 30 days			
Witness	100 a	33.82 c	61.32 c
CMC	100 a	47.81 a	67.93 b
XG	100 a	42.37 b	72.65 a
Time: 90 days			
Witness	100 a	20.80 c	66.93 c
CMC	100 a	41.66 b	71.72 a
XG	100 a	42.09 a	68.96 b
Time: 150 days			
Witness	100 a	32.77 c	56.54 c
CMC	100 a	37.11 b	62.98 a
XG	100 a	42.41 a	61.98 b
Time: 210 days			
Witness	80,40 b	24.01 c	50.26 c
CMC	100 a	28.44 a	54.40 a
XG	100 a	28.31 b	52.01 b

[(1)]Means followed by the same lowercase letter, in columns, do not differ by the Tukey's test at 5%.

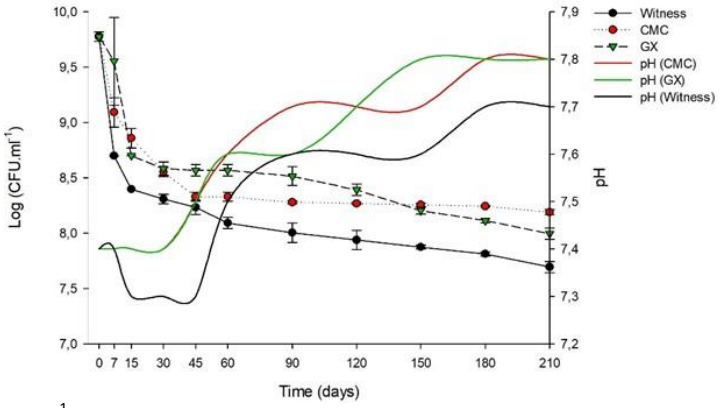

Fig 1. Relation of survival (CFU mL^{-1}) and the hydrogen potential (pH) of *Pseudomonas fluorescens*, during 210 days of storage at 5 °C, using carboxymethylcellulase (CMC) and xanthan gum (GX) biopolymers for conservation.

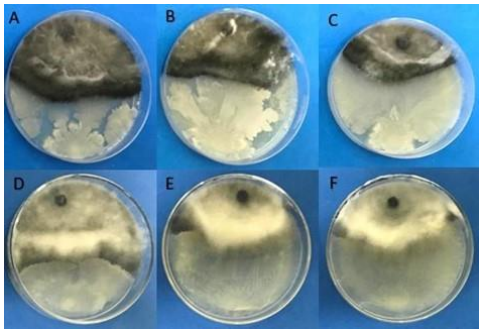

Fig 3. Biocontrol of *Macrophomina* sp. by *Pseudomonas fluorescens* through method 2 with and without addition of biopolymer in 7 and 210 days. Legend: A (7-day witness), B (7-day carboxymethylcellulose), C (7-day xanthan gum), D (210-day witness), E (210-day carboxymethylcellulose), and F (210-day xanthan gum).

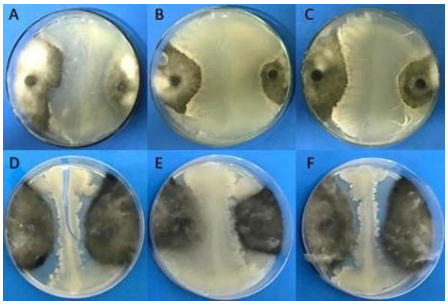

Fig 4. Biocontrol of *Macrophomina* sp. by *Pseudomonas fluorescens* through method 3 with and without addition of biopolymer in 7 and 210 days. Legend: A (7-day witness), B (7-day carboxymethylcellulose), C (7-day xanthan gum), D (210-day witness), E (210-day carboxymethylcellulose), and F (210-day xanthan gum).

The authors reported that the antagonist modes of action include competition for nutrients and space, which appeared in the three methods tested in this research, in addition to producing inhibitory metabolites and biofilm formation. It has also been found that *P. fluorescens* produced antibiosis through diffusible metabolites in agar, controlling sporulation and mycelial growth of *Alternaria ricini* (Silva et al., 1998).

Some biopolymers are used for the formation microcapsules through different processes, but with the function of forming a polymeric matrix, which has advantages in direct inoculation into soil or seeds, reducing predation and competition with native microorganisms, in addition to the release gradation of cells in the soil (Shoebitz et al., 2013). Using compounds with adhesive and emulsion-forming capacity in microorganism formulations for biological control proved effective at protecting cells against desiccation and consequent death. In this sense, our study corroborates the efficiency of using biopolymers to protect cells during the storage period (Green et al., 1998).

Materials and methods

Microorganisms

This study was conducted at the Laboratory of Applied Agromicrobiology and Biotechnology, at the Federal University of Tocantins – Campus of Gurupi.

Macrophomina sp. as the pathogen of choice is justified by its behavior of attacking several cultures, thus requiring control. In turn, the bacterium *Pseudomonas fluorescens* was the alternative of choice to control the pathogen since the biological control of other diseases is based on this microorganism.

We sourced the microorganisms *Pseudomonas fluorescens* and *Macrophomina* sp. from the laboratory strain bank to be stored at 5 °C in conservation tubes with culture mediumand trimmed in a Petri dish with CCY culture environment (0.0222 g L^{-1} L-glutamine, 1.0 g L^{-1} hydrolyzed casein, 1.0 g L^{-1} peptone, 0.4 g L^{-1} yeast extract, 0.6 L L^{-1} glycerin bi-distilled, 0.068 g L^{-1} of $ZnCl_2$, 0.11 g L^{-1} of MgCl, 0.0196 g L^{-1} of $MnCl_2$, 0.0294 g L^{-1} of $CaCl_2$, 0.0134 g L^{-1} of $FeCl_3$, 0.0884 g L^{-1} KH_2PO_4, 0.25 g L^{-1} of K_2HPO_4, 20 g L^{-1} agar) for the bacteria, and BDA (200 g L^{-1} potato, 20 g L^{-1} dextrose, 20 g L^{-1} agar) for the fungus (Abbas et al., 2014; Smith and Onions, 1983).

Inoculant production

The inoculant was produced from the culture medium composed of 5.0 g L^{-1} of peptone, 5.0 g L^{-1} of yeast extract, 3.0 g L^{-1} of sodium chloride, 3.0 g L^{-1} of magnesium sulphate, 0.5 g L^{-1} of potassium sulphate, 16 mL^{-1} of soy molasses. The environment was prepared in a 1 L Erlenmeyer flask, and pH adjusted to 6.0. Sterilization was performed in autoclave at 121 °C for 30 min. After reaching room temperature, the culture medium was inoculated with the bacteria, transferring the strain with the aid of a sterile inoculation loop in a laminar flow cabin. Soon after inoculation, the Erlenmeyer flask was placed in a Shaker incubator (Novatecnica®) at 28°C, and 120 rpm for 96 hours.

The fermentation process to produce the inoculant lasted 96 hours with the *Pseudomonas fluorescens* bacterium initially inoculated into the sterile culture medium, where the microorganism began to consume the nutrients from the culture medium, thereby favoring its multiplication, which occurs in stages. Initially, in the "lag" step, the microorganism undergoes a process of adaptation to the environment, and then in the "log" step, it begins to carry out the cell multiplication process, which continues for a certain period until it reaches the stationary phase. Multiplication speed then decreases until the start of decay stage, when the microorganisms begin to die, followed by the interruption of the fermentation process for the cells to be preserved.

Quantification and pH control

Soon after inoculation, a sample was taken from each Erlenmeyer, the pH was measured and the surface plating was performed using the *Spread Plate* technique (Taylor et al., 1993) to quantify the microorganism in colony-forming units ($CFUmL^{-1}$) at time 0 h, in dilutions 10^4,10^6, and 10^8. The Petri dishes were incubated in a bacteriological incubator at 28 ºC for 72 hours for the colonies to be counted. The same procedure was performed every 24 h to control the fermentation from the samples using a digital pH meter (Hanna Instruments®).

Preparation and addition of biopolymers

Soon after fermentation, the biopolymers carboxymethylcellulose (CMC) and xanthan gum (XG) were added to the inoculant. A preservative solution was prepared separately for each biopolymer by adding 0.1 g of the biopolymer in 100 mL of distilled water, followed by sterilization at 121 ºC for 30 minutes. After cooling the preservative solution, 15 mL were added to 150 mL of fermented inoculum and stored in Falcon tubes at 5 °C. The treatments were then divided into T1 (witness without preservative), T2 (added with carboxymethylcellulose), and T3 (added with xanthan gum).

Assessment of inoculants at different storage times

The treatments were stored at 5 °C to ensure better preservation of the microorganism, and a sample was sourced at each assessing period, as follows: 0, 7, 15, 30, 45, 60, 90, 120, 150, 180, and 210 days.

For each assessment, plating was performed through the Spread Plate technique, in CCY culture medium, at dilutions 10^4,10^6, and 10^8, in triplicate, incubating the plates in a bacteriological incubator at 28 ºC, for 72 hours for the colony to be counted.

The pH of the samples was also measured using a digital pH meter (Hanna Instruments®).

Methods of antagonism by P. fluorescens

The tests were performed to assess the maintenance of the viability and antagonistic activity of *P. fluorescens* cells using biopolymers. Therefore, the tests used the inoculant produced in five different storage times, namely 7, 30, 90, 150, and 210 days.

Three methods assessed the efficiency of using biopolymers in the antagonistic activity of the bacteria.

In the fungal culture method on antagonist culture (method 1), 100 µL of the inoculant were placed and spread using a previously sterilized Drigalski loop on BDA culture medium in a Petri dish. Subsequently, a disk of *Macrophomina* sp. grown for 10 days was added to the dish center.

In the paired culture method (method 2), 10 µL of the inoculant were added to the BDA culture medium with one dish end at 5 mm from the edge, and another end added to a disk with the fungal culture of *Macrophomina* sp. grown for 10 days.

In the central risk matching method (method 3), a scratch divided the dish in the middle using 40 µL of the inoculant, placed using an automatic micropipette. Subsequently, a disk of the fungal culture of *Macrophomina* sp. grown for 10 days was added in each bipartition of the dish at 5 mm from the edge.

The control treatment used only a 10-day-old pathogen disk placed in the center of the dish culture medium. All methods were performed in triplicate. The procedures were followed by incubation in a growth chamber (BOD) under a photoperiod of 12 hours and 28 °C.

Assessments and statistical analysis

For CFU mL^{-1} and pH control, all assessments were performed in triplicate. Data were assessed in double factorial in a completely randomized design: one factor as the culture medium, and another as biopolymers. Subsequently, the results were subjected to analysis of variance and means test (Tukey test at 5% significance level) on the statistical software Rstudio version 1.3. The graphs were generated on the SigmaPlot software, version 12.0.

In turn, the assessments of the antagonism tests were carried out 24, 48, and 72 hours after the tests, measuring the diameter of the pathogens in opposite directions with the aid of a millimeter caliper, defining an average for each colony. The percentage of mycelial growth inhibition (MGI) was calculated using the formula by Meten et al. (1976), where: $MGI = \left[\frac{(\text{Control growth} - \text{Treatment growth})}{\text{Control growth}}\right] x100$. The collected data were subjected to analysis of variance and means test (Tukey test at 5% significance level) on the SISVAR statistical software, version 5.6.

Conclusion

Using carboxymethylcellulose and xanthan gum to preserve *Pseudomonas fluorescens* cells and maintain their antagonistic activity against *Macrophomina* sp. proved efficient. Thus, it represents an alternative to ensure inoculant viability for a period of 210 days, a period over which the bacteria remained at an inhibition percentage of mycelial growth superior to the treatment without biopolymer addition.

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