



## Biochemical characterization of systemic bacteria in bananas, sensitivity to antibiotics and plant phytotoxicity during shoot proliferation

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**ABSTRACT.** The objective of this work was to characterize the biochemically systemic bacterial isolated from banana plants, to evaluate the bacterial sensitivity to antibiotics, and to determine the phytotoxicity of banana shoots during in vitro proliferation. Systemic bacteria belonging to the *Klebsiella* and *Aeromonas* genera were isolated from the “Maravilha” (FHIA 01 AAAB), “Preciosa” (PV 4285 AAAB) and “Thap Maco” (AAB) varieties and were then characterized. Tests of shoot sensitivity to antibiotics were performed, and the minimum inhibitory concentration (MIC) and phytotoxic effects of selected antibiotics to plants were determined. Among the 20 antibiotics evaluated, the strains showed sensitivity to cefaclor, cefalexin, cefalotin, nalidixic acid, chloramphenicol, and vancomycin. However, during MIC determination, the best results were obtained with cefaclor, vancomycin or nalidixic acid alone in concentrations ranging from 512 to 1,024 mg L<sup>-1</sup>. In culture medium, cefaclor at 1,024 mg L<sup>-1</sup> was the only antibiotic to affect the multiplication and the shoot survival in culture.

**Keywords:** *Musa* spp., micropropagation, contamination, endophytic microorganisms, antimicrobial control.

## Caracterização bioquímica de bactérias sistêmicas em bananeiras, sensibilidade a antibióticos e fitotoxicidade de plantas durante a proliferação de brotos

**RESUMO.** O objetivo do trabalho foi caracterizar bioquimicamente bactérias sistêmicas isoladas de plantas de bananeiras, avaliar a sensibilidade das bactérias a antibióticos e determinar a fitotoxicidade de brotos de bananeiras durante a proliferação in vitro. Bactérias sistêmicas pertencentes aos gêneros *Klebsiella* e *Aeromonas* foram isoladas a partir das variedades “Maravilha” (FHIA 01 AAAB), “Preciosa” (PV 4285 AAAB) e “Thap Maco” (AAB), sendo em seguida caracterizadas. Testes de sensibilidade das brotações aos antibióticos foram desenvolvidos e a mínima concentração inibitória (MIC) e os efeitos fitotóxicos dos antibióticos selecionados em relação aos brotos foram determinados. Entre os 20 antibióticos avaliados, verificou-se que as bactérias mostraram sensibilidade para o cefaclor, cefalexina, cefalotina, ácido nalidíxico, cloranfenicol e vancomicina. Entretanto, durante a determinação da MIC os melhores resultados foram obtidos com cefaclor, vancomicina e ácido nalidíxico em concentrações entre 512 a 1.024 mg L<sup>-1</sup>. Em meio de cultura, o cefaclor na concentração de 1.024 mg L<sup>-1</sup> foi o único a afetar a multiplicação e a sobrevivência de brotos em cultivo.

**Palavras-chave:** *Musa* spp., micropropagação, contaminação, microrganismos endofíticos, controle microbiano.

### Introduction

In the culture of plant cells, tissues, and organs, the main reasons for the loss of plant material are contaminations caused by fungi, bacteria, and yeasts. These microbial contaminations in growth media may be attributed to ineffectiveness in the process of explant disinfection or to inefficient aseptic practices in the handling of the culture. However, the greatest evidence of the source of microbial contamination in the multiplication stage relates to endophytic organisms, as these contaminants are not generally eliminated by disinfectant agents because they are

hosted inside the vegetal tissues and are protected from the action of surface disinfectants (Thomas, 2007; Scherwinski-Pereira & Costa, 2010). In the case of bananas, the initial explant for in vitro establishment, and therefore, the raw material used for micropropagation, is usually isolated from apical buds, which are protected from contact with the soil by various layers of tissue (immature leaves), which are eliminated during the sterilization process (Oliveira, Costa, & Scherwinski-Pereira, 2008). However, despite rigorous processes of sterilization of the explants and removal of the outer leaves, these

explants can present high levels of contamination during the establishment and multiplication phases, constituting the major cause of loss of material during micropropagation of the species (Thomas, Swarna, Patil, Prakash, & Rawal, 2008; Thomas & Soly, 2009).

The most practical measure to be taken to prevent the spread of these contaminations, which can lead to total loss of the material under cultivation, is the autoclaving and disposal of the contaminated material. However, in cases where maintenance of the contaminated plant material is necessary, complete control of the contamination is essential (Scherwinski-Pereira, Mattos, & Fortes, 2003).

One alternative for the reduction of contamination problems is the application of curative treatments, and several experiments using antimicrobial substances to complement the action of disinfectants, thereby improving the efficiency during disinfection of the material, have been published (Kulkarni, Kelkar, Watve, & Krishnamurthy, 2007; Mbah & Wakil, 2012; Msogoya, Kanyagha, Mutigitu, & Mamiro, 2012). However, the success of efforts to control microorganisms using substances, in particular those intended for controlling bacteria, the main sources of contamination during large-scale micropropagation, depends on the isolation, identification, and testing of the sensitivity of the bacteria to antibiotics. This is because control of these contaminants is only possible through the use of substances that are within the spectrum of effectiveness against these microorganisms (Thomas et al., 2008; Donnarumma et al., 2011).

During *in vitro* culture, it is also important that the selected antibiotic is effective against the contaminating bacteria without compromising the normal development of the plants. For this reason, it is essential to conduct tests to evaluate the phytotoxicity of the antibiotic on the explants (Mittal, Gosal, Senger, & Kumar, 2009; Grzebelus & Skop, 2014).

Although contaminations during *in vitro* culture are considered by researchers in the field to be the main cause of loss of material, studies in this area have made very few advances in recent years, and much remains to be done, particularly in banana culture, due to the growing demand for micropropagated plantlets.

The objective of this work was to characterize the biochemically systemic bacteria isolated from banana plants, to evaluate the bacterial sensitivity to antibiotics, and to determine the phytotoxicity of banana shoots during *in vitro* proliferation.

## Material and methods

Systemic bacteria were isolated from the propagative material of the Maravilha (FHIA 01 AAAB), Preciosa (PV 4285 AAAB) and Thap Maeo (AAB) varieties, previously established *in vitro* by Oliveira, Costa, and Scherwinski-Pereira (2008). Contaminated material was selected approximately thirty days following establishment, and using a flame-sterilized platinum loop, the contaminants were then transferred individually to Petri dishes containing Nutrient Agar medium (NA) (peptone, 5 g L<sup>-1</sup>; meat extract 3 g L<sup>-1</sup>; glucose, 5 g L<sup>-1</sup>; agar, 15 g L<sup>-1</sup>; pH 7.0 ± 0.2) for the purpose of purification, based on the morphological characteristics of the bacteria, particularly pigmentation, texture, surface, and border. Once inoculated on the NA medium, the bacterial material was incubated at 28 ± 1°C for five days until complete growth of the colonies was observed. The sowing of bacterial material was repeated in new media using the cross-streak method until purification was observed.

Once isolated and purified, the bacteria were initially evaluated in terms of shape, pigmentation, surface, and texture and were then assessed by Gram staining. They were sent to the Fundação André Tosselo (André Tosselo Foundation, Campinas, São Paulo State) to be identified at the level of family, genus, and species by standard biochemical tests (Krieg & Holt, 1994).

Following identification, susceptibility testing of the isolated bacteria was performed using the disk diffusion sensitivity method, by means of paper disks impregnated with twenty different types of antibiotics: cefalexin (30 µg mL<sup>-1</sup>), chloramphenicol (30 µg mL<sup>-1</sup>), streptomycin (10 µg mL<sup>-1</sup>), amikacin (30 µg mL<sup>-1</sup>), ampicillin (10 µg mL<sup>-1</sup>), penicillin (10 µg mL<sup>-1</sup>), rifampicin (5 µg mL<sup>-1</sup>), sulfonamide (300 µg mL<sup>-1</sup>), cefaclor (30 µg mL<sup>-1</sup>), cefotaxime (30 µg mL<sup>-1</sup>), cefoxitin (30 µg mL<sup>-1</sup>), nalidixic acid (30 µg mL<sup>-1</sup>), oxacillin (1 µg mL<sup>-1</sup>), cefalotin (30 µg mL<sup>-1</sup>), vancomycin (30 µg mL<sup>-1</sup>), tetracycline (30 µg mL<sup>-1</sup>), amoxicillin (10 µg mL<sup>-1</sup>), gentamicin (10 µg mL<sup>-1</sup>), erythromycin (15 µg mL<sup>-1</sup>), and novobiocin (5 µg mL<sup>-1</sup>).

To perform the test, a portion of each culture was first transferred to individual Erlenmeyer flasks containing 50 mL of nutrient broth medium (NB) (5 g L<sup>-1</sup> of peptone, 3 g L<sup>-1</sup> of meat extract, 5 g L<sup>-1</sup> of glucose, pH 7.0 ± 0.2, without the addition of agar) and agitated continuously for eighteen hours in an orbital shaker at 100 rpm. After the growth period, an aliquot of 100 µL of each microbial agent was spread over the surface of NA medium contained in Petri dishes using a glass Drigalski loop, followed by

the distribution of paper disks impregnated with the antibiotics. In this phase, the cultures remained under incubation at  $28 \pm 1^\circ\text{C}$  without light, in accordance with the methodology described by Scherwinski-Pereira, Mattos, and Fortes (2003).

The sensitivity of the bacterial isolates to the antibiotics was evaluated for up to forty-eight hours of incubation, as determined by measuring the inhibition zone formed in millimeters. The isolates that presented the formation of an inhibition zone of a minimum of eight millimeters were considered sensitive to the antibiotics tested. In total, six observation units, each made up of one disk impregnated with a specific antibiotic, were evaluated for each isolate, to determine the average inhibition zone. The Petri dishes used to evaluate the sensitivity of the bacterial isolates to the antibiotics were completely randomly arranged during the cultivation.

Having determined the most effective antibiotics, the minimum inhibitory concentration (MIC) was determined using the method proposed by Scherwinski-Pereira et al. (2003). To this end, the isolates were transferred to new NA media and were incubated at  $28^\circ\text{C}$  for a period of eighteen to twenty-four hours. Using a platinum loop, aliquots of the incubated isolates were transferred individually to Erlenmeyer flasks containing 50 mL of a solution of NB medium and were maintained under agitation at 100 rpm for twenty-four hours at a temperature of  $28 \pm 1^\circ\text{C}$ . After this period, 1 mL of the bacterial suspension was removed and transferred to test tubes containing 9 mL of saline solution to perform serial dilutions to obtain the most probable number of cells in a  $10^{-5}$  suspension.

Eleven test tubes with saline solution were prepared in duplicate, and the six antibiotics previously selected in the disk diffusion sensitivity test were added: cefaclor, vancomycin, nalidixic acid, cefalotin, chloramphenicol, and cefalexin. The antibiotics were cold-sterilized by means of filtration (Millipore® 0,22  $\mu\text{m}$ ) and were individually added to test tubes containing 2 mL of solution to obtain a dilution of from  $1/2$  to  $1/1,024$ , corresponding to concentrations of the test antibiotic ranging from 2 to 1,024  $\text{mg L}^{-1}$ . For each treatment, an aliquot of 100  $\mu\text{L}$  of a bacterial suspension ( $10^{-5}$ ) was added, using tubes containing only saline solution as controls.

Subsequently, the tubes were maintained at  $28 \pm 1^\circ\text{C}$  in the dark and under agitation (100 rpm). The turbidity of the media was tested for up to ninety-six hours of incubation. To confirm the results of the inhibition of bacterial growth, aliquots of 100  $\mu\text{L}$  of the clear dilutions of the trial agents were smeared onto Petri dishes containing NA medium, in accordance with the methodology developed by Scherwinski-

Pereira et al. (2003). This process was performed in triplicate, evaluating the growth of colonies for up to seventy-two hours of incubation.

Having determined the MIC, the phytotoxicity of the antibiotics to cultivation was then assessed. Banana shoots of the Preciosa variety, obtained during the in vitro multiplication phase and measuring approximately 1.2 cm, were cultivated in MS (Murashige & Skoog, 1962) medium with the addition of 4  $\text{mg L}^{-1}$  N<sup>6</sup>-benzylaminopurine (BAP). The three most effective antibiotics for bacterial control (nalidixic acid, cefaclor, and vancomycin) were individually added to this growth medium at concentrations of 0 (control), 512, and 1,024  $\text{mg L}^{-1}$ .

As described above, the antibiotics were cold-sterilized using 0.22  $\mu\text{m}$  filters (Millipore®) and were then added to growth medium during the cooling process (40 to  $50^\circ\text{C}$ ). After adding the antibiotics to the medium, the explants were inoculated and kept in a growth room at  $25 \pm 2^\circ\text{C}$ , with a photoperiod of 16 hours and radiation of 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Explant survival percentage, shoot height, and multiplication rate were evaluated in two successive subcultures of thirty days each.

The statistical design used for the test was totally randomized with five replications. The treatments were arranged in a 3 x 3 factorial scheme, with three types of antibiotics tested in three concentrations (0, 512, and 1,024  $\text{mg L}^{-1}$ ) for a total of nine treatments with four explants per batch. The data obtained were submitted for variance analysis, and the averages were compared using Tukey's test at a 5% probability.

## Results and discussion

Although species of the genus *Aeromonas* are often involved in studies focusing on their pathogenicity in humans, and although they most frequently occur in water and fish, there are studies in the literature that claim that soil is the most important reservoir of these species because the soil is where the strains survive for the longest periods of time and are capable of being transmitted through plant material (Janda & Abott, 2010), which may explain their presence in banana culture explants. Bacteria of the species *Klebsiella* are more commonly found in association with banana culture. Braga, Sá, and Mustafá (2001) reported losses of up to 75% of banana explants of the Caipira variety during the establishment phase caused by four different species of bacteria – among them, *Klebsiella*.

Although some mechanisms related to the interaction between endophytic organisms and plants, particularly fruit-producing plants, are still poorly understood, under ex vitro conditions, many

endophytic microorganisms have a beneficial symbiotic relationship with the host plant, which may be related to the production of growth hormones or even biological nitrogen fixation (Ryan, Germaine, Franks, Ryan, & Dowling, 2008). Among these endophytic organisms, bacteria of the genus *Klebsiella* have been routinely used as model organisms for genetic and biochemical studies of biological nitrogen fixation (BNF). Therefore, the presence of a nitrogen-fixing species associated with different banana varieties may be a strong indicator that the banana is a valuable endophytic flora that needs to be further studied, both for its BNF potential and for other possible symbiotic activities.

In terms of morphological and biochemical characteristics, *Aeromonas* colonies are light cream in color, with irregular borders, smooth, shiny surfaces, creamy texture, and characteristic odor (Table 1). The cells are in the form of short rods with convex circular form and are facultative anaerobic, mobile, nonspore-forming, catalase and oxidase positive, urease and Gram negative (Table 2). In plant tissue culture, eradication of this genus is considered to be problematic because it is very resistant to and difficult to eliminate with chlorine-based agents (Sisti, Alabano, & Brandi, 1998), such as hypochlorite, a substance commonly used in the disinfection of explants cultivated in vitro.

**Table 1.** Morphological characterization of the systemic bacteria *Aeromonas hydrophila* and *Klebsiella pneumoniae* isolated from banana plants.

Morphological characteristics	<i>Aeromonas hydrophila</i>	<i>Klebsiella pneumoniae</i>
Color <sup>1</sup>	Light cream	Cream
Shape <sup>1</sup>	Circular and convex	Circular and elevated
Texture	Creamy	Creamy
Motility	+	-
Cellular morphology	rod	Short rod

<sup>1</sup>After 24 hours in nutrient agar at 28°C, pH 7.0 ± 0.2.

Among the morphological and biochemical characteristics of *Klebsiella* colonies are their cream color, elevated circular form, smooth, shiny, regular surface, and creamy texture (Table 1). The cells are

short rods, Gram negative, facultative anaerobic and immotile, without spores, catalase positive, and oxidase negative (Table 3).

According to Scherwinski-Pereira et al. (2003), this genus belongs to the *Enterobacteriaceae* family and is directly linked to high rates of loss of materials during in vitro cultivation.

The two genera isolated and identified in this study both presented negative Gram stain test results, corroborating with the results obtained by Nietzsche et al. (2006), who reported that the highest percentages

of contaminating bacteria found in banana explants from the varieties Prata Anã and SH36-40 were Gram negative, with values of 62 and 57%, respectively.

**Table 2.** Morphological and biochemical characterization of the systemic bacteria *Aeromonas hydrophila* isolated from banana plants.

Morphological and biochemical characteristics	<i>Aeromonas hydrophila</i>
O <sub>2</sub> utilization	Anaerobic facultative
Gram	-
Endospore	-
Catalase	+
Nitrate	+
Hydrolysis	
Arginine	+
Descarboxilation	
Ornithine	-
Oxidase	+
Gelatinase	+
Assimilation of amino acids as carbon sources	
L-proline	+
L-alanine	+
L-histidine	+
DL-lactate	+
Assimilation of fatty acids as carbon sources	
Sodium acetate	+
Gluconic	+
Propionic	-
Heptanoic	-
Aspartic acid	-
Assimilation of sugars and derivatives as carbon sources	
D-mannitol	+
Trehalose	+
Maltotriose	+
N-acetyl-glucosamine	+
Myo-inositol	+
Maltose	+
D-mannose	+
D-galactose	+
Sucrose	+
Glucose	+
Arabinose	-
D-xylose	-
Acid assimilation of β-hydroxy β-methyl butyrate (HMB)	-
Assimilation of carboxylic acids as carbon sources	
Azelaic	-
Sebacic	-
Suberic	-
Adipic	-
Citric	-
Poritaconic	-
Urease	-
Triple-agar sugar iron	+

When the antibiotic sensitivity tests were conducted, the genus *Aeromonas* showed more pronounced sensitivity to the antibiotics cefalotin, chloramphenicol, cefotaxime, nalidixic acid, erythromycin, cefalexin, tetracycline, and cefaclor. The antibiotics most effective in forming the largest halos of inhibition against the genus *Klebsiella* were vancomycin, chloramphenicol, cefotaxime, ceftoxitin, tetracycline, and cefaclor (Table 4). Nogueira et al. (2006), studying microorganisms belonging to the *Enterobacteriaceae*

family, including organisms of the genus *Klebsiella*, also found that they were especially sensitive to cefotaxime.

**Table 3.** Morphological and biochemical characterization of the systemic bacteria *Klebsiella pneumoniae* isolated from banana plants.

Morphological and biochemical characteristics	<i>Klebsiella pneumoniae</i>
O <sub>2</sub> utilization	Anaerobic facultative
Gram	-
Endospore	-
Catalase	+
Nitrate	+
Oxidase	-
H <sub>2</sub> S production	-
Indole production	-
Hydrolysis	
ONPG	+
Esculin	+
Arginine	nr
Glucose oxidation	+
Acid production	
Sucrose	+
Inositol	+
Glucose	+
Raffinose	+
Mannitol	+
Sorbitol	+
Raffinose	+
Lactose	+
Maltose	+
Xylose	+
Ramnose	+
Malonate	+
Adonitol	-
Deamination	
Ornithine	-
Lysine	+
Citrate	+
Descarboxilation	
Arginine	-
pH change from acetamide	-
Fermentation on 2,4,4-trichloro-2-hydroxy-diphenylether	-
Growth in presence of Polymixina B	-
Urease	+
Triple-agar sugar iron	+

These results enabled the selection of antibiotics for use in a subsequent research to determine the minimum inhibitory concentration (MIC) of the contaminants, a step considered fundamental by Scherwinski-Pereira et al. (2003), who stated that the success of procedures with antibiotics for in vitro culture can only be achieved by isolating and identifying the bacteria and performing tests to determine their sensitivity to antimicrobial substances. According to Scherwinski-Pereira and Costa (2010), due to the high cost of treatment and their phytotoxicity, antibiotics should only be used for culture-specific contaminants because only the bacteria within the spectrum of action of each antibiotic will be controlled.

Of the six antibiotics tested, only three inhibited bacterial growth: nalidixic acid, cefaclor, and

vancomycin. However, nalidixic acid was only effective for one of the genera in the study and produced bactericidal effects only in treatments containing the highest concentration tested (1,024 mg L<sup>-1</sup>). Cefaclor and Vancomycin inhibited growth of both isolates at half of this concentration (512 mg L<sup>-1</sup>) (Table 5).

**Table 4.** Culture susceptibility test of the identified bacterial contaminants to different antibiotics.

Antibiotics	Concentration ( $\mu\text{g mL}^{-1}$ )	Inhibition halo (mm)	
		<i>Klebsiella</i>	<i>Aeromonas</i>
Cefalotin	30.0	18.0 ± 1.0s	30.0 ± 1.0s
Gentamicin	10.0	11.0 ± 0.0s	15.0 ± 1.0s
Rifampicin	5.0	10.0 ± 1.0s	16.0 ± 2.0s
Vancomycin	30.0	20.0 ± 4.0s	19.0 ± 2.0s
Penicillin	10.0	0.0 ± 0.0r	13.0 ± 2.0s
Chloramphenicol	30.0	21.0 ± 8.0s	23.0 ± 3.0s
Ampicillin	10.0	6.0 ± 4.0r	6.0 ± 1.0r
Cefotaxime	30.0	29.0 ± 3.0s	27.0 ± 8.0s
Streptomycin	10.0	13.0 ± 1.0s	16.0 ± 1.0s
Novobiocin	5.0	12.0 ± 1.0s	13.0 ± 7.0s
Nalidixic acid	30.0	19.0 ± 1.0s	29.0 ± 1.0s
Amoxicillin	10.0	0.0 ± 0.0r	17.0 ± 1.0s
Erythromycin	15.0	2.0 ± 4.0r	20.0 ± 3.0s
Sulfonamide	300.0	0.0 ± 0.0r	2.0 ± 4.0r
Cefalexin	30.0	17.0 ± 1.0s	31.0 ± 4.0s
Cefoxitin	30.0	26.0 ± 3.0s	19.0 ± 10s
Oxacillin	1.0	8.0 ± 1.0s	12.0 ± 6.0s
Amikacin	30.0	13.0 ± 1.0s	17.0 ± 1.0s
Tetracycline	30.0	20.0 ± 1.0s	21.0 ± 2.0s
Cefaclor	30.0	26.0 ± 2.0s	39.0 ± 4.0s

\*The sensitivity of contaminants was determined after up to 48 hours incubation, determining the inhibition halo size (mm). It were considered susceptible to antibiotics the contaminants that showed the formation of a halo of inhibition of 8 mm according to Scherwinski-Pereira et al. (2003); s = susceptible; r = resistant.

In general, in spite of both isolates presenting sensitivity only at the higher concentrations tested (512 and 1,024 mg L<sup>-1</sup>), the growth of *Klebsiella* was also inhibited in a medium containing Cefaclor at a concentration of 256 mg L<sup>-1</sup>, demonstrating that this microorganism is more susceptible to this product than the genus *Aeromonas*.

During phytotoxicity testing, the survival rates of the propagative material cultivated in vitro were 100%, with phytotoxic effects only being reported in the treatment with 1,024 mg L<sup>-1</sup> Cefaclor, which led to the death of 65% of the initially inoculated material (Table 6). This treatment also produced the lowest rate of multiplication of the material, with an average of approximately 0.2 shoots per explant, a value significantly lower than those of the other treatments, which reached rates between 1.3 and 2.9 shoots per explant. Lima and Moraes (2006) also observed a reduction in multiplication rates when working with banana explants of the Caipira variety and with the antibiotic Rifampicin added to the MS medium, reporting a reduction in the rate over the subcultures from 2.9 shoots per explant to approximately 1.5.

**Table 5.** Minimum inhibitory concentration (MIC) of antibiotics for bacterial strains *Klebsiella* (Kleb) and *Aeromonas* (Aer) isolated from banana during micropropagation.

MBCI (mg L <sup>-1</sup> )	Nalidixic acid		Cefaclor		Cefalexin		Chloramphenicol		Vancomycin		Cefalotin	
	Kleb	Aer	Kleb	Aer	Kleb	Aer	Kleb	Aer	Kleb	Aer	Kleb	Aer
0	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+	+	+	+
64	+	+	+	+	+	+	+	+	+	+	+	+
128	+	+	+	+	+	+	+	+	+	+	+	+
256	+	+	-	+	+	+	+	+	+	+	+	+
512	+	+	-	-	+	+	+	+	-	-	+	+
1,024	+	-	-	-	+	+	+	+	-	-	+	+

+: growth; -: absence of growth.

**Table 6.** Multiplication and survival rate of banana shoots in MS (Murashige & Skoog, 1962) medium with the addition of 4 mg L<sup>-1</sup> N<sup>6</sup>-benzylaminopurine (BAP)<sup>(1)</sup>.

Concentration (mg L <sup>-1</sup> )	Multiplication rate			Survival rate (%)		
	Cefaclor	Nalidixic Acid	Vancomycin	Cefaclor	Nalidixic Acid	Vancomycin
0,0	2.2 aA	2.2 aA	2.2 aA	100.0 aA	100.0 aA	100.0 aA
512	1.3 bB	2.9 aA	2.5 aA	100.0 aA	100.0 aA	100.0 aA
1,024	0.2 bB	2.1 aA	2.6 aA	35.0 bB	100.0 aA	100.0 aA
CV (%)		24.5			17.8	

<sup>(1)</sup>Means followed by equal letters, lower case in the columns and upper case in the lines, within each variable, do not differ by Tukey's test, at 5% probability.

However, the same behavior was not observed in treatments with vancomycin and nalidixic acid. With these antibiotics added to the medium, the multiplication values of cultures reached approximately 2.0 shoots per explant, similar to the results obtained by Costa, Scherwinski-Pereira, Pereira, and Oliveira (2006) and Oliveira et al. (2008) in multiplication experiments of bananas without the addition of any antibacterial agent. Additionally, no symptoms of phytotoxicity were visually observed in the shoots cultivated in medium containing these antibiotics, regardless of the concentrations used.

Although costly, the addition of antibiotics to the growth medium can provide efficient results with respect to the loss of plant material. Lima and Moraes (2006) observed a reduction in bacterial contamination of up to 66.6% when antibiotics were added to MS medium during the cultivation of bananas of the Caipira variety. However, the effectiveness of the use of an antibiotic to control bacteria depends on its form and spectrum of action. Vancomycin, for example, belongs to the group of glycopeptides, which act as inhibitors of bacterial cell wall synthesis, weakening and causing the death of the bacteria (Kohanski, Dwyer, & Collins, 2010). Despite its spectrum of action being limited to Gram positive bacteria and its usage at generally high concentrations in this study, vancomycin was effective in the control of *Klebsiella* and *Aeromonas* - both Gram negative bacteria, which indicates that the spectrum of action of this bactericide can be enhanced when its concentration is increased, as shown by Scherwinski-Pereira et al. (2003), who suggested

increased concentrations of antibiotics in the growth medium when they present low toxic effects on crops.

As with vancomycin, cefaclor also acts as an inhibitor of bacterial cell wall synthesis. This bactericide belongs to the group of cephalosporins and can be effective both for Gram positive and Gram negative bacteria. Despite the positive action in the control of bacteria during in vitro cultivation (El-Shaboury, Saleh, Mohamed, & Rageh, 2007), in this study the bactericidal potential of cefaclor negatively affected the development of banana explants when added to growth medium in high concentrations.

Nalidixic acid belongs to the group of quinolones that hinder bacterial replication by affecting the DNA synthesis of the bacteria. Soon after identification and due to its activity against aerobic Gram-negative bacteria, Lescher, Froelich, Gruett, Bailey, & Brundage (1962) reported that this antibiotic was effective against species of the genus *Klebsiella*, whereas Jacoby (2005) and Minarini and Darini (2012) reported that several mechanisms make *Klebsiella* resistant to quinolones, including nalidixic acid.

In this context, there are many factors that should be observed prior to control of in vitro contaminants through the addition of antibiotics to the growth medium. These include the appropriate concentration, the form and spectrum of action of the bactericide, and the phytotoxic effect on cultivation (Scherwinski-Pereira et al., 2003). However, the contrasting results found in the literature when

antibiotics are used in MS medium lead us to infer another factor that may influence the rate of phytotoxicity of a product: the genotype. This hypothesis can be confirmed by comparing the work of Lima and Moraes (2006) with that of Carneiro, Silva, Ximenes, Carneiro, and Borges (2000). The former, working with the antibiotic rifampicin, reported no anomalies in banana plants of the Caipira cultivar propagated in MS medium, whereas the latter, using equivalent concentrations of the same antibiotic, detected phytotoxic effects on plants of the Maçã variety, as evidenced by deformation of the aerial part and a reduction in the final size of the shoots.

### Conclusion

Bacteria of endophytic origin belonging to the genera *Klebsiella* and *Aeromonas* are contaminants of banana plants during micropropagation;

The most effective antibiotics for controlling them are cefaclor, vancomycin, and nalidixic acid, at concentrations between 512 and 1,024 mg L<sup>-1</sup>;

Among the three antibiotics selected, the rate of multiplication and survival of banana shoots is only affected by cefaclor when added to the multiplication medium at concentrations greater than or equal to 512 mg L<sup>-1</sup>;

At concentrations between 512 and 1,024 mg L<sup>-1</sup>, vancomycin and nalidixic acid do not affect either the multiplication or the survival rate of banana shoots during in vitro multiplication.

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