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Original article

Isolation and characterization of bacteria from tissue culture of *Cenchrus purpureus* CT-608

Aislamiento y caracterización de bacterias presentes en el cultivo de tejidos de *Cenchrus purpureus* CT-608

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ABSTRACT: The objective of the present work consisted of reporting advances in the isolation and characterization of bacteria present in the tissue culture of *Cenchrus purpureus* CT-608. The study was developed in the Biotechnology Laboratory of the Agrarian University of Havana. Fragments of bacterial colonies from contaminated microplants were used and isolated in test tubes with Nutrient Agar medium. The colonies were described according to their macroscopic and microscopic characteristics through Gram staining and optical microscopy at 1000× magnification. The following descriptors were evaluated: cellular shape, size, grouping arrangement, reaction to Gram staining, cyst production, capsule production, endospore production. Bacterial sensitivity to the set of antibiotics was determined: Penicillin, Amoxicillin, Ampicillin, Cephalexin, Cefixime, Ceftriaxone, Nalidixic Acid, Gentamicin, and Chloramphenicol. Antibiotic response was assessed using the Kirby-Bauer technique. Two bacterial colonies were isolated, where one Gram-positive morphotype (MF1) and two Gram-negative morphotypes (MF2 and MF3) were obtained from one colony. The bacillary cellular form predominated, with a size of 3-4 mm, and the grouping arrangement varied from bacillus to irregular rosette-shaped. The morphotypes showed sensitivity to Nalidixic Acid and Cephalexin, and resistance to Penicillin, Amoxicillin, and Ampicillin. Three bacterial morphotypes with diverse morphological and physiological characteristics were isolated from *C. purpureus* CT-608 microplants. Their late appearance in the culture medium and their confinement around the explant indicate they may be endophytic microorganisms, an aspect that should be verified through chemotaxis.

Key words: colonies, endophytes, macroscopic and microscopic characteristics, morphotypes.

RESUMEN: El objetivo del presente trabajo consistió en reportar los avances del aislamiento y caracterización de bacterias presentes en el cultivo de tejidos de *Cenchrus purpureus* CT-608. El estudio se desarrolló en el Laboratorio de Biotecnología de la Universidad Agraria de la Habana. Se empleó fragmentos de colonias de bacterias provenientes de microplantas contaminadas y se aislaron en tubos de ensayos con medio Agar Nutriente. Las colonias se describieron según sus características macroscópicas y microscópicas mediante tinción de Gram y microscopio óptico con aumento 1000X. Se evaluaron los descriptores: forma celular, forma de agrupación, tamaño, reacción a la tinción de Gram, producción de quistes, producción de cápsula, producción de endosporas. Se determinó la sensibilidad bacteriana al set de antibióticos: Pencillina, Amoxacillina, Ampicillina, Cefalexina, Cefixime, Ceftrioxima, Ácido nalidíxico, Gentamicina y Cloranfenicol. La respuesta a los antibióticos se realizó mediante la técnica de Kirby-Bauer. Se aislaron dos colonias bacterianas, donde se obtuvo un morfotipo Gram positivo (MF1) y dos morfotipos Gram negativo (MF2 y MF3) en una de las colonias. Predominó la forma celular bacilar, con tamaño de 3-4 mm y la forma de agrupación varió de bacilo a irregular arrocelada. Los morfotipos mostraron sensibilidad al Ácido nalidíxico y la Cefalexina, y resistencia a Penicillina, Amoxacillina y Ampicillina. Se aislaron tres morfotipos bacterianos en microplantas de *C. purpureus* CT-608 con características morfológicas y fisiológicas diversas. Su aparición tardía en el medio de cultivo y su circunscripción alrededor del explante indican que pueden tratarse de microorganismos endófitos, aspecto que debe ser comprobado mediante quimiotaxis.

Palabras clave: características macroscópicas y microscópicas, colonias, endófitos, morfotipos.

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INTRODUCTION

Plant tissue culture is a biotechnological method that allows the production of whole plants from fragments of plant organs and tissues established under controlled environmental conditions, with the absence of associated microorganisms, heterotrophic nutrition, and plastic or glass containers (1). Generally, the micropropagation technique is carried out under completely aseptic conditions; therefore, bacteria have often been described as contaminants that can cause plant tissue death by competing for nutrients and modifying the culture medium (2,3). However, due to current knowledge about the benefits of endophytic microorganisms and associative bacteria in promoting growth and inducing stress tolerance, interest in their use has increased in some stages of micropropagation (4).

Cenchrus purpureus (Schumach.) Morrone, known as elephant grass, is cultivated throughout tropical and subtropical regions due to its high biomass accumulation potential, nutritional value, vigor, and persistence, making it a highly demanded forage for feeding ruminant species (5). It has been reported that this plant has the potential to associate with plant growth-promoting microorganisms, including endophytic diazotrophic bacteria (6). In this regard, four bacterial genera have been isolated from the roots of this species: *Sphingomonas*, *Bacillus*, *Pantoea*, and *Enterobacter*, associated with the alleviation of salt stress and plant growth promotion (7).

Currently, assays are being developed to optimize the micropropagation of *C. purpureus* CT-608 using segments of plants grown *in vitro*. As a first step, disinfection and *in vitro* establishment experiments were carried out, followed by studies to regenerate plantlets through direct organogenesis, during which bacteria associated with the microplants were found. In the consulted literature, research describing the genera and species of bacteria present in tissue culture of *C. purpureus* is scarce. Therefore, the objective of the following study is to report progress in the isolation and characterization of bacteria present in tissue culture of *C. purpureus* CT-608.

MATERIALS AND METHODS

The following study was carried out at the plant tissue culture facilities of the Biotechnology Laboratory of the Faculty of Agronomy at the Agrarian University of Havana (UNAH), San José de las Lajas, Mayabeque, Cuba.

Biological material

This consisted of bacteria detected in *C. purpureus* CT-608 plantlets cultured *in vitro*, obtained from the second subculture in regeneration experiments. The plants were established in test tubes containing 15 mL of culture medium, under controlled conditions in a growth chamber with a photoperiod of 16 hours of light and 8 hours of darkness, relative humidity of 70 ± 5 %, light intensity of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and temperature of 25 ± 2 °C.

Bacterial isolation

A fragment of the colony was extracted directly from the contaminated test tube and cultured in test tubes containing 15 mL of Nutrient Agar medium composed of 5.0 g L bacteriological peptone, 1.0 g L nutrient extract, 2.0 g L yeast extract, 5.0 g L sodium chloride, and 15.0 g L agar, with the pH adjusted to 5.7. After inoculation, the tubes were incubated for 48 hours at 30 ± 2 °C.

Description of bacterial colonies

Following isolation, macroscopic description was carried out visually, determining the shape, color, surface, and margin of the bacterial colonies according to Bergey's Manual of Bacteriology (8). Subsequently, microscopic description was performed to determine colony morphology using Gram staining. The following descriptors were evaluated: cell shape, grouping pattern, size, reaction to Gram staining, cyst production, capsule production, and endospore production. Observations were made using an optical microscope at 1000X magnification.

Antibiogram

Bacterial sensitivity was determined using a set of antibiotics: Penicillin, Amoxicillin, Ampicillin, Cephalexin, Cefixime, Ceftriaxone, Nalidixic acid, Gentamicin, and Chloramphenicol. Antibiotic susceptibility was assessed using the Kirby-Bauer disk diffusion method (9). For this purpose, bacterial colonies subcultured on Petri dishes with Nutrient Agar medium were suspended until achieving a standard turbidity of 0.5 on the McFarland scale and incubated for 24 hours. After this period, five antibiotic-impregnated disks were placed per plate, with a minimum separation of 24 mm to avoid interference, and incubated for 24 hours at 35 ± 2 °C. The inhibition zone diameter, including the disk, was measured in millimeters (mm) and compared with the EUCAST breakpoint table to classify bacteria as sensitive, intermediate, or resistant.

RESULTS AND DISCUSSION

Two bacterial colonies were isolated, appearing approximately 30 days into the multiplication phase across multiple replicates of the *C. purpureum in vitro* culture. They always appeared separately in the test tubes, with their growth confined to the immediate vicinity of the explant without colonizing the rest of the medium. Nevertheless, their presence did not affect the development and growth of the microplants. This result could indicate that the microorganisms present are endophytic, thus withstanding the disinfection treatments applied to the explant.

The bacteria under study are shown in (Figure 1A and 1B). Initially, two colonies were isolated (Figure 1C and 1D), which differ from each other in their macroscopic characteristics (Table 1). Microscopic observation of the isolates revealed the presence of two bacterial morphotypes (MT) in Colony 1 and one morphotype in Colony 2.

Colony 1 was purified using the streak plate method to separate the isolated bacterial morphotypes, which were designated as MT1 and MT2, and MT3 from Colony 2.

Microscopic characterization shows one Gram-positive morphotype (MT1) and two Gram-negative morphotypes (MT2 and MT3) (Figure 1E and 1F). Bacillary shape predominates, although MT3 exhibits a typical irregular, star-shaped form characteristic of the *Phylum Firmicutes*. No evidence of cysts, endospores, or capsules was found in any of the isolated morphotypes. All three morphotypes are catalase-positive, with a marked aerobic metabolism (Table 2).

The antibiogram showed sensitivity to nalidixic acid and cephalexin (Rocephin) for morphotypes MT1 and MT2, and resistance to penicillin, amoxicillin, and ampicillin. The assay could not be performed for MT3, as it did not grow after the first subculture, which prevented the application of antibiotic disks.

Some authors state that endophytic microorganisms develop part of their life cycle within a plant (10). Most colonize different compartments of the plant, such as the apoplast, including intercellular spaces of the cell walls and xylem vessels. On the other hand, some researchers report that disinfection methods do not always eliminate microorganism populations in *in vitro* tissues; many are capable of remaining latent in vascular bundles and are protected from chemical agents (11). Furthermore, factors that may contribute to contamination include deficient explant disinfection, problems with culture medium sterilization, environmental contaminants, and deficiencies in sterile area handling.

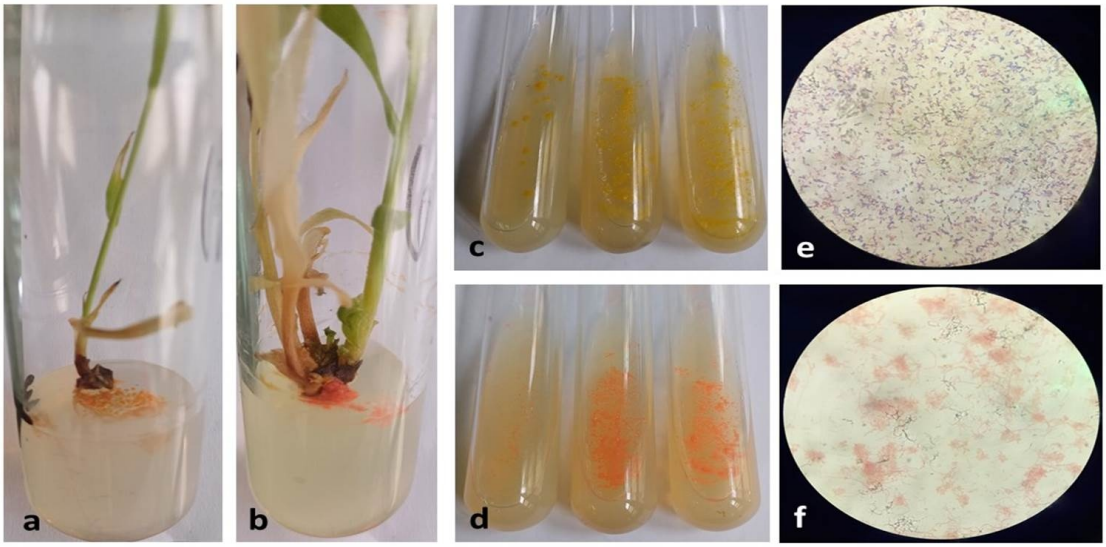

Figure 1. A. *C. purpureus* CT-608 microplants showing bacterial colony growth (MT1-MT2). B. *C. purpureus* CT-608 microplants showing growth of bacterial colony MT3. C. Bacteria isolated on Nutrient Agar medium (MT1-MT2). D. Bacteria isolated on Nutrient Agar medium (MT3). E. Microscopic characteristics of morphotypes MT1 and MT2. F. Microscopic characteristics of morphotype MT3

Table 1. Macroscopic characteristics of bacterial morphotypes isolated on Nutrient Agar

Macroscopic characteristics	Bacterial colonies		
	MF1	MF2	MF3
Common	Medium size after 48 hours of growth. Circular shape with tendency toward irregularity. Diffuse elevation. Creamy texture. Catalase-positive.		
Different	Slightly undulate margin. Intense yellow color	Slightly undulate margin. Light yellow color	Entire margin. Intense orange color

Source: Prepared by the authors

Table 2. Microscopic characteristics of the bacterial morphotypes isolated on Nutrient Agar medium

Microscopic characteristics	Bacterial morphotypes		
	MF1	MF2	MF3
Cell shape	bacillary	bacillary	bacillary
Size	3-4 mm	3-4 mm	3-4 mm
Grouping pattern	bacillus	bacillus	irregular, star-shaped
Gram reaction	+	-	-

Source: Prepared by the authors

The macroscopic and microscopic analyses performed to characterize the three bacterial morphotypes are of great importance. In the case of macroscopic analysis, it allows the determination of specific characteristics of the strains and constitutes the most common primary method for identifying colony phenotypes (12). In this regard, some authors state that this method is based on observable macroscopic characteristics of colonies, such as size, colony type, texture, color, and appearance (13). On the other hand, microscopic analysis reveals key characteristics for bacterial identification, such as the cell wall type of the microorganism or the presence of distinctive structures.

Among these methods, Gram staining allows classification of bacteria based on differences in cell wall composition. Knowing whether bacteria are Gram-positive or Gram-negative enables appropriate measures to be taken to identify or even combat bacteria if they are pathogenic (14).

The optical microscope observation results determined the predominance of bacillary shape in the bacterial morphotypes. Bacterial cells can vary in shape and, accordingly, can be classified as cocci (spherical), bacilli (cylindrical, rod-shaped), spirilla (spiral or helical), and filaments (complex forms). Knowing cell shape provides information about survival, activity in the environment, and motility. In the case of bacilli, they have a larger surface area to volume ratio and can obtain nutrients more efficiently from dilute solutions (15). Additionally, they produce and release hydrogen peroxide (H_2O_2) and lactic acid, which prevents the development of other bacterial and fungal forms (16).

The antibiogram showed sensitivity to nalidixic acid and cephalexin for morphotypes MT1 and MT2, and resistance to penicillin, amoxicillin, and ampicillin. The responses found in this assay are of great importance for designing disinfection strategies combined with nalidixic acid or cephalexin. Antibiotics are a subgroup of antimicrobials with antibacterial activity that cause the death or inhibit the growth of bacteria and fungi in culture media and *in vitro* grown plants (17). However, it is important to note that inappropriate use of antibiotics is the main driver of bacterial resistance (18); therefore, it is important to use antibiotics responsibly and in combination with other control measures, such as proper sterilization of culture media, tools, and equipment.

Antibiotics such as nalidixic acid and ampicillin have effective activity against a broad spectrum of Gram-negative bacteria (19). Nalidixic acid is a quinolone that acts inside bacteria on DNA gyrase, an enzyme essential for DNA coiling and supercoiling, an action that prevents DNA replication and promotes its breakage (20). On the other hand, penicillin, amoxicillin, and ampicillin inhibit reproduction and growth, and cause elongation and lysis of susceptible bacteria (21).

There is a group of Gram-negative bacilli that possess enzymes conferring resistance to amoxicillin and ampicillin, as well as some first-generation cephalosporins. Second- and third-generation cephalosporins are stable against these enzymes and can therefore be used as an alternative treatment. Furthermore, resistance in some Gram-positive bacilli is predominantly associated with structural changes in the cell wall or cytosolic components such as ribosomes, rather than enzymatic mechanisms, making them resistant to penicillin or cephalosporins (22).

CONCLUSIONS

Three bacterial morphotypes were isolated from *C. purpureus* CT-608 microplants, exhibiting diverse morphological and physiological characteristics. Among these, one Gram-positive morphotype (MT1) and two Gram-negative morphotypes (MT2 and MT3) were identified.

Bacillary shape predominates in MT1 and MT2, although MT3 exhibits a typical irregular, star-shaped form. No evidence of cysts, endospores, or capsules was found in any of the cases. All three morphotypes are catalase-positive, with a marked aerobic metabolism. The antibiogram showed sensitivity to nalidixic acid and cephalexin (Rocephin) for MT1 and MT2, and resistance to penicillin, amoxicillin, and ampicillin.

The late appearance of the bacterial morphotypes in the culture medium and their confinement around the explant suggest that they may be endophytic microorganisms. This aspect should be verified through chemotaxis techniques in subsequent assays.

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