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***In vitro* inhibition of *Xanthomonas* spp. by lemon-scented gum extracts.** Inibição *in vitro* de *Xanthomonas* spp. por extratos de eucalipto-limão.

Iramar V. S. Mendes[1], Eder Marques[2*]

[1-] Agronomist student, UPIS Integrated Colleges. E-mail: iramarviniciusss@gmail.com
[2*-] Agronomist, Full Professor II, UPIS Integrated Colleges, Fazenda Lagoa Bonita, BR 020, KM 12, DF 335, KM 4,8 – Planaltina, Brasília – DF. E-mail: eder.marques.08@gmail.com

Resumo

Foi objetivo deste trabalho avaliar extratos de eucalipto-limão (*Corymbia citriodora*) na inibição do crescimento *in vitro* de *Xanthomonas* spp. Os tratamentos consistiram dos extratos aquoso, infusão, alcoólico e fervura, além da testemunha com antibióticos. A metodologia utilizada para avaliar o antagonismo foi a de difusão em dupla camada. Os extratos foram testados a 50, 35 e 15% de concentração. Nenhum dos extratos inibiu o crescimento de *X. citri* pv. *glycines*, apenas o antibiótico bacitracina. Quanto a *X. vesicatoria*, observou-se que somente o extrato alcoólico de folhas de *C. citriodora* inibiu seu crescimento, com destaque para o extrato alcoólico a 35%, além dos antibióticos estreptomicina e bacitracina. O crescimento de *X. campestris* foi inibido por extratos alcoólicos e aquosos, com destaque dos extratos alcoólicos a 50 e 35% e do aquoso a 50%, assim como por estreptomicina e penicilina. Os extratos aquoso e alcoólico de eucalipto-limão mostraram potencial no controle *in vitro* de *Xanthomonas* e novos estudo serão realizados com novas concentrações e no controle *in vivo* de doenças causadas por estas bactérias.
Palavras-chave: Bactérias fitopatogênicas. Extrato de plantas. Manejo alternativo.

Abstract

The aim of this work was to evaluate lemon-scented gum (*Corymbia citriodora*) extracts in inhibiting the *in vitro* growth of *Xanthomonas* spp. The treatments consisted of aqueous, infusion, alcoholic and boiling extracts, in addition to the control with antibiotics. The methodology used to assess the antagonism was the double layer diffusion. The extracts were tested at 50, 35 and 15% concentration. None of the extracts inhibited the growth of *X. citri* pv. *glycines*, only the antibiotic bacitracin. As for *X. vesicatoria*, it was observed that only the alcoholic extract of *C. citriodora* leaves inhibited its growth, with emphasis on the 35% alcoholic extract, in addition to the antibiotics streptomycin and bacitracin. The growth of *X. campestris* was inhibited by alcoholic and aqueous extracts, highlighted on 50% and 35% alcoholic and 50% aqueous extracts, as well as by streptomycin and penicillin. The aqueous and alcoholic extracts of lemon-scented gum showed potential in the *in vitro* control of *Xanthomonas* and new studies will be carried out with new concentrations and *in vivo* control of diseases caused by these bacteria.
Keywords: Alternative management. Plant extract. Plant pathogenic bacteria.

Introduction

Xanthomonas is a numerous genus of Gram-negative plant-associated bacteria. They are mandatory aerobics and their optimum temperature for growth can vary between 25° and 30°C (SADDLER; BRADBURY, 2005). The pathogenic species of this bacterium exhibit a high degree of specificity with the host plant and can be further differentiated into pathovars (abbreviation pv.), which are defined based on the characteristic host range and/or tissue specificity, invading the elements of the xylem of the vascular system or the intercellular spaces of the host tissue mesophilic parenchyma (RYAN et al., 2011). They infect around 390 botanical species, 120 monocots and 270 dicots (LEYNS et al., 1984).

Among the economically important species of *Xanthomonas* stand out: *X. citri* pv. *glycines* - which is the causal agent of bacterial pustule in soybean (CONSTANTIN et al., 2016); *X. vesicatoria* - associated with the complex of bacterial spots in tomato and pepper (JONES et al., 2004) and *X. campestris* pv. *campestris* - causal agent of black rot in crucifers (VAUTERIN et al., 1995).

Such bacterioses have been controlled, basically, using chemical compounds and, in great majority, cuprics, for which cases of resistance have already been described (BENDER et al., 1990, MARQUES; UESUGI; FERREIRA, 2009, LUGO et al., 2013). In this context, the adoption of alternative measures, in the integrated management for disease control, has become a necessity for farmers (EL KHOURY; MAKKOUK, 2010), especially those who seek sustainable and environmentally safe management.

Corymbia citriodora (Hook.) K. D. Hill & L. A. S. Johnson (Myrtaceae), is an arboreal aromatic plant, due to the essential oils present in its leaves. Originally from Australia, it is commonly known as lemon-scented gum, citron-scent gum and lemon gum tree (BOLAND; BROPHY; HOUSE, 1991).

Antimicrobial principles (ESTANISLAU; BARROS; PEÑA, 2001, MAHMOUD; EBRAHIM; ALY, 2004, MISSANJO; MKWEZALAMBA, 2016, MUHAMMED; DADA; ALO, 2018, MIGUEL et al., 2018, INSUAN; CHAHOMCHUEN, 2020.), insecticides (NEGRINI et al., 2018) and anthelmintic (ARAÚJO-FILHO et al., 2019) have already been described in this species. Its main by-product, the essential oil has a main component is citronellal. Other components with antibacterial action have already been described as aromadendrene, 1,8-cineol and citronellol (MULYANINGSIH et al., 2011).

Some works have reported the alternative management, using plant extracts, of diseases caused by bacteria of the *Xanthomonas* genus (SATISH; RAVEESHA; JANARDHANA, 2002). Regarding eucalyptus extracts, Lucas et al. (2012) studied the essential oil (did not specify which species) to inhibit *X. vesicatoria in vitro* and *in vivo*, as well as Negi and Kumar, (2015) to *X. citri* subsp. *citri*. The extract of *E. camaldulensis* showed activity against *X. citri* subsp. *malvacearum* (RASHID et al., 2016). In this same sense, Yemata et al. (2019) evaluated the effect of *C. citriodora* to *X. citri* pv. *musacearum*. Abdurrahman, Ahamed, Amein (2020) also studied the antimicrobial potential of *Eucalyptus* sp. to *X. vesicatoria*.

This study aimed to evaluate the *in vitro* growth inhibition of *Xanthomonas* spp. by extracts of *Corymbia citriodora*.

Materials and methods

Bacterial strains

The bacterial strains (Table 1) used in this study were provided by the Plant Pathogenic Bacteria Collection, Department of Phytopathology, University of Brasília. Such bacteria are stored in sterile distilled water, in a working collection, and have been reactivated in NA (Nutrient Agar) medium.

Table 1 - Description of the plant pathogenic bacteria strains used in this study.

Strain	Species	Host
UnB 306	*Xanthomonas citri* pv. *glycines*	Soybean (*Glycine max* L.)
UnB 828	*Xanthomonas vesicatoria*	Tomato (*Lycopersicum esculentum* Mill.)
UnB 831	*Xantomonas campestris* pv. *campestris*	Kale (*Brassica oleracea* var. *acephala*)

Place of performance of the experiment and obtaining extracts of Corymbia citriodora

The study was conducted in the Federal District, central Brazil (15.58 ºS, 47.73 ºW), consisting of the Cerrado biome, during the month of March 2021. According to the Köppen classification, the location has a Tropical seasonal climate of megathermic savannah, with an average annual precipitation of 1,400 mm (CARDOSO; MARCUZZO; BARROS, 2014).

Corymbia citriodora seedling leaves of approximately six months old were harvested from a seedbed. To make the extracts, 5 g of leaves were used, which were macerated, boiled, or infused in 10 mL of distilled water or ethyl alcohol. The maceration (Treatment 1) was carried out with the aid of a crucible and mortar, boiling (Treatment 2) took place in a water bath for 15 minutes and infusion for 10 minutes (Treatment 3). These preparations were used immediately. The alcoholic extraction (Treatment 4), in 70% ethyl alcohol, took place by maceration and was kept for 24 hours until its use.

Double layer inhibition test

To evaluate the antagonism of lemon-scented gum extracts to *Xanthomonas* spp., the double layer diffusion inhibition method was used, which consisted of making a base layer of NA medium, placing four paper discs at equidistant points per plate of Petri and pipette 10 µL of each treatment (50, 35 and 15% extracts). Then, a suspension of approximately 10 x 10^8 CFU/mL (equivalent to McFarland Scale 7) was prepared from which 25 µL were removed and added to 5 mL of semi-solid NA medium (0.8%) flux (48 ºC), forming a covering layer in the base medium, according to Romeiro (2007, with modifications).

Experimental design and statistical analysis

The design used was completely randomized (CRD), in a factorial arrangement (4 x 3 or 3 x 2) with three replications, composed of three plates, containing four paper discs (350 g/m^2), including the four treatments (composed by the respective extract/dilution), plus the control (discs with the antibiotics bacitracin, penicillin and streptomycin 10 μg/disc). Based on the test, the formation of inhibition zones was observed, which had their diameters (in cm) measured with the aid of a millimeter ruler.

The experiment data were subjected to analysis of variance (ANOVA), using the SISVAR 5.6 program (FERREIRA, 2014). The means of the values of the inhibition parameters were compared by the Tukey test, at 5% probability.

Results

None of the plant extracts inhibited the growth of the UnB 306 strain (*X. citri* pv. *Glycines*). Among the three antibiotics tested, only bacitracin inhibited this plant pathogenic bacterium, with an average of inhibition zones of 0.4 cm.

Only the alcoholic extract of lemon-scented gum inhibited the strain UnB 828 (*X. vesicatoria*), although at all concentrations tested. Interestingly, the highlight was the 35% concentration, where inhibition zones of 2.4 cm were observed, which differed significantly from the other concentrations (Figure 1). Even so, the 50% alcoholic extract inhibited more than the antibiotics used as control, with mean inhibition zone of 1.48 cm. Treatment with 15% alcoholic extract did not differ significantly from the antibiotic streptomycin and bacitracin. The antibiotic penicillin did not inhibit this bacterial strain (Figure 2).

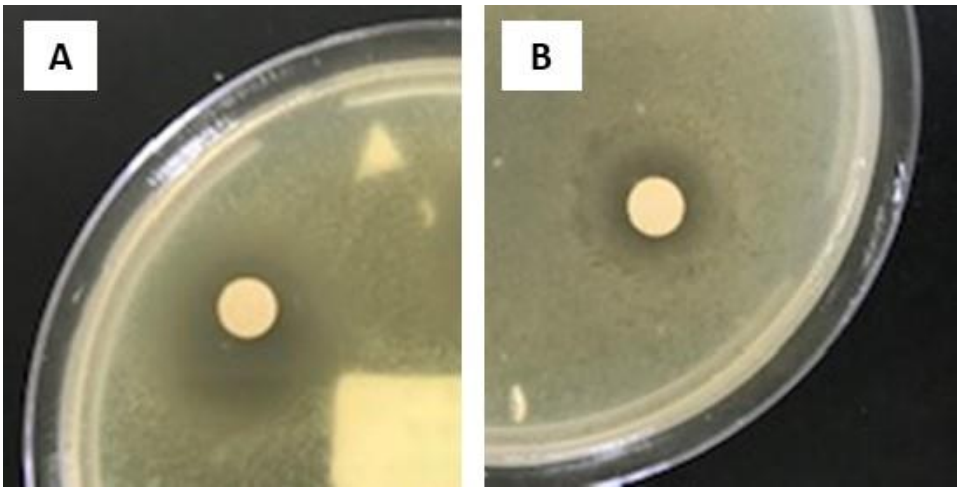

Figure 1 – Results of the assay of alcoholic extract of *Corymbia citriodora* at 35% inhibiting plant pathogenic bacteria, where: A) UnB 828 (*Xanthomonas vesicatoria*) and B) UnB 831 (*Xanthomonas campestris* pv. *campestris*).

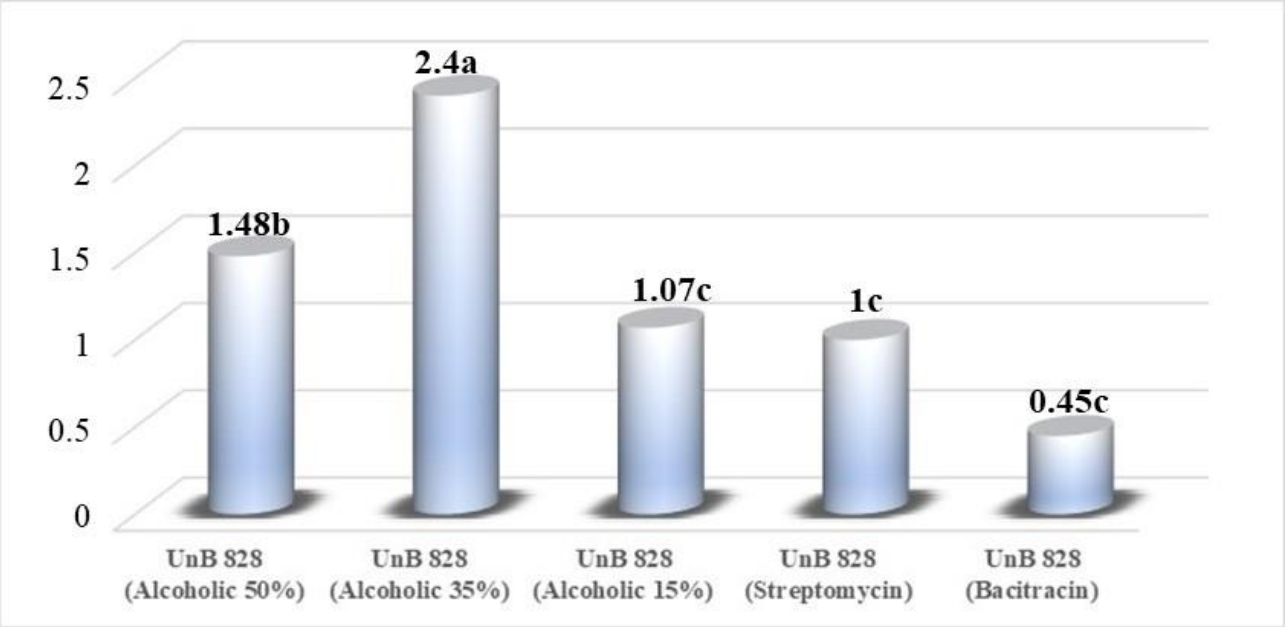

Figure 2 – Result of antagonism (Y axis – inhibition zones in cm) of *Corymbia citriodora* extracts on the growth of plant pathogenic bacteria *Xanthomonas vesicatoria* (X axis).

Regarding strain UnB 831 (*X. campestris* pv. *campestris*), both aqueous and alcoholic extracts were able to inhibit this bacterium. The highlight was for the 50% alcoholic extract, followed by 35% and the 50% aqueous extract, with the largest inhibition zones of 1.3, 1.2 and 1 cm, with no significant difference between them. Treatments with 35% and 15% aqueous extracts did not differ from controls with streptomycin and penicillin, with inhibition zones below 0.6 cm (Figure 3). The extracts obtained by infusion and boiling did not inhibit this bacterial strain, as did the antibiotic bacitracin.

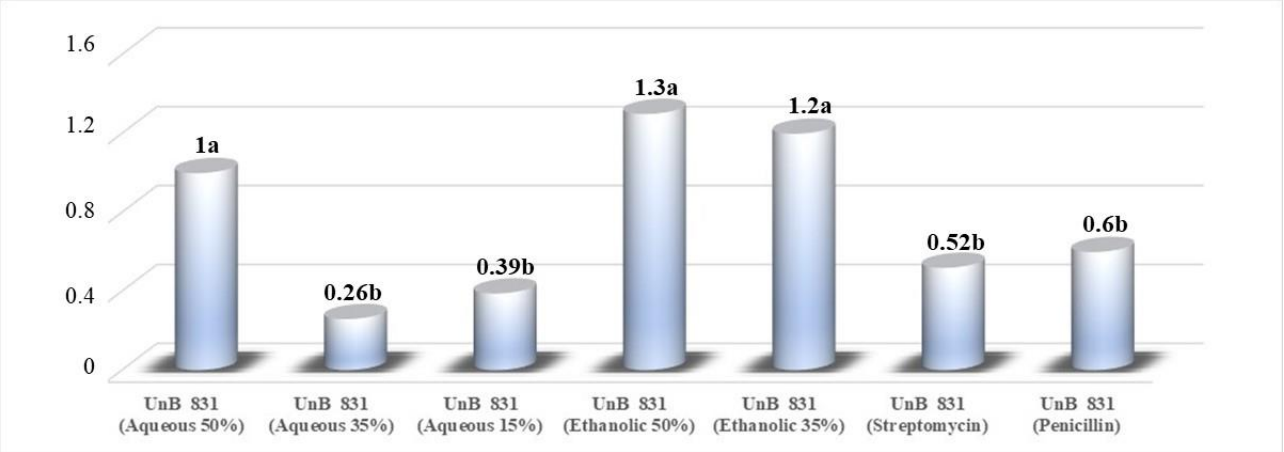

Figure 3 - Results of antagonism (Y axis – inhibition inhibition zones) *Corymbia citriodora* extracts on the growth of plant pathogenic bacteria *Xanthomonas campestris* pv. *campestris* (X axis).

Discussion

The antibacterial potential of *Eucalyptus* spp. has been investigated. Lucas et al. (2012) reported that eucalyptus essential oil (did not discriminate the species) inhibited the *in vitro* growth of *X. vesicatoria* at concentrations of 0.1; 1; 10 and 100%. Negi and Kumar (2015) observed inhibition of *X. citri* subp. *citri* by aqueous extracts of *Eucalyptus* sp. leaves, with larger inhibition

zones at the maximum concentration (20%). The aqueous extract of *E. camaldulensis* significantly inhibited the *in vitro* growth of *X. citri* pv. *malvacearum* and the incidence of the bacterial blight of cotton in the field (RASHID et al., 2016). Similarly, Abdurrahman, Ahamed, Amein (2020) observed that the ethanol extract of *Eucalyptus* sp. showed an inhibitory effect on *X. vesicatoria in vitro* and on tomato leaf spot severity.

Likewise, *C. citriodora* is reported to have antimicrobial properties (ESTANISLAU; BARROS; PEÑA, 2001, MAHMOUD; EBRAHIM; ALY, 2004, MISSANJO; MKWEZALAMBA, 2016, MUHAMMED; DADA; ALO, 2018, MIGUEL et al., 2018, INSUAN; CHAHOMCHUEN, 2020), insecticide (NEGRINI et al., 2018) and anthelmintic (ARAÚJO-FILHO et al., 2019). According to Mulyaningsih et al. (2011), some active and bactericidal compounds present in lemon-scented gum essential oil are aromadendreno, 1,8-cineol, citronellal and citronellol, which explains the potential for inhibiting *Xanthomonas* spp. observed in the present study.

According to Missanjo and Mkwezalamba (2016), the essential oil of *C. citriodora* inhibits the growth of the human pathogens *Escherichia coli* and *Staphylococcus aureus*. Similarly, Muhammed, Dada, Alo (2018) report that ethanol extracts of lemon-scented gum at 200-500 mg/ml also inhibited the human pathogen *E. coli*. Later, Insuan and Chahomchuen (2020) also observed antibacterial activity of its essential oil against *Bacillus subtilis, S. aureus, S. intermedius, E. coli* and *Pseudomonas aeruginosa*.

Studies of *C. citriodora* extracts on the *in vitro* inhibition of *Xanthomonas* spp. are less frequent, although Yemata et al. (2019) have evaluated the effect of extracts (dry powdered leaves macerated in methanol) of this plant on *X. citri* pv. *musacearum* (causing agent of bacterial wilt in cotton) and, corroborating the results of the present work, they observed that lower concentrations of the extract also revealed greater antibacterial activity.

Regarding the antibiotics tested, Shenge et al. (2007) also reported that bacitracin and streptomycin inhibited *X. vesicatoria*, although with variation among isolates of this plant pathogenic bacterial species. In this same sense, Sain et al. (2008) observed that the antibiotics streptomycin and penicillin also inhibited the growth of *X. campestris* pv. *campestris*, with inhibition potential varying among isolates.

Conclusions

The aqueous and alcoholic extracts inhibit the *in vitro* growth of *Xanthomonas vesicatoria* and *campestris*. The results evidence that the inhibition can be species or isolated-specific and dependent on the type of extract and concentration. New studies will be carried out with new concentrations and *in vivo* control of diseases caused by these bacteria.

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