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Research Article

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In Vitro Growth Control Assays of Bacteria Isolated from Lemon Fruit Lesions

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Abstract

Citriculture finds favorable climate conditions for its development in the northwest region of the state of São Paulo - Brazil, effectively contributing to the regional economy, generating income for producers and employment for its inhabitants. Citrus cancer is an important disease that affects citrus. Caused by the bacterium *Xanthomonascitri*, subspecies citri, this phytopathology is spread by the action of nature, contaminated seedlings and mainly human action during cultural treatments. Cancer lesions can be found on leaves, branches, and fruits, and their spread in the orchard can occur to neighboring plants, contaminating the entire cultivated area if proper phytosanitary care is not taken. There is no specific control for citrus cancer, and the use of management techniques and the use of some products that mitigate economic losses are suggested. Among these management practices, we can mention the eradication of the infected plant, preventing it from contaminating the neighborhood. Chemical control uses copper-based products to protect the fruits from injury, bactericides to disinfect the boxes used in the harvest so that there is no greater contamination in the rest of the orchard. In this work, we isolated microorganisms from lesions found in lemon fruits. Samples extracted from the lesions were inoculated in nutrient agar by striatal technique to isolate colonies. After growth, two of these strains were selected for the growth inhibition tests by the minimum concentration in the liquid medium. The results demonstrated the efficiency in growth inhibition when increasing concentrations of peracetic acid and copper were used. Aqueous extracts of pomegranate (*Punicagranatum* L.) showed a potential inhibitory effect on bacterial growth.

Keywords

Citriculture; Plant pathology; Xanthomonascitr

Introduction

Citriculture is of great economic importance worldwide, with citrus fruits having the largest production in the world compared to other fruits, with a global production of approximately 98.3 million tons, including oranges, mandarins, lemons and grapefruits [1]. In the state of São Paulo, citrus cultivation finds favorable climatic conditions for its development, which, according to Embrapa, is the main citrus-producing state in Brazil. Among the main producing regions of the state, we can highlight the northwest of São Paulo, which includes the municipalities of Barretos, Araraquara, Jaboticabal, Catanduva, and São José do Rio Preto. Thus, we can define that citrus cultivation contributes effectively to the economy of this region, producing income for producers and employment for its inhabitants.

Several pathogens are reported to cause significant losses, leading to wasted citrus fruits and economic losses [1] such as citrus cancer. Citrus cancer is one of the most important diseases in the world's citrus industry [2]. Caused by the *bacterium Xanthomonascitri*, subspecies citri, a compulsory, Gram-negative, rod-shaped aerobic bacterium with the presence of a single polar flagellum and do not form spores [3]. The bacterium forms yellow colonies in the culture medium as a result of xantomonadine production [4]. The appearance of these lesions enables the development of other opportunistic bacteria in the site. Citrus cancer is a serious disease in regions where rainfall and high temperatures are frequent during the sprouting period and early fruit development [5].

The infected site is characterized by the occurrence of necrosis. In the leaves appear first small oily spots, later the lesions rupture by pathogen-induced tissue hyperplasia. A white or yellow spongy growth is then observed. These pustules darken and thicken with a consistency of brown and rough cork [6]. This phytopathology is easily disseminated and occurs due to the action of nature, contaminated seedlings and mainly human action during the cultural treatments [7], its spread in the orchard can occur in neighboring plants, and can contaminate the entire cultivated area if proper phytosanitary care. not taken. The disease manifests as wartlike lesions on leaves, branches, and fruits [7]. The main consequences of citrus canker in areas where the disease is endemic are premature leaf and fruit fall, consequently decreasing production [2,7]. Defoliation due to the high incidence of citrus can seriously compromise plant development, especially in the first years after planting resulting in lower yield [8].

According to Fundecitrus [11], symptoms become visible on leaves two to five weeks after infection. At first, darkened spots are formed, often with yellowing around, resulting from the multiplication of bacteria and soaking of plant tissue. Symptoms develop into light brown pustules. Lesions are first seen on the inferior surface. As the disease progresses, they become larger and may reach more than an inch in diameter. In Brazil, its first finding was in the São Paulo municipality of Presidente Prudente, in 1957 [9]. In the same year, it was also found in the state of Paraná, in the municipality of Lupionópolis [10]. There is no specific control for citrus cancer, and the use of management techniques and the use of some products that could mitigate economic losses are suggested [12]. The specific use of insoluble fixed copper-based products, due to their cost-effective plant protection, encourages excessive applications throughout the year [12]. There is great environmental concern about repeated copper applications and their transformation, availability, and mobility in the soil [13].

A concern that has long occurred in the selection of varieties less sensitive to the bacillus that causes citrus canker. The sensitivity found in plants is known to be different. Approaches ranging from conventional breeding methods to the production of transgenic plants

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are being used to produce resistant plants [14]. The hybridization consists of the combination of organelles and the nuclear genomes of different species [15].

In this sense, the objective of this work was to test the Minimum Inhibitory Concentration of the main commercial products used in the control of citric cancer, such as peracetic acid and copper hydroxide. The efficacy of the natural extract of pomegranate (*Punicagranatum L.*) in the control of bacterial growth *in vitro* was also tested.

Materials and Methods

Bacterial isolation

Lemon fruits with suspected lesions of citrus canker (Figures 1A and B) were isolated and taken to the microbiology laboratory of the Centro Universitário do Norte Paulista - UNORP. Microorganisms from lesions found on the fruits were isolated using a bacteriological loop. These were inoculated into Petri dishes containing nutrient agar. From the plate striation technique, it was used to obtain the isolated colonies (Figures 2A and B). The culture was kept in a bacteriological greenhouse for 48 hours at 30ºC. After growth, two of these colonies were selected for the growth inhibition tests in the liquid medium.

Preparation of pomegranate extract

The pomegranate fruit macerate was prepared with the aid of a mortar and pistil. Fruit segments were ground and diluted in aqueous solution with a 20% stock concentration (20 g/100 mL).

Minimum Inhibitory Concentration Assays

Cultivation took place in liquid TSB medium in the presence of different concentrations of acetic perchloric acid, copper hydroxide, chlorhexidine and pomegranate extract. After 70 hours of growth, the bacteria were again plated on nutrient agar to determine the Minimum Inhibitory Concentration for bacterial growth.

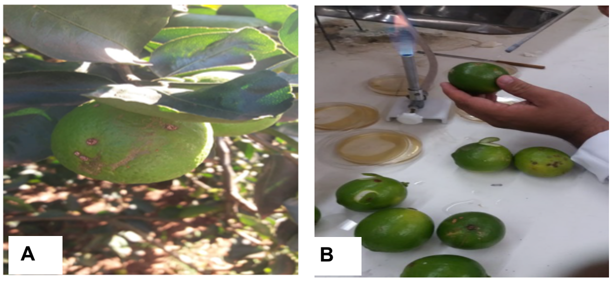

Figure 1: A = Figure shows the lesions observed on the lemon fruit. B: Bacterial isolation procedure.

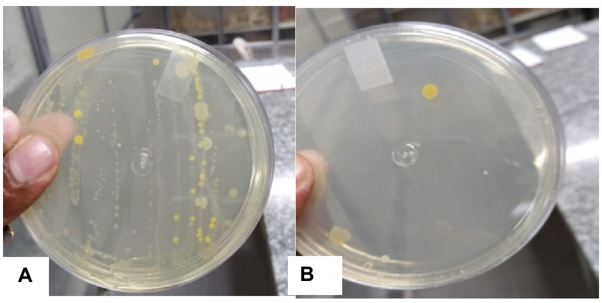

Figure 2: A and B. Isolated colonies that were selected for inhibition tests.

Results and Discussion

Peracetic acid is considered a potent biocide, even at low concentrations (0.0001% to 0.2%). Its advantages are to remain effective even in the presence of organic waste, and not to have a toxic or carcinogenic residual form (acetic acid and oxygen). Its action provides excellent disinfection in a short time [16]. Peracetic acid has been suggested to chemically control the growth of the *bacterium Xanthomonascitri*, subspecies citri. Our growth inhibition tests showed that it is effective for this control in the development of the two samples of bacteria isolated from the lesions of the fruit. Final concentrations of 13.3, 33.3, 66.6 μg/mL of peracetic acid in 3 mL of liquid medium were used. When the cultivation of these microorganisms was in the solid medium, it was observed that the growth was totally inhibited when the acid concentration was 13.3 μg/mL (Figure 3A and B).

Several copper-based products are reported to have some effectiveness in controlling citrus cancer. The concentrations of copper hydroxide tested were 23, 69 and 115 μg/mL. In our inhibition studies, copper hydroxide proves its efficiency in controlling the development of both samples. When cultivation was performed in a solid medium, it was observed that growth was inhibited with concentration from 23 μg/mL (Figures 4A and B).

Chlorhexidinegluconate 0.12%, the mechanism of action of this substance is broad-based, encompassing Gram-positive and Gram-negative bacteria, yeasts, dermatophytes and some lipophilic viruses [16]. Gram-positive microorganisms are more susceptible to chlorhexidine than Gram-negative. In our tests, 0.12% chlorhexidinegluconate was efficient in inhibiting bacterial growth. The culture performed in Petri dishes shows the control in concentrations above 36 μg/mL (Figures 5A and B). We tested the

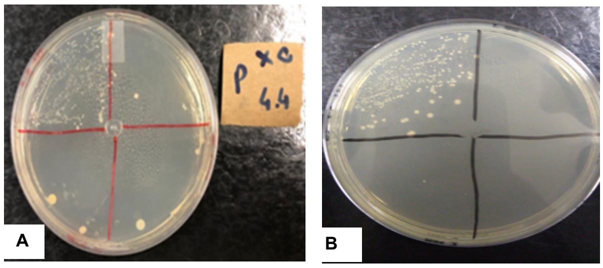

Figure 3: A and B. Shows the efficiency of peracetic acid in inhibiting bacterial growth at increasing concentrations.

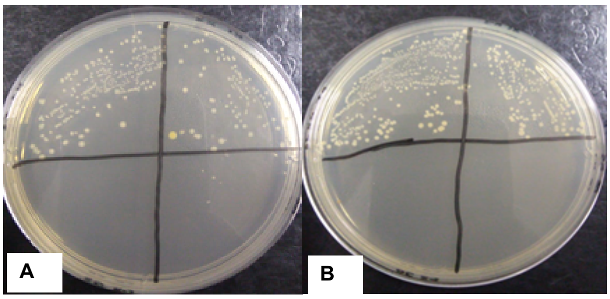

Figure 4: A and B. Efficiency of copper hydroxide in inhibiting bacterial growth at increasing concentrations.

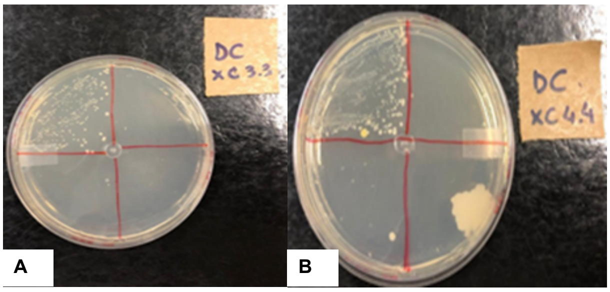

Figure 5: A and B. Effect of 0.12% chlorhexidine glyconate on bacterial growth control.

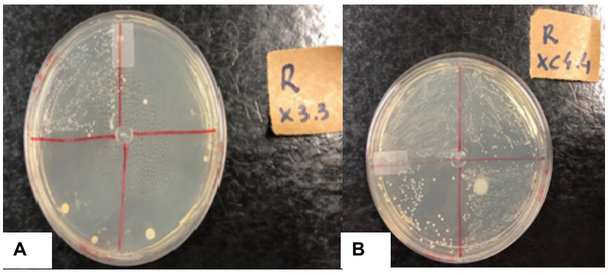

Figure 6A and B: Growth inhibition with pomegranate extract.

inhibitory effect for the final concentrations of 36, 72 and 144 µg/mL of chlorhexidinegluconate.

The pomegranate extract (*Punicagranatum L.*) was used in the formulation of 20% diluted in distilled water. Pomegranate has been popularly used since ancient times, empirically. Currently, scientific studies *in vitro* and in vivo with different preparations of this plant have supported its antimicrobial and anti-inflammatory properties [17,18]. These activities have demonstrated the therapeutic potential of its fruit from parts such as the rind, leaves, and seeds. We tested bacterial growth in the presence of 1.33, 2.33 and 3.33 mg / mL of pomegranate extract (final concentration). The extract showed its ability to inhibit bacterial growth of the cultivated samples, indicating that an increase in concentration promotes greater inhibition of growth (Figures 6 A and B).

Conclusion

According to our *in vitro* results, we observe the effectiveness of peracetic acid in the effective control of bacterial growth. Copper-based products that are most commonly used today for citrus cancer control, in our *in vitro* assays with copper hydroxide have excellent results in controlling bacterial growth. Chlorhexidinegluconate was effective in growth control when present at 36 µg / mL. Pomegranate extract appears to be effective at high concentrations, tending to complete inhibition of bacterial growth.

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