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Delving into Chemical Control Options for Bacterial Canker (*Clavibacter michiganensis* subsp. *michiganensis*) in Tomatoes: An *In-vitro* Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The 2022/2023 tomato crop witnessed the emergence of *Clavibacter michiganensis* subsp. *michiganensis* in a tomato field, leading to symptoms resembling bacterial canker. Identification of the suspected bacterium, *C*. *michiganensis* subsp. *michiganensis*, utilized specific primers (CMM5 and CMM6) for PCR reaction, resulting in a 614 bp fragment. Several fungicides and bactericides were tested for their ability to control bacterial growth in Petri dishes. Fungicides and bactericides that completely inhibit the bacterial growth in Petri dishes included benzalkonium chloride (250 mg a.i./L), copper oxychloride (1680 mg a.i./L with 1000 mg metallic copper/L), copper hydroxide (2764

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mg a.i./L with 1800 mg metallic copper/L), fluazinam (500 µL a.i./L), difenoconazole + pidiflumetofen (200 + 120 µL a.i./L), cuprous oxide (1344 mg a.i./L with 1200 mg metallic copper /L), mancozeb + famoxadone (1000 + 100 mg a.i./L), mancozeb (4000 mg a.i./L) and metiram + pyraclostrobin (2200 + 200 mg a.i./L). The packaged dose of casugamycin (60 µL a.i./L) failed to completely inhibit *C*. *michiganensis* subsp. *michiganensis* growth, necessitating doses exceeding 140 µL a.i./L for complete inhibition. Only at a dosage of 140 µL a.i./L was there no observable growth on the Petri dish containing YDC. Label doses of casugamycin did not prevent the growth of any bacteria, albeit partially controlling *Clavibacter* and *Pectobacterium* populations. At the dose of 140 µL a.i./L, the sole bacterium that proliferated was *Xanthomonas hortorum* pv. *gardneri*. The other bacteria were included in this study focusing on *Clavibacter* solely to understand the effect of certain products on other important bacteria in tomato cultivation. The active ingredients, difenoconazole + pidiflumetofen (200 + 120 µL/L active ingredient) and fluazinam (500 µL/L active ingredient) effectively suppressed *C*. *michiganensis* subsp. *michiganensis* growth. The study indicates that various tested fungicides and bactericides were effective in curbing *C*. *michiganensis* subsp. *michiganensis* growth under laboratory conditions. Nonetheless, efficacy may fluctuate based on dose and specific product used. Further research, including field trials, is imperative to evaluate product efficacy under real-world conditions and devise comprehensive management strategies for tomato bacterial canker control.

Keywords: Bacteria control; casugamicin, fluazinam; difenoconazole + pidiflumetofen; Pectobacterium; Xanthomonas euvesicatoria pv. Perforans.

1. INTRODUCTION

Tomatoes (*Solanum lycopersicum* L.) are among the most economically important vegetable crops worldwide, contributing significantly to global food security and agricultural economies with approximately 6,059,197ha and a production of 254,449,772.15t worldwide [1]. However, the cultivation of tomatoes is often challenged by various pathogens, including bacteria such as *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), which causes the bacterial wilt and canker in tomato plants first reported in 1910 [2]. It has caused catastrophic epidemics in most tomato-growing areas of the world [3,4]. Commercial fields in Ontario, Canada, have experienced yield losses of up to 84% [5], while in France, losses range from 20% to 30% [6], and in Illinois, USA, they amount to 46% [7].

The Cmm is a gram-positive bacterium known for its devastating effects on tomato plants. This is the only gram-positive bacterium that causes canker in tomato plants. Upon infection, it colonizes the vascular tissues of the plant, leading to wilting, stunting, leaf chlorosis, and the formation of cankers on stems and fruits [8]. These symptoms not only reduce yield but also compromise the quality and marketability of the produce. The bacteria have the potential to generate proteases such as tomatinase and serine proteases [9] as well as wall-degrading enzymes including cellulases [10], xylanases [11], and pectinases [12]. Moreover, the bacterium can persist in seeds, soil and plant debris [13], posing a long-term threat to subsequent crops.

Tomatoes are the primary host of Cmm, but the bacteria can also infect other plant species, albeit with varying degrees of severity. Pepper (*Capsicum* spp.) and potato (*Solanum tuberosum* L.) are among the cultivated plants susceptible to Cmm infection [14,15]. Moreover, certain weed species can serve as alternative hosts (*Solanum nigrum* L., *Solanum americanum* Mill., *Solanum sarrachoides* Sedntner, *Amaranthus blitoides* S. Wats, *Amaranthus albus* L., *Lactuca serriola* L., *Amaranthus retroflexus* L., *Malva parviflora* L. and *Sisymbrium irio* L.), facilitating the persistence and spread of the pathogen in agricultural ecosystems [16]. Symptomless tomato plants also may harbour the bacteria [17], and the bacteria may endophytically colonize non-host plants [8].

Cultural practices such as crop rotation, sanitation, and the use of certified disease-free seedlings are essential for managing Cmm outbreaks. In fact, Cmm survived for at least 24 months in infested debris at the soil surface, but for only 7 months in buried debris [18]. Furthermore, efforts have been made seeking for genetic resistance through breeding programs which offers a promising long-term solution to combatting Cmm in tomato crops. For while, the only manage available in field is the pulverization of bactericides or fungicides with bactericides

effect. Thus, the study aims to evaluate fungicides and bactericides to control *C*. *michiganensis* subsp. *michiganensis in vitro*.

2. MATERIALS AND METHODS

2.1 *Clavibacter* Place of Origin

The bacterium *Clavibacter michiganensis* subsp. *michiganensis* was isolated at coordinates 26°49'03.0"S and 50°59'26.0"W following an epidemic in 2023 in Caçador, Santa Catarina, Brazil.

2.2 Molecular Identification with Specific Primers

The bacteria were isolated on YDC medium. After DNA extraction, the PCR was performed using the specific primers CMM5 (GCGAATAAGCCCATATCAA) and CMM6 (CGTCAGGAGGTCGCCTAATA) for Cmm specific identification [19].

2.3 Effect of Bactericides and Fungicides on Bacterial Canker Control

The chemicals used in this test were: benzalkonium chloride (250 mg/L active ingredient), copper oxychloride (1680 mg/L a.i. with 1000 mg/L of metallic copper), copper hydroxide (2764 mg/L a.i. with 1800 mg/L of metallic copper), fluazinam (500 µL/L a.i.), difenoconazole + pidiflumetofen (200 + 120 µL/L a.i.), cuprous oxide (1344 mg/L a.i. with 1200 mg/L of metallic copper), mancozeb + famoxadone (1000 + 100 mg/L a.i.), mancozeb (4000 mg/L a.i.), metiram + pyraclostrobin (2200 + 200 mg/L a.i.), probineb (2100 mg/L a.i.), metiram (2100 mg/L a.i.), mancozeb + cymoxanil (1920 + 240 mg/L a.i.), casugamicin (60 µL/L a.i.), acetic acid (2000 µL/L per comercial product), oxathiapiproline + famoxadone (150 + 1500 µL/L a.i.).

The products were individually mixed with YDC (base medium n.º31) (glucose 20 g/L, yeast extract 10 g/L, $CaCO_3$ 20 g/L, and agar 20 g/L). Twelve streaks were made using the bacterium *C*. *michiganensis* subsp. *michiganensis* on each plate. Each streak was observed and assessed for bacterial growth. These data were then transformed into percentages relative to the control, which consisted solely of the YDC culture medium without products.

2.4 Effect of Different Doses of Casugamicin against *Clavibacter*

Casugamicin was individually mixed with YDC medium at doses of 0, 60, 80, 100, 120, 140, 160, 180 and 200 µL a.i./L. Stretch marks were made using the bacterium *C*. *michiganensis* subsp. *michiganensis* on each plate. Each streak was observed and assessed for bacterial growth. The resulting data were then transformed into percentages relative to the control, which consisted solely of the YDC culture medium without products.

2.5 Effect of Casugamicin against *Clavibacter*, *Xanthomonas* and *Pectobacterium*

In a Petri dish containing YDC medium, casugamicin was added at doses of 60 and 140 µL a.i./L. The bacteria *Clavibacter*, *Pectobacterium*, *Xanthomonas gardneri*, and *Xanthomonas perforans* were streaked at each edge of the Petri dish. After seven days, the plates were qualitatively evaluated for bacterial growth. The control consisted solely of the YDC culture medium without product. Specific primers were employed to identify other bacterial species: *X*. *gardneri* (Bs-XgF/Bs-XgR), *X*. *perforans* (Bs-XpF/Bs-XpR), and *Pectobacterium* (Y1/Y2). The other bacteria were included in this study focusing on *Clavibacter* solely to understand the effect of certain products on other important bacteria in tomato cultivation.

2.6 Effect of Difenoconazole + Pidiflumetofen and Fluazinam against *Clavibacter*, *Xanthomonas* and *Pectobacterium*

In a Petri dish containing YDC médium, difenoconazole + pidiflumetofen and fluazinam were added at doses of 200 + 120 µL a.i./L and 500 µL a.i./L, respectively, according to the treatment. The bacteria *Clavibacter*, *Pectobacterium*, *X*. *gardneri*, and *X*. *perforans* were streaked at each edge of the Petri dish. After seven days, the plates were qualitatively evaluated for bacterial growth. The control consisted solely of the YDC culture médium without product. The other bacteria were included in this study focusing on *Clavibacter* solely to understand the effect of certain products on other important bacteria in tomato cultivation.

3. RESULTS AND DISCUSSION

3.1 *Clavibacter* Place of Origin

In the 2022/2023 tomato crop, conditions were favorable for the appearance of *Clavibacter michiganensis* subsp. *michiganensis*. The symptoms of the disease were typical of bacterial canker: drying of the edge of the leaves and generalized burning as the disease progressed (Fig. 1).

3.1.1 Molecular identification with specific primers

The identification of the species of bacteria found in the tomato crop in which the suspect was *Clavibacter michiganensis* subsp. *michiganensis* was confirmed with the specific primers for the bacteria CMM5 and CMM6, generating a fragment with 614 bp (Fig. 2).

3.1.2 Effect of bactericides and fungicides on bacterial canker control

The fungicides and bactericides that completely inhibit the bacterial growth in Petri dishes were: benzalkonium chloride (250 mg/L a.i.), copper oxychloride (1680 mg/L a.i. with 1000 mg/L a.i. of metallic copper), hydroxide copper (2764 mg/L a.i. with 1800 mg/L), fluazinam (500 µL/L a.i.). difenoconazole + pidiflumetofen (200 + 120 µL/L a.i.), cuprous oxide (1344 mg/L a.i. with 1200 mg/L of metallic copper), mancozeb + famoxadone (1000 + 100 mg/L a.i.), mancozeb (4000 mg/L a.i.), metiram + pyraclostrobin (2200 + 200 mg/L a.i.) (Fig. 3 and Fig. 4).

3.1.3 Effect of different doses of *casugamicin* against *Clavibacter*

The packaged dose of casugamycin was not able to complete inhibit the growth of *C. michiganensis* subsp. *michiganensis* and this was only achieved at doses above 140 µL a.i./L (Fig. 5 and Fig. 6).

3.1.4 Effect of casugamicin against *Clavibacter*, *Xanthomonas* and *Pectobacterium*

The label dose of casugamicin did not prevent the growth of any bacteria, although it controlled part of the *Clavibacter* and *Pectobacterium* population. At the dose of 140 µL a.i./L, the only bacteria that grew was *X*. *hortorum* pv. *gardneri* (Fig. 7).

Fig. 1. Area of tomato affected by bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*)

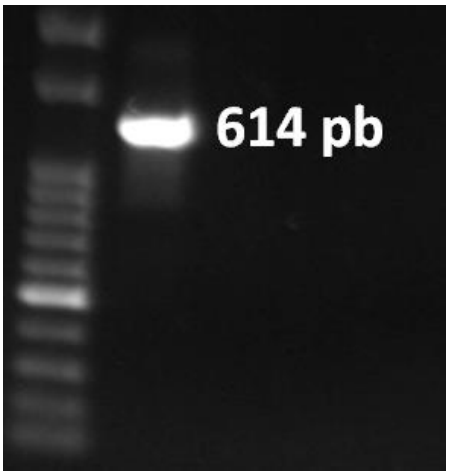

Fig. 2. Molecular identification of *Clavibacter michiganensis* subsp. *michiganensis* with specific primers CMM5 and CMM6. A - 50 bp DNA Ladder, Size range: 50 bp - 1000 bp, B - *Clavibacter michiganensis* subsp. *michiganensis* (CCM5 and CMM6 - 614 bp)

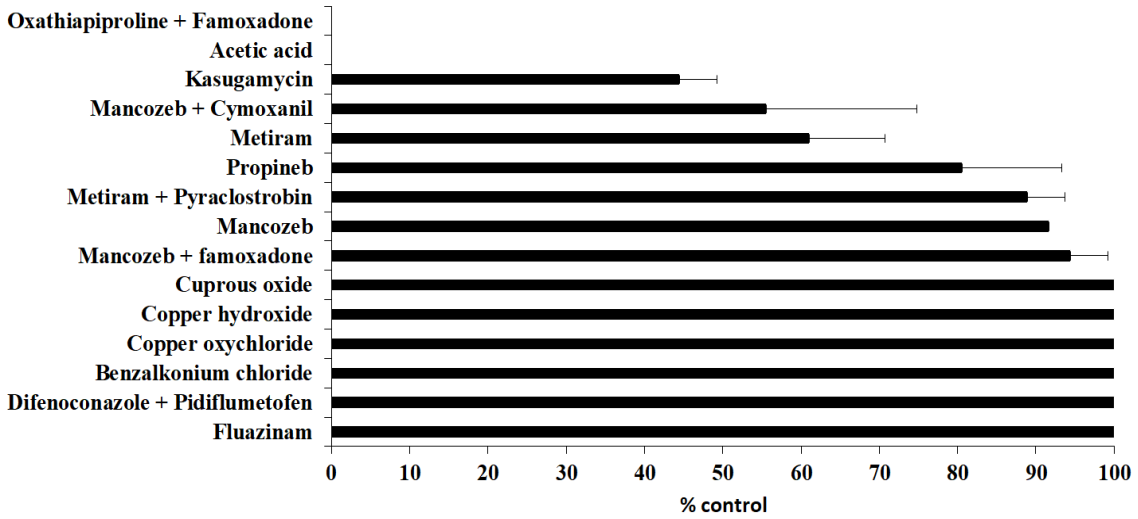

Fig. 3. Effect of bactericides and fungicides on the control of *Clavibacter michiganensis* subsp. *michiganensis in vitro*

3.1.5 Effect of difenoconazole + pidiflumetofen and fluazinam against *Clavibacter, Xanthomonas* and Pectobacterium

The active ingredients difenoconazole + pidiflumetofen (200 + 120 µL/L a.i.) and fluazinam (500 µL/L a.i.) controlled *C. michiganensis* subsp. *michiganensis* (Fig. 8).

3.2 Discussion

The emergence of *Clavibacter michiganensis* subsp. *michiganensis* during the 2022/2023 tomato crop underscores the significance of understanding and managing bacterial diseases in agriculture. The symptoms observed, characterized by leaves edge drying and generalized burning, are indicative of bacterial canker, a well-known threat to tomato crops. Identification of the suspected bacterium using specific primers in PCR provided crucial insights into the microbial composition of the affected tomato crop, confirming the presence of *C. michiganensis* subsp. *michiganensis* by the use of specific primers CMM5 and CMM6 [19].

The evaluation of various fungicides and bactericides for their efficacy in controlling bacterial growth offers valuable information for disease management strategies. Among the tested compounds, benzalkonium chloride,

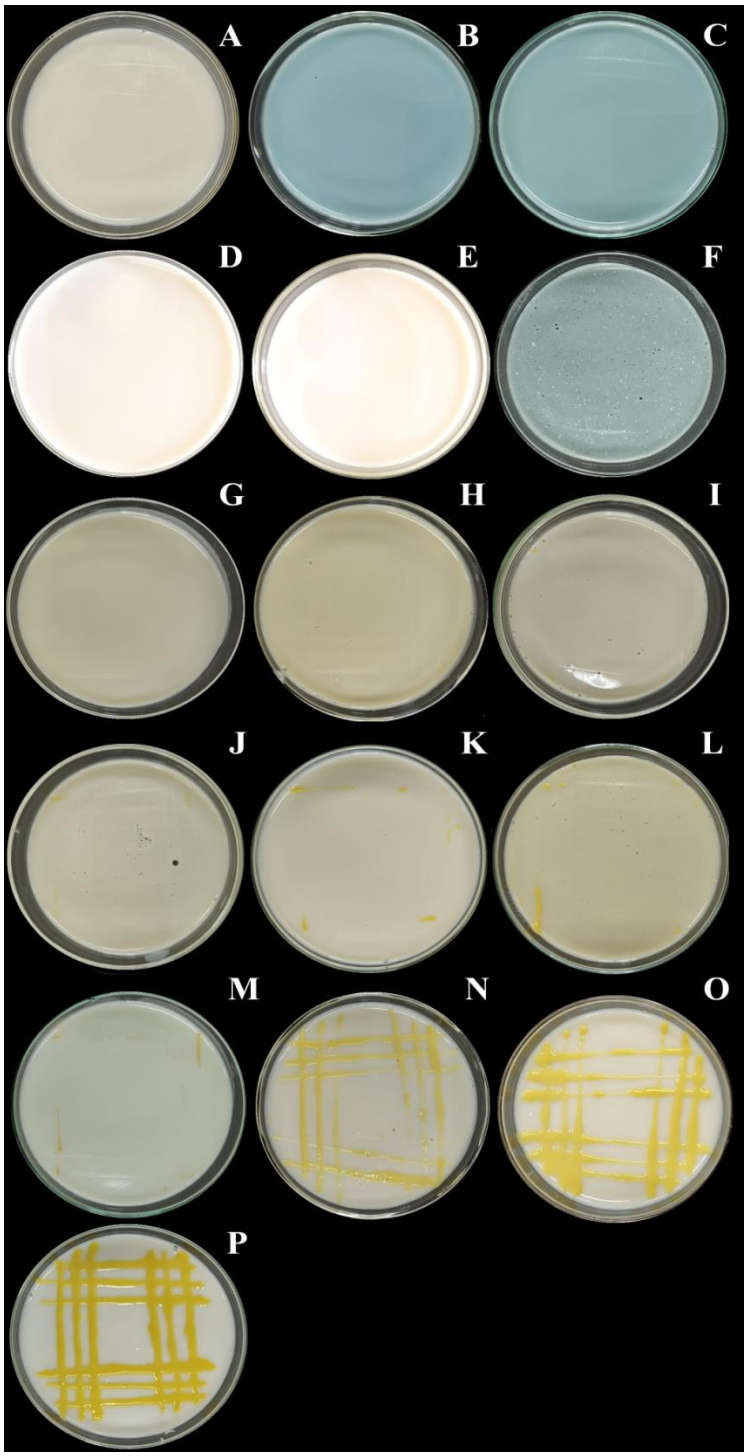

Fig. 4. Effect of bactericides and fungicides on bacterial canker control. A – Benzalkonium chloride (250 mg/L a.i.). B – Copper oxychloride (1680 mg/L a.i. with 1000 mg/L a.i. of metallic copper). C – Copper hydroxide (2764 mg/L a.i. with 1800 mg/L). D – Fluazinam (500 µL/L a.i.). E – Difenoconazole + Pidiflumetofen (200 + 120 µL/L a.i.). F – Cuprous oxide (1344 mg/L a.i. with 1200 mg/L of metallic copper). G – Mancozeb + Famoxadone (1000 + 100 mg/L a.i.). H – Mancozeb (4000 mg/L a.i.). I – Metiram + Pyraclostrobin (2200 + 200 mg/L a.i.). J – Probineb (2100 mg/L a.i.). K – Metiram (2100 mg/L a.i.). L – Mancozeb + Cymoxanil (1920 + 240 mg/L a.i.). M – Casugamicin (60 µL/L a.i.). N – Acetic acid (2000 µL/L p.c.). O – Oxathiapiproline + Famoxadone (150 + 1500 µL/L a.i.). P – Control

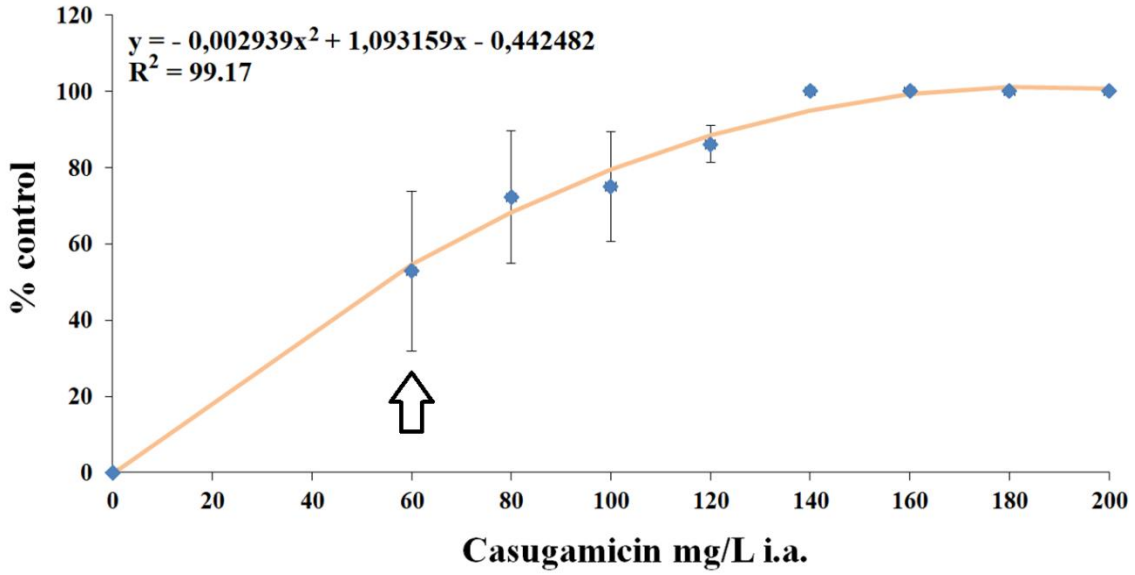

Fig. 5. Effect of increasing doses of casugamicin against *Clavibacter michiganensis* subsp. *michiganensis*. The empty black arrow indicates the label dose

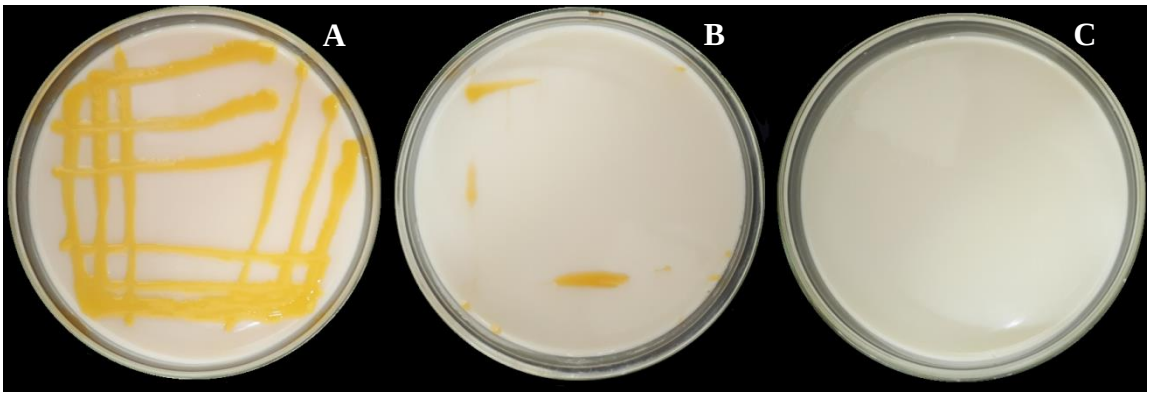

Fig. 6. Effect of casugamycin on *Clavibacter michiganensis* subsp. *michiganensis*. A – Control. B – 60 µL casugamycin a.i./L C – 140 µL casugamycin a.i./L

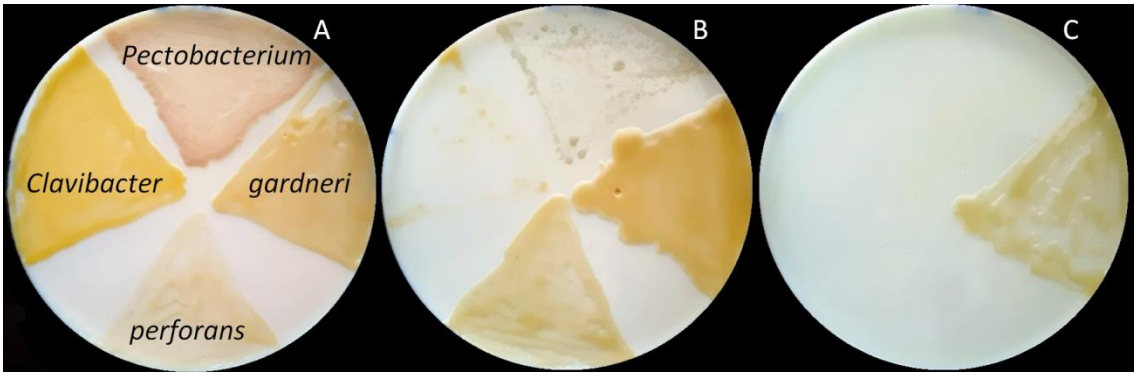

Fig. 7. Effect of casugamycin against *Xanthomonas hortorum* pv. *gardneri*, *X. euvesicatoria* pv. *perforans*, *Clavibacter michiganensis* subsp. *michiganensis*. and *Pectobacterium*. A – Check. B – 60 µL casugamicin a.i./L (label dose). C – 140 µL casugamicin a.i./L

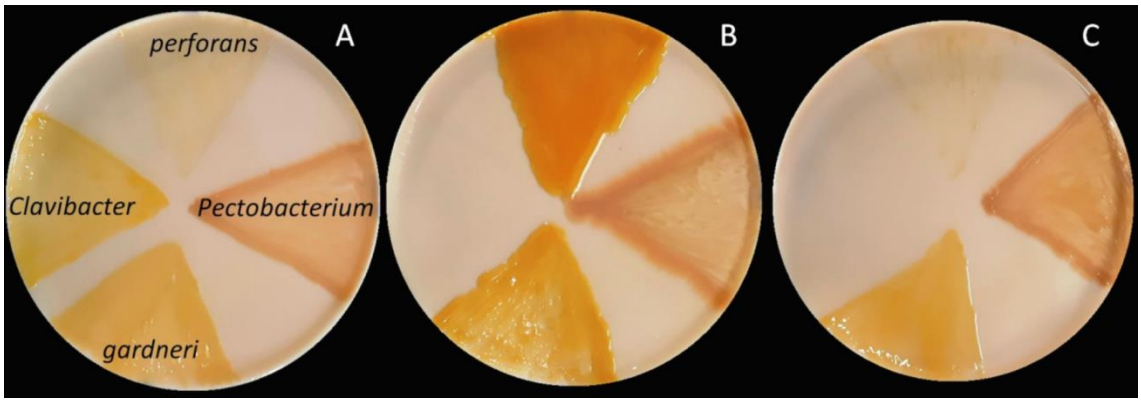

Fig. 8. Effect against *Xanthomonas hortorum* pv. *gardneri*, *X. euvesicatoria* pv. *perforans*, *Clavibacter michiganensis* subsp. *michiganensis*. and *Pectobacterium*. A – Check. B – *Difenoconazole* + *Pidiflumetofen* (200 + 120 µL a.i./L). C – *Fluazinam* (500 µL a.i./L)

copper-based compounds (copper oxychloride and copper hydroxide), fluazinam, difenoconazole + pidiflumetofen, cuprous oxide, mancozeb + famoxadone, mancozeb, and metiram + pyraclostrobin demonstrated promising results by achieving more than 90% inhibition of bacterial growth in Petri dishes. The use of copper compounds such as copper hydroxide and copper sulfate, as well as bactericides like streptomycin and mancozeb, alone or in combination, has been highlighted by other authors as effective in managing Cmm [20,21,22,23].

The inability of the packaged dose of casugamycin to achieve complete control of bacterial growth, necessitating higher doses for efficacy, suggests limitations in its application for managing this particular bacterial strain. Notably, only at a dose of 140 µL a.i./L did the casugamycin exhibit satisfactory inhibition of bacterial growth, emphasizing the importance of dose optimization in disease management protocols. While casugamycin has demonstrated efficacy at elevated doses for managing Cmm, we advise against exceeding the recommended dosage specified in the product insert, as applications must adhere to current regulations.

Furthermore, the differential effects of casugamycin on various bacterial populations, with partial control over *Clavibacter* and *Pectobacterium* but allowing the growth of *X. hortorum* pv. *gardneri* even at the 140 µL a.i./L dose, indicate complex interactions between the applied treatments and the microbial community. Such findings underscore the need for a nuanced approach to disease management, considering not only the target pathogen but also the broader microbiome dynamics within the agricultural ecosystem. Researchers working with broth macrodilution method found that even the dose of 500 $\mu g.ml^{-1}$ was not suficient to prevent the growth of Cmm [22]. Here in, mixing the casugamycin into the media using the dose of 140 µL a.i./L prevent the Cmm growth (Fig. 6). It is hightlighted that results can vary according to the method chosen.

The effectiveness of difenoconazole + pidiflumetofen and fluazinam in controlling *C. michiganensis* subsp. *michiganensis* highlights the potential utility of these compounds as part of integrated disease management strategies. However, their performance under field conditions warrants further investigation to assess real-world efficacy and potential impacts on non-target organisms and environmental health. The success of fluazinam in controlling *X. perforans* has been previously reported [24]. Despite the bactericidal effect of fluazinam, in Brazil, this fungicide is only recommended for the control of fungi and oomycetes. The difenoconazole + pidiflumetofen, on the other hand, is recommended in the label for *Septoria*, *X. perforans*, and *X. vesicatoria*. We believe that the putative reason why the fungicides pidiflumetofen and fluazinam may have bactericidal action is the presence of chlorine and fluorine in their molecules, elements known to be bactericidal.

The study provides valuable insights about the sensibility of Cmm to some active ingredients, highlighting the importance of early detection, reliable species identification, and targeted application of bactericides or known fungicides able to control bacteria. Moving forward,

continued research efforts are essential to refine disease management strategies and ensure sustainable agricultural practices.

4. CONCLUSION

Doses of casugamycin exceeding 140 µL a.i./L, surpassing the recommended dosage, were required for complete inhibition of *Clavibacter michiganensis* subsp. *michiganensis* growth. Additionally, fungicides and bactericides such as difenoconazole + pidiflumetofen and fluazinam were effective in suppressing the growth of this bacterium under laboratory conditions.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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