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Eucalyptus species extracts inhibiting *in vitro* growth of the phytobacterium *Xanthomonas campestris* pv. *campestris*

Extratos de espécies de *Eucalyptus* inibindo o crescimento *in vitro* da fitobactéria *Xanthomonas campestris* pv. *campestris*

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Abstract

This study was carried out to evaluate the antibacterial potential of *Eucalyptus* spp. against two strains of the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* (*Xcc*). The treatments consisted of aqueous and hydroethanolic extracts, at concentrations of 50% (experiment 1) and 35% and 15% (experiment 2), which were studied by the double layer inhibition test. The evaluated species were *E. camaldulensis, E. cloeziana, E. grandis, E. "urograndis"* and *E. urophylla*. Based on the results of experiment 1, it was observed that most of the extracts that inhibited the growth of the bacterial strains tested were hydroethanolic, except for the aqueous extract of the *E. "urograndis"* hybrid. Regarding experiment 2, it was observed that the greatest inhibition was with the concentrated extracts (35%), but the difference for the antibacterial effect was not significant in this case. Comparing the experiments, it was observed that in general the concentrated extracts (50%) had greater inhibition than the diluted ones. These results represent the potential of aromatic plants in the ecological management of *Xcc*, with emphasis on the aqueous and hydroethanolic extracts of *E. cloeziana*. Subsequently, bioassays will be performed to verify the effectiveness of such extracts *in vivo*.

Keywords: Alternative management of plant bacteriosis; Black rot; Arboreal plant extracts; Eco-friendly measures; Plant pathogenic bacteria.

Resumo

Este estudo foi realizado para avaliar o potencial antibacteriano de extratos de *Eucalyptus* spp. contra duas estirpes da bactéria fitopatogênica *Xanthomonas campestris* pv. *campestris (Xcc)*. Os tratamentos consistiram em extratos aquosos e hidroetanólicos, nas concentrações de 50% (experimento 1) e 35% e 15% (experimento 2), os quais foram avaliados pelo teste de inibição em dupla camada. As espécies estudadas foram: *E. camaldulensis, E. cloeziana, E. grandis, E. "urograndis"* e *E. urophylla.* Com base nos resultados do experimento 1, observou-se que a maioria dos extratos que inibiram o crescimento das estirpes bacterianas testadas foram hidroetanólicos, exceto o extrato aquoso do híbrido de *E. "urograndis"*. Em relação ao experimento 2, observou-se que a maior inibição foi com os extratos concentrados (35%), mas a diferença do efeito antibacteriano não foi significativa neste caso. Comparando os experimentos, observou-se que os extratos concentrados (50%) maior inibição do que os diluídos. Estes resultados representam o potencial de plantas aromáticas para o manejo ecológico de *Xcc*, com destaque para os extratos aquoso e hidroetanólico de *E. cloeziana.* Posteriormente serão realizados bioensaios para verificação da efetividade de tais extratos *in vivo*.

Palavras-chave: Manejo alternativo de bacterioses de plantas; Podridão negra; Extratos de plantas arbóreas; Medidas ecologicamente corretas; Bactérias fitopatogênicas.

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INTRODUCTION

Xanthomonas is a large genus of numerous plant-associated proteobacteria (Saddler & Bradbury, 2005). Among the economically important species or pathovars of these bacteria, *X. campestris* pv. *campestris* (*Xcc*) is the causal agent of black rot (Vauterin et al., 1995), considered the most important cruciferous (Brassicaceae) diseases worldwide (Vicent & Holub, 2013), causing losses of up to 60% (Dzhalilov & Tiwari, 1995). Spread by seeds (Van Der Wolf & Van Der Zouwen, 2010) and able to survive for more than 255 days in crop residues (Silva Júnior et al., 2020), the bacteriosis is difficult to manage. Recent genomic studies have revealed that variations in exopolysaccharide (EPS) and lipopolysaccharide (LPS) composition help the bacterium to evade plant defense systems, in addition to a direct correlation between genetic variability and virulence (Chen et al., 2022).

The main form of control for this disease is through exclusion, preventing the introduction of inoculum in unaffected areas (Vicent & Holub, 2013), but genetic resistance is also important (Santos et al., 2008). The chemical control of *Xcc* is done mostly with copper-based products, for which resistance in this bacterium has already been described (Lugo et al., 2013; Behlau et al., 2017). In this context, the adoption of “alternative” or “eco-friendly” measures, in the integrated management for disease control, has become a reality for producers (El Khoury & Makkouk, 2010), especially those seeking sustainable and environmentally safe management.

Eucalyptus is a botanical genus that includes more than 800 species. This forest tree is not only cultivated to obtain wood, cellulose and firewood, but also has numerous medicinal and aromatic properties (Coppen, 2002). Eucalyptus is the most planted tree in Brazil. This is due to its successful adaptation to different environmental conditions in the country, which is located at a latitude similar to that of Australia, the tree’s center of origin, and due to properties that give it several different uses (Castro et al., 2016).

The antimicrobial effect of *Eucalyptus* spp. has been studied, demonstrating its potential (Sabo & Knezevic, 2019; Mendes & Marques, 2021). The application of plant extracts emerged as a new approach to plant pathogen management, since plant extracts have several bioactive compounds capable of inhibiting the growth of microorganisms (Gurjar et al., 2012; Choudhury et al., 2018; Müller-Heupt et al., 2022). Some studies have reported the use of plant extracts to protect plants against diseases caused by *Xanthomonas* (Satish et al., 1999). With reference to eucalyptus, Lucas et al. (2012) investigated how its essential oil (without identifying the species) inhibited *X. vesicatoria* (bacterial spot) *in vitro* and *in vivo*, as also reported by Negi & Kumar (2015), who studied the inhibition of *X. citri* subsp. *citri* (citrus canker). It has already been shown that the extract of *E. camaldulensis* showed activity against *X. citri* subsp. *malvacearum*, the causal agent of angular cotton spot (Rashid et al., 2016). Abdurrahman et al. (2020) also studied the antimicrobial potential of *Eucalyptus* sp. against *X. vesicatoria*, and Abo-Elyousr et al. (2020) observed that the extract of *E. camaldulensis* inhibited the growth of this bacterium and induced resistance to tomato leaf spot.

Concerning *Xcc*, Vigo-Schultz et al. (2006) evaluated the efficacy of the ethanolic tincture of guaco (*Mikania glomerata* Spreng) in the control of black rot of crucifers both *in vitro* and *in vivo*. Similarly, Franzener et al. (2007) studied the antibacterial activity of hydrolates (by-products of distillation in the extraction of essential oils) from medicinal plants against this bacterium. Sain et al. (2007) also examined several plants for *in vitro* control of *Xcc*. Popović et al. (2018) assessed 30 essential oils in inhibiting phytobacteria, including *Xcc*. Fontana et al. (2021) recently investigated the effect of moringa extract (*Moringa oleifera* Lam.), both *in vitro* and *in vivo*, on the control of black rot. Additionally to these studies, Mendes & Marques (2021) evaluated the effect of lemon-scented gum extracts on this phytobacterium.

Based on the above, this study aimed to evaluate the *in vitro* growth inhibition of *Xcc* by aqueous and hydroethanolic extracts of five species of *Eucalyptus*.

MATERIAL AND METHODS

Bacterial isolates

The two bacterial strains of *X. c.* pv. *campestris* used in this study were provided by the Plant Pathogenic Bacteria Collection from the Department of Plant Pathology at the University of Brasília, identified as UnB 831 and UnB 1175, both isolated from kale (*Brassica oleracea* var.

acephala). These strains are stored in sterile distilled water, in a working collection, and were reactivated in NA (Nutrient Agar) medium.

Where the experiment was performed and how extracts of *Eucalyptus* spp. were obtained

The study was conducted in Goiânia, Goiás, at the laboratory of the Núcleo de Pesquisa em Fitopatologia, Escola de Agronomia, Universidade Federal de Goiás, *Campus* Samambaia.

The plants were obtained from seeds provided by IPEF – the Institute for Forestry Research and Studies. Leaves of seedlings that were approximately five months old were harvested from the seedbed. To make the extracts, 5 g of leaves were used, which were macerated in 10 mL of distilled water and hydroalcoholic solution (ethanol:water, 70:30). The maceration was carried out with the aid of a crucible and mortar. The eucalyptus species tested were *E. camaldulensis, E. cloeziana, E. grandis, E. urophylla* and hybrid *E. "urograndis"* (*E. urophylla* x *E. grandis*).

Before use, the extracts were stored at 4ºC and at the time of use they were sterilized by filtration with a 0.22 µm membrane.

Double layer inhibition test

Experiment 1

To assess the antagonism of *Eucalyptus* spp. extracts to *X. c.* pv. *campestris*, the double layer diffusion inhibition method was used, which consisted of making a base layer of Nutrient Agar medium, placing two paper discs (350 g/m^2) at equidistant points per Petri dish and pipetting 5 µL of each extract. Then, a suspension of approximately 10 x 10^8 CFU/mL (equivalent to McFarland Scale 7) was prepared, from which 25 µL were withdrawn and then added to 5 mL of semi-solid NA medium (0.8%) fluxing (48 °C), forming a covering layer in the middle base, according to Romeiro (2007, with modifications). As a control treatment, discs containing the antibiotic streptomycin (10 µg/disc) were used.

Experiment 2

The second experiment was carried out in the same way as experiment 1; however, extracts that inhibited bacterial growth by 50% were selected from the previous experiment, now being tested at 15% and 35% concentration for one of the strains, as already performed by Mendes & Marques (2021).

Experimental design and statistical analysis

The design used was completely randomized, in a factorial arrangement (5 x 2 x 2 or 5 x 2) with three replications, consisting of three plates, containing two paper discs, including the five treatments (composed of the respective extract/dilution), two bacterial strains (UnB 831 and UnB 1175), two extracts (aqueous and hydroethanolic) and the controls (discs with the antibiotic streptomycin). In the evaluation, the formation of inhibition zones was observed, which had their diameters (in cm) measured with the aid of a millimeter ruler.

The experimental data were subjected to an analysis of variance (ANOVA), using the SISVAR 5.6 Program (Ferreira, 2014). The mean values of the inhibition parameters were compared by the Scott-Knott test, at 5% probability.

RESULTS AND DISCUSSION

Based on the results of experiment 1 (Figure 1), most of the extracts that inhibited the growth of *X. c.* pv. *campestris* strains tested were hydroethanolic, except for the aqueous extract from plant 4 (P4 - *E. urograndis* hybrid). The zone of inhibition averages (Figure 2) ranged between 0.85 and 1.43 cm for bacterial strain UnB 831 and 0.88 and 1.53 cm for UnB 1175. The control treatments with streptomycin exhibited inhibition zones of 1.77 and 1.44 cm for UnB 831 and UnB 1175, respectively. The treatments with extracts exhibited smaller inhibition zones than the control treatment (streptomycin), except for the treatment with the hydroethanolic extract of *E. cloeziana*, against the bacterial strain UnB 1175, which exhibited an average inhibition (1.53 cm) greater than the control (1.44 cm), standing out in this stage of the bioassay.

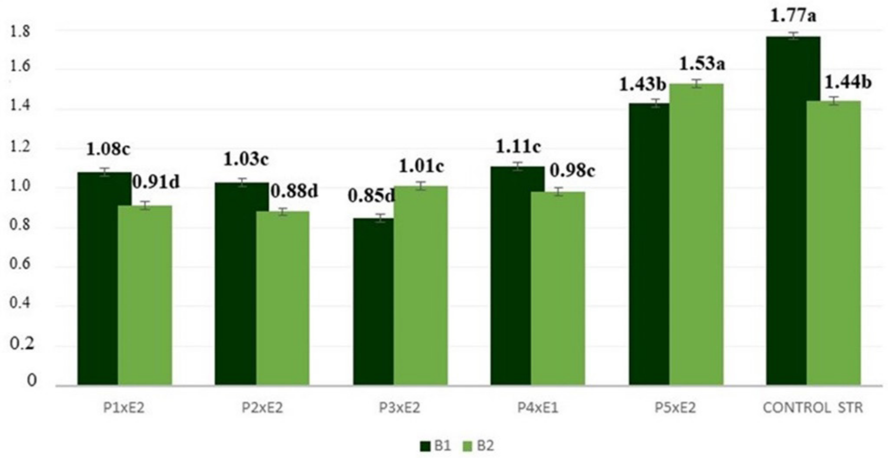

Figure 1. Positive results of the antagonism bioassay (Y axis - inhibition zones in cm) of *Eucalyptus* spp. 50% extracts, in the growth of two strains of the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* (X axis), where: B1 (UnB 831 strain), B2 (UnB 1175 strain), E1 (aqueous extracts), E2 (hydroethanolic extracts), P1 (*E. camaldulensis*), P2 (*E. grandis*), P3 (*E. urophylla*), P4 (*E. urograndis*), P5 (*E. cloeziana*) and STR (Control with streptomycin).

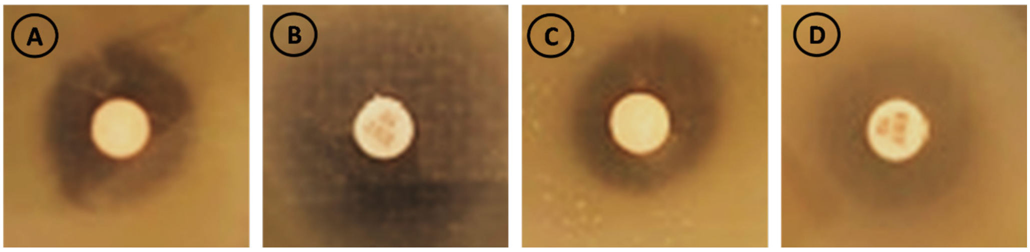

Figure 2. Inhibition zones of hydroethanolic extracts from *Eucalyptus cloeziana* to *Xanthomonas campestris* pv. *campestris* in experiment 1 (50%), where: A) UnB 831 strain (1.43 cm) B) UnB 831 streptomycin control (1.77 cm), C) UnB 1175 strain (1.53 cm) and D) UnB 1175 streptomycin control (1.44 cm).

Based on the results of experiment 2 (Y axis in Figure 3), now comparing extracts at 15% and 35% concentration, it was observed that the selected strain (UnB 831) was inhibited most by the more concentrated extracts (at 35%), although not significantly. On the other hand, at the concentration of 15%, the extract of *E. cloeziana* stood out and differed significantly from the others. The inhibition zones' averages (Figure 4) ranged between 0.71 and 1.03 cm for 15% and 0.95 and 1.13 cm for 35% concentration.

Comparing statistically the results of experiments 1 and 2, it was only for the twice-tested strain UnB 831 (Figure 5) that concentrated extracts (50%) were seen to inhibit more than the diluted ones, with a significant difference in treatments with the species *E. camaldulensis* (P1), *E. urograndis* (P4) and *E. cloeziana* (P5). In the case of *E. grandis* (P2) extracts, there was no significant difference between 50% and 35% concentrations. Moreover, for the treatment with *E. urophylla* (P3) extracts, it was observed that there was no difference in inhibition between 15 and 35% concentrations.

As shown, the demand for and study of plant extracts that can be used in the management of diseases in agriculture is growing (Gurjar et al., 2012; Choudhury et al., 2018). In Brazil, for example, there is already a commercial botanical product, registered in the Ministry of Agriculture, Livestock and Supply, based on the extract of *Melaleuca alternifolia* Cheel leaves (tea tree), recommended for the management of various fungi and phytopathogenic bacteria, including some *Xanthomonas* species (Agrofit, 2022).

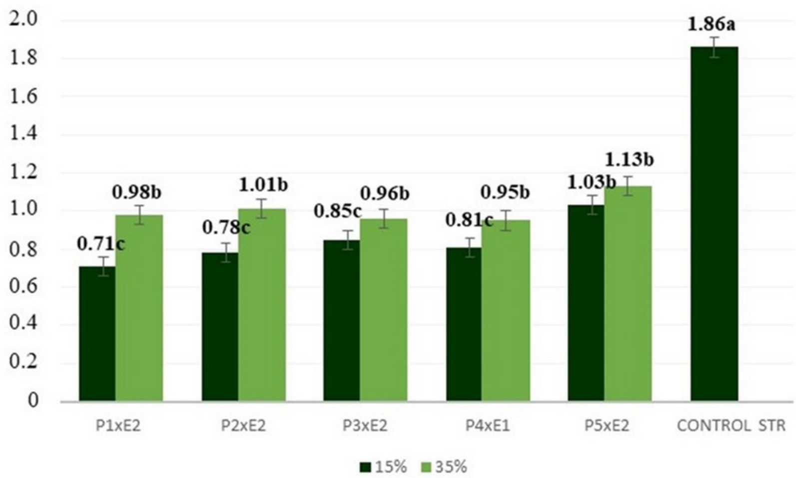

Figure 3. Result of antagonism (Y axis – inhibition zones in cm) of *Eucalyptus* spp. extracts at 15% and 35%, in the growth of bacterial *Xanthomonas campestris* pv. *campestris* strain UnB 831 (X axis), where: E1 (aqueous extracts), E2 (hydroethanolic extracts), P1 (*E. camaldulensis*), P2 (*E. grandis*), P3 (*E. urophylla*), P4 (*E. urograndis*), P5 (*E. cloeziana*) and STR (Control with streptomycin).

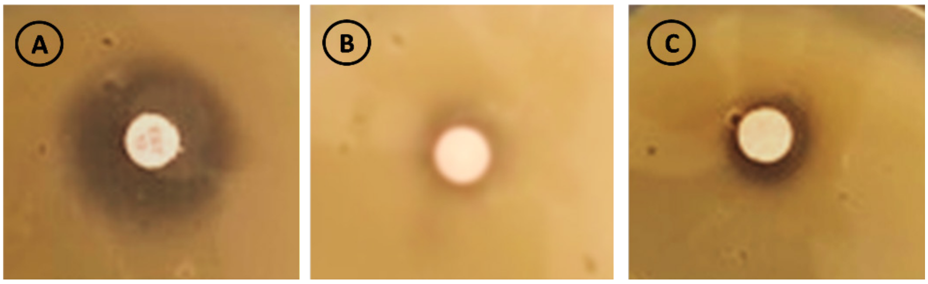

Figure 4. Inhibition zones of *Eucalyptus cloeziana* extracts in relation to *Xanthomonas campestris* pv. *campestris* (UnB 831) in experiment 2, where: A) Control treatment with streptomycin (1.86 cm), B) Extract at 15% (1.03 cm) and C) Extract at 35% (1.13 cm).

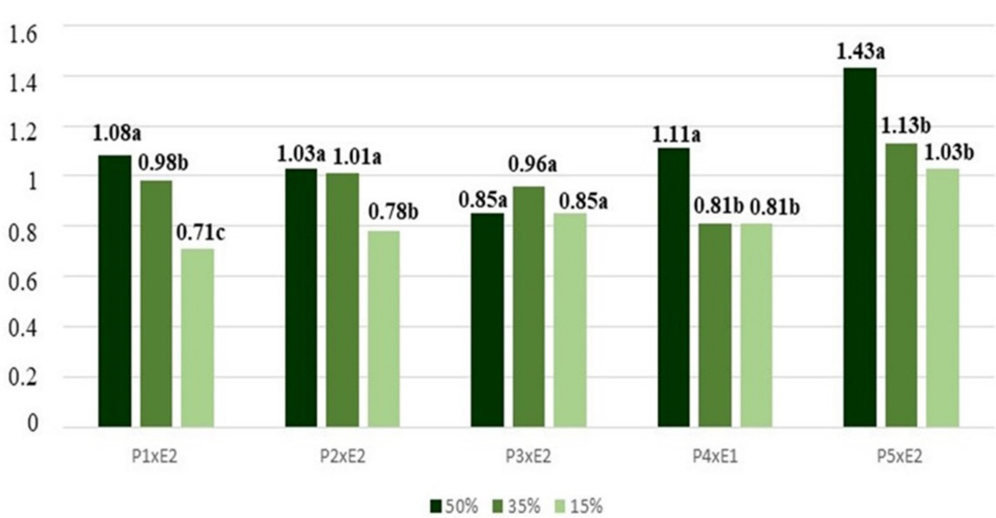

Figure 5. Joint analysis of the inhibition results of *Eucalyptus* spp. extracts in the growth of the strain of *Xanthomonas campestris* pv. *campestris* (UnB 831), where: E1 (aqueous extracts), E2 (hydroethanolic extracts), P1 (*E. camaldulensis*), P2 (*E. grandis*), P3 (*E. urophylla*), P4 (*E. urograndis*) and P5 (*E. cloeziana*).

As regards the plants used in this study, it is already known that *Eucalyptus* is a botanical genus with recognized medicinal, aromatic (Coppen, 2002) and also antimicrobial properties (Sabo & Knezevic, 2019; Müller-Heupt et al., 2022), supporting the results observed here.

Among the known antibacterial active molecules in *Eucalyptus* species are aromadendrene, citronellal, citronellol, cineol (Mulyaningsih et al., 2011) and α-pinene (Sebei et al., 2015).

Thus, the antibacterial potential of eucalyptus has been investigated and assessed. Lucas et al. (2012) reported that eucalyptus essential oil (species not informed) inhibited the *in vitro* growth of *X. vesicatoria* at concentrations of 10 and 100%, with inhibition zones of 3.0 and 2.5 cm, respectively. Negi & Kumar (2015) observed inhibition of *X. citri* subp. *citri* by aqueous extracts of *Eucalyptus* sp. leaves, with larger zones at the maximum concentration of 20% (between 1.13 and 1.73 cm). The aqueous extract of *E. camaldulensis* significantly inhibited the *in vitro* growth of *X. citri* pv. *malvacearum* (between 0.34 and 1.12 cm) and the incidence of the cotton bacterial blight in the field (Rashid et al., 2016). Similarly, Abdurrahman et al. (2020) observed that the hydroethanolic extract of *Eucalyptus* sp. showed an inhibitory effect on *X. vesicatoria in vitro* (between 0.87 and 1.1 cm) and on tomato leaf spot severity. Supporting the present study, Abo-Elyousr et al. (2020) also observed that ethanolic extracts of *E. camaldulensis* inhibited the *in vitro* growth of *X. vesicatoria* more than the aqueous ones, besides inducing resistance to leaf spot (increase in peroxidase activity).

As for *Xcc*, Vigo-Schultz et al. (2006) evaluated the efficacy of the ethanolic tincture of guaco (*Mikania glomerata* Spreng) in the control of black rot of crucifers both *in vitro* and *in vivo*, observing greater inhibition of the bacterium at a maximum concentration of 0.1%. In this same sense, Franzener et al. (2007) studied the antibacterial activity of hydrolates from medicinal plants against this plant pathogenic bacterium and reported that citronella hydrolates (*Cymbopogon nardus* (L.) Rendle) at a concentration of 25% promoted inhibition close to 30%. Popović et al. (2018) examined 30 essential oils in inhibiting phytopathogenic bacteria, including *Xcc*. The researchers observed zones of inhibition ranging between 7.25 and 3.5 cm. In addition to these studies, Fontana et al. (2021) assessed the effect of moringa extract (*Moringa oleifera* Lam.), both *in vitro* and *in vivo*, on the control of black rot, with concentrations of 0.25%, 0.5% and 0.1% exhibiting an antibacterial effect.

Although the antimicrobial potential of *E. cloeziana* against *Xcc,* which stood out in this study, was not found in the literature, Tian et al. (2020) reported the inhibitory effect of its essential oil on human pathogenic bacteria *Staphylococcus aureus, Salmonella typhi, Bacillus subtilis* and *Escherichia coli*.

Corroborating the results of the present study, Mendes & Marques (2021) observed inhibition zones varying between 0.26 and 1.3 cm when using hydroethanolic and aqueous extracts of lemon-scented gum (*Corymbia citriodora* (Hook.) K.D. Hill & L.A.S. Johnson) against *Xcc*. There, the 35% alcoholic extract inhibited the *X. vesicatoria* strain more than 50%, but the more concentrated extracts (50%) also showed greater inhibition of *X. c.* pv. *campestris* (also UnB 831) as was observed here. According to Mostafa et al. (2018), the difference in the inhibitory concentration of plant extracts is due to the variation in their chemical constituents and the volatile nature of some of their components. Thus, in general, it is observed that the ethanolic extracts exhibit lower inhibitory concentrations than most of the corresponding aqueous extracts.

CONCLUSIONS

The hydroethanolic extracts of the five eucalyptus species exhibit inhibitory activity *in vitro* against *Xanthomonas campestris* pv. *campestris*, with emphasis on *Eucalyptus cloeziana* extracts, which can represent an eco-friendly management strategy for this bacterium. The results, once again, demonstrate that the inhibition is isolate-specific and dependent on the plant extract concentration, in general higher in the more concentrated treatments. New studies will be carried out with new concentrations and in the *in vivo* preventive control of cruciferous black rot.

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