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# Antibacterial Activity of Alkyl Gallates against *Xanthomonas citri* subsp. *citri*

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**The plant-pathogenic bacterium *Xanthomonas citri* subsp. *citri* is the causal agent of Asiatic citrus canker, a serious disease that affects all the cultivars of citrus in subtropical citrus-producing areas worldwide. There is no curative treatment for citrus canker; thus, the eradication of infected plants constitutes the only effective control of the spread of *X. citri* subsp. *citri*. Since the eradication program in the state of São Paulo, Brazil, is under threat, there is a clear risk of *X. citri* subsp. *citri* becoming endemic in the main orange-producing area in the world. Here we evaluated the potential use of alkyl gallates to prevent *X. citri* subsp. *citri* growth. These esters displayed a potent anti-*X. citri* subsp. *citri* activity similar to that of kanamycin (positive control), as evaluated by the resazurin microtiter assay (REMA). The treatment of *X. citri* subsp. *citri* cells with these compounds induced altered cell morphology, and investigations of the possible intracellular targets using *X. citri* subsp. *citri* strains labeled for the septum and centromere pointed to a common target involved in chromosome segregation and cell division. Finally, the artificial inoculation of citrus with *X. citri* subsp. *citri* cells pretreated with alkyl gallates showed that the bacterium loses the ability to colonize its host, which indicates the potential of these esters to protect citrus plants against *X. citri* subsp. *citri* infection.**

Brazil is the main producer of concentrate orange juice in the world. Exporting 98% of its production, Brazil holds the leading position in the orange juice business, representing 85% of the international market in this sector (1). Brazilian citriculture, an economic activity that generates over $2 billion/year in export revenues, contributes to more than half of the orange juice produced globally. Despite great success, the culturing of citrus faces constant threats such as diseases, which in the past decade forced the eradication of 40 million trees, resulting in the suppression of the spread of the disease.

Among the threats, citrus canker represents one of the most serious diseases of citrus, which is present in the main sweet-orange-producing areas of the world, the state of São Paulo, Brazil, and Florida. The etiological agent is the Gram-negative bacterium *Xanthomonas citri* subsp. *citri* (2). *X. citri* subsp. *citri* is responsible for Asiatic citrus canker, a severe form of the disease that affects all the commercially important citrus species and cultivars in use (reviewed in reference 3). Infected trees exhibit crater-like lesions on aerial tissues, and with time, infection decreases orange production. In susceptible citrus hosts and in the absence of disease control measures, it causes premature fruit drop, defoliation, and shoot dieback. *X. citri* subsp. *citri* penetrates the host plant by natural openings (stomata) and wounds (3). Bacteria spread in the orchards through the action of wind-blown rain, and the colonization of the leaf mesophyll is exacerbated by the concomitant presence of the citrus leaf miner *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) (4).

The state of São Paulo, which produces 75% of the oranges in Brazil, had the first official detection of *X. citri* subsp. *citri* in 1957 (5), and since then, it adopted a strict eradication program still considered the only effective strategy to avoid the advance of citrus canker in areas where it is not endemic (6). From 1999 until 2009, growers in the state of São Paulo were obligated to eliminate all the plants in an orchard if the incidence of symptomatic trees exceeded 0.5%; below this figure, the growers had to eradicate only the affected trees and the neighboring ones contained in a radius of 30 m. From mid-2009, the legislation in this state was relaxed, and only the 30-m radius is being applied. As a consequence, 6 months after the legislation change, the number of new cases of the disease increased 80%, and there is now a real risk of citrus canker becoming endemic in the state of São Paulo. Citrus canker is already endemic in other orange-planting areas in the south of Brazil. There, as well as in Argentina, producers use integrated management approaches such as the cultivation of less-susceptible citrus genotypes produced in *X. citri* subsp. *citri*-free nurseries, the planting of windbreaks as barriers against the spread of the disease, and the spraying of copper bactericides (7). These practices increase the cost of production and are not as effective as eradication to prevent new infections and the spread of *X. citri* subsp. *citri*; besides, copper sprays are known to leave residuals on fruits and soil, and the emergence of resistant strains is an issue (8). Current research is focused on developing new strategies to minimize losses and to make citriculture profitable. The use of a combination of disease control measures is recommended, which includes the induction of innate plant defenses (9, 10), the generation of more resistant citrus varieties (11, 12), and, perhaps, the

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development/amelioration of chemical inhibitors to fight the bacterium and yet display reduced environmental impacts.

Supporting this idea, we initiated a search for natural and synthetic compounds that are able to perturb the growth of *X. citri* subsp. *citri*. We evaluated the anti-*X. citri* subsp. *citri* activity of gallic acid (3,4,5-trihydroxybenzoic acid), an intermediate of the hydrolysable tannin biosynthesis pathway in plants (13–15) which, together with its natural and semisynthetic derivatives, has been associated with a broad spectrum of biological actions (16–21). Moreover, gallic acid and derivatives demonstrated antimicrobial properties. Octyl gallate exhibited fungicidal activity against *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* at any stage of their growth (22, 23). Leal et al. previously reported the potent fungitoxicity of nonyl gallate against yeasts, dermatophytes, and hialohyphomycetes (24). Lauryl gallate was found to show antibacterial activity against Gram-positive and Gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) (25, 26).

Here we demonstrate that alkyl gallates are potent inhibitors of *X. citri* subsp. *citri*. The anti-*X. citri* subsp. *citri* activity of such compounds can be correlated with the size of their carbon side chain. We show evidence for the possible intracellular targets of these compounds, as bacteria treated with alkyl gallates exhibited a disruption of the divisional septum and/or the bacterial centromere. We also show that the treatment of the bacterium with selected alkyl gallates reduces viability and host infection and that these compounds may possess postinfection activity, indicating their potential for use in disease management.

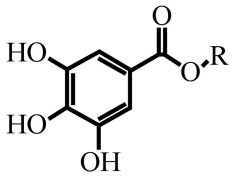
## MATERIALS AND METHODS

**Synthesis of the alkyl gallates.** Synthetic esters of gallic acid were prepared as described previously, with minor modifications (16, 17). A 3.0-ml solution of *N*,*N*′-dicyclohexylcarbodiimide (DCC) (1.0 mmol) in dried *p*-dioxane was added to a cooled (5°C) solution of 0.2 mmol gallic acid (Sigma) (compound 1) (Table 1) and 20 mmol alkyl alcohol in 6.0 ml of dried *p*-dioxane. After 48 h, the solvent was removed under reduced pressure. The residue was partitioned 3 times with ethyl acetate (EtOAc) and filtered. The filtrate was washed successively with a saturated aqueous citric acid solution (3 times) and saturated aqueous $NaHCO_3$ (3 times), dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The crude products were purified over a silica gel column eluted with mixtures of hexanes and EtOAc, furnishing alkyl gallates (compounds 2 to 16) (Table 1). The molecular structures of these compounds were established by $^1H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectral analysis (16, 17).

**Bacterial strains and media.** Wild-type *Xanthomonas citri* subsp. *citri* (2) strain 306 (IBSBF 1594), formerly designated *X. axonopodis* pv. *citri*, was used in the present work. The *X. citri* subsp. *citri* 306 mutant strains expressing green fluorescent protein (GFP)-ZapA (27) and ParB-GFP (A. P. Ucci and H. Ferreira, unpublished data) were *X. citri* subsp. *citri amy*::pPM2a-*zapA* and *X. citri* subsp. *citri parB*::pPM7g-*parB*, respectively. Cells were cultivated at 30°C under rotation (200 rpm) in LB/LB agar medium (28), and kanamycin was added, when required, at 15.6 μg/ml.

**REMA.** The resazurin microtiter assay (REMA) plate method was performed as previously described (29, 30), with modifications. Stock solutions of chemicals at 2 to 5 mg/ml were prepared by dissolving the alkyl gallates (dried-powder samples) in 10% dimethyl sulfoxide (DMSO) (diluted in LB). Test suspensions of gallates were prepared by diluting the stock solutions in LB using a 2-fold scheme; after serial dilution, the most concentrated sample had ~1% DMSO (which did not alter *X. citri* subsp. *citri* growth in preliminary analyses) and an initial concentration of a given compound at 1,000 μg/ml. The concentration range prepared for each of the compounds was 15 to 1,000 μg/ml. In 96-well microtiter plates, 100 μl of each of the compound dilutions was added to a mixture of 90 μl of LB and 10 μl of bacterial inoculum (standardized to $10^5$ CFU/well). The negative control consisted of 190 μl of LB and 10 μl of cell suspension; the positive control had the addition of kanamycin (15.6 μg/ml). Upon the incubation of the test plates at 30°C for 24 h, cell viability was determined by the addition of 15 μl of a 0.01% (wt/vol) resazurin solution to each of the wells, following an extra incubation period of 2 h at 30°C. Viable microorganisms reduced the blue dye to a pink color, which was detected by fluorescence scanning using a SPECTRAfluor Plus (Tecan) microfluorimeter set to an excitation/emission profile of 530 nm/590 nm. Three independent experiments were conducted, and the data were used to construct plots of chemical concentration versus cell growth inhibition in order to determine the $MIC_{50}$ and $MIC_{80}$.

**TABLE 1** Anti-*Xanthomonas citri* activities of gallic acid and alkyl gallates



| Compound | Name | R[c] | MIC (μg/ml) (MIC in μM) | C log *P*[a] |
|---|---|---|---|---|
| 1 | Gallic acid | H | 500 (2,940) | 0.89 |
| 2 | Methyl gallate | $CH_3$ | 62.5 (340) | 0.92 |
| 3 | Ethyl gallate | $CH_2CH_3$ | 62.5 (315) | 1.27 |
| 4 | *n*-Propyl gallate | $(CH_2)_2CH_3$ | 62.5 (295) | 1.73 |
| 5 | *i*-Propyl gallate | $CH(CH_3)_2$ | 62.5 (295) | 1.55 |
| 6 | *n*-Butyl gallate | $(CH_2)_3CH_3$ | 62.5 (276) | 2.13 |
| 7 | *i*-Butyl gallate | $CH_2CH(CH_3)_2$ | 62.5 (276) | 1.95 |
| 8 | *n*-Pentyl gallate | $(CH_2)_4CH_3$ | 31.2 (130) | 2.53 |
| 9 | *n*-Hexyl gallate | $(CH_2)_5CH_3$ | 31.2 (123) | 2.92 |
| 10 | *n*-Heptyl gallate | $(CH_2)_6CH_3$ | 31.2 (116) | 3.32 |
| 11 | *n*-Octyl gallate | $(CH_2)_7CH_3$ | 31.2 (110) | 3.72 |
| 12 | *n*-Nonyl gallate | $(CH_2)_8CH_3$ | 15.6 (53) | 4.11 |
| 13 | *n*-Decyl gallate | $(CH_2)_9CH_3$ | 15.6 (50) | 4.51 |
| 14 | *n*-Undecyl gallate | $(CH_2)_{10}CH_3$ | 15.6 (48) | 4.90 |
| 15 | *n*-Dodecyl gallate | $(CH_2)_{11}CH_3$ | 62.5 (185) | 5.30 |
| 16 | *n*-Tetradecyl gallate | $(CH_2)_{13}CH_3$ | 1,000 (2,732) | 6.09 |
| Kanamycin[b] | | | 15.6 (32) | |

[a] Theoretical lipophilicity (20).
[b] Positive control.
[c] R, radical.

**Microscopy.** Cells of wild-type and mutant strains of *X. citri* subsp. *citri* were treated with the alkyl gallates for 6 h at 30°C and immobilized on agarose-covered slides for observations by microscopy, as previously described (27). Cells were visualized by using an Olympus BX-61 microscope and documented with a monochromatic XM-10 camera. Image processing and analyses were conducted by using Cell-F (Olympus).

**Pathogenicity tests.** The host citrus used was cultivar Pera Rio of sweet orange (*Citrus sinensis* L. Osbeck), kept under greenhouse conditions at 25°C to 35°C. For the infiltration tests, *X. citri* subsp. *citri* cells were cultivated in LB until the optical density (OD) at 600 nm reached ~1; cells were diluted to $10^5$ CFU/ml in LB and treated with the compounds at the $MIC_{80}$ for 6 h at 30°C. After this period, cell suspensions were infiltrated on the abaxial surface of leaves by using hypodermic syringes. Symptoms were observed during the course of 3 weeks. All the tests were performed in triplicates.

For the postinfection test, *X. citri* subsp. *citri* cell suspensions were diluted to $10^8$ CFU/ml in LB; leaves were inoculated by the puncture

method, in which needles are rapidly immersed into the cell suspension following the perforation of the leaves from the abaxial surface. After the development of symptoms, in up to 4 weeks, ~5 μl of the compounds at 2 mg/ml was dropped onto the lesions, and exposure took place for 30 min at room temperature. Forty-eight hours after the treatment, lesions were removed individually from the leaves and macerated in 1 ml of 1× phosphate-buffered saline (PBS), and the number of viable cells in the suspensions was determined by plating onto NYG agar (5 g/liter peptone, 3 g/liter yeast extract, and 20 g/liter glycerol).

**Statistics.** Statistical analysis was performed by using one-way analysis of variance (ANOVA) followed by a Tukey posttest ($P < 0.05$). MIC values were determined by using logarithmic regression analyses.

## RESULTS

**Alkyl gallates inhibit *X. citri* subsp. *citri* growth.** In order to evaluate the potential of alkyl gallates to inhibit the growth of *X. citri* subsp. *citri*, cells were cultivated and exposed to different concentrations of gallic acid (compound 1) (Table 1) and a collection of gallic acid alkyl esters (3,4,5-trihydroxybenzoates) (compounds 2 to 16), followed by an evaluation of their inhibitory activities using the REMA (see Fig. S1 in the supplemental material). In Table 1, we also summarize the MICs of these compounds, which were determined within the concentration range of 3.9 to 1,000 μg/ml. Of the 16 compounds tested, 14 alkyl gallates (compounds 2 to 15) were able to inhibit bacterial growth. Gallic acid itself was not active against *X. citri* subsp. *citri*, exhibiting an MIC of 500 μg/ml (2,940 μM). On the other hand, methyl gallate (compound 2) showed an MIC of 62.5 μg/ml (340 μM), which was approximately 8 times more potent than gallic acid (compound 1). This observation supported the idea that the esterification of the carboxyl group on gallic acid structure was an important criterion for the anti-*X. citri* subsp. *citri* activity, which encouraged us to further investigate other derivatives esterified with linear and ramified alcohols from $C_2$ to $C_{14}$. Among these, the compounds that carried side chains ranging from 9 to 11 carbon atoms (compounds 12 to 14) displayed the most potent activities against *X. citri* subsp. *citri*. Undecyl gallate (compound 14), for instance, showed a more prominent effect, with an MIC of 15.6 μg/ml (48 μM), which resembles the inhibitory activity of our positive control, kanamycin (MIC = 15.6 μg/ml, or 32 μM). Altogether, our results indicate a clear and positive correlation among MIC values, alkyl chain length, and lipophilicity.

Considering that lipophilicity is an important property for the development of bioactive compounds (31), the theoretical lipophilicity values of the alkyl gallates were expressed as calculated partition coefficients (C log *P*), as previously reported (20) (Table 1). By comparisons of the determined MIC values with the C log *P* values, we noticed that the anti-*X. citri* subsp. *citri* activity of the alkyl gallates was associated with their lipophilicity. Highly lipophobic gallates (compounds 2 to 7), which exhibit C log *P* values ranging from 0.92 to 1.95, as well as the highly lipophilic dodecyl gallate (compound 15) (C log $P = 5.30$) had an overall weak antibacterial effect. The anti-*X. citri* subsp. *citri* activity then increased in conjunction with an increase in the number of side-chain carbons until reaching a peak, here represented by undecyl gallate (compound 14) (C log $P = 4.90$). Subsequently, we documented a diminished effect for dodecyl gallate (compound 15) (C log $P = 5.30$), which preceded the approach of a cutoff represented by tetradecyl gallate (compound 16) (C log $P = 6.09$).

Finally, in order to test if the compounds had bacteriostatic or bactericidal activities, we attempted to propagate the cells in LB agar after the treatments. As a result, we could not detect cellular growth following treatment with chemicals in concentrations where the REMA indicated 100% inhibition; hence, we conclude that the alkyl gallates eliminated bacterial viability.

***X. citri* subsp. *citri* cells treated with alkyl gallates are unable to induce citrus canker lesions.** The colonization of the host is a vital process to the survival of any pathogen; therefore, we wanted to evaluate if the treatment of *X. citri* subsp. *citri* cells with the alkyl gallates would impair their ability to multiply in artificially inoculated plant tissues and to produce the typical symptoms of citrus canker. Leaves of sweet orange, cultivar Pera Rio, a susceptible host, were infiltrated with *X. citri* subsp. *citri* cell suspensions ($10^5$ CFU/ml) after treatment with the substances at the $MIC_{80}$ and monitored for a period of 3 weeks in order to score for the appearance of symptoms. The reason for the use of the $MIC_{80}$ was to ensure that after treatment, there would be enough live cells, although exposed to a fairly high concentration of the compounds, to be evaluated in their ability to colonize the host citrus.

The typical canker lesions induced by the untreated cells (control) can be characterized by hyperplasia and hypertrophy on the region of the inoculum, which is frequently surrounded by a chlorotic halo (Fig. 1A). Six of the substances tested (compounds 6 to 10 and 12) (Table 1) completely precluded the ability of *X. citri* subsp. *citri* to produce disease symptoms (Fig. 1B). Alkyl gallate compound 11, although active, induced only a partial reduction of the symptoms. The remaining chemicals did not induce any detectable alteration of the colonization/virulence phenotype of *X. citri* subsp. *citri*. Note that the infiltration of the compounds alone or with 1% DMSO did not produce any lesions on leaves. In addition, the treatment of *X. citri* subsp. *citri* with DMSO (without any alkyl gallate) did not reduce the virulence of the bacterium (Fig. 1A).

Irrespective of their mechanisms of action, alkyl gallate compounds 6 to 10 and 12 directly or indirectly impaired the ability of *X. citri* subsp. *citri* to colonize a susceptible host, demonstrating that these compounds confer substantial protection against *X. citri* subsp. *citri* infection. In order to evaluate their potential to heal diseased leaves, we treated citrus canker lesions individually with compounds 6 to 10 and 12 (Fig. 2). Host leaves were perforated with needles previously immersed in *X. citri* subsp. *citri* cell suspensions, and upon the appearance of symptoms, ~5 μl of alkyl gallate compounds 6 to 10 and 12 (at 2 mg/ml) was dropped onto the cankers. At 48 h posttreatment, alkyl gallates 6, 7, 9, 10, and 12 caused a significant reduction of the bacterial population inside the lesions. Compounds 6, 7, 10, and 12 showed a reduction of the bacterial count of practically 1 order of magnitude and were as effective on the lesions as the positive control, kanamycin (also at 2 mg/ml) (compare the log CFU/ml determined for the controls and DMSO-treated and untreated lesions with the ones for kanamycin and the compounds). Compound 9 was the most prominent of the alkyl gallates tested, showing a reduction of the bacterial population of almost 2 orders of magnitude. The only exception was compound 8, which was active at precluding host colonization and unable to act postinfection. Altogether, the data show the potential of alkyl gallates to treat affected plant tissues.

**Alkyl gallates induce an increase in cell length.** To investigate the possible mechanisms of action of the alkyl gallates, we studied the effects of these compounds on cell morphology. In these analyses, we looked for signs of division arrest and/or chromosome

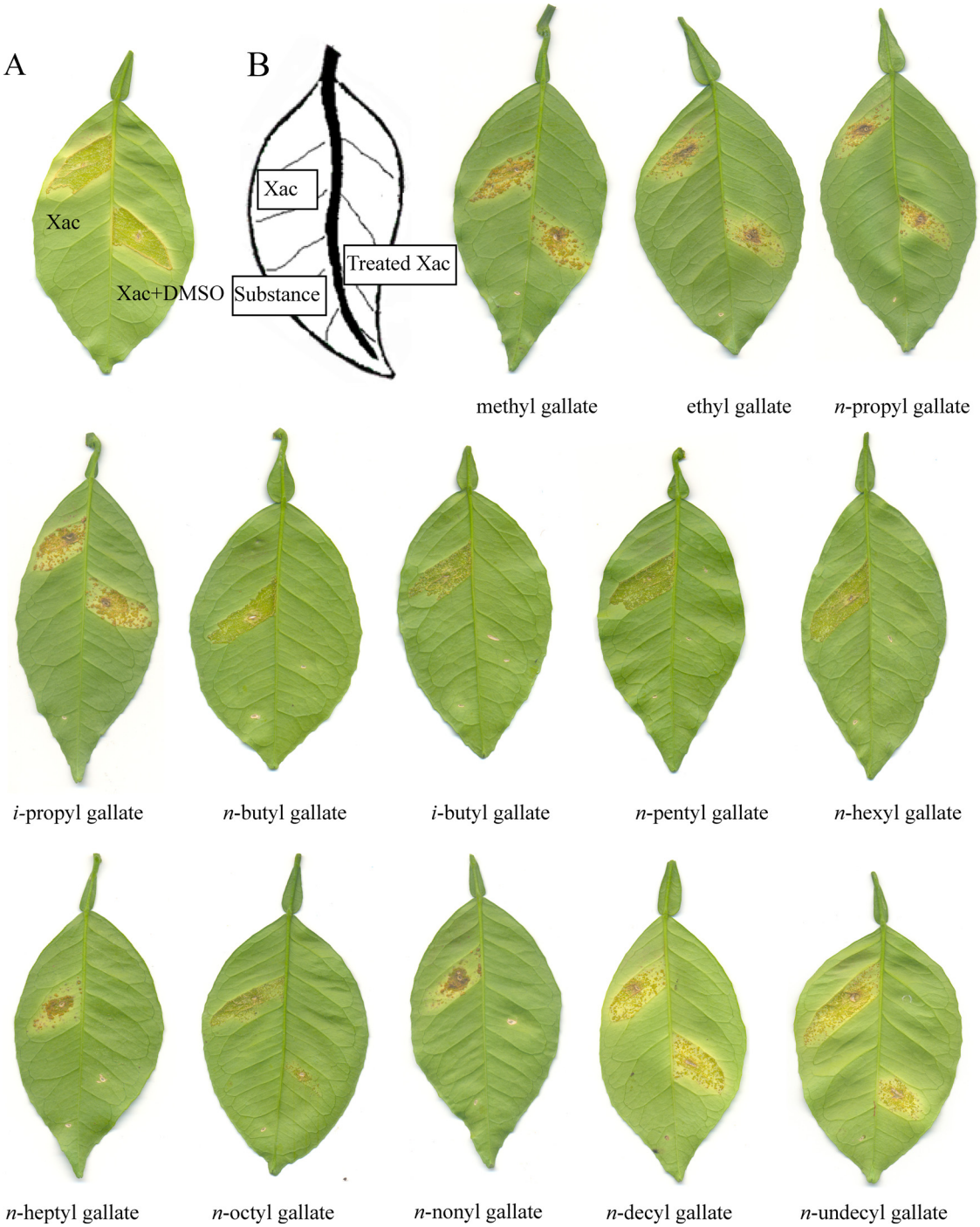



**FIG 1** Effects of alkyl gallates on the ability of *X. citri* subsp. *citri* to induce citrus canker symptoms. Cells were subjected to treatment with the compounds at concentrations corresponding to the $MIC_{80}$ for a period of 6 h and subsequently infiltrated into leaves of *Citrus sinensis* L. Osbeck (Pera Rio). (A) Leaf infiltrated with untreated *X. citri* subsp. *citri* cells and *X. citri* subsp. *citri* cells treated with the vehicle 1% DMSO. Results correspond to a period of 3 weeks of incubation. (B) Schematics of infiltration for the remaining leaves. Xac, untreated *X. citri* subsp. *citri* cells; Substance, alkyl gallate dissolved in 1% DMSO; Treated Xac, treated *X. citri* subsp. *citri* cells. The compounds used are indicated below each picture. Here we show a representative experiment from three independent tests.

segregation defects, which can normally be translated as increased-length and cell filamentation phenotypes.

Wild-type cells of *X. citri* subsp. *citri* were treated for 6 h with the 14 compounds for which we observed a concentration-response pattern in the REMA described above (compounds 2 to 15) (Table 1). The treatment of *X. citri* subsp. *citri* with the alkyl gallates generated aberrant phenotypes, such as cells showing septum misplacement, judged by the fact that constrictions were formed in places other than in the middle of the rod, and some rods with extremely increased cellular volumes (data not shown). Since

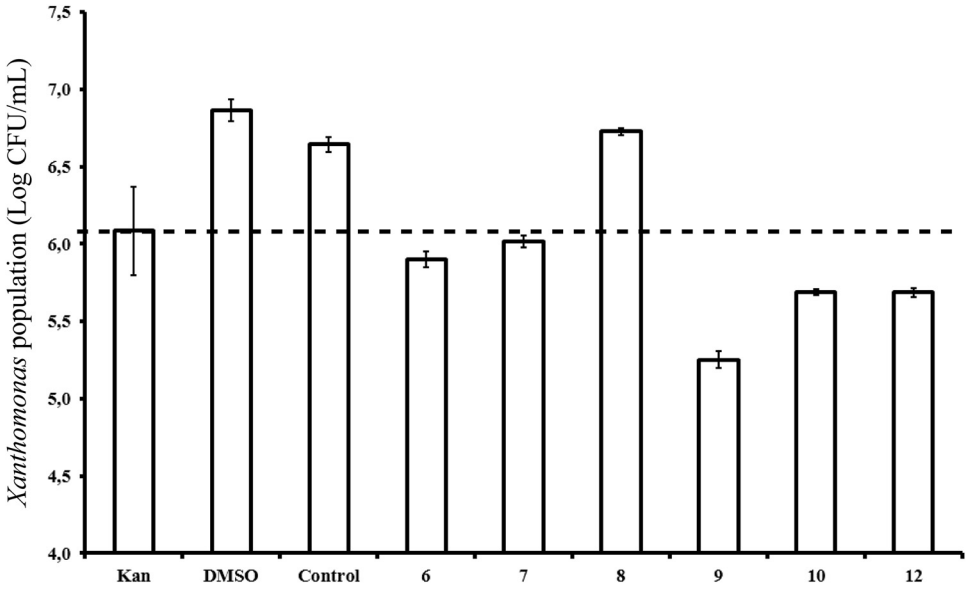



**FIG 2** Bacterial population is reduced in lesions treated with the alkyl gallates. Lesions of citrus canker were treated with the alkyl gallates at 2 mg/ml for 30 min; 48 h after exposure to the compounds, lesions were removed individually from the leaves and macerated in 1× PBS, and the number of viable cells in the suspensions was determined by plating onto NYG agar. Each bar corresponds to the average of the bacterial population per milliliter (log CFU/ml) from 6 lesions per treatment (± standard deviations). Kan, kanamycin (2 mg/ml); DMSO (1%), vehicle in which the compounds were diluted; Control, untreated lesions.

these phenotypes were documented rarely following an exhaustive inspection of the samples, we decided to use a more tractable measurement to detect morphological variations. First, we performed pairwise visual comparisons of treated and untreated cells, which enabled us to detect alterations in cell length (see Fig. S2 in the supplemental material). Second, to facilitate the evaluations, we measured individually 100 cells for each concentration of the chemical compounds tested (the concentrations used for morphological analyses varied from 3.9 to 60 μg/ml, the last one being the maximum concentration for which we could detect cells under phase-contrast microscopy without the accumulation of cell debris). Measurements were performed in two independent experiments, giving a total of 200 cells, and used to calculate the average size of the cells (Table 2). Treatment with substances 2 to 5 and 9 to 14 led to a significant increase in the average cell size. For each value marked with an asterisk in Table 2, compare the value expressed as the average cell size for the internal experimental control (IC) (untreated *X. citri* subsp. *citri* cells) with that for treatment (note that each treatment had its own IC to avoid wrong estimates due to small fluctuations in cell size that may occur naturally; ICs were performed alongside and under the same conditions of treatment without the compounds). Alkyl gallates 6 to 8 and 15 were the only ones that did not differ statistically compared with their internal controls. Taken together, these results show that 10 out of 14 substances that inhibited the growth of *X. citri* subsp. *citri* in REMA (~72% of the alkyl gallate series) were able to induce morphological changes in this bacterium, detected as significant increases in cell length. The observation that *X. citri* subsp. *citri* continued to grow after treatment with the alkyl gallates argues against an inhibition of the transcription/translation processes in these cells, and it might be indicative of perturbations of other cellular processes, such as cell division and/or chromosomal segregation. On the other hand, compounds 6 to 8 and 15 might cause growth inhibition by acting on other targets.

**Alkyl gallates may target the chromosome segregation and cell division machineries.** As described above, we raised the possibility that some alkyl gallates could perturb the processes of cell division and/or chromosome segregation based on the observation that *X. citri* subsp. *citri* cells treated with these compounds exhibited an increased cell length. To further investigate this, we looked at the ability of such compounds to disrupt the centromere and/or septum structures with the help of fluorescent markers. Two *X. citri* subsp. *citri* mutant strains were used for these analyses: one was the *X. citri* subsp. *citri amy*::pPM2a-*zapA* mutant expressing extra copies of ZapA as GFP fusions (GFP-ZapA) (27),

**TABLE 2** Morphological analysis of cells treated with the alkyl gallates[a]

| Compound[b] | Avg cell length (μm) ± SD | |
|---|---|---|
| | IC[c] | Treated[d] |
| 2 | 1.44 ± 0.31 | 1.74 ± 0.32* |
| 3 | 1.44 ± 0.31 | 1.65 ± 0.65* |
| 4 | 1.44 ± 0.31 | 1.57 ± 0.21* |
| 5 | 1.44 ± 0.31 | 1.61 ± 0.28* |
| 6 | 1.49 ± 0.23 | 1.52 ± 0.21 |
| 7 | 1.49 ± 0.23 | 1.47 ± 0.18 |
| 8 | 1.72 ± 0.3 | 1.85 ± 0.48 |
| 9 | 1.72 ± 0.3 | 1.97 ± 0.62* |
| 10 | 1.39 ± 0.27 | 1.62 ± 0.3* |
| 11 | 1.39 ± 0.27 | 1.71 ± 0.32* |
| 12 | 1.39 ± 0.27 | 1.71 ± 0.38* |
| 13 | 1.39 ± 0.27 | 1.6 ± 0.29* |
| 14 | 1.52 ± 0.24 | 1.62 ± 0.32* |
| 15 | 1.57 ± 0.29 | 1.60 ± 0.30 |

[a] $n = 200$. Data correspond to averages ± standard deviations.
[b] Numbers correspond to the numbers shown in Table 1.
[c] Average length of the internal control (IC) (untreated cells).
[d] Cells were treated for 6 h at 30°C. *, $P < 0.05$ by one-way ANOVA with a Tukey posttest.

and the other was the *X. citri* subsp. *citri parB*::pPM7g-*parB* mutant, which expresses ParB-GFP alongside the native ParB proteins (A. P. Ucci and H. Ferreira, unpublished).

ZapA is a conserved bacterial factor that acts as a positive regulator of the cell division protein FtsZ (32). Considering that the site of ZapA action is right at the site of Z-ring formation, GFP-ZapA constitutes an excellent septum marker. In order to evaluate if the alkyl gallates could perturb septum assembly, we subjected *X. citri* subsp. *citri amy*::pPM2a-*zapA* cells to treatment with the compounds that induced altered cell morphology (compounds 2 to 5 and 9 to 14) (Table 1). Normally growing *X. citri* subsp. *citri amy*::pPM2a-*zapA* cells, or even cells treated with the vehicle 1% DMSO, exhibited the standard GFP-ZapA localization pattern, which corresponds to a fluorescent septum between dividing rods (Fig. 3A, arrows). When the cells were exposed to the alkyl gallates, compounds 9, 10, and 11 induced a complete disruption of the septa (Fig. 3B), where the GFP-ZapA fluorescence signal was dispersed throughout the cytoplasm. Note that treatment with some compounds (e.g., alkyl gallate 11) induced an apparent contraction of the cytoplasmic content in a few cells (Fig. 3B, asterisks). Only cells with a normal aspect (Fig. 3B, black and white crosses) were considered in our analyses. The time course of exposure/documentation was 6 h of total exposure with observations every 30 min; all the compounds mentioned above disrupted the septum within the first 30 min. As usual for a division arrest phenotype, the cells treated with alkyl gallates 9, 10, and 11 continued to grow (see above), which indicates that they are metabolically active but not dividing normally.

Given the fact that, by REMA, we noticed a clear peak of anti-*X. citri* subsp. *citri* activity around compound 13, which could be related to its carbon side chain, we wondered if it was possible to extend our window of compounds targeting the septum by evaluating alkyl gallates with shorter carbon side chains as well. As a result, we found that compound 8 was also able to disrupt the septal structure (Fig. 3B), even though this substance did not promote any significant alteration in cell length (see above).

Rod elongation may also occur in response to chromosome segregation defects, and in this sense, the ParB-GFP/DNA complex, which is known to form a centromere-like structure in bacteria, may serve as a marker for the disruption of the segrosome (the chromosome segregation machinery). The bacterial centromere is constituted by ParB bound to DNA at specific *cis*-acting elements (*parS*) located around the origin of replication of the chromosomes (e.g., *Bacillus subtilis* [33]). As proposed previously, ParB functions by interacting with other cellular factors to facilitate chromosome partitioning toward the polar regions, enabling cell division to proceed (34–36). Thus, ParB-GFP colocalizes with the origins, and the centromere structure formed can be seen by fluorescence microscopy as two foci, each close to a cell pole (37, 38). *X. citri* subsp. *citri* also exhibits a ParB-GFP localization profile (Fig. 4A, arrows) (Ucci and Ferreira, unpublished) that resembles the ones documented previously for *B. subtilis* and, perhaps, *Caulobacter crescentus* (37–39). Upon treatment with the alkyl gallates, we found that four substances (compounds 9 to 11 and 13) (Table 1) showed activity against the ParB-GFP/DNA complex (Fig. 4B). The time course of treatment showed that this complex is fairly stable, taking an average of 4 h to be disrupted by the compounds (the equivalent of two doubling times for *X. citri* subsp. *citri*). This is consistent with the observation that in *B. subtilis*, Spo0J-GFP (a ParB-like protein) can be seen throughout

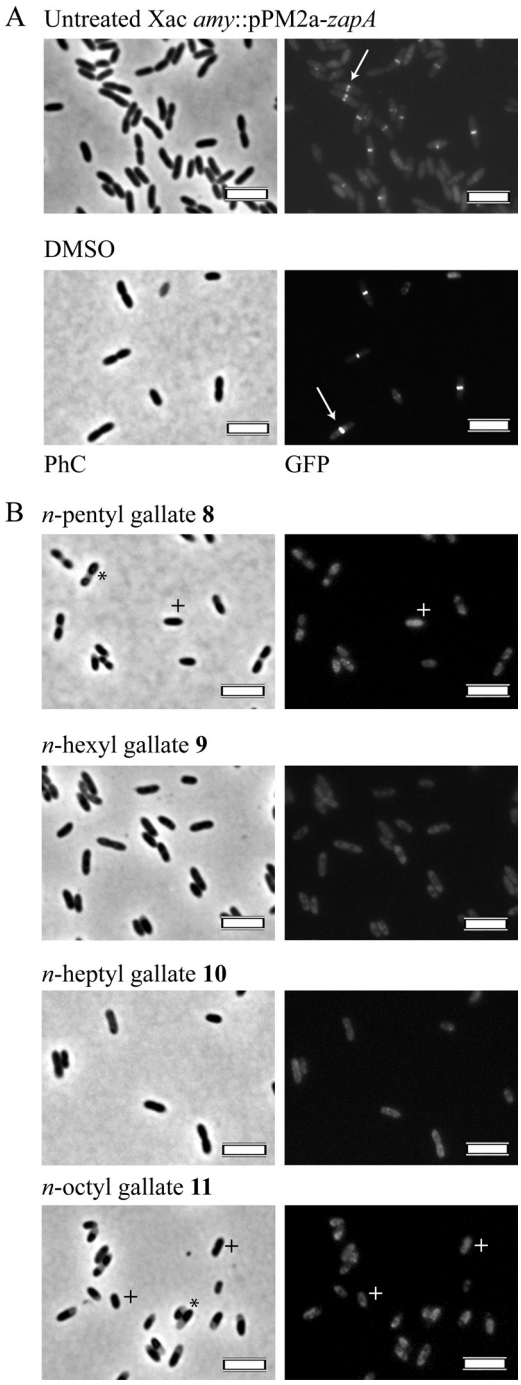



**FIG 3** Alkyl gallates disrupt the divisional septum of *X. citri* subsp. *citri*. Mutant cells of *X. citri* subsp. *citri* expressing GFP-ZapA were cultivated until the OD at 600 nm reached ~0.3 and then subjected to treatments with the alkyl gallates at concentrations corresponding to the $MIC_{50}$. The presence or absence of GFP-ZapA septal labeling was monitored by fluorescence microscopy after 30 min of exposure to the alkyl gallates. (A) Cells cultivated in the absence (untreated) or in the presence of 1% DMSO. Septa are marked with arrows. (B) Cells treated with the alkyl gallates (we show only data for the compounds that perturbed septa). Note that in our analyses, we excluded cell types which resembled the ones labeled with asterisks and considered those similar to the ones marked with crosses. DMSO, vehicle in which substances were dissolved; PhC, phase-contrast microscopy. The scale bars correspond to 4 μm. Magnification, ×100.

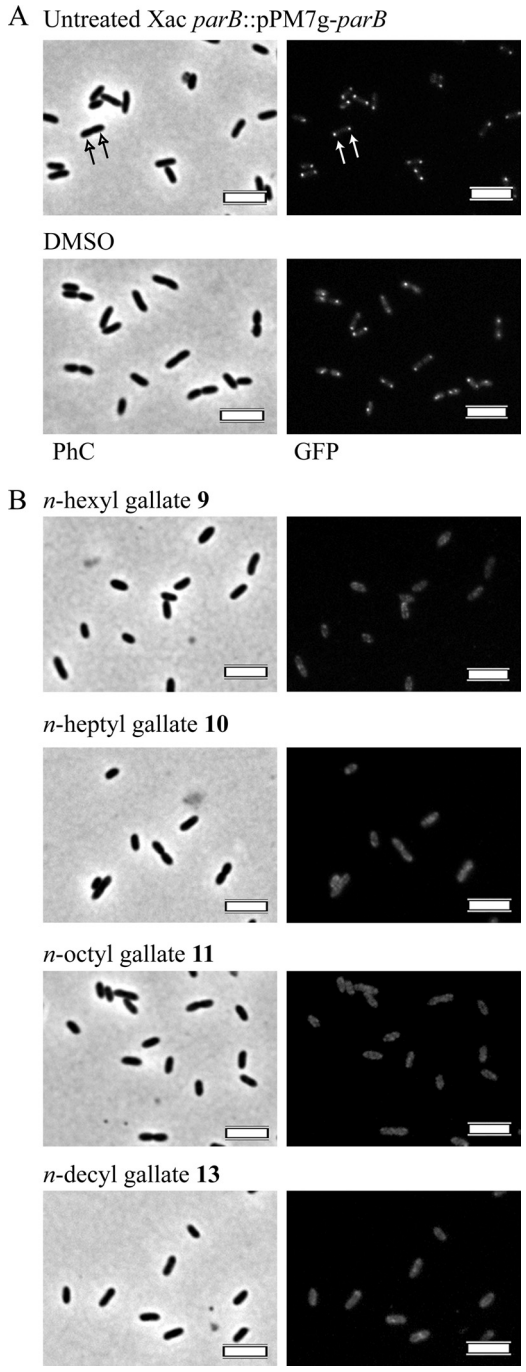



**FIG 4** Alkyl gallates target the centromere of *X. citri* subsp. *citri*. *X. citri* subsp. *citri* mutants expressing ParB-GFP were cultivated until the OD at 600 nm reached ~0.3 and then treated with the alkyl gallates at the $MIC_{50}$ for a period of 6 h at 30°C. The presence or absence of ParB-GFP foci was monitored by fluorescence microscopy every 30 min from the start of exposure to the compounds and documented after 2 h. (A) Cells cultivated in the absence (untreated) or in the presence of 1% DMSO. The ParB-GFP signal is marked with white arrows on the GFP panel. (B) Cells exposed to the alkyl gallates. Data are relative only to compounds that disrupted the centromere. DMSO, vehicle in which substances were dissolved; PhC, phase-contrast microscopy. The scale bars correspond to 4 μm. Magnification, ×100.

**TABLE 3** Summary of the activities displayed by alkyl gallates against *X. citri* subsp. *citri*

| Compound | Presence of activity | | | | |
|---|---|---|---|---|---|
| | Growth inhibition | Impairs host colonization | Increases cell length | Septum/centromere disruption | |
| | | | | Septum | Centromere |
| 1 | − | − | − | − | − |
| 2 | + | − | + | − | − |
| 3 | + | − | + | − | − |
| 4 | + | − | + | − | − |
| 5 | + | − | + | − | − |
| 6 | + | + | − | − | − |
| 7 | + | + | − | − | − |
| 8 | + | + | − | + | − |
| 9 | + | + | + | + | + |
| 10 | + | + | + | + | + |
| 11 | + | − | + | + | + |
| 12 | + | + | + | − | − |
| 13 | + | − | + | − | + |
| 14 | + | − | + | − | − |
| 15 | + | − | − | − | − |
| 16 | − | − | − | − | − |

the cell cycle, and occasionally, a Spo0J-GFP focus splits into two, which happens when an origin of replication fires (40). When we compared the pictures taken using phase-contrast microscopy with the GFP ones after treatment with the alkyl gallates, we saw that cells looked fairly normal, although enlarged, but without a clear sign of death and/or an accumulation of inclusion bodies. Our results indicate an action of the alkyl gallates on the centromere complex instead of a simple aggregation of ParB-GFP in *X. citri* subsp. *citri* cells.

Among the four compounds that disrupted the septum (compounds 8 to 11), three also acted on the centromere (compounds 9 to 11), which raises the possibility that they may target a factor(s) or system(s) that is common to both processes of chromosome segregation and cell division.

## DISCUSSION

The plant pathogen *Xanthomonas citri* is the causal agent of citrus canker, a severe disease that affects cultivars of citrus worldwide and represents a major threat to one of the most important economic activities in Brazil. The eradication of entire orchards of trees infected by *X. citri* subsp. *citri* is the only effective control to prevent the spread of the disease to new areas; however, the obligation to do so based on legal governmental actions has led to many disputes, generating too much discontent, especially among orange growers (3). Citriculture in the largest orange-producing area in the world, the state of São Paulo, Brazil, seems to be shifting from eradication to an integrated management system (7), where any help to control citrus canker will make a difference. Here we show the potential use of alkyl gallates to prevent *X. citri* subsp. *citri* growth and dissemination, and in addition, some of the compounds tested displayed an ability to reduce the bacterial population within lesions of diseased leaves. The compiled activities of gallic acid and the alkyl gallates against *X. citri* subsp. *citri* are summarized in Table 3. The colorimetric REMA indicated that the alkyl gallates are potent anti-*X. citri* subsp. *citri* agents exhibiting MICs comparable to those of our positive control, kanamycin

(<50 μg/ml). Furthermore, our data are in agreement with data from many other studies of homologous series (41–43) showing that the biological behavior of the alkyl gallates could be correlated with the cutoff phenomenon, which has frequently been attributed to their amphiphilic properties. Structurally, the amphiphile of the alkyl gallates is associated with the presence of two groups, phenolic hydroxyls (hydrophilic moiety) and an alkyl side chain (lipophilic tail). Therefore, we believe that the length of the carbon side chain determines the release of the bioactive portion of the alkyl gallates (the gallic acid unit) inside the cells.

At present, the only substances used in the field to confer some degree of protection against *X. citri* subsp. *citri* are copper-based formulations. Copper sprays confer a relative control of citrus canker, reducing new infections on young, immature plant tissues (leaves, stems, and fruits) (44, 45). The toxic effect of copper against *X. citri* subsp. *citri* takes place only at the plant surface, without any internal and/or systemic action (46). As a consequence, infected tissues may act as a source of bacterial inoculum, even though plants receive frequent sprays of copper formulations (44, 46). Although useful and frequently applied in agriculture, copper accumulates in the environment and may lead to the development of copper-resistant bacterial populations (46, 47). Here we showed that the exposure of *X. citri* subsp. *citri* cells to alkyl gallates (compounds 6 to 12, except compound 11) (Table 3) prevents the pathogen from colonizing and/or inducing citrus canker symptoms *in planta*, which has a potential disinfectant action. To our knowledge, this is the first report showing the effect of organic compounds against *X. citri* subsp. *citri*. Moreover, the treatment of citrus canker lesions on the leaves of a susceptible host with alkyl gallates 6, 7, 9, 10, and 12 impaired the proliferation of the pathogen within the plant tissue. Further analyses are now required in order to evaluate the leaf surface retention and subsequent penetrability of the compounds into the mesophyll, which are important measures to judge their efficacy in the field.

There have been several reports in the literature of cell division inhibitors that were identified and/or developed based on assisted screenings for natural, semisynthetic, and synthetic compounds that are able to disrupt FtsZ function in bacteria (for a comprehensive review, see reference 48). Several of these compounds have been included in patent-claiming processes, since cell division has become one of the ideal targets for the development of antibacterial agents. What supports this view is the fact that protein factors that operate on bacterial cell division, an essential process for life, are in general unique to *Bacteria*. Moreover, these factors are widespread and conserved among this domain of life, and for those proteins which show some degree of homology to eukaryotic counterparts (e.g., bacterial FtsZ and eukaryotic tubulins [49, 50]), there are still considerable differences among them to allow for the development of bacterium-specific inhibitors. Here we found that compounds 8 to 11 were able to disrupt septum formation in *X. citri* subsp. *citri* (Table 3). Moreover, we demonstrated that *X. citri* subsp. *citri* cells treated with such substances were unable to colonize the host citrus, which showed their potential to protect against this plant pathogen. At the moment, it remains unknown whether the disappearance of the septum induced by these compounds was a direct effect on FtsZ or indirect by targeting any of the other cell division proteins that are recruited to the Z ring during cytokinesis (51). Future experiments to assess the direct action of alkyl gallates on FtsZ are needed and will be carried out shortly.

Three out of the four gallates that disrupted the septum also targeted the centromere of *X. citri* subsp. *citri* (compounds 9 to 11) (Table 3). To show this, we used an *X. citri* subsp. *citri* mutant strain expressing ParB-GFP (Ucci and Ferreira, unpublished) in which the ParB-GFP/DNA complex (the bacterial centromere) has an easily recognizable localization pattern of 2 foci per rod, each occupying a region close to a cell pole (Fig. 4). Upon treatment with alkyl gallates 9 to 11, the two discrete centromere foci disappeared, and only dispersed fluorescence could be detected within the cells. This observation raised the possibility that a factor/system common to both chromosome segregation and cell division could be the real target for these compounds in *X. citri* subsp. *citri*. Chromosome segregation and cell division are two essential and interlinked processes in bacteria (34, 52, 53). Direct links between components of the two processes (e.g., FtsZ and ParB via an FtsZ inhibitor such as MipZ in *C. crescentus*) have been demonstrated (34, 36). Curiously, we found recently that *X. citri* subsp. *citri* has an overall chromosome segregation/cell division scheme that resembles what was proposed previously for the model *C. crescentus* (54; Ucci and Ferreira, unpublished). In the latter, the correct site for septum assembly within the cell is defined by the action of MipZ, which in turn has its localization dependent on the centromere (ParB/DNA) complex. The disruption of MipZ and/or the centromere function leads to a cell division defect in *C. crescentus* (34). Finally, cytoskeletal proteins, such as the actin-like protein MreB, contribute to chromosome partitioning in bacteria (55). Based on these observations, we speculate that compounds 9 to 11 may disrupt cell division by targeting the chromosome segregation machinery of *X. citri* subsp. *citri*, which also requires further investigation.

The present study shows that the alkyl gallates could be grouped according to two main different biological profiles, which probably reflect their actions on distinct targets. The first group is represented by those compounds that induced altered cell morphology (compounds 2 to 5) (Table 3), but they were unable to interfere with host colonization; furthermore, they showed no detectable influence on the processes of chromosome segregation and/or cell division. The second group (compounds 9 to 11) precluded the ability of *X. citri* subsp. *citri* to colonize the host citrus, where chromosome segregation and cell division were implicated as the intracellular targets for such compounds.

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(ex Riker et al., 1935) sp. nov. nom. rev.; and "var. fuscans" of *X. campestris* pv. phaseoli (ex Smith, 1987) dye 1978 as *X. fuscans* subsp. *fuscans* sp. nov. Syst. Appl. Microbiol. **28**:494–518.
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