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Research article

Oxidative foliar photo-necrosis produced by the bacteria *Pseudomonas cedrina*

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ABSTRACT

Background: Although bioactive metabolites capable of causing oxidative photo-necrosis in plant tissues have been identified in fungi, little is known about this type of mechanism in bacteria. These metabolites act as photosensitizers that generate reactive oxygen species (ROS) capable of causing damage to cells. In addition, these metabolites can pass into an energetically excited state when they receive some luminous stimulus, a condition in which they interact with other molecules present in the environment, such as molecular oxygen (O_2), also known as triplet oxygen ($^3O_2$), generating ROS.
Results: The suspension of the bacterial culture of *Pseudomonas cedrina* was shown to produce foliar necrosis in papaya leaves (*Carica papaya* L.) only in the presence of sunlight, which is evidence of photosensitizing mechanisms that generate singlet oxygen ($^1O_2$). From the chemical study of extracts obtained from this bacteria, 3-(4-(2-carboxipropyl) phenyl) but-2-enoic acid (1) was isolated. This compound, in the presence of light and triplet oxygen ($^3O_2$), was able to oxidize ergosterol to its peroxide, since it acted as a photosensitizer producing $^1O_2$, with which it was corroborated that a photosensitization reaction occurs, mechanism by which this bacterium could prove to cause oxidative foliar photo-necrosis.
Conclusions: P. cedrina was able to induce oxidative foliar photo-necrosis because of its potential ability to produce photosensitizing metabolites that generate singlet oxygen in the plants it colonizes. Based on the above, it can be proposed that some bacteria can cause oxidative foliar photo-necrosis as an important mechanism in the pathogenesis of host species.
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1. Introduction

In ecosystems, plants are continuously exposed to biotic stress generated by different types of micro and macroscopic organisms. One of the consequences of such stress is disease caused by pathogenic microorganisms [1,2]. These phytopathogens cause changes in their structural or physiological processes which are observed in leaves, stems and/or roots [3]. One of the symptoms of disease is the development of necrosis, which is the death of tissue cells, plant organs and sometimes the entire plant, caused by the degeneration of the protoplasm [4,5].

Some phytopathogenic fungi have been reported to cause necrosis by the production of metabolites that act as photosensitizers and generate reactive oxygen species (ROS) [6,7,8,9]. These metabolites are capable of causing damage to the plasma membranes of cells, formation of adducts in the guanine present in deoxyribonucleic acid, degradation of proteins, generation of endo-peroxides that contribute to the peroxidation of lipids, among others [10,11,12,13]. Because of their chemical structure, photosensitizing metabolites are capable of passing to an energetically excited state when they receive some luminous stimulus, a condition under which they interact with other molecules present in the environment, such as molecular oxygen (O_2), also known as triplet oxygen ($^3O_2$), thus generating reactive oxygen species (ROS). When the photosensitizer directly transfers an electron to molecular oxygen or triplet (type I reactions), a superoxide (O_2•-) anion is formed, from which hydroxyl free radical (•OH) and hydrogen peroxide (H_2O_2) originate. However, if in the process of photosensitization a change

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occurs in the spin of the outermost orbitals of $^3O_2$ (type II reactions) then singlet oxygen ($^1O_2$) is formed [14,15].

In the case of stimuli related to ROS capable of generating plant necrosis, most research has focused on the study of hydrogen peroxide (H_2O_2) and superoxide anion (O_2•-), leaving aside non-radical mechanism ROS through $^1O_2$ [16]. Although bioactive metabolites capable of causing oxidative photo-necrosis in plant tissues have been identified in fungi, little is known about this type of mechanism produced by bacteria [6,7,8]. Our group has reported the photosensitizing capacity of macrosporin, an anthraquinone produced by the phytopathogenic fungus *Stemphylium lycopersici*, as a causal agent of oxidative foliar photo-necrosis, produced by the generation of $^1O_2$, the same mechanism presented by cercosporin [8]. It is for this reason that we report the bacterial oxidative foliar photo-necrosis produced by *P. cedrina* through the generation of singlet oxygen ($^1O_2$), a mechanism that has already been demonstrated that is present in phytopathogenic fungi but had not been reported in bacteria until now.

2. Materials and methods

2.1. Biological material

Samples of plant tissues with necrosis areas or symptoms of bacterial disease were collected for the isolation and subsequent purification of bacteria. The first criterion for selecting the bacteria was to select those colonies that produced some pigmentation when inoculated in King's medium B (KB, Mast Group Ltd., UK) because it has been reported that this characteristic is usually associated with phytopathogenic bacteria [17]. From a total of 43 strains isolated from plant tissue with necrosis, only 16 strains developed coloration, the strain of *P. cedrina* being selected because of the production of fluorescent pigments that diffused in the culture medium, as well as presenting the ability to cause a notorious hypersensitivity response (necrosis) when a bacterial suspension of this strain was infiltrated on leaves of *Nicotiana tabacum*. This strain was identified based on its colonial morphology, biochemical tests and molecular studies, as previously reported [18]. Tobacco plants (*N. tabacum* L.) and papaya plants (*C. papaya* L.) grown at the Centro de Investigación en Micología Aplicada (CIMA, Mexico) were used for the subsequent bioassays.

2.2. Evaluation of the necrotic effect in tobacco and papaya

The *P. cedrina* strain was cultured in five Erlenmeyer flasks (500 mL) with 100 mL of liquid medium Luria Bertani (LB, DIBICO, Mexico) incubated in agitation (150 rpm) for 48 h at 27°C ± 1 [18]. Subsequently, 250 μL of the bacterial suspension adjusted to a concentration of 1×10^{-6} CFU/mL were inoculated on the midribs of tobacco and papaya leaves using a syringe (NIPRO of 3 mL). As negative control, 250 μL of the medium LB were infiltrated. The plants were kept in greenhouse conditions at room temperature (~25°C). Treatments were performed in triplicate and plants were observed every 24 h [8].

2.3. Evaluation of foliar photo-necrosis in papaya leaves

The strain was cultivated under the same conditions as mentioned above. At the end of the incubation period, the cultures were sterilized with moist heat (121.5°C, 15 lb/in^2, 15 min), followed by sonication for 1 h and subsequent filtration [19]. From the resulting solution, 250 μL were infiltrated with a syringe (NIPRO of 3 mL) into the midribs of papaya leaves. Sterilized LB medium was used as the negative control. Treatments were performed in triplicate in the presence and absence of sunlight. The evolution of the damage caused was monitored every 24 h for 20 d [8].

2.4. Larger-scale cultivation and extraction of metabolites

The *P. cedrina* strain was inoculated into a total volume of 40 L of liquid medium LB in Erlenmeyer flasks (500 mL) with 100 mL of medium, which were kept in agitation (115 rpm) at 27°C ± 1 for 48 h [18]. At the end of the incubation period, the culture was frozen and lyophilized. The dehydrated fraction was continuously extracted with a mixture of chloroform:methanol (1:1), and the extract obtained was concentrated in a rotary evaporator at reduced pressure [20].

2.5. Isolation and characterization of the compound

The bacterial extract was purified by column chromatography (Merk silica gel, 0.2–0.5 mm and 0.040–0.063 mm) eluted in an ascending polarity gradient (mixtures of hexane and ethyl acetate). The monitoring of the compounds was performed by thin layer chromatography (TLC MERCK, 60 F_{254}), revealed with UV light (254 and 366 nm), Iodine vapors and phosphomolybdic acid. The compound was identified by analysis of ^1H and ^{13}C and two-dimensional (COSY, DEPT, HSQC, HMBC) NMR experimental data [21].

3-(4-(2-carboxypropyl) phenyl) but-2-enoic acid (**1**): from the fraction eluted with ethyl acetate of the bacterial extract, a white amorphous crystalline compound (Mp 269–274°C), TLC (ethyl acetate, silica gel): RF = 0.65, was obtained with the following spectroscopic data: ^1H RMN ($CDCl_3$, 600 Hz) δ, ppm: 7.53 (2 H, d, J = 7.7 Hz, H-5); 7.36 (1 H, sa, H-2); 5.80 (2 H, d, J = 7.7 Hz, H-6); 3.49 (1 H, dd, J = 12.7, 6.2 Hz, H-10), 3.18 (1 H, dd, J = 12.7, 10.0 Hz, H-10′); 2.79 (1 H, dp, J = 10.1, 6.7 Hz, H-11); 1.87 (1 H, sa, H-13); 1.20 (1 H, d, J = 7.1 Hz, H-14). ^{13}C RMN ($CDCl_3$, 600 Hz) δ, ppm: 177.4 (C-12); 167.4 (C-1); 156.1 (C-7); 153.0 (C-4); 143.4 (C-5); 139.0 (C-2); 110.2 (C-3); 101.1 (C-5, C-6); 101.0 (C-9); 41.9 (C-10); 34.1 (C-11); 11.9 (C-14); 11.2 (C-13).

2.6. In vitro photosensitizing test

Following the Lagunes and Trigos methodology [22], a solution containing 10 mg of ergosterol and 5 mg of the compound (**1**) in 30 mL of ethanol was prepared using a test tube (Pyrex 9825, 16 × 125 mm) that was placed inside a photo-oxidation chamber (30 × 30 cm). The solution was irradiated for 2 h with a continuous flow of 75 mL/s of oxygen (medical grade). Four compact fluorescent lamps (CFLT4-GE 20 W) with a light emission between the visible light spectrum (380–780 nm) were used as the light source. The temperature inside the photo-oxidation chamber was 32°C. The photo-oxidation reaction was carried out in triplicate and the ergosterol photo-oxidation reaction without a photosensitizer was established as a control. After 2 h, the reaction was monitored by TLC (8:2 n-Hex:AcOEt) against real samples of ergosterol and ergosterol peroxide. TLC was developed in chambers with Iodine vapors. To confirm the presence of ergosterol peroxide in the reaction mixture, ^1H NMR (300 MHz, $CDCl_3$) was identified. The fluorescence of the compounds was demonstrated with UV light (λ = 360 nm).

3. Results and discussion

3.1. Evaluation of the necrotic effect in tobacco and papaya

A total of 43 bacterial strains were isolated from plant tissues with symptoms of disease, of which only 16 strains developed coloration (Fig. 1A), the strain of *P. cedrina* being selected because of the production of fluorescent pigments that diffused in the culture medium (KB), as well as presenting the ability to cause a notorious necrosis when it was infiltrated in tobacco leaves (Fig. 1B). It was observed that a bacterial suspension of the *P. cedrina*

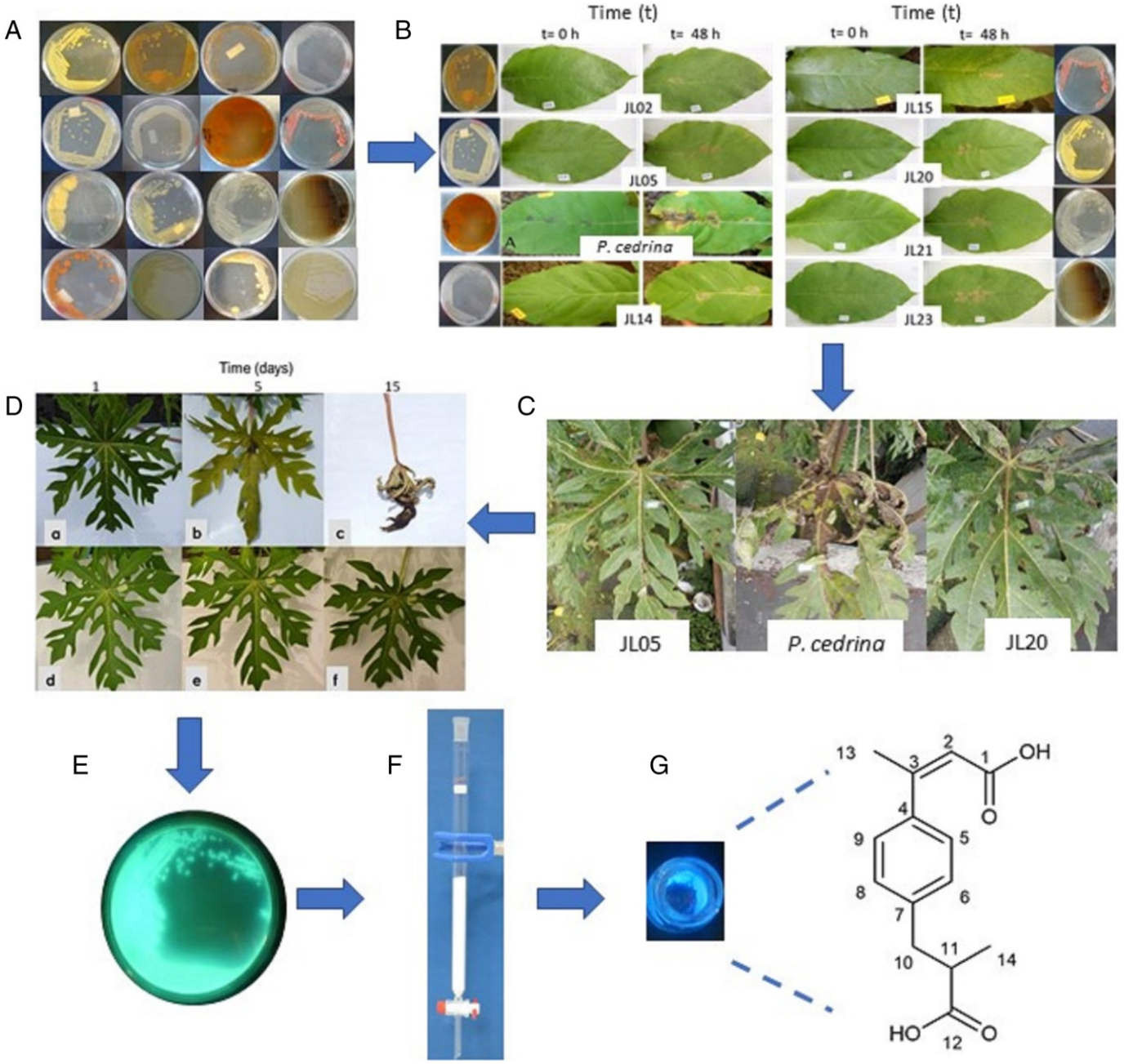

Fig. 1. Schematic overview on steps involved in the isolation and selection of the bacteria strain *Pseudomonas cedrina* causative oxidative foliar photo-necrosis and the isolation of the possible responsible photosensitizer metabolite. (**A**) Sixteen bacteria strains isolated from necrotic plant tissue that produced some pigmentation in KB medium; (**B**) Eight bacteria strains that caused necrosis when it were infiltrated in tobacco leaves; (**C**) Three bacteria strains that caused necrosis when it were infiltrated in papaya leaves; (**D**) Evaluation of foliar photo-necrosis in papaya leaves infiltrated with *P. cedrina* suspension in sunlight conditions (a, b, c) and absence of sunlight (d, e, f) on 1, 5 and 15 d; (**E**) Fluorescence of strain *P. cedrina* observed with UV light ($\lambda = 360$ nm); (**F**) Isolation and purification by column chromatography of fluorescent fractions from the extract chloroform:methanol (1:1) from the larger-scale cultivation of *P. cedrina*; (**G**) Structure of compound fluorescent and photosensitizer isolated from *P. cedrina*: 3-(4-(2-carboxipropyl) phenyl) but-2-enoic acid.

strain caused foliar necrosis in the presence of sunlight when infiltrated in both tobacco (Fig. 1B) and in papaya leaves (Fig. 1C) after 48 h.

3.2. Evaluation of foliar photo-necrosis in papaya leaves

When we evaluated the necrosis in papaya leaves infiltrated with the suspension of the bacterial culture, it was observed that only in the presence of sunlight foliar necrosis was produced after 24 h, which started at the infiltration point, appreciating a gradual increase of the necrosed area, being notorious after 5 d. It was also observed that from the fifth day the leaves became chlorotic. After 15 d, the generalized leaf death was fully expressed (Fig. 1D **a, b, c**). The plants that were kept in the absence of sunlight did not present necrosis 20 d after infiltration (Fig. 1D **d, e, f**).

When examining the effect caused in the infiltrated leaves, it was observed that they showed necrosis only in the presence of sunlight, giving evidence that it occurs probably through photosensitizing mechanisms generating singlet oxygen, a similar mechanism that has already been reported for the fungus *S. lycopersici*, which produces macrosporin, a compound capable of photosensitizing and producing singlet oxygen $^1O_2$, causing damage to plant cell

membranes and consequently, the development of necrosis in plant tissues [8].

3.3. Isolation and characterization of compound

A chemical study was made of the fractions with photonecrotic activity obtained from the larger-scale cultivation of *P. cedrina* in order to identify the metabolite or metabolites with photosensitizing capacity produced by the bacterium, obtaining a compound, which was identified based on the analysis of its spectroscopic data as 3-(4-(2-carboxipropyl) phenyl) but-2-enoic acid (**1**) (Fig. 1G).

The compound (**1**) showed ability to fluoresce with UV light (360 nm) and from which 18 mg of a white fluorescent powder was obtained from the chloroform:methanol (1:1) extract (Fig. 1G). The nuclear magnetic resonance spectra of ^{1}H and ^{13}C of (**1**) yielded a total of 16 protons and 14 carbons, respectively. The ^{1}H spectrum showed a pair of aromatic signals at δ 7.53 and 5.80 (d, J = 7.7 Hz, 2 H) that each integrated for two protons and were assigned to H-5 and H-9, as well as H-6 and H-8 respectively as well as a simple signal to δ 7.36 (s, 1H) that was assigned to H-2. In the high field, the following signals were highlighted: δ 3.49 (dd, J = 12.7, 6.2 Hz, 1 H) corresponding to H-10, 3.18 (dd, J = 12.7, 10.0 Hz, 1 H) attributable to H-10′; 2.79 (dp, J = 10.1, 6.7 Hz, 1 H) assignable to H-11. Finally, two signals to δ 1.87 (sa, 3 H) assigned to H-13 and 1.20 (d, J = 7.1 Hz, 3 H) assigned to H-14. According to the 2D COSY experiment, the couplings between H-2 and H-13 stand out; as well as that of H-5 with H-6 that by the symmetry of the molecule are also equivalent to H-8 and H-9, thus evidencing a di-substituted aromatic system in "*para*". In addition, the links between H-11 with H-14, as well as H-11 with H-10 and H-10′ were observed. The ^{13}C data confirm the presence of 12 signals, of which two of them are equivalent to two carbons, due to the symmetry of the molecule by an aromatic system di-substituted in "*para*", which assumes the presence of 14 carbon atoms in the structure. Thus, two weak signals at 177.4 and 167.4 ppm showed the presence of an acid group α, β unsaturated and another simple saturated acid. At 156.1, 153.0, 143.4 and 101.0 four aromatic carbon signals were observed, of which the first two are attributed to quaternary carbons and the other two correspond to two carbons, evidencing the presence of a di-substituted aromatic ring; additionally, on the one hand, at 139.0, 110.2 and 11.2 and on the other hand at 41.9, 34.1 and 11.9, they showed the complementary information of the two existing lateral chains. The HMBC experiment mainly confirmed the connectivities between the two side chains and the aromatic ring through the interaction of three links between C-4 (δ 153.0) and H-2 (δ 7.36), as well as the connectivity of two links between C-7 (δ 156.1) and H-10′ (δ 3.49 and 3.18 respectively). Connections between C-1 with H-2 and H-5, C-4 with H-2 and H-5, C-5 with H-6, C-7 with H-10, C-8 with H-9, C-9 with H-8, C-12 with H-10, C-13 with H-2 and C-14 with H-10′ were observed, confirming the proposed structure for this acid that had not been previously isolated.

3.4. In vitro photosensitizing test

When we evaluated the ability of the isolated compound as a photosensitizer testing ergosterol photo-oxidation, the compound (**1**) was able, together with oxygen, to oxidize ergosterol to ergosterol peroxide, confirming the transformation of ergosterol to its peroxide with TLC (Fig. 2A) and the H^{1} NMR spectrum of the reaction mixture (Fig. 2B). Thus, it was possible to identify a low field displacement of the two vinyl protons at 6.53 and 6.25 ppm corresponding to the ergosterol peroxide ring [22].

With this result, it was determined that the compound (**1**) in the presence of light and triplet oxygen ($^{3}O_2$), was able to oxidize ergosterol to its peroxide, and it acted as a photosensitizer producing singlet oxygen ($^{1}O_2$), with which it was corroborated that a photosensitization reaction occurs, mechanism by which the bacteria

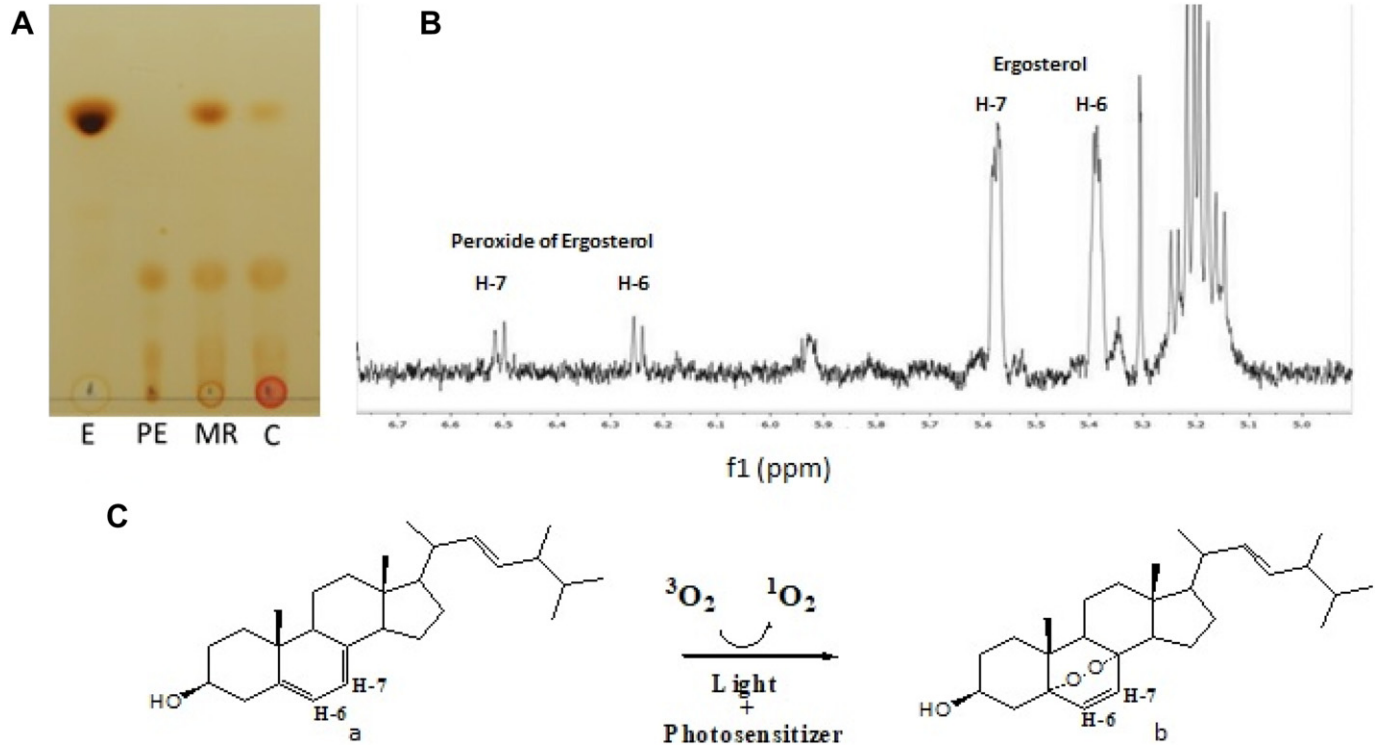

Fig. 2. *In vitro* photosensitizing test of 3-(4-(2-carboxipropyl) phenyl) but-2-enoic acid. (**A**) TLC monitoring (8:2 n-Hex:AcOEt, Silica Gel 60 F_{254}, revealed in iodine vapors) of the photo-oxidation of ergosterol, (E) Ergosterol (reference), (PE) Ergosterol peroxide (reference), (MR) photo-oxidation of ergosterol using 3-(4-(2-carboxypropyl) phenyl) but-2-enoic acid as a photosensitizer, (C) photo-oxidation of ergosterol using eosine as photosensitizer. (**B**) Detail of ^{1}H RMN spectrum (300 MHz, $CDCl_3$) of the reaction mixture of the photo-oxidation of ergosterol. (**C**) Conversion reaction of (a) ergosterol to (b) ergosterol peroxide.

P. cedrina, like some phytopathogenic fungi, could prove to cause oxidative foliar photo-necrosis.

4. Conclusions

The ability of *P. cedrina* to produce photosensitizing compounds was demonstrated *in vivo* by inoculation on papaya leaves and observation of its effects in the absence or presence of sunlight, as well as *in vitro* of one of its metabolites 3-(4-(2-carboxipropyl) phenyl) but-2-enoic acid, using ergosterol as substrate. We can conclude that this bacterium was able to induce oxidative foliar photo-necrosis because of its potential ability to produce photosensitizing metabolites that generate singlet oxygen in the plants it colonizes. Based on the above, it can be proposed that some bacte cause oxidative foliar photo-necrosis as an important mechanism in the pathogenesis of host species.

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Conflict of interest

None.

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