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# Photodynamic inactivation of the phytopathogenic bacterium *Xanthomonas citri* subsp. *citri*

Anastasia Ndemueda[1], Inês Pereira[1], Faustino, M.A.F.[2], Cunha, Â. [1]

[1]CESAM and Department of Biology, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal

[2]LAQV-REQUIMTE and Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

Corresponding author: Ângela Cunha acunha@ua.pt



## Statement of contribution

All authors contributed substantially to the conception and design of the work or the acquisition and analysis of data, to the drafting or critical revision of the manuscript and approved the final submitted version.

## Abbreviated running headline

Photoinactivation of X*anthomonas citri*

## Significance and impact of the study (100 words)

This study demonstrates for the first time that the causative agent of citrus canker, *Xanthomonas citri* subsp. *citri* is susceptible to photodynamic inactivation and that biofilms can be eradicated with the phenothiazine dye Toluidine Blue O (50 μM) in presence of a non-toxic concentration of KI (100 mM) upon exposure to natural sunlight.

## Abstract

The present work intended to evaluate the applicability of photodynamic inactivation (PDI) of *Xanthomonas citri* subsp. *citri* with toluidine blue O (TBO), a commercial photosensitizer, as a strategy to control citrus canker.

Assays were conducted with cell suspensions and biofilms constructed either on polypropylene microtubes (*in vitro* assays) or on the surface of orange leaves (*ex vivo* assays), in the presence of TBO and under artificial white light irradiation or natural sunlight.

PDI assays using TBO alone caused a maximum 5.8 log reduction of *Xanthomonas citri* viable cells in suspensions, and much smaller inactivation (1.5 log) when in biofilm forms. However, concomitant use of KI potentiated the TBO photosensitization.

Biofilms were completely eradicated (> 6 log reduction) with 5.0 µM TBO + 10 mM KI (*in vitro*) or 5.0 µM TBO + 100 mM KI (*ex vivo*) after artificial white light irradiation.

Under natural sunlight irradiation conditions, complete eradication was achieved with 50 µM TBO and 100 mM KI.

PDI has potential to be applied in the control of citrus canker in field conditions although further studies are needed to show that there are no risks to plant physiology or fruit quality.


Keywords: citrus canker; toluidine blue O; potassium iodide; phytopathogens; photosensitization;

## Introduction

Citrus cancer is a globally occurring infectious disease caused by the gram-negative phytopathogenic bacterium *Xanthomonas citri* subsp. *citri* [syn. *X. campestris* pv. *citri*, *Xanthomonas axonopodis* pv *citri* (Behlau & Belasque, 2014)]. The disease causes defoliation and premature fruit drop. Leaf loss compromises the photosynthetic area resulting in lower productivity and the appearance of injuries and fall of fruits before harvest causes losses of up to 80% (Lanza et al. 2018).

Cupric bactericides still represent the most popular chemical approach (Behlau et al. 2017). However, they have a strictly preventive character, showing neither curative efficacy nor systemic activity in the plant (Lamichhane et al. 2018). On the other hand, the application of copper-based biocides may induce bacterial resistance to biocides in soil microbes (Glibota et al. 2019) and phytopathogens (Roach et al. 2020). In fact, the first copper-resistant *X. citri* strain (*X. citri* CuR) was reported in nurseries that received regular applications of copper-based bactericides (Canteros et al. 2008). With the aim of increasing the efficiency of treatments while reducing the impact of undesirable side-effects, compounds with low copper concentration, such as copper sulphate pentahydrate (Favaro et al. 2017), gallic acid esters (Savietto et al. 2018), zinc oxide nanoparticles (Graham et al. 2016), systemic neonicotinoid biocides and acquired resistance inducers (Graham and Myers 2016), biological control with endophytic bacteria producing quorum sensing inhibitors or toxic secondary metabolites (Daungfu et al. 2019; Villamizar and Caicedo 2019) and bacteriophages (Ibrahim et al. 2017) have been tested. However, a treatment that is operationally viable while ensuring high efficiency, low environmental toxicity and good economic sustainability has not yet been achieved.

Photodynamic inactivation (PDI) of microorganisms relies on the interaction of a non-toxic photosensitive molecule (photosensitizer, PS), light and molecular oxygen. Once activated by light, the PS catalyzes the formation of reactive oxygen species (ROS) that will induce oxidative damage to lipids, proteins, including enzymes, and nucleic acids, leading to cell death (Wainwright et al. 2017). PDI has several advantages when compared to traditional antimicrobial approaches: it is effective against different types of microorganisms regardless of their antibiotic or antifungal resistance profile; it addresses different molecular targets mainly in external structures and does not depend on the accumulation of PS in the intracellular compartment; it involves cytotoxic agents, particularly singlet oxygen, for which cells lack effective defense or detoxification mechanisms. The development of specific resistance to oxidative stress is not yet demonstrated and only a few dyes are considered as potential substrates of efflux pumps (Cieplik et al. 2018).

Although antitumoral and antimicrobial therapies are historically the most prominent biological applications, PDI has been progressively expanding to fields not directly related to human health (Alves et al. 2015; Jesus et al. 2018; Glueck et al. 2019). PDI targets all groups microorganisms, but they are not evenly susceptible. Gram-positive bacteria are more susceptible to PDI than gram-negative bacteria due to differences in cell wall structure and composition (Almeida et al. 2015) and microbial biofilms are less susceptible than planktonic cells because the extracellular polymeric substances behave as diffusion barriers and ROS quenchers (Beirão et al. 2014; Gambino and Cappitelli 2016). Nevertheless, PDI with synthetic (porphyrins, chlorins, phtalocyanines, among others) and natural (curcumin, riboflavin, etc) PSs has been successfully applied against bacterial, fungal or mixed biofilms (Vilela et al. 2012; Beirão et al. 2014; Bonifácio et al. 2018). Recently, it has been discovered that the

addition of non-toxic concentrations of inorganic salts like KI, KBr, KSCN or $NaNO_2$ significantly potentiates the photosensitization process to the level of the complete eradication of the otherwise less susceptible microbial targets (Hamblin and Abrahamse 2018; Santos et al. 2019).

Despite the recognized advantages of PDI as an antimicrobial approach, the transposition into phytosanitary applications is still poorly explored. There are, however, promising reports of PDI of the fungi *Colletotricum abscissum* in citrus (Gonzalez et al., 2017) and *Fusarium oxysporum* in sprouted wheat seeds (Žudytė and Lukšienė 2019) and the Gram negative bacterium *Pseudomonas syringae* pv. *actinidiae* in kiwi plants (Jesus et al. 2018; Martins et al. 2018).

This work aimed at contributing to the development of a photodynamic control protocol for citrus canker. For this purpose, the efficiency of photosensitization of *X. citri* subsp. *citri* with toluidine blue O (TBO), in presence of KI, was assessed in cell suspensions and biofilms upon irradiation with artificial or natural sunlight.

## Results and discussion

As a first approach, the efficiency of photodynamic inactivation (PDI) was accessed in cell suspensions, by calculating the logarithmic reduction factor of the concentration viable cells determined before and after irradiation. Exposure to white artificial light (400-700 nm, 150 mW $cm^{-2}$) for 60 min (0.540 kJ $cm^{-2}$) in the presence of 80 μM TBO caused a 5.8 log inactivation (Fig. 1). The PS concentration increase failed to improve the photodynamic inactivation efficiency. *X. citri* subsp. *citri* revealed lower susceptibility to PDI than other Gram negative bacteria. For instance, a lower concentration of TBO (44 μM) and smaller light dose (40 J $cm^{-2}$) caused >8 log inactivation in cell suspensions of *Escherichia coli* (Usacheva et al. 2001). The lower

susceptibility of *X. citri* subsp. *citri* to PDI may be related to genetically encoded mechanisms of protection against oxidative stress, important in the infection process (Loprasert et al. 1996; Fuangthong et al. 2015).

[Fig 1]

The type of PSs determines affinity to target cells, prevailing photosensitization mechanism and ultimately, inactivation efficiency (Alves et al. 2015). When comparing results obtained with cells suspensions using the same platting method (pour platting), porphyrins and chlorins have been reported as attaining higher inactivation factors against some Gram negative phytopathogenic bacteria. *X. anoxopodis* suffered a 7 log reduction with 10 μM of a mixture of cationic derivatives of chlorin $e_6$ and an energy dose of 27 J $cm^{-2}$ (Glueck et al. 2019). PDI of *Pseudomonas syringae* pv. *actinidiae* (Psa) with a cationic porphyrin produced an inactivation factor equivalent to that obtained in this work (6 log) with half the concentration of porphyrinic PS (50 μM) and a slightly higher light dose (Jesus et al. 2018) and when a mixture of several porphyrin derivatives was used, the factor raised to 7.4 log with only 5.0 μM of PS mixture after a total light dose of 14 J $cm^{-2}$ (Martins et al. 2018). However, for the moment, these synthetic PSs are neither affordable nor commercially available for large-scale use.

There was no significant variation in light (LC) and dark (DC) controls (ANOVA, $p > 0.05$), indicating that neither the TBO nor KI exhibited cytotoxic effect in the absence of light.

The PDI *X. citri* subsp. *citri* biofilms attached to polypropylene microtubes (Fig. 2) with 100 μM of TBO caused a much smaller reduction (1.5 log) than observed in cells suspensions, in similar irradiation conditions. The decreased susceptibility of biofilms to PDI, in relation to the planktonic form, has been recurrently reported and leads to the need for higher light doses and PS concentrations (Beirão et al. 2014;

Bonifácio et al. 2018). The effect is usually attributed to barrier imposed by the extracellular matrix, which limits PS and $O_2$ access to target cell structures, and the ROS-scavenging effect of extracellular polymeric substances (Kishen 2017).

However, in presence of 10 mM of KI there was inactivation of the biofilm cells to the limit of detection of the method (6.3-6.6 log), with TBO concentrations as low as 5.0 µM.

[Fig. 2]

The potentiating effect of non-toxic concentration of KI on the photosensitization of biofilms has been demonstrated for several PSs in various target microorganisms, although in general, with higher KI concentrations. Effective PDI of *Candida albicans* biofilms required 100 µM of methylene blue (MB) and 100 mM KI (Freire et al. 2016). The addition of 100 mM KI increased by about 2 log the inactivation factor of biofilms of *Enterococcus faecalis* with 100 µM TBO (Ghaffari et al. 2018) and complete eradication of biofilms of *Escherichia coli* was attained by combining 1.0 µM of a mixture of porphyrinic PS with 100 mM KI (Vieira et al. 2019). In this study, the combination of 5.0 µM TBO and 10 mM KI was sufficient to reduce the concentration of viable cells in biofilms of *X. citri* subsp. *citri* to the detection limit, which corresponds to a lower KI concentration than usually used for the PDI of biofilms of other Gram negative bacteria. However, the Miles-Misra method used in the assays with biofilms has a much higher limit of detection than the pour-platting technique. Complete inactivation would correspond to a >8 log reduction that cannot be demonstrated with the former method. With 1.0 µM TBO + 1.0 mM KI, there was a small (1.3 log), albeit significant (ANOVA, $p < 0.05$) photodynamic inactivation of biofilm cells. Light (LC) and dark (DC) controls confirm that like planktonic cells,

biofilms are not susceptible to light and that neither TBO nor KI have inhibitory effect in the dark.

[Fig. 3]

The *ex vivo* assays of PDI of biofilms of *X. citri* subsp. *citri* on the surface of orange tree leaves had the dual purpose of evaluating if under these conditions the same inactivation efficiency as in the *in vitro* assays could be achieved and also detecting eventual damages in the integrity of the leaves. Under artificial light, complete eradication of biofilms (log reduction ~ 6 log) occurred in presence of 100 mM KI, with TBO concentrations ranging 5-100 µM (Fig. 3) despite the fact that the photodynamic inactivation efficiency was lower than *in vitro*. Although the combination of 5.0 µM TBO + 10 mm KI that produced complete inactivation of biofilms *in vitro* was not tested, the combination corresponding to a higher PS concentration (10 µM TBO + 10 mM KI) caused a reduction of only 6.3 log (>99.9999%). Nevertheless, complete eradication of the biofilms was achieved in presence of 100 mM KI. The reduction in PDI efficiency in *ex vivo* conditions, compared to *in vitro* assays, was also observed in PDI of Psa on kiwi leaves (Jesus et al. 2018) and may be related to protection and shading caused by leaf micro-texture and sheltering provided by the stomata. Another effect may be associated with the experimental design. In the *in vitro* assays, the biofilms were immersed in the PS-containing solution during irradiation PS whereas in the *ex vivo* assays, the biofilms were pre-exposed to the PS during the dark incubation period but later removed from the PS-containing solution. Therefore, in the ex vivo assays, the PS availability is restricted to the molecules that actually bound the biofilms during the dark exposure period. Nevertheless, complete eradication of the biofilms with cationic porphyrins was achieved without visible leaf damage (Jesus et al. 2018) as

196 opposed to what is observed with anionic porphyrins that can cause plant cell death
197 (Leroy-Lhez et al. 2019).

198 Envisaging the transposition of PDI protocol to field conditions, the biofilm PDI
199 on orange leaves was tested under natural sunlight irradiation (Fig. 4). Since sunlight
200 irradiance was lower (23-60 mW $cm^{-2}$), irradiation time was extended up to 4 h to reach
201 a similar total energy dose (~ 0.6 kJ $cm^{-2}$) to that used in the experiments under artificial
202 white light. Under these experimental conditions with 100 mM KI, there was complete
203 inactivation of the biofilms with TBO concentrations higher than 50 μM and a 3.6 log
204 reduction with concentration of 20 μM of TBO. The results indicate that under natural
205 sunlight irradiation, the photosensitization process was less efficient than under artificial
206 light conditions. Whereas with artificial light complete eradication (~6 log reduction) of
207 the biofilms was achieved with 5.0 μM TBO + 100 mM KI, with natural sunlight a 10-
208 fold higher of PS concentration was required. This may be a consequence of the
209 different spectra of the artificial light source and natural sunlight (Supporting
210 Information). TBO has a peak of absorbance at 630 and the peak of energy of artificial
211 light is centered around 580 nm, whereas in natural sunlight the peak occurs closer to
212 500 nm. Therefore, artificial light is likely to deliver more energy to the PS than natural
213 sunlight. Also, natural sunlight contains UV, which is absent from the emission
214 spectrum of artificial light, and may induce the photodegradation of the PS.
215 Phenothiazine dyes are susceptible to photo-reduction (Koizumi et al., 1964) that in the
216 case of prolonged exposure to solar irradiation, could cause an effective loss of PS.
217 However, complete eradication of the biofilm was still attained within 4 h, without
218 visible damage to the leaves and, in this case, better preserving the freshness and
219 turgidity of the leaves.

So far, no leaf damage caused by the direct contact with the PS was detected in long experiments (2 week-trials without PS re-application) of PDI of *Colletotrichum* on young orange trees exposed to sunlight (de Menezes et al. 2014). Also, leaf damage caused by KI has not been reported, for concentrations up to 100 mM. However, the effect of long term exposure to TBO and KI has not yet been tested.

[Fig. 4]

As conclusion, the phytopathogenic *X. citri* subsp. *citri* is less susceptible to PDI than other Gram negative bacteria, particularly in the biofilm forms. However, photosensitization with TBO in presence of KI produces a significant reduction in viable cell concentration. It was possible to completely eradicate leaf biofilms with 50 μM TBO and 100 mM KI after 4 h of exposure to indirectly applied natural sunlight. These results allow us to expect that complete photodynamic inactivation may be reached with even lower PS concentrations in a full day light exposure (~12 h). Visual inspection of leaves revealed no damage, which indicates good TBO compatibility with plant tissue. However, it will also be important to evaluate other physiologic parameters, namely chlorophyll content, that may provide information on potential negative effects of TBO on photosynthetic performance. The fact that TBO is susceptible to photo-reduction upon prolonged irradiation can represent an advantage considering the use for phyto-sanitary applications as it reduces the risks of accumulation in the environment. Therefore, PDI with TBO represents a promising perspective for a cost-effective and environmentally sustainable alternative to chemical biocides for control of citrus cancer.

## Materials and methods

### Photosensitizer and coadjutant solutions

The stock solution (500 µM) of TBO (Sigma-Aldrich, St. Louis, Missouri, EUA) was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, Missouri, EUA) and stored at 4 ºC in the dark. The stock solution (5 mM) of potassium iodide (KI, Atom Scientific, Cheshire, UK) was prepared in distilled water and stored at room temperature. Prior to each assay, TBO and KI stock solutions were sonicated for 30 min to ensure complete homogeneity of the solution.

**Preparation of bacterial cultures, cells suspensions and biofilms**

*Xanthomonas citri* subsp. *citri* Xcc 306 (Gonçalves-Zuliani et al. 2015) was provided by Prof. William Nunes (State University Maringá, Brazil). Prior to each assay, a fresh stationary-phase culture ($OD_{600}$ ~ 0.8) in Tryptic Soy Broth (TSB, Liofilchem, Roseto degli Abruzzi, Italy) was prepared (18 h at 37 °C under 180 rpm).

Cell suspensions were obtained by diluting (1:10) the fresh culture in phosphate saline buffer (PBS, pH 7.2). For the preparation of biofilms on the internal surface of microtubes (*in vitro* assays), 1 mL-aliquots of fresh culture were transferred to sterile 1.5 mL polypropylene microtubes. After 24 h incubation at 37 ºC (without agitation) the medium was discarded, the microtube walls were gently rinsed with 1.0 mL PBS for removal of loosely attached cells and 1.0 mL of fresh TSB was added to each tube. Biofilms were allowed to mature for 48 h at 37 ºC, without agitation, and before the PDI assays, the medium was discarded and the biofilm was gently rinsed with sterile PBS. For the *ex vivo* assays, freshly harvested orange tree leaves were washed with distilled water and dried with filter. On the upper page of each leaf, three squares (2 x 2 cm) were drawn with permanent marker. The leaves were sterilized by60 min of immersion in 1% hydrogen peroxide and 15 min UV irradiation on each page. Sterilized leaves were immersed (30 min at 37 °C) on a 1:10 dilution in PBS of a fresh TSB culture of

the bacterium. Inoculated leaves were individually transferred to petri dishes containing 15 mL PBS and incubated for 48 h at 37 °C for biofilm maturation. Whenever necessary, sterile PBS was added to the bottom of the plate to prevent leaf dehydration.

**Photodynamic inactivation experiments**

For the PDI of cell suspensions, the solution of TBO was added directly to the suspension to achieve the work concentration of 80 μM. For the *in vitro* assays of biofilm PDI, 1.0 mL of PBS was added to each microtube and the solutions of TBO and KI were added to achieve the work concentrations (1-100 μM TBO and 1-100 mM KI). For the *ex vivo* assays of biofilm PDI, the leaves on which biofilms developed were immersed in PBS containing the work concentrations of TBO and KI (5-100 μM TBO and 10 or 100 μM KI). Once in contact with the PS, cell suspensions and biofilms were incubated in the dark, at 37 ºC, during 30 min. After dark incubation, leaves used in the *ex vivo* assays were removed from the solutions and placed on petri plates containing 15 mL of PBS (underneath the leaf) to avoid excessive desiccation during irradiation.

For PDI assays with artificial light, cell suspensions and biofilms, either in microtubes or on the surface of orange tree leaves, were irradiated during 60 min with artificial white light (400-800 nm;150 mW $cm^{-2}$) delivered by a 250 W quartz/halogen lamp system equipped with a fiber optic probe (LumaCare s Model 122, Newport Beach, USA). In PDI assays with natural sunlight (350-850 nm; 23-60 mW $cm^{-2}$, according to CliM@UA; http://climetua.fis.ua.pt/weather/solar_radiation/aveiro), irradiation was conducted for 4 h.

**Calculation of the logarithmic reduction factor**

The concentration of viable cells was determined before and after irradiation, in cell suspensions and biofilms. For the quantification of viable cells in suspensions, aliquots were serially diluted in PBS and pour-plated in triplicate in Tryptic Soy Agar (TSA, Liofilchem, TSB, Liofilchem, Roseto degli Abruzzi, Italy). For the analysis of biofilms in microtubes, each microtube was sonicated for 90 s (Silvercrest®, Neckarsulm, Germany) and homogenized in the vortex for 60 s. The homogenized suspensions were serially diluted in PBS and platted in triplicate in TSA by the Miles-Misra method. Biofilms on leaves were scrapped from the 2 x 2 cm squares with a swab, resuspended in 1.0 mL PBS, and thereafter treated as biofilms in microtubes. Cultures were incubated for 24 h at 37 ºC and colony forming units (CFU) were counted in the replicates of the most suitable dilution. The inactivation efficiency was calculated as the logarithmic reduction in the concentration of viable cells during the period corresponding to irradiation of the tests.

Controls were included in all assays. Light controls (LC) were exposed to the same irradiation conditions as the tests, without addition of the TBO and KI. Dark controls received the highest tested concentrations of TBO, KI or TBO + KI and were incubated in the dark during a period equivalent to the irradiation of the tests. Each experimental condition was tested in 3 independent assays.

Significance of the differences between treatments was assessed by one-way ANOVA, performed with the GraphPad Prism 6 package, considering $p < 0.05$ as significant.

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## Conflict of interest

No conflict of interest declared.

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439

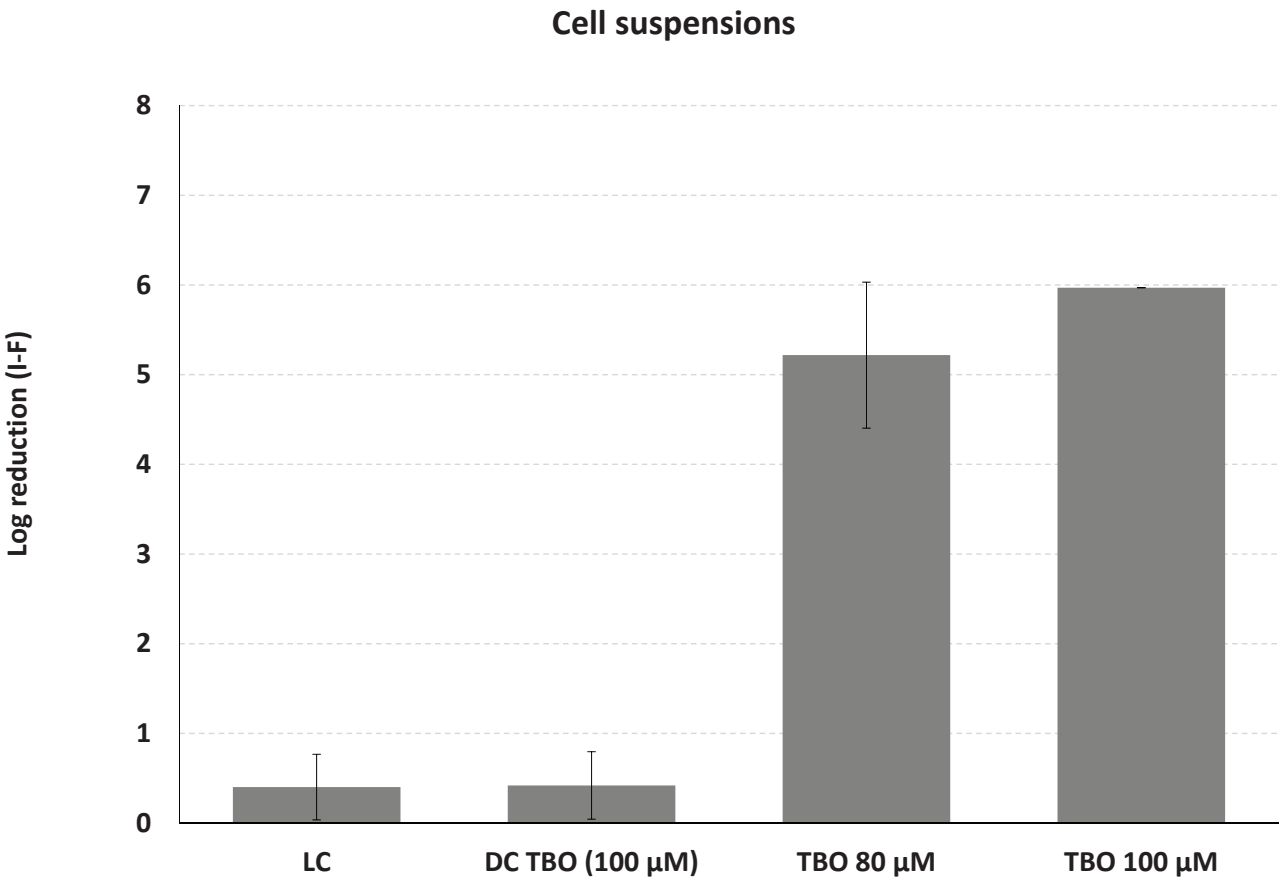
## Figure Legends

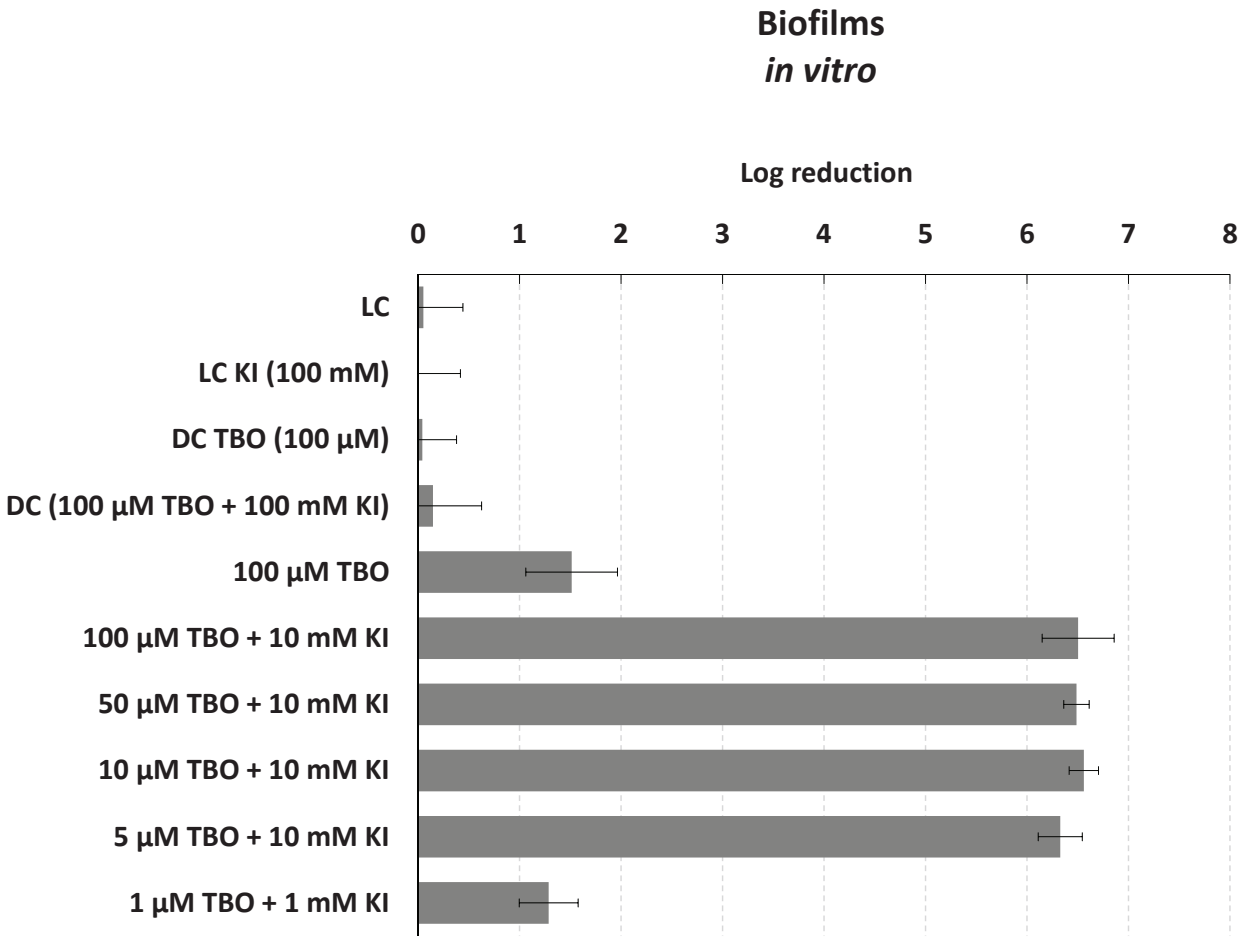
Figure 1. Logarithmic reduction of the concentration of viable cells in suspensions of *Xanthomonas citri* subsp. *citri* irradiated with artificial white light (150 mW $cm^{-2}$) for 60 min (0.540 kJ $cm^{-2}$), in presence of TBO. Error bars represent the standard deviation of 3 independent assays with replicates.

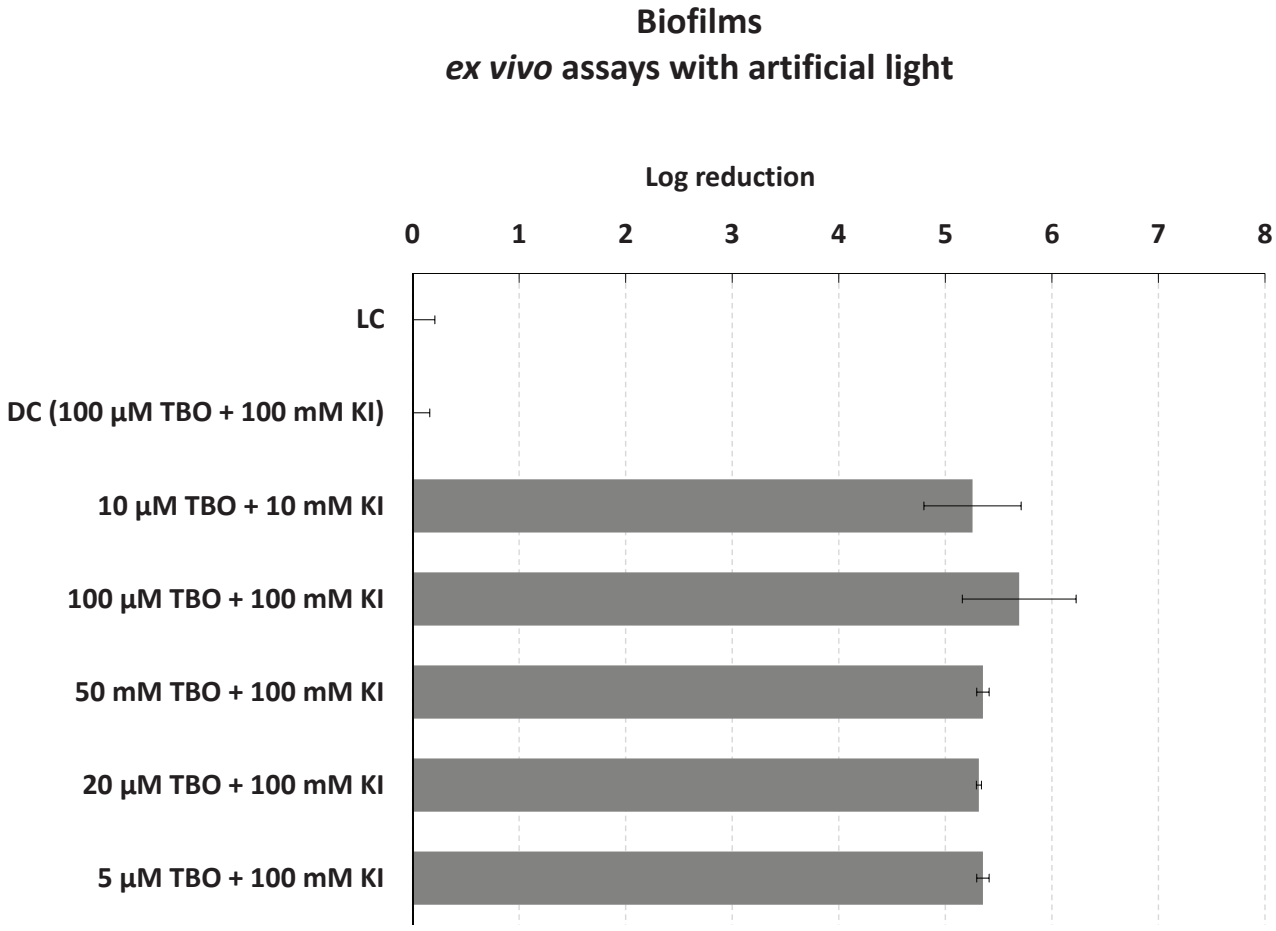
Figure 2. Logarithmic reduction of the concentration of viable cells in biofilms of *Xanthomonas citri* subsp. *citri* adherent to polypropylene microtubes, irradiated with artificial white light (150 mW $cm^{-2}$) for 60 min (0.540 kJ $cm^{-2}$), in presence of TBO and KI. Error bars represent the standard deviation of 3 independent assays. * indicates absence in the 10 µL aliquots (complete eradication).

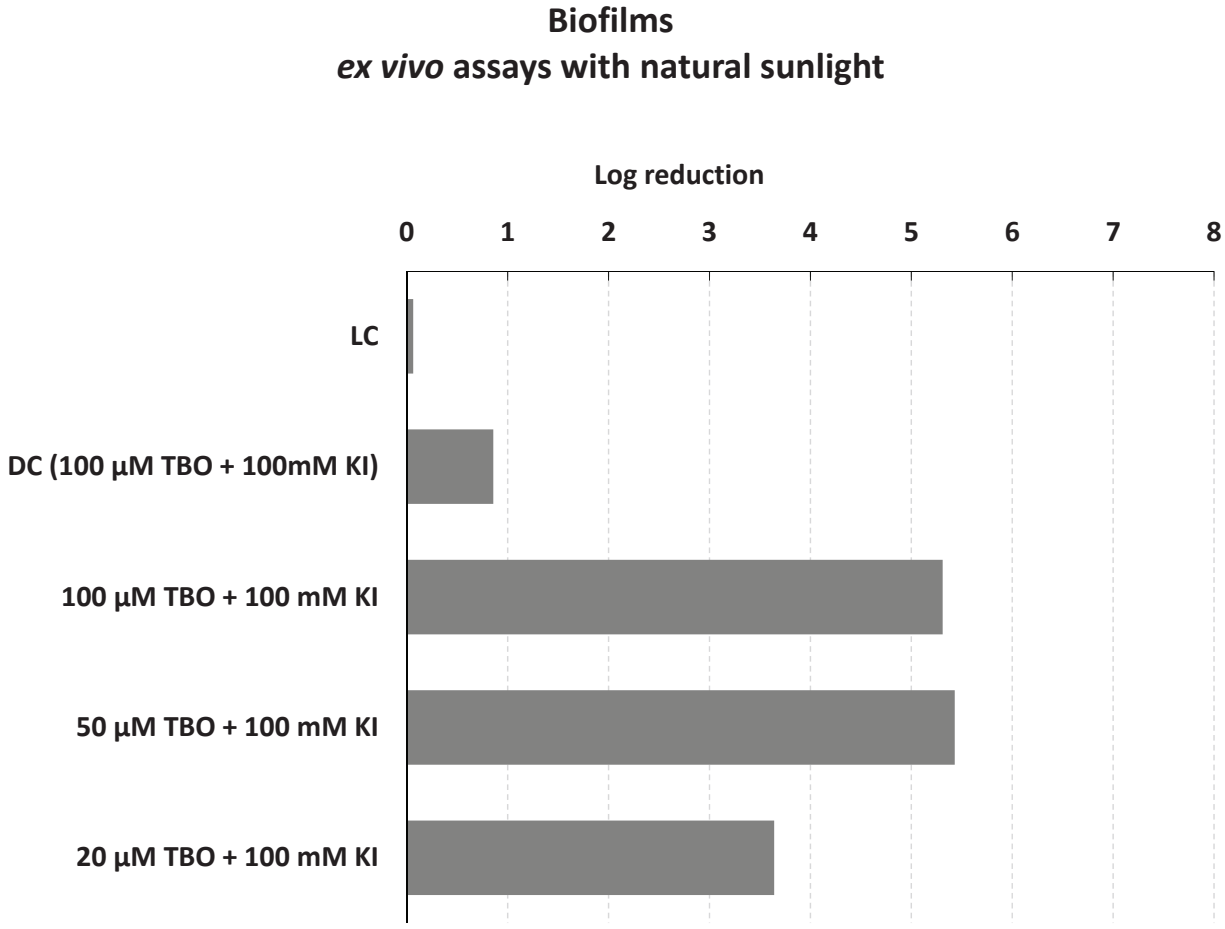
Figure 3. Logarithmic reduction of the concentration of viable cells in biofilms of *Xanthomonas citri* subsp. *citri* on the surface of orange tree leaves, irradiated with artificial white light (150 mW $cm^{-2}$) for 60 min (0.540 kJ $cm^{-2}$), in presence of TBO and KI. Error bars represent the standard deviation of 3 independent assays. * indicates absence in 10 µL aliquots (complete eradication).

Figure 4. Logarithmic reduction of the concentration of viable cells in biofilms of *Xanthomonas citri* subsp. *citri* on the surface of orange tree leaves, irradiated with natural sunlight for 4 h (~0.6 kJ $cm^{-2}$), in presence of TBO and KI. Error bars represent the standard deviation of 3 independent assays. * indicates absence in 10 µL aliquots.


Cell suspensions
Log reduction (I-F)
8
7
6
5
4
3
2
1
0
LC
DC TBO (100 µM)
TBO 80 µM
TBO 100 µM


Biofilms
in vitro
Log reduction
0
1
2
3
4
5
6
7
8
LC
LC KI (100 mM)
DC TBO (100 µM)
DC (100 µM TBO + 100 mM KI)
100 µM TBO
100 µM TBO + 10 mM KI
50 µM TBO + 10 mM KI
10 µM TBO + 10 mM KI
5 µM TBO + 10 mM KI
1 µM TBO + 1 mM KI


Biofilms
ex vivo assays with artificial light
Log reduction
0
1
2
3
4
5
6
7
8
LC
DC (100 µM TBO + 100 mM KI)
10 µM TBO + 10 mM KI
100 µM TBO + 100 mM KI
50 mM TBO + 100 mM KI
20 µM TBO + 100 mM KI
5 µM TBO + 100 mM KI


Biofilms
ex vivo assays with natural sunlight
Log reduction
0
1
2
3
4
5
6
7
8
LC
DC (100 µM TBO + 100mM KI)
100 µM TBO + 100 mM KI
50 µM TBO + 100 mM KI
20 µM TBO + 100 mM KI

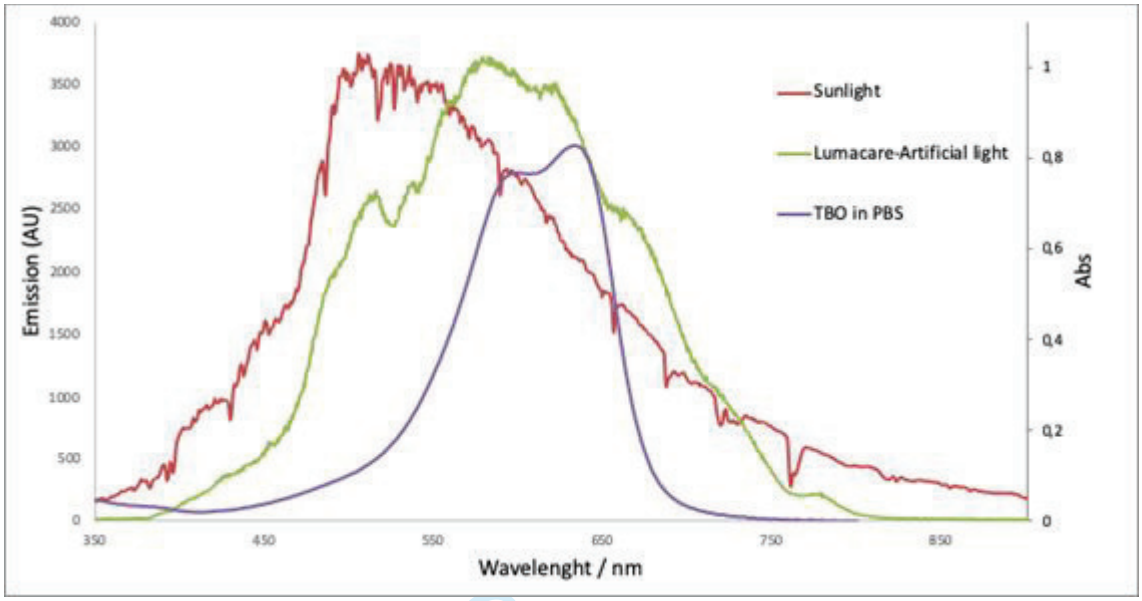



Figure S1 Absorption spectrum of Toluidine Blue O (TBO) dissolved in phosphate saline buffer (PBS) and emission spectra of natural sunlight and artificial white light delivered by the Lumacare 122 system coupled to a light fiber probe (400-800 nm).