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In vitro photodynamic inactivation of conidia of the phytopathogenic fungus *Colletotrichum graminicola* with cationic porphyrins†

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Photodynamic inactivation (PDI) is an efficient approach for the elimination of a series of microorganisms; however, PDI involving phytopathogenic filamentous fungi is scarce in the literature. In the present study, we have demonstrated the photoinactivating properties of five cationic *meso*-(1-methyl-4-pyridinio)porphyrins on conidia of the phytopathogen *Colletotrichum graminicola*. For this purpose, photophysical properties (photostability and $^1O_2$ singlet production) of the porphyrins under study were first evaluated. PDI assays were then performed with a fluence of 30, 60, 90 and 120 J cm^{-2} and varying the porphyrin concentration from 1 to 25 μmol L^{-1}. Considering the lowest concentration that enabled the best photoinactivation, with the respective lowest effective irradiation time, the *meso*-(1-methyl-4-pyridinio)porphyrins herein studied could be ranked as follows: triple-charged **4** (1 μmol L^{-1} with a fluence of 30 J cm^{-2}) > double-charged-*trans* **2** (1 μmol L^{-1} with 60 J cm^{-2}) > tetra-charged **5** (15 μmol L^{-1} with 90 J cm^{-2}) > mono-charged **1** (25 μmol L^{-1} with 120 J cm^{-2}). Double-charged-*cis*-porphyrin **3** inactivated *C. graminicola* conidia in the absence of light. Evaluation of the porphyrin binding to the conidia and fluorescence microscopic analysis were also performed, which were in agreement with the PDI results. In conclusion, the cationic porphyrins herein studied were considered efficient photosensitizers to inactivate *C. graminicola* conidia. The amount and position of positive charges are related to the compounds' amphiphilicity and therefore to their photodynamic activity.

Introduction

Photodynamic inactivation (PDI) is a technique based on the association of a photosensitizer, light and molecular oxygen with the objective of eliminating microorganisms by destroying their cell structures.[1–3] PDI can be employed for the inactivation of various microorganisms, including bacteria,[4,5] viruses,[6] fungi[3,7–9] and parasites.[10,11]

Among the photosensitizers employed for PDI, porphyrins have been extensively evaluated, especially their cationic derivatives. The attachment of positively charged groups to the macrocycle enhances porphyrin water solubility[12,13] and improves its interaction with microorganism cell structures.[3,13–15] In this way, cationic porphyrins have been evaluated as photosensitizers[14,16–18] against Gram positive and Gram negative bacteria,[5,19–22] yeast[14,17,20,23] and filamentous fungi.[9,18] It is important to mention that due to the resistance offered by the fungal cell structures (*e.g.* cell wall), the inactivation of such organisms usually demands high photosensitizer concentrations, as well as high light intensities.[18]

Fungus *Colletotrichum graminicola* is the etiologic agent of corn (*Zea mays*) anthracnose,[24–26] which is the most important disease that affects corn plants.[25,27–29] Anthracnose causes a substantial decrease in the corn crop yield, which can be as large as 40%.[26,29,30] *C. graminicola* is capable of surviving on corn residues previously infected,[31] specially in the soil surface,[32] which turns a cultivating area into a potential inoculum site.[26] In addition, the great genetic variability shown by *C. graminicola* makes the employment of hybrid plants ineffective as a tool for anthracnose control.[33–35] The use of fungicides has only been partially effective for the treatment of anthracnose. In addition, microorganism resistance against the most common fungicides has also been reported.[36,37] Not least important is the fact that pesticide-based control of plant

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diseases has several negative implications for the environment and human health.[38]

Studies involving PDI of filamentous fungi have been described in the literature.[18,39] In the case of phytopathogenic fungi, PDI using hematoporphyrin has been utilized to eliminate a number of filamentous fungi (*Alternaria alternata*, *Aspergillus flavus*, *Fusarium avenaceum*, *Fusarium poae*, *Fusarium culmorum*, *Acremonium strictum*, *Trichothecium roseum* and *Rhizopus oryzae*) which are generally relevant to the food industry.[9] Considering phytopathogens of the genus *Colletotrichum*, there are two reported PDI studies. Menezes *et al.* (2014)[40] employed phenothiazine dyes as photosensitizers for the inactivation of *C. acutatum* and *C. gloeosporioides*. The same authors[41] also utilized the furocoumarins 8-methoxypsoralen and isopimpinellin, and a mixture of two coumarins to photoinactivate *C. acutatum*.[41] As far as we are aware, porphyrins have never been utilized as photosensitizers for the photoinactivation of *Colletotrichum* spp.

In view of the potential application of photosensitizers in agriculture, as well as the lack of studies devoted to this approach, here we evaluated the photoinactivation of the conidia of *C. graminicola* using cationic porphyrins. The choice of the photosensitizers herein utilized (Fig. 1) was based on the fact that tetra-methylpyridinio porphyrin (**5**) has been largely utilized for the photoinactivation of bacteria,[4,19,21,42–46] filamentous fungi[18] and yeast.[23,47–49] Considering that different charge numbers and distributions could greatly affect the photoinactivating properties of this type of porphyrin, we included in the present study all five possible cationic porphyrin bearing phenyl or 1-methyl-4-pyridinio groups at the macrocycle *meso* positions.

Experimental

Reagents, materials and general methods

All reagents and solvents utilized were of reagent grade and they were used as obtained. Pyrrole, 4-pyridinecarboxaldehyde, *N*,*N*-dimethylformamide (DMF) and 3-diphenylisobenzofuran (DPBF) were purchased from Sigma-Aldrich®. Benzaldehyde, propionic acid and ethyl ether were purchased from VETEC®.

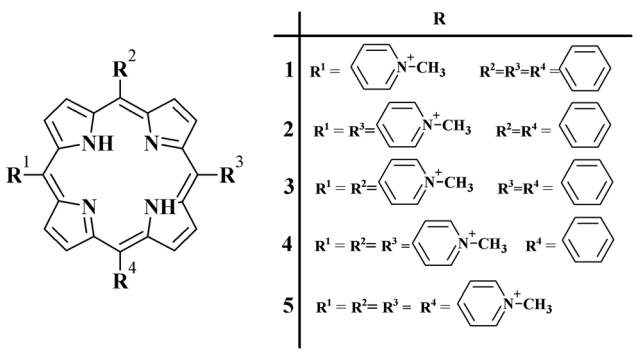

Fig. 1 Structure of the porphyrins used for the photoinactivation studies of *Colletotrichum graminicola* conidia.

Dimethyl sulfoxide (DMSO) and methyl iodide were from Merck®. Thin-layer chromatography (TLC) was performed on silica gel coated aluminum sheets 60 F_{254} (Merck®) using solvent mixtures measured on a v/v basis. Column chromatography was carried out using silicagel 60 35–70 mesh (Fluka®).

Porphyrin characterization was performed by means of ^{1}H NMR spectroscopy, MALDI-TOF mass spectrometry and ultraviolet-visible spectroscopy with a Bruker AVANCE III 400 NMR spectrometer, a MALDI-TOF/MS model Autoflex II Bruker Daltonics and UV-1800 Shimadzu spectrophotometer, respectively.

Materials used for the culture media preparation were oatmeal (Nestlé®), bacteriological agar (VETEC®) and Sabouraud dextrose agar (Kasvi®). Sterile 6-well plates (well volume: 15.53 mL) for photodynamic inactivation were purchased from TPP®.

The white light source used for the photodynamic and photophysical assays was a Lumacare LC 122A with a compatible fiber optic probe (400–800 nm) attached to a 250 W quartz/halogen lamp (LumaCare®, USA). Fluorescence measurements for the binding assay were performed with a RF-5301PC Shimadzu spectrofluorometer. Centrifugation employed for the photosensitizer binding assay and microscopic analysis was performed with the centrifuge Sigma, model 1-14. Microscopy images were recorded using a fluorescence microscope (Olympus BX51TF) with a FITC filter and with microscope magnification of eighty. Images were evaluated with Cell^F software, 5.0 version (Olympus Europe Software Information).

Porphyrin synthesis

The five cationic porphyrins evaluated (Fig. 1) were synthesized according to a methodology that has been previously described,[50] with modifications. Briefly, a mixture of pyrrole, benzaldehyde, 4-pyridinecarboxaldehyde (4 : 1 : 3) in propionic acid was refluxed for 1 h. After the reaction time, the reaction mixture was concentrated by heating and porphyrins were precipitated by adding acetone to the concentrated mixture. The purple solids obtained corresponded to a mixture of six compounds, including tetraphenylporphyrin and the desired *meso*-pyridyl porphyrins. The porphyrin mixture was fractionated by flash chromatography on a silica gel column with chloroform : methanol (99 : 1) as the mobile phase. Porphyrins containing one (0.9% yield), two-*trans* (1.1% yield), two-*cis* (1.0% yield), three (3.1% yield) and four (2.5% yield) *meso*-pyridyl groups were then obtained separately. Each of the *meso*-pyridyl porphyrins was subsequently subjected to a methylation reaction with excess methyl iodine, in DMF, to give cationic *meso*-(1-methyl-4-pyridinio)porphyrins **1** (iodide salt, 90% yield), **2** (diiodide salt, 38% yield), **3** (diiodide salt, 27% yield), **4** (triiodide salt, 60% yield) and **5** (tetraiodide salt, 64% yield).[51] Synthesis procedures and compound characterization are detailed in the ESI.† Spectral properties of porphyrins **1–5** coincided with literature data.[52,53]

Photosensitizer stock solutions

Photosensitizer stock solutions used in the photophysical and biological studies were prepared in DMSO at a concentration of 1 mmol L^{-1} and stored at 4 °C.

Photostability assays and singlet oxygen generation

Photostability experiments were performed as previously described,[18] differing in the concentration of the stock solutions for the porphyrin derivatives (1 mmol L^{-1} in DMSO). Photostability was estimated by irradiating the porphyrins dissolved in distilled water (2 mL) in a quartz cuvette under stirring, with the following parameters coinciding with those utilized for the PDI assays: irradiance of 100 mW cm^{-2}, at room temperature. After periods of 0, 1, 2, 3, 4, 6, 8, 10, 15 and 20 min, the absorbance of the Soret band was measured spectrophotometrically and referenced in percentage from the original absorbance (100%).

Singlet oxygen generation was determined using DPBF as the singlet oxygen quencher, with an irradiance of 9 mW cm^{-2} in a glass cuvette, using a cut-off filter for wavelengths ≤540 nm. A solution of each porphyrin (0.5 mmol L^{-1}) and DPBF (50 mmol L^{-1}) in DMF/H_2O (9 : 1) was utilized for the experiments. The absorption decay of DPBF at 415 nm was measured at irradiation intervals up to 20 min. The percentage of the DPBF absorption decay is proportional to the production of $^1O_2$.[18]

Preparation of conidia stock suspension

The strain of *Colletotrichum graminicola* was isolated at Campo Mourão, Paraná State, Brazil and it was obtained from the collection of phytopathogenic fungi at EMBRAPA Milho e Sorgo, Minas Gerais, Brazil. Fungus access was authorized by Conselho de Gestão do Patrimônio Genético, with the number: 010850/2013-9. Microorganisms were cultivated in oatmeal agar (FAA, 60 g oatmeal flour, 15 g bacteriological agar, 1000 mL distilled water). Cultures were incubated at 27 °C for approximately one week until the development of a mycelium. The cultures were then removed from incubation and mycelium was scrubbed to stimulate fungus sporulation. A new incubation using the same period of time was performed. After this period, for the preparation of conidia suspensions, a volume of 5 mL of phosphate buffered saline (PBS, 4 g of NaCl, 0.1 g of KCl, 0.72 g of Na_2HPO_4 and 0.12 g of KH_2PO_4 to a final volume of 500 mL and pH 7.4 ± 0.2)[18] was added to the sterile glass tube and a portion of conidia was inserted and homogenized. To determine the concentration of conidia in the initial suspension, a hemocytometer was used and the concentration of conidia was expressed as colony-forming unit per milliliter (CFU per mL). The photoinactivation assays were performed with a concentration of conidia of 10^5 CFU per mL.

PDI experimental setup

Initially, a series of experiments using porphyrin **5** were conducted in order to determine the most suitable concentration for the following comparative photoinactivation assays. Aliquots of conidia suspension (0.1 mL) were transferred to 6-well plates. Different volumes of the stock solution of **5** were added in order to obtain final concentrations of 5, 25, 50 and 75 μmol L^{-1} in the final volume of 5 mL, fulfilled with PBS. The resulting mixtures were kept under stirring on melting ice to prevent heating during irradiation. Two controls were included in each irradiation experiment: a light control (LC) subjected to the same fluence as the samples, but without a photosensitizer, and a dark control (DC) containing the photosensitizer at the highest concentration evaluated, in the absence of light. After initial experiments, higher concentrations, 50 and 75 μmol L^{-1}, demonstrated complete inactivation after a fluence of 30 J cm^{-2}. Therefore, the concentrations evaluated were 5, 10, 15 and 25 μmol L^{-1} for all derivatives under study. Samples of 100 μL were collected in the beginning of the test and after application of a fluence of 30, 60 and 90 J cm^{-2} and spread-plated on Sabouraud dextrose agar for the determination of the concentration of viable conidia. Colonies formed after 48 h of incubation at 27 °C were counted. The average value of the duplicates was used as an estimate of the concentration of viable conidia in the suspension and expressed as CFU per mL. Three independent assays were conducted for each porphyrin under study, the profile of inactivation of different photosensitizers being constructed with the average and the standard deviation of the results obtained. The survival of conidia of *C. graminicola* was plotted as the logarithm of the concentration of viable conidia (log CFU per mL) *versus* fluence (J cm^{-2}).[18]

Fluorescence microscopy studies

A suspension of 10^7 conidia per mL was incubated with each photosensitizer, in the dark for 20 min, at selected concentrations (based on preliminary microscopy results): 25 μmol L^{-1} for compounds **1** and **5** and 10 μmol L^{-1} for compounds **2**, **3** and **4**. Then, the unbound photosensitizer was removed by centrifugation at 11 000*g* for 5 min. Two washings with PBS were performed before fresh centrifugation. After the addition of 1 mL of PBS in tubes, the slides were prepared with 50 μL of the resulting suspension, inserted between the slide and coverslip.

Photosensitizer binding

Photosensitizer binding experiments were performed according to a methodology previously described,[18] with modifications. The conidia (suspension of 10^6 conidia per mL) were incubated in the dark at 30 °C in the presence of 10 μmol L^{-1} (**2**, **3** and **4**) or 25 μmol L^{-1} (**1** and **5**) of cationic porphyrins (as defined for the fluorescence microscopy assay). After the incubation periods (0, 20 and 60 min), the unbound photosensitizer was removed out of the suspension by centrifugation for 5 min at 11 000*g*. In order to evaluate the strength of the attachment of the porphyrin to the biological material, two series of aliquots were prepared: one set of conidia was digested immediately after centrifugation and the other was further washed with PBS, prior to digestion. For digestion, the pellets were resuspended in 1 mL of the digesting solution (aqueous NaOH 0.1 mol L^{-1}) and incubated at room temperature for 24 h.[18] The fluorescence of the resulting solutions was then directly measured. The excitation wavelengths were 445 nm (**1**), 419 nm (**2**), 421 nm (**3**), 422 nm (**4**) and 426 nm (**5**), with the emission being measured in between 600–750 nm.

Statistical analysis

Statistical analysis was performed by using Graphpad Prism 5.0 for Windows. Data normality was evaluated and attested by the Shapiro–Wilk test. The significance of the PDI effect for each porphyrin derivative and of the irradiation time on the inactivation of conidial cells was assessed by the one-way univariate analysis of variance (ANOVA) model with the Newman–Keuls multiple comparison test. A value of $p < 0.05$ was considered significant.

Results

Porphyrin photostability and singlet oxygen generation

Table 1 shows the photostability and $^1O_2$ generation results presented by porphyrins **1–5**. Photostability was estimated according to the percentages of the Soret band decay, which were recorded under an irradiance of 100 mW cm^{-2}. The photostability results in Table 1 refer to the remaining absorbance after an irradiance of 100 mW cm^{-2}, where lower percentages reflect lower photostabilities. All porphyrins evaluated were considerably photostable. Porphyrin **1** was the least stable photosensitizer evaluated, which showed 88% of remaining absorbance after 20 min of irradiance.

The capacity of $^1O_2$ production was determined by the indirect photooxidation method, which was based on the measurement of the DPBF absorbance decay at 415 nm, in the presence of each of the porphyrins herein studied, over 20 min at 9 mW cm^{-2} of irradiance. According to Table 1, porphyrins **1** and **4** were the best $^1O_2$ producers, which decreased DPBF absorbance by 87 and 88%, respectively.

Photodynamic inactivation of the conidia of *Colletotrichum graminicola*

Results of the preliminary PDI experiments, which were performed using 25 μmol L^{-1} of the porphyrin concentration, are shown in Fig. 2. These first experiments were also performed in order to evaluate if porphyrins **1–5** would be adequate photosensitizers against *C. graminicola* conidia, namely, if the tested porphyrins were active only in the presence of light and photosensitizers, simultaneously. For this purpose, conidia susceptibility was first evaluated with light, in the absence of a photosensitizer (light control) and without light, in the presence of a photosensitizer (dark control). At this stage, light control and PDI were conducted with a fluence of 120 J cm^{-2}. This evaluation indicated that porphyrins **2**, **4** and **5** efficiently eliminated *C. graminicola* in the PDI assay, showing no significant conidia killing in the dark. Porphyrin **1** presented a decrease in conidia viability in the PDI assay that was considered to be low, taking into account the high porphyrin concentration utilized.

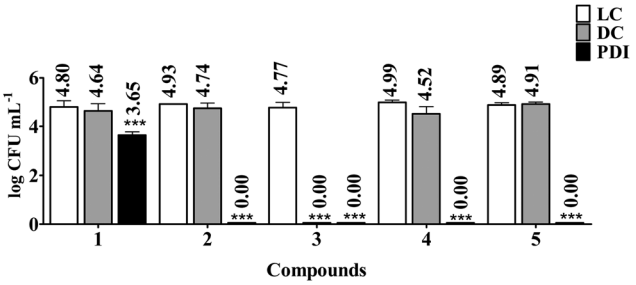

Fig. 2 Controls and preliminary PDI tests on *Colletotrichum graminicola* using cationic porphyrins **1–5** as photosensitizers. LC: light control using a fluence of 120 J cm^{-2}. DC: dark control, conducted with a porphyrin concentration of 25 μmol L^{-1}. PDI experiments were performed with the same concentration and fluence used for controls. Results are expressed as the logarithm of viable colony-forming unit per milliliter (log CFU per mL). The average values are indicated above the bars. Asterisks indicate the level of significance of conidia inactivation (***: $p <$ 0.001). Error bars represent standard deviation.

Differently, porphyrin **3** eliminated *C. graminicola* for both PDI and dark control assays. Porphyrin **3** was further evaluated under lower concentration values (1–15 μmol L^{-1}) in the dark; however, a complete conidia killing was observed for all cases (data not shown). In this way, this porphyrin was not considered an appropriate photosensitizer and it was not employed for the following PDI assays.

Based on the results shown in Fig. 2, porphyrins **2**, **4** and **5** were then more thoroughly studied (Fig. 3) by varying porphyrin concentrations with lower values (5, 10 and 15 μmol L^{-1}) and using fluences of 30, 60 and 90 J cm^{-2}. Porphyrins **2** and **4** promoted complete conidia inactivation under all porphyrin concentrations and fluences evaluated. On the other hand, porphyrin **5** promoted a photoinactivation that was effective for most of the conditions tested, although it was considerably lower in comparison with porphyrins **2** and **4**, especially for fluences studied in Fig. 3.

In order to differentiate between the photosensitizing efficiency of porphyrins **2** and **4**, even lower concentrations of these porphyrins were employed for the PDI experiments (1 μmol L^{-1} and 2.5 μmol L^{-1}) (Fig. 4). This evaluation indi-

Table 1 Photostability and singlet oxygen generation of porphyrins **1–5**

Compound	Photostability[a] (%)	DPBF absorbance decay[b] (%)
DPBF	—	12
1	88	87
2	95	82
3	90	80
4	96	88
5	99	78

[a] Photostability results are expressed as percentages from the initial absorbance of the porphyrin Soret band, after 20 min of irradiance (100 mW cm^{-2}). Higher percentage values reflect higher photostabilities. [b] Singlet oxygen generation was assessed by the DPBF photooxidation (50 mmol L^{-1}; in DMF/H_2O 9 : 1) upon irradiation for 20 minutes with white light filtered through a cut-off filter for wavelengths <540 nm (9 mW cm^{-2}), with or without a photosensitizer (0.5 mmol L^{-1}). Percentage values indicate DPBF absorbance decay from the initial absorbance. Higher percentage values reflect higher $^1O_2$ generation. Complete monitoring (1–20 min) of photostability and $^1O_2$ assays, is shown in the ESI.

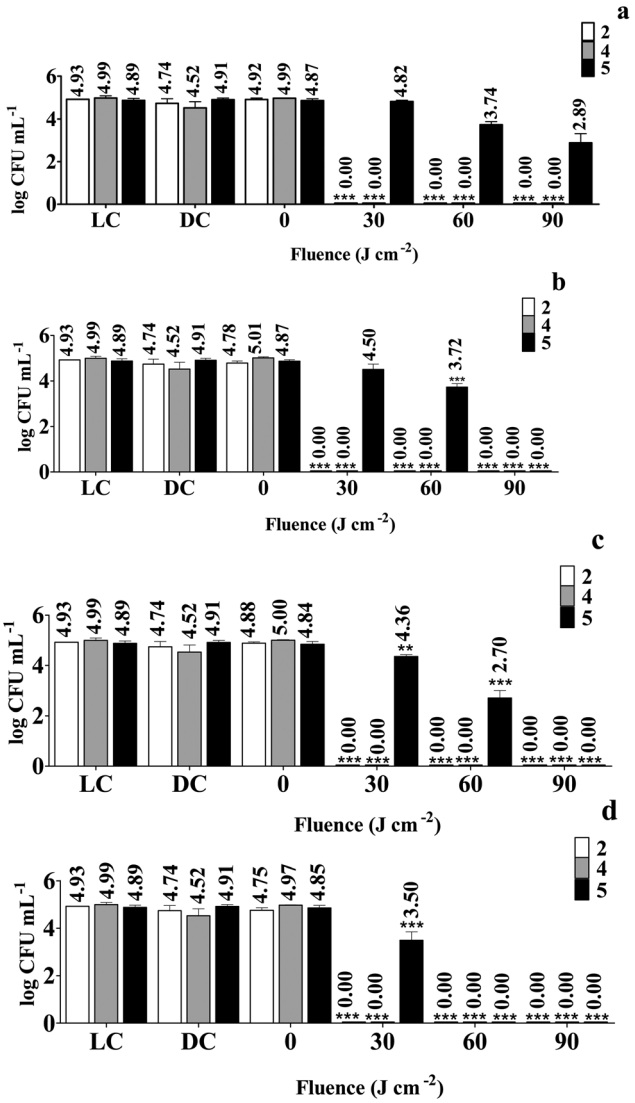

Fig. 3 Controls and PDI of *Colletotrichum graminicola* employing porphyrins **2**, **4** and **5** at concentrations of 5 μmol L^{-1} (a), 10 μmol L^{-1} (b), 15 μmol L^{-1} (c) and 25 μmol L^{-1} (d), with a fluence of 0, 30, 60 and 90 J cm^{-2}. Results are expressed as the logarithm of viable colony-forming unit per milliliter (log CFU per mL). The average values are indicated above the bars. Asterisks indicated the level of significance of conidia inactivation (**p < 0.01, ***: p < 0.001). Error bars represent standard deviation.

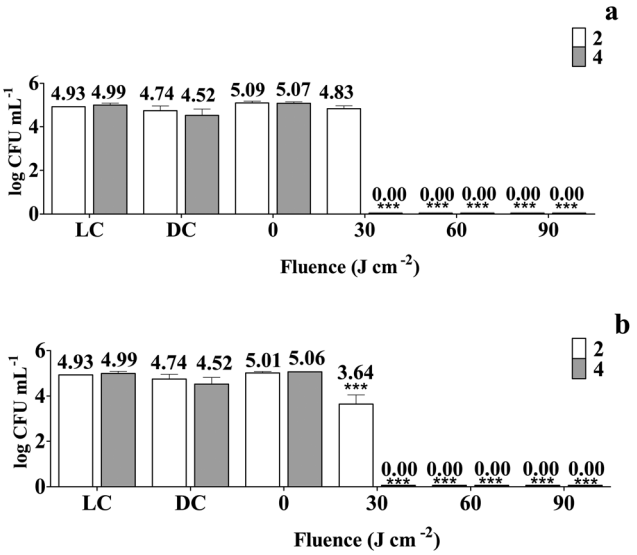

Fig. 4 Controls and PDI of *Colletotrichum graminicola* employing porphyrins **2** and **4** at concentrations of 1 μmol L^{-1} (a) and 2.5 μmol L^{-1} (b), with a fluence of 0, 30, 60 and 90 J cm^{-2}. Results are expressed as the logarithm of viable colony-forming unit per milliliter (log CFU per mL). The average values are indicated above the bars. Asterisks indicated the level of significance of conidia inactivation (***: p < 0.001). Error bars represent standard deviation.

cated that porphyrin **4** photoinactivated *C. graminicola* with a higher efficiency in comparison with **2**. Porphyrin **4** was able to provide a complete inactivation of the conidia, even at 1 μmol L^{-1} under a fluence of 30 J cm^{-2} while porphyrin **2** required 60 J cm^{-2} using the same concentration.

Fluorescence microscopic analysis

The fluorescence microscopy experiments shown in Fig. 5 were performed with 25 μmol L^{-1} (porphyrins **1** and **5**) or with 10 μmol L^{-1} (porphyrins **2**, **3** and **4**). These concentrations were defined based on preliminary experiments that showed that the most active porphyrins (**2**, **3** and **4**) promoted an intense fluorescence when using concentrations higher than 10 μmol L^{-1}, which impaired a good visualization of the conidial structures. The images collected from the microscopic evaluation of the *C. graminicola* conidia and their interactions with the cationic porphyrins **1**–**5** are shown in Fig. 5. The green image areas are related exclusively to the conidial structures and denoted the absence of porphyrins. The red color is related to the fluorescence emitted by the porphyrin molecules that are not interacting with conidial structures. The yellow-colored areas denote interaction between porphyrin and conidial structures. Fig. 5b indicates that porphyrin **1** was heavily present on the surface of and inside the conidia; however, the whole content of **1** was not necessarily interacting with conidial structures. All the other porphyrins seem to interact with conidial structures to some extent, with porphyrin **3** (Fig. 5d) causing evident conidial disruption.

Porphyrin binding to the conidia of *Colletotrichum graminicola*

The porphyrin concentrations utilized for the binding experiments were exactly the same as those utilized for the fluorescence microscopy experiments, namely, porphyrins **1** and **5** were assayed at 25 μmol L^{-1} and porphyrins **2**, **3** and **4** at 10 μmol L^{-1}. Compounds **2** and **3** presented the higher binding values after 60 min of incubation, which were 4.35×10^9 and 5.16×10^9 molecules per CFU, respectively. Porphyrin **4**, at 10 μmol L^{-1}, showed comparable binding values with porphyrins **1** and **5**, both at 25 μmol L^{-1}, which were 8.46×10^8, 1.48×10^9, and 1.20×10^9 molecules per CFU (60 min of incu-

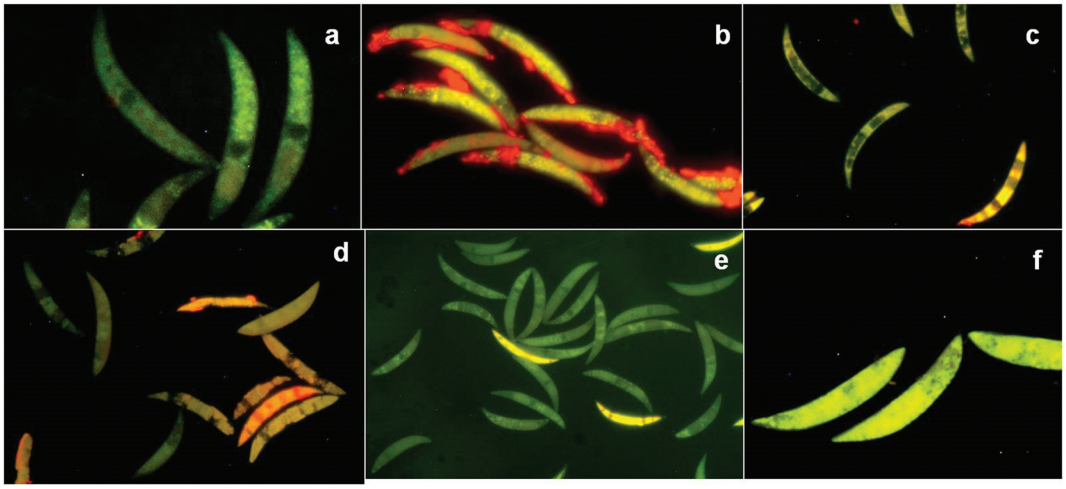

Fig. 5 Fluorescence microscopy assays of *Colletotrichum graminicola* conidia in the absence (a) and in the presence of cationic porphyrins **1** (b), **2** (c), **3** (d), **4** (e), **5** (f) at a concentration of 10 μmol L^{-1} for porphyrins **2**, **3** and **4** and 25 μmol L^{-1} for **1** and **5**. The green color is related to the conidial structures, red to porphyrins and yellow to the interaction between the microorganisms and porphyrins.

bation), respectively. The washing procedure served to eliminate those photosensitizer molecules that were not firmly associated with the fungal structures. As shown in Fig. 6, the washings caused a reduction in the binding values for all porphyrins and incubation times evaluated.

Discussion

Cationic porphyrins have been shown to be suitable photosensitizers for the inactivation of diverse microorganisms by PDI. The photoinactivating properties of porphyrin **5** have been extensively evaluated in the literature.[4,17,18,21,23] For this reason, we decided to study porphyrin **5**, together with other closely related porphyrins, in order to verify if cationic *meso*-(1-methyl-4-pyridinio)porphyrins could be potentially useful as an alternative for the control of fungal phytopathogens, specifically for *C. graminicola*. As **5** presents four identical cationized *meso*-pyridyl groups, we synthesized porphyrins having a different number or positioning of the phenyl and 1-methylpyridinio groups at the *meso* positions of the macrocycle. It is well known that the above-mentioned structural differences play an important role in porphyrin physicochemical, photophysical and photodynamic properties.[54]

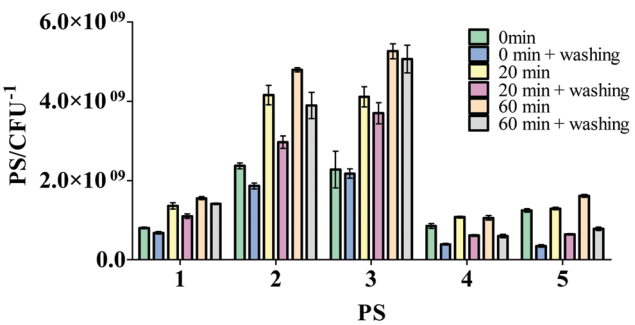

Fig. 6 Binding of the tested porphyrins to *Colletotrichum graminicola* conidia after incubation in the dark for 0, 20 and 60 min at 30 °C (with and without washings with PBS), using 25 μmol L^{-1} of porphyrins **1** and **5** and 10 μmol L^{-1} of porphyrins **2**, **3** and **4**. Binding values are expressed as the number of porphyrin molecules per colony-forming unit (PS per CFU). PS: photosensitizer. Error bars represent standard deviation.

Preliminary PDI assays (Fig. 2), which were performed with a high porphyrin concentration (25 μmol L^{-1}) demonstrated the suitability of the fluence utilized and also proved that porphyrins **1**, **2**, **4** and **5** could be considered suitable photosensitizers, since they were active only in the presence of light. On the other hand, porphyrin **3** promoted a complete inactivation of *C. graminicola* in the absence of light, even using lower porphyrin concentrations. This behavior suggests a light-independent mode of action (and consequently a $^1O_2$-independent action) that also operates for this porphyrin. This observation was supported by the results shown in Fig. 6, which indicated a large binding of porphyrin **3** to *C. graminicola* conidia, with subsequent cell disruption (Fig. 5d). Simões *et al.* (2016),[55] Alves *et al.* (2009)[5] and Kessel *et al.* (2003)[56] also comparatively studied the PDI of cationic porphyrins presenting different charge numbers and positioning. For the three studies mentioned, the porphyrin presenting two positive charges at adjacent *meso*-positions was the most active photosensitizer. This behavior was explained by the distortion of the porphyrin ring, which was directly caused by the electrostatic repulsion of the two close positive charges.[55,56] In the present work, the high photoactivity expected for the doubly-charged *cis*-porphyrin (**3**) was somehow translated as the cytotoxicity observed in the dark. Considering the different PDI/dark susceptibilities shown by distinct microorganisms, a previous study[9] reported that *Aspergillus flavus* was inactivated by hematoporphyrin, in the absence of light, at a concentration of 5 μmol L^{-1}, while the other fungi evaluated were only eliminated under PDI con-

ditions. These literature data could indicate that the light independent inactivation promoted by porphyrins is related to species of the microorganism under study.

The preliminary PDI results (Fig. 2) also showed porphyrin **1** as exhibiting a photoactivity that was considerably lower than the other porphyrins. This compound (**1**), also differently from the other porphyrins evaluated, is poorly soluble in aqueous medium. This property is directly related to the presence of only one positively charged group attached to the highly hydrophobic tetrapyrrole ring. The lack of water solubility can trigger an aggregative behavior, which has been recurrently observed for this type of compound.[12,18,55,57] In this way, the lower photostability and the high estimate of $^1O_2$ singlet production presented by **1** (Table 1) could be, in part, artefacts raised from aggregation events. Aggregation could also explain the lower photoactivity observed for **1**. Even though this porphyrin presented a considerable binding value (Fig. 6), most of the photosensitizers aggregated on the surface of the conidia (Fig. 5b). This probably caused an inaccessibility of $^1O_2$ or other oxygen reactive species to important conidial structures, which reflected the low photoactivity observed.

Considering PDI experiments with varying fluence and porphyrin concentration (Fig. 3 and 4), porphyrins **2** and **4** were arguably more photoactive than **5**. Porphyrin **5** required a fluence of 90 J cm^2 at 15 μmol L^{-1} in order to completely inactivate *C. graminicola*, while porphyrins **2** and **4** provided the same effect under all concentrations and fluences outlined in Fig. 3. Fig. 4 experiments ultimately allowed the differentiation between porphyrins **2** and **4**, which indicated the latter as the most efficient photosensitizer herein evaluated. Taking into account the lowest concentration that enabled the best photoinactivation with the respective lowest effective fluence, the photosensitizers could be ranked as follows: **4** (1 μmol L^{-1} with a fluence of 30 J cm^{-2}) > **2** (1 μmol L^{-1} with 60 J cm^{-2}) > **5** (15 μmol L^{-1} with 90 J cm^{-2}) > **1** (25 μmol L^{-1} with 120 J cm^{-2}).

Taking into account the $^1O_2$ production and binding shown by porphyrins **2**, **4** and **5**, compound **4** owes most of its activity to the production of high levels of $^1O_2$ (Table 1). This observation is sustained by the low binding results presented by this porphyrin (Fig. 6). In this way, it is presumable that the high level of killing provided by a small amount of porphyrin **4** inside the conidia was made possible by its outstanding $^1O_2$ production per molecule. On the other hand, in comparison with porphyrin **4**, compound **2** presented a lower level of $^1O_2$ production, which was counterbalanced by an excellent porphyrin binding to the conidia. The lower photoactivity of porphyrin **5**, in comparison with **2** and **4**, could be explained by its $^1O_2$ production (comparable to **2**) and its low binding values (comparable to **4**).

According to Engelmann and coworkers (2007)[58] and Simões *et al.* (2016),[55] a suitable photosensitizer should present both a hydrophilic and hydrophobic character in order to trespass the biological membranes. In the present work, the sole evaluation of the binding results (Fig. 6) clearly indicated that the porphyrins presenting two positively charged groups (**2** and **3**) were the ones that bound effectively to *C. graminicola*, two methylpyridinio groups (hydrophilic) and two phenyl groups (hydrophobic) being the best ratio of those substituents at *meso*-positions of the macrocycle. The fact that porphyrin **2** gave the best binding value reinforces the importance of the amphiphilic character, which is more pronounced for the *cis*-porphyrin that possesses both hydrophobic and hydrophilic groups on the same side of the molecule.

The number of bound molecules herein determined by the binding assay was from 846 million up to 5.16 billion of molecules per conidia. These numbers are substantially higher than the ones determined by Gomes *et al.* (2011)[18] for *Penicillium chrysogenum*, which reached approximately 20 million per conidia. Considering that porphyrin **5** was included in the cited paper, it is presumable that the type of microorganism plays an important role in terms of photosensitizer binding.

PDI studies utilizing the same structurally related porphyrins herein evaluated are described in the literature, mainly for bacteria. Porphyrins **3**, **4** and **5** were evaluated in terms of their photoinactivating properties against the marine bacteria *Enterococcus seriolicida* and *Vibrio anguillarum*, and *Escherichia coli*.[46] In this case, compounds **3** and **4** were efficient in eliminating *V. anguillarum*, while only compound **4** effectively eliminated *E. coli.* The photoinactivation of *E. coli* and *Staphylococcus warneri*,[19] which used porphyrin **5** as a photosensitizer, served as the reference for the evaluation of an analog containing three *meso* methylpyridino cationic groups and one pentafluorophenyl ring. For *E. coli*, both photosensitizers completely inactivated bacteria at 5 μmol L^{-1}. In the case of *S. warneri*, only the tricationic derivative was effective at a concentration of 0.5 μmol L^{-1}. In general, these studies are consistent with a higher photoinactivating efficiency of the unsymmetrical cationic porphyrins, which is in agreement with the study herein presented.

The PDI studies involving the genus *Colletotrichum* available in the literature indicated that phenothiazine dyes and coumarines require a concentration ranging from 5 to 50 μmol L^{-1} to photoinactivate these microorganisms.[40,41] In this way, cationic porphyrins **2** and **4** can be considered better photosensitizers than the compounds previously evaluated for the genus *Colletotrichum*.

Regarding the potential application of porphyrin derivatives in agriculture, it is important considering the alleged photodamage to the host plant (*Zea mays*). There are a number of publications indicating that superior plants are quite resistant in the face of light-activated photosensitizers. In fact, photosensitizing processes naturally occur in plants – *via* endogenous phytoalexins,[59] thiophens,[60] root ketoalkenes and ketoalkynes[61] as defense mechanisms against phytopathogens and as natural physiological processes.[62] To avoid photodamage, vegetal cells minimize oxygen reactive species and singlet oxygen action *via* superoxide dismutase, ascorbate peroxidase and glutathione reductase, which are present in the plant chloroplasts.[63] In addition, xanthophylls (plant secondary metabolites) dissipate the excess of energy from light through non-photochemical mechanisms.[64] These natural protecting

mechanisms presented by plants could spare the host plant against the photodamage promoted by porphyrins, which could favour the exclusive photoinactivation of the infecting *C. graminicola.* Nevertheless, these are just indications of the potentiality of porphyrins for such a purpose. The *in vivo* evaluation of porphyrins using *Zea mays* plants affected by *Colletotrichum* is an ongoing project in our laboratory and it will be reported soon.

Concluding remarks

The present study demonstrated that cationic porphyrins structurally related to tetra-charged porphyrins (**5**), especially the *trans*-doubly-charged (**2**) and triply-charged (**4**) porphyrins, are promising photosensitizers for the PDI of phytopathogen *Colletotrichum graminicola*. The *cis*-doubly-charged porphyrin (**3**) showed fungicide properties in the absence of light by disrupting conidial structures. Singly-charged **1** presented a low photoactivity, probably due to its lower solubility in aqueous medium.[54,65] The doubly-charged porphyrins evaluated (**2** and **3**) appeared to subside due to a more efficient binding to *C. graminicola* conidia. In general, porphyrins presenting a high $^1O_2$ singlet production allied to a good binding value, which was mostly provided by an appropriate balance between the hydrophilic and hydrophobic moieties of the porphyrin structure, were the most promising photosensitizers.

These results confirm the applicability of cationic porphyrins for the PDI of filamentous fungi and open the possibility of using these types of compounds as photosensitizers to be applied in agriculture.

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