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ORIGINAL PAPERS

Photodynamic Inactivation of plant pathogens part II: fungi

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Abstract

The constantly increasing demand for agricultural produce from organic and conventional farming calls for new, sustainable, and biocompatible solutions for crop protection. The overuse of fungicides leading to contamination of both produce and environment and the emergence of plant pathogenic fungi that are resistant to conventional treatments warrant the need for new methods to combat fungal infections in the field. We here deliver the follow-up study to our research on the Photodynamic Inactivation (PDI) of plant pathogenic bacteria (Glueck et al. in Photochem Photobiol Sci 18(7):1700–1708, 2019) by expanding the scope to fungal pathogens. Both fungal species employed in this study—*Alternaria solani* and *Botrytis cinerea*—cause substantial crop and economic losses. Sodium magnesium chlorophyllin (Chl, approved as food additive E140) in combination with Na_2EDTA and the chlorin e6 derivative B17-0024 holding cationic moieties serve as eco-friendly photoactive compounds. Effectiveness of the antifungal PDI was measured by inhibition of growth of mycelial spheres (average diameter 2–3 mm) after incubation with the photosensitizer for 100 min and subsequent illumination using a LED array (395 nm, 106.6 J cm^{-2}). One hundred micromolar Chl combined with 5 mM Na_2EDTA was able to successfully photokill 94.1% of *A. solani* and 91.7% of *B. cinerea* samples. PDI based on B17-0024 can completely inactivate *A. solani* at 10 times lower concentration (10 µM); however, for *B. cinerea*, the concentration required for complete eradication was similar to that of Chl with Na_2EDTA (100 µM). Using a plant compatibility assay based on *Fragaria vesca,* we further demonstrate that both photosensitizers neither affect host plant development nor cause significant leaf damage. The plants were sprayed with 300 µL of treatment solution used for PDI (one or three treatments on consecutive days) and plant growth was monitored for 21 days. Only minor leaf damage was observed in samples exposed to the chelators Na_2EDTA and polyaspartic acid, but overall plant development was unaffected. In conclusion, our results suggest that sodium magnesium chlorophyllin in combination with EDTA and B17-0024 could serve as effective and safe photofungicides.

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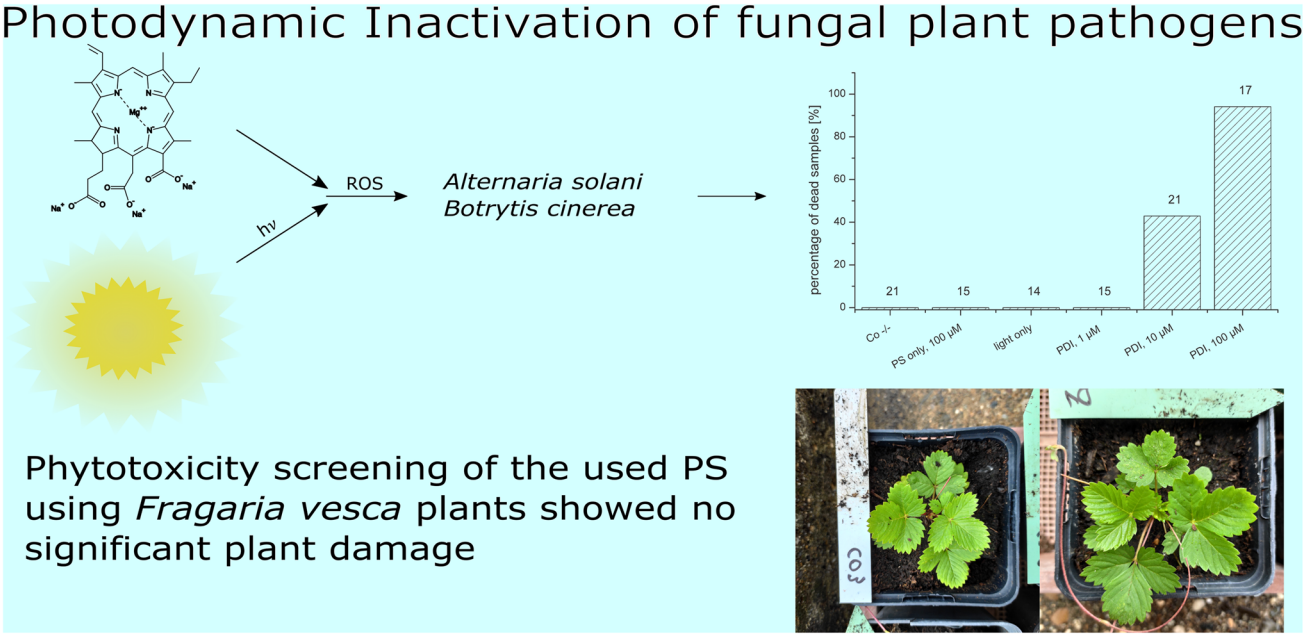
Graphical abstract



Abbreviations

A. solani	*Alternaria solani*
B. cinerea	*Botrytis cinerea*
CFU	Colony forming unit(s)
DCFDA	2′,7′-Dichlorofluorescin diacetate
DPBS	Dulbecco's phosphate-buffered saline
PDI	Photodynamic Inactivation
PS	Photosensitiser
ROS	Reactive oxygen species
PA	Polyaspartic acid
Na_2EDTA	Ethylenediaminetetra-acetic acid disodium salt dihydrate
Chl	Sodium magnesium chlorophyllin
Chl–CHS	Chlorophyllin–chitosan
B17-0024	B17-0024 Ce6-15,17-DMAE
Cu	Copper
Ce6	Chlorin e6
SOSG	Singlet oxygen sensor green
S. lycopersicum	*Solanum lycopersicum*
F. vesca	*Fragaria vesca*
EPPO	European and Mediterranean Plant Protection Organization
PDI-PCA	PDI-plant compatibility assay

1 Introduction

Since the industrial revolution, our society must cope with an ever-growing world population on one hand and a decline in available arable land per person on the other hand [1, 2]. The rising need for food in industrialised countries has mainly been satisfied by high-density industrial monocultures. According to the American Phytopathological Society, the global production of tomato was about 159 million tons and the production of potato 375 million tons in 2011 [3]. However, industrialized farming poses the risk of high crop losses in the case of disease outbreaks or pest infestations and overuse of pesticides comes at a high cost for the environment. Phytopathogenic fungi have the potential to spread by air, animals, contaminated soil or water and therefore have the potential to obliterate entire harvests. This situation is further aggravated by the rise of resistant fungal strains.

The list of fungal species that trouble growers is extensive. Two phytopathogens, *Altanaria solani* (causing early blight in *Solanum lycopersicum*, tomato) and *Botrytis cinerea* (responsible for grey mold) are among the top threats as they affect important and valuable crops. *Altanaria solani* is responsible for estimated 78% of annual financial tomato yield losses [4]. Early blight symptoms include lesions on aboveground organs that as disease progresses may result in complete defoliation of infected plants [5]. *Botrytis cinerea* is able to infect about 200 different plant species and causes financial damage of 10–100 billion USD per year [6] with grapevines representing the most important host for the pathogen where it induces grey rot/mold and noble rot. Other target crops with severe outcomes are e.g.: strawberry, raspberry and vegetables like broccoli or lettuce. Both fungi are considered necrotrophic as they sustain themselves of nutrients from dead plant tissue.

As current management strategies in conventional farming for infections with *A. solani*, the EPPO recommends the use of anilazine, azoxystrobin, chlorothalonil, Cu compounds, cymoxanil, difenoconazole, famoxadone, oxadixyl, procymidone in their EPPO Standards for good plant protection practice for solanaceous crops under protected cultivation [7] and azoxystrobin, chlorothalonil, copper, difenoconazole, famoxadone, iprodione, procymidone, tolylfluanid for outdoor crops [8].

In case of organic farming, copper compounds are widely used to fight fungal disease. Kuehne et al. [9] estimate the amount of copper compounds used in German conventional apple farming in the year 2013 to 1.4 kg ha^{-1}, and, nearly identical, to 1.5 kg ha^{-1} in organic farming. However, in other crops, e.g. grapes, the usage of copper compounds increases from 0.8 in conventional agricultural farming to 2.29 kg ha^{-1} in organic farming. In total, 84.4 t of copper compounds were applied in conventional farming and 26.5 t in organic farming [9]. This massive input of copper—natural Cu concentrations range from 20 up to 100 mg Cu kg^{-1} soil depending on soil type [10, 11] may lead to accumulation in the environment: data from vineyards in France prove that concentrations up to 1500 mg Cu kg^{-1} soil can be reached [12]. This accumulation of Copper in the soil can have toxic effects on the microbiome, groundwater and plants [13]. Although considered controversial, the European Commission extended the approval of copper compound usage (up to 28 kg Cu ha^{-1}) until 2025 as stated in the Commission implementing regulation (EU) 2018/1981 of 13 December 2018 [14], as effective and sparing alternatives are lacking.

Photodynamic Inactivation (PDI) utilizes photosensitizer molecules (PS) that when activated by light produce reactive oxygen species that can rapidly oxidize multiple cellular targets resulting in nearly instantaneous pathogen cell death. Advantages of PDI include no known resistance and the broad range of pathogens that can be targeted [15]. To determine the ROS and/or singlet oxygen production capabilities of the used PS system we performed a DCFDA and SOSG assay.

The objective of this study is to assess the utility of two chlorin PS previously shown to have great efficacy against bacterial plant pathogens [16]. We use the anionic water-soluble semi synthetic chlorophyll derivative sodium magnesium chlorophyllin (Chl; registered as food additive E140) combined with a chelator (Ethylenediaminetetra-acetic acid disodium salt dihydrate, Na_2EDTA) and the chlorin e6 derivative B17-0024 Ce6-15,17-DMAE (B17-0024), carrying cationic moieties (kindly provided by Suncor AgroScience), as PS. In addition we examine the effects of PDI on strawberry leaves under natural sunlight in two application scenarios:"type S"—similar to applications in the field, or "type T"—maximizing the amount of PS delivered to the foliage to assess the potential effect of PDI treatment on the host plant.

2 Experimental

2.1 Preparation of stock solutions

Sodium magnesium chlorophyllin (Chl, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and B17-0024 (kindly provided by Suncor AgroScience, Mississauga Ontario, Canada) were dissolved in ultrapure water to get stock solutions with 10 mM PS concentration and stored at − 20 °C in the dark until use. Polyaspartic acid (PA; Baypure®DS100; Kurt Obermeier GmbH & Co. KG, Bad-Berleburg, Germany) was acquired as 40% solution. A solution of 400 mM Na_2EDTA (EDTA disodium salt dihydrate, VWR International, B-3001 Leuven) was obtained by dissolving in ultrapure water, adjusting the pH to 7.8 and subsequent autoclaving. All solutions for the plant compatibility assays were prepared using tap water.

2.2 ROS and singlet oxygen detection

For the measurement of the absorption spectra of Chl, Chl with 5 mM Na_2EDTA or B17-0024 (PS concentration 100 µM in DPBS) 100 µl were transferred into a 96 well plate (µ-clear, Greiner Bio-One GmbH, Kremsmünster, Austria). The absorption signal was measured using a Infinite M200 microplate reader (Tecan, Grödig, Austria). To detect the singlet oxygen generation the Singlet Oxygen Sensor Green (Invitrogen by Thermo Fisher Scientific) assay was perfomed. Ninety microliters of Chl (100 µM), Chl (100 µM) with 5 mM Na_2EDTA orB17-0024 (100 µM) were mixed with 10 µM SOSG (stock solution 500 µM in 100% methanol kept on ice until use) and illuminated for up to 1 J cm^{-2} at 395 nm (irradiance 8 mW cm^{-2} measured with a LI-180 Spectrometer, LI-COR Biosciences GmbH, Bad Homburg, Germany) in a 96 well plate (µ-clear, Greiner Bio-One GmbH). Additionally the production of reactive oxygen species (ROS) was investigated using 2′,7′-Dichlorofluorescin diacetate (DCFDA, Sigma-Aldrich Handels GmbH, Wien, Austria). The 10 mM DCFDA stock was prepared in DMSO. Fifty microliters of dissolved DCFDA were mixed with 200 µl of 10 mM sodium hydroxide and incubated in the dark at room temperature for 30 min to form dichlorofluorescin (DCFH). After the incubation period, 1 ml of DPBS buffer was added to neutralize the solution. The DCFH solution was kept on ice until use. [17] DCFH (10 µM) was mixed with 100 µM PS (with and without 5 mM Na_2EDTA for Chl). Ninety microliters of the mixture were placed in a single well of a 96 well plate (µ-clear, Greiner Bio-One GmbH) and subsequently illuminated at 395 nm for up to

1 J cm^{-2}. The fluorescence of the illuminated SOSG and DCFH samples was measured using an Infinite M200 microplate reader with excitation wavelength 488 nm and emission wavelength 525 nm. The gain was set to 70.

2.3 Fungal culture

Botrytis cinerea was grown on agar plates containing 30 g l^{-1} malt extract (Roth), 3 g l^{-1} peptone (Peptone ex casein, Roth) and 15 g l^{-1} Agar–Agar (Kobe I, Roth) at 26 °C. Spores were transferred from the plates into liquid medium and incubated at 26 °C and 200 rpm in a shaking incubator (MaxQ 4000, Thermo Scientific, Marietta Ohio, USA) for 48 h to obtain spherical patches of mycelia with an average diameter of 1–2 mm. *Alternaria solani* was grown on agar plates containing 200 ml l^{-1} tomato mash (Passata di Pomodoro, S Budget, Spar Österreichische Warenhandels-AG, Salzburg, Austria), 3 g l^{-1} $CaCO_3$ (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 15 g l^{-1} Agar–Agar (Roth) at 26 °C for 3–4 days. Parts of the mycelium from the edge of the growing mycelial disc were transferred to liquid medium and incubated at 26 °C under constant agitation at 200 rpm for 24 h in a shaking incubator to obtain mycelial spheres of approximately 2 mm diameter.

2.4 PDI of B. cinerea and A. solani

Mycelial spheres were transferred into 24-well plates (one per well) using a pipette. Five hundred microliters of DPBS containing no PS (double negative control, Co −/−, light only) or either 1 µM, 10 µM or 100 µM of the respective photoactive compound were added to the wells. The samples were incubated for 100 min in the dark at room temperature under constant agitation and subsequently illuminated from below using a LED array (irradiance 14.8 mW cm^{-2}, radiant exposure 106.6 J cm^{-2}) consisting of 432 light emitting diodes (diode type L-7113UVC, dominant wavelength 395 nm, Kingbright Electronic Europe GmbH, Issum, Germany). After illumination, the mycelial spheres were carefully transferred to tomato mash agar plates (*A. solani*) or malt peptone agar plates (*B. cinerea*) to evaluate the photokilling efficiency. Samples showing no growth after 7 days of incubation at 26 °C were considered as dead. The percentage of dead mycelial spheres was calculated by dividing the number of dead samples by the number of treated samples (multiplied by 100).

2.5 PDI-plant compatibility assay

To assess possible damage of the compounds employed in this study (B17-0024 [Ce6 15, 17, DMAE], Chl and PA/Na_2EDTA) to the host plant a PDI-plant compatibility assay as shown in Fig. 1 was established [18]. *Fragaria vesca* (small woodland strawberry) was chosen as model plant. *Fragaria vesca* is a high value crop susceptible to various fungal pathogens that can cause economically devastating disease outbreaks [19]. The foliage is pale green, which allows for easy evaluation of damage. *Fragaria vesca* seeds (indigenous to Salzburg, Austria) were obtained from the seed collection of the botanical garden (Paris Lodron University of Salzburg) and germinated in a greenhouse (temperature range: min 18 °C, max 43 °C, 50–70% rel. humidity) using CL ED73 "Einheitserde" (> 50% white peat, ~ 30% clay, ~ 20% block peat [20]) as plant substrate. The plants were not additionally fertilized. The seedlings were later pricked out to individual pots and subsequently transferred outside under a transparent rain cover (mean temp. 16 °C). The plants were grown up to BBCH stage 14 and all runners were pruned to ensure a comparable experimental baseline. Plants that showed

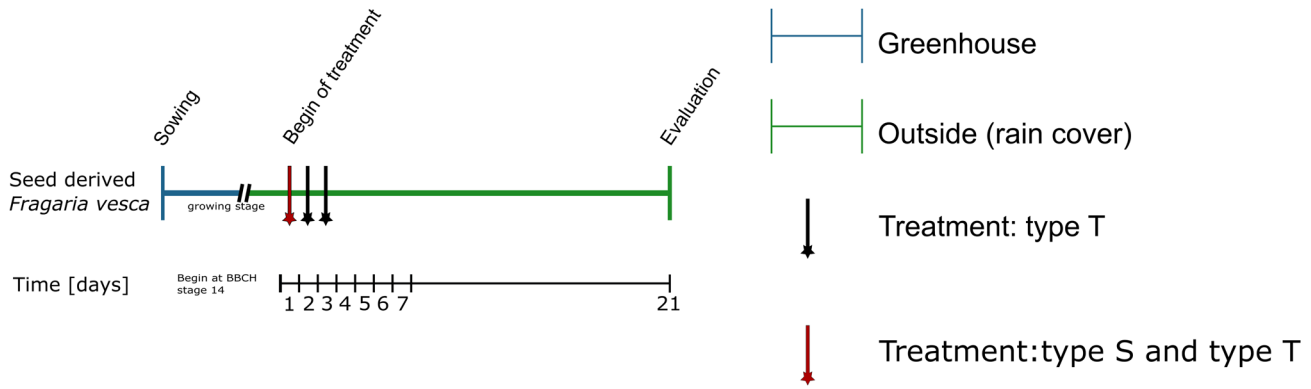


Fig. 1 Schematic timeline of the PDI-plant compatibility assay on *Fragaria vesca*. Blue line: plants were grown inside a greenhouse. Green line: plants were grown outside under a transparent rain cover. Red star: start of treatment for type S (treated one time) and type T (treated three times); black stars: treatment days for the three times treated samples (type T). Evaluation was done at day 21 after BBCH14

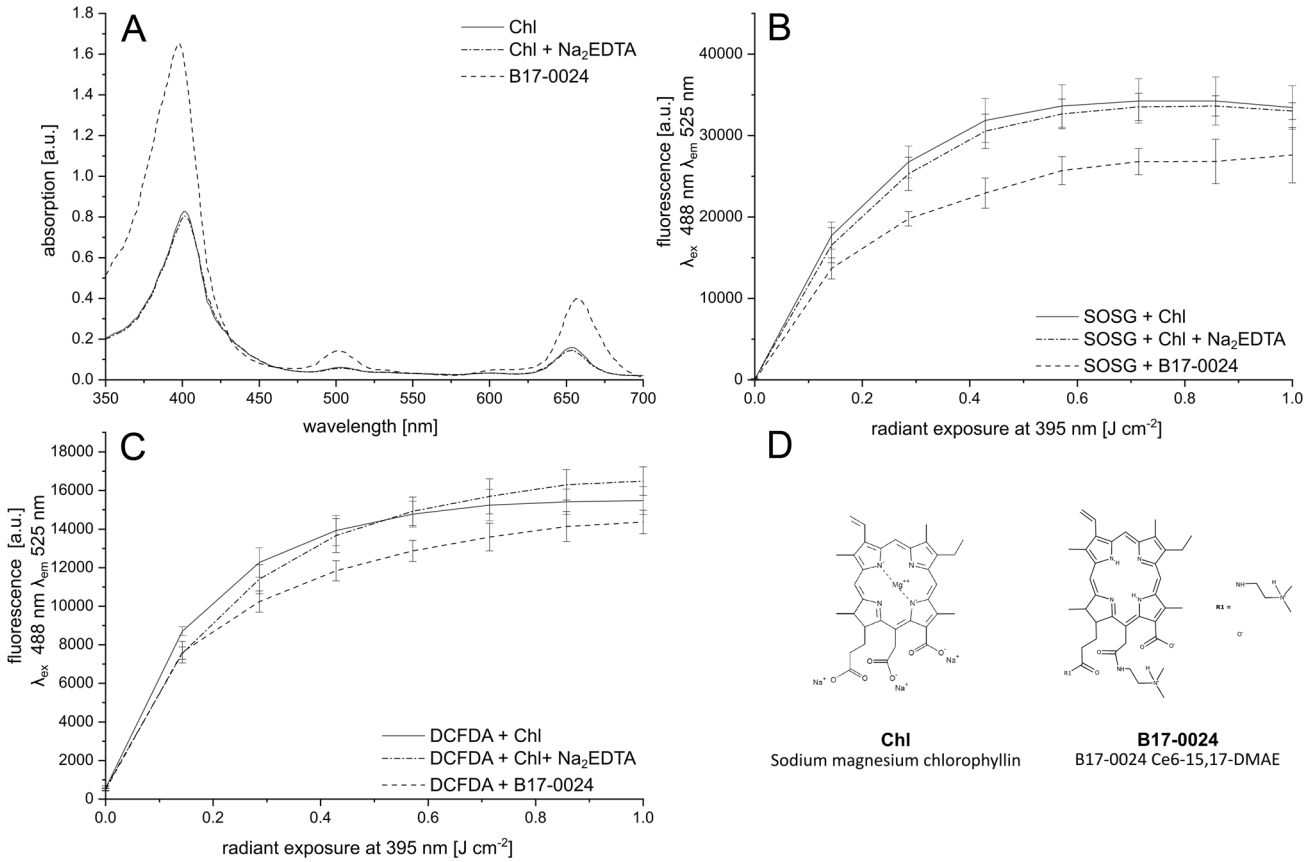


Fig. 2 Photophysical properties of the photosensitizers and PS mixtures used in this study. Straight line: Chl, dash-dot: Chl + Na_2EDTA, dashed line: B17-0024. **A** Absorption specta Chl, Chl + 5 mM Na_2EDTA and B17-0024 (PS concentration for all samples 100 µM, measured in triplets). **B** and **C** SOSG and DCFDA assay of Chl (100 µM), Chl (100 µM) + Na_2EDTA (5 mM) and B17-0024 (100 µM). Excitation (λ_{ex})488 nm; emission (λ_{em}) 525 nm, gain 70. Illuminated up to 1 J cm^{-2} using a 395 nm LED array. The mean of three experiments is shown. **D** Chemical structure of Sodium magnesium chlorophyllin (Chl) and B170024 Ce6-15,17-DMAE (B17-0024)

signs of pest damage before the first treatment were removed from the experiment. Two different application methods were evaluated: "type S", a single treatment and "type T", a total of three treatments on subsequent days, aiming to maximize exposure of plant foliage to the PS. Samples were divided into the following test groups: (1) H_2O—"type T", (2) Na_2EDTA—"type T", (3) Chl + 5 mM Na_2EDTA—"type S", (4) Chl + 5 mM Na_2EDTA—"type T", (5) B17-0024—"type S" and (6) B17-0024—"type T" and one complete set where Na_2EDTA was replaced with a stronger chelating agent (Baypure DS 100® (PA)). For each test group a total of four plants were used. Each plant was sprayed with 300 µL of the respective solution from above according to the treatment regime. Plant health and growth was monitored and documented (Nikon D40, Objective: Nikon DX AF-S NIKKOR 18–55 mm 1.3.5–5.6 GII ED DSLR camera) for 21 days. After this period a qualitative plant compatibility evaluation according to the following parameters was performed: (1) leaf or plant death, (2) growth inhibition, (3) leaf deformations and (4) leaf discoloration as well as brown spots or leaf margins.

3 Results and discussion

This study is a continuation of our previous work published in Photochemical and Photobiological Sciences in 2019 [16] by extending the application spectrum of Photodynamic Inactivation with chlorin molecules from bacterial to fungal phytopathogens. We used sodium magnesium chlorophyllin (approved as food additive "natural green" E140) with and without Na_2EDTA and B17-0024, a synthetic derivative of Ce6 with cationic moieties without additives as photoactive compounds.

The absorption spectra (Fig. 2A) of both PS (Fig. 2D) show an absorption maximum at 398 nm for B17-0024 (absorption 1.66) and at 401 nm for Chl (absorption 0.83). The addition of Na_2EDTA does not alter the absorption

spectrum of Chl (Fig. 2A) (absorption 0.81 at 401 nm). At the same PS concentration (100 µM) the absorption of B17-0024 is twice as high as the one of Chl. Upon illumination of a mixture of the PS with SOSG, SOSG turns fluorescent depending on the radiant exposure applied. Similar to the absorption spectrum—the addition of Na_2EDTA to Chl does not influence the SOSG fluorescence signal (Fig. 2B) and thus does not interfere with the photodynamic reaction. The SOSG fluorescence signal of B17-0024 is lower than the signal of Chl. The DCFDA assay leads to a similar outcome. The fluorescence signal increases with increasing radiant exposure (Fig. 2C). As expected Na_2EDTA does not influence the outcome of the assay. The results of both the SOSG and DCFDA assay suggest, that Chl and B17-0024 form singlet oxygen as well as ROS. According to the literature, chlorin-based PS tend to be characterized to function for the most part by the type II photochemical reaction [30–32]. The additive Na_2EDTA does not influence the photophysical processes of the photodynamic reaction.

For treatment of planktonic bacteria incubation with PS for 5 min was sufficient to achieve a more than 6 log killing of pathogens. However, for antifungal activity, as small three-dimensional mycelial spheres were treated, the incubation period was extended to 100 min to allow for diffusion of the photosensitizers into the 3D structures and the radiant exposure was increased to 106.6 J cm^{-2} to ensure light saturation.

One key difference between bacteria and fungi relevant for the PDI photokilling process is the composition of fungal cell walls, which are comprised of mannan, glucan and chitin—the latter two form interchain hydrogen bonds and basically constitute the exoskeleton of the fungal cell—as well as proteins and further polysaccharides, depending on fungal species [21]. This structure and the larger size of fungal cells call for adjustments of the PDI treatment regime for fungi in comparison to bacteria. Both, the incubation period and the delivered light energy were increased for antifungal PDI.

As a result of this different cell wall composition, photoinactivation of *A. solani* (Fig. 3A) showed only a 11.5% killing and was ineffective against *B. cinerea* (Fig. 4A) when using the negatively charged Chl without cell wall permeabilizing agents. In contrast, *A. solani* was successfully photoinactivated with a killing efficiency of 94.1% when using 100 µM Chl in combination with 5 mM Na_2EDTA (Fig. 3B) and B17-0024 (Fig. 3C), which induced complete inactivation of the mycelia with (100% killing) at concentrations of 10 µM and above. The used photosensitizers (PS only, 100 µM) showed no signs of dark or light toxicity (light only) after 120 min. Sample photographs of the experiment are shown in supplementary data (Figure S1).

Experiments using *B. cinerea* as model fungus resulted in very comparable photoinactivation. Again, PDI using Chl without additives (Fig. 4A) had no effect on fungal growth

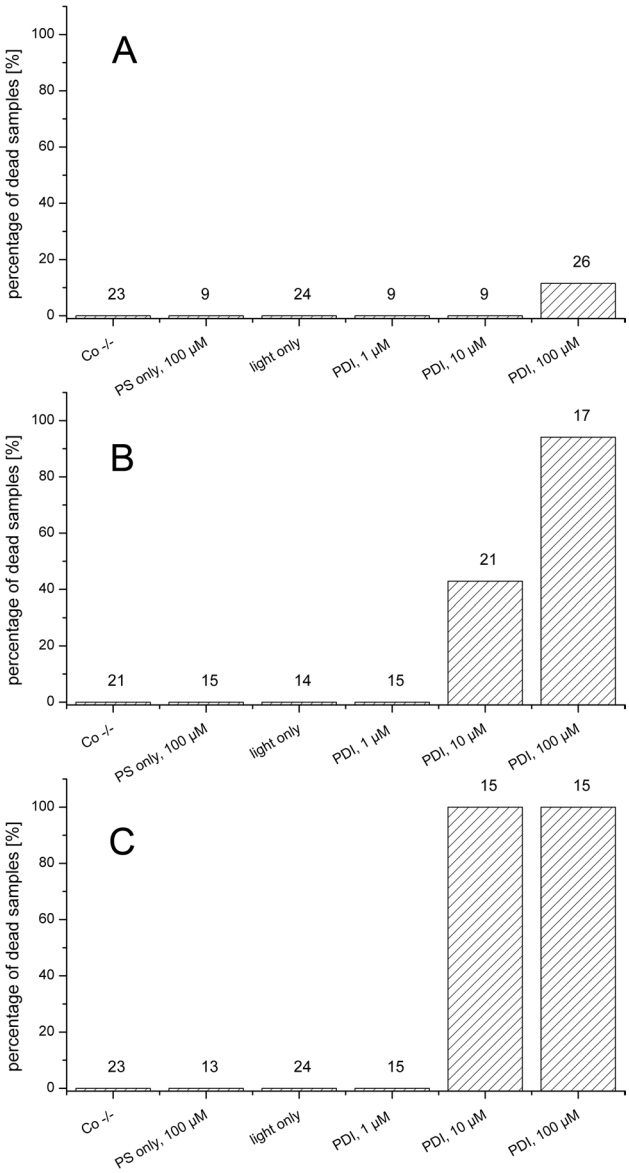


Fig. 3 Percentage of dead *Alternaria solani* samples after Photodynamic Inactivation using: **A** Chl, **B** Chl with 5 mM Na_2EDTA and **C** B17-0024 at concentrations of 1 µM, 10 µM and 100 µM. The samples were incubated for 100 min and subsequently illuminated for 120 min @395 nm (radiant exposure of 26.6. J/cm^2). The number above the bars indicates the number of treated samples. Co–/–, negative control; PS only, PS dark control; light only; PDI, PS + illumination

resulting in 100% viable samples. Combination of 10 µM Chl with 5 mM of Na_2EDTA induced photokilling of 33.3% and after increasing the concentration of Chl to 100 µM (+5 mM Na_2EDTA) almost complete eradication (91.7%) was achieved (Fig. 4B). B17-0024 was able to kill 66.7% of all samples at a concentration of 10 µM and resulted in complete photokilling at 100 µM (Fig. 4C). Once more, neither the photosensitizers, nor the activating light influenced the

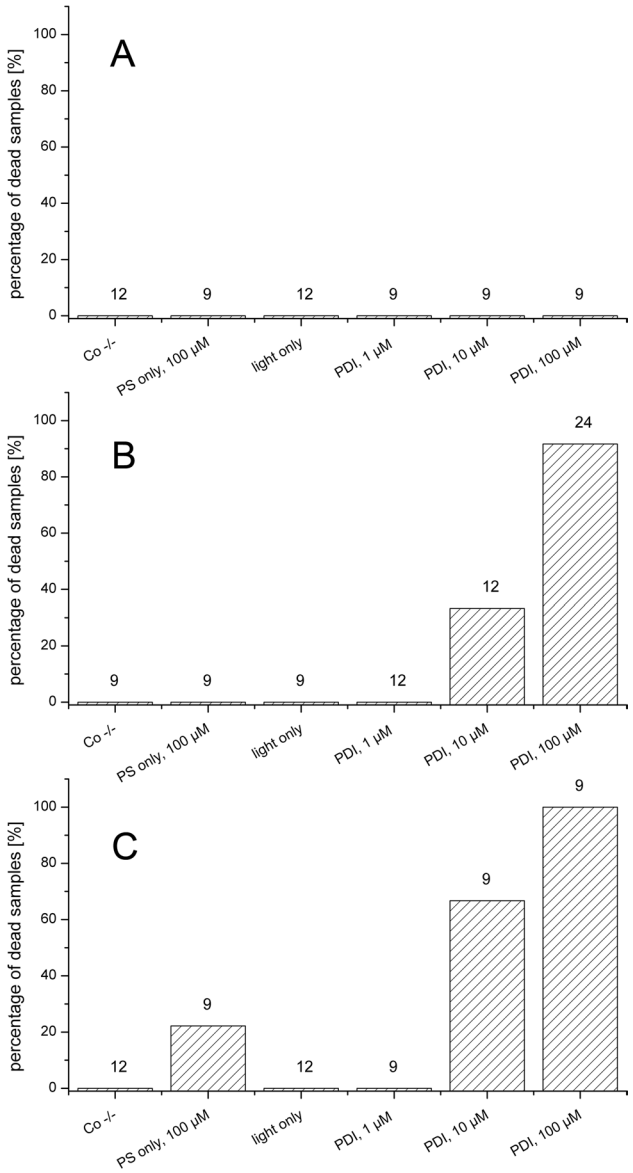


Fig. 4 Percentage of dead *Botrytis cinerea* samples after Photodynamic Inactivation using: **A** Chl, **B** Chl with 5 mM Na_2EDTA and **C** B17-0024 at concentrations of 1 µM, 10 µM and 100 µM. The samples were incubated for 100 min and subsequently illuminated for 120 min @395 nm (radiant exposure of 26.6. J/cm^2). The number above the bars indicates the sample size. Co−/−, negative control; PS only, PS dark control; light only; PDI, PS + illumination

growth of the mycelial patches. The photodocumentation of the experiment can be found in the supplementary data (Figure S1).

A different approach to treat bacteria and fungi with Chl was suggested by Luksiene and coworkers in 2011 [22]. The authors did not study the effects on plants but performed Chl-based PDI on strawberry fruits to extend the shelf life of the produce. After inoculation of the fruits with *Listeria monocytogenes*, incubation with 1 mM Chl for 5 min the illumination of the samples was performed using blue light (400 nm, 12 mW cm^{-2}, 30 min). By this, the shelf life of the strawberries was increased by two days [22].

To overcome the poor interaction of Chl with the fungal cell wall a Chlorophyllin-Chitosan (Chl-CHS) complex (0.001–0.1%) was used for photokilling of *Botrytis cinerea*. Illumination at 405 nm with 38 J cm^{-2} resulted in up to 60% growth inhibition of the fungus [23].

Buchovec et al. [23], studied inactivation of naturally occurring yeasts/molds on the surface of strawberries by 1.4 log achieved again based on the photoactivated Chl–CHS complex (1.5×10^{-5} M Chl–0.1% CHS; incubated for 30 min and illuminated with 38 J cm^{-2}).

EDTA has been shown to be an efficient cell membrane permeabilizer being able to aid the uptake of various molecules into the cell [24] and thus may facilitate improved delivery of the anionic PS in fungal cells. The higher efficacy of B17-0024 may be due to the cationic moieties facilitating its own entry into the cells, as previously shown for cationic porphyrins [25].

Chl and Na_2EDTA are readily available, can simply be mixed together without the need of complexation, and economic. This is especially of importance if the substances are applied in agriculture, where considerable amounts of PS and additives are needed to successfully photoinactivate entire fields or orchards. Chl is also registered as food additive E140 without upper limit in the European Union and shall therefore be easy to register as plant protection agent.

To exclude negative effects of PDI on host plants a phototreatment with the same PS was performed using a plant compatibility assay on woodland strawberries (*F. vesca* at growing stage BBCH14 [26]). All used PS as well as Na_2EDTA were tested in the same concentrations as in the fungal experiments described above. We also tested an alternative chelator PA (Baypure DS 100®) with higher chelation power for phytotoxic effects on leaves. To give a better overview, a comparison between representative samples (day 0 and day 21 for 2 plants per treatment group) is given in Figs. 5, 6 and 7. In Fig. 5, the results of the first PDI-plant compatibility assay (PDI-PCA) are shown. B17-0024 (Type S and T treatment), Chl (type S and T), Na_2EDTA (type T) and tap water (type T) were tested. In Fig. 5 only type T treatments are shown since the type S treatments did not result in any leaf damage, deformation or discolouration. The results of the PDI-PCA applying type T treatment showed no signs of leaf damage for B17-0024, Chl and the water control after 21 days but plant one in the Na_2EDTA test group + 21d showed minimal deformations (arrows) in leaves which were in contact with the solutions containing Na_2EDTA. The photoactive compounds do not seem to have a negative effect on plant health and development per se, and the use of Na_2EDTA caused only minor leaf damage in one sample.

Fig. 5 PDI-plant compatibility assay on young *Fragaria vesca* plants. Two representative plants per treatment group as indicated with "**A**" and "**B**" at day zero indicated by "0" and 21 days after treatment start "+21d" are shown. Samples were sprayed (300 µl from above) three times (type T) with B17-0024, Chl or H_2O (control) and 5 mM Na_2EDTA (chelator only). Leaf damage sites or deformations are indicated by arrows

Fig. 6 PDI-plant compatibility assay on young *Fragaia vesca* plants outside. Two representative plants per treatment group as indicated with "**A**" and "**B**" at day zero indicated by "0" and 21 days after treatment start "+21d" are shown. Samples have been sprayed (300 µl from above) once (type S) or three times (type T) with Baypure DS 100 (PA) well as baseline control (H_2O) and a completely untreated control (Co–/–). Slight leaf deformations are visible in the type T PA samples. Leaf damage sites or deformations are indicated by arrows. Pest damage indicated by asterisk

Fig. 7 PDI-plant compatibility assay on young Fragaria vesca plants outside the greenhouse. Two representative plants per treatment group as indicated with "**A**" and "**B**" at day zero and (indicated by "0" and 21 days after treatment start "+21d" are shown. Samples have been sprayed (300 µl from above) three times (type T) and once (type S) with 100 µM Chl + 1.2% PA as well as a type T PA control. Observable leaf damage in the three times treated Chl + PA samples as well as in the PA control. Arrows indicate leaf damage sites or deformations

Increasing the chelation power by replacing Na_2EDTA with PA (Baypure DS 100®) induced minimal leaf deformations in the type S and T test groups (Fig. 6). However, this was also observed in the water and Co –/– groups. It is assumed that these leaf deformations may partly arise due to heat stress, osmotic/salt stress or lensing effects of dew or spray droplets, but could also be attributed to treating plants in a very early stage of development. Figure 7 shows a combination of PA with Chl (type S + T) and a PA control (type T). All four plants in the type T treatment group of PA + Chl showed leaf damage in the actually treated leaves indicated by arrows (necrosis, brown leaf margins,

leaf deformations), but overall plant development was unaffected.

Only a very limited number of studies focus on Photodynamic Plant Protection. Fracarolli et al. [27] showed that sunlight activation of 50 µM 8-methoxypsoralen (8-MOP) was able to completely kill conidia of *Colletotrichum acutatum* conidia. They also tested the effects on the host plant leaves *Citrus sinensis)* and reported no observable damage to the citrus plants. Ambrosini et al., applied the anionic porphyrin Tetra-4-sulfonatophenyl porphyrin tetra-ammonium (TPPS) and were able to inhibit the mycelial growth of *Botrytis cinerea* at 1.5 µM PS concentration. They also were able to show in a trial with grapevine clones (Chardonnay, Merlot, Sauvignon), that 12.5 µM TPPS has no adverse effect on grapevine roots. *Botrytis cinerea* on grapevine vs − 1.5 µM TPPS was shown to completely inhibit mycelial growth [28]. Gonzales et al. [29] used 25 and 50 µM Methylene blue and 30 min sunlight illumination to treat petals and leaves of sweet orange plants infected with conidia of *Colletotricum abscissum* and were able to obtain a 3 $\log_{10}$ reduction—and complete eradication of conidia in some samples.

The results of these studies are very promising and show good efficacy of the photoactive substances at lower concentrations when compared to our experiments. However, as different model organisms, PS and experimental conditions were used, direct comparison is difficult. We achieved 94.1% growth inhibition of *A. solani* and *B. cinerea* after photoinactivation based on 100 µM Chl with 5 mM Na_2EDTA or complete killing of mycelial sheres with 100 µM B17-0024. For evaluation of these results, one must consider that the samples treated in this study are tissoid structures that might be more difficult to treat than single hyphae or conidia due to the need for the photosensitizers and activating light to penetrate the mycelial spheres.

Even though the killing efficiency is somewhat lower for Chl when compared to B17-0024, the natural origin of Chl might allow for an economic and eco-friendly application in agriculture (given a typical spraying volume of 2000 L per hectare in orchards and a concentration of the photoactive compound of 100 µM, 137 g of Chl are needed). This aspect requires special consideration for more expensive photosensitizers such as TPPS or the ecologically more problematic phenothiazines.

In conclusion, Photodynamic Inactivation based on chlorin e6 derivatives adds to the grower's toolbox to fight plant diseases caused by fungal and bacterial [16] plant diseases. The synthetic B17-0024 shows somewhat higher photokilling efficiency when compared to Chl. The natural compound, however, allows for economic and eco-friendly application. Both substances do not induce negative effects on the development of strawberry plants.

4 Conclusion

In face of expiring approvals for substances used in plant protection as well as the rising demand of organically farmed agricultural produce and considering the strict regulations regarding plant protection that come with it, PDI might emerge at exactly the right time to present an alternative approach for fighting fungal phytopathogens.

The data presented in this study prove that Photodynamic Inactivation based on two derivatives of chlorin e6 is a feasible tool to kill fungi relevant in agriculture in lab experiments. Considering the fact that the compounds will be applied in agricultural practice in high amounts, the use of Chl as PS should allow for economical and eco-friendly treatment, given its approval as food additive. The good water-solubility of Chl is also advantageous for this specific application, as the substances are typically applied in the field in aqueous solution.

However, to be effective against fungi and Gram-negative bacteria [16], addition of Na_2EDTA is required. As this additive does neither influence the absorption properties of Chl, nor the generation of ROS, the approach of combining a natural PS with a cell wall permeabilizer appears to be valid. As alternative PS, B17-0024 allows significant killing of both fungal species without additives and, at least for *Alternaria*, at a concentration one order in magnitude lower (10 µM) when compared to Chl (100 µM). Successful phototreatment of small mycelial spheres, as we demonstrate here, proves that both PS are able to diffuse into these mycelial structures to sensitize hyphae inside the spheres.

The experimental protocols based on either Chl + Na_2EDTA or B17-0024 appear to be safe for plant leaves. Extension of plant compatibility testing, field trials as well as further fine-tuning of the PS/additive solutions are needed to ensure a successful transition of the technology from the lab into the farmer's toolkit. Hopefully, the model system introduced here for determination of photokilling of mycelial spheres and the plant compatibility assay based on young strawberries will serve as guide for future investigations and will help to encourage researchers involved in Photodynamic Inactivation to get involved in plant protection by photosensitizers and light.

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Declarations

Conflict of interests The authors declare no competing interests.

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