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Photodynamic Inactivation in agriculture: combating fungal phytopathogens resistant to conventional treatment

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Abstract

Botrytis cinerea is a severe threat in agriculture, as it can infect over 200 different crop species with gray mold affecting food yields and quality. The conventional treatment using fungicides lead to emerging resistance over the past decades. Here, we introduce Photodynamic Inactivation (PDI) as a strategy to combat *B. cinerea* infections, independent of fungicide resistance. PDI uses photoactive compounds, which upon illumination create reactive oxygen species toxic for killing target organisms. This study focuses on different formulations of sodium–magnesium–chlorophyllin (Chl, food additive E140) as photoactive compound in combination with EDTA disodium salt dihydrate (Na_2EDTA) as cell-wall permeabilizer and a surfactant. In an in vitro experiment, three different photosensitizers (PS) with varying Chl and Na_2EDTA concentrations were tested against five *B. cinerea* strains with different resistance mechanisms. We showed that all *B. cinerea* mycelial spheres of all tested strains were eradicated with concentrations as low as 224 µM Chl and 3.076 mM Na_2EDTA (LED illumination with main wavelength of 395 nm, radiant exposure 106 J cm^{-2}). To further test PDI as a *Botrytis* treatment strategy in agriculture a greenhouse trial was performed on *B. cinerea* infected bell pepper plants (*Capsicum annum* L). Two different rates (560 or 1120 g Ha^{-1}) of PS formulation (0.204 M Chl and 1.279 M Na_2EDTA) and a combination of PS formulation with 0.05% of the surfactant BRIJ L4 (560 g Ha^{-1}) were applied weekly for 4 weeks by spray application. Foliar lesions, percentage of leaves affected, percentage of leaf area diseased and AUDPC were significantly reduced, while percentage of marketable plants were increased by all treatments compared to a water treated control, however, did not statistically differ from each other. No phytotoxicity was observed in any treatment. These results add to the proposition of employing PDI with the naturally sourced PS Chl in agricultural settings aimed at controlling *B. cinerea* disease. This approach seems to be effective regardless of the evolving resistance mechanisms observed in response to conventional antifungal treatments.

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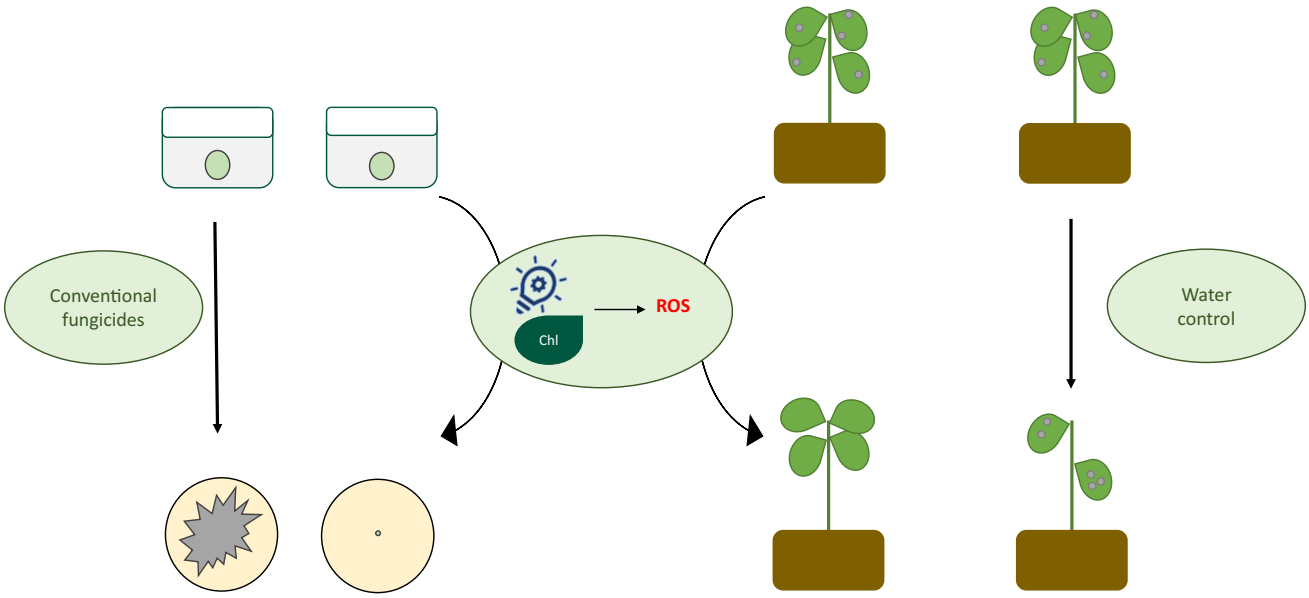
Graphical abstract



Keywords Photodynamic Inactivation · *Botrytis cinerea* · Antimicrobial resistance · Chlorophyllin · Greenhouse trial

1 Introduction

Phytopathogens cause worldwide crop yield losses of up to 40%, thereby leading to severe economic losses for the agricultural industry. These shortfalls lead to waste of valuable resources, such as water, energy, fertilizers, and labor. In addition, the fall in crop yields is anticipated to cause increasing food prices, posing a threat to adequate access to nutritionally beneficial food and endangering worldwide food supply [1, 2]. Global warming will further endanger crop yields, especially in the Northern Hemisphere, due to shifts in pathogen distributions and microbial species compositions. Moreover, changes in physiologic, biochemical, and evolutionary processes of the host and pathogen interaction will be affected by climate change, leading to the rise of new pathogens [3, 4].

Phytopathogenic fungi are a serious hazard in agriculture. *Botrytis cinerea*, a necrotrophic plant pathogen, especially concerns farmers as it causes substantial damage in horticultural crops. Therefore, it gained attention as a critical target for the development of novel management strategies in agricultural research [5]. Following *Magnaporthe oryzae, B. cinerea* was ranked the second most important fungal plant pathogen in 2012 [6]. And still, a decade later, *B. cinerea* is an issue of concern, due to its many different routes of attack and multi-layered and multifunctional infection pathways [7]. As a generalist pathogen it is capable of causing gray-mold disease in over 200 different crop species, making it a universal challenge to most sectors of agricultural industry [8]. Pathogenicity begins with its ability to persist as mycelia, conidia or sclerotia in crop remnants. Upon entering the fresh host plants during early development stages, it remains quiescent until more favourable growth conditions appear. Consequently, damages caused by *B. cinerea* are frequently detected post-harvest [9]. Since the gray mold disease affects such a wide range of crop plants and pathogenicity occurs in all parts of the plant, the costs of damage control are difficult to assess. However, chemical control measures against *Botrytis* species are estimated to cost up to 40 € per hectare, accounting for 10% of the global fungicide market [6].

Chemical control agents in form of synthetic fungicides are the main measure for management of *B. cinerea* [10]. Yet, the overuse of synthetic fungicides in agriculture since the 1950s lead to an increasing number of resistant strains also within the *Botrytis* genus. Managing resistance in *B. cinerea* is particularly challenging because, in addition to single target site resistance, certain strains have developed multi-drug resistance (MDR) [11]. Multidrug resistant *B. cinerea* strains exhibit enhanced efflux activity of fungicides and overexpression of membrane transporter genes. The incidence of MDR strains is rapidly increasing in French and German vineyards. In contrast, the development of fungicides is lagging behind due to common obstacles, such as off-target toxicity or food safety issues, emphasizing the need for alternative

management strategies against resistant *B. cinerea* strains [12]. Alternating several fungicides, varying doses, and using drug mixtures can decelerate, but not prevent the development of resistances [13]. Particularly for organic agriculture, the number of approved fungicide compounds is limited. Consequently, copper-based formulations were introduced against *Botrytis* infections [14]. Yet, the extensive use of copper may lead to accumulation in soils, compromising the groundwater, soil microbiomes and plants [15]. Moreover, besides residual pesticides, copper accumulates in the fruit and hence in the processed product, as recently demonstrated for European organic wines [16].

As an alternative method to combat fungal phytopathogens in agriculture, Photodynamic Inactivation (PDI) was recently introduced [17]. PDI utilizes photosensitizers (PS). These photoactive molecules generate reactive oxygen species upon illumination which are then capable of oxidizing various cellular components, such as cell wall and DNA, leading to death of pathogenic cells. Through PDI a wide spectrum of pathogens can be targeted, without any known development of resistance [18]. Sodium–magnesium–chlorophyllin (Chl) is a natural, water-soluble PS, approved as a food additive in the European Union (E140), thereby proving its safety in humans. Chl was already proven to show a phototoxic effect against fungal and bacterial phytopathogens [17, 19]. Recently, Chl has achieved an antibacterial effect against a Streptomycin resistant strain of the Gram-negative bacteria *Erwinia amylovora* [20].

The aim of this study is to investigate Chl for its potential as a PS in PDI of fungicide-susceptible versus -resistant strains of *B. cinerea*. The formulations SUN-D-05, -06 and -07, containing the naturally derived Chl in different concentrations, are used in addition with the chelator EDTA disodium salt dihydrate (NA_2EDTA) and a surfactant (Alkylsulfosuccinate). As we successfully demonstrated the effectiveness of SUN-D-06 in vitro, this formulation was further examined within a greenhouse trial using the pepper 'California Wonder'.

2 Experimental

2.1 Fungal culture

Botrytis cinerea strains for the in vitro study were purchased from the DSMZ (Braunschweig, Germany [21]) or were kindly provided and resistance typed by Anne-Sophie Walker (Palaiseau, France) (Table 1). Cultivation and PDI were preformed according to the protocol of Hamminger et al. [17]. The fungal strains were grown on malt extract peptone (MEP) agar plates (30 g L^{-1} malt extract (Carl Roth GmbH & Co. KG), 3 g L^{-1} peptone (Peptone ex casein, Roth) and 15 g L^{-1} Agar–Agar (Kobe I, Roth)) at 26 °C. The imides resistant strain Bc-80 was cultivated on MEP agar plates supplemented with 5 mg L^{-1} cycloheximide (Sigma-Aldrich Chemie GmbH), while the benzimidazole-resistant strain (Bc-842) and the MDR strains (Bc-VA101 and Bc-VA664) were cultured on MEP agar supplemented with 1 mg L^{-1} carbendazim (Sigma-Aldrich Chemie GmbH). The fungicides carbendazim and cycloheximide were diluted in 2.5 mL sterile ddH_2O and added after autoclaving at 75 °C. For growing mycelial spheres, fungal material was transferred from agar plates into 20 mL liquid MEP medium at 26 °C and 200 rpm in a shaking incubator (MaxQ 4000, Thermo Scientific) for at least 48 h until mycelial spheres of 2–3 mm formed.

2.2 In vitro Photodynamic Inactivation

PS formulations were received as powders from Suncor AgroScience (Mississauga, ON, Canada). Aqueous solutions of the dry material were prepared (for concentrations refer Table 2) and stored at − 20 °C until use. The absorption signal of the PS formulation with medium Chl concentration (SUN-D-06) was measured at 0.23% concentration using an Infinite M200 microplate reader (Tecan Trading AG). The mycelial spheres were transferred into 24-well plates (one sphere per well) using a pipette. To each well 500 µL of treatment were added. Samples were incubated for 100 min in the dark at room temperature under constant agitation. Negative controls were kept in Dulbecco's Phosphate

Table 1 PDI-treated *Botrytis cinerea* strains including isolate name, known resistances, date and origin of collection

Isolate	Resistances	Date collection	Origin of collection
WT (DSM 877)	–	Before 1977	Unknown
80	Imides, benzimidazoles, hydroxyanilides	2006	France, Champagne vineyard
842	Benzimidazoles	2007	France, Champagne vineyard
VA101	Imides, benzimidazoles, MDR	2005	France, Champagne vineyard [22]
VA664	Imides, benzimidazoles, MDR	2005	France, Champagne vineyard [22]

MDR multi drug resistance

Table 2 Formulation composition for in vitro Photodynamic Inactivation of *B. cinerea* spheres including concentrations of active photosensitizer compound sodium–magnesium–chlorophyllin (Na–Mg–Chl), concentrations of the chelator EDTA disodium salt dihydrate (Na_2EDTA), surfactant Alkylsulfosuccinate and dilution percentages

Treatment	Active compound	Used dilution in ddH_2O [%]	Conc. Na–Mg–Chl [M]	Conc. Na_2EDTA [M]	Mass percentage alkylsulfosuccinate [%]
Negative control	DPBS	–	–	–	–
Positive control	H_2O_2	3	–	–	–
SUN-D-05	Na–Mg–Chl	0.28	0.394	1.101	36
SUN-D-06	Na–Mg–Chl	0.23	0.204	1.279	43
SUN-D-07	Na–Mg–Chl	0.22	0.102	1.398	46

Buffered Saline (DPBS, Sigma-Aldrich Chemie GmbH) and positive controls in hydrogen peroxide (H_2O_2, Roth). After incubation, each sphere was washed once in 500 µL DPBS. Samples were illuminated in fresh 500 µL DPBS. The illumination was performed using an LED array (22 mW cm^{-2}, 106 J cm^{-2}, diode type L-7113UVC, dominant wavelength 395 nm, Kingbright Electronic Europe GmbH). Dark controls of each treatment were kept in the dark for the same time period. Subsequently, each sphere was transferred to 6 cm MEP agar plates (without fungicide) and incubated at 26 °C for 7 days. The photokilling effect was evaluated by counting the samples for which no growth was observed. The percentage of dead samples was calculated as follows:

$$\text{Percentage of dead samples [\%]} = \frac{\text{treated samples without growth}}{\text{amount treated samples}} \times 100$$

2.3 Greenhouse trial

A greenhouse trial was conducted on pepper plants (*Capsicum annum* L., California Wonder, Houwelings Nursery Ltd.) in a research greenhouse located at Frost Environmental, Abbotsford, BC, Canada. The greenhouse contained 24 LED lights (each 0.9 m long, 120 W per light (~ 10,800 lm per light, Monios-L)) on a 12 h timer as well as supplemental heat. Temperature and humidity logs are given in the Supplementary Materials. The 7-week-old plants at the 6–8 leaf stage were transplanted into coco fiber bags (Richgrow™, Terralink) in the greenhouse. Each plot of a one m^2 area contained two bags with a total of four plants laid out in a randomized complete block (RCB) design with four replicates. Each plant was fertigated with a single emitter (0.5 gph low flow rate, Netafim Emitter). Foliar sprays of PS were prepared in a solution volume of 1000 L ha^{-1}, accounting for 100 mL per plot. Controls were sprayed with water. Treatments are summarized in Table 3, water control was used as negative control, while RHAPSODY ASO (Plant Products) was chosen as a positive control. Besides SUN-D-06 in different rates also a combination of SUN-D-06 with BRIJ L4 was tested. This ethylene oxide-based surfactant is a spray additive which enables the foliar uptake of compounds into leaves and is already used in emulsifier systems in agriculture [23]. Foliar applications were made with a CO_2 backpack sprayer at 276 kPa, equipped with a single nozzle (Tip 8002VS, TeeJet Technologies). Minor foaming was observed when mixing the PS products, approximately 0.5 cm per 100 mL. Foaming dissipated within a minute of mixing and posed no problem to the application.

Table 3 Treatments, product rates and solution volumes for the bell pepper greenhouse trial

	Product rate [g Ha^{-1}]	Product per plot [m^{-2} 100 mL^{-1}]
Water control	–	–
SUN-D-06 1 × rate	560	0.056 g
SUN-D-06 2 × rate	1120	0.112 g
SUN-D-06 1 × rate + 0.05% BRIJ L4	560	0.056 g (+ 0.05 mL BRIJ L4)
RHAPSODY ASO	1–2 L/100 L	2 mL

Treatment was applied weekly for a total of 4 weeks (Fig. 1). Forty-eight h after the first treatment, wetted leaves were inoculated under cool, cloudy conditions in the late evening. Sporulating diseased leaves from extra plants were brushed against the trial plants to dislodge the conidia from the diseased plants onto the healthy plants. Conidia from the diseased plants were confirmed under the microscope as *B. cinerea*. A high level of natural inoculum was present in the greenhouse as well.

Gray-mold symptoms were assessed weekly, prior to each application and at 7 days after the last application for a total of 5 assessments. Foliar symptoms, such as leaf lesions, wilting, stem lesions, and leaf drop, were counted and recorded. The number of lesions were recorded at each date as well as the number of leaves affected and total leaves lost. Visual assessments of diseased leaf area

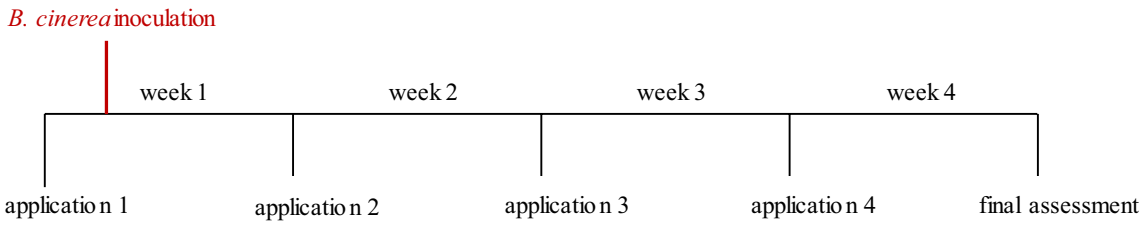


Fig. 1 Experimental schedule of the greenhouse trial. Four applications of treatments were done in 7-day intervals. The inoculation of *Botrytis cinerea* was done 48 h after the first treatment application

Table 4 Horsfall–Barratt (HB) scale for the determination of diseased leaf area including transformed HB scale

Leaf area affected [%]	HB scale	Transformed HB scale [%]
0	0	1.17
0–3	1	2.34
3–6	2	4.68
6–12	3	9.37
12–25	4	18.75
25–50	5	37.5
50–70	6	62.5
70–88	7	81.25
88–94	8	90.63
94–97	9	95.31
97–100	10	97.66
100	11	98.82

(leaf area covered by *B. cinerea* lesions) and plant vigor were also recorded at each assessment. The percentage of leaf area diseased was rated weekly on an Horsfall-Barratt (HB) scale. The HB ratings were transformed to percentages following the standard grade formula by Redman, King and Brown [24] (Table 4).

Vigor was rated on a 1–10 scale where 10 = healthy, green and vigorous and 1 = dead. The area under the disease progress curve (AUDPC) [25] was calculated at the end of the trial using the percent leaf area data. Due to the high level of disease pressure in combination with cool winter conditions, the plants did not mature to fruiting stage, so final yield was assessed on the plant's marketability and quality. Each plant was determined if it was marketable or non-marketable, one week after the final treatment, based on the plant vigor, number of *Botrytis* spots and percent leaf area covered by the spots. Plants with vigor ratings of 7 or higher and with a percentage of leaf area covered of 1% or less were considered marketable.

The mean disease severity for all evaluated plant characteristics was calculated using following equation:

$$\text{Mean disease severity } [\%] = \frac{\text{mean value (treatment)}}{\text{mean value (water control)}} \times 100$$

3 Results

3.1 In vitro Photodynamic Inactivation

The absorption spectrum of the PS formulation SUN-D-06 and the structure of the photoactive molecule Chl are shown in Fig. 2. The percentage of dead samples was measured after a 7-day incubation period by evaluating if growth of mycelial spheres occurred on agar plates. Sample images of mycelial patches on agar plates are provided in Fig. 3A. In all experiments, untreated controls (co-/-) of all strains showed clear growth, resulting in a complete coverage of the dishes with mycelia after 7 days. Illumination with 395 nm

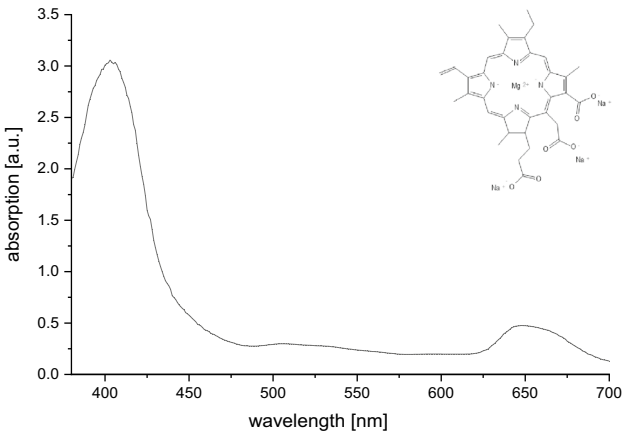


Fig. 2 Absorption spectrum of the photosensitizer formulation SUN-D-06 at a 0.23% dilution and the chemical structure of the photoactive molecule sodium–magnesium chlorophyllin

LED light without PS had no effect on all samples (light only control).

A complete eradication (100%, no growth detected on agar plates) was given for the wild-type strain (Bc-WT,

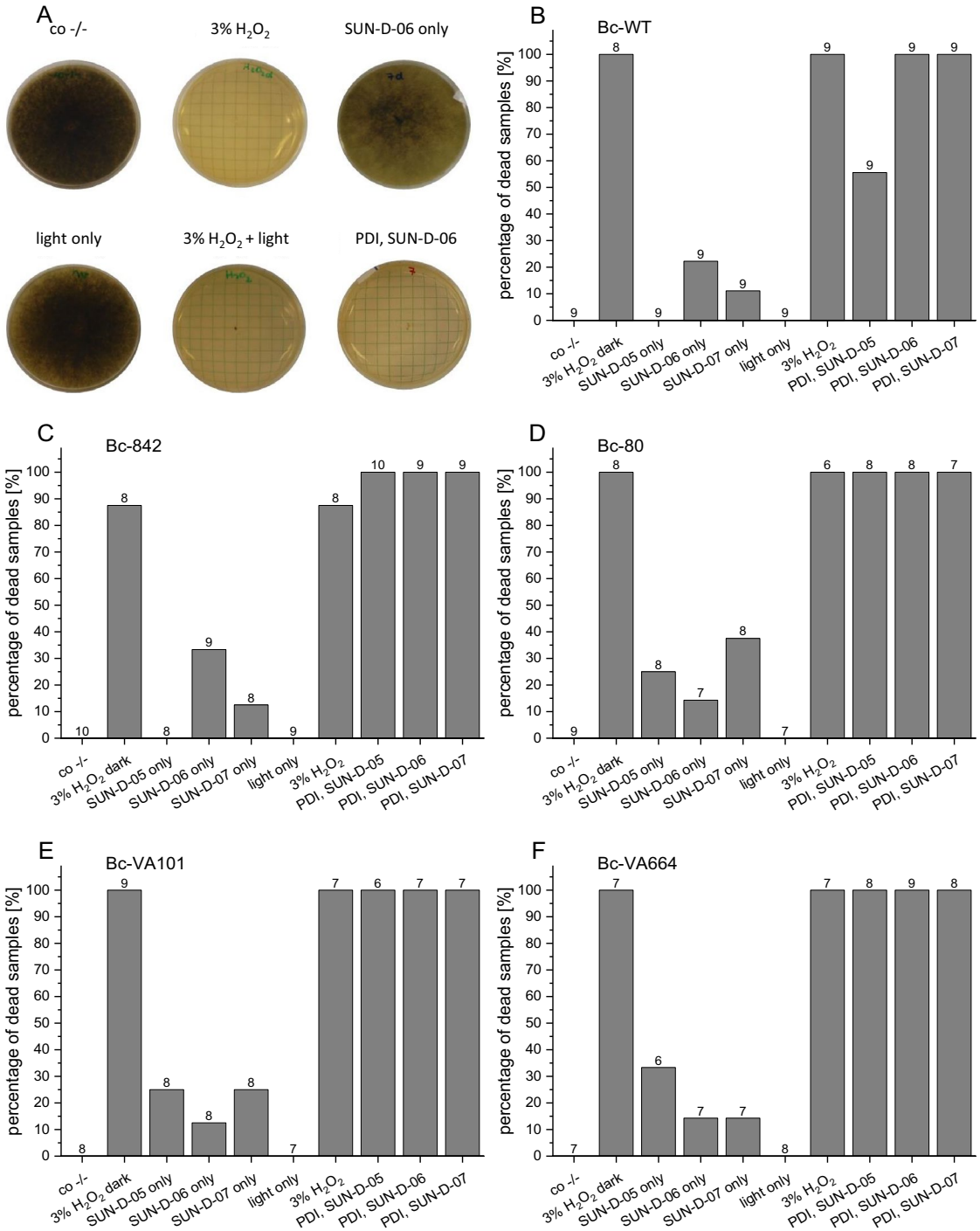


Fig. 3 Results of in vitro tests for five tested *B. cinerea* strains after SUN-D-05, -06 and -07 PDI treatment. **A** Sample photographs of agar plates of the wild-type *B. cinerea* 7 days post PDI treatment using the PS SUN-D-06. Mean percentages of dead samples [%]: **B** wild-type strain (Bc-WT). **C** Strain Bc-842, carbendazim-resistant (**D**) a cycloheximide resistant strain Bc-80 and two multidrug resistant strains BC-VA101 (**E**) and Bc-VA664 (**F**). PDI treatment was done using an LED array (395 nm, 22 mW cm^{-2}, 106 J cm^{-2}). Co-/-: double negative control, DPBS, no illumination; light only: DPBS with illumination; 3% H_2O_2 only: positive control, no illumination; 3% H_2O_2: positive control with illumination. The numbers above bars represent the number of tested fungal spheres per treatment. Summarized numerical values can be found in the Supplementary Materials

Fig. 3B) after PDI with a radiant exposure of 106 J cm^{-2} and the PS formulations SUN-D-06 and SUN-D-07. Even though SUN-D-05 contains the highest concentration of the photoactive compound Chl, it eradicated only 55.5% of samples. The positive control 3% hydrogen peroxide induced killing of all mycelial spheres. As for dark toxicity of the PS formulations, incubation of Bc-WT spheres with SUN-D-06 without illumination resulted in growth inhibition of 22.2% of spheres. SUN-D-07 was less toxic and SUN-D-05 did not show dark toxicity at all.

As for the benzimidazole resistant strain Bc-842, treatment with all three tested-PS formulations resulted in complete photokilling (100%, Fig. 3C). Dark toxicity of SUN-D-06 without illumination was 33.3%, and again, SUN-D-05 was not dark-toxic. SUN-D-07 showed less toxicity than SUN-D-06. The positive control (H_2O_2, illuminated and in the dark) achieved a somewhat lower toxicity than PDI (87.5%).

Likewise, phototreatment of the imides resistant strain Bc-80 was successful with all tested-PS formulations (100% killing of mycelial spheres, Fig. 3D). Equally, the positive control H_2O_2 eradicated all tested mycelial spheres. The highest dark toxicity was detected for SUN-D-07 (37.5% killing of spheres). SUN-D-05 (14.3%) and SUN-D-06 (25.0%) showed lower toxicity in the dark.

PDI with all tested PS formulations effectively photokilled the MDR strain Bc-VA101 (100% killing of spheres, Fig. 3E), as did the positive control H_2O_2. The dark toxicity of the tested formulations did not exceed the values achieved for strain Bc-80 (< 25%).

In like manner, all samples of the second MDR strain Bc-VA664 were entirely eradicated by SUN-D-05, SUN-D-06 and SUN-D-07 (Fig. 3F). Likewise, the positive control H_2O_2 showed an antifungal effect (100% killing). Toxicity of SUN-D-05 alone without illumination was 33.3%, not exceeded by the other formulations (14.3% for SUN-D-06 and SUN-D-07).

3.2 Greenhouse trial

The number of *Botrytis* leaf spots (Fig. 4A) after 3 weeks showed significant differences in Tukey's HSD at $P = 0.05$ compared to the water control. All tested products and the standard RHAPSODY ASO were significantly different from the water-treated control. However, SUN-D-06 at the 2× rate, and the 1× rate in combination with 0.05% BRIJ L4, performed the best with zero spots (100% reduction from the water control). At the final assessment, 7 days after the 4th application, all test products were significantly different from the water-treated control, but not significantly different from each other or the standard RHAPSODY ASO. Plants treated with RHAPSODY ASO had the lowest average number of spots (86.2% percent of inhibition).

At each assessment, the number of affected leaves and total leaves was counted per plant to calculate the percentage of diseased leaves (Fig. 4B). Significant differences in Tukey's HSD at $P = 0.05$ were first observed in the third week. All test products and the standard RHAPSODY ASO were significantly different from the water control. However, the 2× rate of SUN-D-06 and SUN-D-06 in combination with 0.05% BRIJ L4 performed the best with 100% of inhibition compared to the water-only treated control. After the final assessment, all test products were significantly different from the water control but not significantly different from each other or the standard product RHAPSODY ASO. Plants treated with RHAPSODY ASO had the lowest average percentage of leaves affected (5%), with SUN-D-06 in combination with BRIJ L4 the next best-performing product at an average of 9.5% leaves affected, lower than all the other test products.

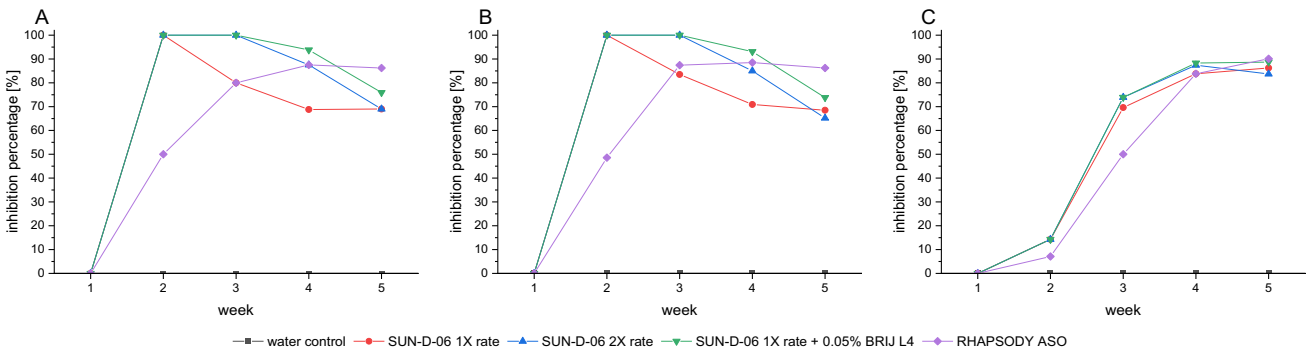


Fig. 4 **A** Mean disease severity percentage of *Botrytis* leaf spots per plant in respect to the water control. **B** Mean disease severity percentage of number of diseased leaves in respect to the water control. **C** Mean disease severity percentage of diseased leaf area in respect to the water control. Each characteristic was evaluated in 7-day intervals before the next treatment application. All numerical values in tables can be found in the Supplementary Materials

Significant differences in diseased leaf area were first observed in week 4 (Tukey's HSD, $P = 0.05$) (Fig. 4C). The 1× rate of SUN-D-06 in combination with BRIJ L4 and the 2× rate of SUN-D-06 were the highest performing products with an average 1.3–1.4% leaf area diseased (87.4–88.3% less than the water control), both treatments were significantly different from the water-treated control, but not significantly different from the other test products or the standard RHAPSODY ASO. For the final assessment, all test products and the standard RHAPSODY ASO were significantly different from the water control in Tukey's HSD at $P = 0.05$, but not significantly different from each other. The standard RHAPSODY ASO performed the best with an average percent of inhibition of 90.1%, while SUN-D-06 in combination with BRIJ L4 was the next best treatment with an average 88.7% percent of inhibition compared to the water control.

SUN-D-06 in combination with BRIJ L4 performed the best for AUDPC with the lowest AUDPC of 37.6 but it was not statistically different from the other product treatments or the standard RHAPSODY ASO (Table 5). All product treatments including the standard RHAPSODY ASO were significantly different from the water control in Tukey's HSD at $P = 0.05$.

Table 5 Integrated disease progress curve (AUDPC), visual rating of plant vigor and the mean inhibition percentage of dropped leaves compared to the water control

	AUDPC	Vigor rating	Number of dropped leaves
Water control	194.8 ± 294.5	6.0 ± 1.8	0.8 ± 0.9
	a	a	a
	–		–
SUN-D-06 1X rate	44.6 ± 14.8	7.8 ± 1.0	0.4 ± 0.7
	b	b	ab
	(− 77.1%)		(− 50.0%)
SUN-D-06 2X rate	41.7 ± 11.1	7.7 ± 0.9	0.1 ± 0.2
	b	b	b
	(− 78.6%)		(− 87.5%)
SUN-D-06 1X rate + 0.05% BRIJ L4	37.6 ± 6.2	7.9 ± 0.8	0.1 ± 0.3
	b	b	b
	(− 80.7%)		(− 87.5%)
RHAPSODY ASO	48.4 ± 54.1	8.4 ± 0.7	0.1 ± 0.2
	b	b	b
	(− 75.2%)		(− 87.5%)

Each characteristic was calculated after the final assessment in week 5. Plant vigor and number of dropped leaves for earlier evaluations and all other numerical values in tables can be found in the Supplementary Materials

Mean and standard deviation of four plants per plot, four replicates per treatment, RCB design. Numbers followed by the same letter in each column are not significantly different in Tukey's HSD at $P = 0.05$. Percentage change in comparison to water control

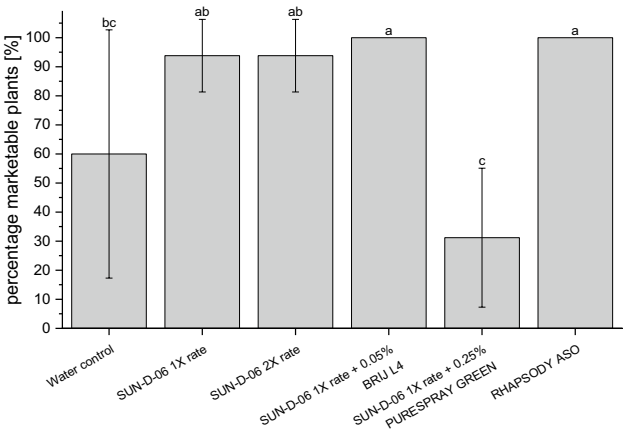


Fig. 5 Total marketable plants. Numbers were evaluated after the final assessment. Plants with vigor ratings of 7 or higher and with a percentage of leaf area covered of 1% or less were considered marketable. Mean and standard deviation of four plants per plot, 4 replicates per treatment, RCB design. Letters indicate Tukey's HSD at $P = 0.05$ results, bars labeled with same letters are not significantly different. All numerical values in tables can be found in the Supplementary Materials

Each plant was rated for vigor on a 1–10 scale where 10 = healthy, vigorous, and green in color and 1 = dead (Table 5). Significant differences from the water control in Tukey's HSD were first observed after the first treatment. Plants treated with SUN-D-06 in combination with BRIJ L4 had the highest average plant vigor rating of 8.7, a 10.1% increase in vigor from the control. After the final assessment, SUN-D-06 in combination with BRIJ L4 was the highest performing treatment with an average vigor rating of 7.9. SUN-D-06 in combination with BRIJ L4 was significantly different from the water control but not significantly different from the standard RHAPSODY ASO or the 2× rate of SUN-D-06.

Leaf drop is a common symptom of *Botrytis* gray mold on greenhouse pepper. Each week the number of leaves per plant dropped was counted (Table 5) Significant differences were first observed in Tukey's HSD at $P = 0.05$ after the final assessment. At least one leaf had dropped in all treatments however, the 1× rate of SUN-D-06 had the highest average leaf drop per plant (0.4), 50.0% less than the water control but not significantly different from the control or the other test products.

The percentage of marketable plants was evaluated after the final assessment (Fig. 5). SUN-D-06 in combination with BRIJ L4 and the standard RHAPSODY performed the best for total marketable yield (100%) with the highest percentages of marketable plants but were not significantly different from the 1× or 2× rate of SUN-D-06.

4 Discussion

The need for environmentally friendly *Botrytis* management agents is expected to rise over the next few decades [26]. Classic treatments using regular fungicides bear the risk of novel resistant strains and as the world population grows, crop protection must be prioritized to ensure food safety globally [27]. We successfully demonstrated that PDI using the natural photosensitizer Na-Mg-Chlorophyllin (Chl, food additive E140), combined with a surfactant and Na_2EDTA, represents a promising approach to combat *B. cinerea* (Fig. 3). This is especially meaningful, as Chl-based PDI proved highly effective also against strains with challenging fungicide resistances. When illuminated, photoactive substances will undergo a change in their energy state, resulting in the production of toxic species of either oxygen radicals (type-I photochemical reaction) or singlet oxygen molecules (type II photochemical reaction). Chlorin-based molecules, such as Chl, favour to produce singlet oxygen molecules rather than radicals, therefore are considered type II PS [28–30]. In vitro experiments could show a 100% photokilling effect against all tested strains, independent on resistance to conventional treatment. Our observations indicate that SUN-D-06 and -07 are the most promising formulations with little to no dark toxicity. The SUN-D-05 formulation could achieve a killing effect of 100% for the resistant strains, however only a 55.5% killing rate for the wild-type. As working hypothesis, we suggest that due to the darker color of the solution resulting from the higher Chl concentration in SUN-D-05, a filter effect is likely in the treated mycelial spheres. The stained outside hinders the light penetration into in the inner sphere, most likely leading to a potential decreased photokilling effect. This indicates that Chl-based PDI, preferably in low concentrations, provides one universal management strategy regardless of the resistance mechanisms of *B. cinerea.*

Furthermore, under high disease pressure in a research greenhouse in British Columbia, Canada, SUN-D-06 in combination with BRIJ L4 was the best-performing treatment against *B. cinerea*. Reduction of *Botrytis* lesions in number and in diameter *in planta* was best achieved in our study by addition of BRIJ L4. BRIJ L4 is an ethylene oxide-based surfactant enabling the foliar uptake of compounds into leaves and is presently used in agricultural settings [23]. Compared to the water-treated control, plants treated with SUN-D-06 in combination with 0.05% BRIJ L4 had 75.9% fewer *Botrytis* lesions per plant, 73.8% fewer leaves affected, and 88.7% lower leaf area diseased (Fig. 4), an AUDPC that was 80.7% less, and the highest plant vigor (Table 5) and percentage of marketable plants at the end of the trial (Fig. 5).

The need for a cell permeabilizing agents in antifungal PDI-based approaches results from fungal cell-wall peculiarities [31]. The negatively charged Chl alone cannot permit the wall and therefore cannot introduce reactive oxygen species to the pathogen [17]. Other studies overcome this problem by using positively charged synthetic PS. For example, the cationic PS Tetra-4-sulfonatophenyl porphyrin tetraammonium, in concentrations as low as 1.5 µM inhibited the growth of *B. cinerea* cultures completely [32]. However also another naturally occurring photoactive compound, curcumin, can kill *B. cinerea* effectively (4 $\log_{10}$ steps of *B. cinerea* spores and reduced cell viability). Nevertheless, the highly hydrophobic molecule must laboriously be dissolved in ethanol [33]. In our study, Na_2EDTA was used to achieve cell-wall penetration. The effect is supported by Hamminger et al., who achieved an almost complete *Botrytis* eradication (91.7%) using 100 µM Chl in combination with 5 mM Na_2EDTA and the same light conditions as in this study, indicating that Chl is a suitable antifungal agent, if used in combination with the cell permeabilizing agent. Moreover, the same study revealed a 94.1% photokilling effect against *Alternaria solani*, the inducing pathogen of early blight, under the same experimental conditions. Furthermore, it could be shown that Na_2EDTA does not influence Chl absorption, nor its ability to generate reactive oxygen species. Hence, the addition of cell permeabilizing agents is not altering the photoactive effects of Chl, but rather enhance the toxicity against microbes [17].

The antimicrobial effect of Chl for agricultural applications has been shown against other pathogens already, however mainly against bacteria. Chl at a concentration of 100 µM showed a 7 $\log_{10}$ step reduction against a streptomycin resistant strain of *Erwinia amylovora* [20]. The application of Chl-based PDI for human-relevant pathogens mainly focuses on food disinfections, as Chl is approved as a food additive in the European Union (E140). PDI using Chl was introduced as promising method to ensure food safety on sprouts and cherry tomatoes [34, 35]. Using 100 µM Chl, a 4 $\log_{10}$ step reduction of *Listeria innocua* could be achieved in mung bean seeds, whereby no alteration of the further germination of the seed was reported [34]. The Gram-positive food pathogens *Bacillus cereus* and *Listeria monocytogenes* were reduced by 1.5 $\log_{10}$ steps in 150 µM Chl on tomato fruits with no harm to the tomato plants [35]. The copper salt of Na-chlorophyllin was found as an effective antimicrobial on food package surfaces against *Listeria monocytogenes* and *Bacillus cereus* [36, 37]. Additionally, Chl has an antibacterial effect against *Staphylococcus aureus* on lettuce [38]. Chl concentrations of as little as 15 µM were also already tested against Gram-negative food pathogens, in order to establish edible coatings for strawberry fruits. While Chl alone was not able to eradicate *Salmonella enterica*, a chlorophyllin-chitosan complex could reduce the bacteria

by 7 $\log_{10}$ steps [39]. Recently, the application range of Chl used in antibacterial materials was further expanded to hospital settings. Fabrics based on Chl achieved an 99.998% and 99.994% inactivation against clinically relevant and antibiotic resistant strains of *Enterococcus faecium* and *Staphylococcus aureus,* respectively [40].

To our understanding, until now little is known to the effect of PDI against resistant phytopathogenic fungi. To date, one single other study has introduced PDI against a fungicide-resistant *Penicillium digitatum* strain using a water-soluble vitamin K3 analog as a PS illuminated by natural sunlight [41]. The results presented in this study and available results from previous studies conclude that PDI using Chl-based PS is a working tool against phytopathogenic *B. cinerea*. The in vitro experiments of this study could show that SUN-D-06 and SUN-D-07, PS formulations based on the naturally derived Chl can achieve a 100% photokilling effect in several *B. cinerea* strains, independent on their resistance to conventional fungicides. A total eradication was achieved with PS formulations containing Chl at a concentration as little as 470 µM.

Moreover, because of the limited number of *in planta* studies of PDI applications, knowledge about the effect in actual agricultural use is limited. So far, studies implicate that Chl-based PS do not harm plant growth or integrity and do not alter the quality of edible plants, e.g., tomatoes [17, 34, 35, 42]. The data we present here show a good compatibility of the used PS and plant growth. After four applications of the PS formulations on greenhouse pepper plants through spraying, the marketability of the plants was enhanced compared to the control and was equally high as for the competitor product RHAPSODY ASO. Due to the growth conditions in the greenhouse and the reduced temperature of the area of experiment, the growth of fruits could not be assessed and might be a question for further research. RHAPSODY ASO uses the strain QST 713 of *Bacillus subtilis* as an active ingredient and was shown to be active against *B. cinerea* [43]. However, pesticides based on replacement microbes possess several downsides. Proper storage environments and culture conditions appropriate for the used active microorganism must be kept in mind. Also, the production of microorganism-based products serves several challenges for example the missing standardization [27]. Likewise, the production of ready-to-use PS for fungal control comes with challenges [44]. Photostability of the product is welcome, however a degradation of the product through light into products that plants can easily metabolize is adding to the eco-friendliness of the product. Chl degrades into natural products, which do not tend to be accumulated in the soil [45]. This study, including *in vitro* and *in planta* results give a promising outlook to future field applications of Ch-based PDI. The spectrum of SUN-D-06, given in Fig. 2, indicates by the absorption peaks in the visible light range that an excitation via sunlight is a possible option for a cost-effective use of the tested-PS formulations. Larger field trials on different model plants, such as grapes, and against different phytopathogens are however needed for a better insight into Chl as a PS in organic agriculture.

To conclude, PDI using a natural, water-soluble Chl-based PS provides a reliable antifungal effect in vitro against *B. cinerea* irrespective of resistance to conventional antifungals. Chl-based PS are effective for photoinactivation of fungal spheres. In addition, a good control of gray mold of greenhouse peppers was granted using the same PS, comparable to the standard RHAPSODY ASO, with no visible crop injury. The efficacy of the product was enhanced by the addition of the surfactant BRIJ L4 at 0.05%. Our findings strengthen the idea of using tailored formulations of Chl as photofungicides, allowing for economic and eco-friendly disease control.

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Declarations

Conflict of interest The authors declare no competing interests.

References

1. Fisher, M. C., Gurr, S. J., Cuomo, C. A., Blehert, D. S., Jin, H., Stukenbrock, E. H., Stajich, J. E., Kahmann, R., Boone, C., Denning, D. W., Gow, N. A. R., Klein, B. S., Kronstad, J. W., Sheppard, D. C., Taylor, J. W., Wright, G. D., Heitman, J., Casadevall, A., & Cowen, L. E. (2020). Threats posed by the fungal kingdom to humans, wildlife, and agriculture. *MBio*. https://doi.org/10.1128/mbio.00449-20
2. Flood, J. (2010). The importance of plant health to food security. *Food Security, 2*, 215–231. https://doi.org/10.1007/s12571-010-0072-5
3. Chaloner, T. M., Gurr, S. J., & Bebber, D. P. (2021). Plant pathogen infection risk tracks global crop yields under climate change. *Nat Clim Change, 11*, 710–715. https://doi.org/10.1038/s41558-021-01104-8
4. Singh, B. K., Delgado-Baquerizo, M., Egidi, E., Guirado, E., Leach, J. E., Liu, H., & Trivedi, P. (2023). Climate change

impacts on plant pathogens, food security and paths forward. *Nature Reviews Microbiology*. https://doi.org/10.1038/s41579-023-00900-7
5. Doehlemann, G., Ökmen, B., Zhu, W., & Sharon, A. (2017). Plant pathogenic fungi. *Microbiology Spectrum, 5*, 1. https://doi.org/10.1128/microbiolspec.funk-0023-2016
6. Dean, R., Van Kan, J. A. L., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J. J., Dickman, M., Kahmann, R., Ellis, J., & Foster, G. D. (2012). The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology, 13*, 414–430. https://doi.org/10.1111/j.1364-3703.2011.00783.x
7. Bi, K., Liang, Y., Mengiste, T., & Sharon, A. (2023). Killing softly: A roadmap of Botrytis cinerea pathogenicity. *Trends in Plant Science, 28*, 211–222. https://doi.org/10.1016/j.tplants.2022.08.024
8. Williamson, B., Tudzynski, B., Tudzynski, P., & Van Kan, J. A. L. (2007). *Botrytis cinerea*: The cause of grey mould disease. *Molecular Plant Pathology, 8*, 561–580. https://doi.org/10.1111/j.1364-3703.2007.00417.x
9. Roca-Couso, R., Flores-Félix, J. D., & Rivas, R. (2021). Mechanisms of action of microbial biocontrol agents against *Botrytis cinerea*. *Journal of Fungi, 7*, 1045. https://doi.org/10.3390/jof7121045
10. Hua, L., Yong, C., Zhanquan, Z., Boqiang, L., Guozheng, Q., & Shiping, T. (2018). Pathogenic mechanisms and control strategies of *Botrytis cinerea* causing post-harvest decay in fruits and vegetables. *Food Quality and Safety, 2*, 111–119. https://doi.org/10.1093/fqsafe/fyy016
11. Leroux, P., Gredt, M., Leroch, M., & Walker, A.-S. (2010). Exploring mechanisms of resistance to respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold. *Applied and Environment Microbiology, 76*, 6615–6630. https://doi.org/10.1128/AEM.00931-10
12. Kretschmer, M., Leroch, M., Mosbach, A., Walker, A.-S., Fillinger, S., Mernke, D., Schoonbeek, H.-J., Pradier, J.-M., Leroux, P., Waard, M. A. D., & Hahn, M. (2009). Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea*. *PLoS Pathogens, 5*, e1000696. https://doi.org/10.1371/journal.ppat.1000696
13. Walker, A.-S., Micoud, A., Rémuson, F., Grosman, J., Gredt, M., & Leroux, P. (2013). French vineyards provide information that opens ways for effective resistance management of *Botrytis cinerea* (grey mould). *Pest Management Science, 69*, 667–678. https://doi.org/10.1002/ps.3506
14. Marín-Guirao, J. I., Páez-Cano, F. C., García-García, M. D. C., Katsoulas, N., & de Cara-García, M. (2023). Evaluation of copper-free alternatives to control grey mould in organic Mediterranean greenhouse tomato production. *Agronomy, 13*, 137. https://doi.org/10.3390/agronomy13010137
15. Fagnano, M., Agrelli, D., Pascale, A., Adamo, P., Fiorentino, N., Rocco, C., Pepe, O., & Ventorino, V. (2020). Copper accumulation in agricultural soils: Risks for the food chain and soil microbial populations. *Science of the Total Environment, 734*, 139434. https://doi.org/10.1016/j.scitotenv.2020.139434
16. Čuš, F., Česnik, H. B., & Bolta, ŠV. (2022). Pesticide residues, copper and biogenic amines in conventional and organic wines. *Food Control, 132*, 108534. https://doi.org/10.1016/j.foodcont.2021.108534
17. Hamminger, C., Glueck, M., Fefer, M., Ckurshumova, W., Liu, J., Tenhaken, R., & Plaetzer, K. (2022). Photodynamic inactivation of plant pathogens part II: Fungi. *Photochemical & Photobiological Sciences, 21*, 195–207. https://doi.org/10.1007/s43630-021-00157-0
18. Wainwright, M., Maisch, T., Nonell, S., Plaetzer, K., Almeida, A., Tegos, G. P., & Hamblin, M. R. (2017). Photoantimicrobials—Are we afraid of the light? *The Lancet Infectious Diseases, 17*, e49–e55. https://doi.org/10.1016/S1473-3099(16)30268-7
19. Glueck, M., Hamminger, C., Fefer, M., Liu, J., & Plaetzer, K. (2019). Save the crop: Photodynamic Inactivation of plant pathogens I: Bacteria. *Photochemical & Photobiological Sciences, 18*, 1700–1708. https://doi.org/10.1039/C9PP00128J
20. Wimmer, A., Glueck, M., Ckurshumova, W., Liu, J., Fefer, M., & Plaetzer, K. (2022). Breaking the rebellion: Photodynamic inactivation against *Erwinia amylovor*a resistant to streptomycin. *Antibiotics, 11*, 544. https://doi.org/10.3390/antibiotics11050544
21. Leibniz Institute. (2023). German collection of microorganisms and cell cultures GmbH. In: *Botrytis cinerea* DSM 877. Retrieved September 8, 2023 from https://www.dsmz.de/collection/catalogue/details/culture/DSM-877
22. Walker, A.-S., Gladieux, P., Decognet, V., Fermaud, M., Confais, J., Roudet, J., Bardin, M., Bout, A., Nicot, P. C., Poncet, C., & Fournier, E. (2015). Population structure and temporal maintenance of the multihost fungal pathogen *Botrytis cinerea*: causes and implications for disease management. *Environmental Microbiology, 17*, 1261–1274. https://doi.org/10.1111/1462-2920.12563
23. Baales, J., Zeisler-Diehl, V. V., Narine, S., & Schreiber, L. (2023). Interaction of surfactants with *Prunus laurocerasus* leaf surfaces: Time-dependent recovery of wetting contact angles depends on physico-chemical properties of surfactants. *Chemical and Biological Technologies in Agriculture, 10*, 81. https://doi.org/10.1186/s40538-023-00455-y
24. Horsfall, J. G., & Barratt, R. W. (1945). An improved grading system for measuring plant diseases. *Phytopathology®, 35*, 655.
25. The American Phytopathological Society (APS). (2023). Disease progress over time. In: Calc. Area Dis. Prog. Curve Quantify Dis. Prog. Retrieved 21 November, 2023 from https://www.apsnet.org/edcenter/disimpactmngmnt/topc/EcologyAndEpidemiologyInR/DiseaseProgress/Pages/AUDPC.aspx
26. AbuQamar, S., Moustafa, K., & Tran, L. S. (2017). Mechanisms and strategies of plant defense against *Botrytis cinerea*. *Critical Reviews in Biotechnology, 37*, 262–274. https://doi.org/10.1080/07388551.2016.1271767
27. Abbey, J. A., Percival, D., Abbey, L., Asiedu, S. K., Prithiviraj, B., & Schilder, A. (2019). Biofungicides as alternative to synthetic fungicide control of grey mould (*Botrytis cinerea*)—Prospects and challenges. *Biocontrol Science and Technology, 29*, 207–228. https://doi.org/10.1080/09583157.2018.1548574
28. Huang, L., Xuan, Y., Koide, Y., Zhiyentayev, T., Tanaka, M., & Hamblin, M. R. (2012). Type I and type II mechanisms of antimicrobial photodynamic therapy: An in vitro study on gram-negative and gram-positive bacteria. *Lasers in Surgery and Medicine, 44*, 490–499. https://doi.org/10.1002/lsm.22045
29. Uliana, M. P., Pires, L., Pratavieira, S., Brocksom, T. J., de Oliveira, K. T., Bagnato, V. S., & Kurachi, C. (2014). Photobiological characteristics of chlorophyll a derivatives as microbial PDT agents. *Photochemical & Photobiological Sciences, 13*, 1137–1145. https://doi.org/10.1039/c3pp50376c
30. Abrahamse, H., & Hamblin, M. R. (2016). New photosensitizers for photodynamic therapy. *The Biochemical Journal, 473*, 347–364. https://doi.org/10.1042/BJ20150942
31. Gow, N. A. R., Latge, J.-P., & Munro, C. A. (2017). The fungal cell wall: Structure, biosynthesis, and function. *Microbiol Spectr, 5*, 1. https://doi.org/10.1128/microbiolspec.funk-0035-2016
32. Ambrosini, V., Issawi, M., Sol, V., & Riou, C. (2020). Photodynamic inactivation of *Botrytis cinerea* by an anionic porphyrin: An alternative pest management of grapevine. *Science and Reports, 10*, 17438. https://doi.org/10.1038/s41598-020-74427-9
33. Seididamyeh, M., Netzel, M. E., Mereddy, R., Harmer, J. R., & Sultanbawa, Y. (2023). Photodynamic inactivation of *Botrytis cinerea* spores by curcumin—Effect of treatment factors and characterization of photo-generated reactive oxygen

species. *Food and Bioprocess Technology*. https://doi.org/10.1007/s11947-023-03150-w
34. Fellner, A., Hamminger, C., Fefer, M., Liu, J., & Plaetzer, K. (2023). Towards microbial food safety of sprouts: photodynamic decontamination of seeds. *Photonics, 10*, 239. https://doi.org/10.3390/photonics10030239
35. Paskeviciute, E., Zudyte, B., & Luksiene, Z. (2018). Towards better microbial safety of fresh produce: Chlorophyllin-based photosensitization for microbial control of foodborne pathogens on cherry tomatoes. *Journal of Photochemistry and Photobiology B: Biology, 182*, 130–136. https://doi.org/10.1016/j.jphotobiol.2018.04.009
36. Luksiene, Z., Buchovec, I., & Paskeviciute, E. (2010). Inactivation of Bacillus cereus by Na–chlorophyllin-based photosensitization on the surface of packaging. *Journal of Applied Microbiology, 109*, 1540–1548. https://doi.org/10.1111/j.1365-2672.2010.04780.x
37. Luksiene, Z., Buchovec, I., & Paskeviciute, E. (2010). Inactivation of several strains of *Listeria monocytogenes* attached to the surface of packaging material by Na–chlorophyllin-based photosensitization. *Journal of Photochemistry and Photobiology B: Biology, 101*, 326–331. https://doi.org/10.1016/j.jphotobiol.2010.08.002
38. Yan, Y., Tan, L., Li, H., Chen, B., Huang, J., Zhao, Y., Wang, J., & Ou, J. (2021). Photodynamic inactivation of planktonic Staphylococcus aureus by sodium magnesium chlorophyllin and its effect on the storage quality of lettuce. *Photochemical & Photobiological Sciences, 20*, 761–771. https://doi.org/10.1007/s43630-021-00057-3
39. Buchovec, I., Lukseviciute, V., Marsalka, A., Reklaitis, I., & Luksiene, Z. (2016). Effective photosensitization-based inactivation of Gram (−) food pathogens and molds using the chlorophyllin—chitosan complex: Towards photoactive edible coatings to preserve strawberries. *Photochemical & Photobiological Sciences, 15*, 506–516. https://doi.org/10.1039/c5pp00376h
40. Jiang, C., Scholle, F., Jin, F., Wei, Q., Wang, Q., & Ghiladi, R. A. (2024). Chlorophyllin as a photosensitizer in photodynamic antimicrobial materials. *Cellulose, 31*, 2475–2491. https://doi.org/10.1007/s10570-024-05758-3
41. Li, X., Sheng, L., Sbodio, A. O., Zhang, Z., Sun, G., Blanco-Ulate, B., & Wang, L. (2022). Photodynamic control of fungicide-resistant *Penicillium digitatum* by vitamin K3 water-soluble analogue. *Food Control, 135*, 108807. https://doi.org/10.1016/j.foodcont.2021.108807
42. Islam, M. T., Ng, K., Fefer, M., Liu, J., Uddin, W., Ckurshumova, W., & Rosa, C. (2023). Photosensitizer to the rescue. In planta and field application of photodynamic inactivation against plant-pathogenic bacteria. *Plant Disease, 107*, 870–878. https://doi.org/10.1094/PDIS-05-22-1152-RE
43. Elmhirst, J. F., Haselhan, C., & Punja, Z. K. (2011). Evaluation of biological control agents for control of botrytis blight of geranium and powdery mildew of rose. *Canadian Journal of Plant Pathology, 33*, 499–505. https://doi.org/10.1080/07060661.2011.630758
44. Islam, M. T., Sain, M., Stark, C., Fefer, M., Liu, J., Hoare, T., Ckurshumova, W., & Rosa, C. (2023). Overview of methods and considerations for the photodynamic inactivation of microorganisms for agricultural applications. *Photochemical & Photobiological Sciences*. https://doi.org/10.1007/s43630-023-00466-6
45. Heaton, J. W., & Marangoni, A. G. (1996). Chlorophyll degradation in processed foods and senescent plant tissues. *Trends in Food Science & Technology, 7*, 8–15. https://doi.org/10.1016/0924-2244(96)81352-5