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Braga, GÚL, Silva-Junior, GJ, Brancini, GTP, Hallsworth, JE and Wainwright, M (2022) Photoantimicrobials in agriculture. Journal of photochemistry and photobiology. B, Biology, 235. p. 112548. ISSN 1011-1344

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1 **Photoantimicrobials in agriculture**

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20

21

22 **ABSTRACT**

23 Classical approaches for controlling plant pathogens may be impaired by the development of
24 pathogen resistance to chemical pesticides and by limited availability of effective antimicrobial
25 agents. Recent increases in consumer awareness of and/or legislation regarding environmental
26 and human health, and the urgent need to improve food security, are driving increased demand
27 for safer antimicrobial strategies. Therefore, there is a need for a step change in the approaches
28 used for controlling pre- and post-harvest diseases and foodborne human pathogens. The use of
29 light-activated antimicrobial substances for the so-called antimicrobial photodynamic treatment is
30 known to be effective not only in a clinical context, but also for use in agriculture to control plant-
31 pathogenic fungi and bacteria, and to eliminate foodborne human pathogens from seeds,
32 sprouted seeds, fruits, and vegetables. Here, we take a holistic approach to review and re-
33 evaluate recent findings on: (i) the ecology of naturally-occurring photoantimicrobials, (ii)
34 photodynamic processes including the light-activated antimicrobial activities of some plant
35 metabolites, and (iii) fungus-induced photosensitization of plants. The inhibitory mechanisms of
36 both natural and synthetic light-activated substances, known as photosensitizers, are discussed
37 in the contexts of microbial stress biology and agricultural biotechnology. Their modes-of-
38 antimicrobial action make them neither stressors nor toxins/toxicants (with specific modes of
39 poisonous activity), but a hybrid/combination of both. We highlight the use of photoantimicrobials
40 for the control of plant-pathogenic fungi and quantify their potential contribution to global food
41 security.

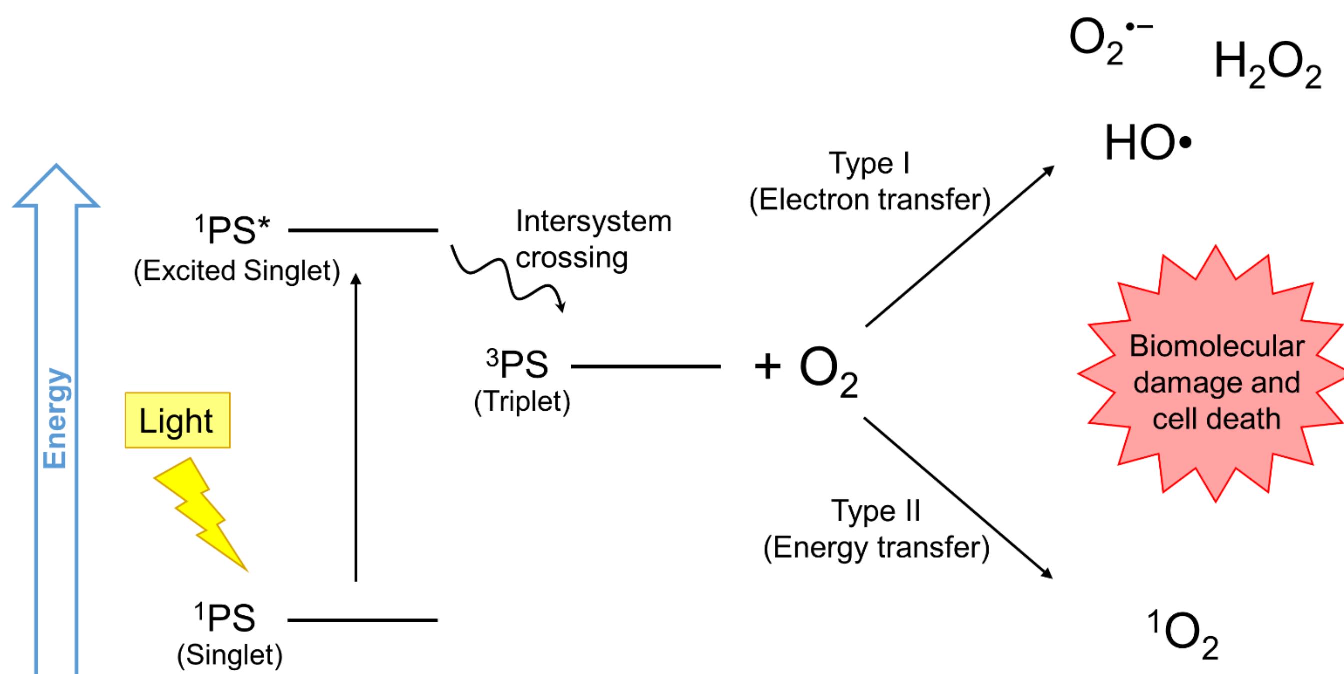
42 *Keywords*: antimicrobial photodynamic treatment (APDT); cellular toxicity versus stress;
43 foodborne pathogens; global food security; photoantimicrobials; plant-pathogenic fungi

44

45 **1. Introduction**
46

47 The progressive increase in the numbers of fungi and bacteria that are tolerant to currently
48 used antimicrobials is a major threat to human health (Fisher *et al.*, 2018; Revie *et al.*, 2018;
49 Sabino *et al.*, 2020). Indeed, the intensive antimicrobial use raises concerns regarding both
50 human and environmental health. Furthermore, there is an acute need to improve food security
51 on a global scale (Kettles and Luna, 2019). Against this backdrop, it is imperative to develop new
52 and effective strategies for the control of plant-pathogenic microorganisms. Antimicrobial
53 photodynamic treatment (APDT) is a promising alternative to conventional antifungal and
54 antibiotic agents which can be used for the treatment of localized infections in animal and human
55 hosts or to kill plant- or human/animal pathogens in the environment (Calzavara-Pinton *et al.*,
56 2012; Dai *et al.*, 2011; de Menezes *et al.*, 2014b; de Menezes *et al.*, 2014a; de Menezes *et al.*,
57 2016; Gonzales *et al.*, 2017; Hamblin, 2016; Rodrigues *et al.*, 2012a; Rodrigues *et al.*, 2013;
58 Smijs and Pavel, 2011; Vera *et al.*, 2012; Wainwright *et al.*, 2017).

59 To achieve microbial killing, APDT uses three primary components, namely a
60 photosensitizer, light, and molecular oxygen. The accumulation of a photosensitizer in the cell
61 (either inside or at the surface) of the target microbe is followed by exposure to light that, at an
62 appropriate wavelength, excites the photosensitizer. This causes the production of reactive
63 oxygen species (ROS), such as singlet oxygen ($^1O_2$) and hydroxyl radicals (•OH), which cause
64 biomolecular damage to the cell, effectively killing it with little to no side effects on the host (Fig.
65 1) (Brancini *et al.*, 2016; Calzavara-Pinton *et al.*, 2012; de Menezes *et al.*, 2014a; de Menezes *et*
66 *al.*, 2014b; Gonzales *et al.*, 2010; Gonzales *et al.*, 2017; St. Denis *et al.*, 2011; Wainwright *et al.*,
67 2017).



68

69 **Figure 1 –** The principle of antimicrobial photodynamic treatment. A photosensitizer (PS), upon
70 exposure to light, is initially excited to a higher-energy electronic state. Then, via intersystem crossing
71 (transitioning between different electronic states), the excited PS transitions to a triplet state, which
72 reacts with molecular oxygen via either an electron transfer or energy transfer reaction; Type I or Type II
73 reactions, respectively. The former produces reactive oxygen species such as superoxide anion radical
74 ($O2^{\bullet-}$), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2); the latter generates singlet oxygen ($^1O_2$).
75 These reactive oxygen species cause biomacromolecular damage and pathogen cell death.

76

77 Several types of photosensitizers have been used successfully to perform APDT. These
78 include plant-produced, microbe-produced, and synthetic or semi-synthetic photoantimicrobials:
79 chlorins, curcumins, flavins, furocoumarins, hypericins, indocyanines, phenothiazines,
80 phthalocyanines, porphyrins, xanthenes, and others (Hamblin, 2016; Hasenleitner and Plaetzer,
81 2020; Temba *et al.*, 2016; Wainwright *et al.*, 2017). When reactive species such as $^1O_2$ are

produced at plant surfaces, either via natural plant-produced photosensitizer or via agriculturally applied photosensitizer, they damage fungal spores and mycelia, yeasts, bacteria, as well as the ovipositors of insects that are embedded in the plant tissue (Berenbaum and Larson, 1988; Flors and Nonell, 2006; Gonzales *et al.*, 2017).

The chronic and inevitable drawback of conventional (chemical) antifungals, which, like antibiotics, have target-specific modes-of-action, is the development of microbial resistance (Wainwright *et al.*, 2017). By contrast, there is little evidence of the development of resistance to biophysical stressors (e.g. chaotropic, hydrophobic, and oxidative stressors) that act as antimicrobials at multiple target sites and/or via sites-of-action within the cell (Ball and Hallsworth, 2015; Bhaganna *et al.*, 2010; Cray *et al.*, 2013b; Cray *et al.*, 2013a; Cray *et al.*, 2014; Cray *et al.*, 2015b; Hallsworth, Heim and Timmis, 2003). Furthermore, most of the photosensitizers used in APDT exhibit low mammalian toxicity and are environmentally-friendly relative to conventional pesticides (Andrade *et al.*, 2022; Hamblin, 2016; Wainwright *et al.*, 2017). The APDT has the additional advantage of, unlike most conventional fungicides and antibiotics, being able to kill both metabolically-active and -inactive cells, including bacterial and fungal spores (de Menezes *et al.*, 2014a; de Menezes *et al.*, 2014b; de Menezes *et al.*, 2016; Eichner *et al.*, 2015; Fracarolli *et al.*, 2016; Gomes *et al.*, 2011; Gonzales *et al.*, 2010; Gonzales *et al.*, 2017; Luksiene, Buchovec and Paskeviciute, 2009; Luksiene, Buchovec and Paskeviciute, 2010a; Rodrigues *et al.*, 2012a).

Additionally, APDT is not only able to control plant pathogens pre- and post-harvest (Ambrosini *et al.*, 2020; de Menezes *et al.*, 2014a; de Menezes *et al.*, 2014b; Fracarolli *et al.*, 2016; Gonzales *et al.*, 2017; Luksiene and Paskeviciute, 2011; Tang *et al.*, 2021) but can kill foodborne pathogens and inactivate microbial toxins (Huang *et al.*, 2021; Jančula *et al.*, 2010). This said, the identification of effective photosensitizers, and evaluation of potential side-effects on plant- and environmental health, are imperative to the further development of APDT for use in agriculture (de Menezes *et al.*, 2014a; de Menezes *et al.*, 2014b; Luksiene and Paskeviciute, 2011; Tang *et al.*, 2021; Vol'pin *et al.*, 2000).

108 Here, we take a compound-oriented approach, but one based on diverse lines of evidence.
109 We evaluate the natural ecology of photosensitizer-driven antimicrobial processes in plants,
110 including the importance of photosensitizers for phytopathogens and for plant defenses.
111 Additionally, we examine the use of photoantimicrobials in an agricultural context to determine
112 the potential to improve global food security. We discuss inhibitory mechanisms of
113 photosensitizers, in relation to microbial stress biology and agricultural biotechnology, with
114 emphasis on their use for the control of plant-pathogenic fungi, preventing spoilage of foods and
115 feeds, and for controlling mycotoxin-producing fungi and foodborne pathogens, and global food
116 security.

117

118 **2. Photodynamic inactivation of plant-pathogens**

119 As opposed to topical applications in a clinical setting, the use of APDT to control
120 agricultural plant-pathogens would require bulk application of photosensitizers over considerable
121 areas of land and at reasonable prices, and environmental safety is paramount. Some
122 photosensitizers can be obtained directly from plants, algae, and cyanobacteria or from by-
123 products of processing of fruits such as Tahiti acid lime (*Citrus aurantifolia*) and grapefruit (*Citrus*
124 *× paradisi*) (Asthana *et al.*, 1993; de Menezes *et al.*, 2014a; Fracarolli *et al.*, 2016; Hudson and
125 Towers, 1991; Temba *et al.*, 2016). Use of APDT in the field can take advantage of solar radiation,
126 so does not need artificial light for photosensitizer activation. The high irradiances and broad
127 emission spectrum of solar radiation can activate diverse types of photosensitizers, whether they
128 are excited by visible light or by UV radiation (de Menezes *et al.*, 2014a; Hudson and Towers,
129 1991). Unlike controlled lighting provided by lasers, LEDs, or other artificial sources, incident solar
130 radiation in the field fluctuates. This is due to factors such as climate and weather, time of year,
131 distance from the equator, altitude, atmospheric humidity, dust, and pollution. Furthermore, the
132 periods of illumination in the field are lengthy and the light cycles follow a diurnal pattern (Braga
133 *et al.*, 2015).

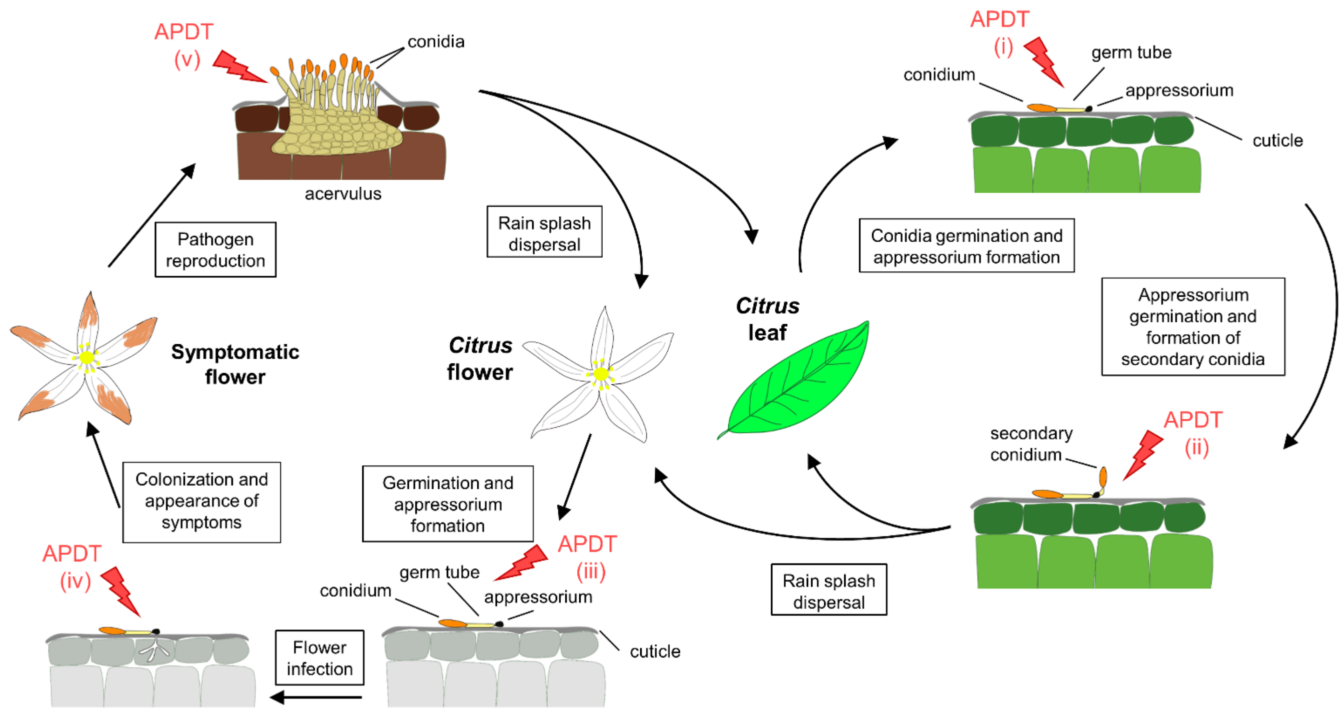
134 In clinical settings, APDT can be designed to target the pathogen rather than the host by
135 applying the photosensitizer topically on a localized area of infection and by restricting delivery of
136 light to that area of infection (Hamblin, 2016; Wainwright *et al.*, 2017). On agricultural crops,
137 however, such a protocol would not be feasible as the photosensitizer is applied indiscriminately
138 on pathogen and plant, which are both exposed to solar radiation. Preventing damage to the crop
139 plant, therefore, must be achieved by other means. Nonetheless, fruits and grains can be readily
140 processed post-harvest, using APDT, to reduce the populations of spoilage microbes and
141 foodborne pathogens under controlled conditions and using artificial light sources (Buchovec *et*
142 *al.*, 2016; Luksiene and Paskeviciute, 2011).
143
144 **2.1 Photodynamic inactivation of plant-pathogenic fungi**
145 Widespread application of synthetic fungicides which have modes-of-action based on site-
146 specific targets within the pathogen cell has been the treatment-of-choice for pre- and post-
147 harvest control of most plant-pathogenic fungi (Ishii and Holloman, 2015; Kretschmer *et al.*, 2009;
148 Oliver and Hewitt, 2014). However, fungicide resistance has been reported for decades in
149 commercially important pathogens of agricultural crops, including *Alternaria*, *Aspergillus*,
150 *Colletotrichum*, *Erysiphe*, *Fusarium*, *Mycosphaerella*, *Plasmopara,* and *Pythium* (Andrivon *et al.*,
151 1997; Bartlett *et al.*, 2002; Chitolina *et al.*, 2021; Deising, Reimann and Pascholati, 2008; Ishii and
152 Holloman, 2015; Jensen *et al.*, 2016; Peres *et al.*, 2005; Ribas e Ribas *et al.*, 2016; Wong and
153 Midland, 2007; Wong *et al.*, 2008). Current concerns about environmental and human health have
154 given rise to recent legislation restricting the use of many of the more dangerous agrochemicals
155 in some regions of the world. Combined with microbial resistance, this has been accompanied by
156 decreasing numbers of commercial fungicides that are approved for agricultural use. For instance,
157 top agricultural producing countries around the world have banned the use of or limited the access
158 to a series of harmful pesticides (Ding *et al.*, 2019; Donley, 2019; Gunnell *et al.*, 2017). This
159 included restrictions on the concentrations and overall quantity of approved pesticides that can

160 be applied (Jess *et al.*, 2014). The need for novel and/or integrated strategies to control fungi both
161 pre- and post-harvest is now, therefore, more urgent than ever.

162 Taxonomically diverse fungi have been effectively killed by APDT, including human
163 pathogens of the genera *Candida* (Dai *et al.*, 2011; Gonzales and Maisch, 2012; Rodrigues *et al.*,
164 2013; Rodrigues *et al.*, 2020a; Rodrigues *et al.*, 2020b) and *Trichophyton* (Rodrigues *et al.*,
165 2012a; Romagnoli *et al.*, 1998; Smijs *et al.*, 2014; Trigos and Ortega-Regules, 2002);
166 entomopathogens used for biological control of insects, such as *Beauveria* (Martin, Mischke and
167 Schroder, 1998) and *Metarhizium* (Gonzales *et al.*, 2010); saprophytic fungi that also act as
168 opportunistic pathogens of humans, such as *Acremonium* (Lukšiene *et al.*, 2005), *Aspergillus*
169 (DiCosmo, Towers and Lam, 1982; Friedberg *et al.*, 2001; Gilaberte *et al.*, 2011; Gonzales *et al.*,
170 2010; Temba *et al.*, 2016), *Cryptococcus* (Bourque *et al.*, 1985; Rodrigues *et al.*, 2012b),
171 *Emericella* (Trigos and Ortega-Regules, 2002), *Exophiala* (Gao *et al.*, 2016), *Neurospora* (Blanc,
172 Tuveson and Sargent, 1976; Shimizu, Egashira and Takahama, 1979), *Penicillium* (Asthana *et*
173 *al.*, 1993; Gomes *et al.*, 2011), and *Rhizopus* (Liu *et al.*, 2019; Luksiene, Peciulyte and Lugauskas,
174 2004); endophytes, such as *Papulaspora* (Trigos and Ortega-Regules, 2002); and plant-
175 pathogens, such as *Alternaria*, *Cladosporium* (DiCosmo, Towers and Lam, 1982; Luksiene,
176 Peciulyte and Lugauskas, 2004; Lukšiene *et al.*, 2005; Tegegne, Pretorius and Swart, 2008),
177 *Botrytis* (Ambrosini *et al.*, 2020; Hamminger *et al.*, 2022; Kairyte, Kadys and Luksiene, 2013;
178 Luksiene and Buchovec, 2019; Mares *et al.*, 2004; Tang *et al.*, 2021; Tegegne, Pretorius and
179 Swart, 2008), *Botryosphaeria*, *Mycosphaerella*, *Rhizoctonia*, and *Sclerotium* (Tang *et al.*, 2021;
180 Tegegne, Pretorius and Swart, 2008), *Colletotrichum* (de Menezes *et al.*, 2014a; de Menezes *et*
181 *al.*, 2014b; DiCosmo, Towers and Lam, 1982; Fracarolli *et al.*, 2016; Gonzales *et al.*, 2017),
182 *Fusarium* (Asthana *et al.*, 1993; Bourque *et al.*, 1985; de Menezes *et al.*, 2016; Gao *et al.*, 2016;
183 Kashiwabuchi *et al.*, 2013; Lazzaro *et al.*, 2004; Lukseviciute and Luksiene, 2020; Luksiene,
184 Peciulyte and Lugauskas, 2004; Lukšiene *et al.*, 2005; Mares *et al.*, 2002; Mares *et al.*, 2004;
185 Tegegne, Pretorius and Swart, 2008; Vorobey and Pinchuk, 2008), *Magnaporthe* (Vol'pin *et al.*,

186 2000), *Trichothecium* (Luksiene, Peciulyte and Lugauskas, 2004), as well as the oomycetes
187 *Pythium* and *Saprolegnia* (DiCosmo, Towers and Lam, 1982; Mares *et al.*, 2004; Tang *et al.*,
188 2021; Tegegne, Pretorius and Swart, 2008). The majority of studies for plant-pathogens have
189 been performed *in vitro*; only a handful of assays have been conducted on a plant host, few
190 experiments have emulated field conditions, and even fewer trials have assessed efficacy in the
191 field. The small number of field trials carried out to test APDT may be explained by the need for
192 wide-scale application of photosensitizers across large areas (where environmental safety is
193 paramount) as opposed to topical applications in a clinical setting.

194 As explained above, effective APDT of plant-pathogenic fungi relies on the presence of the
195 photosensitizer, simultaneous exposure to solar radiation, and the lifestyle of the fungal species.
196 Some pathogens develop distinct and specialized structures such as asexual spores (e.g.,
197 conidia), sexual spores (e.g., ascospores, basidiospores) and other structures (appressoria,
198 fruiting bodies, hyphae/mycelium, sclerotia, biofilms, etc). Invasion and colonization of plant tissue
199 is carried out by hyphae of pathogenic fungi, but spores are usually produced on host-plant
200 surface (Agrios, 2005; Lucas, Dyer and Murray, 2000; Mukherjee *et al.*, 2021; Peres *et al.*, 2005).
201 Thus, these spores are usually exposed to sunlight, so are a vulnerable structure, among others,
202 that can be targeted by APDT (Fig. 2) (de Menezes *et al.*, 2014a; de Menezes *et al.*, 2014b; de
203 Menezes *et al.*, 2016; Fracarolli *et al.*, 2016).



204

205 **Figure 2 –** Stages of the pathogen lifecycle at which antimicrobial photodynamic treatment (APDT; red
206 arrows) can prevent conidial germination and formation of appressorium (i), production of secondary
207 conidia (ii) penetration/infection of the host-plant (iii and iv), and pathogen reproduction/dispersal (v).
208 This schematic is based on *Citrus* infection by *Colletotrichum abscissum*.

209

210 Therefore, it is fortunate that studies into APDT of fungi have focused on conidia rather
211 than hyphae. The antifungal assays with conidial suspensions can be readily standardized, since
212 conidia are produced by most filamentous fungi, and the inoculum suspension is easy to prepare
213 (Arnason *et al.*, 1986; Aver'yanov *et al.*, 2011; Braga *et al.*, 2015; Clsi, 2017; de Menezes *et al.*,
214 2014a; de Menezes *et al.*, 2014b; DiCosmo, Towers and Lam, 1982; Gonzales *et al.*, 2010;
215 Kairyte, Kadys and Luksiene, 2013; Luksiene, Peciulyte and Lugauskas, 2004; Mares *et al.*, 2004;
216 Vorobey and Pinchuk, 2008). Conidia act as agents of dispersal for the majority of plant-
217 pathogens, and can exhibit robust stress biology upon germination (Araújo *et al.*, 2020;
218 Dijksterhuis *et al.*, 2018; Dijksterhuis, 2019; Stevenson *et al.*, 2017b; Stevenson *et al.*, 2017c). In
219 pathogenic species, conidia are also involved in recognition and infection of the host (Barros *et*
220 *al.*, 2010; Braga, Destéfano and Messias, 1999; Braga *et al.*, 2015; Nascimento *et al.*, 2010;

221 Peres *et al.*, 2005). The biophysical properties (electrostatic charge, hydrophobicity, etc) and
222 chemical composition of the conidia surface differ greatly from those of hyphae (or the vegetative
223 cells of yeast species) (Barros *et al.*, 2010; Gonzales *et al.*, 2010). Furthermore, conidial
224 properties can vary between developmental stages, nutritional history and physiological status,
225 fungal species and strain, and even within populations of the same strain (Rangel *et al.*, 2005;
226 Wyatt *et al.*, 2015a; Wyatt *et al.*, 2015b). Such factors, particularly surface structure/chemistry,
227 can influence or determine the outcomes of exposure to photosensitizers (de Menezes *et al.*,
228 2014b; Gonzales *et al.*, 2010; Rodrigues *et al.*, 2012a). Phototreatment of conidia-producing
229 lesions on plant surfaces can cause a marked reduction in the viability of the fungal propagules
230 present, thereby reducing disease transmission (Fig. 2) (Agrios, 2005; Timer and Zitko, 1991;
231 Zulfiqar, Brlansky and Timmer, 1996).

232 A genus that has been the subject of APDT studies is *Colletotrichum* (de Menezes *et al.*,
233 2014a; de Menezes *et al.*, 2014b; Fracarolli *et al.*, 2016; Gonzales *et al.*, 2017), an ascomycete
234 genus of common plant pathogens of both wild- and crop-plant species (Ciampi-Guillardi *et al.*,
235 2022; Gama *et al.*, 2022; Gonçalves *et al.*, 2021; Wharton and Diéguez-Uribeondo, 2004).
236 *Colletotrichum* species are potent pathogens, responsible for major economic losses, especially
237 on temperate, subtropical, and tropical fruits (Wharton and Diéguez-Uribeondo, 2004). During the
238 asexual stage, *Colletotrichum* species produce acervuli on plant surfaces, which release mucilage
239 containing vast numbers of unicellular conidia (Ben *et al.*, 2021; Dowling *et al.*, 2020; Zulfiqar,
240 Brlansky and Timmer, 1996). This mucilage is readily dissolved by water, so conidia spread via
241 rain-splash to other plants, albeit only short distances from the source (Fig. 2) (Madden, Yang
242 and Wilson, 1996; Ntahimpera, Madden and Wilson, 1997). Strategies to minimize *Colletotrichum*
243 epidemics are based on preventive conventional fungicide sprays during the blooming period,
244 particularly on rainy seasons (Gama *et al.*, 2020; Silva-Junior *et al.*, 2014). However, fungicide-
245 resistant *Colletotrichum* isolates have been reported (Deising, Reimann and Pascholati, 2008;

246 Dowling *et al.*, 2020; Forcelini *et al.*, 2016; Peres, Seijo and Turechek, 2010; Wong and Midland,
247 2007; Wong *et al.*, 2008).

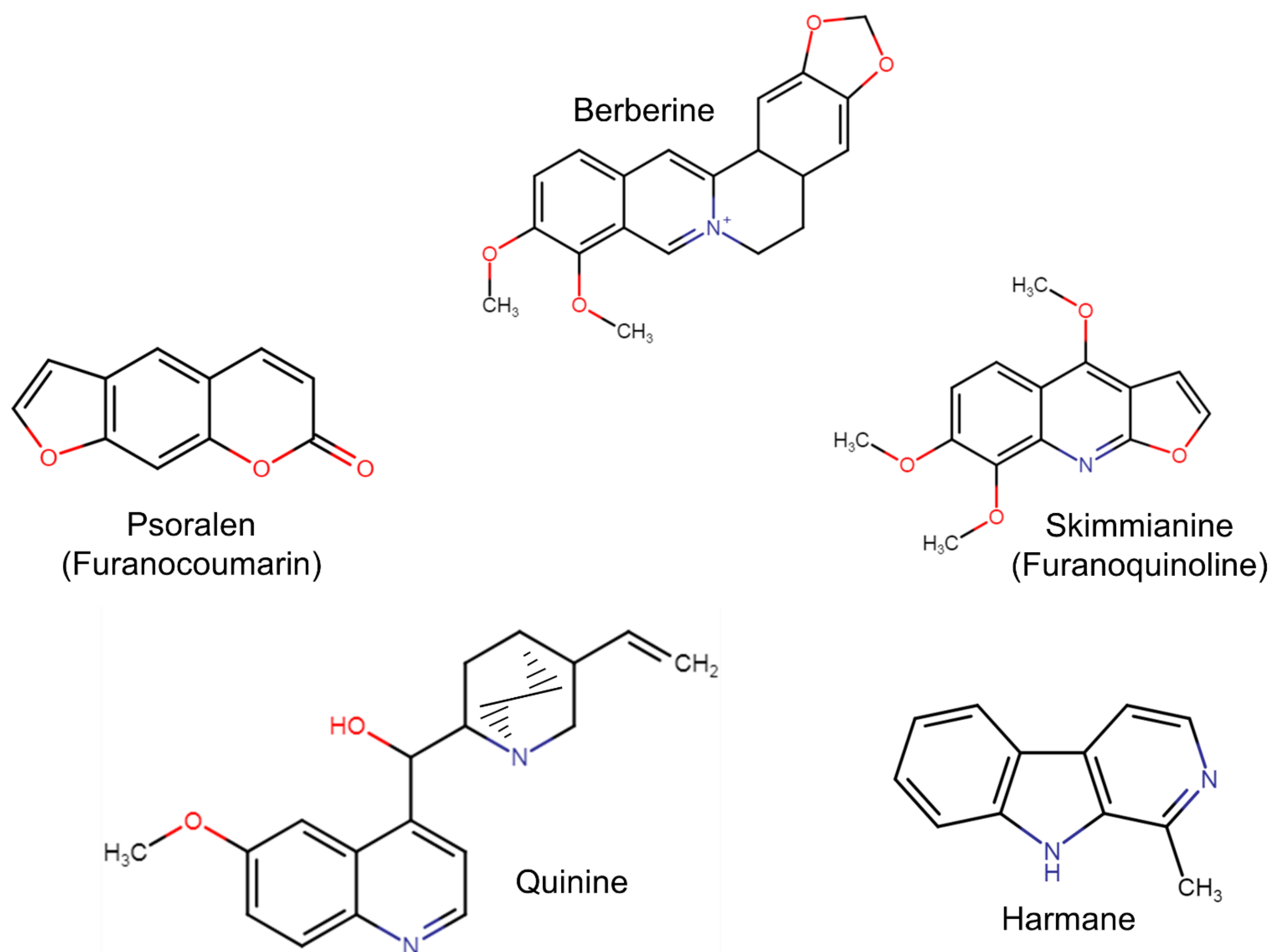
248 Chemically diverse photosensitizers have been used in APDT to kill conidia of plant-
249 pathogens *in vitro*, including: (i) plant metabolites, such as coumarins and furocoumarins (de
250 Menezes *et al.*, 2014a; Fracarolli *et al.*, 2016), curcumin (Al-Asmari, Mereddy and Sultanbawa,
251 2017; Temba *et al.*, 2016), phenylheptatriyne (Bourque *et al.*, 1985), phenylphenalenone
252 (Lazzaro *et al.*, 2004), polyacetylenes (Christensen and Brandt, 2006), and thiophenes (DiCosmo,
253 Towers and Lam, 1982); (ii) semi-synthetic compounds, such as chlorophyllins (Hamminger *et*
254 *al.*, 2022; Luksiene and Paskeviciute, 2011) and porphyrins (Tang *et al.*, 2021; Vandresen *et al.*,
255 2016; Vorobey and Pinchuk, 2008); phthalocyanine metal complexes (Vol'pin *et al.*, 2000); and
256 (iii) synthetic compounds, such as phenothiazinium dyes (e.g., methylene blue, new methylene
257 blue N, and toluidine blue O) (de Menezes *et al.*, 2014b; de Menezes *et al.*, 2016; Gao *et al.*,
258 2016; Gonzales *et al.*, 2017; Liu *et al.*, 2019; Paziani *et al.*, 2019; Tonani *et al.*, 2018) and
259 xanthenes [e.g., rose bengal (RB)] (Arboleda *et al.*, 2014). For each of these classes, we closely
260 examine photodynamic inactivation of plant-pathogenic fungi.

261

262 **2.1.1. Photodynamic inactivation of fungi by plant metabolites**

263 Plants employ various strategies to protect themselves against pathogens, including the
264 constitutive and inductive production of secondary metabolites. Some of these compounds exhibit
265 antimicrobial activities upon photoactivation (de Menezes *et al.*, 2014a; Fracarolli *et al.*, 2016;
266 Hudson and Towers, 1991; Larson and Berenbaum, 1988). Some plants, even those not generally
267 considered to be phototoxic, can rapidly synthesize photosensitizers upon infection by a pathogen
268 (Flors and Nonell, 2006; Kourany, Arnason and Schneider, 1988). Photosensitizers of plant origin
269 include alkaloids with a structure that can be based on tryptamine (e.g., hermane), phenylalanine
270 and tyrosine (e.g., berberine, sanguinarine) or anthranilic acid (e.g., skimmianine and other

271 furanoquinolines); cinnamate derivatives (e.g., coumarins and furocoumarins); polyketides (e.g.,
272 polyenes, thiophenes, quinines, and chromenes); and porphyrins that are precursors and
273 degradation products of chlorophylls (Fig. 3) (de Menezes *et al.*, 2014a; Flors and Nonell, 2006;
274 Fracarolli *et al.*, 2016; Hudson and Towers, 1991).



275

276 **Figure 3 –** The chemical structures of common plant-produced photosensitizers. Chemical classes are
277 shown in brackets. Structures were drawn with Marvin JS (ChemAxon).

278 Coumarins and furocoumarins (e.g., psoralens, angelicins) are found in the oil ducts and
279 cuticles of species within the Apiaceae (e.g., carrots), Fabaceae (e.g., beans and lentils),
280 Moraceae (e.g., figs), Rutaceae (e.g., *Citrus* species), among others (Asthana *et al.*, 1993; de
281 Menezes *et al.*, 2014a; Hudson and Towers, 1991; Manderfeld *et al.*, 1997; Nigg *et al.*, 1993).
282 They exhibit antimicrobial or insecticidal activities, via either light-independent or light-dependent

283 mechanisms (Bintsis, Litopoulou-Tzanetaki and Robinson, 2000; Bogucka-Kocka and Krzaczek,
284 2003). Both coumarins and furocoumarins are typically synthesized continuously (albeit at low
285 levels), so are constitutive. However, their synthesis is upregulated when plants experience
286 bacterial and fungal infection or abiotic stresses (Asthana *et al.*, 1993; Desjardins, Spencer and
287 Plattner, 1989; Manderfeld *et al.*, 1997). In general, the highest concentrations of furocoumarins
288 within the leaf occur at the surface, in the epidermal layer (Zobel and Brown, 1989) and in oil
289 glands within the peel of *Citrus* fruits (Fisher and Trama, 1979).

290 Treatment of cells using psoralens and UV-A radiation induces pyrimidine monoadducts
291 and interstrand crosslinks within DNA (Bordin *et al.*, 1976; Dardalhon *et al.*, 2007; Grant, Von
292 Borstel and Ashwood-Smith, 1979). The phototoxicity of psoralens was initially thought to be a
293 consequence of light-activated conjugation with DNA. However, Joshi and Pathak (1983)
294 revealed that, whether linear or angular in their molecular configuration, furocoumarins can trigger
295 production of reactive oxygen species upon exposure to light (Joshi and Pathak, 1983). It is likely
296 that both $^1O_2$ and superoxide radicals contribute to the phototoxicity of these compounds,
297 especially via their adverse effects on the plasma membrane (Joshi and Pathak, 1983; Llano,
298 Raber and Eriksson, 2003). The photoactivation of furocoumarins, and associated damage to
299 membrane systems have been reviewed previously (Dall'Acqua and Martelli, 1991). It is widely
300 recognized that the damage by furocoumarins occurs via multiple mechanisms (Llano, Raber and
301 Eriksson, 2003; Potapenko, 1991; Sumorek-Wiadro *et al.*, 2020). However, in the context of
302 fungal photosensitization, the relative importance of each of these processes has yet to be
303 determined.

304 Carotenoids and other (blue-green) pigments of *Fusarium oxysporum* and *Fusarium solani*
305 which cause root-rot and wilt in *Citrus* trees, respectively,) and *Penicillium digitatum* and
306 *Penicillium italicum* (which cause fruit rot, as agents of green mold and blue mold, respectively)
307 were evaluated as protectants against APDT using the plant-derived photosynthesizers 8-

308 methoxypsoralen (8-MOP) and α-terthienyl (α–T; a thiophene). For each of these fungal species,
309 mutants in conidial pigmentation and wild-type strains were treated with each photosensitizer (at
310 10 μg mL^{-1}) and exposed to UV radiation (broad-spectrum source; emission from 300 nm to 425
311 nm; irradiance of 40-43 W m^{-2}). Phototreatment of conidia using α–T was effective, killing most of
312 them, regardless of fungal species. Mutants of *F. oxysporum* and *F. solani* that cannot accumulate
313 carotenoids in their conidia were highly vulnerable to APDT. Likewise, conidial-pigment mutants
314 of *P. digitatum* and *P. italicum* were more sensitive than the wild-type to APDT with α–T.
315 Comparisons of *Fusarium* wild-type conidia and the carotenoid-deficient mutants showed that
316 carotenoids are less effective at protecting against APDT with 8-MOP than APDT with α–T. A
317 different result was observed in the study of *Penicillium*. The heavily pigmented blue-and-green
318 wild-type conidia of *P. digitatum* and *P. italicum*, and a rust-colored mutant of *P. digitatum* were
319 more tolerant to APDT with 8-MOP than their (white) mutant counterparts (Asthana and Tuveson,
320 1992). The authors hypothesized that carotenoids in wild-type *Fusarium* conidia protect against
321 damage by UV-A-activated α–T by quenching $^1O_2$, while the blue-green pigment(s) of wild-type
322 *P. italicum* conidia (located in the cell wall) prevent DNA damage caused by 8-MOP by filtering
323 out UV wavelengths that would otherwise activate the photosenstitizer.

324 In a similar study, *Citrus jambhiri* leaf extracts, and the pure furocoumarins bergapten (5-
325 methoxypsoralen; 5-MOP) and psoralen, were evaluated for phototoxicity against wild-type
326 conidia of *F. oxysporum*, *F. solani*, *P. digitatum*, and *P. italicum* and their color-mutant strains
327 (Asthana *et al*., 1993). The wild-type strains of both of these *Penicillium* species were less
328 vulnerable than their mutant strains to APDT using furanocoumarins plus UV-A radiation. A 5-
329 log_{10} reduction in conidia of *F. oxysporum* viability was observed both in the wild-type strain and
330 the pigmentation-mutant strains. However, wild-type conidia of *F. solani* were at least two orders
331 of magnitude less susceptible than the white mutant conidia. Additionally, Asthana *et al.* (1993)
332 compared ADPT treatment with bergapten of wild-type strains and mutant strains of each

333 *Penicillium* species, and observed different outcomes. In *P. italicum*, conidia of the wild-type and
334 the mutant with altered brown coloration survived with minimal inactivation, whereas mutant white
335 conidia were extremely susceptible. In *P. digitatum*, killing of wild-type and rust-mutant conidia
336 reached 5 log_{10}. For both *Penicillium* species, the white mutant was highly susceptible to
337 phototreatment (with survival decreasing by as much as six orders of magnitude) (Asthana *et al.*,
338 1993). Similar results were observed with psoralen activated by UV-A radiation (Asthana *et al.*,
339 1993). Phototreatment using bergapten was one order of magnitude less effective than treatment
340 using psoralen.

341 A study of APDT using either 8-MOP + isopimpinellin (both furocoumarins) or a mixture of
342 citropten + 7-methoxy coumarin (both coumarins) compared efficacies against conidia of
343 *Colletotrichum abscissum* (former *C. acutatum sensu lato*) and *Aspergillus nidulans* (de Menezes
344 *et al.*, 2014a). Isopimpinellin and the mixture of coumarins were obtained from liquid residues
345 after the industrial processing of *C. aurantifolia*. Upon treatment with the photosensitizers, conidia
346 were exposed to solar radiation. Phototreatment with 8-MOP (50 µM) reduced survival by
347 approximately 2 and 4 log_{10} for *C. acutatum* after 1 and 2 h of exposure, respectively; and by
348 approximately 4 log_{10} for *A. nidulans*, regardless of the duration of light exposure. Also, APDT
349 using the mixture of coumarins reduced survival by approximately 1 and 3 log_{10} for *C. acutatum*
350 after 1 and 2 h of light exposure, respectively. As observed for 8-MOP, phototreatment with the
351 coumarin mixture was more effective for *A. nidulans* conidia, for which the reduction in survival
352 was approximately 4 log_{10}, regardless of the duration of light exposure. For *C. acutatum* conidia,
353 isopimpinellin was the least effective treatment, reducing survival by less than 2 log_{10} after a 2-h
354 light exposure. Nonetheless, isopimpinellin was effective against *A. nidulans* conidia, reducing
355 survival by approximately 4 log_{10}. This study also reported that 8-MOP penetrates conidia and
356 accumulates within cytoplasmic vesicles (de Menezes *et al.*, 2014a). Furthermore, APDT using
357 crude extracts from *C. aurantilfolia*, red grapefruit, and white grapefruit at 12.5 mg L^{-1} were

358 performed and killed from 20% to 70% of the conidia. The *C. aurantilfolia* extract was the most
359 effective (Fracarolli *et al.*, 2016).

360 Given that APDT with furocoumarins and coumarins was effective against *C. abscissum*,
361 an imporant question is whether the host plant would tolerate such treatment. To address this
362 issue, the effects of phototreatment on the leaves of plant hosts *Citrus sinensis* (sweet orange),
363 *Citrus reticulate* × *C. sinensis* hybrid (Murcott tangerine), and *Fragaria ananassa* (strawberry)
364 were evaluated using furocoumarins and coumarins combined with solar radiation (de Menezes
365 *et al.*, 2014a; Fracarolli *et al.*, 2016). Phototreatment with 8-MOP, isopimpinellin, and coumarins
366 did not damage the leaves of *C. sinensis* or Murcott tangerine. However, successive daily
367 applications of phototreatment (for 2 weeks) using the individual furocoumarins and the coumarin
368 mixture caused considerable damage to the leaves of strawberry, with the death of epidermis-
369 and parenchyma cells and oxidation of leaf pigments (de Menezes *et al.*, 2014a). This result is
370 interesting because the photosensitizers used were isolated from *Citrus* spp., so these plants
371 might be expected to have some tolerance to the photosensitizers in order to avoid self-induced
372 damage. Conversely, strawaberry plants do not produce these photosensitizers, so self-induced
373 damage is not an issue and mechanisms to avoid it are not necessary. Nonetheless, these results
374 show that host damage can occur, depending on plant species, so that the safety of
375 phototreatment must be assessed on a case-by-case basis.

376 Polyacetylenes (polyenes) are a highly effective class of photosensitizers that occur in
377 flowers, leaves, stems, and roots of species in the plant families Apiaceae, Asteraceae, and
378 Campanulaceae (Binns *et al.*, 2000; Christensen and Brandt, 2006; Hudson and Towers, 1991;
379 Mares *et al.*, 2004). Several plant species are known to produce and accumulate acetylenes,
380 polyacetylenes, and thiophenes in response to infection by microbial pathogens (Arnason *et al.*,
381 1986; Bourque *et al.*, 1985; Kourany, Arnason and Schneider, 1988). They are synthesized in
382 plant cells via the desaturation and chain shortening of fatty acids. Derivatives of polyacetylenes
383 include the sulfur-containing thiophenes (Hudson and Towers, 1991). Many polyacetylenes

384 exhibit antifungal activity, and these are greatly enhanced by solar radiation or near-UV radiation
385 (Arnason *et al.*, 1986; Bourque *et al.*, 1985; DiCosmo, Towers and Lam, 1982; Mares *et al.*, 2002;
386 Mares *et al.*, 2004). For diverse biological systems, studies show that the phototoxicity of
387 polyacetylenes depends on oxygen availability (Gong *et al.*, 1988). Acetylenes, especially
388 polyacetylenes, are linear, rigid (inflexible), and lipophilic molecules that accumulate in cellular
389 membranes. *In vitro* experiments suggested that the fungal plasma membrane is the primary site-
390 of-action for photoactivated acetylenes, and that they are not genotoxic (Arnason *et al.*, 1986).
391 The biological activities of four thiophene photosensitizers on *Saccharomyces cerevisiae* cells
392 (potential genotoxicity and kill rates) were evaluated in the dark or combined with exposure to
393 UV-A radiation (irradiance of 5 W m^{-2} and emission peak at 350 nm) (Muzzoli and Sacchetti,
394 2001). None of these four tiophenes were found to be genotoxic: α-terthienyl (α-T); 5-(4-hydroxy-
395 1-butenyl)-2,2´-bithienyl (BBT-OH); 5-(3-buten-1-ynyl)-2,2´-bithienyl (BBT); and 5-(4-acetoxy-1-
396 butinyl)-2,2´-bithienyl (BBT-OAc).

397 Phototreatments were performed *in vitro* using three naturally-occurring thiophene
398 derivatives as photosensitizers: 5-(3-buten-1-ynyl)-2,2´-bithienyl (Compound I, BBT); 2,2´:5´,2´´-
399 terthienyl (Compound II); and 2-chloro-4-[5-(penta-1,3-diynyl)-2-thienyl]but-3-ynyl acetate
400 (Compound III), combined with exposure to UV-A (320-380 nm) against some plant-pathogenic
401 ascomycetes, oomycetes, and zigomycetes (DiCosmo, Towers and Lam, 1982). Compounds I
402 and II were obtained from *Echinops sphaerocephalus* and compound III from *Tagetes erecta* (both
403 members of the Asteraceae). Conidia of *Alternaria alternata*, *Aspergillus niger*, *Cladosporium*
404 *variable*, and *Colletotrichum* spp., as well as sporangiospores of *Rhizopus nigricans* were placed
405 on media containing 0.01, 0.1, 1, and 10 μg mL^{-1} of the photosensitizers and exposed to UV-A
406 radiation either immediately or after incubations of 17 and 24 h. In all cases, APDT reduced
407 mycelial growth by 50-100% regardless of the photosensitizer or fungal/oomycete species. The
408 oomycetes were the most susceptible, irrespective of the photosensitizer used. Phototreatment
409 using Compound II repressed conidiogenesis in *A. niger* and sporangiogenesis in *R. nigricans*.

410 Germlings were generally more susceptible to APDT than non-germinated propagules. However,
411 the viability of ungerminated conidia of *A. niger* and *R. nigricans* was unaffected by APDT with
412 Compound II. A previous study reported that the UV-mediated cytotoxicity of Compound II occurs
413 in *Escherichia coli* and *S. cerevisiae* only in the presence of available oxygen, which is consistent
414 with the photodynamic basis for its mode-of-action (Arnason *et al.*, 1986).

415 The APDT was conducted on conidia and mycelia of the cereal pathogen *Fusarium*
416 *culmorum* using phenylheptatriyne combined with near-UV radiation (300-400 nm, 5 W m^{-2}),
417 which was extracted from the plant *Bidens pilosa* (Asteraceae). The treatment strongly inhibited
418 both germination of macroconidia and growth of mycelia (Bourque *et al.*, 1985). Phenylheptatriyne
419 disrupts membrane function in *F. culmorum* via both light-dependent and light-independent
420 mechanisms (Arnason *et al.*, 1986). Phototreatment of mycelia or macroconidia with
421 phenylheptatriyne (10 ppm) led to increasing granulation of the cytoplasm as exposure to near-
422 UV radiation (300-400 nm, 5 W m^{-2}) was increased (indicating cellular damage), inhibited ^{14}C-
423 phenylalanine uptake and respiration, and enhanced K^+ leakage, confirming that the plasma
424 membrane is the primary target site of phenylheptatriyne (Arnason *et al.*, 1986).

425 Furthermore, the accumulation of phototoxic thiophenes was studied in *T. erecta* that was
426 infected with *F. oxysporum* (Kourany, Arnason and Schneider, 1988). The naturally occurring
427 thiophenes BBT-OH, BBT-OAc, α-T, BBT, and 5-(3,4-diacetoxy-1-butinyl)-2,2´-bithienyl (BBT-
428 20Ac) completely inhibited spore germination at 5 μg mL^{-1} (*in vitro*) in the presence of near-UV
429 radiation (300-400 nm, 4 W m^{-2}). Also, α-T was strongly phototoxic against mycelia (Kourany,
430 Arnason and Schneider, 1988).

431 The plant *Targetes patula* (French marigold; Asteraceae) also accumulates the thiophenes
432 α-T and BBT-OH (Romagnoli *et al.*, 1998). The APDT was carried out against the plant-
433 pathogenic fungi *Botrytis cinerea*, *Fusarium moniliforme*, and *Pythium ultimum* using the pure
434 thiophenes α-T and BBT-OH, and a methanol extract of *T. patula* (Mares *et al.*, 2002; Mares *et*
435 *al.*, 2004). Mycelia were placed on media containing 5, 10, and 50 μg mL^{-1} of each pure thiophene,

436 or *T. patula* extract at a range of dilutions, and then exposed to UV-A radiation (peak at 350 nm,
437 5 W m^{-2}) or simulated solar radiation. Each of these treatments inhibited growth in a concentration-
438 dependent manner and regardless of pathogen species. In terms of reduction of growth-rate, *P.*
439 *ultimum* was the most susceptible species, and *F. moniliforme* was the least susceptible (Mares
440 *et al.*, 2002). The use of scanning electron microscopy and transmission electron microscopy
441 revealed structural alterations to the plasma membrane of *P. ultimum*, disorganization of the
442 cytoplasm, destruction of the nuclear envelope, and damage to the cell wall (Mares *et al.*, 2004).
443 Comparable damage was observed in the dermatophyte fungus *Nannizia cajetani* following APDT
444 using BBT-OH (Romagnoli *et al.*, 1998).

445 Phenylphenalenones are phototoxic polycyclic aromatic compounds found mainly in
446 Haemodoraceae and Musaceae families (Hidalgo, Kai and Schneider, 2015). They protect
447 against pathogens, and their accumulation is upregulated in response to several fungal species
448 (Flors and Nonell, 2006; Luis *et al.*, 1994). Light-induced $^1O_2$ production and antifungal activity
449 was reported for phenylphenalelones extracted and purified from pathogen-infected *Musa*
450 *acuminate* (dwarf banana) (Lazzaro *et al.*, 2004). Conidia of *F. oxysporum* were spread onto
451 potato dextrose agar supplemented with each of the purified phenylphenalenones and then either
452 kept in the dark or exposed to visible light. For each photosensitizer obtained this way, antifungal
453 activity was observed in both light and darkness; but was highest with exposure to light for the
454 majority of the photosensitizers. Furthermore, antifungal activity was proportional to the amount
455 of $^1O_2$ produced by phenalenones. Experiments conducted in D_2O-based culture media confirmed
456 the participation of $^1O_2$ in phenylphenalenone phototoxicity (Lazzaro *et al.*, 2004). Interestingly,
457 the synthesis of 4-phenylphenalenone, which exhibited both the highest $^1O_2$ yield and greatest
458 antifungal activity of the phenylphenalenones assayed, occurs only in infected plants. By contrast,
459 the less potent 9-phenylphenalenones occur in both healthy and infected plants from other
460 families. Given the adverse effects of $^1O_2$ on cellular systems, plants could have evolved to
461 minimize $^1O_2$ generation whenever possible (Lazzaro *et al.*, 2004). Also, some fungi have evolved

462 partial resistance to photosensitizers produced by plants for self-defense: for instance, 463 *Mycosphaerella fijiensis*, the causative agent of the black sigatoka leaf-spot disease of bananas, 464 can convert phenylphenalenones to sulfate conjugates that are inactive (Hidalgo *et al.*, 2016).

465 Decontaminating fungi-infected grain, maize, peanuts, or other seeds (whether used for 466 sowing or consumption) mitigates against dispersal and mycotoxin contamination of the food 467 supply chain. *Aspergillus flavus* is a commonly-occurring seed-borne pathogen that produces 468 mycotoxins, including aflatoxin that is a potent carcinogen (Temba *et al.*, 2016). Thus far, 469 chemical antifungals have been used to kill seed-borne fungal pathogens, but with varying levels 470 of success (Dweba *et al.*, 2017). Furthermore, seed-decontamination treatments must kill fungi 471 and inactivate mycotoxins without reducing seed viability or vigour (Lukšienė *et al.*, 2007).

472 Phototreatment of *A. flavus* conidia was evaluated both *in vivo* and *in vitro* using curcumin 473 as the photosensitizer (Temba *et al.*, 2016). Curcumin, a yellow polyphenol, is obtained from the 474 tubers of the plant *Curcuma longa*. Conidia were treated with different photosensitizer 475 concentrations (from 5 to 100 µM) and exposed to light at 420 nm, both in phosphate buffered 476 saline (PBS) solution and on maize kernels. Fluences used ranged from 12 to 84 J cm^{-2} and were 477 obtained using a xenon arc lamp with adjustable wavelength selection as the light source. 478 Reductions of conidial viability were up to 3 log_{10} in suspensions and 2 log_{10} in maize kernels 479 when optimal combinations of photosensitizer concentration and light fluence were used (Temba 480 *et al.*, 2016). Also, APDT using curcumin (100 to 1000 µM) combined with white light (24 to 96 J 481 cm^{-2}) were evaluated on conidia of *A. flavus*, *A. niger*, *F. oxysporum, Penicillium crysogenum*, 482 and *Penicillium griseofulvum* (Al-Asmari, Mereddy and Sultanbawa, 2017). Conidia were killed by 483 curcumin whether in spore suspensions or on the surface of agar plates.

484 Curcumin was also shown to be effective against *B. cinerea*: phototreatment of spores with 485 a concentration of 800 µM and a light fluence of 120 J cm^{-2} (430 nm wavelength) completely killed 486 the conidia. Furthermore, the toxins botrydial and dihydrobotrydial, which accumulate in spores 487 under normal conditions, could not be detected in treated conidia (Huang *et al.*, 2021). These

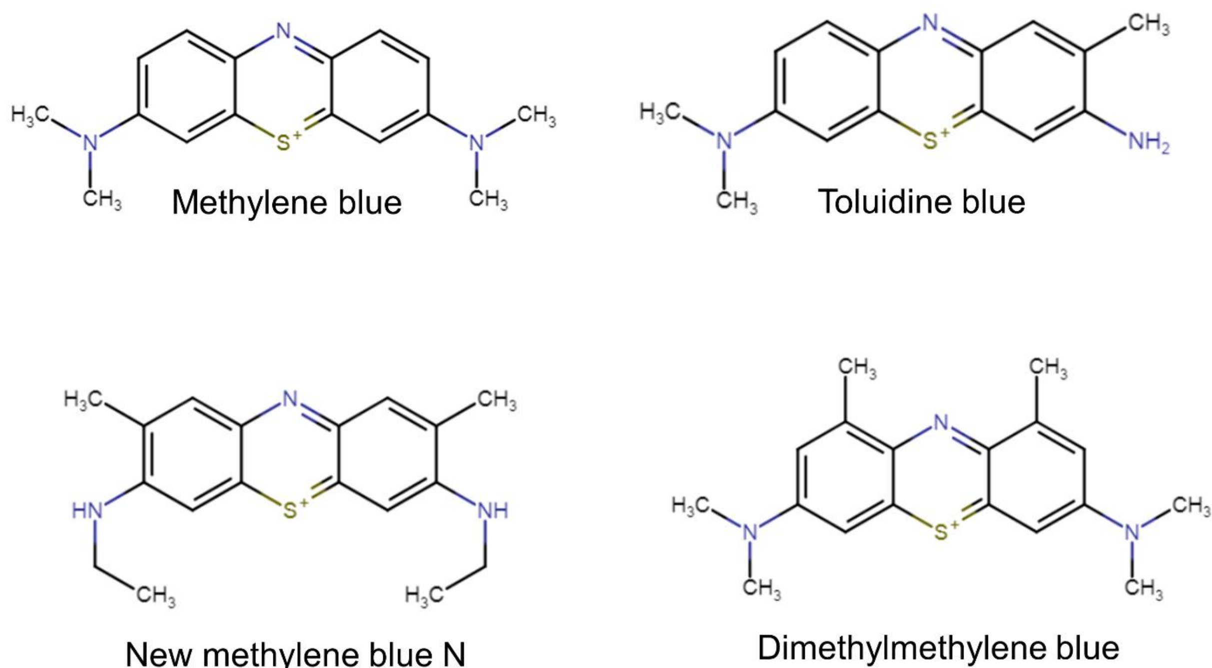
488 results further emphasize the application of APDT not only for pathogen killing, but also for toxin
489 inactivation or destruction.

490

491 **2.2 Photodynamic inactivation of plant-pathogenic fungi using synthetic and semi-**
492 **synthetic photosensitizers**

493 **2.2.1 Phenothiazines**

494 Phenothiazines are tricyclic organic compounds derived from a thiazine heterocyclic (Fig.
495 4). For control of fungi in both agriculture and medicine, phenothiazinium dyes are among the
496 most-commonly used photosensitizers. Generally, they are not toxic to mammals (Wainwright *et*
497 *al.*, 2017) and are environmentally safer than widely-used commercial fungicides (Andrade *et al.*,
498 2022).



499

500 **Figure 4 –** Chemical structure of phenothiazinium dyes commonly used for photodynamic treatment.
501 Structures were drawn with Marvin JS (ChemAxon).

502

503 Methylene blue and other phenothiazinium photosensitizers are used for tracing cell
504 malignancy and to treat methemoglobinemia (a blood disorder arising from the oxidation of Fe^{2+}

505 to Fe^{3+} within hemoglobin) at concentrations that are orders of magnitude higher than the
506 minimum concentrations required to kill microbes (Shatila, Verma and Adam, 2017; Wainwright,
507 2010). Novel phenothiaziniums, such as the pentacyclic S137, new methylene blue N, and
508 derivatives with basic side-chains, exhibit higher levels of antifungal activity compared to
509 methylene blue (Dai *et al.*, 2011; de Menezes *et al.*, 2016; Rodrigues *et al.*, 2012a; Wainwright,
510 Meegan and Loughran, 2011; Wainwright *et al.*, 2015). Recent studies have shown that the
511 antimicrobial activity of phenothiaziniums can be enhanced by urea or inorganic salts such as
512 potassium iodide (Nuñez *et al.*, 2015; Vecchio *et al.*, 2015), which is also observable for other
513 photosensitizers (Bispo, Suhani and van Dijl, 2021; Castro *et al.*, 2020).

514 In terms of interaction between photosensitizers and target pathogen, the negatively-
515 charged surfaces of both the fungal plasma membrane and fungal cell wall promote interactions
516 with cationic phenothiaziniums, and several lines of evidence suggest that the plasma membrane
517 is the primary site of damage following light-activation of these molecules (de Menezes *et al.*,
518 2016; Ito, 1978; Paardekooper *et al.*, 1992; Paardekooper *et al.*, 1995).

519 A study of *Saccharomyces fragilis* revealed that the photosensitizer toluidine blue O, which
520 is commonly used for APDT, interacts with polyphosphates localized outside the plasma
521 membrane without entering the cells (Tussen, Beekes and Van Steveninck, 1981). Also, it was
522 reported that toluidine blue O does not enter cells of *S. cerevisiae* and that the photodynamic
523 activity a consequence of its action on the extracellular medium and/or on the outer surface of the
524 plasma membrane (Ito, 1977). A study of APDT on *S. cerevisiae* showed that toluidine blue O
525 causes rapid oxidation of ergosterol and the subsequent accumulation of oxidized ergosterol
526 within the plasma membrane (Bocking *et al.*, 2000). The damaged plasma membrane facilitates
527 entry of the photosensitizer into the cytosol, which further damages intracellular membranes and
528 biomolecules. This, in turn, leads to impaired mitochondrial function and, ultimately, cell death
529 (Bocking *et al.*, 2000).

530 Indeed, APDT with methylene blue, toluidine blue O, new methylene blue N, or S137
531 increased the plasma membrane permeability of *F. moniliforme*, *F. oxysporum*, and *F. solani*
532 conidia. However, only the most-lipophilic photosensitizers, new methylene blue N and S137,
533 caused peroxidation of membrane lipids (de Menezes *et al.*, 2016), which could indicate that
534 photsensitizer localization is heavily dependent on fungal species and/or developmental stage.
535 Furthermore, a recent study compared the mechanism of *C. albicans* photoinactivation with new
536 methylene blue N and S137 (Rodrigues *et al.*, 2020b). Whereas new methylene blue N targets
537 mitochondria and reduce their membrane potential, S137 partitions into the cell membrane due
538 to its high log *P* (6.26), where it causes destabilization and increased permeability (Rodrigues *et*
539 *al.*, 2020b). Upon light exposure, S137 already present within the cell membrane increases
540 photokilling, especially at lower light fluences (Rodrigues *et al.*, 2020b).

541 Gonzales and coworkers evaluated the effects of APDT with methylene blue or toluidine
542 blue O on conidia of the saprophyte *A. nidulans* and the entomopathogen *Metarhizium robertsii*
543 (formerly *M. anisopliae lato sensu*) (Gonzales *et al.*, 2010). Conidia of *Metarhizium* species have
544 long been used as inoculum for control of agricultural insect pests, and are still one of the most
545 effective fungal species for this purpose (Braga *et al.*, 2015; Brancini *et al.*, 2022; Fernandes *et*
546 *al.*, 2015; Rangel *et al.*, 2015). In the study, concentrations of methylene blue and toluidine blue
547 O ranged from 1 to 400 μM and conidia were exposed to broad-spectrum visible light (irradiance
548 of 50 W m^{-2}) for 30 or 60 minutes. Mortality rates of up to 99.7% were achieved according to CFU
549 counts, and germination of conidia which remained viable was delayed, suggesting considerable
550 stress or damage to surviving conidia (Hamill *et al.*, 2020). Washing conidia prior to light exposure
551 slightly reduced the effect of APDT on *M. robertsii* but strongly reduced the effect on *A. nidulans*.
552 These findings suggest that methylene blue and toluidine blue O are taken up by each type of
553 conidia at different rates or interact with conidia via different mechanisms (Gonzales *et al.*, 2010).

554 Additionally, when APDT of *A. nidulans* and *M. robertsii* was attempted for conidia in potato
555 dextrose broth, no loss of viability occurred, indicating that some constituents of the medium may

556 act as antioxidants and scavengers of ROS (Gonzales *et al.*, 2010). Furthermore, conidial
557 pigments conferred some protection against APDT with phenothiazinium photosensitizers. The
558 conidia of the *M. robertsii* dark green wild-type and yellow-colored mutants were more resistant
559 to APDT compared with white (albino)- or violet-colored mutants (Gonzales *et al.*, 2010). Similarly,
560 dark green wild-type conidia of *A. nidulans* were more tolerant to APDT using methylene blue or
561 toluidine blue O than mutants with diverse conidial pigmentation (Al-Rubeai and El-Hassi, 1986).

562 The efficacy of APDT of *C. abscissum*, *Colletotrichum gloesosporioides*, and *A. nidulans*
563 conidia was determined *in vitro* using the using phenothiazinium photosensitizers methylene blue,
564 toluidine blue O, new methylene blue N, and S137 (de Menezes *et al.*, 2014b). Minimum inhibitory
565 concentration (MIC) was determined for each photosensitizer at different light fluences and S137
566 was found to be the most effective. At fluences ≥ 20 J cm^{-2}, for example, an S137 concentration
567 of only 10 μM was sufficient to prevent fungal growth, regardless of species. The dark toxicity of
568 S137 was also greater than that of the other photosensitizers assayed, regardless of the species.
569 Superior activity was also reported for S137 and new methylene blue N relative to methylene blue
570 against *Candida* (Dai *et al.*, 2011; Rodrigues *et al.*, 2013) and *Trichophyton* spp. (Rodrigues *et*
571 *al.*, 2012a). Furthermore, APDT of conidia with new methylene blue N or S137 and solar radiation
572 or red light (emitted by LEDs) was highly effective, regardless of the photosensitizer, light source
573 or species. When conidia were washed prior to light exposure, APDT using new methylene blue
574 N or S137 was about as effective as APDT without washing, indicating that these photosensitizers
575 were taken in by conidia.

576 Consistent with this, microscopic examination of *C. abscissum* conidia revealed that new
577 methylene blue N and S137 accumulated in cytoplasmic vesicles (de Menezes *et al.*, 2014b).
578 Photosensitizer entry and accumulation begins upon contact with conidia, and is independent of
579 light exposure. This study also compared localization of these photosensitizers with the
580 localization of the dyes Sudan III and FM4-64®, which have affinity for lipid bodies and vacuolar
581 membranes, respectively. Both new methylene blue N and S137 accumulated in lipid bodies and

582 small vacuoles. Conidial treatment in the dark with S137 at concentrations ≥ 50 μM modified the
583 structures of the cytoplasmic organelles and caused the formation of large vesicles (de Menezes
584 *et al.*, 2014b).

585 One concern about using these photosensitizers on crop plants in the field is their loss of
586 photosensitizing potential after extended light exposure. If new methylene blue N or S137 are
587 exposed to solar radiation prior to application to conidia, their APDT potency is reduced (de
588 Menezes *et al.*, 2014b), a phenomenon known as photobleaching (Nassar, Wills and Harriman,
589 2019). For instance, when new methylene blue N and S137 were exposed to solar radiation for 3
590 h and then used for APDT, conidial survival was reduced only about 3 $\log_{10}$ relative to controls,
591 compared with a reduction of 5 $\log_{10}$ if the photosensitizers were not exposed to solar radiation
592 prior to APDT. After exposure to solar radiation for 12 h, S137 was only weakly active against *C.*
593 *abscissum* conidia, whereas new methylene blue N retained more of its activity, with an ability to
594 kill 90% (1 $\log_{10}$) of the conidia (de Menezes *et al.*, 2014b). However, it is important to note that
595 these experiments were performed under harsh conditions: photosensitizer solutions were
596 exposed continuously to solar radiation at a tropical site (21.2° latitude S) during cloudless, early-
597 autumn days. Thus, the longevity of phenothiazinium photosensitizers is likely to be greater for
598 most agricultural scenarios, especially if geographical location or climatic conditions involve less-
599 intense solar exposure (de Menezes *et al.*, 2014b).

600 The effects of photodynamic treatment on the leaves of *C. sinensis* were evaluated using
601 methylene blue, new methylene blue N, toluidine blue O, and S137 (each at 50 μM) and solar
602 radiation (de Menezes *et al.*, 2014b). There was no apparent damage to the plant (regardless of
603 the photosensitizer used), presumably because the photosensitizer could not penetrate the 4-μm-
604 thick leaf cuticle (de Menezes *et al.*, 2014b). As ROS generated during APDT have relatively short
605 half-life, their diffusion can be very limited, thereby restricting damage to the immediate vicinity of
606 the photosensitizer (Skovsen *et al.*, 2005). Therefore, APDT of plant-pathogenic microbes located
607 on the host-plant surfaces proceeds without compromising the integrity of the latter.

608 In the last decades, human mycoses caused by species considered to be plant pathogens
609 or fungal saprophytes (rather than human pathogens) increased dramatically (Guarro, 2013).
610 Among the causative agents are species of *Aspergillus*, *Exophila*, *Fusarium*, and *Rhizopus* (Gao
611 *et al.*, 2016; Guarro, 2013; Liu *et al.*, 2019; Woo *et al.*, 2013). Invasive human infections by these
612 fungi are usually refractory to treatment with conventional antifungals (Guarro, 2013; Liu *et al.*,
613 2019; Paulussen *et al.*, 2017), so APDT of these fungi may have clinical potential. In this sense,
614 a detailed study to evaluate APDT using methylene blue, new methylene blue N, toluidine blue
615 O, and S137 on both ungerminated and germinated microconidia of *F. moliniforme*, *F. oxysporum*,
616 and *F. solani* were evaluated (de Menezes *et al.*, 2016). The intracellular localization of the
617 photosensitizers as well as potential consequences of APDT were determined, including lipid
618 peroxidation, plasma-membrane permeability, and conidial survival. Regardless of the
619 photosensitizer used, APDT killed both ungerminated and germinating microconidia efficiently for
620 all three *Fusarium* species (de Menezes *et al.*, 2016).

621 Another strategy to control and treat fungi-caused human diseases is the combination of
622 APDT and antifungals. In this sense, the effects of APDT with methylene blue at concentrations
623 of 8 to 32 μg mL^{-1} either alone or in combination with standard antifungal compounds were
624 evaluated (Gao *et al.*, 2016). The treatment was carried out for both planktonic cells and biofilms
625 of clinical isolates of *Exophiala dermatitidis*, *F. oxysporum*, and *F. solani*. Phototreatment with
626 methylene blue reduced survival by up to 3.8 log_{10} and 6.4 log_{10} of planktonic *Exophiala* spp. and
627 *Fusarium spp.*, respectively. The reductions for biofilms were 4.2 log_{10} and 5.6 log_{10}, respectively.
628 However, light fluence used had to be two-fold higher than that used against planktonic cells.
629 Application of APDT prior to the use of standard antifungals resulted in dramatic reduction of MICs
630 when compared to antifungal treatment alone irrespective of fungal species (Gao *et al.*, 2016).

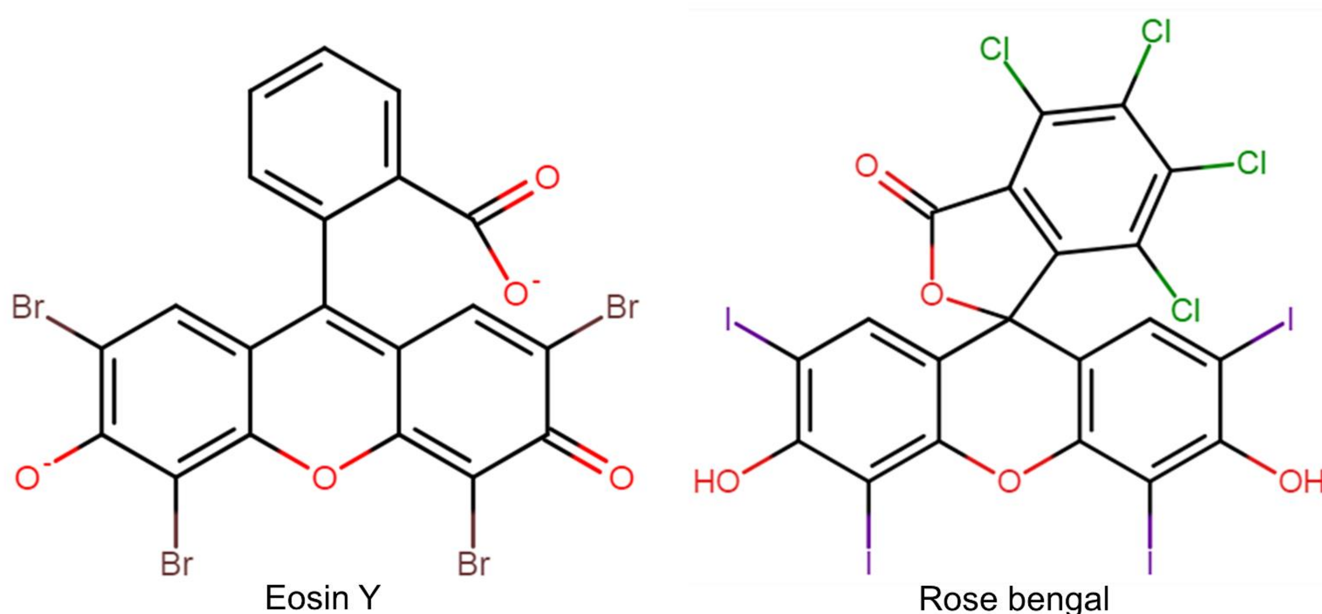
631 The mucoromycete *Rhizopus oryzae* causes post-harvest fruit rot and is also a common
632 cause of mucormycosis, an aggressive and frequently fatal opportunistic fungal infection in
633 immunocompromised individuals (Uyar and Uyar, 2018; Walther, Wagner and Kurzai, 2020).

634 Phototreatment with methylene blue (32 µg mL^{-1}) and red light (LED, 635 nm, 12 J cm^{-2})
635 completely inhibited growth and the reduction in CFU counts was up to 4.3 log_{10}. Also, APDT
636 reduced the MIC for the antifungals itraconazole, posaconazole, and amphotericin B (Liu *et al.*,
637 2019).

638

639 **2.2.2 Xanthenes**

640 Xanthene is a tricyclic dibenzopyran organic compound that, while not possessing useful
641 photodynamic properties, has many derivatives that are used in APDT, such as eosin Y and rose
642 bengal (Fig. 5). The *in vitro* effects of APDT with eosin Y on the endophyte *Papulaspora immersa*
643 and the plant-pathogen *Emericella rugulosa* were evaluated (Trigos and Ortega-Regules, 2002).
644 Ergosterol oxidation and survival were assessed after APDT. Mycelia of these fungi were no
645 longer viable after the treatment and cell death correlated with ergosterol photooxidation,
646 indicating that ergosterol damage may be an effective way of achieving photoinactivation.



647

648 **Figure 5 –** Chemical structure of the two best known and most used xanthene dyes. Structures were
649 drawn with Marvin JS (ChemAxon).

650

651 In integrated pest management programs, photoactive insecticides, such as xanthene
652 derivatives, may be combined with microbial biocontrol agents (Kim, Je and Choi, 2010; Mischke,
653 Martin and Schroder, 1998). However, only a few studies have addressed the potential issue of
654 APDT causing harm to biological control agents. Such biocontrol agents include viruses (e.g.
655 baculoviruses, entomopoxviruses), bacteria (e.g. *Bacillus thuringiensis*), and entomopathogenic
656 fungi (e.g. *Beauveria bassiana*, *Isaria fumosorosea*, and *Metarhizium* spp.).

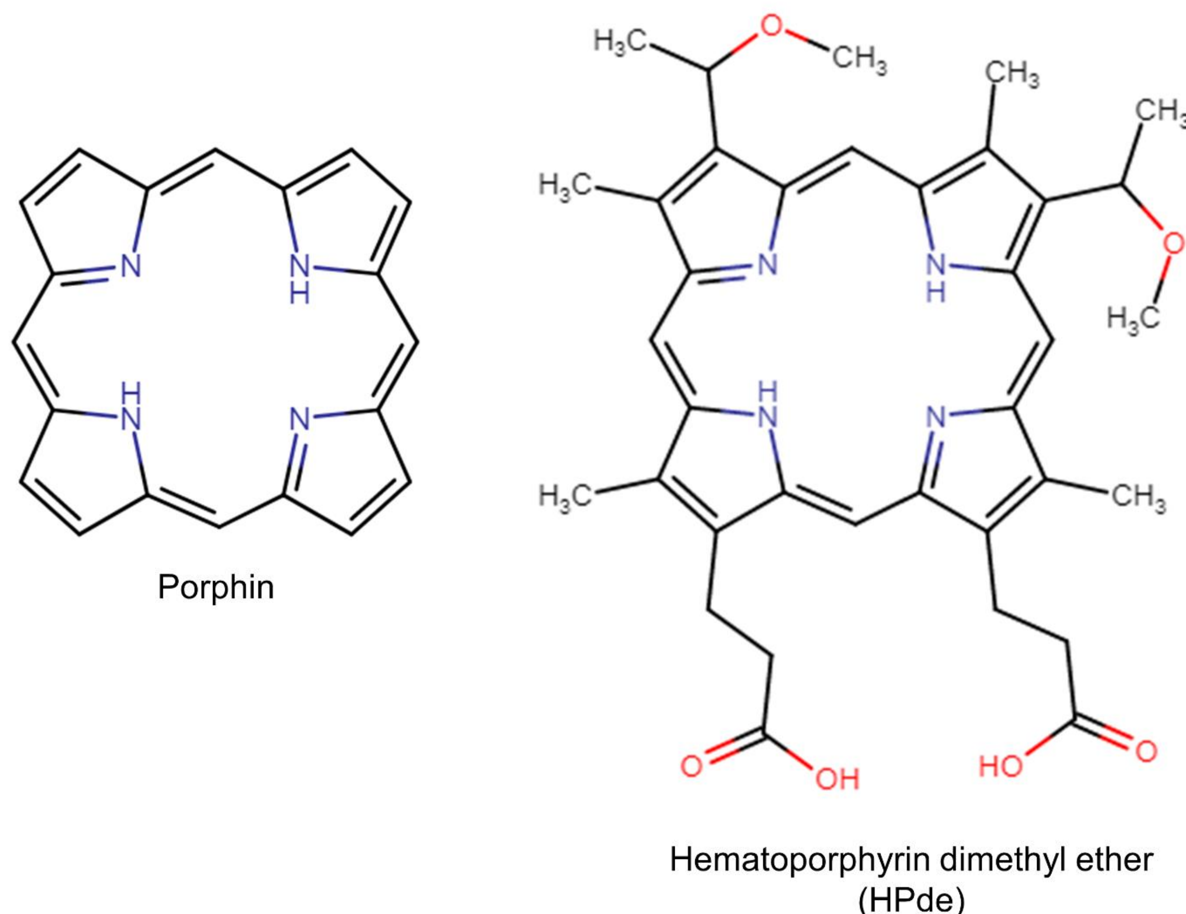
657 One study evaluated APDT using the xanthene dyes phloxine B (an insecticidal
658 photoactive compound), rose bengal, fluorescein, or eosin Y combined with white light and
659 observed inhibited growth of the biocontrol agents *B. thurigiensis* and *B. bassiana* (Martin,
660 Mischke and Schroder, 1998). Also, phloxine B was evaluated for compatibility with selected
661 biocontrol fungi to determine its potential for use in integrated pest management programs
662 (Mischke, Martin and Schroder, 1998). These studies found evidence that phloxine B, like other
663 photosensitizers (see below), inhibit some biocontrol fungi. Phloxine B at 0.01% (w/v) inhibited
664 the growth of *B. bassiana*, *Coniothyrium minitans*, and *Verticillium lecanii* in the presence of light.
665 Growth of the fungus *Trichoderma virens* was inhibited by phloxine B both with and without light
666 exposure, while growth of *Stilbella erythrocephala* was not affect by phloxine B, irrespective of
667 illumination (Mischke, Martin and Schroder, 1998). Treatment with phloxine B at 0.005, 0.01, and
668 0.02 g L^{-1} combined with visible light did not affect conidia germination of the entomopathogen *I.*
669 *fumosorosea* and, interestingly, a complementary effect of phloxine B on the insecticidal efficacy
670 of the fungus against the greenhouse whitefly, *Trialeurodes vaporariorum*, was observed (Kim,
671 Je and Choi, 2010). Collectively, these data suggest that chemically diverse photosensitizers can
672 inhibit phylogenetically diverse biocontrol agents such that each photosensitizer/biocontrol
673 organism combination must be evaluated prior to consideration for use in pest-management
674 programs.

675 Fungal infections of the human cornea, frequently caused by species of the genera
676 *Aspergillus* and *Fusarium*, are termed fungal keratitis, a disease potentially leading to blindness

677 (Thomas, 2003). Fungal keratitis is more common in agricultural communities, and its
678 management is restricted by the availability of effective antifungal agents, which must be able to
679 penetrate corneal tissue (Thomas, 2003). The effects of APDT on clinical isolates of *A. fumigatus*
680 and *F. solani* using rose bengal and riboflavin as photosensitizers were compared (Arboleda *et*
681 *al.*, 2014). Rose bengal and other xanthene dyes have been routinely used in ophthalmology
682 clinics to visualize degeneration of, or other defects in, the surface epithelium of the eye (Feenstra
683 and Tseng, 1992; Pellosi *et al.*, 2012). Conidia suspensions were treated with rose bengal or
684 riboflavin, both at 0.1% (w/v), and were then exposed to green or UV-A light (375 nm, 29.1 W m^{-2}
685). Phototreatment with rose bengal prevented the growth of both *A. fumigatus* and *F. solani*, but
686 no photoinactivation was observed with riboflavin (Arboleda *et al.*, 2014). The success of APDT
687 with rose bengal at 0.1 or 0.2% and green light (15 min at 5.4 J cm^{-2}) were reported in a pilot
688 clinical study with patients with progressive keratitis caused by *Fusarium spp.* and *Curvularia spp.*
689 (Naranjo *et al.*, 2019).
690

691 **2.2.3 Porphyrins**

692 Porphyrins are heterocyclic macrocycles composed by four pyrrole subunits
693 interconnected via methane bridges (Fig. 6) and have been widely evaluated for APDT of
694 phytopathogenic organisms.



695

696 **Figure 6 –** Chemical structure of porphin, the simplest porphyrin, and the hematoporphyrin dimethyl
697 ether (HPde) derivative. Structures were drawn with Marvin JS (ChemAxon).

698 *Fusarium* is a genus of filamentous fungi that contains many agriculturally important plant
699 pathogens, mycotoxin producers, and opportunistic human pathogens (Dong *et al.*, 2020; Lysøe
700 *et al.*, 2014; Ma *et al.*, 2013; Stenglein, 2009). The effects of APDT with hematoporphyrin dimethyl
701 ether (HPde) on spores of plant pathogens (*Fusarium avenaceum* and *Trichothecium roseum*)
702 and saprotrophic opportunistic human pathogens (*A. flavus* and *R. oryzae*) were evaluated
703 (Luksiene, Peciulyte and Lugauskas, 2004). Sporangiospores of *R. oryzae* and conidia of the
704 other fungal species were treated with HPde (0.25 to 71 µM) and exposed to visible light at 300
705 W m^{-2} for 15 minutes. HPde accumulated within the spores and exhibited dark toxicity regardless
706 of fungal species, though its potency as an inhibitor of germination varied with fungal species,
707 with *A. flavus* being more susceptible than the other species tested. The APDT using HPde and

708 visible-light exposure inhibited spore germination for all species, but *A. flavus* and *R. oryzae* were
709 more susceptible than the other fungi. *In-vitro* APDT using HPde and visible-light was also
710 effective at killing conidia of the plant-pathogen *A. alternata* and saprotrophic/human pathogen
711 *Acremonium strictum* (Lukšiene *et al.*, 2005).

712 APDT of *F. culmorum* and *Fusarium poae* conia was evaluated *in vitro* using protoporphyrin
713 IX, which is a hydrophobic dye that localizes to cell membranes (Vorobey and Pinchuk, 2008).
714 Conidia were treated with protoporphyrin IX (1 to 4 µM) and were then exposed to visible light at
715 150 W m^{-2}. Phototreatment resulted in protein and lipid oxidation, increased plasma-membrane
716 permeability, and reduced conidial viability. At 4 µM protoporphyrin IX and a fluence of 20 J cm^{-}
717 ^{2}, germination decreased by 55 and 96% for *F. culmorum* and *F. poae*, respectively.

718 Many studies have attempted to improve the efficiency of APDT with porphyrins by
719 modifying their structure and producing a series of cationic derivatives. For instance, APDT of
720 conidia from the saprotrophic fungus *Penicillium chrysogenum* was carried out using five cationic
721 porphyrins (each at 50 µM) and white light (irradiance 2,000 W m^{-2}, 20 min) (Gomes *et al.*, 2011).
722 The most effective porphyrin, 5,10,15,20-Tetrakis(*N*-methylpyridinium-4-yl)porphyrin tetraiodide,
723 caused a 4.1 log_{10} reduction in conidial viability. The size of the *N*-alkyl chain was shown to
724 correlate with photoinactivation efficiency, mainly by affecting the solubility of the photosensitizer
725 and its biding to conidia. In this sense, the best photosensitizer was the molecule with the shortest
726 carbon chain, suggesting that the increase of the *N*-alkyl length of all four alkyl chains does not
727 improve the photodynamic efficiency. The amount of photosensitizer incorporated by conidia was
728 a determinant for photoinactivation efficiency and varied among the different porphyrins.
729 Accordingly, examination using light microscopy revealed that all of the porphyrins penetrated
730 conidia, but some showed a more uniform distribution within cells whereas others localized to the
731 plasma membrane (Gomes *et al.*, 2011).

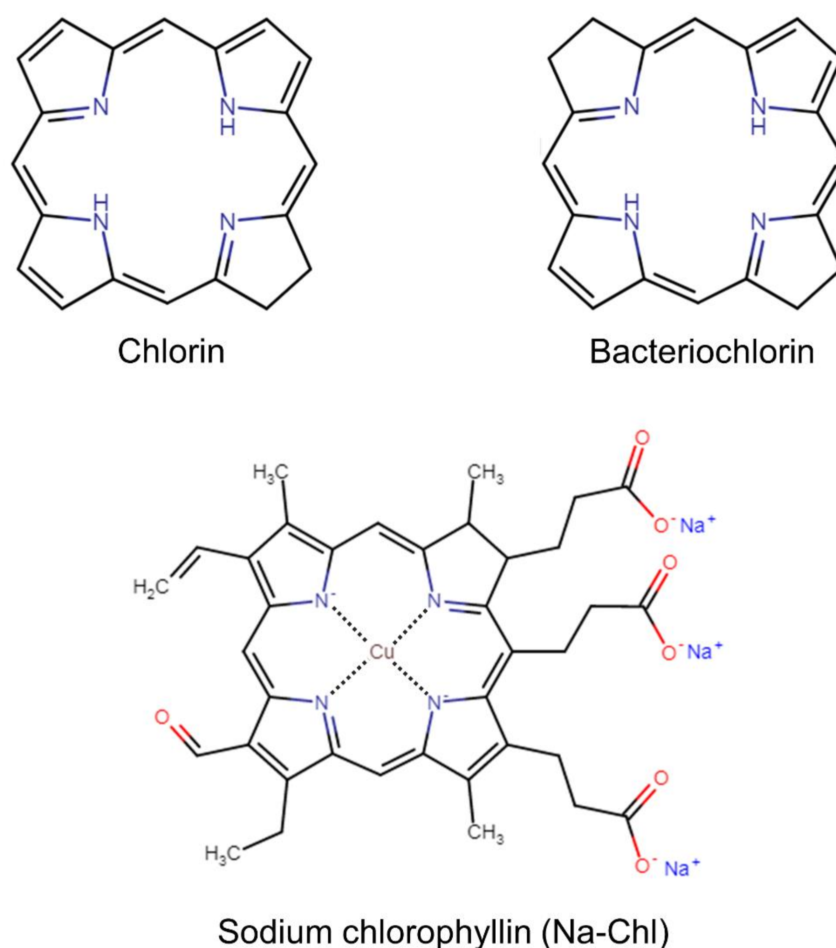
732 *Colletotrichum graminicola* is a destructive pathogen of maize causing both stalk rot and
733 leaf blight (Damm *et al.*, 2010). Treatment of *C. graminicola* conidia was carried out using five

734 cationic *meso*-(1-methyl-4-pyridinio)porphyrins which have phenyl or 1-methyl-4-pyridinio group
735 at the macrocycle *meso* position (Vandresen *et al.*, 2016). This was performed using porphyrin
736 concentrations from 1 to 25 µM and fluences ranging from 30 to 120 J cm^{-2} (emitted from a 250-
737 W halogen lamp). Considering the lowest photosensitizer concentration and the lowest light
738 fluence that enabled photoinactivation, the porphyrins efficiencies were ranked as triple-charged
739 (1 µM with a fluence of 30 J cm^{-2}) > double-charged-*trans* (1 µM with a fluence of 60 J cm^{-2}) >
740 tetra-charged (15 µM with a fluence of 90 J cm^{-2}) > mono-charged (25 µM with a fluence of 120
741 J cm^{-2}). The APDT using the triple-charged porphyrin at 1 µM and 30 J cm^{-2} killed all conidia.
742 Double-charged-*cys*-porphyrin killed conidia in the dark, i.e without light-activation. The
743 porphyrins that presented high $^1O_2$ quantum yields and accumulated to a high degree in conidia
744 were the best photosensitizer (Vandresen *et al.*, 2016).

745 APDT with a porphyrin (TMPyP) metal-organic framework (PS@MOF) was evaluated
746 against the plant-pathogenic fungi *Sclerotinia sclerotiorum*, *Pythium aphanidermatum* and *B.*
747 *cinerea* both *in vitro* and *in planta* (Tang *et al.*, 2021). *In vitro* APDT with PS@MOF strongly
748 inhibited mycelia growth of the three fungal species at photosensitizer concentrations of 6, 12,
749 and 24 mg L^{-1}. Also, APDT was able to control *S. sclerotiorum* on cucumber with efficiency equal
750 to the dicarboximide fungicide dimethachlon without causing damage to the host plant (Tang *et*
751 *al.*, 2021). Also, APDT with the anionic porphyrin tetra-4-sulfonatophenyl porphyrin tetra-
752 ammonium (TPPS) was tested against *B. cinerea* both *in vitro* and *in planta* (Ambrosini *et al.*,
753 2020). *In vitro* APDT with TPPS at 1.5 µM combined with white light caused potent inhibition of
754 mycelium growth. Also, mycelium pre-treated with TPPS was unable to infect detached leaves of
755 any of the three grapevine clones from Chadornnay, Merlot, and Sauvignon. Importantly,
756 treatment with the photosensitizer at 12.5 M did not damage the plants (Ambrosini *et al.*, 2020).
757

758 **2.2.4 Chlorins, bacteriochlorins, chlorophyllins, and chitosan**

759 A chlorin, the core chromophore of a chlorophyll, is a dihydroporphyrin macrocycle that
760 contains three pyrrole rings and one pyrroline ring (Fig. 7) (Taniguchi and Lindsey, 2017). Several
761 of the clinically important photosensitizers are chlorins, including m-tetrahydroxyphenylchlorin,
762 benzoporphyrin derivative, radachlorin, and chlorin e6 (Abrahamse and Hamblin, 2016).
763 Structurally, chlorins have a double bond in one pyrrole ring reduced (Fig. 7) whereas
764 bacteriochlorins have two pyrrole rings with reduced double bonds (Fig. 7) (Martinez De Pinillos
765 Bayona *et al.*, 2017). The bacteriochlorin group also includes important clinical photosensitizers
766 (Abrahamse and Hamblin, 2016) and both chlorins and bacteriochlorins have been evaluated as
767 photosensitizers for use in APDT against plant pathogens and foodborne human pathogens
768 (Lopez-Carballo *et al.*, 2008; Luksiene and Paskeviciute, 2011; Uliana *et al.*, 2014).



769

770 **Figure 7 –** Chemical structures depicting the differences between chlorin (20 π electrons) and
771 bacteriochlorin (18 π electrons). The structure of a chlorin derivative, sodium chlorophyllin, is also
772 shown. Structures were drawn with Marvin JS (ChemAxon).

773

774 As mentioned earlier, positively-charged photosensitizers tend to be more efficient as
775 antimicrobials because of their affinity for the negatively-charged cell surfaces of bacteria and
776 fungi (Hamblin, 2016). Indeed, the attachment of polycationic polymers such as poly-L-lysine and
777 polyethylenimine to chlorins (that otherwise do not possess any intrinsic positive charge)
778 enhanced their efficiency in APDT (Hamblin *et al.*, 2002; Tegos *et al.*, 2006). Interestingly, it was
779 reported that an asymmetric dicationic bacteriochlorin was significantly more active against Gram-
780 positive bacteria and fungi than a symmetrically-substituted tetracationic bacteriochlorin (Huang
781 *et al.*, 2014). Recently, thiopyridinium and methoxypyridinium chlorin derivatives were tested

782 against *F. oxysporum* (Sierra-Garcia, Cunha and Lourenço, 2022). Among these, a free-base
783 thiopyridinium chlorin was shown to be the most effective compound, achieving complete conidial
784 killing after 15 min (45 J cm^{-2}) of white-light exposure at a concentration of 15 μM. The other
785 compounds required either higher concentrations and/or longer exposure to light (Sierra-Garcia,
786 Cunha and Lourenço, 2022). These results show that chlorin derivatives can be potent
787 photosensitizers against plant-pathogenic fungi.

788 Chlorophyllins are semi-synthetic, water-soluble salts derived from chlorophyll and are also
789 approved as food colorants in both the EU and the USA (Fig. 7) (Wrolstad and Culver, 2012).
790 Chlorophyllins have been used as photosensitizers for photodynamic treatment of several types
791 of cancers as well as for APDT (Afrasiabi *et al.*, 2020; Luksiene and Paskeviciute, 2011; Luksiene
792 and Buchovec, 2019; Lukseviciute and Luksiene, 2020). Treatment using sodium salts of
793 chlorophyllin (Na-Chl) and visible light was evaluated for post-harvest control of spoilage
794 microbes on strawberries (Luksiene and Paskeviciute, 2011). Naturally-contaminated strawberry
795 fruits were soaked in Na-Chl at 1 mM for 5 min and illuminated for 20 min with visible light (400
796 nm and irradiance of 120 W m^{-2}). The growth of fungi and total aerobic mesophiles was reduced
797 by 86 and 97%, respectively. Consequently, shelf life of treated fruits was extended by two days.
798 Also, APDT increased total antioxidant activity of the fruit extracts by almost 20% but did not
799 impact the amounts of either anthocyanins or phenols, nor caused changes to fruit color (Luksiene
800 and Paskeviciute, 2011).

801 Chitosan is a cationic linear polysaccharide produced commercially by deacetylation of
802 chitin. Additionally, chitosan can form films and exhibits antimicrobial activity against a wide range
803 of microorganisms (Dutta, Tripathi and Dutta, 2012; Ke *et al.*, 2021). A chlorophyllin-chitosan
804 complex (Chl-CHS) has been used in APDT to kill microorganisms on fruit and grains surfaces.
805 The APDT using Chl-CHS was assessed for the microbiota of strawberries (Luksiene and
806 Paskeviciute, 2011). Naturally-contaminated strawberries were soaked for 30 min in 0.1% (w/v)
807 chitosan, 1.5 10^{-5} M chlorophyllin/0.1% chitosan or 1.5 10^{-5} M chlorophyllin and were exposed to

808 405-nm radiation for 60 min (fluence of 38 J cm^{-2}). Chitosan combined with light exposure reduced
809 colony forming units of fungi by 0.4 log_{10}; chlorophyllin-based APDT reduced colony forming units
810 by as much as 0.9 log_{10}; and APDT using Chl-CHS reduced colony forming units by 1.4 log_{10}
811 (Luksiene and Buchovec, 2019), showing the superior performance of the complex. No additional
812 photosensitization-induced free radical was found in the strawberry matrix and no changes were
813 caused to color, texture, and nutritional or visual quality of the fruits (Luksiene and Buchovec,
814 2019).

815 Furthermore, APDT using Chl-CHS was evaluated against fungi present on the surface of
816 wheat grains (Buchovec and Lukšienė, 2015). Wheat grains were soaked in 0.1% Chl-0.001%
817 chitosan solution and were then exposed to 405-nm radiation for 30 min (at a fluence of 30 J cm^{-2}
818). This treatment reduced the number of colony forming units of fungi by 0.68 log_{10} (mortality
819 ~80%). APDT with Chl-CHS was also evaluated to inactivate *Fusarium graminearum* mycelia *in*
820 *vitro* and conidia on artificially-contaminated wheat grains. *In vitro* APDT with 0.005%/Chl-0.5%
821 chitosan combined with exposure to 405-nm radiation inhibited mycelium growth but did not kill
822 the fungus. The results of the study did not make it clear what percentage of conidia was
823 inactivated by APDT on the grain surface, but the treatment did not affect the vigor and viability
824 of the grains (Buchovec and Lukšienė, 2015).

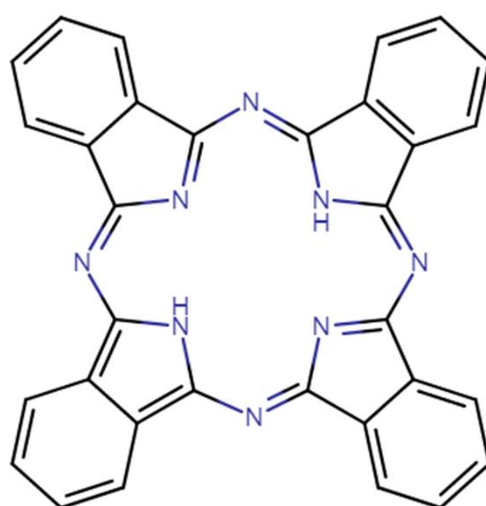
825 Chitosan has also been chemically combined with other photosensitizers, such as
826 protoporphyrin XI and riboflavin, yielding the conjugates PPIX-CHS and RF-CHS, respectively
827 (Dibona-Villanueva and Fuentealba, 2021; Dibona-Villanueva and Fuentealba, 2022). Both
828 conjugates were used for the APDT of *P. digitatum*. The PPIX-CHS compound inhibited fungal
829 growth by 100% at 0.005% (w/v) after one hour of white-light exposure. Interestingly, using a
830 mixture of unconjugated protoporphyrin IX and chitosan did not result in fungal killing, showing
831 the improved properties of the conjugate (Dibona-Villanueva and Fuentealba, 2022). The RF-
832 CHS conjugate was also effective against the fungus, albeit only at higher concentrations (0.5-
833 0.9%) compared to PPIX-CHS (Dibona-Villanueva and Fuentealba, 2021). Nonetheless, the

834 conjugation of riboflavin and chitosan greatly improved the photodynamic properties of the former:
835 the RF-CHS conjugate had higher $^1O_2$ yield and improved interaction with fungal cells compared
836 to riboflavin alone (Dibona-Villanueva and Fuentealba, 2021).

837

838 **2.2.5 Phthalocyanines**

839 Phthalocyanines are two-dimensional, 18 π-electron aromatic porphyrin analogues
840 consisting of four isoindole subunits linked together via nitrogen atoms (Fig. 8) (Claessens, Hahn
841 and Torres, 2008). Phototreatment using different types of phthalocyanines can kill various fungi,
842 including plant-pathogenic species (Prandini *et al.*, 2022; Rodrigues *et al.*, 2020a; Rodrigues *et*
843 *al.*, 2012b; Vol'pin *et al.*, 2000).



844

845 **Figure 8 –** Basic chemical structure of a phthalocyanine. The structures were drawn with Marvin JS
846 (ChemAxon).

847

848 The filamentous ascomycete *Magnaporthe oryzae* (formely *Magnaporthe grisea*) causes
849 rice blast, the most economically devastating disease of cultivated rice (Wilson and Talbot, 2009).
850 Rice blast occurs throughout crop areas and is typically responsible for a 10 to 30% reduction of
851 rice yield (Martin-Urdiroz *et al.*, 2016; Yan and Talbot, 2016). Use of conventional antifungals to

852 control *M. oryzae* has proven expensive and of limited efficacy (Yan and Talbot, 2016). Therefore,
853 the effects of 20 phthalocyanine-metal complexes on conidia germination of *M. grisea* (= *M.*
854 *oryzae*) both with and without light exposure were evaluated (Vol'pin *et al.*, 2000). Photosensitizer
855 concentrations tested ranged from 0.5 to 100 µg mL^{-1} and light was provided by a xenon lamp
856 with water filter. Inhibition of conidia germination varied between zero and 78% depending on the
857 type of phthalocyanine-metal complex. The authors also evaluated the phytotoxicity of these
858 complexes using rice-plant leaves. Most of the phthalocyanine-metal complexes did not damage
859 the leaves, but some did cause chlorotic or necrotic lesions that were however local and negligible
860 at the low photosensitizer concentrations required to control the pathogen. Interestingly, some of
861 these complexes also protected rice plants from blast disease. The authors hypothesize that, *in*
862 *planta*, the phthalocyanine redox activity in the dark along with its photosensitizing ability promote
863 the generation of ROS, which damage the fungus and, consequently, favor disease resistance
864 (Vol'pin *et al.*, 2000).

865 The chemical derivatization of phthalocyanine-metal complexes is an important process to
866 obtain better photosensitizers. For instance, a thiopyridinium derivative of Zn(II) phthalocyanine
867 was shown to be very effective against *C. abscissum*, achieving complete conidial killing at only
868 5 µM and a fluence of 37.5 J cm^{-2} (640-nm red light) (Prandini *et al.*, 2022). Furthermore,
869 ammonium derivatives of Si(IV) phthalocyanines were shown to be effective against *E. coli* and
870 *S. aureus*, also at low concentrations (3 and 6 µM) and a white-light fluence of 540 J cm^{-2}
871 (Gamelas *et al.*, 2022).

872

873 **2.2.6 5-aminolevulinic acid**

874 Microbial contamination of seed- and bean sprouts that are produced for human
875 consumption is a chronic problem in the food supply chain (Mir *et al.*, 2021; Symes, Goldsmith
876 and Haines, 2015). Sprouts are produced from plants such as legumes (e.g. beans, chickpeas,
877 lentils, peas, and soybean), cereals (e.g. rye, wheat, barley, and oats), and vegetables (including

878 alfalfa, radish, mustard, and other *Brassica* species). The methods currently employed to
879 decontaminate seeds and beans prior to sprouting (e.g. washing with chlorine, chlorine dioxide,
880 sodium and calcium hypochlorite, and hydrogen peroxide) have a number of drawbacks
881 (Lukšienė *et al.*, 2007; Mir *et al.*, 2021).

882 Studies have been carried out to evaluate APDT of seeds (prior to sprouting) using 5-
883 aminolevulinic acid (5-ALA) (Lukšienė *et al.*, 2007; Luksienė and Zukauskas, 2009). 5-ALA is a
884 naturally-occurring precursor to heme synthesis in eukaryotic and prokaryotic cells, which induces
885 the production of the endogenous photosensitizers protoporphyrin IX, uroporphyrin, and
886 coproporphyrin (Kamp *et al.*, 2005). When present in the extracellular milieu, 5-ALA is taken up
887 by cells of bacteria, yeast, and filamentous fungi. This can in turn stimulate synthesis of porphyrin-
888 type photosensitizers which can be light-activated to enable control of the microbes (Harris and
889 Pierpoint, 2012; Kamp *et al.*, 2005; Luksienė and Zukauskas, 2009; Polmickaitė-Smirnova *et al.*,
890 2022).

891 The APDT using 5-ALA has proved effective to control fungal contaminants of wheat grains
892 (Lukšienė *et al.*, 2007). Wheat grains naturally contaminated with fungi were soaked for 4 h in a
893 solution of NaCl (5%) with 5-ALA (6 mM) at 26 ^{o}C and were then exposed to light (522 nm, emitted
894 by an incandescent lamp equipped with optical filters). Twelve hours after exposure, grains were
895 examined for the presence of viable fungi, which revealed that *Apergillus* spp., *Fusarium* spp.,
896 *Mucor* spp., and *Rhizomucor* spp. were susceptible to APDT with 5-ALA while *Acremonium* was
897 not. Treatment with 5-ALA not only reduced fungal contamination but also stimulated the growth
898 of wheat seedlings and roots during the subsequent sprouting procedure, all without impairing
899 grain germination and viability (Lukšienė *et al.*, 2007). A similar molecule, methyl aminolevulinate,
900 is the methyl ester of 5-ALA and has also been used as photosensitizer in APDT. Methyl
901 aminolevulinate-based APDT was used against finger nail infections of *F. oxysporum* and
902 *Aspergillus terreus* (Gilaberte *et al.*, 2011). This treatment cured refractory onychomycosis
903 caused by these fungi.

904

905 **2.2.7 Riboflavin and riboflavin derivatives**

906 Riboflavin, widely known as vitamin B_2, is a water-soluble compound which can be
907 synthesized by plants and microorganisms, but is essential for animals as they lack an
908 endogenous biosynthetic pathway (Schwechheimer *et al.*, 2016). Riboflavin acts as cofactors for
909 oxidoreductases as well as prosthetic groups for enzymes in the β-oxidation pathway (Massey,
910 2000). The vitamin is synthesized biotechnologically using microorganisms, and is mainly used
911 as feed and food addictive as well as for pharmaceutical applications (Schwechheimer *et al.*,
912 2016).

913 When exposed to visible light, riboflavin acts as a potent photosensitizer by producing $^1O_2$
914 (Bäumler *et al.*, 2012; Cardoso, Libardi and Skibsted, 2012; Fuentealba *et al.*, 2015). However,
915 due to the lack of positive charge, riboflavin is not a very effective photosensitizer for use in APDT
916 (Nielsen *et al.*, 2015). For instance, riboflavin combined with UV-A could not inhibit the growth of
917 either hyphae or conidia of *A. fumigatus*, *F. solani*, and other *Fusarium* spp. (Arboleda *et al.*, 2014;
918 Kashiwabuchi *et al.*, 2013; Sauer *et al.*, 2010). However, the addition of riboflavin at 250 μM
919 significantly enhanced the efficacy of simulated solar disinfection at 150 W m^{-2} against a variety
920 of microorganisms, including *F. solani*, with mortality rates of 100% being achieved after a 6-h
921 exposure (Heaselgrave and Kilvington, 2010). Pretreatment of fungi with amphotericin B can
922 increase the effectiveness of APDT using riboflavin and UV-A (365 nm, 30 W m^{-2}), according to
923 an *in vitro* study of *A. fumigatus*, *C. albicans*, and *Fusarium* spp. (Sauer *et al.*, 2010). Also,
924 synthetic riboflavin derivatives that are positively charged have been successfully used in APDT
925 against *Bacillus* endospores (Eichner *et al.*, 2015). In this sense, and as mentioned earlier, the
926 conjugation of riboflavin with chitosan improved the interaction of the former with *P. digitatum*
927 cells (Dibona-Villanueva and Fuentealba, 2021).

928

929 **3. Post-harvest photoinactivation of foodborne pathogens and microbial contaminants**

930 Plant surfaces are typically subject to extreme fluctuations in water activity (Stevenson *et* 931 *al.*, 2015b), solar radiation, temperature, and other parameters. As such, they tend to be nutrient-932 poor (Lievens *et al.*, 2015) and can be inhospitable for human pathogens such as enteric bacteria. 933 Damage to plant tissues, such as that caused by plant pathogens or food processing, can create 934 a habitable substrate for, and so promote the growth of, enteric pathogens of humans (Heaton 935 and Jones, 2008; Weiman, 2014).

936 Among the main foodborne bacterial pathogens are *Bacillus cereus*, *E. coli*, *Listeria* 937 *monocytogenes*, and *Salmonella enterica*, none of which is capable of growth on low water-938 activity surfaces of < 0.850-800 (do Prado-Silva *et al.*, 2022; Santos *et al.*, 2015; Stevenson *et* 939 *al.*, 2015a). Microbial contamination of fruits and vegetables and other types of food/feeds can 940 also greatly shorten their shelf life, especially by fungal psychrophiles and xerophiles, some of 941 which are capable of growth even at subzero temperatures and at ≤ 0.585 water activity (Chin *et* 942 *al.*, 2010; Collins and Buick, 1989; Stevenson *et al.*, 2017c; Stevenson *et al.*, 2017b; Stevenson 943 *et al.*, 2017a). Several studies, carried out *in vitro* using different photosensitizers, have 944 established that APDT can efficiently kill diverse foodborne pathogens and spoilage microbes, 945 including cells and spores of bacteria (Aponiene *et al.*, 2015; Buchovec *et al.*, 2017; Eichner *et* 946 *al.*, 2015; Gulías *et al.*, 2020; Luksiene and Buchovec, 2019; Luksiene and Brovko, 2013; do 947 Prado-Silva *et al.*, 2021; do Prado-Silva *et al.*, 2022; Silva *et al.*, 2018; Sobotta *et al.*, 2019).

948 Natural and semi-synthetic photosensitizers such as 5-ALA (Buchovec, Vaitonis and 949 Luksiene, 2009; Luksiene, Buchovec and Paskeviciute, 2009), sodium magnesium and sodium 950 copper chlorophyllin (approved as food addictives E-140 and E-141, respectively) (Buchovec *et* 951 *al.*, 2016; Buchovec *et al.*, 2017; Hasenleitner and Plaetzer, 2020; Luksiene and Buchovec, 2019; 952 Luksiene and Paskeviciute, 2011; Luksiene, Buchovec and Paskeviciute, 2010a; Luksiene, 953 Buchovec and Paskeviciute, 2010b), curcumin (approved as the food additive E-100) (Gong *et* 954 *al.*, 2020; Glueck *et al.*, 2017; Hu *et al.*, 2018; Temba *et al.*, 2016; Tortik, Spaeth and Plaetzer, 955 2014), furocoumarins (de Menezes *et al.*, 2014a; Fracarolli *et al.*, 2016; Ulate-Rodríguez *et al.*,

956 1997), hypericin (Aponiene *et al*., 2015; Kairyte *et al*., 2012), and riboflavin derivatives (Eichner
957 *et al*., 2015) are among the most studied in relation the food microbiology.

958 APDT of the Gram-negative *S. enterica* using 5-ALA efficiently kills the bacterium,
959 according to *in vitro* studies (Buchovec, Vaitonis and Luksiene, 2009). Bacterial cells were taken
960 from an exponential-phase culture and incubated with 7.5 mM 5-ALA for up to 1 h in the dark,
961 and then exposed to visible light (LED, with emission at 400 nm and irradiance of 200 W m^{-2}).
962 Bacterial photodynamic inactivation was dependent on the incubation time with 5-ALA and
963 fluence. Viable cell number was reduced by up to 6 log_{10}. *In vitro* APDT with 5-ALA was also able
964 to kill the Gram-positive *B. cereus*, regardless of whether vegetative cells or spores were tested
965 (Luksiene, Buchovec and Paskeviciute, 2009). Photoinactivation of *B. cereus* on the surfaces of
966 grains and packaging materials was also observed (Luksiene, Buchovec and Paskeviciute, 2009).
967 Cells and spores of *B. cereus* suspended in PBS or on the surface of food-packaging material
968 and wheat grains were treated with 5-ALA (3 to 7.5 mM) and then exposed to visible light. Survival
969 of vegetative cells in suspension was reduced by 6.3 log_{10} and on the surface of food packing by
970 4 log_{10}. *B. cereus* spores both in suspension and on packaging material were also susceptible to
971 APDT and survival was reduced by 3.7 and 2.7 log_{10}, respectively. Similar results were observed
972 for the highly pathogenic *L. monocytogenes* which was killed by 5-ALA-based APDT both in
973 suspension (survival reduction up to 4 log_{10}) and as biofilm on the surface of packaging material
974 (survival reduction up to 3.1 log_{10}) (Buchovec, Paskeviciute and Luksiene, 2010). *B. cereus*
975 spores were also efficiently inactivated by APDT (> 3 log_{10} reduction in survival) when a tricationic
976 porphyrin was used as the photosensitizer. However, the susceptibility of spores to porphyrin-
977 based APDT was highly variable among different species of *Bacillus* (da Silva *et al*., 2012).

978 Sodium magnesium chlorophyllin (E-140) and sodium copper chlorophyllin (E-141) were
979 incorporated into gelatin films, and their potential to inhibit *Staphylococcus aureus* and *L.*
980 *monocytogenes* was evaluated (Lopez-Carballo *et al*., 2008). Bacterial cell suspensions were
981 spread on the surface of tryptone soy agar. Control gelatin films (no photosensitizer) and gelatin

982 films supplemented with E-140 or E-141 were placed on the surface of the inoculated agar, and
983 then irradiated for 5 or 15 min. Following these treatments, plates were incubated for 24 h and
984 bacterial colonies were then counted. Results showed that the E-140- and E-141-based APDT
985 reduced the number of colony forming units of *S. aureus* and *L. monocytogenes* by 5 and 4 log_{10},
986 respectively. *In vitro* APDT with sodium magnesium chlorophyllin at 5 µM combined with blue light
987 (433 nm, 6.6 J cm^{-2}) reduced the survival of *S. aureus* by more than 7 log_{10} (Hasenleitner and
988 Plaetzer, 2020). However, APDT with the two chlorophyllins had no effect on the viability of the
989 Gram-negative bacteria *E. coli* and *Salmonella* spp. (Lopez-Carballo *et al.*, 2008).

990 APDT using Na-Chl on survival of cells and spores of *B. cereus* (Luksiene, Buchovec and
991 Paskeviciute, 2010a) and *L. monocytogenes* (Luksiene, Buchovec and Paskeviciute, 2010b) was
992 evaluated *in vitro*, both in suspension and on the surface of yellow packaging trays (polyolefin-
993 mixture of polyethylene and polypropylene). Cells and spores of *B. cereus* suspended in PBS or
994 on packing material were incubated with Na-Chl (7.5×10^{-8} to 7.5×10^{-5} M) and then exposed to
995 visible light (peak emission at 400 nm and irradiance of 200 W m^{-2}). Treatment with 7.5×10^{-7} M
996 of Na-Chl reduced the survival up to 7 log_{10} of the cells of *B. cereus* suspended in PBS and those
997 on the surface of packaging trays. *B. cereus* spores were more tolerant to APDT than cells, but
998 were also killed depending on photosensitizer concentration and light-exposure time. Treatment
999 with 7.5×10^{-5} M of Na-Chl reduced the survival of the spores suspended in PBS up to 4 log_{10}.
1000 Reduction of the survival of the spores on packaging tray was 5 log_{10} at 7.5×10^{-5} M of Na-Chl.

1001 Also, APDT using Na-Chl was tested on the survival of thermosensitive and thermotolerant
1002 strains of *L. monocytogenes* both in suspension and on the surface of yellow packaging trays
1003 (Luksiene, Buchovec and Paskeviciute, 2010b). Phototreatment reduced the survival of both
1004 strains up to 7 log_{10} when cells were suspended in PBS and killed all the cells when they were on
1005 packaging trays. The APDT of *B. cereus* and *L. monocytogenes* using Na-Chl was considerably
1006 more effective than washing with 200 ppm sodium hypochlorite (Luksiene, Buchovec and
1007 Paskeviciute, 2010a; Luksiene, Buchovec and Paskeviciute, 2010b). Mechanistic studies

1008 conducted with Gram-negative bacteria has shown that during Na-Chl-based APDT, chlorin binds
1009 to the surface of the bacterial cell causing $^1O_2$-mediated membrane damage and cell wall
1010 disruptions, increased release of intracellular components, and cell death (Žudytė *et al.*, 2020).
1011 As presented earlier, APDT using Na-Chl and visible light was tested in strawberries that
1012 had been inoculated with *L. monocytogenes* (Luksiene and Paskeviciute, 2011). Strawberries
1013 were inoculated with *L. monocytogenes*, soaked in 1 mM Na-Chl solution for 5 min, and then
1014 exposed to visible light (400 nm, irradiance of 120 W m^{-2}) for 30 min. The treatment reduced the
1015 viability of the cells by 1.8 log_{10} compared to control samples.
1016 The effect APDT with the Chl-CHS chlorophyllin-chitosan complex on survival of *L.*
1017 *monocytogenes* on the surface of wheat grains was evaluated (Buchovec and Lukšienė, 2015).
1018 Also, coating of strawberries with Chl-CHS and illumination with visible light at 76 J cm^{-2}
1019 inactivated yeast/microfungi on the fruits by 1.4 log and prolonged the shelf life by 3 days without
1020 any negative effect on the fruits (Luksiene and Buchovec, 2019). Details for these two studies
1021 were discussed above and can be found on section 2.2.4. Furthermore, the effects of APDT using
1022 Na-Chl alone and combined with chitosan or high-power pulsed UV (200-1000 nm, peak at 260
1023 nm) on the survival of *S. enterica* were determined *in vitro* (Buchovec *et al.*, 2017). The APDT
1024 alone reduced the survival of the bacteria by 2.05 log_{10} while APDT combined with chitosan or
1025 pulsed UV reduced the viability by 7.28 and 7.5 log_{10}, respectively. Interestingly, Na-Chl-based
1026 APDT induced the transcription of genes responsible for ROS inactivation in *S. enterica*
1027 (Buchovec *et al.*, 2017).
1028 Hypericin-based APDT and hypericin-based APDT combined with high power pulsed light
1029 (HPPL) were evaluated for *L. monocytogenes* and *S. enterica* (Kairyte *et al.*, 2012). Cells were
1030 incubated with hypericin (10^{-5} or 10^{-7} M) in PBS and exposed to visible light (peak emission at
1031 585 nm and irradiance of 38.4 W m^{-2}). For the combined treatment, after APDT, bacteria were
1032 exposed to 350 pulses of HPPL (UV fluence of 0.023 J cm^{-2}). Hypericin interacted with the cells
1033 of both species and APDT reduced the survival of *Listeria* and *Salmonella* by 7 and 1 log_{10},

1034 respectively. Electron microscopy studies showed that APDT induced total collapse of the *Listeria*
1035 cell wall, but not that of *Salmonella*. Combined treatment of APDT and pulsed light reduced the
1036 survival of *Listeria* and *Salmonella* by 6.7 to 7 $\log_{10}$, respectively. The effect of APDT with
1037 hypericin (1.5×10^{-5} to 1×10^{-8} M) and visible light (585 nm, irradiance of 38.4 W m^{-2}, and fluences
1038 up to 9.2 J cm^{-2}) on the survival of *B. cereus* both *in vitro* and inoculated on the surface of fruits
1039 (apricots and plumes) and vegetables (cauliflowers) were also investigated (Aponiene *et al.*,
1040 2015). Hypericin-based APDT reduced the survival of the bacteria up to 4.4 $\log_{10}$ *in vitro*.
1041 Inactivation of mesophilic bacteria on the surface of fruits and vegetables reached up to 1.3 $\log_{10}$.
1042 The use of APDT employing curcumin bound to polyvinylpyrrolidone (PVP-C) and
1043 NovaSol®-curcumin for the decontamination of *S. aureus* from cucumber, peper, and chicken
1044 meat was evaluated (Tortik, Spaeth and Plaetzer, 2014). Both curcumin and PVP-C have been
1045 approved as food additives. Vegetables and meat were contaminated with the bacteria, sprinkled
1046 with PVP-C and NovaSol®-curcumin at concentrations of 50 and 100 μM, respectively, and
1047 illuminated immediately using visible light (emission peak at 435 nm, irradiance 94 W m^{-2} and
1048 fluence 33.8 J cm^{-2}). Photodynamic inactivation of *S. aureus* caused a mean reduction of 2.6 $\log_{10}$
1049 on cucumbers, 2.5 $\log_{10}$ on pepper, and 1.7 $\log_{10}$ on chicken meat relative to controls. Also, no
1050 visible changes of the exterior appearance of the foodstuff after APDT were observed (Tortik,
1051 Spaeth and Plaetzer, 2014).
1052 APDT using phenotiazinium photosensitisers, porphyrins, and xanthenes have also been
1053 tested against foodborne pathogens and microbial contaminants post-harvest. The spore-forming
1054 bacterium *Alicyclobacillus acidoterrestris* can cause great losses to fruit juice industries due to its
1055 thermal and chemical resistance and spoilage potential. Phototreatment with new methylene blue
1056 or tetracationic porphyrin combined with white light inactivated the spores both in suspension
1057 (PBS and orange juice) and on orange peels. Reductions in viability reached up to 7.3 $\log_{10}$ in
1058 suspensions and 2.8 $\log_{10}$ on peels. The presence of potassium iodide increased the effect of
1059 APDT (do Prado-Silva *et al.*, 2020).

1060 APDT with cationic porphyrins were also used to photoinactivate the Gram-negative
1061 phytopathogenic bacterium *Pseudomonas syringae* pv. *actinidiae* both *in vitro* and in kiwifruit
1062 plants under solar radiation. Photoinactivation reached up to 7.4 log_{10} *in vitro* and 6.2 log_{10} on
1063 leaves (Martins *et al.*, 2018). Also, APDT with eosin Y combined with green light was evaluated
1064 against the pathogenic bacteria *S. enterica*, *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. cereus*. *P.*
1065 *aeruginosa* was completely inactivated at 10 μmol L^{-1}, and reductions in viability reached 2.7 and
1066 1.7 for *B. cereus* and *S. enterica*, respectively. *E. coli* viability was slightly reduced while *S. aureus*
1067 was the most susceptible, being completely inactivated by eosin at 5 μmol L^{-1} and 5 min of light
1068 exposure (Bonin *et al.*, 2018).
1069

1070 **4. Fungal tolerance to photoantimicrobials**

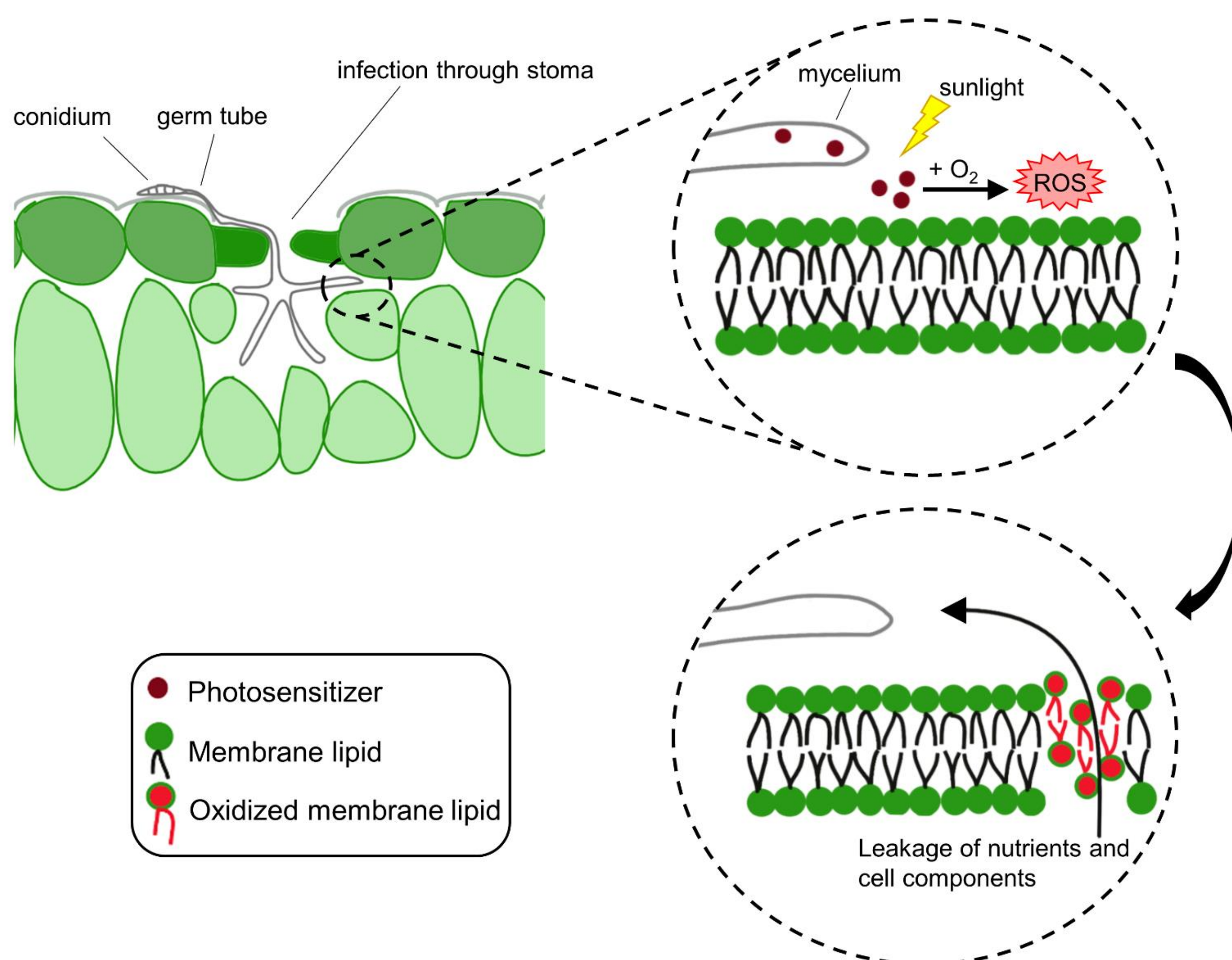
1071 Plants that produce furocoumarins and other potent photosensitizers can still experience
1072 severe microbial infections, and this is due to the development of tolerance or resistance to
1073 photosensitization in some specialized pathogens. *Fusarium sambucinum*, as well as some other
1074 plant pathogens, can metabolize, and thereby detoxify, xenobiotics such as furocoumarins. *F.*
1075 *sambucinum* is cosmopolitan in terms of habitat and lifestyle (both soil saprophyte and plant-
1076 pathogen), but is not generally regarded as any more stress tolerant than comparable species of
1077 fungi (Cray *et al.*, 2016). Circumstantial evidence from ecophysiological/toxin-resistance studies
1078 suggests that individual strains may preferentially inhabit either soils or the plant host (Desjardins,
1079 Spencer and Plattner, 1989). *F. sambucinum* tolerance to the furocoumarin xanthotoxin has been
1080 tested *in vitro* for 62 strains obtained from soils and diseased plants. As all the experiments were
1081 conducted in the dark, only direct inhibition by compounds was evaluated. Twenty-one out of 24
1082 *F. sambucinum* strains isolated from plants and only two out of 38 strains isolated from soil were
1083 found to be highly tolerant to xanthotoxin. Of 16 *F. sambucinum* strains tested against 16
1084 furocoumarin precursors and furocoumarins, all those that had been isolated from plants were
1085 highly tolerant and, in most cases, completely able to metabolize all of the compounds assayed.

1086 Conversely, most of the soil-derived strains tested were tolerant to furanocoumarin precursors
1087 but sensitive to certain furocoumarins (Desjardins, Spencer and Plattner, 1989).

1088 Metabolic inactivation of phototoxic plant metabolites is not the only mechanism that plant
1089 pathogens use to protect themselves against host-induced photosensitization. For instance,
1090 conidia of some fungi contain high concentration of pigments, such as carotenoids, that scavenge
1091 reactive species and can mitigate stresses such as those induced by solar UV radiation and
1092 photodynamic processes (Blanc, Tuveson and Sargent, 1976; Braga *et al.*, 2015; Thomas,
1093 Sargent and Tuveson, 1981; Shimizu, Egashira and Takahama, 1979). Pigments such as
1094 carotenoids are usually located within the plasma membrane and are able to quench both singlet
1095 oxygen and other types of reactive species produced by photosensitizers (Thomas, Sargent and
1096 Tuveson, 1981; Shimizu, Egashira and Takahama, 1979; Will, Newland and Reppe, 1984). Other
1097 pigments, which are structural components of the conidial cell wall (including melanins and
1098 melanin-like compounds), can selectively absorb solar radiation at the wavelengths required to
1099 activate the plant's photosensitizers (Asthana and Tuveson, 1992). The wild-type conidia of
1100 several *Colletotrichum*, *Fusarium*, and *Neurospora* species that are yellowish accumulate
1101 carotenoids, while *Alternaria*, *Aspergillus*, *Metarhizium, and Penicillium* conidia that are dark
1102 colored (brown-, gray- green- or bluish) are characterized by high levels of melanins or melanin-
1103 like pigments within the cell wall (Asthana and Tuveson, 1992; Gonzales *et al.*, 2010).

1104 The above discussion contains examples of plants which produce photosensitizers that act
1105 as photoantimicrobials, protecting them against infections by pathogenic microbes. However, in
1106 plant-microbe interactions, there are also cases in which the opposite occurs. Plant-pathogenic
1107 fungi of the genera *Alternaria*, *Cladosporium*, *Elsinoë* and *Mycosphaerella* produce
1108 perylenequinone pigments during host infection, such as cercosporin, elsinochromes,
1109 hypocrellins, calphostin, and rubellin which are potent photosensitizers and damage the plant
1110 (Chung, 2011; Daub, Herrero and Chung, 2005; Daub, Herrero and Chung, 2013; Heiser, Sachs
1111 and Liebermann, 2003; Świderska-Burek *et al.*, 2020; Thomas *et al.*, 2020). Most of the

1112 characterized perylenequinones produced by fungi share a common 4,9-dihydroxy-3,10-
1113 perylenequinone core and differ in side chain composition (Daub, Herrero and Chung, 2013).
1114 These pigments are very potent $^1O_2$-generating photosensitizers that have a crucial role in the
1115 establishment of pathogenic association between fungi and their plant host (Chung, 2011; Daub,
1116 Herrero and Chung, 2013). The production of these photosensitizers during infection causes lipid
1117 peroxidation and damage to the plasma membrane of the host cells, leading to leakage of
1118 nutrients into the intercellular spaces colonized by the pathogen (Fig. 9) (Daub, 1982; Daub and
1119 Briggs, 1983; Daub, Herrero and Chung, 2013).



1120

1121 **Figure 9 –** Schematic mechanism depicting the mode-of-action of phytopathogenic fungi that use
1122 photosensitizers for pathogenesis. After penetration through stomata, the fungus releases a
1123 photosensitizing molecule in the intercellular space. Activation of this molecule by light results in the
1124 production of reactive oxygen species that damage lipids at the cell membrane, releasing plant nutrients

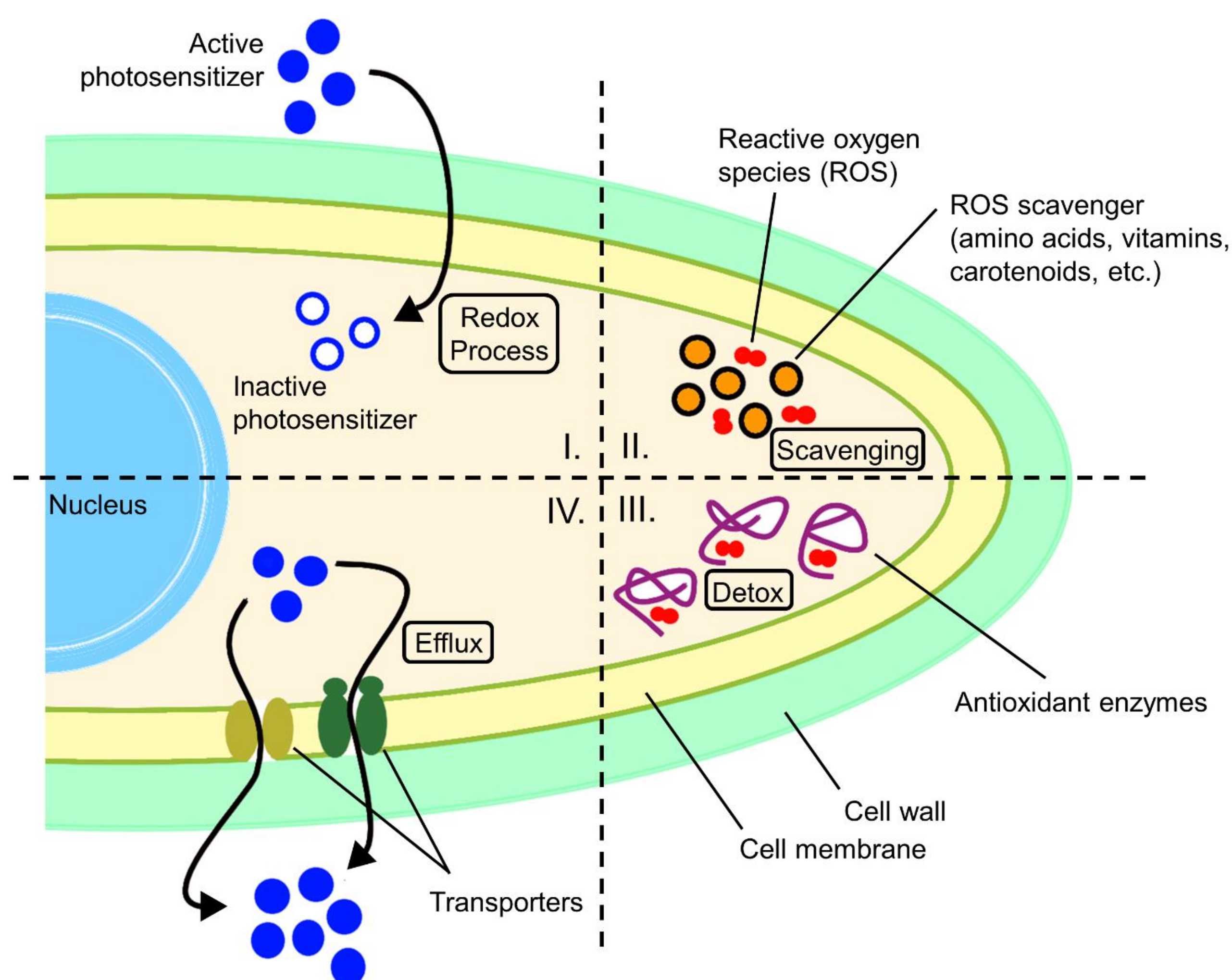
1125 into the medium and allowing sustained fungal growth. The depicted mechanism is based on that of
1126 *Cercospora* fungi.

1127

1128 Interestingly, the producing fungi are resistant to these photoactivated toxins (Daub,
1129 Herrero and Chung, 2013). Among these fungi, those of the genus *Cercospora* are the best
1130 studied (Daub, Herrero and Chung, 2013; Świderska-Burek *et al.*, 2020). *Cercospora* species
1131 cause devastating leaf-blighting disease on a wide range of important plant host species
1132 worldwide (Beseli *et al.*, 2015). Several species of the genus *Cercospora* produce large quantities
1133 of the phototoxin cercosporin, a lipid-soluble 4,9-hydroxyperylene-3,10-quinone derivative, which
1134 absorbs light and reacts with oxygen, generating reactive oxygen species, mostly $^1O_2$ (Daub and
1135 Hangarter, 1983). Unlike free-radical forms of ROS against which resistance mechanism are well
1136 characterized, the cellular bases of $^1O_2$ resistance are still being elucidated (Beseli, Noar and
1137 Daub, 2015; Daub, Herrero and Chung, 2013; Thomas *et al.*, 2020). Light is required, not only for
1138 cercosporin activation, but also for cercosporin production (Ehrenshaft and Upchurch, 1991).
1139 *Cercospora* species can, under light, produce and thrive in concentrations of cercosporin up to
1140 1000-fold higher than that which is lethal to other organisms (Ehrenshaft *et al.*, 1998). These fungi
1141 are highly tolerant not only to cercosporin but also to a broad range of structurally unrelated $^1O_2$-
1142 generating photosensitizers, including porphyrins, xanthenes, and phenothiazinium dyes
1143 (Ehrenshaft, Jenns and Daub, 1995). Some fungi other than *Cercospora* species, such as
1144 *Alternaria solani*, *Cladosporium cucumerinum*, *Cladosporium fulvum*, *Colletotrichum lagenarium*,
1145 *Verticillium* sp., *S. cerevisiae* and *Sporobolomyces* sp. are also highly resistant to cercosporin
1146 and other $^1O_2$-generating photosensitizers (Daub, 1987). Due to their high intrinsic tolerance,
1147 these fungi are excellent models for the elucidation of molecular and genetic bases of resistance
1148 to $^1O_2$-generating photosensitizers.

1149 Much of what is known about the tolerance of *Cercospora* to cercosporin and to other $^1O_2$-
1150 generating photosensitizers came from Daub's group and some of their reviews are

1151 recommended for an in-depth approach on this subject (Daub and Ehrenshaft, 2000; Daub, 1152 Herrero and Chung, 2005; Daub, Herrero and Chung, 2013; Świderska-Burek *et al.*, 2020). 1153 *Cercospora* auto-resistance to light-activated cercosporin is a complex and yet not completely 1154 understood characteristic mediated by multiple mechanisms including the reversible reduction 1155 and detoxification of the cercosporin inside the fungal cells, the production of $^1O_2$ quenchers, and 1156 the transport of the toxin out of the cells (Fig. 10) (Beseli *et al.*, 2015; Daub *et al.*, 1992; Daub, 1157 Herrero and Chung, 2013).



1158

1159 **Figure 10 –** Examples of mechanisms via which cells may be either tolerant or resistant to 1160 photodynamic inactivation. These mechanisms include: (I) inactivation of photosensitizers via redox 1161 reactions; (II) scavenging of reactive oxygen species by specialized molecules (e.g., carotenoids, vitamin 1162 B6); (III) detoxification of reactive oxygen species by antioxidant enzymes (e.g., superoxide dismutase); 1163 and (IV) efflux of photosensitizers from the intracellular medium by transporters. These mechanisms are

1164 based on current knowledge on tolerance/resistance to APDT on *Cercospora* fungi, but they are
1165 potentially present in all microorganisms.

1166 Carotenoids are the most efficient quenchers of $^1O_2$ identified in biological systems, and
1167 are able to quench not only $^1O_2$, but also the activated triplet state of $^1O_2$-generanting
1168 photosensitizers (Daub and Payne, 1989; Daub, Herrero and Chung, 2005; Kruk and Szymańska,
1169 2021). Carotenoids were also the first endogenous compounds identified that can protect fungi
1170 against cercosporin-based APDT (Daub and Payne, 1989). Carotenoid-deficient mutants of
1171 *Neurospora crassa* are significantly more sensitive to cercosporin than the carotenoid-producing
1172 wild-type isolates (Blanc, Tuveson and Sargent, 1976). Carotenoids also protect fungi against
1173 other $^1O_2$-generanting photosensitizers that damage the plasma membrane. Conidia of *N. crassa*
1174 and mycelia of *Ustilago violaceae* mutants lacking carotenoids are less tolerant to methylene
1175 blue- and toluidine blue O-based APDT than the wild-type strains (Thomas, Sargent and Tuveson,
1176 1981; Will, Newland and Reppe, 1984). However, experiments performed with *Cercospora*
1177 *nicotianae* showed a different and unexpected result (Ehrenshaft, Jenns and Daub, 1995). Target
1178 gene disruption was used to create carotenoid-minus mutants of a wild-type and two cercosporin-
1179 sensitive *C. nicotianae* mutants. These carotenoids-deficient mutants had similar sensitivity to
1180 either photoactivated cercosporin or five others $^1O_2$-generanting photosensitizers (rose bengal,
1181 hematoporphyrin, methylene blue, toluidine blue O, eosin Y) compared to the parent strains.
1182 Together, these data suggested that carotenoids are important to fungal tolerance to
1183 photoactivated cercosporin but they are not involved or have only a minor effect on *Cercospora*
1184 resistance to $^1O_2$-generating photosensitizers (Daub and Payne, 1989).

1185 The term vitamin B_6 is used to describe all biologically interconvertible forms of pyridoxine
1186 (Bilski *et al.*, 2000). Vitamin B_6 and its derivatives are good $^1O_2$ quenchers; also, the pyridoxine
1187 moiety can function as a redox quencher for excited cercosporin by forming the cercosporin
1188 radical anion (Bilski *et al.*, 2000). Mutants of *C. nicotianae* deficient in biosynthesis of vitamin B_6
1189 are highly sensitive to cercosporin and other $^1O_2$-generanting photosensitizers, such as

1190 hematoporphyrin, rose bengal, eosin Y, methylene blue, and toluidine blue O (Ehrenshaft *et al.*,
1191 1998; Ehrenshaft *et al.*, 1999b; Ehrenshaft *et al.*, 1999a). Analysis of cellular levels of vitamin B_6
1192 also showed that *C. nicotianae* has 2- to 3-fold higher levels of B_6 vitamers than the cercosporin-
1193 sensitive fungi *A. flavus* and *N. crassa* (Herrero and Daub, 2007).

1194 In contrast to $^1O_2$ quenchers, there is little evidence for a role of antioxidant enzymes in
1195 cercosporin resistance in *Cercospora*. *C. nicotianae* does not have higher levels of superoxide
1196 dismutase (SOD), catalase, or peroxidase activities as compared to cercosporin-based APDT
1197 sensitive fungi and there is also no difference in overall antioxidant activity (Daub, 1987).

1198 The ability to transport cercosporin and other photosensitizers out of the cell also
1199 contributes to fungal resistance to these compounds (Beseli *et al.*, 2015; Daub, Herrero and
1200 Chung, 2013). Both the Major Facilitator Superfamily (MFS) and ATP-binding cassette (ABC)
1201 family of transporters are able to transport cercosporin out of *Cercospora* cells and provide partial
1202 resistance against cercosporin-based APDT (Beseli *et al.*, 2015). Targeted disruption of the gene
1203 for CFP (*C*ercosporin *F*acilitator *P*rotein), an MFS transporter, in the soybean pathogen
1204 *Cercospora kikuchii* drastically reduced the production of cercosporin, greatly impaired virulence
1205 of the fungus, and increased sensitivity to exogenous cercosporin in comparison to the wild-type
1206 strain (Callahan *et al.*, 1999). Also, the transgenic expression of *CFP* gene in the cercosporin-
1207 sensitive fungus *Cochiobolus heterostrophus* resulted in increased tolerance to cercosporin due
1208 to its export out of the fungus (Upchurch *et al.*, 2002).

1209 The importance of MFS transporters to the resistance to $^1O_2$-generating photosensitizers
1210 was also observed in other fungal pathogens. *Bcmfs1*, an MFS transporter from *B. cinerea*,
1211 provides tolerance to cercosporin-based APDT (Hayashi, Schoonbeek and De Waard, 2002).
1212 Deletion mutants showed increased sensitivity to photoactivated cercosporin, while
1213 overexpression mutants displayed decreased sensitivity (Hayashi, Schoonbeek and De Waard,
1214 2002). Mutants of the citrus pathogen *A. alternata* lacking the *AaMFS19* gene, which encodes an

1215 MFS transporter, display profound sensitivity to the $^1O_2$-generating photosensitizers eosin Y, rose
1216 bengal, hematoporphyrin, methylene blue, and cercosporin (Chen *et al.*, 2017).

1217 The importance of ABC transporters to cercosporin resistance was demonstrated in *C.*
1218 *nicotianae*. Mutants with disruption of *ATR1*, which is an ABC transporter gene, had dramatic
1219 reductions in cercosporin production and also showed moderately higher sensitivity to
1220 cercosporin indicating that ATR1 acts as a cercosporin efflux pump and has a partial role in
1221 cercosporin-based APDT resistance (Amnuaykanjanasin and Daub, 2009). CnATR2, another
1222 ABC transporter involved in partial resistance to cercosporin, was recently characterized.
1223 Transformation and expression of *CnATR2* in the cercosporin-sensitive fungus *N. crassa*
1224 significantly increased cercoporin resistance. However, target gene disruption of *CnATR2* in the
1225 wild type *C. nicotianae* did not decrease resistance. The overexpression of the gene that codes
1226 for Snq2p, a well-characterized multidrug, ABC-type, efflux protein conferred resistance to
1227 cercosporin and to other $^1O_2$-generating photosensitizers such as methylene blue and toluidine
1228 blue O to an otherwise sensitive *S. cerevisiae* strain (Ververidis *et al.*, 2001). In contrast, the *snq2*
1229 null mutant was not more sensitive to methylene blue and toluidine blue O than a wild-type control
1230 strain (Ververidis *et al.*, 2001).

1231 Studies with redox-sensitive dyes, reducing agents, and with detection of reduced and
1232 oxidized forms of cercosporin indicated that the most relevant mechanism responsible for
1233 *Cercospora*'s resistance to cercosporin is the ability of these fungi to maintain cercosporin within
1234 the hyphae in a reduced form (Fig. 10) (Daub *et al.*, 1992; Daub and Ehrenshaft, 2000; Daub,
1235 Herrero and Chung, 2005; Daub, Herrero and Chung, 2013; Jenns and Daub, 1995; Świderska-
1236 Burek *et al.*, 2020). Reduced cercosporin is a poor generator of $^1O_2$, particularly in aqueous
1237 solution (Leisman and Daub, 1992). As the reduced form is labile, and readily reoxidizes on
1238 aeration or removal from the reducing agents, cercosporin that diffuses away from the fungal cell
1239 spontaneously reoxidizes to the photoactive form needed for the infection of the host plant (Daub
1240 *et al.*, 1992; Leisman and Daub, 1992).

1241 Other fungal species were also tested for cercosporin-reducing ability (Daub *et al.*, 1992).
1242 *A. alternata*, which is cercosporin-resistant, was able to reduce cercosporin but *A. flavus* and *N.*
1243 *crassa*, which are cercosporin-sensitive, had only limited ability. Evidence suggested that the
1244 reduction of the photosensitizer may be a generalized mechanism of resistance for *Cercospora*
1245 to other $^1O_2$-generating photosensitizers (Daub *et al.*, 1992). *Cercospora* species were also
1246 resistant to eosin Y- but not to rose bengal-based APDT. Microscopic observation showed that
1247 *Cercospora* species were not capable of reducing rose bengal but were capable of reducing eosin
1248 Y. The reduction of the photosensitizer as a protective mechanism was also observed in other
1249 fungal species. The over-expression of the gene *CPD1* (*C*ercosporin *P*hotosensitizer
1250 *D*etoxification) that codes for a putative plasma membrane-associated reductase conferred
1251 resistance to cercosporin, methylene blue and toluidine blue O in *S. cerevisiae* (Ververidis *et al.*,
1252 2001).

1253 It is often stated that, due to its multiple-target mode of action, the selection of fungi
1254 displaying resistance to APDT is unlikely. However, the existence of several fungal species that
1255 are intrinsically resistant to APDT with $^1O_2$-generating photosensitizer, indicates that the
1256 possibility of the emergence of tolerance to APDT in fungal species of medical or agricultural
1257 importance deserves more attention. A recent study performed by da Cruz and coworkers has
1258 showed that *C. abscissum* submitted to successive cycles of APDT can become more tolerant to
1259 the treatment, although the decrease in susceptibility was small. Also, the study revealed that the
1260 more-tolerant strain also accumulated higher amounts of carotenoids (da Cruz et al., in
1261 preparation). Unfortunately, other studies performing long-term experimentation with filamentous
1262 fungi to determine whether or not it is possible to select resistant strains during successive cycles
1263 of APDT are still lacking. There is no doubt that the understanding of the mechanisms responsible
1264 for the intrinsic resistance to APDT of some fungal species, such as of the genus *Cercospora*, will
1265 be important to understand and eventually anticipate a possible emergence of resistance to APDT
1266 in species of medical and agricultural importance, which may occur with the expansion of its use.

1267 The discussion above may seem heavily based on Type II reactions, i.e., those producing
1268 $^1O_2$. However, some of the mechanisms presented (Fig. 10) can also operate to avoid cell damage
1269 arising from Type I reactions. For instance, the redox processes that render photosensitizers not
1270 reactive to light would also prevent Type I reactions from occurring. Similarly, efflux pumps that
1271 prevent photosensitizers from being light activated inside cells do not discriminate between Type
1272 I and Type II photosensitizers.

1273 The same cannot be said about the other two mechanisms, i.e., detoxification and
1274 scavenging. Cells have known lines of defense against Type I ROS –such as superoxide anion
1275 radical and hydrogen peroxide– in the form of the enzymes superoxide dismutase, catalase, and
1276 glutathione reductase, as well as specific scavengers for these reactive species. Both the
1277 expression of the enzymes and the production/accumulation of scavengers can be modulated to
1278 achieve increased tolerance to photosensitizers operating via Type I reactions. On the other hand,
1279 there is no known first line of defense against $^1O_2$, so an effective antioxidant system may prevent
1280 exclusively against Type I photosensitizers. Evidence of this can be found on extremophilic
1281 microorganisms. The bacterium *Deinococcus radiodurans* has a remarkable antioxidant system
1282 that effectively protects the proteome from ionizing radiation, desiccation, and oxidative stresses
1283 at high levels (Qi *et al.*, 2020), making the microbe very tolerant to Type I ROS. Nonetheless, *D.*
1284 *radiodurans* cannot sustain the damages imposed by $^1O_2$-producing photosensitizers, with at
1285 least one report showing that its tolerance falls bellow that of *E. coli* (Nitzan and Ashkenazi, 1999;
1286 Schafer, Schmitz and Horneck, 1998).

1287 At present, it is not possible to say whether the tolerance mechanisms to Type I and to
1288 Type II photosensitizers would emerge and occur concomitantly (if at all). However, if tolerance
1289 to APDT is a multifactorial process operating at many levels, then alternating chemically-diverse
1290 photosensitizers that operate via different reactions will provide some protection against the
1291 emergence of tolerance.

1292

5. Conclusions and unresolved questions

Plants, as do microbes, produce a mechanistically (and chemically) diverse array of antimicrobials which can vary with species, habitat, and environmental conditions (Cray *et al.*, 2015a; Lievens *et al.*, 2015; Oren and Hallsworth, 2014; Wecke and Mascher, 2011; Suryawanshi *et al.*, 2015). Of these, photosensitizers can have elegant mechanisms and are some of the most potent antimicrobials; yet, they are relatively undervalued in relation to their biotechnological potential.

The above discussion covers the ecologies of naturally-occurring, photodynamic processes including the light-activated antimicrobial activities of some plant metabolites, and the intriguing use of the photodynamic process by some plant-pathogenic fungi as an important virulence factor. The use of natural and synthetic photosensitizers to kill plant-pathogenic fungi and foodborne pathogens were also reviewed and discussed. The inhibitory mechanisms of both natural and synthetic light-activated substances were covered in the contexts of microbial stress biology and agricultural biotechnology. Implications were also made in relation to treatment of clinical infections caused by opportunistic fungi pathogens, once considered only plant pathogens and/or saprotrophic.

The development of conventional pesticides is a complex, costly, and time-consuming process that can be divided into three main steps: (i) research on the synthesis and screening of molecules, (ii) product development; and (iii) registration. The research evaluates the biological, chemical, toxicological, environmental, and commercial characteristics of candidate molecules to be registered. The development includes several processes, such as optimization of formulation, assessment of products in field trials against different biological targets in a variety of crops, and evaluation of toxicological and environmental impacts. Finally, product data are submitted to different regulatory agencies, which may agree or disagree with the registration and commercialization (McDougall, 2016). Therefore, as in the development of conventional

pesticides, photosensitizers may be submitted to similar processes before large-scale use in agriculture. In addition, the average time spent by a company to develop a conventional pesticide is approximately 11 years and the cost is about US$ 286 million (McDougall, 2016). Currently, the cost for obtaining a photosensitizer-based product is unknown, and future research on economic feasibility is needed. Furthermore, there is still a considerable knowledge gap due to numerous unresolved questions. For example, although it is well established that APDT with most of the photosensitizers tested is able to kill, to a greater or lesser extent, most of the different species of fungi *in vitro*, little is known about the efficacy of the treatment in the field on different crops. An important issue that needs attention is the negative effect of shadowing, which may be caused by the plant canopy and/or by an extensive cloud cover lasting many days. Similarly, little is known about the side effects of the different photosensitizers on the host plants and in the environment. Also, formulations containing photosensitizers will have to be developed and approved for use in the field.

Despite its great potential, it seems that the development and use of APDT in agriculture has been delayed by the fact that this antimicrobial approach is unknown to the majority of agricultural professionals and by the apparent lack of interest by the chemical and pharmaceutical industries in the development of photosensitizer-based products for agricultural use. This parallels a similar lack of interest from these industries in the development of clinical anti-infectives based on this approach for both human and veterinary application. Here we showed that the use of photoantimicrobials is a viable and needed alternative to control plant- and foodborne pathogens, and has the potential to contribute to improving global food security.

Acknowledgments

This work was supported by grants 2012/15204-8 and 2016/11386-5 from the State of São Paulo Research Foundation (FAPESP) to GULB. We also thank the National Council for Scientific

and Technological Development for research grants to GULB (425998/2018-5 and 307738/2018-3) and to GJSJ (308202/2017-1 and 316910/2021-0), and for a post-doctoral fellowship (165191/2020-1) to GTPB.

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