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Light and Singlet Oxygen in Plant Defense Against Pathogens: Phototoxic Phenalenone Phytoalexins†

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ABSTRACT
Plants defend themselves from pathogen infections or mechanical injury by a number of mechanisms, including the induced biosynthesis of antimicrobial secondary metabolites. These compounds, termed phytoalexins, represent a very economical way to counteract hazard, because the carbon and energy resources are diverted to phytoalexin synthesis only at the early period of attack and only at its site. The occurrence of phenalenone chromophores in phytoalexins of plants originally nonphototoxic suggests that these plants respond to pathogen attacks by biosynthesizing singlet oxygen photosensitizers able to use solar energy for defense. This concept may have implications for the development of novel crop protection strategies.

Introduction

Plants cannot move to avoid environmental challenge such as pathogen infections, tissue damage, or adverse environmental conditions, and they have therefore evolved sophisticated mechanisms to perceive and respond to these threats. They protect themselves through a wide array of constitutive and induced defense mechanisms, which include, for example, programmed cell death, surface-to-air signaling, expression of defense proteins, and production of antimicrobial secondary metabolites.[1] The latter compounds, which have attracted the attention of chemists from different fields, can be divided into two groups: phytoalexins, which are synthesized de novo upon biotic or abiotic stress, and phytoanticipins, which are preformed antimicrobial metabolites.[2,3] The classification of an antimicrobial secondary metabolite in one or the other group does not depend on its chemical structure but on the mechanism of production. Hence, some compounds may be phytoalexins in one species and phytoanticipins in others.

Phytoalexins have several characteristics worth noting:[4] (i) prior to infection, they are generally undetectable in the plants; (ii) they are synthesized very rapidly, within hours following a microbial attack or stress situation; (iii) their formation is restricted to a local region around the infection site; (iv) they are toxic to a broad spectrum of fungal and bacterial pathogens of plants. Globally, phytoalexin production represents a very economical way to counteract certain hazards because the carbon and energy resources are diverted to phytoalexin synthesis only at the early period of attack and only at its site.[5] Moreover, this strategy avoids autotoxicity until a challenge arises, because the biological activity of these compounds is inherent in the definition of phytoalexins.[6]

Phytoalexin production is a common mechanism of resistance in a wide range of plants, although plant families vary in the types of secondary products biosynthesized for this purpose. To respond effectively to a pathogen invasion, plants must quickly recognize its presence and rapidly start phytoalexin production. Polysaccharide fragments from fungal cell walls are sometimes

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† Dedicated to the memory of Professor Christopher S. Foote, whose work and attitudes inspired so many of us.

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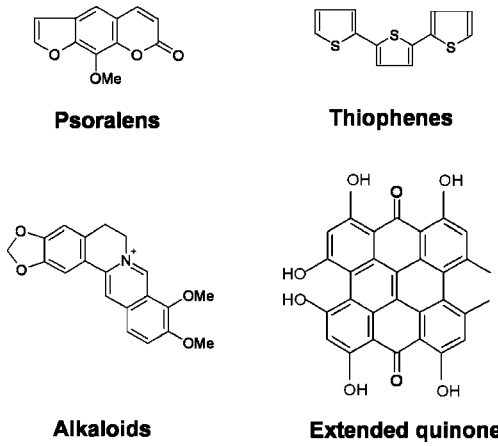


FIGURE 1. Common constitutive phototoxins (phytoanticipins) from different plant families.

involved in the recognition of fungal pathogens. These fragments, which probably arise from the action of hydrolytic defense enzymes secreted by the plant, trigger the synthesis of phytoalexins. In general, substances that stimulate the synthesis of phytoalexins are called "elicitors". Other fungal molecules, such as glycoproteins, peptides, and fatty acids, can also be elicitors of phytoalexin biosynthesis. It is noteworthy that pectin fragments from the plant's own cell wall, which may act as a signal for the presence of a wound (e.g., produced by an insect bite), also elicit phytoalexins in some species.[4]

Plant Phototoxins

To enhance the efficacy of their defense compounds, plants can also make use of light energy. Phototoxins, or photosensitizers, are secondary metabolites that catalyze biological actions following the absorption of light energy.[7] These reactions are often lethal toward organisms that are harmful to those plants, such as microorganisms, herbivores, or other competing plants.[7,8] A large number of constitutive phototoxins, that is, phytoanticipins with broad-spectrum biocidal action, have been isolated from different phytochemical classes. They can be grouped into a limited number of structural types, namely, acetophenones, furanocoumarins (or psoralens), polyacetylenes, thiophene derivatives, extended quinones, and alkaloids, reflecting a limited number of biosynthetic pathways (Figure 1).[9] In particular, psoralens from Apiaceae and the extended quinone hypericin from *Hypericum* sp. are among the most studied phototoxins. For some of these compounds, particularly thiophenes and furanocoumarins, substantial concentration increases are found in diseased plants, that is, they are also phytoalexins.[10,11] Thus, the plant's phototoxicity is enhanced upon pathogen attack.

According to the general scheme for photosensitized oxidation reactions,[12] two mechanisms of phototoxin action have been proposed (Figure 2): direct photooxidation of biologically relevant molecules by either electron or hydrogen abstraction and subsequent reaction of the radical species formed with nearby oxygen molecules (type I mechanism) and electronic excitation energy

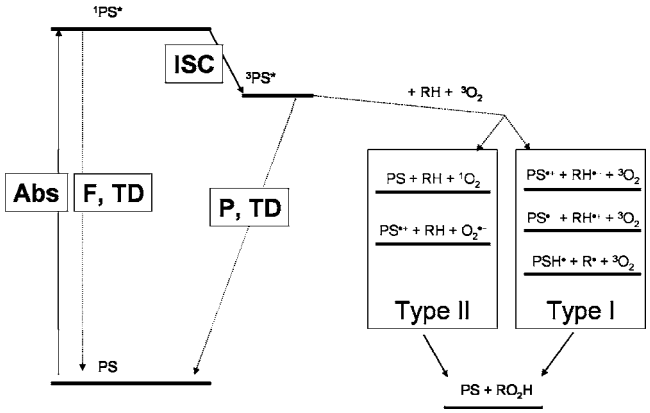


FIGURE 2. Energy diagram showing the relevant photophysical pathways involved in the photosensitized oxidation of a biological substrate (RH): Upon absorption of light energy, a photosensitizer (PS) in its electronic ground state is promoted to a higher-energy excited singlet state ($^1PS^*$), which may deactivate by either fluorescence (F) or thermal decay (TD) back to the ground state or may undergo intersystem crossing (ISC) to the triplet state ($^3PS^*$). The thus-produced triplet excited sensitizer may subsequently either deactivate back to the ground state by phosphorescence (P) or thermal decay (TD) or react primarily with RH (type I mechanism) or oxygen (type II mechanism) to finally produce the oxidation product RO_2H.

transfer from the phototoxin to molecular oxygen to form the metastable, highly oxidizing singlet molecular oxygen species ($O_2(^1\Delta_g)$, hereafter $^1O_2$), type II mechanism.[13] Both mechanisms rely primarily on the absorption of light energy by the photosensitizer, which promotes it to a higher-energy electronic excited state. The extra energy allows it to undergo the oxidation reactions outlined above.

Type I reactions are generally localized around the site of photosensitizer excitation given the low diffusivity and high reactivity of the intermediate species formed. Type II reactions, in contrast, may spread several millimeters around the site of singlet oxygen production, particularly since many plant phototoxins are present primarily in epidermal tissues and thus are in close contact with atmospheric oxygen. This enables the formation of $^1O_2$ in the gas phase, where it is 1000-fold more persistent than in the liquid phase, allowing it to travel over much longer distances.[14] Because $^1O_2$ reacts with a wide range of biological substrates ranging from membrane lipids to proteins to nucleic acids,[15] it is generally accepted that this reactive oxygen species participates in plant defense.[16] When present at the leaf surface, $^1O_2$ can interact with invaders such as fungal spores, bacteria, yeasts, and insect ovipositors. In fact, light-induced formation of $^1O_2$ in phototoxic plants has already been demonstrated.[17]

Phototoxic Phytoalexins: Biological Role of the Phenalenone Chromophore

Because they are not always present in plants, phytoalexins have been largely overlooked in standard screening programs searching for new natural products. Of specific interest here, we were surprised to discover that a number of plants belonging to the Haemodoraceae, Musaceae,

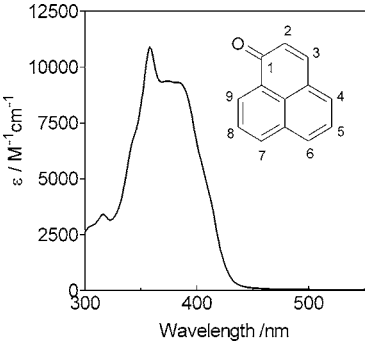


FIGURE 3. Molecular structure of phenalenone (PN) and its absorption spectrum in benzene.

Pontederiaceae and Strelitziaceae produce secondary metabolites containing the aromatic ketone phenalenone (PN, cf. Figure 3).[18−22] Of special interest are the Musaceae, in which these metabolites are exclusively phytoalexins, that is, are absent in healthy plants.[21−27]

Phenalenone (also called perinaphthenone) is a molecule very well known to photochemists working on $^1O_2$. It is a unique photosensitizer due to its ca. 100% quantum yield of $^1O_2$ production (Φ_Δ, defined as the number of $^1O_2$ molecules produced per absorbed quanta of light), a property that remains in all solvents investigated so far ranging from water to cyclohexane.[28−30] This advantage, added to its photostability and its low ability to deactivate $^1O_2$, have made PN one of the most widely used $^1O_2$ photosensitizers. Its absorption spectrum is shown in Figure 3. This outstanding aromatic ketone has been proposed as the universal reference for the determination of $^1O_2$ quantum yields, and it has attracted the interest of our laboratory for several years.[30−34]

We became thus immediately interested in these natural phenalenone derivatives, because the presence of this exceptional $^1O_2$ photosensitizer in their structure suggested that electronic excitation of the PN moiety (by exposure to light or as the result of enzymatic processes[35,36]) might be a component in the process of plant protection by these metabolites. Particularly for Musaceae, plants not listed as phototoxic, the confirmation of this hypothesis would provide new insights into the role of light in plant protection: the plant would become phototoxic in response to pathogen attack through the rapid biosynthesis of a photosensitizer. We explored the photophysics, photochemistry, and photobiology of these phenalenone phytoalexins as a means to document the role of the PN chromophore in plant−pathogen interactions.

The first compounds that we studied were phenylphenalenone phytoalexins from banana plants (Musaceae) (Figure 4). Biosynthesis of 4- and 9-phenyl-substituted phenalenones in banana plants is stimulated upon infection by *Mycosphaerella fijiensis,* a pathogen that greatly reduces the growth of the leaves,[23] or with *Fusarium oxysporum*, the agent responsible for the Panama disease.[24−26] They are biosynthesized from phenylalanine, tyrosine, and acetate units[37] and are found in leaves, fruits, and rhizomes of the infected plants (Figure 4).

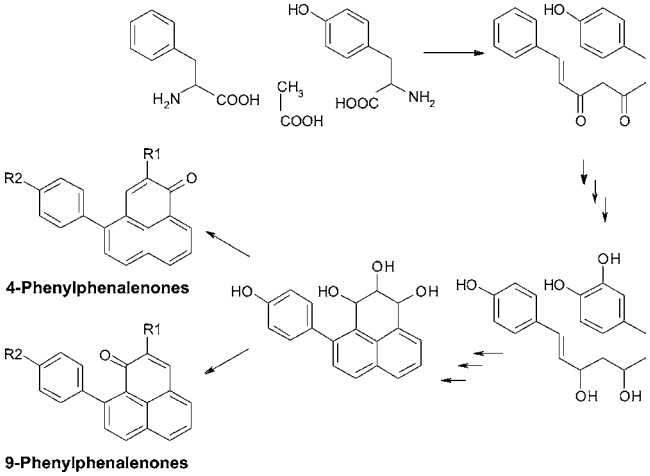


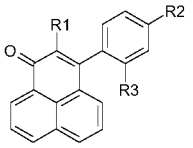
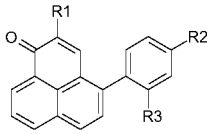
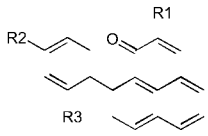
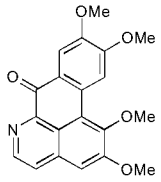
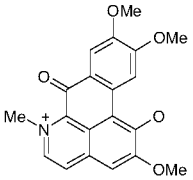
FIGURE 4. Biosynthesis of 4- and 9-phenylphenalenones.[37] R1 and R2 can vary between OH/OMe and H/OH/OMe, respectively.

Indeed, we found that, upon absorption of light energy, all phenylphenalenones examined sensitized the production of $^1O_2$ in polar and nonpolar media. Photobiological tests revealed that antifungal activity of these compounds toward *F. oxysporum* is enhanced in the presence of light. Moreover, the IC_{50} values under illumination correlated well with the Φ_Δ values, and the antifungal activity was enhanced to a maximum of 4-fold in D_2O-supplemented growth medium, which is a fingerprint for $^1O_2$-mediated reactions. For comparison, a 5-fold increase was observed for the parent PN with $\Phi_\Delta = 1$. These facts together suggest the intermediacy of $^1O_2$ produced by electronic excitation of the phenylphenalenone phytoalexins.[38]

Remarkably, 4-phenylphenalenones have been described only as phytoalexins and only in Musaceae species, whereas 9-phenylphenalenones are phytoalexins in Musaceae but exist also as phytoanticipins in other plant species, for example members of family Haemodoraceae, where they were first isolated,[18,39] and of family Strelitziaceae.[22] They confer toxicity to plants such as *Lachnanthes tinctoria* (Haemodoraceae), an effect soon recognized to be induced by light.[40] Kornfeld and Edwards further showed that the pigments in *Lachnanthes tinctoria* are photodynamic agents.[41] As a side note, phototoxicity of species of *Lachnanthes* was first suggested in Charles Darwin's treatise "On the Origin of Species"[42] in which he reported that effects of ingestion of this plant in pigs depend on their skin color, although no explicit connection to light exposure was made at the time.

From the photophysical point of view, two clearly distinct behaviors were found in these two series of phenylphenalenones. Like the parent PN, phenylphenalenones substituted in the 3- and 4-position generally have large Φ_Δ values (3-phenylphenalenones are compounds of synthetic origin that we added for completeness of our photophysical studies). However, for the 9-phenylphenalenones, values of Φ_Δ are significantly smaller (Table 1). Time-resolved absorption spectroscopy and $^1O_2$ phosphorescence detection, as well as cyclic voltammetry, among other techniques, suggest that this difference is caused by a photoinduced intramolecular charge-transfer

Table 1. Φ_Δ Values for Phenalenone and Phenalenone-like Photosensitizers in Benzene[38,43]

		R_1	R_2	R_3	Φ_Δ
3-Phenylphenalenones		H	OMe	H	0.94
4-Phenylphenalenones		OMe	H	H	0.72
		OH	OMe	H	0.12
9-Phenylphenalenones		H	H	NO_2	0.51
		H	H	H	0.08
		OMe	H	H	0.08
		H	OMe	H	0.01
		OH	H	H	0.03
		OH	OH	H	0.01
		OH	OMe	H	0.002
Oxoglaucine		--	--	--	1.0
Corunnine		--	--	--	0.025[a]

[a] In acetonitrile.

process from the electron-rich phenyl to the PN moiety, which operates efficiently only when the phenyl group is the 9-position and involves both the singlet and triplet excited states of PN.[43] This is best inferred by inspection of the Φ_Δ data in Table 1: for compounds with identical R_1, Φ_Δ decreases as the electron-donating ability of R_2 or R_3 increases (OMe > OH > H > NO_2). One may speculate that plants in which 9-phenylphenalenones are constitutive use this effect to balance the ability to defend themselves against pathogens with the autotoxicity that a high quantum yield of $^1O_2$ might inflict. In this sense, it is remarkable that 4-phenylphenalenones, with large Φ_Δ, are found only in infected plants, that is, are not biosynthesized unless necessary. Our observation that phytoalexins are more efficient $^1O_2$ photosensitizers than structurally related phytoanticipins opens the door to search for a similar trend in other groups of photoactive secondary metabolites.

While our data suggest the main intermediacy of $^1O_2$, that is, type II mechanism, type I photodynamic reactions, in particular photoinduced charge-transfer reactions, cannot be ruled out in anaerobic environments, because the rather low reduction potential of PN (−1.1 V vs SCE) should facilitate its reactivity toward electron-rich substrates. Indeed, a rate constant $k_q = 3.0 \times 10^9\ M^{-1}{\cdot}s^{-1}$ has been measured for the reaction between triplet PN and diazabicyclo[2.2.2]octane, DABCO.[34]

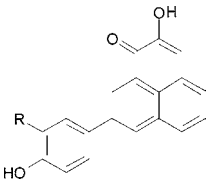


FIGURE 5. Structure of recently isolated 8-phenylphenalenones,[44] R = H or OH.

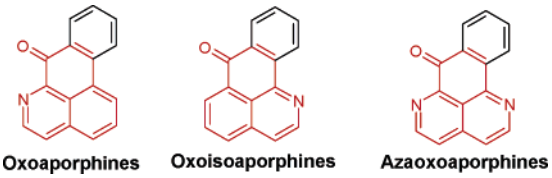


FIGURE 6. Molecular structure of phenalenone-like alkaloids.

We note that a new group of constitutive phenylphenalenones, this time substituted in the 8-position (Figure 5), have very recently been isolated from *Eichhornia crassipes* (Pontederiaceae).[44] It will be interesting to find out whether these compounds are also phytoalexins and can act as $^1O_2$ photosensitizers.

Phenalenone-like Phytoalexins

Alkaloids structurally related to PN, that is, oxoaporphines, oxoisoaporphines, and azaoxoaporphines (Figure 6), have also been isolated from plants belonging to different families such as Annonaceae,[45−47] Lauraceae,[48] Magnoliaceae,[49] Fumariaceae,[50,51] Menispermaceae,[52] and Papaveraceae.[53,54] Of these, only oxoaporphines have thus far been positively identified as phytoalexins. Oxoglaucine (OG; Figure 7) is a phytoalexin from the Magnoliaceae family that is elicited in the plant after mechanical injury but is absent in the healthy plant.[49] The reduced form of OG, glaucine, is the main alkaloid found in healthy plants.[49,55] Upon mechanical injury, glaucine is rapidly oxidized to OG (major product) and to other alkaloids such as corunnine and pontevedrine (minor products) by chemical and photochemical processes, in particular by $^1O_2$,[53,56−58] which suggests that OG is formed *in planta* by (photo)chemical or enzymatic oxidation of glaucine (Figure 7).

When we examined OG for its photosensitization ability, we found that it is an efficient $^1O_2$ photosensitizer, indeed much better than the minor product corunnine (Table 1).[59] The efficiency of $^1O_2$ photosensitization by OG is close to unity in nonpolar environments, similarly to PN, which is, to the best of our knowledge, an unprecedented value for a natural alkaloid. As shown in Figure 8, Φ_Δ decreases when the polarity of its environment increases, a behavior that has been rationalized in terms of the solvent effects on the energy of the excited states.[59] Therefore, in the case of OG, the modulation of Φ_Δ arises from the particular effect of polarity and hydrogen-bonding ability of its environment, rather than by a photoinduced charge-transfer process as in 9-phenylphenalenones.

One may speculate that the main role of OG is to prevent pathogens from invading the plant through the wounds caused by mechanical injury. Interestingly, OG is a rather inefficient $^1O_2$ quencher ($k_q = 8 \times 10^5$ $M^{-1}\cdot s^{-1}$ in perdeuterated benzene),[59] unlike the related alkaloids boldine and glaucine, for which an antioxidant role has been suggested.[58] Given its low quenching ability, it is unlikely that OG acts as an antioxidant. Furthermore, our results suggest a prooxidant role of glaucine, since OG is one of its oxidation products. It will be interesting to investigate whether other PN-like alkaloids, such as oxoisoaporphines[60−63] and azaoxoaporphines,[45,64,65] play a similar role in other plants.

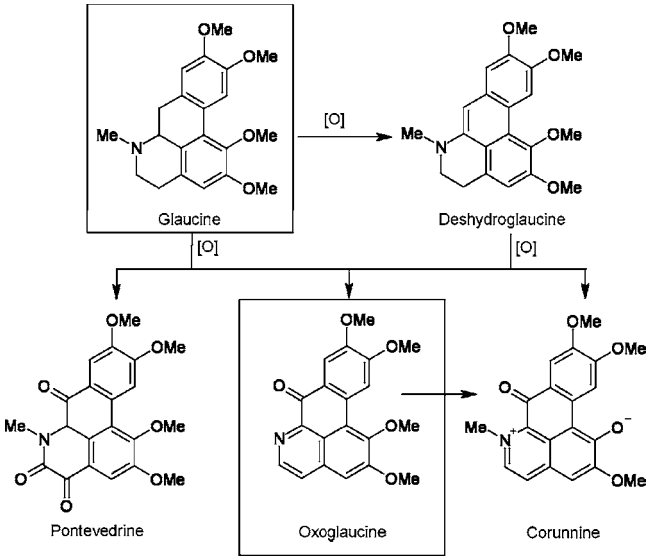


FIGURE 7. Oxidation pathways of glaucine to yield the related phytoalexins oxoglaucine (major product) and corunnine and pontevedrine (minor products).

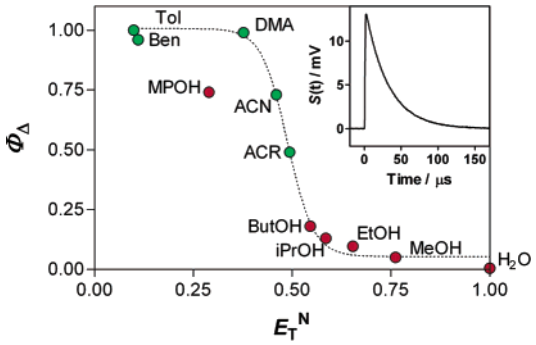


FIGURE 8. $^1O_2$ quantum yields for oxoglaucine (OG) vs solvent polarity (E_T^N) for nonprotic (green) and protic (red) solvents. Tol = toluene; Ben = benzene, DMA = dimethylacetamide; ACN = acetonitrile; MPOH = 2,4-methyl-3-pentanol; ACR = acrylonitrile; ButOH = butanol; iPrOH = 2-propanol; EtOH = ethanol; MeOH = methanol. Inset shows the time profile of the $^1O_2$ 1270 nm phosphorescence upon pulsed laser flash photolysis of OG in benzene.

Other Phototoxic Phytoalexins

The concept of a photofunctional phytoalexin has already been proposed in the literature, even though the phenalenones seem to have gone unnoticed in this context.[10,16,66,67] For instance, the activity of isoflavonoid phytoalexins in vitro is enhanced by the presence of light, A sesquiterpenoid cotton phytoalexin, 2,7-dihydroxycadalene, is photo-

active toward cauliflower mosaic virus.[67] Other well-established phototoxic phytoalexins are the furanocoumarins, the thiophenes, and the β-carbolines.[10,13,68]

A careful literature search unveils other phytoalexins whose activity has never been linked to light or electronic excitation but whose structural similarity with well-known $^1O_2$ photosensitizers leads to the reasonable speculation that photoinduced processes might contribute to their activity. Examples of such compounds are some natural anthraquinones, which have been described as phytoalexins, from several plant families such as Rubiaceae or Malvaceae.[69−72] In parallel, the in vitro $^1O_2$ photosensitizing ability of closely related compounds has been demonstrated.[73−76] In our opinion, it is only a matter of time before the "family" of phototoxic phytoalexins expands, because only a limited number of phytoalexins have been tested so far for their light-dependent activity and, in particular, for their ability to photosensitize $^1O_2$.

Concluding Remarks and Outlook

The data collected so far demonstrate that plants in which the rapid biosynthesis of phenalenone derivatives is induced by fungal attack or mechanical injury are able to use environmental light and oxygen to produce cytotoxic species, in particular, $^1O_2$, to respond to such challenges. We have shown that the ability of these compounds to photosenstize $^1O_2$ can be modulated by solvent- or substituent-induced effects. The latter modulation is correlated with their phototoxicity toward the eliciting agent itself, *F. oxysporum*. In addition to its role as a primary toxic species, $^1O_2$ may also act as a signaling species that triggers a range of responses at the first stages after stress conditions.[77,78] Both processes should probably be regarded as complementary, taking place at different stages of the postinfectional response.

The development of techniques for the detection of $^1O_2$ in plant tissue, in particular imaging techniques, will give further insight on its presence and role in plants. Recent progress in this field includes specific fluorescent probes compatible with plant tissue, such as DanePy[79−82] and Singlet Oxygen Sensor Green,[83,84] that can be combined with fluorescence microscopy. An alternative and highly promising technique is the use of microscopy coupled to NIR detection of the phosphorescence emission of $^1O_2$ at 1270 nm.[85] The study of the events taking place upon absorption of light by phytoalexins holds the potential for the development of novel, environmentally safer crop protection strategies based on sunlight-activated compounds,[86,87] for example, by incorporation of genes encoding for phytoalexin synthesis or by stimulating plants to produce their own phytoalexins.[5]

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