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Light- and singlet oxygen-mediated antifungal activity of phenylphenalenone phytoalexins

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The light-induced singlet oxygen production and antifungal activity of phenylphenalenone phytoalexins isolated from infected banana plants (*Musa acuminata*) are reported. Upon absorption of light energy all studied phenylphenalenones sensitise the production of singlet oxygen in polar and non-polar media. Antifungal activity of these compounds towards *Fusarium oxysporum* is enhanced in the presence of light. These results, together with the correlation of IC_{50} values under illumination with the quantum yield of singlet oxygen production and the enhancing effect of D_2O on the antifungal activity, suggest the intermediacy of singlet oxygen produced by electronic excitation of the phenylphenalenone phytoalexins.

Introduction

Plants protect themselves against pathogen infections, tissue damage, or adverse environmental conditions through a wide array of constitutive and induced defence mechanisms, which include, *e.g.*, programmed cell death, surface-to-air signalling, expression of defence proteins, and production of antimicrobial secondary metabolites.[1–7] The latter compounds can be divided into two groups: phytoalexins, which are synthesized *de novo* upon pathogen infection, and phytoanticipins, which are pre-formed antimicrobial metabolites.[8] In concurrence with the above mechanisms, some plants use light for defence purposes. Thus, a large number of constitutive phototoxins, *i.e.* phytoanticipins, with broad-spectrum biocidal action have been isolated from different phytochemical classes. Phototoxins or photosensitizers are secondary metabolites that catalyse biological actions following the absorption of light energy.[9] These reactions are often lethal towards organisms that are harmful to those plants, like microorganisms, herbivores, or other competing plants.[9,10] Structurally, they can be grouped into a limited number of families, namely acetophenones, furocoumarins, polyacetylenes, thiophene derivatives, extended quinones, and alkaloids, which also reflects a limited number of biosynthetic pathways.[11] Two possible mechanisms of action have been proposed: direct photooxidation of biologically relevant molecules (type I mechanism), and excitation energy transfer from the phototoxin to molecular oxygen, to form the highly oxidising singlet oxygen [$O_2(^1\Delta_g)$, hereafter $^1O_2$] (type II mechanism).[12] This reactive oxygen species can participate in the plant defence mechanisms[13] and light-induced formation of $^1O_2$ in phototoxic plants has already been demonstrated.[14]

Banana plants (*Musa acuminata*) infected with *Mycosphaerella fijiensis*, a pathogen that greatly reduces the growth of the leaves,[15] or with *Fusarium oxysporum*, the agent responsible for the Panama disease,[16–18] contain a group of compounds termed phenylphenalenones (Fig. 1). These metabolites are absent in non-infected susceptible banana plants[16–18] but are constitutively synthesised in the pathogen-resistant SH-348 cultivar.[19] Some of these phenylphenalenones are endowed with strong antifungal activity,[20–22] which has been suggested to be consequence of DNA intercalation, radical formation, or oxygen activation.[22] These findings lead us to propose that these compounds act as protective phytoalexins against different types of phytopathogens in *Musaceae*.

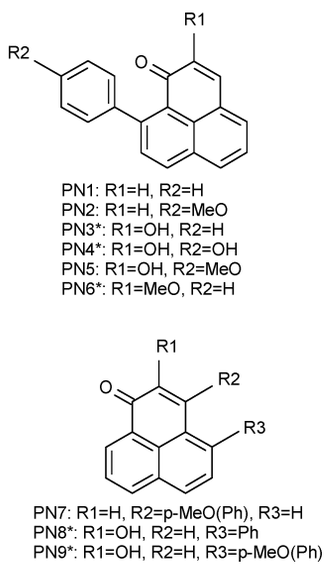

Fig. 1 Structure of phenylphenalenones studied. Natural phytoalexins are marked with *.

Phenylphenalenones are biosynthesised from two phenylpropanoid units,[20,23] and are found in leaves, fruits and rhizomes of the infected plants. Structurally related substances are also found in the same cultivars infected with the burrowing nematode *Radopholus similis*[24] or conidia of *Colletotichium musae*, the casual pathogen of anthracnose in banana fruits.[20,25]

The presence of the potent $^1O_2$ photosensitiser chromophore phenalenone (PN)[26–28] in the skeleton of these phytoalexins suggests that electronic excitation of the PN moiety (*e.g.* by exposure to light but also as the result of enzymatic processes as in α-terthienyl[29,30]) might be an important component in the process of plant protection by these phytoalexins. In fact, similar phenylphenalenones, previously described as constitutive compounds in *Haemodoraceae* and *Pontederiaceae* species,[31] conferred toxicity to plants such as *Lachnantes tinctoria*, an effect later demonstrated to be induced by light.[32] Interestingly,

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Lachnantes phototoxicity was already hinted in Darwin's treatise 'On the Origin of Species'.[33]

Herein we report a photophysical and photobiological study of a series of phenylphenalenone phytoalexins (PN3, PN4, PN6, PN8 and PN9), as well as of other phenalenones of synthetic origin (PN1, PN2, PN5 and PN7) included for completeness (Fig. 1). We show that these compounds inhibit *Fusarium* growth *in vitro* in a light-dependent manner through the intermediacy of $^1O_2$.

Materials and methods

Chemicals

The naturally occurring phenylphenalenones (PN3, PN4, PN6, PN8 and PN9) were isolated from infected *M. Acuminata* cultivars and/or synthesised from the parent PN similarly to the non-natural compounds (PN1, PN2, PN5 and PN7), as reported elsewhere.[15–18,23] PN, deuterium oxide (D_2O), methyl alcohol-d (CH_3OD) and dimethylsulfoxide (DMSO) were obtained from Aldrich (Madrid, Spain). Benzene was purchased from SdS (Peypin, France; Spectrosol quality). Potato-dextrose-agar (PDA) medium was obtained from OXOID (Madrid, Spain).

Characterization of phenylphenalenones

^1H, ^{13}C and bidimensional NMR spectra were recorded on Bruker AMX400 and Wp200SY spectrometers. IR spectra were recorded on a Bruker IFS 28/55 (FTIR) spectrophotometer. UV-VIS spectra were measured using a Jasco V-560 spectrophotometer and low-resolution mass spectra were obtained using a Hewlett-Packard mass-spectrometer model 5995.

Fungal pathogen

Fusarium oxysporum f. sp. *cubense* race 4 was obtained from monosporic cultures (ICIA, Instituto Canario de Investigación Agraria) growing in PDA, potato extract (4 g l^{-1}), glucose (20 g l^{-1}) and agar (15 g l^{-1}), pH 5.6. The fungus was subcultured weekly in Petri dishes containing PDA, cutting sections of 0.5 cm of diameter with a cork borer from the younger edge of another actively growing fungus. Every cylinder of mycelium was put upside down in the centre of a new Petri dish containing PDA. The fungus was grown for 7 days in the dark at 26 ± 2 °C.

Spectroscopic studies

Absorption spectra were recorded with a Varian Cary 4E spectrophotometer. Fluorescence spectra were recorded with a Shimadzu RF540 spectrofluorometer. The fluorescence quantum yields, ϕ_F, were determined comparing the area under the corrected emission curves for the samples and anthracene in ethanol as standard ($\phi_F = 0.27$),[34] and correcting for variations in the solvent refractive index. Transient spectra and kinetics were determined using nanosecond laser flash-photolysis. $^1O_2$ production was studied by time-resolved NIR phosphorescence detection (TRPD). A nitrogen laser (Radiant Dyes Accessories GmbH, RDN 50/25 λ_{exc} = 337 nm) or a Nd:YAG laser (Continuum Surelite 10, 3rd harmonic, 355 nm) were used for excitation. The laser fluence was varied using a variable neutral density filter and measured diverting a small fraction of the beam onto a pyroelectric energy meter. For TRPD, the concomitant NIR luminescence was detected at 90° with a liquid-nitrogen cooled germanium diode (North-Coast, EOL-817P) mounted behind a 1050 nm cut-off silicon filter and a 1270 nm interference filter. For laser flash-photolysis, changes in sample absorbance were detected using a Hamamatsu R928 photomultiplier to monitor the intensity variations of an analyzing beam produced by a 75 W short arc Xe lamp (USHIO). For both series of experiments the signal was fed to a Lecroy 9410 oscilloscope for digitizing and averaging (typically 100 shots) and finally transferred to a PC computer for data storage and analysis. The $^1O_2$ quantum yields, ϕ_Δ, were determined by comparing the $^1O_2$ phosphorescence intensity shown by the compounds to that produced by an optically-matched sample of the reference PN, for which $\phi_\Delta = 0.93$ in benzene and $\phi_\Delta = 0.97$ in CH_3OD.[26–28] The time dependence of the photosensitised $^1O_2$ phosphorescence intensity $S(t)$ is described by eqn. (1), where $S(0)$ is a quantity proportional to ϕ_Δ, and τ_T and τ_Δ are the lifetimes of the sensitiser's triplet state and of singlet oxygen, respectively:

$$S(t) = S(0)\frac{\tau_\Delta}{\tau_T - \tau_\Delta}\left(e^{-t/\tau_T} - e^{-t/\tau_\Delta}\right) \quad (1)$$

$S(0)$ was determined by fitting eqn. (1) to the observed phosphorescence intensity.

Fungitoxicity assays

The fungitoxicity of the phenylphenalenones was assessed from their ability to inhibit the hyphal growth.[35,36] The compounds were dissolved in DMSO and the solutions were filter-sterilised and stored in the dark. An aliquot of 10 µl of each phenalenone solution was added to 1 ml of PDA still liquid (at around 40 °C) in an Eppendorf tube. The resulting supplemented culture medium was then extended on a previously sterilised microscopic slide. The fungus was always used in active-growing phase after 3 to 7 days of subculture. A fresh suspension of spores was prepared by adding 5 ml of sterile water over the fungus and scrapping the mycelium with a sterile spatula. The suspension obtained was then filtered through sterile ashless filter paper (Whatman, 15 to 20 µpore size) and the spore concentration was determined microscopically using a Neubauer chamber, adjusting it when necessary to 1×10^8 spores ml^{-1}. Suspended spores (5 µl) were distributed over the surface of the solidified medium. The slides were then incubated at 26 ± 2 °C in hermetically sealed plastic boxes in order to reach a high humidity percentage, and were maintained during 15 h either in the dark or in the light. In the latter case, the boxes were placed at 40 cm from 4 + 4 white light tubes (Sylvania, ES Standard, 18W, Daylight 154) over and below them in an incubation chamber. Fungal growth was stopped by immersing the culture medium in 0.01% lactophenol cotton blue solution. The hyphal length of typically 20 germinating spores per slide and per replica was measured using a light microscope Zeiss Axiostar with a graduated scale in one of its oculars.

Statistical analysis

For each experiment, the results of three independent replicates were used to calculate the average hyphal length and typical error. The results obtained for each compound and concentration were subjected to an initial exploratory analysis to corroborate the normal distribution of data (Shapiro–Wilk) and the homogeneity of variance, followed by ANOVA analysis and the Tukey *post-hoc* test. The IC_{50} value, defined as the concentration of a compound required to reduce fungal growth to 50% of the control value,[37] was obtained by interpolation in the corresponding hyphal length *vs.* phenalenone concentration plots. All statistical analyses were done using the SPSS software, version 10.0 (www.spss.com).

Results and discussion

Photophysical studies

The absorption spectra of phenylphenalenones PN1–PN9 in benzene are given in Fig. 2. Clearly, two groups can be distinguished. The first one (panels a and b) includes compounds

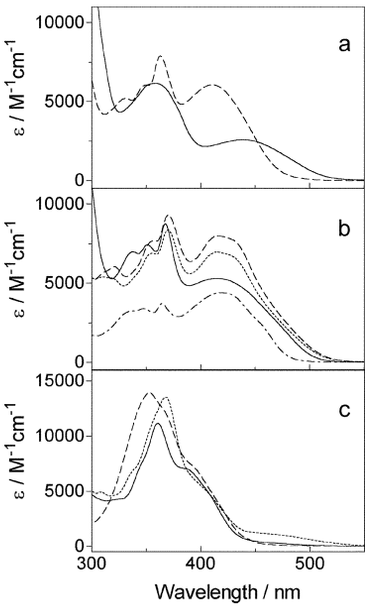

Fig. 2 Absorption spectra of the phenylphenalenones in benzene. (a) MeO-substituted at position 2: PN6 (---) and PN9 (——). (b) OH-substituted at position 2: PN3 (——), PN4 (···), PN5 (---), PN8 (-··-). (c) Unsubstituted at position 2: PN1 (——), PN2 (···) and PN7 (---).

bearing an electron-donating group at position 2 (PN3, PN4, PN5, PN6, PN8 and PN9) characterised by an intense band in the visible spectral region, as already noted for amino- or hydroxy- derivatives of anthraquinone and fluorenone.[38] This band arises from the conjugation of the substituent with the π-electron system of the phenalenone chromophore. The second group of compounds (panel c), which are unsubstituted at position 2, lacks this visible-region band. Substitution at positions 3 or 9 has a minor effect. In all cases bathochromic shifts of the lowest-energy band are observed upon increasing the solvent polarity, consistent with a ππ* excited singlet state. All are essentially non-fluorescent in benzene ($\phi_F < 10^{-3}$).

Using TRPD, $^1O_2$ formation was observed for all compounds in polar and non-polar media. The ϕ_Δ values ranged from 0.94 to 0.002 (Fig. 3 and Table 1). Analysis of the ϕ_Δ data in Table 1 reveals two clearly distinct behaviours depending on the position of the phenyl group. Phenalenones bearing a phenyl group at position 9 (PN1 to PN6) show ϕ_Δ values lower than 0.1, while the ϕ_Δ values for PN7–PN9, which are 3- and 4-phenyl-substitued, are high and similar to that of the bare PN.

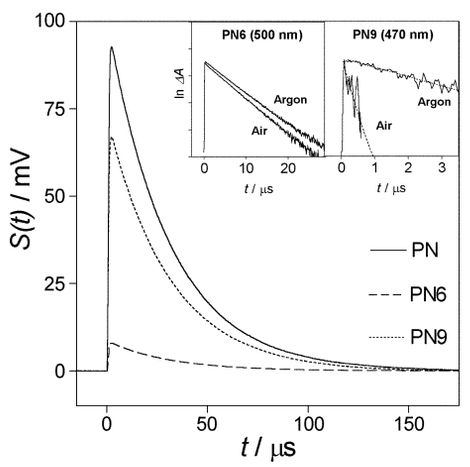

Fig. 3 Time dependence of 1270 nm $^1O_2$ phosphorescence intensity rise and decay upon excitation at 355 nm of a 9-phenylphenalenone (PN6) and a 4-phenylphenalenone (PN9) in benzene. For comparison, the signal for the reference sensitiser phenalenone (PN, $\phi_\Delta = 0.93$) is also shown. Insets: transient absorption changes observed upon nanosecond laser flash-photolysis at 355 nm in benzene.

Table 1 ϕ_Δ values for phenalenones in benzene and CH_3OD (10% error bars)

Phenalenone	Benzene	CH_3OD
PN	0.93	0.97
PN1	0.08	0.07
PN2	0.01	0.01
PN3*	0.03	0.03
PN4*	0.01	0.007
PN5	0.002	0.008
PN6*	0.08	0.04
PN7	0.94	[a]
PN8*	0.12	[a]
PN9*	0.72	[a]

[a] Not measured.

Table 2 Antifungal activities (IC_{50}/μM) of phenylphenalenones in the dark and in the presence of light

Phenylphenalenone	Dark	Light
PN	>1000	<1.5
PN1	83	9.0
PN2	96	15
PN3*	69	[a]
PN4*	72	29
PN5	[a]	[a]
PN6*	>100	3.8
PN7	65	<1
PN8*	74	1.5
PN9*	[b]	[b]

[a] Not active. [b] Not measured.

Interestingly, 9-phenylphenalenones are both phytoanticipins (*i.e.* constitutive) and phytoalexins, whereas the more potent photosensitisers 4-phenylphenalenones are only found in infected susceptible plants (*i.e.* are exclusively phytoalexins).[16,31]

Nanosecond laser flash-photolysis experiments confirmed the above classification (insets Fig. 3). In 3- and 4-phenylphenalenones formation of phenalenone triplet state was observed, as demonstrated by the sensitivity of the transient decay rate to oxygen. The triplet lifetimes in Ar-saturated benzene solutions were 35.0, 10.2 and 3.2 μs for PN7, PN8 and PN9 respectively. Oxygen quenched the transients with rate constants close to the diffusion-control limit (1–2 10^9 M^{-1} s^{-1}).[28] In the case of 9-phenylphenalenones PN1–PN3 and PN6, transients in the microsecond timescale were observed which could not be quenched by oxygen. Clearly, other photochemical processes compete with $^1O_2$ production in 9-phenylphenalenones, yielding lower, albeit non-zero, ϕ_Δ values. A detailed analysis of such competing processes is beyond the scope of this paper and will be reported elsewhere.

Antifungal activity of phenalenones

The IC_{50} values of phenylphenalenones towards *Fusarium oxysporum* were obtained after experiments in the concentration range of 1 to 100 μM, except for PN5, which crystallised in the culture medium at 100 μM and for which the maximum concentration was 30 μM. For commercial PN, concentrations of 300 μM and 1 mM were also tested. The IC_{50} values in the dark and in the presence of light are shown in Table 2.

Phenalenones PN3, PN4 and PN8 (phytoalexins) as well as PN1 and PN7 (non-natural) show antifungal activity already in the dark, consistent with earlier reports.[20,22,25] Their IC_{50} towards *F. oxysporum* is comparable to that of other phytoalexins.[39–41] The phytoalexin PN6 and the non-natural compounds PN and PN2 show little activity, and the non-natural PN5 is totally inactive. With the exception of PN3 and PN5, the presence of light greatly enhances the antifungal activity of all phenalenones (Table 2). The increase is especially dramatic for

PN, PN7 and PN8. The values of IC_{50} in the presence of light show good correlation with the quantum yields of $^1O_2$ production by the phenalenones (Table 1).

To confirm the participation of $^1O_2$ in phenylphenalenone phototoxicity, we conducted experiments in a D_2O-based culture medium, since $^1O_2$ has a much longer lifetime in D_2O than in H_2O.[42] Fig. 4 shows the reduction of hyphal length after irradiation in H_2O and D_2O media. All photoactive phenalenones reduced the length of the hyphae to a greater extent in D_2O than in H_2O. These results, together with the evidence that irradiation produces $^1O_2$, and with the correlation observed between IC_{50} and ϕ_Δ values, point to participation of light and $^1O_2$ in the antifungal activity of phenylphenalenone phytoalexins.

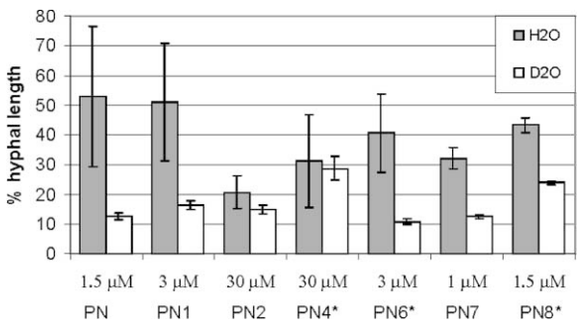

Fig. 4 Fungal growth in response to each compound in normal and in deuterium-supplemented culture medium. The results are expressed as a percentage of the respective control bars to facilitate comparison, since growth was somewhat slower in the deuterium-supplemented medium. Natural phytoalexins are marked with *.

The concept of photoactive phytoalexin has already been proposed in the literature.[13,43–45] For instance, the activity of isoflavonoid phytoalexins *in vitro* is enhanced by light, but their phototoxicity is due to oxygen-independent free radical formation.[43] A sesquiterpenoid cotton phytoalexin, 2,7-dihydroxycadalene, is photoactive towards cauliflower mosaic virus,[44] but the mechanism of the light-dependent inactivation has not been studied. To the best of our knowledge, our report provides for the first time unambiguous evidence linking the defence activity against a pathogen to the light-induced production of $^1O_2$ by the elicited phytoalexin.

As a final note, it is interesting that the 4-phenylphenalenone PN8, the phytoalexin with the highest $^1O_2$ production yield and highest antifungal activity, is released only upon fungal infection in susceptible banana plants, while the less sensitising 9-phenylphenalenones are also encountered in healthy plants from other families. One might speculate that, given the deleterious effects of $^1O_2$ on living organisms, plants have evolved to preclude the generation of large amounts of $^1O_2$ unless necessary. It will be interesting to extend these studies to other photoactive phytoalexins to draw a general picture of the role of $^1O_2$ photosensitization in plant defence.

Acknowledgements

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