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Perinaphthenone phototransformation in a model of leaf epicuticular waxes

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Graphical abstract

ABSTRACT

Perinaphthenone (1*H*-phenalen-1-one, PN) is a reference photosensitizer producing singlet oxygen with a quantum yield close to one in a large variety of solvents. It is also the basic structure of a class of phototoxic phytoalexins. In this work, the PN photoreactivity was studied for the first time in a paraffinic wax, used as model of leaf epicuticular waxes. The PN photodegradation was monitored by UV-Vis spectroscopy. The triplet excited state, singlet oxygen and the hydroxyperinaphthenyl radical were detected by diffuse reflectance laser flash photolysis, near infrared phosphorescence and by EPR spectroscopy, respectively. The PN phototransformation was found to be five-fold faster in the wax than in *n*-heptane under steady-state irradiation. The hydroxyperinaphthenyl radical formation was observed in aerated irradiated paraffin wax while in *n*-heptane solution the radical was observed only in the absence of oxygen. These results show that under continuous irradiation, PN is much more easily phototransformed in a solid environment than in solution. Several photoproducts were identified, in particular phenalanone, PN dimers, and oxidized PN-alkanes adducts. Finally, when pyrethrum extract is added into the wax, the radical concentration was increased by a factor of 2.4. Such photochemical reactions may occur when systemic pesticides enter the plant cuticle.

KEYWORDS. Perinaphthenone; photosensitizer; singlet oxygen; phototransformation; epicuticular wax; phytoalexin; hydroxyperinaphthenyl radical; pesticide; pyrethrum extract.

1. Introduction

Plants synthesize phytoalexin substances for microbial and often oxidative protection (1-3). These toxins are produced *de novo* into the leaf cells (4) and have been identified from higher plant tissues into the upper epidermis and in the leaf epicuticular waxes (5-7). Some phytoalexins are phototoxic due to their ability to produce singlet oxygen under solar irradiation (8-10). Many of these natural products are based on the perinaphthenone (1*H*-phenalen-1-one, PN) skeleton and have been isolated from plants and fungi (11-12). Therefore, the photochemical reaction mechanisms of PN are similar to those of some phytoalexins. PN is used as a reference photosensitizer in photochemistry and photobiology (13). The singlet oxygen production quantum yield of this molecule is close to one over a large solvent range (13-16). Nonetheless, in hydrogen donating solvents, PN can be photodegraded (16-17).

The plant leaf epicuticular waxes are mainly composed of long chain alkanes and of other compounds such as esters, ketones and alcohols (18, 19). Some of them are hydrogen donors. In this study paraffin wax films are used as a model of the leaf epicuticular waxes. Previous studies were performed with this model at the wax surface (20-22). This model allows a first approach of the photochemical processes occurring in the waxes of a broad variety of plants. Indeed, paraffin wax is a weak hydrogen donating medium but PN phototransformation is expected because of the degradation observed in N,N'-dimethylacetamide and 1,4-dioxane (16). In fact, in these media the competition between singlet oxygen production and PN transformation is not trivial.

The ability of phytoalexin to act as a singlet oxygen sensitizer and more generally the photochemistry of phytoalexins can be inconvenient for crop treatments (23, 24). Indeed, some pesticides are oxidized by singlet oxygen (25, 26) and these reactions could occur at the leaf

surface and within the leaf epicuticular waxes. Moreover, to our knowledge, no study has been performed on the possible interaction between the PN triplet excited state and pesticides absorbed by plants (systemic pesticides) which enter the cuticle. Under these conditions, pesticide molecules are expected to be in the close environment of phytoalexins that could induce their degradation.

For the first time, PN photochemistry is studied in a model of leaf epicuticular waxes. The goal of this study is to give a first approach of the PN photoreactivity in a solid medium composed of long-chain alkanes and to evaluate the competition between singlet oxygen production and phototransformation. Additionally, identification of photoproducts was performed. Finally, *pyrethrum* extract, a natural pesticide, was added to the paraffin wax film containing PN to obtain evidence of an interaction between the PN triplet excited state and this pesticide.

2. Materials and methods

2.1. Chemicals

Perinaphthenone (97%), n-heptane (99%), methanol ($\geq 99.9\%$), acetonitrile ($\geq 99.9\%$), paraffin wax (mp 53-57°C) and silica gel (Davisil grade 634, 100-200 mesh, 60 angstrom, purity $\geq 99\%$) were all purchased from Aldrich (Saint-Quentin Fallavier, France). Paraffin wax is a mixture of long-chain alkanes (mainly C₂₁-C₃₄). Pestanal pesticides tembotrione and *pyrethrum* extract were both purchased from Sigma-Aldrich.

2.2 Sample preparations

All the samples were prepared in a sodium lamp light room ($\lambda_{\text{irr}} = 589 \text{ nm}$) to avoid photochemical reactions during handling. Stock solutions of PN in *n*-heptane were prepared at

concentrations ranging from 10^{-3} to 10^{-5} M and stored at room temperature. Paraffinic wax films containing PN were prepared as follows: 72 mg of wax and 16.54 mg of PN were mixed and heated at 100°C under magnetic stirring to obtain a homogeneous liquid. Subsequently, the mixture was poured on aluminum foil and pressure was applied using a plate to produce the films. The 10^{-3} M PN solid concentration in the paraffin wax films was calculated based on a wax density of 0.77 g mL^{-1} . Less concentrated films were obtained by dilution with pure paraffinic wax. In all cases, films containing PN were rinsed with acetonitrile to remove any PN molecules remaining at the film surface. For UV-Visible spectroscopy, PN was mixed with cellulose at a 1/100 ratio in weight. For laser flash photolysis paraffin wax films containing PN were directly used without further modification. Silica gel samples containing adsorbed PN were used to monitor the triplet decay in the absence of oxygen. They were prepared as follows: 2 g of silica gel were dried by heating at 120°C under vacuum for 2 hours to a pressure of 5×10^{-5} mbar. Then, a PN solution at a concentration of $100\text{ }\mu\text{M}$ in methanol was added to the silica. The solvent was evaporated under vacuum at room temperature. Then, the sample was maintained under vacuum for 4 hours to ensure removal of trace methanol, water, and air. Finally, the samples were sealed under vacuum in a quartz cuvette. For EPR analyses the liquid mixture containing wax and PN was directly poured inside pyrex tubes. Pesticides were added to the hot liquid wax-PN mixture under magnetic stirring at amounts calculated to obtain a final concentration of 10^{-3} M for tembotrione and $4.3\text{ }\mu\text{L mL}^{-1}$ for the *pyrethrum* extract.

2.3 UV-Vis spectroscopy

Liquid state absorption spectra were recorded using a Cary 3 UV-Visible spectrometer (Varian). Baselines and spectra were recorded at room temperature in the 800-250 nm spectral range with

a 1-nm resolution and a 600 nm.min⁻¹ scan rate. Solid state UV-Visible spectra were recorded using a DRA-CA-30I integrating sphere accessory (Varian) and a BaSO₄ reflectance standard (Spectralon). PN mixed with cellulose was poured into a circular quartz cell of 1-cm thickness and then the cell was placed in the diffuse reflectance port of the sphere accessory. UV-Vis absorption spectra of paraffin wax films containing PN were recorded by positioning the film in the transmission port of the sphere accessory. The signal intensity measured in percentage of transmittance was converted to absorbance from equation (1):

$$A(\text{PN}) = \log\left(\frac{I_t(\text{Pa})}{I_t(\text{PN+Pa})}\right) \quad (1)$$

where A(PN) is the PN absorbance, I_t(Pa) and I_t(Pa+PN) are the transmitted light through the pure paraffin and paraffin containing PN samples, respectively. For PN dissolved in *n*-heptane, the molar absorption coefficient was calculated from UV-Vis absorption spectra of 10⁻⁴, 2×10⁻⁵, and 10⁻⁵ M solutions. In the case of paraffin wax containing PN, the molar absorption coefficient was calculated from transmission spectra of four different films containing 10⁻⁴ M of PN. The film thickness was measured using a MI20 Micrometer (Messmer Instrument Ltd). The average film thickness was 500 ± 100 μm. Molar absorption coefficient calculations of paraffin wax films containing PN were based on the Beer-Lambert law (27).

2.4 Photodegradation quantum yield and rate constants measurements

All the samples were irradiated at 366 nm (room temperature) using a mercury lamp (Oriel, 200 W) equipped with a monochromator (Photomax). The average photon fluence rate ($E_{p,0} = 2.0 \times 10^{15} \text{ cm}^{-2} \text{ s}^{-1}$) was measured using a QE65000 UV-Visible spectrometer (Ocean Optics). Air-saturated solutions were irradiated in a cubic quartz cell of 1-mm pathlength. The decay of PN was monitored by UV-visible spectroscopy. The paraffin wax films containing PN were placed

on a sample holder that allowed reproducible positioning of the sample for irradiation and UV-Visible analysis. The ~500 μm thickness films were irradiated under the same conditions as the solutions. Irradiation experiments were carried out in triplicate. Quantum yields of PN photodegradation (Φ_{deg}) were determined in air-saturated *n*-heptane and paraffin wax. The Φ_{deg} values were calculated from equation 2:

$$\Phi_{\text{deg}} = \frac{\Delta[\text{PN}]}{P_a \cdot \Delta t} \cdot 10^{-3} \cdot N_A \cdot \ell \quad (2)$$

where Φ_{deg} is the PN photodegradation quantum yield, $\Delta[\text{PN}]$ (mol L^{-1}) is the loss of PN concentration after irradiation, P_a ($\text{cm}^{-2} \text{s}^{-1}$) is the photon fluence rate absorbed by the sample, Δt (s) is the irradiation duration, N_A is the Avogadro number, and ℓ (cm) is the sample pathlength. Calculation of Φ_{deg} was based on a concentration loss of not more than 10% of the initial concentration, in order to avoid underestimation that could be due to overlapping absorption of PN and photoproducts.

2.5 LC-MS analyses

The MS system employed consisted of an LC/QTOF equipped with an orthogonal geometry Z-spray ion source (Waters/Micromass, Manchester, UK). For UV detection, a photodiode array detector Waters Alliance 2695 system was used. A volume of 40 μL was injected on a reversed-phase column (Kinetex, Phenomenex, C18, 2.6 μm , 100 mm x 2.1 mm). A flow rate of 0.2 mL min^{-1} was set. The binary solvent system used was composed of acetonitrile (ACN) and milli-Q water. The gradient elution started with 5% ACN and reached 95% ACN in 15 min linearly. These conditions were kept during 25 min. The desolvation chamber and ion source block temperatures were set at 180°C and 100°C, respectively. N_2 was used as the nebulizer gas (35 L h^{-1}) and the desolvation gas (250 L h^{-1}). The electrospray interface was operating in

positive and negative ion modes. The capillary voltage was 3000 V; the sample cone and the extraction cone were set at 35 V and 1 V respectively. Data were acquired over the m/z 90–1000 range at a scan rate of one second per spectrum. The data recorded were processed with MassLynx (version 4.1). PN irradiated 15 hours in *n*-heptane and 30 minutes in paraffin wax film at 366 nm were analyzed. For the *n*-heptane sample the solvent was evaporated at room temperature and the obtained powder was dissolved in ACN. For the paraffinic wax sample, PN and its photoproducts were extracted by dissolving the wax in *n*-heptane and by adding ACN in a 1:1 (v:v) ratio.

2.6 Laser flash photolysis

Transient absorption experiments were carried out on two different devices. Photolysis of paraffin wax films containing PN was conducted at the Institut de Chimie de Clermont-Ferrand (Clermont-Ferrand, France). The apparatus consists of a laser flash photolysis spectrometer from Applied Photophysics (LKS.60) equipped with a Quanta Ray GCR 130-01 Nd:YAG laser ($\lambda = 355$ nm, pulse width of 5-7 ns). The device has been previously described (28). Diffuse reflectance mode was used for the paraffin wax film sample studies. Time resolved absorption spectra were available in the nanosecond to second timescales. Experiments conducted on the paraffin wax films containing PN were made by measuring the reflectance change (ΔR , equation 3) from the transient species signal intensity:

$$\Delta R = \frac{R_0 - R_t}{R_0} \quad (3)$$

where ΔR is the transient species reflectance change, R_0 and R_t correspond to the diffusely reflected light before exposure to the laser pulse and at time t after excitation, respectively.

Laser flash photolysis experiments on PN adsorbed on silica gel were performed at Loughborough University (Loughborough, UK). The nanosecond diffuse reflectance laser flash photolysis apparatus has been previously described (29, 30) and excitation of the samples was performed with the third harmonic (355 nm, pulse width of 5 ns) of a Surelite I Nd:YAG laser (Continuum). The laser pulse energy at maximum output was 100 mJ per pulse, with pulse energy being controlled by varying the Q-switch delay. Diffusely reflected analyzing light from a 300 W xenon arc lamp (Oriel) was collected and focused onto the entrance slit of an $f/3.4$ grating monochromator (Applied Photophysics) and detected with a side-on photomultiplier tube (Hamamatsu R928). Signals were captured using an LT364 Waverunner digitizing oscilloscope (LeCroy). Rate constants for deactivation of transient species in paraffin wax were calculated using a first order deactivation kinetics law (4):

$$y = A_1 \cdot e^{-kt} + y_0 \quad (4)$$

where y and y_0 are the transient species absorbance after and before the laser pulse, respectively. A_1 is the signal amplitude at t_0 , k (s^{-1}) is the rate constant for deactivation, and t (s) the time after the laser pulse. Since transient decay data obtained from systems in heterogeneous media rarely exhibit mono-exponential behaviour, decay of the phenalenone excited triplet state on silica gel was analysed using the dispersive kinetic model of Albery *et al.* which treats the data as being a log Gaussian distribution of rate constants (31), according to equation (5).

$$\frac{C}{C_0} = \frac{\int_{-\infty}^{\infty} \exp(-t^2) \exp[-\bar{k}t \exp(\gamma t)] dt}{\int_{-\infty}^{\infty} \exp(-t^2) dt} \quad (5)$$

where C and C_0 represent transient concentrations (or ΔR and ΔR_0 at low sample loadings) at times t and t_0 after the laser pulse. The width of the distribution is given by γ and $\bar{k}$ is the mean

rate constant. Fitting was carried out using a modified sequential Simplex algorithm such that a global minimum in the reduced χ^2 parameter space was obtained. This procedure has been described previously in reference (32). A comparison of the suitability of this fitting method and a mono-exponential decay has been discussed previously in reference (33).

2.7 EPR spectroscopy

EPR spectra were recorded on a Bruker EMX EPR spectrometer interfaced to a computer with WinEPR Acquisition system software. The spectrometer had the following general settings: center field 349.583 mT, sweep field 10 mT, frequency 9.81 GHz, power 20.12 mW, sweep time 83.9 s, time constant 5.12 ms, modulation frequency 100 kHz, gain 3.17×10^5 and modulation width 0.05 mT. Each EPR spectrum was recorded at ambient temperature and 10 scans were accumulated. The signal acquisition was performed during sample irradiation from a 1000 W Xe-Hg arc lamp (Schoeffel Instruments). EPR spectra obtained from paraffin wax films containing PN were smoothed with a 9 point adjacent average. EPR analyses of PN dissolved in *n*-heptane at a concentration of 10^{-3} M in air saturated solution and deoxygenated solution (argon purged for 15 min) were performed. The kinetics of the formation of the PN radical were followed during PN irradiation by measuring the signal intensity centered at 3491.42 G as a function of time.

2.8 Singlet oxygen phosphorescence measurements

The equipment for the continuous monochromatic excitation of the sensitizer for the measurements of the $^1\text{O}_2$ phosphorescence at 1270 nm has been previously described in detail (14, 34, 35). The paraffin wax film containing PN at a concentration of 10^{-3} M was placed on a

sample holder oriented at 45° relative to a 1000 W Xe-Hg arc lamp, coupled to a monochromator (6 nm bandwidth) adjusted to 367 nm. A cooled (-80°C) NIR photomultiplier (Hamamatsu) was used as a ¹O₂ detector. The ¹O₂ luminescence was collected with a mirror, chopped and, after passing through a focusing lens, a cut-off filter (1000 nm) and an interference filter (1271 nm), was measured at 90° with respect to the incident beam.

3. Results and discussion

3.1 UV-Vis absorption spectra of perinaphthenone in solution and in solid media

UV-Vis absorption spectroscopy was used to determine whether PN is molecularly dispersed in the paraffin wax films or aggregated. In fact, phototoxic phytoalexins present in the plant leaves and irradiated by sunlight are not aggregated. Photochemistry of non-aggregated and aggregated molecules can be different because of the possibility of intermolecular deactivations in the latter case. As a first step we checked that our model is relevant for the studied system. The molecular aggregation can be clearly identified by UV-Vis absorption spectroscopy. In fact, the increase of intermolecular interactions leads to a shift of the absorption maxima and a broadening of the bands. Figure 1 displays the PN UV-Vis spectra recorded in different media; *n*-heptane, paraffin wax, and cellulose.

In *n*-heptane, where PN is completely solubilized at a concentration of 10⁻⁴ M, the absorption maximum is found at 353 nm with a band full width at half maximum (FWHM) of 57 nm (Figure 1.a). When PN is mixed with cellulose, a broadening of the UV-Vis band is observed compared to that in solution (Figure 1.b). This phenomenon is due to the formation of small PN crystals. The absorption band is broad and centered at 395 nm, and two distinct shoulders are observed at 363 and 465 nm with a band FWHM of 101 nm (Figure 1.b). UV-Vis spectrum of

paraffin wax film containing PN shows an absorption maximum at 354 nm and a band FWHM of 63 nm. Molar absorption coefficients were calculated for PN dissolved in *n*-heptane and in paraffin wax. The obtained values are of $11500 \pm 600 \text{ M}^{-1}.\text{cm}^{-1}$ and $16100 \pm 1600 \text{ M}^{-1}.\text{cm}^{-1}$ at 353 and 354 nm, respectively.

Comparison of the UV-Vis absorption spectra of PN dissolved in *n*-heptane and in paraffin wax shows small differences. The maxima are separated by only 1 nm and the band FWHM (full width at half maximum) is 10% smaller in *n*-heptane. In contrast, the UV-Vis spectra of PN in paraffin wax and cellulose display strong differences. Their maxima are separated by 41 nm and the band FWHM is 60% larger in cellulose. The PN crystals are thus well dissolved in the paraffin wax and no aggregation occurs during film preparations. Taking into account these results, we can conclude that paraffin wax films containing PN can be used as a model system for the photochemical behavior of phytoalexins present in leaf epicuticular waxes. Another important parameter is the molar absorption coefficient. It is 30% larger in the wax film compared to solution. Such differences in the absorptivity between the liquid and the solid phase have been already reported for other molecules (36, 37). At very low PN concentration, which is close to real conditions, this would lead to an amount of absorbed light about 30 % higher than that measured in the liquid alkane.

3.2 Compared perinaphthenone phototransformation in *n*-heptane and paraffin wax film

In this study, *n*-heptane is used as a reference solvent for the comparison of the PN photochemical behavior between liquid and solid paraffinic media. Figure 2 shows the UV-Vis absorption spectra evolution under irradiation at 366 nm of PN dissolved in *n*-heptane and in paraffin wax at 10^{-4} M . In *n*-heptane (Figure 2.a) the PN absorption band decreases by 12 % after

1 h. Simultaneously, a new band is observed at wavelengths lower than 325 nm, indicative of photoproducts formation.

In paraffin wax film (Figure 2.b) PN is photodegraded faster by 42 % after 30 min. Moreover, in paraffin wax film, absorbance increases above 420 nm (Figure 2.b), in addition to the absorption bands below 325 nm. The absorbance above 420 nm is weaker than at shorter wavelengths and results in a yellow coloration of the paraffin wax. The absence of photoproduct bands above 420 nm in *n*-heptane can be explained by the small amount formed relative to the wax film. PN quantum yields of photodegradation (Φ_{deg}) were calculated from the UV-Vis spectra. Values of Φ_{deg} are $3.5(\pm 0.4) \times 10^{-4}$ and of $1.7(\pm 0.4) \times 10^{-3}$ in *n*-heptane and wax film, respectively. Therefore, the PN degradation is five-fold more efficient in paraffin wax containing PN than in *n*-heptane. LC-MS analyses were performed to determine the nature of photoproducts. In deoxygenated *n*-heptane, where PN is readily photodegraded and photoproducts accumulate, several chemicals were identified (Figure 3). We successfully detected in positive mode phenalanone (PL, $m/z=183$) corresponding to $[\text{PN}+2\text{H}+\text{H}]^+$ as well as several adducts. The adduct with $m/z=361$ corresponds to $[\text{2PN}+\text{H}]^+$; it should be formed by the addition of the hydroxyperinaphthenyl radical (HPN) to PN followed by a H elimination. The adduct with $m/z=363$ corresponds to $[\text{PN}+\text{PN}+2\text{H}+\text{H}]^+$, *i.e.* to the addition of two HPN radicals. Photoproducts with $m/z=279$ correspond to $[\text{PN}+\text{H}+\text{C}_7\text{H}_{13}+\text{H}]^+$ and thus to the addition of the HPN radical to a radical deriving from *n*-heptane. For all these coupling products, several isomers were detected. By MS², all lost CO confirming the presence of at least a carbonyl function on each. In addition, none were detectable in negative mode ruling out the presence of OH functions. On the basis that the molar absorption coefficients are approximately the same for each photoproduct, the major ones are the PN-*n*-heptane adducts. When PN was irradiated in air-equilibrated paraffin wax film, LC-

MS positive mode allowed to detect oxidized PN ($[\text{PN}+\text{O}+\text{H}]^+$, $m/z=197$), PL ($m/z=183$), and adduct $[\text{2PN}+\text{H}]^+$ ($m/z=361$). From the LC-MS negative mode analysis $[\text{PN}+\text{H}+\text{C}_n\text{H}_{2n-2}+\text{O}_y]^-$ adducts were identified for $n=5, 7$, and 9 , and $y=1$ or 2 (see supplementary information).

3.3 Triplet state formation

The PN triplet excited state ($^3\text{PN}^*$) has been previously observed in many solvents (38), its UV-Vis absorption spectrum displaying a maximum at around 500 nm. In paraffin wax film, the UV-Vis diffuse reflectance spectrum of $^3\text{PN}^*$ has been recorded after pulsed laser excitation at 355 nm (Figure 4.a). The spectrum displays a maximum at 490 nm with a transient species decay rate constant of $1.7 \times 10^5 \text{ s}^{-1}$ (triplet lifetime of 5.9 μs) in the air-equilibrated conditions. Making the hypothesis that the oxygen concentration is the same in the wax as in *n*-heptane ($2.8 \times 10^{-3} \text{ M}$, 39), one gets $k_{\text{O}_2} = 6.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (scheme 1), which is 46 times lower than in *n*-heptane ($2.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$). This latter value was deduced from the $^3\text{PN}^*$ decay rate constants measured in argon-saturated ($6.0 \times 10^4 \text{ s}^{-1}$), air-saturated ($7.8 \times 10^6 \text{ s}^{-1}$), and oxygen-saturated ($3.6 \times 10^7 \text{ s}^{-1}$) *n*-heptane solutions. This significant difference on k_{O_2} is explained by the lower mobility of oxygen in a solid matrix compared to a liquid alkane. In solid polystyrene for instance, the diffusion controlled rate constant is $\sim 2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and the rate constant for oxygen quenching of triplet state sensitizers was evaluated as to be $\sim 1.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (40). Experiments in the absence of oxygen were conducted using silica gel samples prepared under high vacuum. A rate constant of 174 s^{-1} (5.7 ms) was measured for the transient decay under vacuum (Figure 4.b). When the evacuated sample was exposed to air, the PN transient species was completely quenched on these timescales, confirming the attribution of the transient to $^3\text{PN}^*$. The formation of radical species in the wax is likely, as evidenced by the observed PN degradation. Flors *et al.* have observed

their formation in organic solvents (41). In this system, spectral overlap of the triplet state and radical make direct observation of the latter difficult.

3.4 Singlet oxygen production

The formation of singlet oxygen was monitored by near infrared phosphorescence (Figure 5). A strong signal was observed and continuous irradiation of a paraffin wax film containing PN at 367 nm for 55 minutes leads to a 50% signal loss. Two steps are observed during the decrease of the signal. In fact, the signal loss is 35% in the first 20 minutes of irradiation while it is only 15% during the following 35 minutes. Considering that the quantum yield of singlet oxygen production by PN is close to one in most media and that no singlet oxygen acceptor or quencher is present in the paraffin wax, the $^1\text{O}_2$ concentration should be stable under irradiation. Therefore, the $^1\text{O}_2$ signal loss provides further experimental evidence of the PN photodegradation. Another possible reason for this $^1\text{O}_2$ signal loss may be the decrease of oxygen concentration in the film due to the formation of oxidation photoproducts.

3.5 Hydroxyperinaphthenyl radical formation

The formation of the hydroxyperinaphthenyl radical (HPN $\cdot$) was previously observed in deoxygenated 2-propanol by Rabold *et al.* (17). We monitored this radical formation in *n*-heptane and paraffin wax under irradiation. In air saturated *n*-heptane solution, no EPR signal was observed during PN irradiation, in line with the very low Φ_{deg} in this solvent. In deoxygenated *n*-heptane, the HPN $\cdot$ EPR signal was observed (Figure 6.a). The spectrum consists of six quadruplets separated by 6.19 G. Within each quadruplet, bands are separated by 1.74 G. The intensity ratios within the quadruplets are close to 1:3:3:1 while the ratios between the six

quadruplets are of 1:4.5:8.8:8.8:4.5:1. Interestingly, in irradiated air-equilibrated paraffin wax we observed an EPR signal showing the same coupling constants as those measured in *n*-heptane, only the intensity ratios were changed due to the sample anisotropy (Figure 6b). Thus, the HPN[•] radical is formed in both deoxygenated *n*-heptane and in air equilibrated paraffin wax. Detection of the HPN[•] radical in *n*-heptane was possible because oxygen was partly removed. Indeed, the efficient quenching of ³PN* by oxygen (Reaction (6)) does not compete with its reaction with the solvent (hydrogen abstraction, Reaction (7)). The PN radical is likewise observed in paraffin wax in aerated conditions. In fact, this result is consistent with PN irradiation kinetics monitored by UV-Vis spectroscopy (Figure 2) and singlet oxygen near infrared phosphorescence experiments (Figure 5) where PN is degraded in the paraffin wax under air.

3.6 Comparison of reactivity in *n*-heptane and paraffinic wax

We have shown that PN exhibits a higher photodegradability in paraffinic wax than in *n*-heptane. The mechanism given in Scheme 1 established using our experimental data may explain these differences. Following the electronic excitation of PN, ³PN* is formed from ¹PN* by intersystem crossing and the efficiency of this process (Φ_{ISC}) should be close to one in liquid as in solid phases (14, 42). In the presence of oxygen, ³PN* can either transfer its energy to oxygen or abstract a hydrogen atom from the solvent to yield the hydroxyperinaphthenyl radical (15, 16). In *n*-heptane, the former reaction is dominant as the quantum of singlet oxygen production is close to 1. In paraffin wax, the signal obtained could not be quantified, but its intensity strongly

suggests a high efficiency of singlet oxygen production (Figure 5). Nonetheless, Φ_{deg} is 5-fold higher in the wax compared to *n*-heptane. Although the effect on $^1\text{O}_2$ production could be minor in the first stages of the reaction, the sensitizing properties of PN may be altered significantly in case of extended use. A recent computational study by Segado *et al.* (42) shows that the lowest energy triplet state of PN is of $\pi\pi^*$ character where singlet oxygen formation is the main photochemical process. However, their study also indicates an equilibrium between this $\pi\pi^*$ triplet state and a low lying $n\pi^*$ triplet state, allowing for hydrogen abstraction to occur. This difference of Φ_{deg} between *n*-heptane and wax may be related to differences in the rate constants of the two competing reactions (k_{RH} and k_{O_2}). The lower mobility of molecules in the wax suggests lower rate constants. This was demonstrated in the case of k_{O_2} . In the case of k_{RH} , PN is surrounded by solvent molecules and the mobility may have less importance. If we postulate that k_{RH} is of the same magnitude in the two media, the difference in Φ_{deg} values must be assigned to a lower value of k_{O_2} .

The detection of the hydroxyperinaphthenyl radical (HPN $\cdot$) in wax only in spite of the presence of oxygen was a rather surprising result. The stationary concentration of HPN $\cdot$ is given by comparing the rates of production and of decay. Even though the rate of HPN formation is slower in *n*-heptane than in the wax because the scavenging of $^3\text{PN}^*$ by oxygen is faster, the non-observation of HPN $\cdot$ in *n*-heptane also indicates that the decay rate and thus the reactivity of HPN $\cdot$ radical is probably higher in *n*-heptane than in wax. Nevertheless, there is no parallel between the Φ_{deg} and the HPN $\cdot$ stationary concentration increase from *n*-heptane to wax. This suggests that re-oxidation of HPN $\cdot$ radical occurring as a result of interaction with dissolved oxygen, to regenerate PN, is the dominant pathway. Finally, the identified photoproducts in *n*-

heptane and paraffin wax film media derive from HPN[•] radical which may abstract another H atom and yield phenalanone, add onto PN or on a second HPN radical to give dimers of PN. The main reaction is the coupling of HPN[•] with an oxygenated radical deriving from the solvent. These results show that a reference photosensitizer can be photodegraded in solid media consisting of weak hydrogen donor molecules. By extension, the phototoxic phytoalexins structurally close to PN could be degraded in the leaf epicuticular waxes. Moreover, these leaf waxes include alcohols which are strong hydrogen donors and could increase the degradation efficiency. Another aspect not discussed until now is the impact on pesticides. The pesticide activities could be lowered by the phototoxic phytoalexins reactivity. This is discussed in the last section of the paper.

3.7 Environmental implication: phytoalexins in the epicuticular waxes could photosensitize pesticide degradation

The phototoxic activities of phytoalexins must be taken into account for crop treatments (23, 24). In fact, some pesticides are oxidized by singlet oxygen (25, 26). Moreover, we bring evidence of possible reactions between the ³PN* and/or PN derived radicals with pesticides. In this work, we selected as model compounds tembotrione and *pyrethrum* extract that can be absorbed by plants and therefore can be found in the phytoalexins close environment. The kinetics of formation of the HPN[•] radical was monitored by EPR spectroscopy under continuous irradiation of paraffin wax containing PN (Figure 7.a), PN and tembotrione (Figure 7.b), and PN and pyrethrum extract (Figure 7.c). A Xe-Hg arc lamp was used as irradiation source, the lamp emission being filtered by Pyrex tube. The results show that the HPN[•] concentration was higher in the presence of

pyrethrum extract by a factor of 2.4 while no difference was observed between waxes containing tembotrione and PN alone.

These results brought evidence of an interaction between excited PN and the *pyrethrum* extract while tembotrione showed no apparent effect. At this stage it is not possible to go further in the understanding of the involved mechanisms because of the specific features of each pesticide. However, we could demonstrate the photoreactivity of PN may be modified in the presence of a pesticide. By extension, we can assume that pesticide activities can be modified not only by the singlet oxygen photosensitizing the ability of phytoalexins but also as a result of their photodegradation.

4. Conclusions

In this work, we have studied for the first time the PN photoreactivity in a model of leaf epicuticular waxes. Through this model we have highlighted the potential reactivity of phototoxic phytoalexins absorbing sunlight; especially PN structural analogues. Indeed, even when the estimated PN phototransformation quantum yield is low, PN degradation was observed in paraffin wax films under continuous irradiation. Moreover, alkanes are weak hydrogen donors. A more realistic model of the leaf epicuticular wax would imply addition of alcohols and other functional groups, and an increase of the phototransformation efficiency would be expected under those conditions. Similarly, the phototoxic phytoalexins could be degraded in the leaf epicuticular waxes. The consequence could be a weakening of the plant defense mechanisms against microbial attacks. Evidence of that effect, in the case of paraffin wax film containing PN, was given by the observation of a decrease in singlet oxygen production coupled to the formation of a hydroxyperinaphthenyl radical (HPN[•]). Therefore, even if the radical formation is the minor

process, it may alter significantly the sensitizing properties of PN for prolonged use. PN phototransformation in the wax leads to the formation of phenalanone, dimer species and mainly oxidized PN-alkanes adducts. These species are potential singlet oxygen photosensitizers and reductor agents. In *n*-heptane, the fast reaction of HPN[•] with ground state oxygen to yield back PN and the perhydroxyl radical (HO₂[•]) prevents its detection. Nonetheless, phenalanone, dimer species and PN-alkanes adducts were also identified in deoxygenated solutions as expected. Finally, tembotrione and *pyrethrum* extract pesticides which can be absorbed by plants were added to the paraffin wax containing PN. The concentration of HPN[•] was 2.4 times higher in the presence of *pyrethrum* extract. Transposition of this result to phytoalexins photoreactivity leads to the conclusion that effects on the pesticide activities should be considered not only through singlet oxygen production but also H-atom abstraction.

ASSOCIATED CONTENT

Supporting Information

Irradiated PN dissolved in deoxygenated *n*-heptane and air-equilibrated paraffin wax film containing PN samples analyzed by LC-MS positive and negative ionization modes are reported. Decay kinetics of PN triplet excited state in controlled atmospheres are also reported.

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Figure 1. (a) UV-Vis absorption spectra of PN contained in paraffin wax films normalized to 1-cm thickness (solid line) and dissolved in *n*-heptane (dotted line) at 10^{-4} M. (b) UV-Vis diffuse reflectance spectra of cellulose (dashed line), PN mixed with cellulose at a 1% in weight concentration (dotted line), and difference spectrum (solid line).

Figure 2. UV-Vis absorption spectra of PN at $(1-5) \times 10^{-4}$ M before irradiation, and after different time of irradiation at 366 nm (a) in *n*-heptane (1-mm thickness cell) and (b) in a paraffin wax film (420 μ m thickness).

Figure 3. TOF MS ES⁺ spectrum of PN irradiated in deoxygenated *n*-heptane.

Figure 4. (a) Transient absorption spectrum of $^3\text{PN}^*$ in paraffin wax film obtained upon excitation at 355 nm. (b) Deactivation kinetics of $^3\text{PN}^*$ adsorbed on silica gel under air (solid line) and under primary vacuum (dotted line) obtained after a laser pulse irradiation at 355 nm and monitored at 500 nm.

Figure 5. Singlet oxygen near infrared phosphorescence signal under continuous irradiation (at 367 nm) of a paraffin wax film containing PN at concentrations ranging between 10^{-4} and 10^{-3} M.

Figure 6. EPR spectra of the hydroxyperinaphthenyl radical recorded during the irradiation of PN (a) dissolved in deoxygenated *n*-heptane, and (b) in air equilibrated paraffin wax.

Figure 7. Kinetics of formation of the hydroxyperinaphthenyl radical (HPN^*) under continuous irradiation of paraffin waxes containing (a) PN, (b) PN and tembotrione, (c) PN and *pyrethrum* extract (irradiation using a Xe-Hg arc lamp). Tembotrione and *pyrethrum* extract pesticide structures.

583 Figures

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Figure 3

Figure 4a

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653 **Scheme 1.** Chemical reactions induced by PN irradiation in paraffin wax.

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