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Inhibition of Herbicide Photodegradation by Plant Products

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ABSTRACT: Pesticide reactivity toward light is rarely considered at the leaf surface after crop treatment; regardless, these degradation reactions directly impact the pesticide effectiveness. The use of sunscreen adjuvants to overcome photodegradation has presented some limitations so far. Raw hydroalcoholic plant extracts have been recently proposed to be used as photoprotecting adjuvants; on a model system they significantly decreased the photodegradation of pesticide. Here it is demonstrated that their use makes possible a dose reduction. Sulcotrione, a selective herbicide for use in maize, was tested in a growth chamber equipped with simulated solar light against a typical weed in maize. Sprayed weeds were monitored by biometrical and physiological parameters. Sulcotrione minimum dose required for a good herbicidal efficacy (ED_{50}, corresponding to 50% of chlorophyll content decay) was estimated to be 55 g ha^{-1}. In the presence of grape extract added in a 3-fold excess compared to the herbicide, the ED_{50} decreased to 34 g ha^{-1}. The use of grape extract allows extension of sulcotrione herbicidal activity and reduction of the dose by 35% in controlled conditions. This is a promising result for the effective dose field adjustment.

KEYWORDS: photoprotection, grape pomace, anthocyanins, sulcotrione, phytosanitary formulations

INTRODUCTION

When plants are stressed, they can synthesize a large variety of products, which is partly correlated to the type of stimulus. Most of the products are then related to a plant defense process. They involve different kinds of molecular species that can be cytotoxic,[1] or involved in the development of photoinhibition in plants, like anthocyanins.[2] Terrestrial plants, comprising about 250,000 living species, are an extremely diverse source of chemicals with bioactive properties that are in particular valuable to the pharmaceutical and food industry. Plant-derived products are expected to play an increasingly significant role in the commercial development of new drugs, dietary supplements, and functional food products in the near future.[3] Wine, grapes, and grape seed extracts are a major source of polyphenolic components such as anthocyanins, flavanols, catechins, and proanthocyanidins.[4] Grape pomace is typically regarded as a waste product generated in the winemaking industry. Large amounts of grape pomace accumulate annually, which leads to a waste-management issue.[4] Most phytosanitary treatments are sprayed on plants (except for pre-emergent herbicidal treatment), but the fate of the pesticide from the foliage has to be evaluated. At this stage the dissipation processes would not only result in the scattering of the pesticide toward ecosystems but also strongly alter the pesticide efficiency. A series of laboratory experiments have demonstrated that the half-life of photolysis of some pesticides could be as low as a few hours.[5,6] To overcome the loss of efficiency resulting from these degradation reactions on crops, application rates are often increased without consideration for the environmental consequences. In a search of alternative solutions we proposed the use of raw plant extract as a photoprotecting adjuvant for pesticides.[7] It was demonstrated in laboratory conditions on a model that raw hydroalcoholic plant extracts significantly reduce the photodegradation of pesticides. Distinct chemical families of pesticides have been tested, and the extracts from various plants reduced the pesticide degradation in variable proportions (up to 80%).

Considering these results it was interesting to estimate to what extent the gain in photostability impacts the pesticide effectiveness. This demonstration is certainly dependent on every pesticide and plant extract combination. We have considered the photoprotection of an herbicide (sulcotrione) with grape wine extracts. Sulcotrione was selected because it is widely used on maize to control major annual broadleaf weeds.[8] It is also known to be photoreactive on crops.[6] Sulcotrione (benzoylcyclohexanedione herbicide, member of the triketone family) belongs to the 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD) inhibitor family and was introduced in 1991 as the latest mode of action for herbicides.[9] This mode of action is well described[10,11] and provides many opportunities to assess the efficacy of the treatment[12] (Figure 1). Enzymatic inhibition of 4-HPPD by sulcotrione leads to depletion of plastoquinone and tocopherol precursors (homogentisate). Tocopherol biosynthesis is therefore indirectly but quickly impacted by the action of sulcotrione.[13] Loss of carotenoid resulting from plastoquinone depletion induces rapid oxidation of photosynthetic membranes and leads to the degradation of chlorophyll. Consequently, the excess light energy is no longer quenched.[14,15] Destruction of plant pigments produces a symptomatic bleaching of young leaves.[16]

The grape extract was selected as a photoprotecting adjuvant because of its economic interest as a plant residue and its photoprotecting properties. With laboratory tests on model wax films, the

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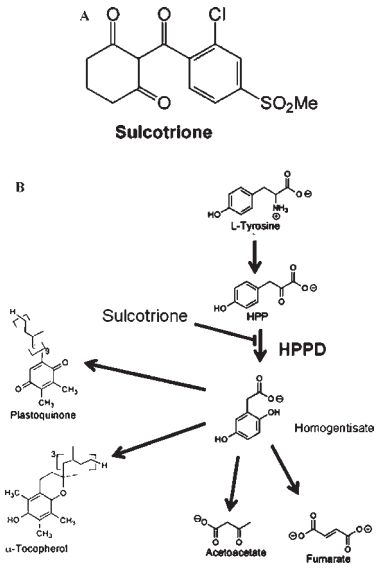

Figure 1. Structure of the active herbicide sulcotrione (2-(2-chloro-4-mesylbenzoyl)cyclohexane-1,3-dione), with the fates of HPP in plants, according to He and Moran.[13] Plants use the product of the HPPD reaction, homogentisate (HGA), to make plastoquinone and tocopherol, both of which are vital to plant metabolism. Tyrosine catabolism catalyzed by 4-hydroxyphenylpyruvate dioxygenase (HppD) converts 4-hydroxyphenylpyruvate (4HPP) to 2, 5-dihydroxyphenylacetate (homogentisate). Sulcotrione inhibits the 4-hydroxyphenylpyruvate dioxygenase (4-HPPD, EC 1.13.11.27) and blocks the formation of homogentisate, the precursor of plastoquinone and tocopherol. The resulting depletion of plastoquinone and tocopherol indirectly impacts chlorophyll integrity and photosynthesis.

addition of grape extract in a 3-fold excess reduced sulcotrione photodegradation by 60%. Grape extract is an anthocyanin-rich mixture typically containing about 60% of polyphenols including 8% anthocyanins. Grape extract is obtained from grape pomace. This solid waste remaining from grape skin constitutes a very abundant and inexpensive source of raw material.[17]

The aim of this study was to estimate if photoprotection can improve sulcotrione herbicide efficacy and to what extent it allows a decrease in the application rate. Sulcotrione efficiency was evaluated by visual observation and by determination of biometrical and physiological parameter measurements of sprayed weeds. The minimum efficient dose was determined by a dose−response curve regression[18] (300, 150, 75, 50, and 37.5 g ha^{-1}) and kinetic study (3, 5, 7, and 10 days after treatments).

MATERIALS AND METHODS

Chemicals. The active ingredient (a.i.) sulcotrione was an analytical standard purchased from Riedel de Haën (Pestanal, Saint-Quentin Fallavier, France). The commercial formulation Mikado (sulcotrione 300 g L^{-1}) was obtained from an agricultural shop. Grape (*Vitis vinifera*) hydroalcoholic extract was provided by Grap'Sud (Cruviers-Lascours, France, lot no. 08010). Tween 20 was purchased from Acros (Saint-Quentin Fallavier, France). Solvents were obtained from Riedel de Haën (methylene chloride, gradient grade and methanol HPLC grade). Formic acid (99%), potassium dihydrogenophosphate (99.5%) and disodium hydrogenophosphate (99%) were obtained from Prolabo (VWR, Fontenay sous Bois, France). Water was purified using a Millipore milli-Q system (Millipore αQ, resistivity 18 MΩ cm, DOC < 0.1 mg L^{-1}). For sunlight actinometry, 4-nitroanisole (97%) was supplied by Aldrich (Saint-Quentin Fallavier, France) and pyridine (99%) by Lancaster (Alfa aesar, Schiltingheim, France). The standard α-tocopherol was purchased from Sigma (L'isle d'Abeau Chesnes, France).

Plant Materials and Spraying Description. Seeds of black nightshade (*Solanum nigrum*) were germinated in moistened vermiculite in a greenhouse. Seedlings were transferred to single plastic pot (one plant per pot) and raised in loam under controlled conditions (26−28 °C; photoperiod 16 h) to the 4−5 leaf stage. At this development stage, the plants uniformly developed were selected and treated in a spraying chamber containing a two meter wide spray boom with four nozzles (Teejet full cone spray TXA800050VK). Each nozzle was delivering 4 mL/s of herbicide solution and covered a round surface of 1256 cm^2. Sulcotrione solutions were prepared by diluting the commercial formulation Mikado (300 g L^{-1}, BayerAgroscience Inc.) in distilled water including a nonionic surfactant (Tween-20) at 0.1% in mass. Sulcotrione concentrations were controlled by HPLC analysis and solutions were applied at doses corresponding to 300, 150, 75, 50, and 37.5 g ha^{-1}. For each treatment, four plants were sprayed for one second. Control plants received the corresponding amount of Tween-20 solution (0.1%) with no adverse effects on plant growth. For each concentration, photoprotection was tested by adding grape extract in formulated solution in a 3-fold excess compared to sulcotrione. Treated plants were grown for 10 days in a controlled growth chamber (24 °C; photoperiod of 16 h) under a simulated sunlight. The irradiation system was equipped with six fluorescent tubes (polychromatic light 300−450 nm, Philips TLD, 40 W) as described earlier.[19] A supplemental irradiation system was set up with 8 actinic neon lamps (TL/05 40 W, Philips), and the irradiance was measured using chemical actinometers.[20] Plants (10 cm height) were placed 30 cm under the system to undergo the same irradiation as that measured in the Suntest reactor during previous photochemical experiments.

Phytotoxicity Evaluation. The toxicity of photoprotector was evaluated on crop and weed. Black nightshade (*Solanum nigrum*) and maize (*Zea mays*) seedlings were grown in the same conditions both to the 5−6 leaf stage. Plants were treated with sulcotrione at 300 g ha^{-1}, grape extract at 900 g ha^{-1} and a mix of sulcotrione (300 g ha^{-1}) and grape extract (900 g ha^{-1}). Treated plants were grown for 10 days in a controlled growth chamber (24 °C; photoperiod of 16 h) under simulated sunlight. Plants were harvested after 10 days for fresh weight and height determination. Leaves were ground with a mortar and a pestle in liquid nitrogen and were lyophilized for dry weight and water content determination. After extraction (20 mg in 5 mL of methanol), total chlorophyll contents were determined using a spectrophotometer and quantified according to the Lichtenthaler equation.[21]

Herbicidal Activity Evaluation. Herbicidal activity of bleaching herbicides can be assessed by different ways measuring biometric or physiological parameters. Leaf injuries were estimated by visual rating of discoloration and necrosis. Leaves were harvested and counted at 3, 5, 7, and 10 days after treatments (DAT). After fresh weight measurement, leaves were ground with a mortar and a pestle in liquid nitrogen and were lyophilized for dry weight and water content determination. The plant death was characterized by the suppression of growth (dry weight stagnation) and the irreversible desiccation of leaves.

The determination of tocopherol and plant pigment content was determined from analysis of the same extraction procedure. The lyophilized sample (20 mg) was extracted in 5 mL of 100% methanol at 4 °C overnight. Samples were mixed for 5 min and centrifuged at 4000*g* for 10 min. Supernatants were split in equal volumes and used immediately for spectrophotometric and HPLC−UV−FL analyses. All sampling procedures were performed in a room equipped with 589 nm Na lights to prevent photodegradation of chemicals. Total chlorophylls and carotenoids content were determined by spectrophotometry and quantified according to the Lichtenthaler equation.[21] Quantification of

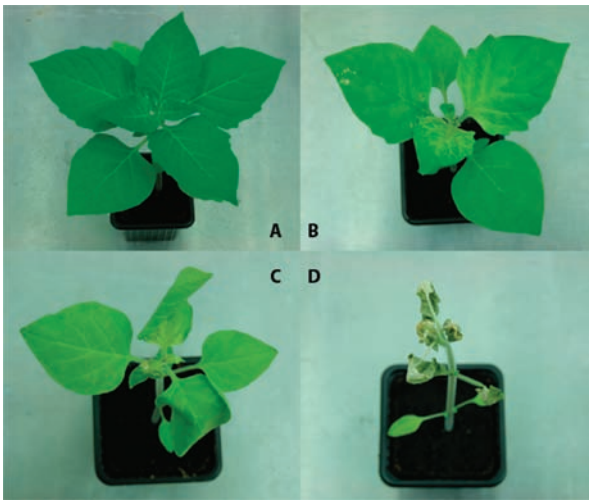

Figure 2. Effect of sulcotrione on plant growth and development. *Solanum nigrum* treated with formulated sulcotrione at 300 g ha^{-1}. A: Healthy. B: 3 days after treatment. C: 5 days after treatment. D: 7 days after treatment.

α-tocopherol was performed using a WATER Alliance HPLC system consisting of a separation module 2695, a multi λ fluorescence detector 2475 and a dual λ absorbance detector 2487. Standard solutions, from 10 to 100 μL mL^{-1} were prepared in methanol. Tocopherol separation (20 μL of sample extract) was performed at 30 °C on an Agilent Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm particle size). The mobile phase was a HPLC grade solution of 96/4% (v/v) methanol/water at a flow rate of 1 mL min^{-1}. Detection of α-tocopherol was conducted with a fluorescence detector set at λ_{ex} = 295 nm and λ_{em} = 330 nm. Tocopherol was identified on the basis of its retention time, and its concentration was calculated using the external standard method.

RESULTS

Determination of a Minimum Efficient Rate in Controlled Condition. Healthy black nightshade (*Solanum nigrum*) plants treated with formulated sulcotrione at doses above 50 g ha^{-1} presented a symptomatic bleaching of young leaves (Figure 2), with a strong concomitant decrease of dry matter weight expressed in % of control (Figure 3A) from 3 days after treatment (DAT). The suppression of plant growth is an indirect consequence of sulcotrione activity and a classical response for stressed plants. These symptoms were followed by plant desiccation at 5 DAT (Figure 3B). The low water content of treated plants indicates the beginning of cell lysis preceding the apparition of the necrosis and plant death at 7 DAT (over 95% of plant death was observed). This sequence of visual and physiological injury has been observed with all the treatments above the dose 60 g ha^{-1}.

At and below 50 g ha^{-1}, the bleaching symptoms still appeared at 3 DAT but no desiccation was observed over the time of the study (Figure 3B). Plant growth was only slowed down until 5 DAT (Figure 3A), but new green leaves appeared at 7 DAT accompanied with an increased of dry matter content. The treatments at 50 and 37 g ha^{-1} are not sufficient to induce plant death.

The chlorophyll content evolution was very similar to biometric parameters. The chlorophyll contents, expressed in percentage of the control of the plants treated at rates above 50 g ha^{-1}, kept decreasing until 10 DAT to reach levels below 50% (Figure 4A). For the treatments of 50 g ha^{-1} and below, a decrease of chlorophyll content was noticed at 3 DAT, but the levels increased after 3 days to

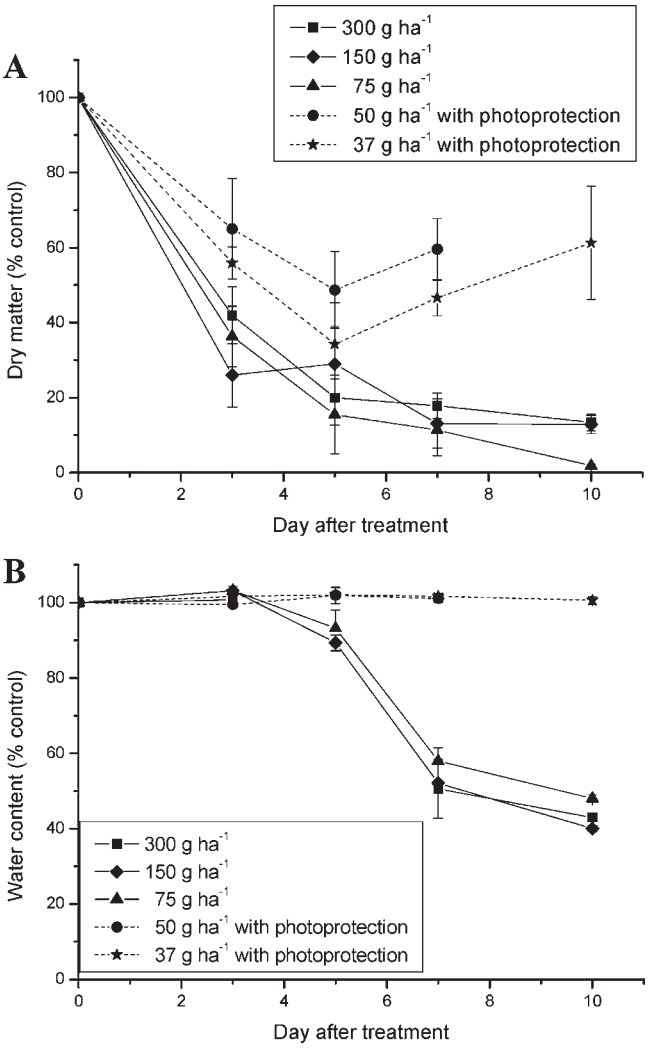

Figure 3. Biomass and water content of plants following sulcotrione treatments. A: Dry matter content of treated plants as a percentage of the control. B: Water content in percentage of control of treated plants. The treatments with sulcotrione were done at following doses shown in the figure; (—) treatments that induce plant death; (- - -) treatments not sufficient to cause plant death.

finally reach the level of the control plants at 10 DAT. In order to determine the minimum dose required for a good weed control, a log−logistic curve was used to describe the dose−response relationships:

$$y = C + \frac{D - C}{1 + 10^{p\log(ED_{50} - x)}}$$

y is chlorophyll content and is expressed as percentage of the control without treatment, D is the upper limit without treatment, C is the lower limit at infinite large doses, ED_{50} is the dose that gives a response halfway between C and D, and p describes the slope around ED_{50}. Dose−response curves were generated by regression analysis in ORIGIN software (dose response, Figure 5). In our controlled laboratory conditions, the ED_{50} without photoprotection was 55 ± 3 g ha^{-1} (the other parameters of the fitting are C = 38 ± 3%, D = 94 ± 4% and p = −0.15 ± 0.04). The adequacy with the biometric parameters confirms that ED_{50} corresponds to a dose giving a weed control with more than 95% of plant death (95% efficacy). The

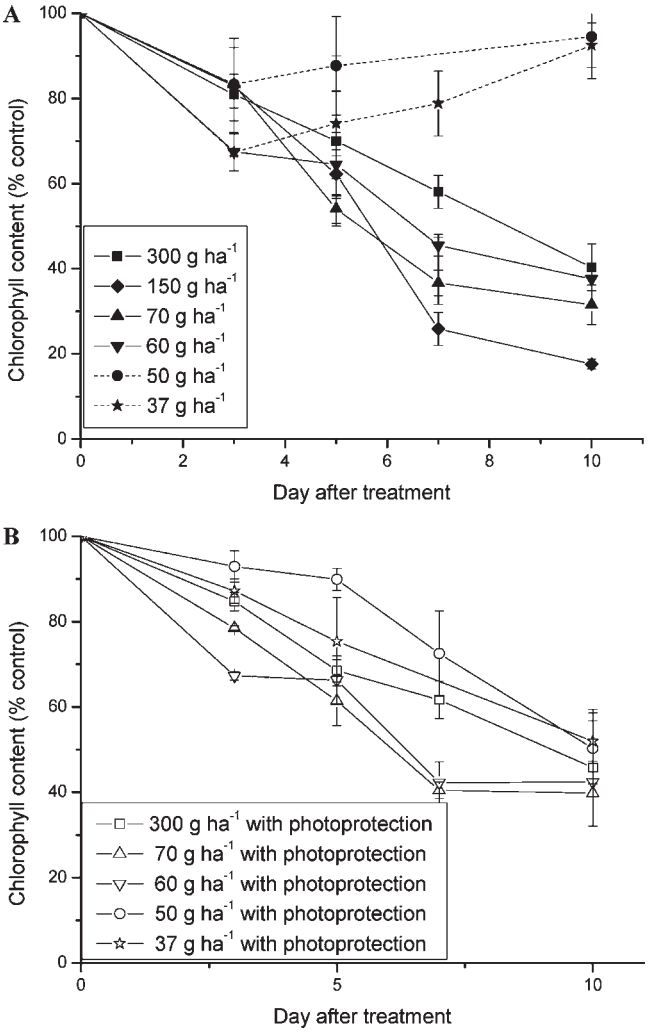

Figure 4. Chlorophyll content of treated plants as a percentage of the control. A: Plants were treated with sulcotrione at the following doses without photoprotection. B: The plants were treated with a mixture of sulcotrione and grape extract (in ratio $R = 3$) with sulcotrione doses shown in the figure.

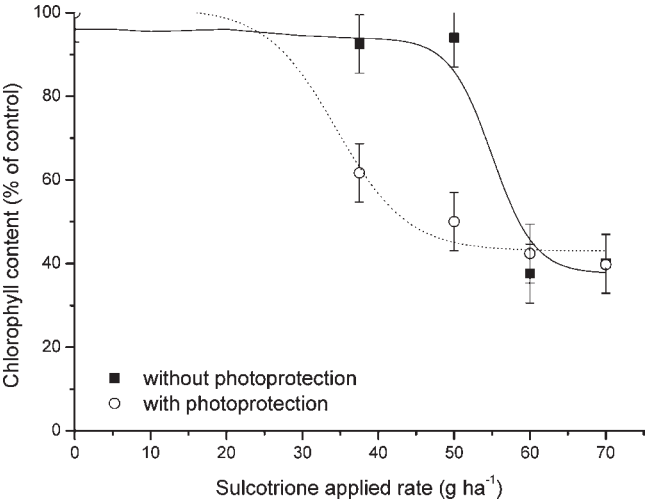

Figure 5. Effect of photoprotection on herbicidal efficiency. Grape anthocyanins were added in 3-fold excess to sulcotrione. Chlorophyll dose response curve 10 days after treatment without photoprotection (dark circles) and with photoprotection (open circles). Data are the results of three replicates.

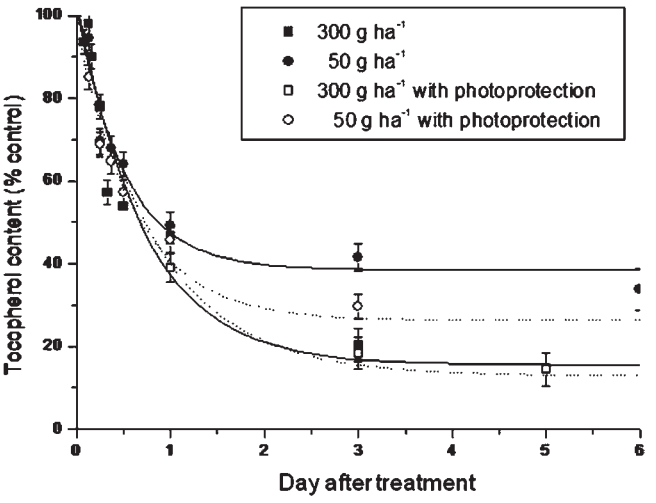

Figure 6. Tocopherol content of treated plants as a percentage of the control. The treatments with sulcotrione without photoprotection correspond to the rates 300 g ha^{-1} (dark squares) and 50 g ha^{-1} (dark circles). For the treatments with a mixture of sulcotrione and grape extract (with the ratio $R = 3$): 300 g ha^{-1} (open squares) and 50 g ha^{-1} (open circles).

sulcotrione recommended rate used in agriculture is 300 g ha^{-1} and also refers to a dose with an efficacy over 95%. As expected the ED_{50} obtained here is well below the value obtained in the field since there is less dissipation in a growth chamber.

Tocopherol content monitoring gives additional information on the quickness of the plant response to the treatments since this content is quickly impacted by the inhibition of HPPD and by the depletion of tocopherol precursors (homogentisate). Tocopherols and plant pigment extraction methods were validated by determination of α-tocopherol recovery in samples spiked with a known amount of α-tocopherol. Recovery was systematically higher than 92%. All samples were simultaneously extracted and immediately analyzed to avoid compound degradation.

The regression analysis of tocopherol content gives exponential decay curves generated using Origin software and the equation

$$y = y_0 + A_1 \exp\left(-\frac{x}{t}\right)$$

y is tocopherol content expressed in percentage of the control without treatment, $1/t$ is the rate of tocopherol content decay, y_0 is the plateau value and A_1 is the amplitude of tocopherol depletion. The plateau y_0 is reached after 3−5 days (Figure 6). Both A_1 and $1/t$ parameters evolve proportionally with the applied sulcotrione dose. For treatments at doses above ED_{50}, A_1 is systematically above 75%, while the treatments below ED_{50} never gave a plateau A_1 above 60%. The rate of tocopherol depletion is also higher for the treatments above ED_{50} ($1/t > 1.38$ $days^{-1}$) than for the treatments below ED_{50} ($1/t < 0.94$ $days^{-1}$). In summary, higher application doses induced a faster response in the plant together with a higher decrease in the maximum amount of tocopherol depletion.

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Reduction of the Minimum Dose Required for a Good Weed Control by Using Photoprotection. The treatments with photoprotection were performed with a 3-fold excess of grape extracts compared to the amount of sulcotrione. Above 55 g ha^{-1}, the addition of grape extract did not generate any increase or decrease in plant response for any of the monitored parameters: dry matter weight, water content (data not shown), chlorophyll (Figure 4B) and tocopherol content. At these doses, the herbicide was in such large excess that the possible gain provided by photoprotection did not impact the effectiveness of the treatments.

The treatments at 50 g ha^{-1} and 37 g ha^{-1} with photoprotection gave a bigger plant response compared to the treatments without photoprotection at the same doses. The chlorophyll content of the plants treated at 50 g ha^{-1} with photoprotection show a decrease starting from 5 DAT to reach 50% at DAT 10 (Figure 4B). The treatment at 37 g ha^{-1} shows the same general tendency. The dose response curve for the chlorophyll content obtained with photoprotection is reported in Figure 5. The new effective dose calculated with photoprotection ($^{PP}ED_{50}$) dropped to 34 ± 2 g ha^{-1} (C = 43 ± 3%, D = 100 ± 5% and p = −0.1 ± 0.03).

The tocopherol content curves also indicate that, below 55 g ha^{-1}, sulcotrione treatment with grape systematically induced higher responses of the plant compared to the treatment without photoprotection. With photoprotection, the amplitude inhibition A_1 was systematically higher than 75% for all the treatments (37 to 300 g ha^{-1}) while it was around 60% for lower rate without photoprotection (Figure 6). The rate of tocopherol decay is also systematically higher than 1.61 day^{-1}, which is quite similar to the dose obtained for the treatments without photoprotection at the doses above ED_{50} = 55 g ha^{-1}.

DISCUSSION

Anthocyanin is a natural plant pigment and a water-soluble natural pigment that appears as red, purple, and blue in plants and belongs to the flavonoid parent class of molecules. It has been shown to mediate antioxidant reactions by stabilizing or inactivating free radicals and preventing cellular oxidative stress. Because grape skins and seeds are the predominant constituents in the pomace, this biomass is speculated to be one of the richest and most cost-effective sources of natural dietary antioxidants.[22] Grape (*Vitis vinifera*) hydroalcoholic extract was shown to have absorption maxima before 400 nm wavelength, with a gap until 650 nm.[23]

Plants often respond to UV light, which affects flavonoid biosynthesis genes.[24] Phenolics can act as UV-protectors e.g. because of their absorption of light between 270 and 290 nm. Anthocyanin pigments are widely used in the food market and medicine, but only a few *in vitro* cultures produced biomass with a high anthocyanin content equal to the plant in nature.[3] Nevertheless, it was shown that in another kind of plants, *Arabidopsis*, a significant ecotype-specific genetic variability in general UV-B responses exist, that can differ according to molecular markers used.[25] Therefore, the contribution of the plant substrate to the herbicide photoprotection through its own products needs to be evaluated.

Phototransformations of the herbicide sulcotrione were earlier described on wax support in laboratory[6] or on maize plants in fields[26] with $k = 1.4 \times 10^{-4}$ s^{-1} and $k = 2.5 \times 10^{-5}$ s^{-1}, respectively. Previously, a good linear correlation was found between the absorption properties of plant extracts at 290 nm and their photoprotecting effects. This correlation demonstrates that the plant extracts certainly act as sunscreens.[23] In dark room, treatments by sulcotrione with or without grape extract display similar plant degradations. Previously, it was observed that plant extracts exposed to light showed differences of anthocyanin stability according to the plant species.[27] In plants, anthocyanins are natural adaptable light screens deployed to modulate light absorption in sensitive tissues such as fruit peel in response to environmental triggers.[28]

The use of natural plant extracts is a very promising strategy to protect active ingredients from photodegradation. In our conditions, it allows the minimum dose required for a good weed control to be decreased by 35%. This demonstration has to be repeated in the field for an adjustment the effective dose with photoprotection in outdoor conditions. Moreover, the use of anthocyanin-rich waste as adjuvant is a good alternative to valorize the solid waste generated by the food industry. These natural products are very abundant and inexpensive raw materials, and they present a potential source to develop sustainable and innovative technology in the plant protection industry bringing both environmental and economical interest.

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