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The Herbicide Saflufenacil (Kixor™) is a New Inhibitor of Protoporphyrinogen IX Oxidase Activity

Klaus Grossmann, Ricarda Niggeweg, Nicole Christiansen, Ralf Looser, and Thomas Ehrhardt*

Saflufenacil (Kixor™) is a new herbicide of the pyrimidinedione chemical class for preplant burndown and selective preemergence dicot weed control in multiple crops, including corn. In this study, the mode of action of saflufenacil was investigated. For initial characterization, a series of biotests was used in a physionomics approach for comprehensive physiological profiling of saflufenacil effects. With the use of treated duckweed plants, metabolite profiling was performed based on quantification of metabolite changes, relative to untreated controls. Physiological and metabolite profiling suggested a mode of action similar to inhibitors of protoporphyrinogen IX oxidase (PPO) in tetrapyrrole biosynthetic pathway. Saflufenacil inhibited PPO enzyme activity in vitro with 50% inhibition of 0.4 nM for the enzymes isolated from black nightshade, velvetleaf, and corn. PPO inhibition by saflufenacil caused accumulations of protoporphyrin IX (Proto) and hydrogen peroxide (H_2O_2) in leaf tissue of black nightshade and velvetleaf. In corn, only slight increases in Proto and H_2O_2 were found, which reflects in planta tolerance of this crop. The results show that saflufenacil is a new PPO-inhibiting, peroxidizing herbicide.

Nomenclature: Bifenox; diuron; saflufenacil; black nightshade, *Solanum nigrum* L. SOLNI; duckweed, *Lemna paucicostata* (L.) Hegelm. LEMPA; velvetleaf, *Abutilon theophrasti* Medik. ABUTH; corn, *Zea mays* L. ZEAMX.

Key words: Herbicide mode of action, metabolite profiling, physiological profiling, protoporphyrinogen IX oxidase inhibitor.

The pyrimidinedione saflufenacil (Kixor™; Figure 1) is a new herbicide being developed by BASF SE for preplant burndown and residual preemergence dicot weed control in multiple crops including corn (Liebl et al. 2008). The compound has preemergence selectivity based on physical placement and in planta crop tolerance. In corn, saflufenacil can be used preemergence at 63 to 125 g ai ha^{-1} for broad dicot weed control, including large-seeded species such as velvetleaf and common cocklebur (*Xanthium strumarium* L.) (Liebl et al. 2008). After application, saflufenacil elicits the same type of phytotoxic symptoms, with rapid light-dependent wilting and necrosis of the shoot tissue, as protoporphyrinogen IX oxidase (PPO)–inhibiting, peroxidizing herbicides in susceptible plant species.

Inhibition of PPO, a key enzyme in tetrapyrrole biosynthesis, is a herbicide mechanism of action that has successfully been used for weed management in agriculture since the introduction of diphenyl ethers such as bifenox in the 1970s (Hirai et al. 2002; Matringe et al. 1993; Nagano 1999). PPO inhibitors can be grouped roughly into two chemical classes: diphenyl ethers, which were the first widely used family of herbicides, and *N*-phenylnitrogen heterocycles, which have become an intensive field of research and development of commercial herbicides in recent years (Grossmann and Schiffer 1999; Hirai et al. 2002; Meazza et al. 2004). PPO catalyzes the conversion of protoporphyrinogen IX to protoporphyrin IX (Proto) (Beale and Weinstein 1990). Blockage of the pathway at this enzymatic step prevents the synthesis of chlorophylls, hemes, and cytochromes in the chloroplast, which are components of many important proteins involved in light harvesting, energy transduction, signal transduction, and detoxification (Beale and Weinstein 1990). As a further consequence, high concentrations of Proto accumulate in the green tissues, which result from an extraplastidic oxidation of protoporphyrinogen IX (Dayan and Duke 1997; Matringe et al. 1993; Matsumoto 2002; Wakabayashi and Böger 1999). Proto is known as a potent photosensitizer, which is the primary photodynamic pigment responsible for the herbicide action. Upon exposure to light, cytosolic Proto molecules interact with oxygen to form singlet oxygen and oxygen radicals, which peroxidize the unsaturated fatty acids of the cell membranes (Dayan and Duke 1997; Matringe et al. 1993; Matsumoto 2002; Wakabayashi and Böger 1999). Lipid peroxidation results in a rapid loss of membrane integrity and function, particularly of the plasmalemma, tonoplast, and chloroplast envelope (Matsumoto 2002; Wakabayashi and Böger 1999). This leads to ion leakage and water loss from the cell. Further effects are inhibition of photosynthesis, formation of ethylene, evolution of ethane from membrane peroxidation, bleaching of chloroplast pigments, tissue necrosis and, ultimately, growth inhibition and plant death (Matsumoto 2002; Wakabayashi and Böger 1999).

The aim of this study was to analyze the herbicidal mode of action of saflufenacil. For initial characterization, an array of bioassays was used in a physionomics approach for comprehensive physiological profiling of saflufenacil effects (Grossmann 2005). The response pattern was compared with profiles of herbicides with known modes of action to classify the biological activity. In addition, metabolite profiling was carried out in treated duckweed plants in order to diagnose the herbicidal mode of action and involved metabolic steps (Fernie et al. 2004; Sauter et al. 1991). Because the physiological and metabolite profiles obtained were in close conformity with those elicited by PPO inhibitors, the effect of saflufenacil on PPO enzyme activity in vitro and Proto and H_2O_2 accumulation in leaf tissue of velvetleaf and black nightshade was investigated.

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Materials and Methods

Chemicals. Saflufenacil (N′-[2-chloro-4-fluoro-5-(3-methyl-2,6-dioxo-4-(trifluoro-methyl)-3,6-dihydro-1(2H)pyrimidinyl)

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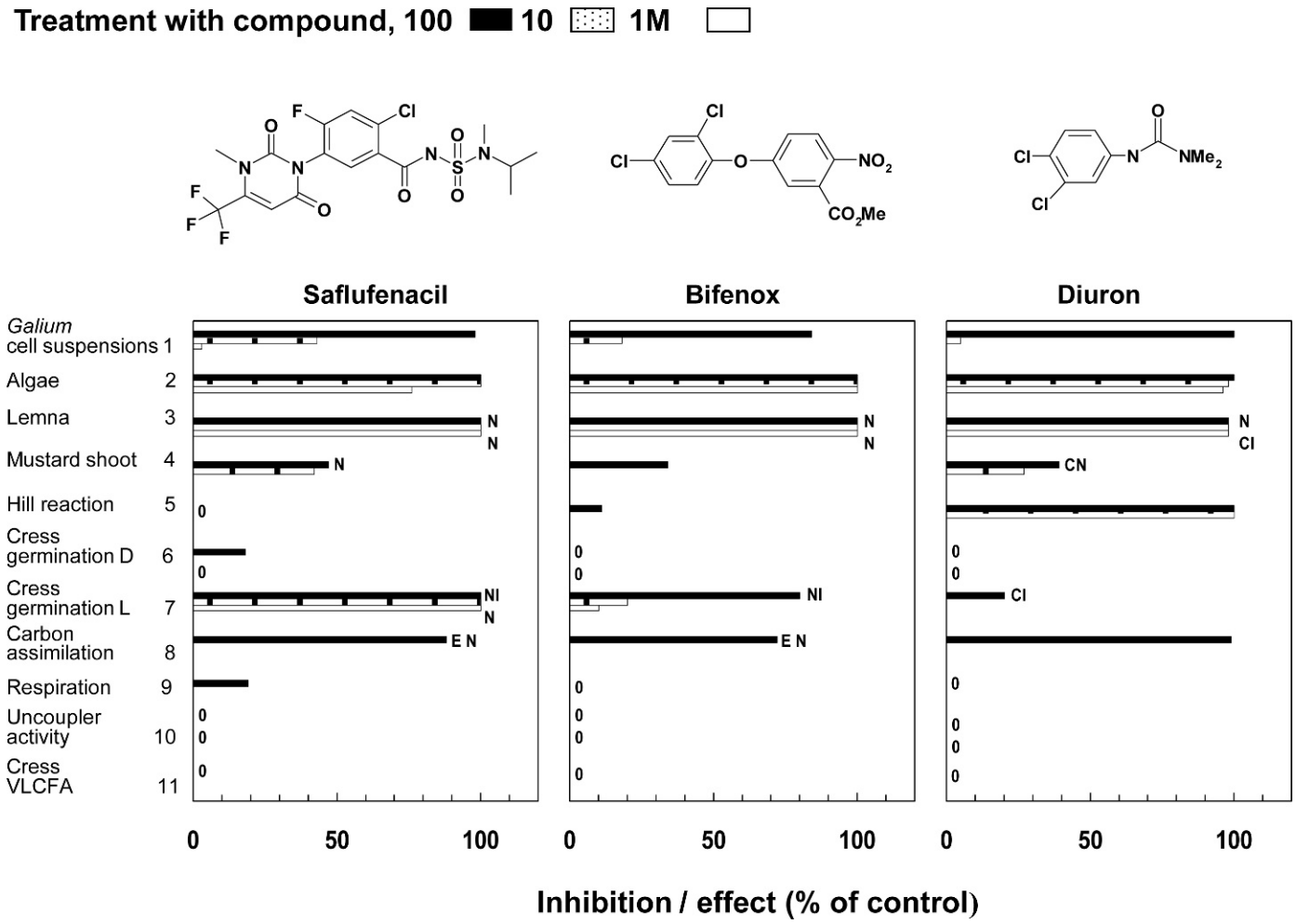


Figure 1. Effects of saflufenacil, bifenox, and diuron in bioassays including *Galium* (cleaver) and algal cell suspensions, *Lemna* (duckweed) plants, isolated mustard shoots, germinating cress seeds, the Hill reaction of isolated wheat thylakoids, respiration by measuring oxygen consumption in heterotrophic cleaver cell suspensions, uncoupler activity in duckweed root mitochondria, carbon assimilation in cleaver plants, and toluidine-blue staining of cress hypocotyls for the detection of inhibition of very-long-chain-fatty-acid (VLCFA) synthesis. SE of the mean in all cases was less than 10%. Symptoms observed: C, chlorosis; E, epinastic leaf deformation; I, root growth inhibition; N, necrosis; WR, intensified green leaf pigmentation.

benzoyl]-N-isopropyl-N-methylsulfamide; BAS 800H; Kixor™; Figure 1) was from BASF SE (Ludwigshafen, Germany). Bifenox (methyl 5-[2,4-dichlorophenoxy]-2-nitrobenzoate; Figure 1) and diuron (N′-[3,4-dichlorophenyl]-N,N-dimethyl-urea; Figure 1) were obtained from Sigma-Aldrich[1] or Dr. Ehrensdorfer.[2]

Physiological Profiling. The bioassays of the physionomics approach were carried out as described elsewhere (Grossmann 2005). In the heterotrophic cell suspension bioassay, freely suspended callus cells from cleaver (*Galium mollugo* L.)[3] were cultivated in a modified Murashige-Skoog medium as described previously (Grossmann 2005). The cells were subcultured at 7-d intervals. Acetone solutions of the compounds were pipetted into plastic tubes and the solvent allowed to evaporate before 2 ml of exponentially growing cell suspensions were added. The tubes (three replicates) were shaken at 400 rpm and 25 C in the dark on a rotary shaker. After incubation for 8 d, the conductivity of the medium was measured as the parameter for cell division growth (Grossmann 2005).

For the algae bioassay, cells of *Scenedesmus acutus* Pringsh.[4] were propagated photoautotrophically (Grossmann 2005). The bioassay was carried out in plastic microtiter dishes containing 24 wells (Grossmann 2005). Before each well was loaded with 0.5 ml cell suspension, 0.5 ml medium and compound in acetone solution were added, and sufficient time was allotted for the organic solvent to volatilize. The 15 additional compartments between the wells were filled with sodium carbonate/bicarbonate buffer. The dishes were sealed with plastic lids and incubated on a shaker under continuous light at 23 C. After 24 h, growth was measured photometrically.

For the duckweed bioassay, stock cultures were propagated mixotrophically in an inorganic medium containing sucrose (Grossmann 2005). The bioassay was conducted under aseptic conditions in plastic petri dishes (5 cm in diameter), which contained 15 ml medium without sucrose. The test compounds were added to the dishes in acetone solution and the organic solvent was allowed to volatilize before four fronds were added to each dish. The culture dishes were then closed with plastic lids and incubated under continuous light (Philips TL white neon tubes, 40 μmol m^{-2} s^{-1} photon irradiance, 400 to 750 nm) in a growth chamber at 25 C. Eight days after treatment, the increase of the area covered by the fronds in each dish was determined as the growth parameter with the use of the image-analyzing system LemnaTec Scanalyzer.[5]

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For the isolated shoot bioassay, seedlings of white mustard (*Sinapis alba* L.) were grown with a nutrient-supplemented peat-based substrate under standardized greenhouse conditions (light/dark: 16/8 h provided by additional illumination with Philips HPI-T Plus lamps, ca. 180 μmol m^{-2} s^{-1}, at 21/19 C and 50 to 80% relative humidity). The shoots were removed, weighed, and placed upright in plastic vials (25 mm in diameter, 38 mm in height; Greiner, Nürtingen, Germany) that contained 12 ml double-distilled water and the test compound added in acetone solution (Grossmann 2005). To reduce evaporation, the vials were closed with plastic covers with slits into which the shoots were fitted (three shoots per vial). The vials were incubated in growth chambers with 16/8-h light/dark photoperiod at 21 C and 75% relative humidity (light: Osram krypton 100-W lamps and Osram universal white neon tubes, 200 μmol m^{-2} s^{-1} photon irradiance, 400 to 750 nm). After 3 d, the changes in fresh weight were measured by weighing the shoots and subtracting the value from the initial weight.

To determine effects on Hill reaction, thylakoids were isolated from shoots of young plants of wheat and assay was performed as previously described (Grossmann 2005). Isolated thylakoids were suspended in a reaction medium (0.75 ml) that contained sucrose (0.1 M), tricine–NaOH (pH 8.0; 50 mM), magnesium chloride (5 mM), and chlorophyll (41 μg ml^{-1}). The assay mixture included thylakoid suspension (0.23 ml), test compound dissolved in acetone + water (80 + 20 by volume; 0.05 ml) and ferrocyanide (0.02 ml). During the subsequent illumination, ferrocyanide was formed in the Hill reaction. Then, in darkness, the ferrocyanide was allowed to react with ferric salt to form the ferrous salt, which produced a complex with phenanthroline. The complex was measured photometrically at 510 nm.

For the germination bioassay, seeds of cress (*Lepidum sativum* L.) were placed in glass petri dishes (5 cm in diameter) filled with a vermiculite substrate (Grossmann 2005). Stock solutions of the test compounds in acetone were added together with 12 ml water. Control seeds were moistened only with water and acetone. The dishes were incubated in a growth chamber at 25 C in the dark for 3 d. Inhibition of germination and seedling development was evaluated visually (0 = no influence, 100 = total inhibition). Afterwards, the dishes were incubated for further 3 d under light conditions (16/8 h light/dark at 25 C and 75% relative humidity, 230 μmol m^{-2} s^{-1} photon irradiance, 400 to 750 nm) and seedling development and plant symptoms were evaluated. For visualization of cuticular defects as an indication for compound-induced inhibition of very-long-chain-fatty-acid (VLCFA) synthesis in cuticular wax formation, seedling shoots were cut off and stained with toluidine blue for 5 min (Grossmann 2005). Epidermal surface of hypocotyl without a functional cuticle is permeable to the hydrophilic dye toluidine blue, which leads to blue staining of hypocotyl tissue.

To determine CO_2 uptake as a parameter for CO_2 assimilation, plants of cleaver (*Galium aparine* L.), which had been raised under greenhouse conditions to the second-whorl stage, were cultivated hydroponically in illuminated glass chambers (four plants per chamber, three replications) that received a constant stream of air (Grossmann 2005). After foliar treatment, the amount of CO_2 assimilated per unit time was determined continuously from the difference between the CO_2 contents of the inflowing and outflowing air streams.

For the determination of respiration, cell suspensions of cleavers (*Galium mollugo* L.)[3] were treated with compound in plastic vessels for 5 h in the dark on a rotary shaker (Grossmann 2005). Then, samples of 5 ml cell suspension were transferred to plastic tubes for measurement of oxygen consumption with the dissolved oxygen measuring system inoLab Oxi Level 3 with the oxygen sensor CellOx 325.[6]

To determine the uncoupler activity of compounds, duckweed plants were pretreated with the mitochondrial potential sensor dye JC-1 (10 μg ml^{-1} medium)[7] for 30 min. JC-1 exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green to red (Grossmann 2005). Mitochondrial membrane depolarization is indicated by a decrease in the red/green fluorescence intensity ration. After staining was accomplished, plants were washed and loaded in 48-well plastic microtiter dishes with each well containing four fronds, 0.5 ml medium, and compound added in acetone solution. After treatment for 10 min, plants were transferred to slides for fluorescence microscopic observation of root mitochondria with the use of an Olympus BX61 epifluorescence microscope.[8]

The results were expressed as percentage inhibition. Mean values of three replicates are given as the percentage inhibition relative to the control. Individual standard errors were less than 10%. All experiments were repeated at least twice and proved to be reproducible. The results of a representative experiment are shown.

Metabolite Profiling. Duckweed plants were cultivated and treated with saflufenacil in plastic petri dishes (three replicates) under continuous light in a growth chamber at 25 C as described. Saflufenacil was added to the dishes, containing 15 ml medium without sucrose, in acetone solution, and the organic solvent was allowed to volatilize before the dishes were loaded with about 120 fronds each. Controls (nine replicates) received corresponding amounts of acetone alone. After 48 and 72 h of treatment with 1 μM saflufenacil, plants from three replicates were washed with distilled water, rapidly dried with filter paper, sampled (200 mg total fresh weight), and frozen in liquid nitrogen. After freeze drying took place, the metabolites were extracted with the use of accelerated solvent extraction (ASE) with polar (methanol + water, 80 + 20 by volume) and nonpolar (methanol + dichlormethan, 40 + 60 by volume) solvents. Subsequent analyses of metabolites by gas chromatography–mass spectrometry (GC-MS) were performed as described elsewhere (Roessner et al. 2000; Walk et al. 2007). In addition, liquid-chromatography–mass-spectrometry (LC-MS/MS; Niessen 2003) analyses were carried out with the use of an Agilent 1100 capillary LC system coupled with an Applied Biosystems/MDS SCIEX API 4000 triple quadrupole mass spectrometer.[9] After reverse-phase high performance liquid chromatography (HPLC) separation, detection and quantification of metabolites were performed in the multiple reaction monitoring (MRM) mode (Gergov et al. 2003).

After GC-MS and LC-MS/MS analyses, data normalization, and data validation, significance between treatment-group values per time and metabolite and respective untreated control-group values were tested with the use of the Student's *t* test (two-sided with inhomogeneous variance; Mead et al.

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1993). Overall, changes in the levels of about 200 identified metabolites and 300 unknown analytes were determined and calculated as numerical ratios, relative to control. Changes of known metabolites are visualized in a biochemical pathway view generated by Metanomics Pathway Explorer. Check for known mode-of-action assignment was performed by comparing the metabolite profile of saflufenacil, including total changes of known and unknown analytes, with those of about 150 previously characterized standard compounds representing a range of about 60 herbicide modes of action. For mode-of-action cluster analysis, prediction analysis of microarrays (PAM, described in Tibshirani et al. 2002) and partial least square–discriminant analysis (PLS-DA, described in van Ravenzwaay et al. 2007) were used as multivariate statistical data analysis. PAM was applied for ranking profile changes elicited by a new compound to profile classes generated by compounds with known mode-of-action class membership with the use of the nearest-shrunken-centroid methodology. The quality of the classification can be estimated by the distance values (dist) and maximum log likelihood values (mml). The ranking is based on the logarithmized probability indicated by mml. The mml values are always below or, in the best case, equal to 0, because P values are between 0 and 1. The dist value is an indicator for the degree of distinctness in the classification, with the highest value as the best, and results from the mml values of the two best ranks of classes. PLS-DA, which was performed with the use of Simca-P+ Version 11 (van Ravenzwaay et al. 2007), is a supervised regression method. It is used to construct an empirical model relating *y*-type variables (e.g., treatments) with *x*-type variables (e.g., analytes) when the variables are many and highly correlated. By applying PLS-DA, a model is obtained that describes maximum separation among predefined classes (Holmes and Antti 2002).

PPO Enzyme Assay and Determination of Tissue Levels of Proto and Hydrogen Peroxide. Enzyme activity of PPO (EC 1.3.3.4) was extracted from coleoptiles or shoots (150 g fresh weight) of dark-grown corn, velvetleaf, and black nightshade seedlings as described previously (Grossmann and Schiffer 1999). Before harvesting took place, the seedlings were allowed to green for 2 h in the light to achieve the highest specific enzyme activities in the thylakoid fractions at low chlorophyll concentrations. At high chlorophyll concentrations significant quenching of fluorescence occurs, which limits the amount of green thylakoids that can be used in the test. Plant materials were homogenized in the cold with a Braun blender with the use of a fresh-weight-to-volume ratio of 1:4. Homogenization buffer consisted of tris(hydroxymethyl)aminomethane (Tris)–HCl (50 mM; pH 7.3), sucrose (0.5 M), magnesium chloride (1 mM), ethylenediaminetetraacetic acid (EDTA) (1 mM), and bovine serum albumin (2 g L^{-1}). After filtration through four layers of Miracloth, crude plastid preparations were obtained after centrifugation at 10,000 × *g* for 5 min and resuspension in homogenization buffer before centrifugation at 150 × *g* for 2 min to remove crude cell debris. The supernatant was centrifuged at 4,000 × *g* for 15 min and the pellet fraction was resuspended in 1 ml of a buffer containing Tris-HCl (50 mM; pH 7.3), EDTA (2 mM), leupeptin (2 μM), pepstatin (2 μM), and glycerol (200 ml L^{-1}) and stored at −80 C until use. Protein was determined in the enzyme extract with bovine serum albumin as a standard. PPO activity was assayed fluorometrically by monitoring the rate of Proto formation from chemically reduced protoporphyrinogen IX under initial velocity conditions. The assay mixture consisted of Tris-HCl (100 mM; pH 7.3), EDTA (1 mM), dithiothreitol (5 mM), Tween 80 (0.085%), protoporphyrinogen IX (2 μM), and 40 μg extract protein in a total volume of 200 μl. The reaction was initiated by addition of substrate protoporphyrinogen IX at 22 C. Saflufenacil was prepared in dimethyl sulfoxide (DMSO) solution (0.1 mM concentration of DMSO in the assay) and added to the assay mixture in concentrations of 0.005 pM to 5 μM before incubation. Fluorescence was monitored directly from the assay mixture with the use of a POLARstar Optima/Galaxy (BMG) with excitation at 405 nm and emission monitored at 630 nm. Nonenzymatic activity in the presence of heat-inactivated extract was negligible. Inhibition of enzyme activity induced by the herbicide was expressed as percentage inhibition relative to untreated controls. Molar concentrations of compound required for 50% enzyme inhibition (IC_{50} values) were calculated by fitting the values to the dose–response equation with the use of nonlinear regression analysis.

Determination of Proto accumulation was performed as described previously (Grossmann and Schiffer 1999). Leaf discs (0.4 cm diameter) were cut from blades of second and third leaves of corn, velvetleaf, and black nightshade plants with a corkborer under dim, green light conditions. Ten randomized leaf discs were then placed adaxial side down on a filter paper in a petri dish (5-cm diameter, four replicates) moistened with 1 ml MES (2-[*N*-morpholino]ethanesulfonic acid) buffer (pH 6.1; 10 mM) containing saflufenacil. Leaf discs in petri dishes were incubated in darkness at 25 C for 24 h before exposure to light for 2 h. Afterwards, leaf discs of replicates were harvested, immediately frozen in solid carbon dioxide and powered under liquid nitrogen. Samples (100 mg) were extracted with methanol + 0.1 M ammonium hydroxide (90 + 10 by volume; 3× 1.25 ml) under a dim, green light source. After centrifugation at 16,000 × *g* for 10 min, the supernatants were combined and evaporated to dryness at 36 C with a rotary evaporator. The residue was dissolved in methanol + 20 mM ammonium phosphate (90 + 10 by volume; pH 5.8; 1 ml) and 100 μl of the sample was separated by reverse-phase HPLC on a NovaPac C18 5-μm column (150 by 4 mm)[10] with the use of a gradient program from methanol + 20 mM ammonium phosphate (80 + 20 by volume; pH 5.8) to methanol + acetonitrile (50 + 50 by volume). The sweep time was 15 min at a flow rate of 1.2 ml min^{-1}. An aliquot of the fractions was removed and fluorescence was monitored for Proto spectrophotometrically. A commercial standard of Proto[1] was used for calibration. Proto levels were expressed on a molar basis per gram fresh weight. The standard deviation of results obtained from parallel extractions of plant material was < 10%.

For determination of H_2O_2 levels, leaf discs in petri dishes were incubated in darkness at 25 C for 22 h before exposure to light for 4 h. Levels of H_2O_2 in the leaf tissue were measured by the modified colorimetrical method of Chang and Kao (1998). Powdered plant material (100 mg, three replications) was extracted at 4 C for 5 min in 1.25 ml phosphate–HCl buffer (50 mM, pH 4.0). The extract was centrifuged at 10,000 × *g* for 10 min and 900 μl of the supernatant was mixed with 400 μl of 0.1% titanium sulphate in H_2SO_4 + water (20 + 80 by volume). After 20 min at 4 C

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and subsequent centrifugation, the intensity of the yellow color of the supernatant was measured at 405 nm. The production of reactive oxygen species was calculated with the use of a calibration curve established in the presence of H_2O_2. Recovery of H_2O_2 was above 60%, as determined by using added standard H_2O_2 to extracts.

Results and Discussion

Physiological Profiling. In initial experiments, a set of bioassays has been used to characterize and classify the mode of action of saflufenacil in more detail. These systems included heterotrophic cleaver and photoautotrophic green algae cell suspensions, duckweed plants, isolated white mustard shoots, and germinating cress seeds. The test panel is completed by assays for monitoring physiological processes, including the Hill reaction of isolated wheat thylakoids, respiration by measuring oxygen consumption in heterotrophic cleaver cell suspensions, membrane function/uncoupler activity determined in duckweed root mitochondria with the use of the potential sensor JC-1, carbon gas-exchange measurements in cleaver plants, and toluidine-blue staining of cress hypocotyls on inhibition of VLCFA synthesis. The response pattern represents a fingerprint of a compound that has proved to be typical of its mode of action (Grossmann 2005). The results can be interpreted directly, or a library of response patterns of compounds with known mode of action can be screened for similarities as an aid to direct further investigations.

The results showed that the inhibitory effects of saflufenacil on growth is triggered through an inhibition of the photosynthetic system (Figure 1). Saflufenacil induced rapid necrosis of plant tissue in light, as observed in duckweed, cress seedlings incubated under light conditions, and cleaver plants treated for the determination of carbon assimilation. Accordingly, inhibition of phototrophic growth in algae and duckweed and reduced carbon assimilation of cleaver plants were found. In addition, growth in heterotrophic cleaver cell suspensions was affected only at high concentrations, and germinating seeds of cress responded only slightly in the dark (Figure 1). No effects were observed on respiratory activity as measured through oxygen consumption in heterotrophic cleaver cell suspensions and on mitochondrial membrane potentials in duckweed roots. Toluidine-blue staining of cress hypocotyls, as an indication of an inhibition of VLCFA synthesis, was also not observed. In contrast to Photosystem II (PSII) electron transport inhibitors, such as diuron, saflufenacil did not directly affect the photosynthetic electron transport in the Hill reaction and induced more rapid tissue necrosis in light, as observed in duckweed and cress seedlings. When compared with the effects of known PPO inhibitors, such as the diphenyl ether bifenox, saflufenacil induced the same type of physiological profile; however, at a higher activity level in the bioassays. This indicates a similar mode of action. Accordingly, in saflufenacil-treated cleaver plants used in the carbon assimilation assay, light-dependent leaf tissue necrotization and reduction in carbon assimilation were accompanied by epinastic leaf deformation (Figure 1). Leaf epinasty is a known phenomenon of PPO inhibitors, because the succeeding series of events induced by these herbicides include Proto-mediated formation of activated oxygen, which elicits synthesis of the growth-regulating phytohormone ethylene (Grossmann and Schiffer 1999; Wakabayashi and Böger 1999).

	moa	dist	mml
1	Protox inhibitors	46.05	-0.00000000022
2	EPSP synthase	2.38	-13.01
3	Cellulose synthesis	2.58	-15.35
4	PSII, microtubule disruption, cellulose synthesis	0.56	-7.21
5	Acetolactate synthase, ALS	5.07	-1.19

Figure 2. Mode-of-action classification by cluster analysis of metabolite changes with the use of prediction analysis of microarrays (PAM). In duckweed plants treated with 1 µM saflufenacil for 48 and 72 h, changes of about 200 identified and about 300 unknown analytes, relative to control, were determined. PAM was applied for ranking profile changes elicited by saflufenacil to profile classes generated by herbicides with known mode-of-action class membership. Ranking of the best five modes of action from 60 is shown (see detailed description of method in the text).

Metabolite Profiling in Duckweed. Metabolite changes to an herbicide can be seen as the end result of alterations induced in the biochemical process steps to the physiological response. Consequently, metabolite profiling is a way to generate profiles that are highly diagnostic of a herbicidal mode of action (Fernie et al. 2004; Sauter et al. 1991). In duckweed, our former studies have shown that this methodology can screen novel compounds for herbicidal mode of action by comparing their metabolite profile with those of previously characterized standards (unpublished data). For this purpose, we have built up a database that includes metabolite profiles of about 150 herbicides from about 60 different modes of action, applied in multiple concentrations and times of treatment. Aquatic duckweed was used because

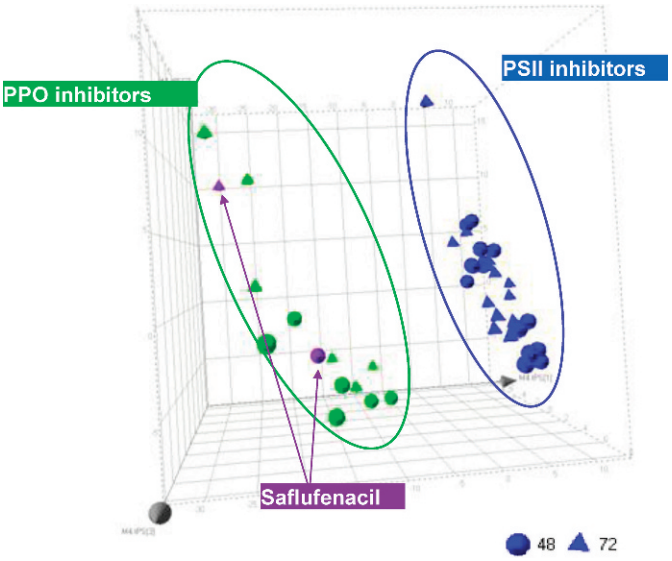


Figure 3. Refinement of mode-of-action classification by cluster analysis of metabolite changes with the use of partial least square–discriminant analysis (PLS-DA). In duckweed plants treated with 1 µM saflufenacil for 48 and 72 h, changes of about 200 identified and about 300 unknown analytes, relative to control, were determined. Testing for profile assignment to the mode of action classes of protoporphyrinogen IX oxidase (PPO) inhibitors or Photosystem II (PSII) inhibitors (represented by seven PSII- and three PPO-inhibitor standard herbicides, applied in three concentrations for 48 and 72 h) was performed with the use of multivariate clustering analysis by PLS-DA. PLS-DA constructs an empirical model that relates treatment as observation (class variable), with analytes as *x*-type variable. The model is visualized as a score plot where each point represents an individual sample. The predictability of the model, indicated by the Q2 (cum) value, is 0.905.

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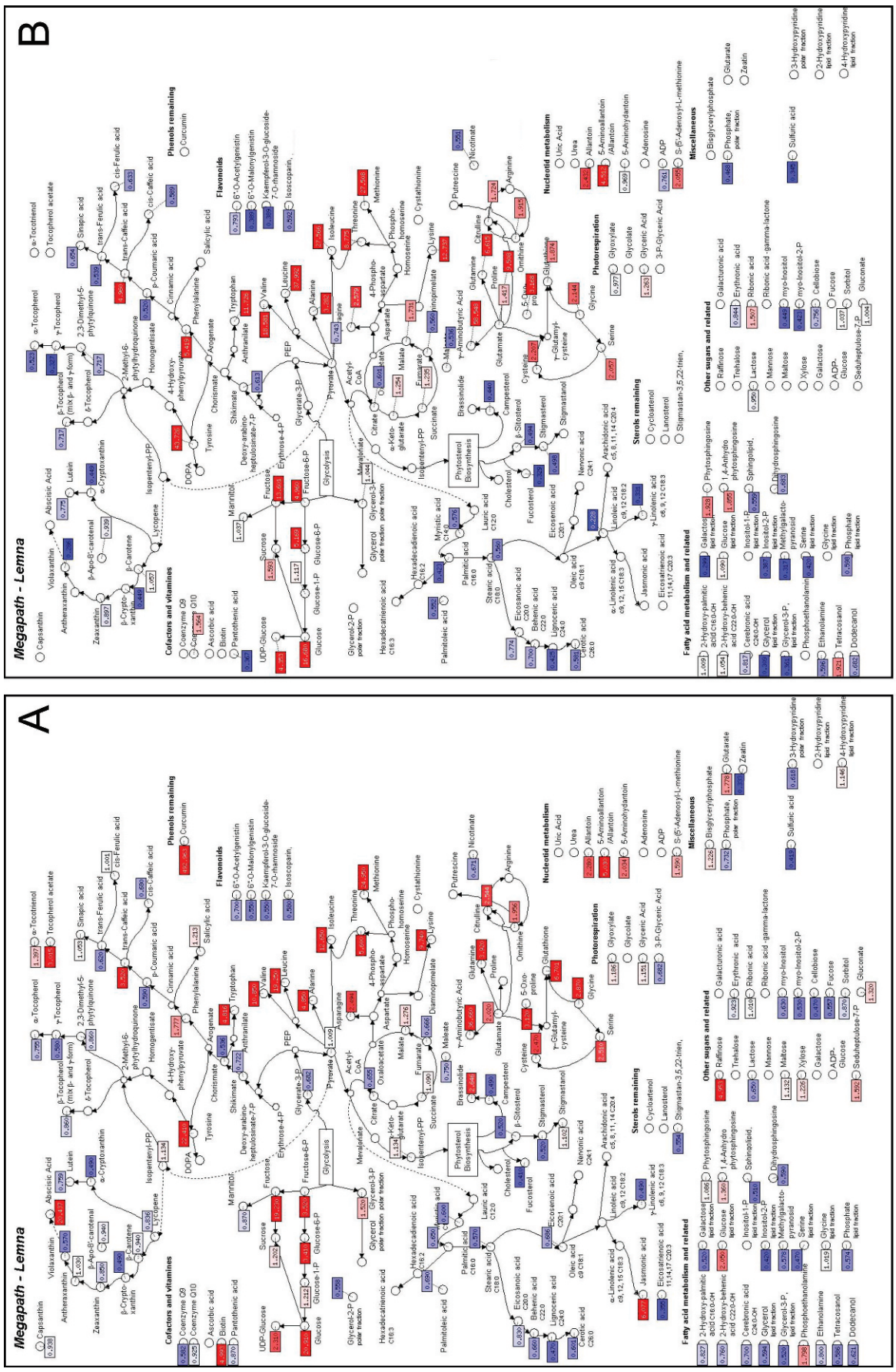


Figure 4. Metabolite profile in duckweed plants treated with 1 µM of saflufenacil (A) and 1 µM bifenox (B) for 48 h. Changes in identified metabolite levels were visualized as ratios, relative to control, in a biochemical pathway view. Metabolites in pathways with flags in red indicate increase in levels, with flags in blue show decrease in levels, related to control. The numerical ratios of changes in metabolite levels are given in the flags.

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Table 1. Effect of saflufenacil on protoporphyrinogen IX oxidase activity extracted and assayed from corn, velvetleaf, and black nightshade.

Plant species	Saflufenacil concentrations required for 50% inhibition (IC_{50}) (nM)
Corn	0.6
Velvetleaf	0.4
Black nightshade	0.2

this small plant, like most important weeds, possesses all organs of a higher vascular plant. The fast growth rate, the genetic uniformity, the facilitated uptake of applied compounds, the small space requirements, and testing under controlled conditions define duckweed as an excellent plant system.

For metabolite profiling of saflufenacil effects, duckweed plants were treated with 1 μM of compound for 48 and 72 h. After solvent extraction and subsequent GC-MS and LC-MS/MS analyses, changes in tissue levels of nearly 200 identified and 300 unknown analytes were quantified and statistically evaluated, relative to control. Check for assignment of saflufenacil profile to known mode-of-action classes was performed with the use of multivariate statistical data analysis by PAM and PLS-DA. First, PAM was applied for ranking profile changes elicited by saflufenacil to profile classes generated by herbicides with known mode-of-action class membership. With excellent maximum log-likelihood value (mml) of 2.2×10^{-10} and distance value (dist) of 46, at first position of ranking, the mode of action of saflufenacil was assigned to PPO inhibitors (Figure 2). The following next-ranked four modes of action from 60, shown in Figure 2, revealed only poor assignment (Figure 2). These included inhibitors of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, cellulose synthesis, and acetolactate synthase (ALS), and triaziflam, which affects multiple target sites with PSII, microtubule disruption, and cellulose synthesis (Grossmann et al. 2001). For refinement of mode-of-action classification, PLS-DA was applied subsequently. The scatter plot in Figure 3 illustrates that the model clearly assigned the metabolite profile induced by saflufenacil to the predefined cluster of those caused by known PPO inhibitors (bifenox, cinidon-ethyl, oxyfluorfen). In contrast, PLS-DA revealed maximum separation of the PPO inhibitor cluster including saflufenacil to the predefined class of known PSII electron transport inhibitors (atrazine, bentazon, bromoxynil, chlorpropham, diuron, metribuzin, phenmedipham). For characterization of saflufenacil effects on plant metabolism in detail, changes in known metabolite levels were visualized as ratios, relative to control, in a biochemical pathway view (Figure 4). Most pronounced changes in metabolite levels were found after treatment with 1 μM saflufenacil for 48 h. At this time, treated duckweed plants had developed only slight symptoms of frond chlorosis, which developed to necrosis, 72 h after treatment. Despite the inconspicuous plant phenotype, 48 h after saflufenacil treatment, strong changes in the metabolite profile typical of PPO inhibitors were obtained. Particularly, strong increases in levels of sugars including fructose and glucose and amino acids of up to 20-fold were observed. This rise in fructose and glucose levels could be explained by sugar recycling from enhanced starch degradation, as an early result from inhibition of photosynthesis by saflufenacil. Subsequent downstream metabolite flow via glycolysis and citrate cycle to the different amino acid pathways could increasingly provide

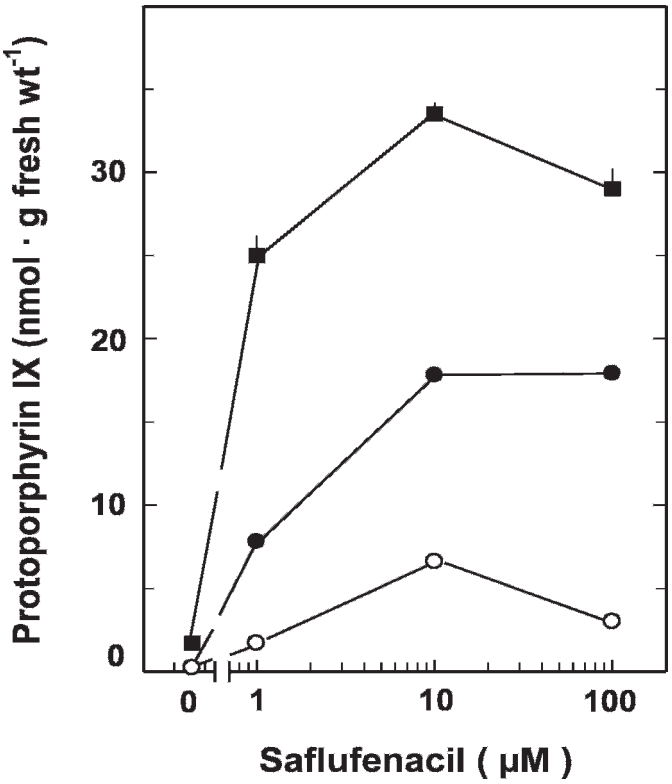


Figure 5. Influence of saflufenacil on protoporphyrin IX (Proto) accumulation in leaf discs from corn (open circle), black nightshade (square), and velvetleaf (closed circle). Vertical bars represent SE of the mean.

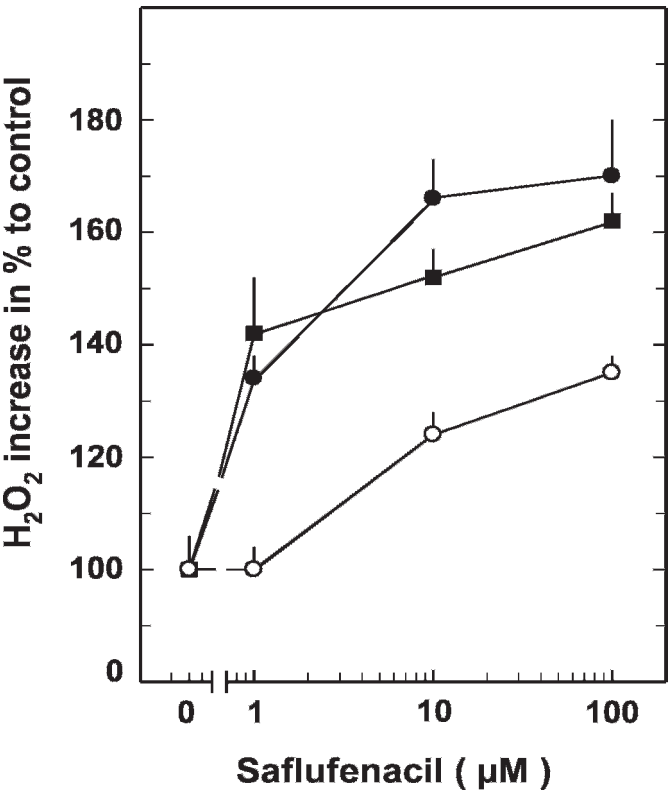


Figure 6. Influence of saflufenacil on H_2O_2 accumulation in leaf discs from corn (open circle), black nightshade (square), and velvetleaf (closed circle). Vertical bars represent SE of the mean. Control levels (100%) in μmol/g fresh weight were 2.34 ± 0.04 for corn, 1.55 ± 0.17 for black nightshade, and 3.71 ± 0.23 for velvetleaf.

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intermediates for enhanced amino acid synthesis. In addition, accelerated protein degradation leading to enhanced levels of amino acids could occur. Concomitantly, saflufenacil-induced decrease in tocopherols and a multitude of lipids, including fatty acids and phytosterols, and carotenoids is typical of reactive oxygen-mediated lipid peroxidation and bleaching of chloroplast pigments. When compared with the effects of bifenox, saflufenacil elicited nearly identical changes in metabolite levels (Figure 4). Overall, metabolite cluster and profile analyses indicated a mode of action of saflufenacil similar to PPO inhibitors.

PPO Enzyme Assay and Determination of Tissue Levels of Proto and H_2O_2. In order to determine the effect of saflufenacil on PPO enzyme activity in vitro, crude plastid preparations from corn coleoptiles and shoots of velvetleaf and black nightshade were assayed. The IC_{50} concentrations obtained for PPO inhibition were around 0.4 nM saflufenacil in all plant species (Table 1) and therefore among the most active inhibitors tested so far (Wakabayashi and Böger 1999). The blockage of PPO prevents the normal enzymatic conversion of protoporphyrinogen IX, which diffuses into the cytoplasm, where it leads to Proto accumulation after oxidation (Dayan and Duke 1997; Wakabayashi and Böger 1999). When leaf discs of corn, velvetleaf, and black nightshade were treated with saflufenacil for 24 h in darkness before exposure for 2 h to light, a concentration-dependent increase in Proto levels was found (Figure 5). At 1 μM saflufenacil, Proto levels increased in corn, velvetleaf, and black nightshade approximately 2-fold, 8-fold, and 25-fold, relative to the control, respectively (Figure 5). In the light, Proto accumulation is known to lead to the formation of activated oxygen (Matringe et al. 1993). Proto increase in saflufenacil-treated leaf discs was accompanied by an accumulation of H_2O_2 (Figure 6). In leaf discs treated with 1 μM saflufenacil for 22 h in darkness before exposure to light for 4 h, H_2O_2 levels increased to 134 and 142% of the control in velvetleaf and black nightshade, respectively. In corn, a rise in H_2O_2 levels was found only at concentrations above 1 μM (Figure 6).

In conclusion, the results from physiological and metabolite profiling and the observed Proto and H_2O_2 accumulation in the green tissue and inhibition of PPO enzyme activity in the nanomolar range indicate that saflufenacil is a new PPO-inhibiting herbicide with peroxidative mode of action.

Sources of Materials

[1] Diuron, Sigma-Aldrich Chemie, 82041 Deisenhofen, Germany.

[2] Bifenox, Dr. Ehrensdorfer, 86199 Augsburg, Germany.

[3] Cleaver cells, DSMZ, Collection of Plant Cell Cultures, 38124 Braunschweig, Germany.

[4] *Scenedesmus acutus* Pringsh. cells, Culture Collection of Algae, University of Göttingen, 37073 Göttingen, Germany.

[5] Image-analyzing system, LemnaTec, 52146 Würselen, Germany.

[6] Oxygen sensor CellOx 325, WTW, 82362 Weilheim, Germany.

[7] Mitochondrial potential sensor dye JC-1, Invitrogen, 76131 Karlsruhe, Germany.

[8] Epifluorescence microscope, Olympus, 20097 Hamburg, Germany.

[9] Triple quadrupole mass spectrometer, Agilent Technologies, 76337 Waldbronn, Germany; Applied Biosystems, 64293 Darmstadt, Germany.

[10] NovaPac C18 5-μm column, Waters, 65760 Eschborn, Germany.

[11] Sigma-Aldrich Chemie, 82041 Deisenhofen, Germany.

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