Saflufenacil (Kixor™): Biokinetic Properties and Mechanism of Selectivity of a New Protoporphyrinogen IX Oxidase Inhibiting Herbicide

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Saflufenacil (Kixor[TM]): Biokinetic Properties and Mechanism of Selectivity of a New Protoporphyrinogen IX Oxidase Inhibiting Herbicide

Klaus Grossmann, Johannes Hutzler, Guenter Caspar, Jacek Kwiatkowski, and Chad L. Brommer*

Saflufenacil (Kixor[TM]) is a new protoporphyrinogen IX oxidase (PPO) inhibiting herbicide for preplant burndown and selective PRE dicot weed control in multiple crops, including corn. The biokinetic properties and the mechanism of selectivity of saflufenacil in corn, black nightshade, and tall morningglory were investigated. After root treatment of plants at the third-leaf stage, the difference in the phytotoxic selectivity of saflufenacil in corn and the weed species has been quantified as approximately 10-fold. The plant species showed similar selectivity after foliar applications; the plant response to saflufenacil was approximately 100-fold more sensitive compared with a root application. PPO enzyme activity *in vitro* was inhibited by saflufenacil, a 50% inhibition lay in a concentration range from 0.2 to 2.0 nM, with no clear differences between corn and the weed species. Treatments of light-grown plants and dark-grown seedlings with [^{14}C]saflufenacil revealed that the herbicide is rapidly absorbed by root and shoot tissue. The [^{14}C]saflufenacil was distributed within the plant systemically by acropetal and basipetal movement. Systemic [^{14}C]saflufenacil distribution can be explained by the weak acid character of saflufenacil and its metabolic stability in black nightshade and tall morningglory. Metabolism of [^{14}C]saflufenacil in corn was more rapid than in the weeds. In addition, low translocation of root-absorbed [^{14}C]saflufenacil in the corn shoot was observed. It is concluded that rapid metabolism, combined with a low root translocation, support PRE selectivity of saflufenacil in corn.

Nomenclature: Butafenacil; flumioxazin; saflufenacil; black nightshade, *Solanum nigrum* L., SOLNI; corn, *Zea mays* L., ZEAMX; tall morningglory, *Ipomoea purpurea* (L.) Roth. PHBPU; velvetleaf, *Abutilon theophrasti* Medik., ABUTH.

Key words: Herbicide mechanism of selectivity, metabolism, uptake and translocation, protoporphyrinogen IX oxidase inhibitor, saflufenacil.

Herbicides that inhibit PPO (Enzyme Classification [EC] 1.3.3.4), which catalyzes the conversion of protoporphyrinogen IX to protoporphyrin IX (Proto) in tetrapyrrole biosynthesis, have successfully been used for weed management in agriculture (Hirai et al. 2002; Matringe et al. 1993; Nagano 1999). Particularly, inhibitors of the *N*-phenylnitrogen heterocycle-type 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 inhibition prevents the synthesis of chlorophylls, hemes, and cytochromes in the chloroplast (for review see Dayan and Duke 1997; Matringe et al. 1993; Matsumoto 2002; Wakabayashi and Böger 1999). As a further consequence, high concentrations of Proto accumulate in the green tissues, which results from an extraplastidic oxidation of protoporphyrinogen IX. 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. Lipid peroxidation results in a rapid loss of membrane integrity and function, bleaching of chloroplast pigments, tissue necrosis, and, ultimately, growth inhibition and plant death.

The pyrimidinedione-type herbicide saflufenacil (Kixor[TM]) was commercially introduced in 2010 for crop desiccation, preplant burndown, and residual PRE control of major dicot weeds in multiple crops, including corn. In corn, saflufenacil can be used PRE at rates of approximately 63 to 125 g ai ha^{-1} for broad dicot weed control, including large-seeded species such as morningglory and velvetleaf (Liebl et al. 2008; Soltani et al. 2009). PRE selectivity of saflufenacil in corn is conferred by physical placement and natural crop tolerance (Liebl et al.

2008). Studies using physiological and metabolite profiling and PPO enzyme activity testing showed that saflufenacil is a PPO-inhibiting, peroxidizing herbicide (Grossmann et al. 2010). Accordingly, accumulations of Proto and hydrogen peroxide (H_2O_2) were observed in treated leaf tissue of sensitive weeds. In corn, only slight increases in Proto and H_2O_2 were found, which indicates a mechanism leading to lower sensitivity to saflufenacil of this crop (Grossmann et al. 2010).

Therefore, the aim of this study was to analyze the mechanism of selectivity of saflufenacil between dicot weeds, such as black nightshade and tall morningglory, and corn. After root and shoot application of [^{14}C]saflufenacil, the uptake, translocation, and metabolism of the herbicide in light-grown young plants and dark-grown seedlings were compared with the effects on PPO activity and plant growth.

Materials and Methods

Chemicals. Saflufenacil; BAS 800H; Kixor[TM]; Figure 1), [*uracil-4-*^{14}C]saflufenacil (98% radiochemical purity, specific activity 4.14 MBq mg^{-1}), and the adjuvant Dash HC© were from BASF SE (Ludwigshafen, Germany). Butafenacil (Figure 1) and flumioxazin (Figure 1) were obtained from Sigma-Aldrich.[1]

Plant Toxicity Studies. For determination of herbicidal selectivity, uniformly developed plants of corn, black nightshade, and tall morningglory were grown with a nutrient-

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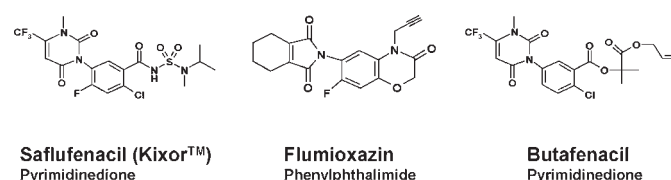

Figure 1. Structures of protoporphyrinogen IX oxidase (PPO)-inhibiting pyrimidinediones saflufenacil (Kixor[TM]) and butafenacil, and phenylphthalimide flumioxazin.

supplemented peat-based substrate to the third-leaf stage under standardized greenhouse conditions (light/dark: 16/8 h with additional illumination by Philips HPI-T Plus lamps, ca. 180 µmol m^{-2} s^{-1}, at 21/19 C and 50 to 80% humidity). For root treatment in hydroponics, plants were transferred into 320 ml glass vessels, which contained 310 ml of half-strength Linsmaier–Skoog (1964) medium, and were maintained at 16/8 h light (400 µmol m^{-2} s^{-1}, 400 to 750 nm)/dark cycles, 22/20 C and 75% relative humidity in climate chambers (three plants per vessel, five replications in randomized position). The solution was aerated throughout the experiments. After 3 d of adaptation, the compound was added to the medium in acetone solution (1 ml L^{-1}, final concentration of acetone). Controls received corresponding amounts of acetone alone, with no adverse effect on the growth of the plants. After incubation for 4 d, fresh weight of shoot parts per plant was determined (mean values from 15 shoots), and molar concentrations of compound required for 50% reduction of shoot fresh weight (IC$_{50}$) were calculated. For foliar treatment, plants were raised in soil in plastic pots (diameter 12.5 cm, volume 500 ml, 1 plant pot^{-1}, 10 replicates) to the third-leaf stage. Plants were sprayed uniformly on a turntable with 400 µl per pot of aqueous solutions (800 L ha^{-1}), which contained saflufenacil in an EC-formulation (120 g L^{-1}) and the adjuvant Dash HC$^{©}$ (0.25%, v/v, equivalent to a field rate of 1 L ha^{-1}), using an airbrush spray gun.[2] Controls received corresponding amounts of the formulation ingredients and adjuvant alone with no adverse effect on plant growth. After incubation for 2 d in climate chambers under the conditions described above, fresh weight of shoot parts per plant was determined (mean values from 10 shoots), and molar concentrations of compound required for IC$_{50}$ were calculated.

For investigation of phytotoxic effects after local leaf application of PPO inhibitors, plants were grown with a nutrient-supplemented peat-based substrate in plastic pots (diameter 12.5 cm, volume 500 ml, 1 plant pot^{-1}, four replicates) to the first (tall morningglory) and third (black nightshade) leaf stage under standardized greenhouse conditions. Ten 1-µl droplets of aqueous solutions, which contained EC-formulated PPO inhibitor and the adjuvant Dash HC$^{©}$ (0.25%, w/v, equivalent to a field rate of 1 L ha^{-1}), were applied with a microsyringe to the adaxial midsection of the first (tall morningglory) or second (black nightshade) leaf. The dose of PPO inhibitors was approximately equivalent to a field rate of 60 g ai ha^{-1} in 400 L of carrier. Afterwards, the pots were placed in growth chambers under continuous low light (70 µmol m^{-2} s^{-1}, 400 to 750 nm, Osram powerstar HQI-R 250W/NDL and Osram krypton 100W lamps) at 22 C and 75% relative humidity for 24 or 48 h. In order to study regrowth behaviour, plants were incubated, following the 24/48 h of continuous light, for additional time at 16/8 h light (400 µmol m^{-2} s^{-1})/dark cycles and 22/20 C.

PPO Enzyme Assay. Enzyme activity of PPO (EC 1.3.3.4) was extracted from coleoptiles or shoots (150 g fresh weight) of dark-grown corn, black nightshade, morningglory, and velvetleaf seedlings as described previously (Grossmann et al. 2010). The seedlings were allowed to green for 2 h in the light before harvesting, in order 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 using 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. Protoporphyrinogen IX was prepared according to the procedure of Jacobs and Jacobs (1982) with the following modifications. All steps were carried out in an argon-flooded tray. Proto (Sigma-Aldrich, Taufkirchen, Germany) stock solution (0.43 mM in 20% ethanol, 10 mM potassium hydroxide [KOH]) was reduced to protoporphyrinogen IX with 20 g of sodium amalgam (Sigma-Aldrich, Taufkirchen, Germany). The resulting colorless solution was adjusted to pH 8.5 by addition of an equal volume of dilution buffer, consisting of 250 mM Tris-HCl (pH 8.5), 5 mM EDTA, and 5 mM reduced L-glutathione (Sigma-Aldrich, Taufkirchen, Germany), and filtered through a 0.2-µm nylon syringe filter. The solution was aliquoted, overlaid with paraffin, and stored in the dark at −80 C. 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 extracted protein in a total volume of 200 µl. The reaction was initiated by addition of substrate protoporphyrinogen IX at 22 C. Saflufenacil, flumioxazin, and butafenacil were 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 using 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 IC$_{50}$ values were calculated by fitting the values to the dose-response equation using nonlinear regression analysis.

Uptake, Translocation, and Metabolism of [^{14}C]Saflufenacil in Young Plants in Light. Plants at the third-leaf stage were placed in glass vials (20 mm diameter, 60 mm height) with 20 ml of half-strength Linsmaier–Skoog (1964) medium. The vials were closed with plastic covers with slits into which the plants were fitted upright (1 plant per vial, 10 replicates). For the study of foliar uptake, 10 µl of an aqueous solution containing [^{14}C]saflufenacil (1.6 µg, 6.67 Kilo-Becquerel [KBq], ca. 400,000 decomposition per minute [dpm]) in an EC-formulation (120 g L^{-1}) was applied per plant. In all cases, the solutions contained the adjuvant Dash HC$^{©}$ (0.25%, v/v,

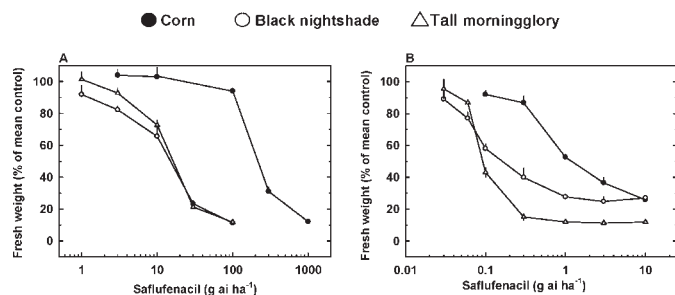Grossmann et al.: Saflufenacil: biokinetics and selectivity • 291

Figure 2. Effect of saflufenacil on shoot fresh weight of young plants of corn, black nightshade, and tall morningglory: (A) 96 h after root treatment in hydroponics and (B) 48 h after foliar treatment. Data were expressed as percent of the mean fresh weight of untreated control plants. Vertical bars represent standard error (SE) of the mean. Calculated 50% reduction of shoot fresh weight (IC_{50}) and selectivity index (SI) values are presented in Tables 1 and 2.

equivalent to a field rate of 1 L ha^{-1}). The dose of saflufenacil was approximately equivalent to a field rate of 60 g ai ha^{-1} in 400 L of carrier. Ten 1-μl droplets were applied with a microsyringe to the adaxial midsection of the second leaf in corn, black nightshade, and tall morningglory. Afterwards, the vials were placed in growth chambers under continuous light (70 μmol m^{-2} s^{-1}, 400 to 750 nm, Osram powerstar HQI-R 250W/NDL and Osram krypton 100W lamps) at 22 C ($\pm$ 1) and 75% ($\pm$ 3) relative humidity. The plants were harvested 16 h after treatment. For the recovery of foliar deposits of the labelled herbicide on or adsorbed to the epicuticular wax layer of the leaf surfaces (nonabsorbed radioactivity), cellulose acetate film stripping was used. This method has been shown to quantitatively remove the platelets of epicuticular wax that cover the intact leaf surface and therefore the labelled herbicide, shortly after drying of the applied droplets (Grossmann and Schiffer 1999). For recovery of the radioactivity taken up in the leaf tissue and translocated within the plant (absorbed radioactivity), each plant was divided into treated leaf (after cellulose acetate film stripping), residual shoot part and root. After determination of fresh weights, plant parts were dried, combusted in a biological materials oxidizer, and radioactivity was quantified by scintillation counting as described (Grossmann and Schiffer 1999). Radioactivity in the cellulose acetate strips was determined after dissolving in acetone. Radioactivity in the plant parts was measured by liquid scintillation counting after combustion. In addition, uptake and translocation were studied by autoradiography. For root treatment, an aqueous solution, which contained [^{14}C]saflufenacil (1.6 μg, 6.67 KBq, ca. 400,000 dpm, dissolved in acetone), was added to the medium of plants cultivated hydroponically, equivalent to a field rate of 60 g ai ha^{-1}. After treatment, the plants were dried and exposed to a film-like radiation image sensor (imaging plate)[3] for 96 h. The autoradiographs were quantified by densitometric scanning using the Bioimage analysis system Analyzer FLA-7000.[4]

For study of metabolism, plants were placed in glass vials and foliar- and root-treated with compounds ([^{14}C] ca. 400,000 dpm per plant) for 16 h as described above. After foliar-treatment, nonabsorbed radioactivity was removed from the leaf surface using cellulose acetate stripping and treated leaf and residual shoot were immediately frozen on solid carbon dioxide and stored at -20 C. After root-treatment, shoot and root material were harvested. Plant material was homogenized in acetonitrile + water (80 + 20 by volume; 10 ml) with a Polytron PT 3100[5] and extracted three times for 1 h at 4 C (three replicate extractions from three plants, respectively). After centrifugation, the combined supernatants were evaporated to dryness and redissolved in acetonitrile + methanol + water (30 + 30 + 40 by volume; 1 ml). A sample of 30 to 100 μl was then separated by reverse-phase high performance liquid chromatography (HPLC) on a Aqua C$_{18}$-5-μm column (250 by 4.6 mm)[6] using a gradient from 40% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid to 90% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid. The gradient sweep time was 30 min at a flow rate of 1.1 ml min^{-1}. Radioactivity of the fractions containing saflufenacil and uracil-ring-N-demethylated and side-chain-N-dealkylated metabolites was determined using an HPLC-coupled radioactivity monitor LB 509.[7] Radiocarbon recovery was above 80% in all cases after the extraction and HPLC procedure. The parent compound and metabolites were identified by their chromatographic retention and by LC-MS/MS Finnigan LTQ FT.[8]

Uptake, Translocation, and Metabolism of [^{14}C]Saflufenacil in Dark-Grown Seedlings.

Seedlings of corn, black nightshade, and tall morningglory were raised in vermiculite substrate at 25 C in darkness. Seedlings with developed coleoptile (2 to 3 cm length in corn) or cotyledons with hypocotyl (4 cm length in black nightshade, 5 cm length in tall morningglory) were placed upright in plastic vials with 10 ml (corn) or 2 ml (black nightshade, tall morningglory) of half-strength Linsmaier–Skoog (1964) medium (one seedling per vial; 30 replicates in corn, 45 replicates in black nightshade and tall morningglory). For shoot treatment,

Table 1. Sensitivity of young plants of corn, black nightshade, and tall morningglory to saflufenacil after root treatment in hydroponics for 96 h.

Plant species	Rate giving 50% reduction in shoot fresh weight (IC_{50}) (g ai ha^{-1})	SI[a]
Corn	190	—
Black nightshade	15	13
Tall morningglory	18	11

[a] The selectivity index (SI) was calculated as follows: SI = IC_{50} (crop)/IC_{50} (weed).

Table 2. Sensitivity of young plants of corn, black nightshade, and tall morningglory to saflufenacil after foliar treatment for 48 h.

Plant species	Rate giving 50% reduction in shoot fresh weight (IC_{50}) (g ai ha^{-1})	SI[a]
Corn	1.30	—
Black nightshade	0.15	9
Tall morningglory	0.09	14

[a] The selectivity index (SI) was calculated as follows: SI = IC_{50} (crop)/IC_{50} (weed).

Table 3. Effects of protoporphyrinogen IX oxidase (PPO) inhibitors on PPO activity extracted and assayed from corn, black nightshade, tall morningglory, and velvetleaf.

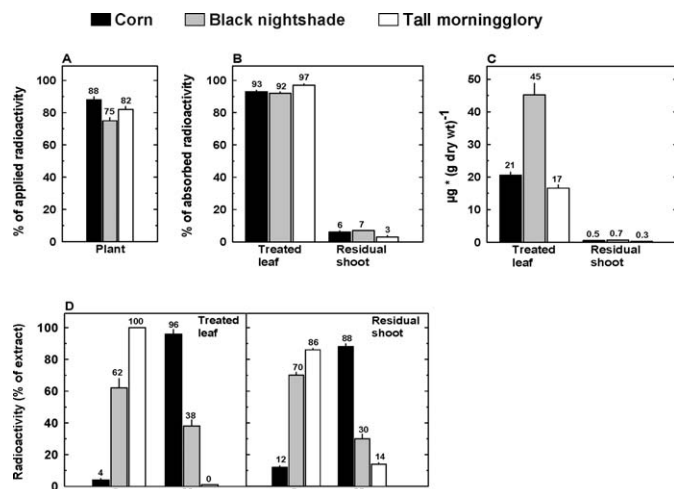| Plant species	Concentrations required for 50% inhibition (IC_{50}) (nM)		
	Saflufenacil	Flumioxazin	Butafenacil
Corn	0.6	0.021	0.005
Black nightshade	0.2	0.018	0.013
Tall morningglory	2.0	0.094	0.020
Velvetleaf	0.4	0.004	0.002

Figure 3. Foliar uptake (A), translocation (B, C), and metabolism (D) of [^{14}C]saflufenacil in light-grown plants of corn, black nightshade, and tall morningglory. Plants at the third-leaf stage were foliar treated with EC-formulated [^{14}C]saflufenacil with the addition of Dash HC$^©$ at equivalents to field rates of 60 g ai ha^{-1} and 1 L ha^{-1}, respectively. After treatment for 16 h, radioactivity taken up in the leaf tissue and translocated beyond was determined by combustion analysis. Data are averages of 10 replicate plants. For study of metabolism (D), radioactivity was extracted from the plant material and analysed by high performance liquid chromatography (HPLC) radiocounting. Radioactivity in % of total extract found in the peaks of saflufenacil (Sa) and totalled metabolites (Me) was shown. Data are averages from three replicate extractions of three plants, respectively. Vertical bars represent standard error (SE) of the mean.

2 μl of an aqueous solution containing [^{14}C]saflufenacil (0.7 μg, 2.84 KBq, ca. 170,000 dpm) and nonlabeled saflufenacil (0.9 μg) in an EC-formulation (120 g L^{-1}), and Dash HC$^©$ (0.25%, v/v) were applied as a droplet to the coleoptile tip in corn or to the cotyledons in morningglory. The dose of saflufenacil was approximately equivalent to a field rate of 60 g ai ha^{-1} in 400 L of carrier. Seedlings of black nightshade were treated with 1.3 μl of an aqueous solution containing EC-formulated [^{14}C]saflufenacil (ca. 0.45 μg, 117,000 dpm) and nonlabeled saflufenacil (0.6 μg), and Dash HC$^©$ by dipping the cotyledons in 150 μl of solution for 30 s. To avoid run off of the applied solution from cotyledon surface, seedlings were placed at an angle against the side of the vials. The vials were incubated at 25 C in darkness and seedlings were harvested 24 h after treatment. Nonabsorbed radioactivity was removed from coleoptile tips of corn and cotyledon surfaces of tall morningglory using cellulose acetate film stripping as described. In black nightshade, nonabsorbed radioactivity was removed from cotyledon surfaces by washing two times with water + acetonitrile (90 + 10 by volume; plus one drop of Tween 20) for 1 min. Radioactivity was extracted from apical and basal halves of coleoptile, seed, and root in corn and from cotyledons, hypocotyl, and root in black nightshade and tall morningglory (three replicate extractions, respectively), and the parent compound and metabolites were determined by HPLC-radiocounting as described above. For root treatment, seedlings were placed upright in plastic vials with nutrient solution containing EC-formulated [^{14}C]saflufenacil, equivalent to a field rate of 60 g ai ha^{-1}. The vials were incubated at 25 C in darkness, and seedlings were harvested 24 h after treatment. Afterwards, nonabsorbed radioactivity was removed from radicles by washing with

water. Radioactivity was extracted from root, seed, and coleoptile in corn and from root, hypocotyl, and cotyledons in black nightshade and tall morningglory (three replicate extractions, respectively), and the parent compound and metabolites were determined by HPLC-radiocounting as described above.

Results and Discussion

Herbicidal Selectivity. Herbicidal selectivity of saflufenacil was studied after root and foliar treatment of light-grown plants at the third-leaf stage. For root application of saflufenacil without the presence of soil, plants were treated with increasing rates of saflufenacil (equivalent to field rates of 1 g ai ha^{-1} to 1,000 g ai ha^{-1}) in hydroponics for 96 h (Figure 2A). In plants of black nightshade and tall morningglory, saflufenacil caused rapid chlorophyll bleaching, desiccation, and necrosis of the green tissues. First phytotoxic symptoms of chlorophyll bleaching were evident within 12 h after treatment. Necrosis developed progressively, first on the youngest leaf blades and petioles and veins of older leaves. Complete necrosis of the entire shoot was observed 48 h after treatment with 60 g ai ha^{-1}. In order to quantify the selectivity between the weed species black nightshade and tall morningglory and the crop species corn, the concentrations of saflufenacil required for IC$_{50}$ values were determined from linear regression equations (Table 1). As shown in Table 1, the selectivity indices of saflufenacil between corn and black nightshade or tall morningglory were 13 and 11, respectively. Foliar application of saflufenacil was carried out by spraying plants with increasing rates of saflufenacil (equivalent to field rates of 0.03 g ai ha^{-1} to 10 g ai ha^{-1}; Figure 2B). In black nightshade, tall morningglory, and corn, saflufenacil caused rapid desiccation and necrosis of the green tissues with first phytotoxic symptoms within 2 to 3 h after treatment. After 48 h under continuous light, IC$_{50}$ values showed that corn and the weed species responded to foliar-applied saflufenacil approximately 100-fold more sensitive (Table 2) than after root exposure (Table 1). The selectivity indices between corn and black nightshade or tall morningglory of 9 and 14, respectively (Table 2), were in a similar range compared to root treatment (Table 1). In summary, the results show that corn exhibits in planta tolerance to saflufenacil.

PPO Enzyme Assay. In order to determine the effect of saflufenacil on PPO enzyme activity *in vitro*, crude plastid preparations from dark-grown corn coleoptiles and shoots of black nightshade, tall morningglory, and velvetleaf were assayed. The IC$_{50}$ concentrations obtained for PPO inhibition by saflufenacil were in a range from 0.2 to 2.0 nM without clear differences in sensitivity to the herbicide between the weed species and corn (Table 3). This indicates a mechanism of selectivity to saflufenacil in corn, which is not target-based. In comparison to the phenylphthalimide flumioxazin and the pyrimidinedione butafenacil, saflufenacil inhibited PPO enzyme activity with lower intensity (Table 3).

Uptake, Translocation, and Metabolism of [^{14}C]Saflufenacil in Young Plants in Light. Foliar uptake of radiolabelled saflufenacil and long distance transport of radioactivity to the meristematic tissues in the shoot were compared in plants at the third-leaf stage of corn, black nightshade, and tall morningglory, which were cultivated in hydroponics during
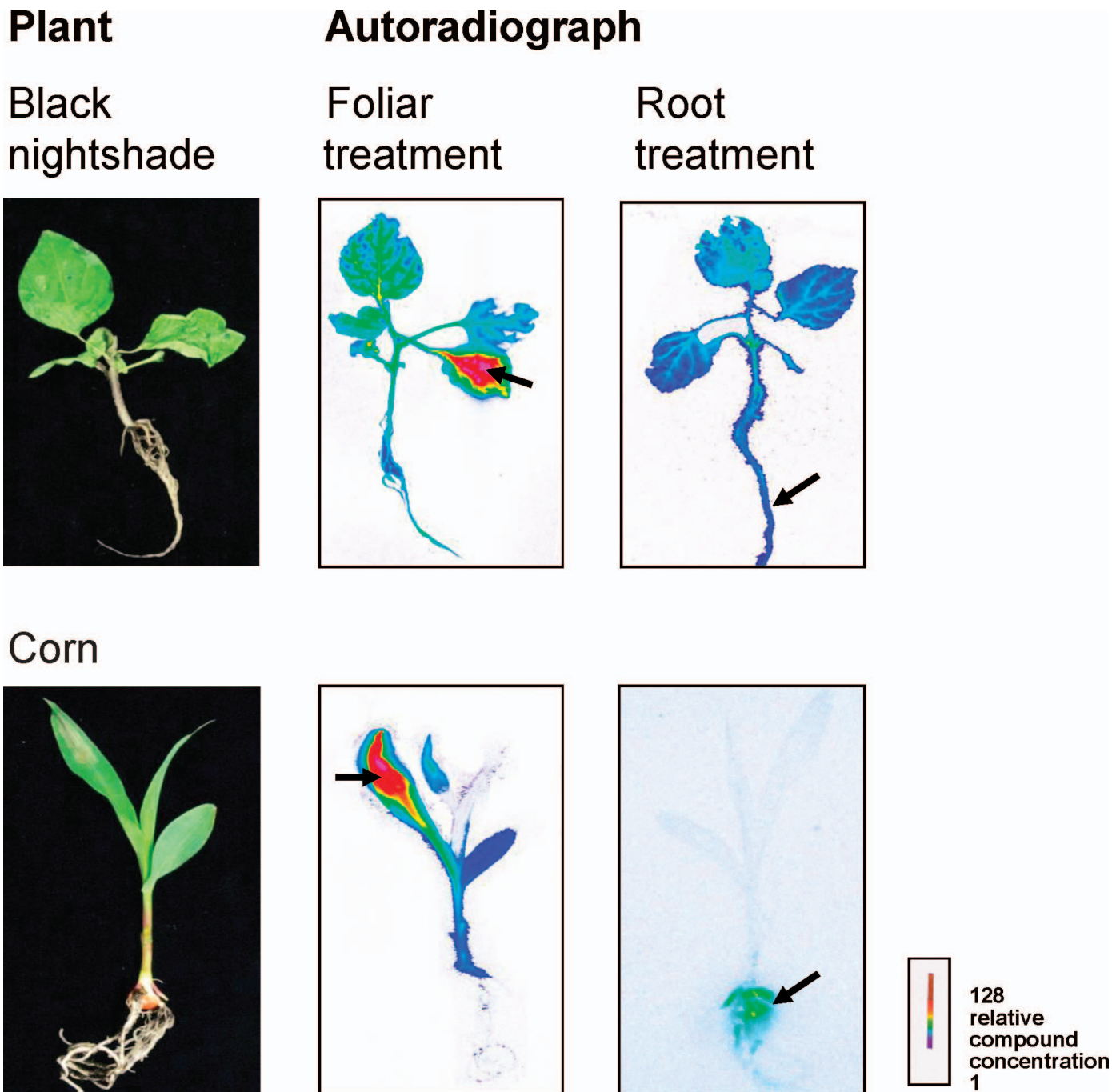
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Figure 4. Autoradiographs of corn and black nightshade plants, which were foliar and root treated (area indicated by arrows) with [^{14}C]saflufenacil, equivalent to field rates of 60 g ai ha^{-1}. After treatment for 16 h, dried plants were exposed to a phosphor imaging plate. The autoradiographs were quantified by densitometric scanning using an image analysis system.

16 h of treatment in light. The [^{14}C]herbicide was applied, equivalent to a field rate of 60 g ai ha^{-1}, to the midsection of the second leaf, and compound behaviour was studied using combustion analysis of plant parts and autoradiography. For foliar treatment, [^{14}C]saflufenacil in EC formulation was used and the adjuvant Dash HC$^©$, equivalent to a field rate of 1 L ha^{-1}, was added to the aqueous solution. In corn, black nightshade, and tall morningglory, approximately 80% of the applied radioactivity was taken up within 16 h of treatment (Figure 3A). During this time, ca. 6, 7, and 3% of the absorbed ^{14}C had been translocated beyond treated leaf to the residual shoot in corn, black nightshade, and tall morningglory,

respectively (Figure 3B). In addition, a portion of ca. 0.8, 0.7, and 0.3% of foliar-absorbed radioactivity was partitioned into the root in corn, black nightshade, and tall morningglory, respectively (not shown). For determination of tissue concentrations, [^{14}C]compound levels were related to tissue dry weight to avoid influence of different levels of tissue desiccation by saflufenacil treatment on calculation (Figure 3C). In black nightshade, tissue concentration of absorbed [^{14}C]herbicide in the treated leaf was approximately twofold higher than those in tall morningglory and corn. Concomitantly, in the residual shoot of black nightshade, [^{14}C]compound concentration was higher than in the other plant species. The higher mobility of
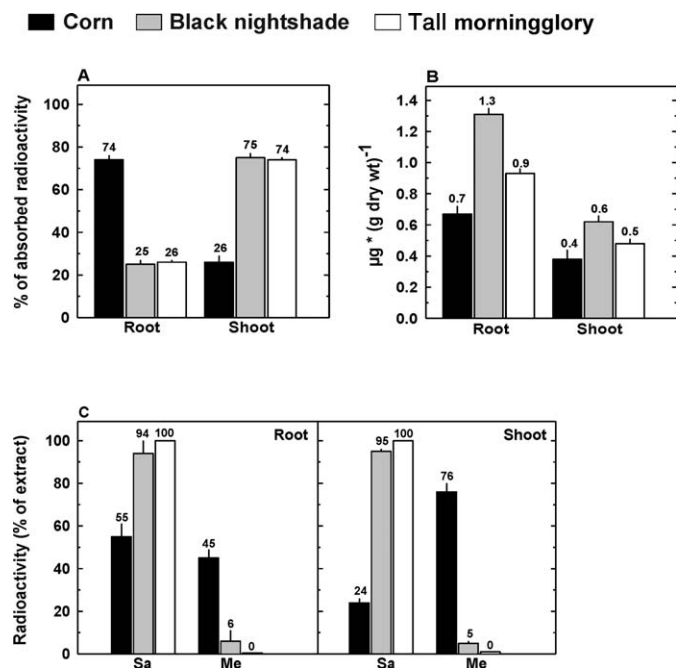
Figure 5. Root uptake and translocation (A, B), and metabolism (C) of [14C]saflufenacil in light-grown plants of corn, black nightshade, and tall morningglory. Plants at the third-leaf stage were root treated with [14C]saflufenacil at equivalents to field rates of 60 g ai ha^{−1}. After treatment for 16 h, radioactivity taken up in the root tissue and translocated to the shoot was determined by combustion analysis. Data are averages of 10 replicate plants. For study of metabolism (C), radioactivity was extracted from the plant material and analysed by high performance liquid chromatography (HPLC) radiocounting. Radioactivity in % of total extract found in the peaks of saflufenacil (Sa) and totalled metabolites (Me) was shown. Data are averages from three replicate extractions of three plants, respectively. Vertical bars represent standard error (SE) of the mean.

saflufenacil in black nightshade was also demonstrated by autoradiography (Figure 4). After foliar uptake of [14C]herbicide, the compound was able to move systemically within the plant, from the treated leaf in phloem vascular tissue beyond to areas of meristematic growth and storage in the residual shoot parts and the root. For study of metabolism after foliar treatment, experimental procedures were identical to those used for the investigation of uptake and translocation. After removal of the nonabsorbed radioactivity from the leaf surface of treated plants, radioactivity, absorbed in the treated leaves and translocated beyond to the residual shoot, was extracted. Saflufenacil and its initial metabolites, uracil-ring-N-demethylated and side-chain-N-dealkylated metabolites, were quantified by HPLC radiocounting. The metabolites are not highly active in PPO inhibition and phytotoxicity (not shown). Therefore, radioactivity found in these peaks were totalled and listed as "metabolites" in the figures. Pronounced differences were found in the metabolism of saflufenacil between the crop and the weed species (Figure 3D). Corn metabolized more of the parent herbicide than black nightshade and particularly, tall morningglory. After 16 h, in the treated leaf tissues of corn, black nightshade, and tall morningglory, 4, 62, and 100% of the absorbed radioactivity, respectively, were found as unchanged herbicide. Similar differences were found in the metabolism of [14C]saflufenacil that had been translocated beyond treated leaves to the residual shoot. After 16 h, 12, 70, and 86% of the extracted radioactivity was identified as unchanged herbicide in corn, black nightshade, and tall morningglory, respectively (Figure 3D).
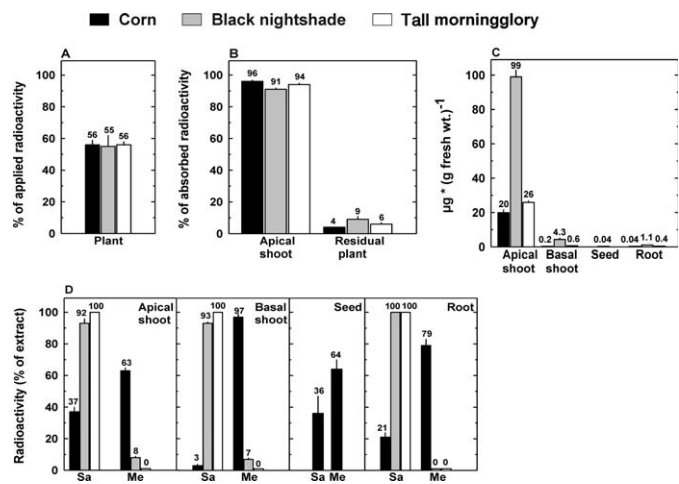

Figure 6. Uptake (A), translocation (B, C), and metabolism (D) of [14C]saflufenacil in dark-grown seedlings after apical shoot treatment (coleoptile tip in corn, cotyledons in black nightshade and tall morningglory). Seedlings were treated with EC-formulated [14C]saflufenacil with the addition of Dash HC[©] at equivalents to field rates of 60 g ai ha^{−1} and 1 L ha^{−1}, respectively. After treatment for 24 h, radioactivity taken up in the shoot tissue and translocated beyond was determined by extraction of radioactivity: in corn, from tip (apical shoot) and basal halves of coleoptile (basal shoot), seed, and root; in black nightshade and tall morningglory, from cotyledons (apical shoot), hypocotyl (basal shoot), and root. Data are averages of three replicate extractions of 30 (corn) and 45 (black nightshade, tall morningglory) seedlings, respectively. For study of metabolism (D), extracted radioactivity was analysed by high performance liquid chromatography (HPLC) radiocounting. Radioactivity in % of total extract found in the peaks of saflufenacil (Sa) and totalled metabolites (Me) was shown. Vertical bars represent standard error (SE) of the mean.
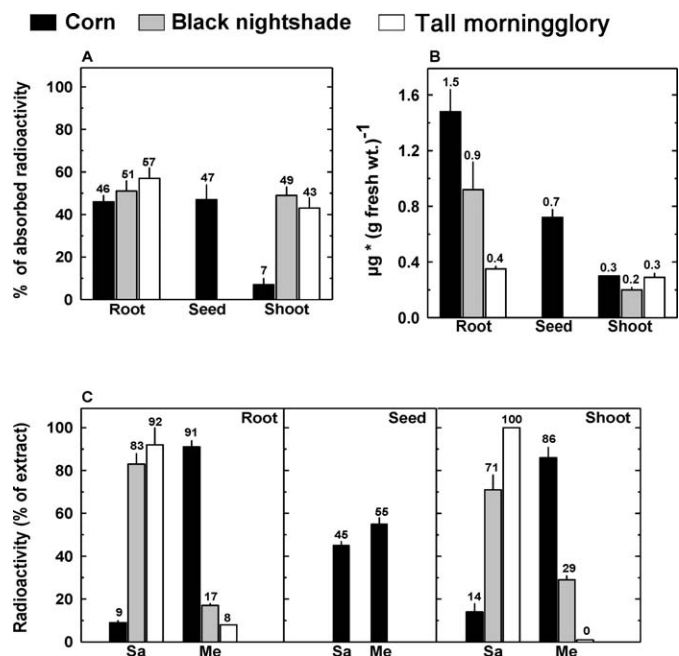
Figure 7. Uptake (B), translocation (A, B), and metabolism (C) of [14C]saflufenacil in dark-grown seedlings of corn, black nightshade, and tall morningglory after root treatment. Seedlings were treated with EC-formulated [14C]saflufenacil, equivalent to field rates of 60 g ai ha^{−1}. After treatment for 24 h, radioactivity was extracted from corn root, seed, and total coleoptile (shoot) and from root and total shoot (hypocotyl, cotyledons) in black nightshade and tall morningglory. Data are averages of three replicate extractions of 30 (corn) and 45 (black nightshade, tall morningglory) seedlings, respectively. For study of metabolism (C), extracted radioactivity was analysed by high performance liquid chromatography (HPLC) radiocounting. Radioactivity in % of total extract found in the peaks of saflufenacil (Sa) and totalled metabolites (Me) was shown. Vertical bars represent standard error (SE) of the mean.

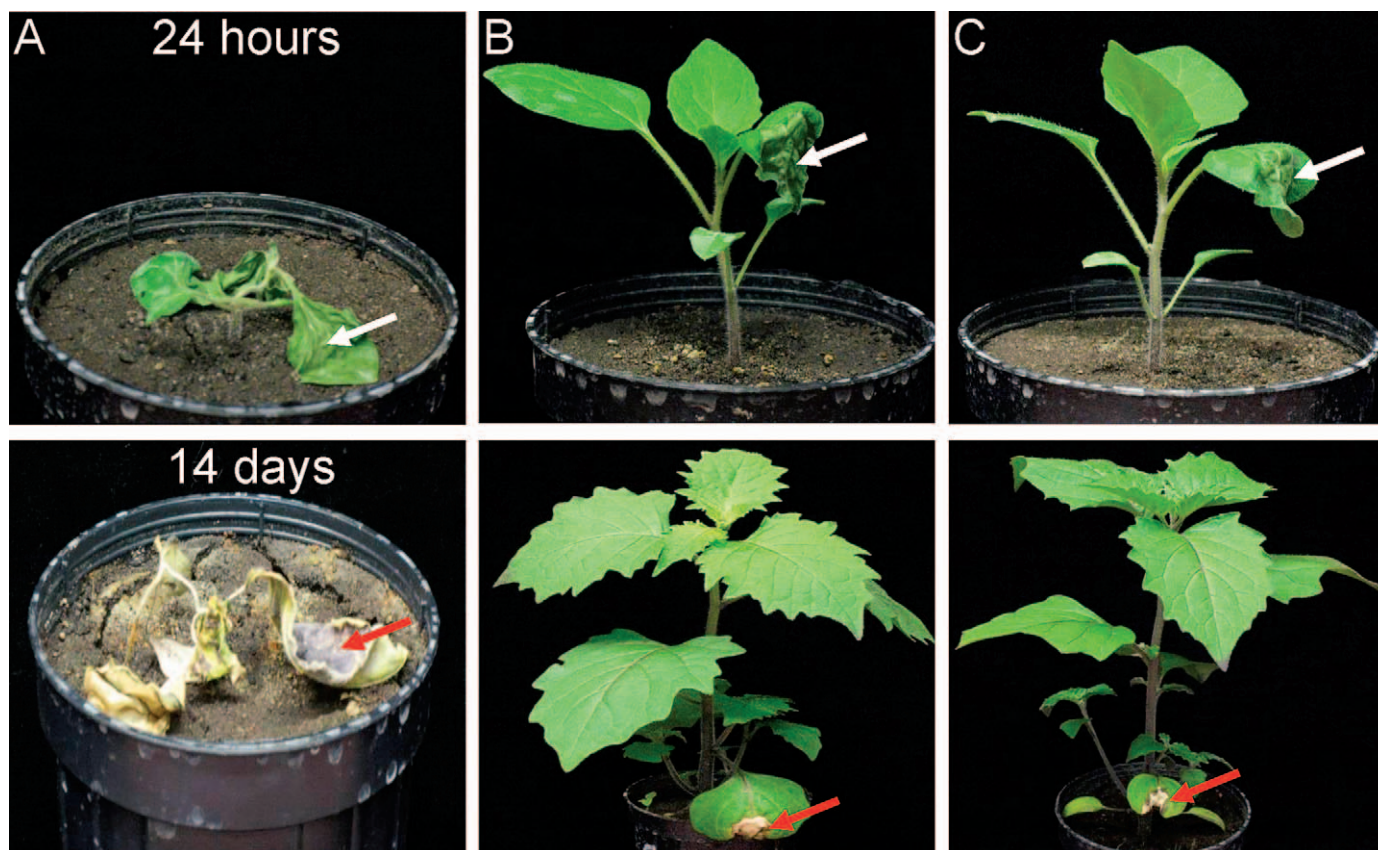Grossmann et al.: Saflufenacil: biokinetics and selectivity • 295

Figure 8. Plant effects after single leaf application of protoporphyrinogen IX oxidase (PPO) inhibitors in black nightshade. Aqueous solutions containing EC-formulated saflufenacil (A), flumioxazin (B), or butafenacil (C), and Dash HC© at equivalents to field rates of 60 g ai ha^{-1} and 1 L ha^{-1}, respectively, were applied as 10 1-µl droplets to the midsection of the second leaf (indicated by arrow) of plants at the third-leaf stage. Then, plants were incubated under continuous light for 24 h, followed by incubation at 16/8 h light/dark cycles for an additional 13 d.

After root exposure of plants to [^{14}C]saflufenacil, equivalent to a field rate of 60 g ai ha^{-1}, for 16 h, acropetal translocation of absorbed ^{14}C to the shoot was considerably higher in the weeds than in corn (Figure 5A). In this time interval, ca. 7% of the applied radioactivity was absorbed by the roots of the plant species (data not shown), and 26, 75, and 74% of the absorbed ^{14}C were translocated acropetally to the shoot in corn, black nightshade, and tall morningglory, respectively (Figure 5A). Based on tissue dry weight, concentrations of [^{14}C]compound in the roots and shoots of the weed species were higher than those in the corn plant parts (Figure 5B). Accordingly, in Figure 4, autoradiographs show that saflufenacil is retained in the root tissue of corn and is, in contrast to black nightshade, only marginally translocated to the shoot. In addition, elevated metabolism of saflufenacil was detected in the shoot tissue of corn (Figure 5C). Here, 24, 95, and 100% of the extracted radioactivity, which had been translocated to the shoot, were detected as unchanged herbicide in corn, black nightshade, and tall morningglory, respectively. In the root tissue of corn, metabolism of absorbed saflufenacil was lower than in the shoot tissue.

Uptake, Translocation, and Metabolism of [^{14}C]Saflufenacil in Dark-Grown Seedlings. In order to simulate the PRE situation of seedlings in contact with saflufenacil without the presence of soil, dark-grown seedlings of corn, black nightshade, and tall morningglory were cultivated in hydroponics in darkness. The seedlings were treated via the shoot or root with [^{14}C]saflufenacil, equivalent to a field rate of 60 g ai ha^{-1}. After 24 h of shoot application via the coleoptile tip (corn) or cotyledons (black nightshade, tall morningglory), approximately 55% of the applied radioactivity was taken up in all plant species (Figure 6A), and 4, 9, and 6% of the absorbed ^{14}C had been translocated beyond to the residual plant parts in corn, black nightshade, and tall morningglory, respectively (Figure 6B). Based on tissue fresh weight, approximately fourfold higher concentrations of ^{14}C were found in the treated shoot parts of black nightshade, compared to tall morningglory and corn (Figure 6C). Concomitantly, tissue concentrations of translocated [^{14}C]herbicide in the basal shoot parts were 0.2, 4.3, and 0.6 µg g fresh weight^{-1} in corn (basal half of coleoptile), black nightshade (hypocotyl), and tall morningglory (hypocotyl), respectively. The lowest concentration of translocated compound was found in corn root tissue. Parent [^{14}C]saflufenacil concentrations were 37, 92, and 100% of the absorbed radioactivity in the corn coleoptile tip and in the cotyledons of black nightshade and tall morningglory, respectively (Figure 6D). Even more pronounced differences were found in the metabolism of [^{14}C]saflufenacil, which had been translocated beyond the treated shoot parts. In the basal part of the corn coleoptile and in the hypocotyl of black nightshade and tall morningglory, 3, 93, and 100% of the translocated radioactivity, respectively, were identified as unchanged herbicide (Figure 6D). Accordingly, in the root tissue of corn, black nightshade, and tall morningglory, 21, 100, and 100% of the translocated radioactivity, respectively, were present as saflufenacil. In the corn seed, 36% of radioactivity was available as unchanged herbicide.
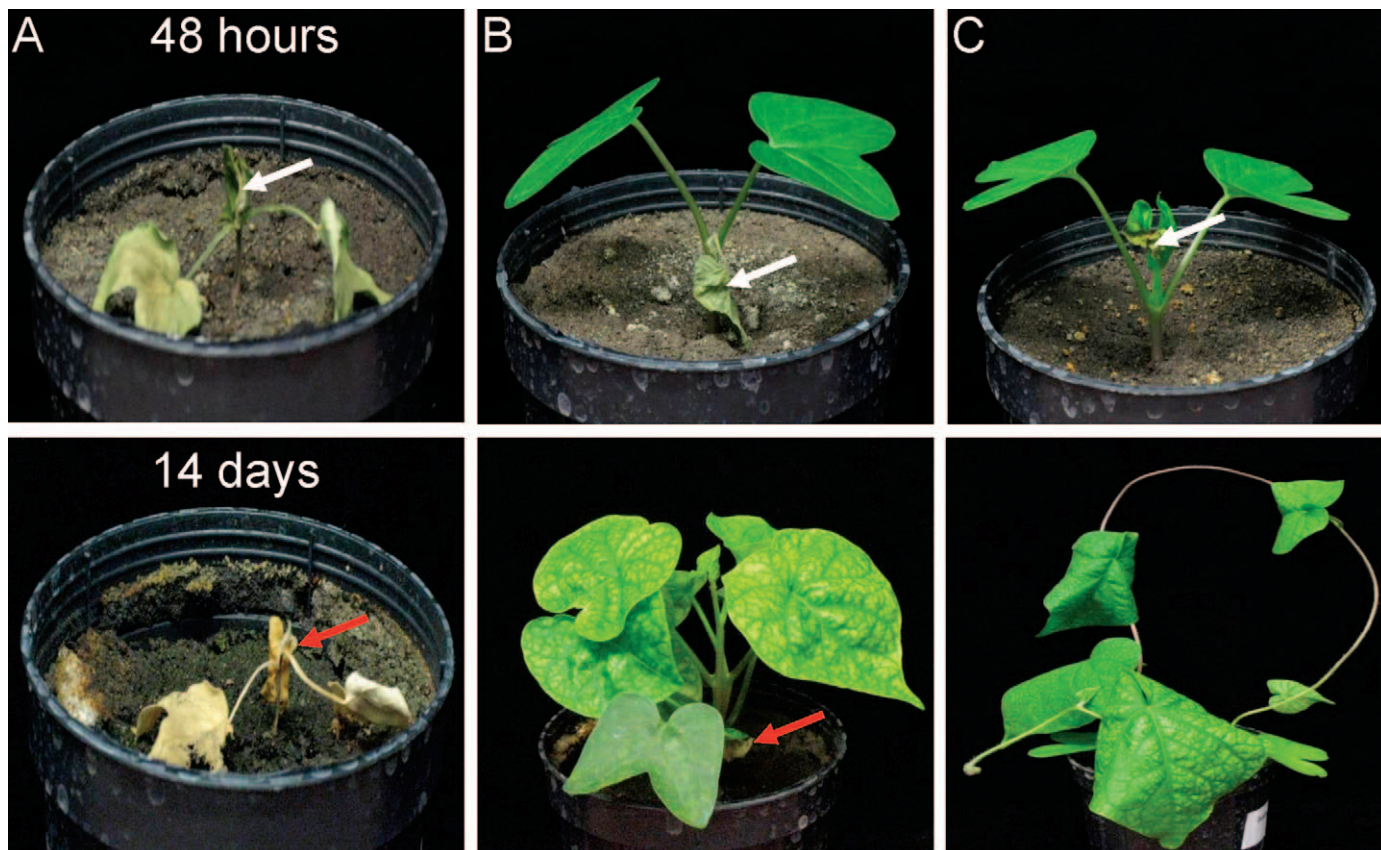
Figure 9. Plant effects after single leaf application of protoporphyrinogen IX oxidase (PPO) inhibitors in tall morningglory. Aqueous solutions containing EC-formulated saflufenacil (A), flumioxazin (B), or butafenacil (C), and Dash HC© at equivalents to field rates of 60 g ai ha^{-1} and 1 L ha^{-1}, respectively, were applied as 10 1-μl droplets to the midsection of the first leaf (indicated by arrow) of plants at the first-leaf stage. Then, plants were incubated under continuous light for 48 h, followed by incubation at 16/8 h light/dark cycles for an additional 12 d.

After root exposure of seedlings for 24 h, based on tissue fresh weight, approximately 1.6- and 4-fold higher concentrations of [^{14}C]compound were found in the roots of corn, compared to black nightshade and tall morningglory, respectively (Figure 7B). Shoot (coleoptile) tissue of corn had concentrations of translocated ^{14}C similar to those found in the shoot parts of the weed species. The reason is that in corn only 7% of the root-absorbed [^{14}C]compound was translocated to the coleoptile, whereas in the weeds, up to 49% of the root-absorbed radioactivity was translocated to the shoots (Figure 7A). The major portions of absorbed radioactivity in corn remained in the root tissue (46%) or in the seed (47%). In accordance with the shoot treatment (Figure 6D), root and coleoptile tissue of corn metabolized root-absorbed and translocated [^{14}C]saflufenacil more efficiently than respective tissues in the weeds (Figure 7C). Portions of 9, 83, and 92% of the absorbed ^{14}C in the root tissue and 14, 71, and 100% of the translocated ^{14}C in the shoot tissue were found as unchanged herbicide in corn, black nightshade, and tall morningglory, respectively. Metabolism of translocated [^{14}C]saflufenacil was lower in corn seed: 45% of the translocated ^{14}C remained as unchanged herbicide.

Conclusion

PRE selectivity of saflufenacil is partly based on physical placement in soil as it affects the amount of herbicide available to the crop in the soil solution (Liebl et al. 2008). Our results show that corn possesses natural tolerance to saflufenacil as a consequence of a more rapid metabolism of the herbicide in the shoot and root tissue, compared to black nightshade and tall morningglory. Low translocation of root-absorbed herbicide into the shoot tissue additionally favours corn tolerance to saflufenacil. Rapid metabolism of saflufenacil to nonphytotoxic metabolites is of particular importance for the developing corn seedling. After possible contact of the seedling with the herbicide in soil, metabolic inactivation of absorbed saflufenacil prevents shoot injury by light exposure following emergence.

As demonstrated in dark-grown seedlings and light-grown young plants of black nightshade and tall morningglory, once absorbed by the plant via the foliage and root, saflufenacil is distributed systemically through acropetal and basipetal movement within the plant. Systemic translocation of saflufenacil was also shown in a variety of other plant species (Ashigh and Hall 2010). This behaviour can be explained by the metabolic stability of saflufenacil in the weeds and its weak acid character, which is unique among commercial PPO inhibitors. This weak acid property of saflufenacil is enabled by the side chain of the molecule, which carries an acidic proton at the nitrogen atom in the amide position. According to Kleier's prediction model, the physical properties of saflufenacil, in terms of ionization constant in aqueous solution value (negative logarithm of acid dissociation constant, Ka [pK_a] of 4.4) and octanol/water partitioning coefficient (logarithm of octanol-water partition coefficient [Log $P_{o/w}$] of 2.6), are nearly

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ideal for phloem systemicity by way of the ion-trap mechanism (Kleier et al. 1998). In the context of the plant vascular system, where pH values typically range from 6 in the xylem to 8 in the phloem (Kleier et al. 1998), the acidic saflufenacil will exist as the anionic form in the xylem and the phloem sap. Saflufenacil is retained in the sieve tube long enough to be transported to remote parts of the plant. Within 16 h of foliar treatment of black nightshade plants at the third-leaf stage, 7% of absorbed ^{14}C had been translocated beyond treated leaf to the residual shoot parts where 70% of the extracted radioactivity was identified as unchanged herbicide (Figures 3B and 3D). However, systemic mobility of a compound also depends on the integrity of the vascular tissue. The general expectation is that the high initial activity of a PPO inhibitor elicits fast necrotic damage of the vascular tissues, which lowers its potential systemic translocation. Saflufenacil's lower or "balanced" intensity of inhibitory action on PPO (Table 3) is thought to lead to slightly delayed injury of vascular tissues after foliar uptake. This enables long-distance transport of the herbicide within the plant before complete tissue destruction occurs.

The consequence is, as demonstrated by treatment of only a single leaf in black nightshade (Figure 8) and tall morning-glory (Figure 9), that saflufenacil elicits contact action on the treated leaf, as well as additional systemic action on the residual shoot parts. The result is the total collapse and necrosis of the entire plant in black nightshade (Figure 8A) and tall morningglory (Figure 9A) within 48 h of saflufenacil treatment. In contrast, PPO inhibitors with limited symplastic phloem movement, such as flumioxazin (Hirai et al. 2002; Senseman 2007; Figures 8B and 9B) and butafenacil (Senseman 2007; Figures 8C and 9C), caused only contact action with tissue necrosis on the treated leaf. Continued growth of the residual shoot parts follows application of these PPO inhibitors. Saflufenacil and butafenacil have a pyrimidine-dione core structure; however, butafenacil does not have a side-chain carrying an acidic proton and, consequently, does not have the same systemic action as saflufenacil. This confirms the importance of the weak acid moiety at the pyrimidinedione structure for systemic mobility and broader herbicidal action.

Sources of Materials

[1] Butafenacil and flumioxazin, Sigma-Aldrich Chemie, 82041 Deisenhofen, Germany.

[2] Airbrush spray gun, Badger Profi 150, Revell, Bünde, Germany.

[3] Imaging plate, Raytest, 75334 Straubenhardt, Germany.

[4] Analyzer FLA-7000, Fuji Photo Film Co., Tokyo 106–8620, Japan.

[5] Polytron PT 3100, Kinematika, 6014 Littau-Luzern, Switzerland.

[6] Aqua C_{18}-5-µm column, Phenomenex, 63741 Aschaffenburg, Germany.

[7] Radioactivity monitor LB 509, Berthold Technologies, 75323 Bad Wildbad, Germany.

[8] LC-MS/MS Finnigan LTQ FT, Finnigan, 355 River Oaks Pkwy, San Jose, CA 95134–1908.

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