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Neonicotinoid Insecticides: Oxidative Stress in Planta and Metallo-oxidase Inhibition

Kevin A. Ford, Alexander G. Gulevich, Tami L. Swenson, and John E. Casida*

Environmental Chemistry and Toxicology Laboratory, Department of Environmental Science, Policy and Management, University of California, Berkeley, California 94720-3112, United States

ABSTRACT: Neonicotinoids not only control insect pests but also sometimes independently alter plant growth and response to stress. We find that imidacloprid, thiacloprid, acetamiprid, thiamethoxam, and clothianidin but not nitenpyram and dinotefuran induce foliar lesions and peroxidative damage in soybean (*Glycine max*) seedlings assayed with the 3,3′-diaminobenzidine stain. The chloropyridinyl-carboxylic acid (COOH) but not the -carboxaldehyde (CHO) metabolites induce peroxidative damage but in a different pattern. Surprisingly, the chlorothiazolyl -CHO and -COOH metabolites induce chlorosis but no clear superimposable peroxidative damage or cell death. Four metallo-oxidases known to modulate reactive oxygen species were not sensitive in vitro to the parent neonicotinoid itself but were to several CHO and COOH metabolites and related compounds, with a sensitivity order of CHO > COOH and tyrosinase > xanthine oxidase and aldehyde oxidase > catalase. Although metallo-oxidase inhibition does not correlate overall with lesion formation, it may play an as yet unknown role in plant response to neonicotinoids.

KEYWORDS: aldehyde oxidase, *Arabidopsis*, catalase, neonicotinoid insecticides, oxidative stress, phytotoxicity, soybean, tyrosinase, xanthine oxidase

INTRODUCTION

Neonicotinoids are a major class of insecticides, with 24% of the total world market value.[1,2] The seven commercial neonicotinoids (Figure 1) are used primarily for crop protection as systemics to control sucking insect pests. They are metabolized by plants into a multitude of oxidation, reduction, cleavage, and conjugation products[3−5] which are generally less toxic than the parent compounds to insects and mammals. The bioactivity of neonicotinoids goes beyond their nicotinic agonist action as the primary target for pest insect control. Neonicotinoid treatment of plants, even in the absence of pests, sometimes enhances growth and protects against abiotic and biotic stress.[6−8]

In our investigation with spinach,[4] we observed neonicotinoid-induced leaf lesions, prompting a survey of several crop species detailed below. Soybean gave a more sensitive and consistent response than spinach, and so it was used to monitor the commercial neonicotinoids and many of their candidate metabolites for structure−activity relationships (SARs) in promoting lesion formation and chlorosis. Soybean was also emphasized because of its major economic importance and the extensive use of neonicotinoids in protecting this crop.

Leaf lesion formation can result from generation of reactive oxygen species (ROS) and peroxidative damage, among a variety of other mechanisms.[9,10] A standard method to examine oxidative stress in plants is the 3,3′-diaminobenzidine (DAB) stain for hydrogen peroxide, which converts DAB to a brown polymer.[11,12] Metallo-oxidases are generally known to modulate ROS levels[13−16] and are potential targets for neonicotinoids and their metabolites.[4,5] Four metallo-oxidases were examined for in vitro inhibition by neonicotinoids and their carboxaldehyde (CHO) and carboxylic acid (COOH) metabolites and related compounds. Tyrosinase (TYR) has a binuclear copper−histidine active site,[14] while xanthine oxidase (XO) and aldehyde oxidase (AOX) have molybdopterin[15,16] and catalase (CAT) has an iron metallo site.[17] Each of these metallo-oxidases is sensitive to some aryl carboxaldehydes and aryl carboxylic acids[14−17] in a pattern that might extend to neonicotinoid metabolites formed in plants. The overall goal of this study is to consider the SAR of neonicotinoids and their metabolites for inducing oxidative stress in crops and for inhibiting metallo-oxidases possibly involved in ROS detoxification (Figure 2).

MATERIALS AND METHODS

Chemicals. Sources for the neonicotinoids and most of the metabolites were reported earlier.[4,18,19] All carboxaldehydes and carboxylic acids were from Aldrich (St. Louis, MO) except the following: **5** from ASDI Global Collections (Newark, DE); **16** from Synthonix (Wake Forest, NC). Raloxifene (**22**) was from Tocris (Ellisville, MO).

Phytotoxicity. The test compounds were administered hydroponically to soybean seedlings (*Glycine max*, Envy variety; $n = 3$) 6−8 cm high, prior to the development of the first trifoliolate leaves, with 50 mL of 100 ppm neonicotinoid made in quarter-strength modified Hoagland's solution for nutritional support (mmol/L: 0.4 $NH_4H_2PO_4$, 2.4 KNO_3, 1.6 $Ca(NO_3)_2$, 0.8 $MgSO_4$, 0.1 Fe as Fe-chelate, 0.023 B as $B(OH)_3$ (boric acid), 0.0045 Mn as $MnCl_2$, 0.0003 Cu as $CuCl_2$, 0.0015 Zn as $ZnCl_2$, 0.0001 Mo as MoO_3 or $(NH_4)_6Mo_7O_{24}$ and Cl as chlorides of Mn, Zn, and Cu). These plants were held in a greenhouse (ambient lighting, 20−25 °C, September to November, Berkeley, CA) for 9 days (neonicotinoids), 9 or 13 days (carboxaldehydes and carboxylic acids), or 17 days (guanidines), photographed to record effects on foliage size and color, and the unifoliolar and trifoliolar leaves were harvested for the DAB assay[11,12] (see below). Other crops were evaluated for phytotoxic effects 7 days after treatment, except for spinach at 11 days. The plants

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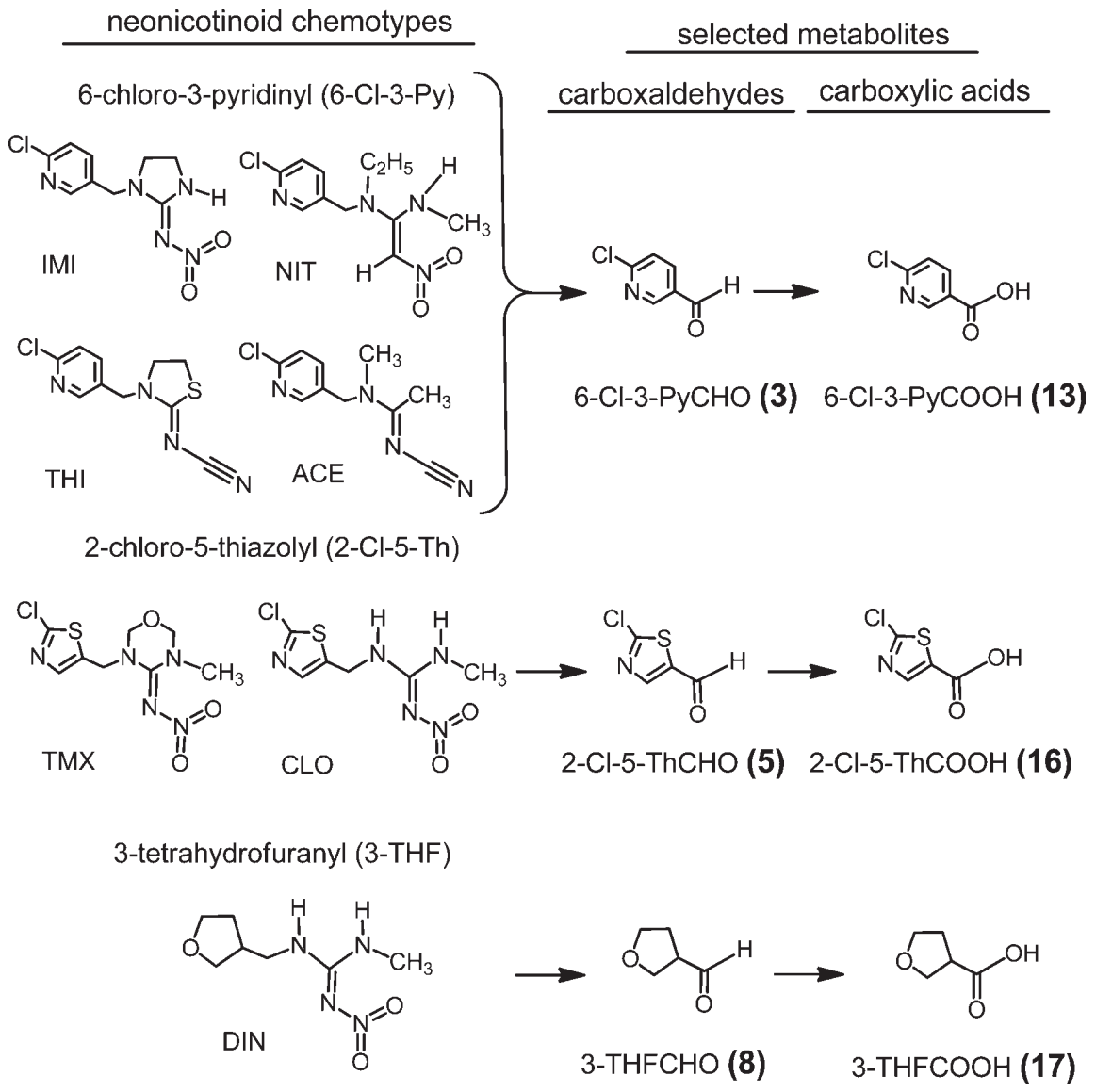

Figure 1. Carboxaldehyde and carboxylic acid metabolites of seven neonicotinoids. Abbreviations for neonicotinoids are as follows: IMI, imidacloprid; NIT, nitenpyram; THI, thiacloprid; ACE, acetamiprid; TMX, thiamethoxam; DIN, dinotefuran; CLO, clothianidin.

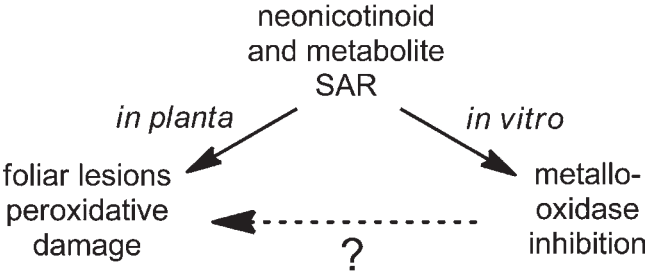

Figure 2. Relationships considered in this investigation.

examined, height, and treatment conditions were as follows: spinach (*Spinacea oleracea*, Tyee variety), 4−6 cm, 100 ppm, hydroponic; grape (*Vitis vinifera*, cabernet sauvignon variety), 6−8 cm, 100 ppm, hydroponic; cotton (*Gossypium hirsutum*), 25−30 cm, 250 ppm, soil; corn (*Zea mays*), 15−20 cm, 100 ppm, soil.

DAB Assay. The DAB assay was used to determine possible peroxidative activity which might result from oxidative stress induced by the test compounds.[10−12] Unifoliolate and trifoliolate soybean leaves (4−6 cm length, 3−4 cm width) as above were cut at the stem and placed in Petri dishes (100 × 15 mm) containing DAB solution (1 mg/mL in distilled water) (adjusted to pH 3.6), positioned 30 cm below a 60 W incandescent light overnight at 20−25 °C, and the DAB solution was taken up through the stems. The DAB-treated leaves were then immersed in 90% ethanol, and the preparation was brought to near boiling in a hot oven for 15 min to clear the green pigment and examined for DAB-positive reddish brown regions compared with the foliar lesions.

Metallo-oxidase Inhibition Assays. *General Considerations.* All reactions were monitored with a 96-well VERSAmax Microplate Reader (Molecular Devices, Sunnyvale, CA). Inhibitor concentrations refer to final solutions used to read absorbance for TYR and CAT and to enzyme plus inhibitor solutions prior to adding chromogenic agents for XO and AOX. Each compound was assayed in triplicate to determine standard deviation (SD) values.

TYR. TYR activity was monitored as 3,4-dihydroxy-L-phenylalanine (L-DOPA) oxidation to L-DOPA-quinone.[20] Solutions of TYR from the

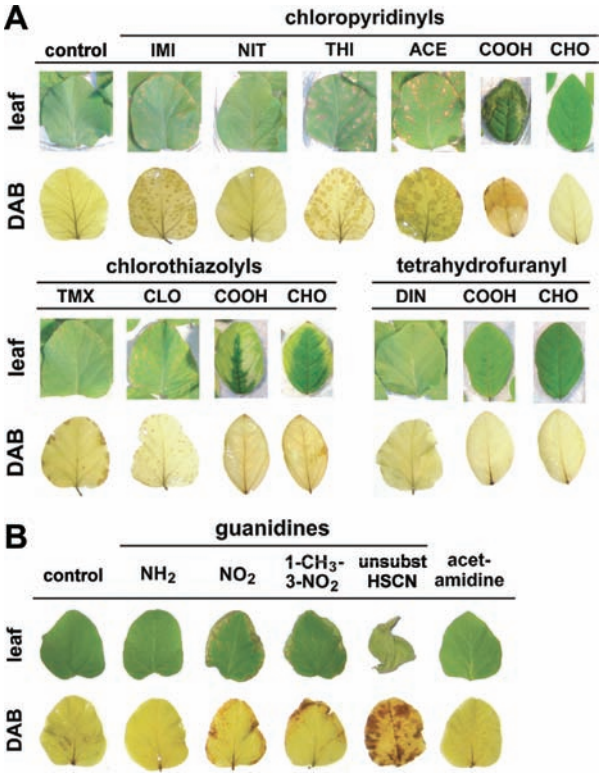

Figure 3. Soybean leaves showing foliar lesions or chlorosis and oxidative damage (DAB) from neonicotinoids (unifoliolate leaves), their carboxaldehyde (CHO) and carboxylic acid (COOH) metabolites (trifoliolate leaves), and guanidine cleavage products (unifoliolate leaves) at 9, 9, or 13 and 17 days, respectively, after hydroponic treatment at 100 ppm. Only the neonicotinoid-treated leaves shown here are from different plants in the same experiment; however, regions of foliar lesions and DAB-positive response were consistently superimposable in all experiments. No apparent phytotoxicity was observed for NIT, DIN, CP-CHO, THF-COOH, THF-CHO, aminoguanidine, and acetamidine treatments.

mushroom *Agaricus bisporus* (Sigma-Aldrich) (1 unit/μL; kept at −20 °C), L-DOPA (1 mM), and test compounds were prepared in 50 mM potassium phosphate buffer pH 6.5. The enzyme preparation (30 units in 30 μL) and test compound (60 μL) were mixed and preincubated for 30 min at 25 °C, and then an aliquot (15 μL) was added to L-DOPA solution (85 μL, 850 μM final concentration) with immediate monitoring of L-DOPA-quinone formation by measuring the absorbance increase for 3 min at 475 nm and 25 °C. Kojic acid was used as the standard inhibitor.[21−23]

XO Assay. XO activity was determined with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium sodium salt (WST-1), which turns from clear to yellow in the presence of ROS.[24,25] Using materials provided in the SOD Determination Kit 19160 from Sigma-Aldrich (St. Louis, MO), solutions of XO (20 μL) were incubated (5 min, 37 °C) with candidate inhibitors in 50 mM potassium phosphate, 0.4 mM EDTA, pH 7.0 buffer (20 μL). WST-1 solution (180 μL) was then added followed by incubation for 20 min at 37 °C and the absorbance (450 nm) recorded versus a no enzyme blank. Allopurinol was used as the standard inhibitor.[26,27]

ppAOX Preparation and AOX Assay. Partially purified AOX (ppAOX) was prepared as described[28,29] from rabbit liver cytosol in potassium phosphate pH 7.4 buffer by precipitation with ammonium sulfate (50% saturation), redissolution of the pellet in phosphate buffer, and dialysis for 21 h at 4 °C. ppAOX activity was stable for at least 3 weeks at 4 °C. For activity assays involving oxidation of 4-(dimethylamino)cinnamaldehyde (DMAC) to the corresponding carboxylic acid,[28−30] ppAOX (20 μg protein, 20 μL) and the test compound solution (20 μL) were incubated for 5 min at 25 °C and then DMAC solution (50 μM, 200 μL) was added and formation of the corresponding carboxylic acid was measured by an absorbance decrease (10 min, 400 nm, 25 °C). The standard inhibitors used were cyanide[30] and raloxifene.[31,32]

CAT. CAT activity was determined using the Catalase Assay Kit from Cayman Chemical Co. (Ann Arbor, MI), which utilizes a solution of lyophilized powder of bovine liver CAT to measure its peroxidatic activity with methanol in the presence of an optimal concentration of hydrogen peroxide. The production of formaldehyde from the CAT reaction was measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromagen, which on oxidation changes from colorless to purple.[33,34] A solution of CAT (Cayman) (80 μL) and test compound in 100 mM potassium phosphate buffer pH 7.0 (96 μL) was incubated for 10 min at 25 °C. An aliquot of the CAT incubation (44 μL) was mixed with methanol (30 μL), 35 mM hydrogen peroxide (20 μL), and 100 mM potassium phosphate buffer pH 7.0 (76 μL) followed by incubation for 20 min at 25 °C. Formaldehyde production was then assayed by addition of Cayman Purpald solution (30 μL), 10 M potassium hydroxide (30 μL), and Cayman potassium periodate solution (10 uL) according to the kit protocol, and the final absorbance was measured at 540 nm and 25 °C. This procedure could not be used with candidate carboxaldehyde inhibitors, because they react directly with Purpald.[35]

RESULTS

Phytotoxicity of Neonicotinoids in Crop Seedlings. Soybean seedlings were treated hydroponically with neonicotinoids at 100 ppm and their phytotoxic effects recorded after 9 days. Lesions were evident in the unifoliolate leaves with IMI, THI, ACE, CLO, and TMX but not with NIT and DIN (Figure 3) or in any of the trifoliolate leaves. We also observed that spinach seedlings treated hydroponically with 100 ppm neonicotinoid solutions for 11 days showed less severe effects than for soybean, with only ACE and CLO producing minor lesions (not shown). This treatment in spinach results in ACE and CLO levels of 50−100 ppm in the leaves.[4] Under all treatment conditions examined the soybean and spinach seedlings were more sensitive to lesions than the cotton, corn, and grape seedlings.

Oxidative Damage of Neonicotinoids and Metabolites in Soybean Seedlings. Soybean was used to compare the localization of leaf lesions and DAB-positive regions for possible oxidative damage at 9, 13, or 17 days (see Materials and Methods) after neonicotinoid and metabolite treatments at 100 ppm. IMI, THI, ACE, CLO, and TMX gave distinct regions of DAB-positive response, whereas NIT and DIN did not damage the unifoliolate leaves (Figure 3) and no effect was observed in trifoliolate leaves. The patterns of damage were somewhat compound dependent at 9 days; however, they essentially followed the same progression of foliar lesions over an extended period of 15 days. Although not illustrated in the neonicotinoid treatments, all DAB-positive regions were consistently superimposable with sites of foliar lesions, with nine plants treated per compound in three independent experiments. The three CHO and three COOH metabolites were included for comparison (Figure 3). None of these CHO or COOH compounds had any effect in the unifoliolate leaves. However, in the

Table 1. Structure−Activity Relationships and Selectivity of Neonicotinoids and Related Carboxaldehydes and Carboxylic Acids as Inhbitors of TYR, XO, and AOX

	$IC_{50} \pm SD$ ($n = 3$) (μM)			
compd no., structure[a]	TYR	XO	AOX	rel sensitivity
		Neonicotinoids		
IMI	>1000 (29)[b]	>1000 (0)	>1000 (6)	
NIT	>1000 (0)	>1000 (−7)	>1000 (1)	
THI	>1000 (22)	>1000 (3)	>1000 (8)	
ACE	1740 ± 60	>1000 (3)	>1000 (−2)	TYR > XO or OX
TMX	>1000 (13)	>1000 (12)	>1000 (4)	
CLO	>1000 (15)	1860 ± 250	>1000 (4)	XO > TYR or AOX
DIN	>1000 (3)	>1000 (6)	>1000 (3)	
		Carboxaldehydes		
3-PyCHO (**1**)	28 ± 8	>1000 (17)	600 ± 98	TYR >AOX > XO
2-Cl-3-PyCHO (**2**)	>1000 (26)	147 ± 15	350 ± 89	XO > AOX > TYR
6-Cl-3-PyCHO (**3**)	770 ± 80	470 ± 70	720 ± 56	XO > TYR or AOX
5-ThCHO (**4**)	7 ± 1	>1000 (1)	890 ± 81	TYR ≫ AOX > XO
2-Cl-5-ThCHO (**5**)	64 ± 36	630 ± 12	360 ± 15	TYR > AOX > XO
2-FuCHO **6**()	43 ± 2	>1000 (37)	700 ± 44	TYR > AOX > XO
3-FuCHO (**7**)	15 ± 6	>1000 (29)	260 ± 10	TYR > AOX > XO
3-THFCHO (**8**)	>1000 (15)	>1000 (0)	360 ± 82	AOX > TYR or XO
PhCHO (**9**)	177 ± 60	260 ± 74	660 ± 80	TYR > XO > AOX
2-HOPhCHO (**10**)	∼1000 (51)	340 ± 67	1140 ± 310	XO > TYR or AOX
4-HOPhCHO (**11**)	770 ± 300	35 ± 3	570 ± 50	XO > TYR or AOX
		Carboxylic Acids		
3-PyCOOH (**12**)	>1000 (39)	>1000 (0)	>1000 (5)	
6-Cl-3-PyCOOH (**13**)	>1000 (46)	>1000 (0)	>1000 (3)	
2-HS-3-PyCOOH (**14**)	80 ± 10	>1000 (4)	>1000 (5)	TYR > XO or AOX
6-HS-3-PyCOOH (**15**)	42 ± 3	>1000 (7)	>1000 (−1)	TYR > XO or AOX
2-Cl-5-ThCOOH (**16**)	>1000 (18)	>1000 (17)	>1000 (−2)	
3-THFCOOH (**17**)	>1000 (16)	>1000 (31)	>1000 (2)	
PhCOOH (**18**)	270 ± 100	>1000 (9)	>1000 (4)	TYR > XO or AOX
2-HO-4-Cl-PhCOOH (**19**)	520 ± 140	>1000 (0)	>1000 (6)	TYR > XO or AOX
		Standard Inhibitors		
kojic acid (**20**)	9 ± 3	>1000	757 ± 87	TYR > AOX > XO
allopurinol (**21**)	>1000 (4)	17 ± 1	>1000 (18)	XO ≫ TYR or AOX
raloxifene (**22**)	*c*	>1000	1533 ± 325[d]	
KCN (**23**)	24 ± 1	>1000 (6)	69 ± 8	TYR > AOX > XO

[a] Substituent abbreviations: Fu, furanyl; Ph, phenyl; Py, pyridinyl; THF, tetrahydrofuranyl; Th, thiazolyl. [b] The percent inhibition at the indicated concentration is given in parentheses. [c] Poorly soluble in buffer and interference by organic carrier solvents. [d] In contrast to rabbit (this study), mouse, rat, and monkey AOX, the human enzyme is very sensitive to raloxifene ($IC_{50} = 0.008$ μM).[31,32]

trifoliolate leaves chloropyridinyl-COOH but not -CHO showed clear superimposable cell death and peroxidative damage in a pattern that differed from neonicotinoid-induced foliar lesions. In the case of chlorothiazolyl-CHO and -COOH, chlorosis was induced and extended toward the central vein with little or no response to DAB. The corresponding tetrahydrofuranyl-CHO and -COOH did not damage the leaves.

Metabolic cleavage of the neonicotinoid heterocyclyl-CH_2-N linkage yields a series of guanidinium metabolites.[5] Four guanidine cleavage products and the analogous acetamidine portion from ACE showed distinctly higher activity and superimposable DAB-positive response for nitroguanidine, 1-methyl-3-nitroguanidine, and guanidine thiocyanate than for aminoguanidine and acetamidine (Figure 3). Overall, a better correlation of foliar lesions and oxidative damage was observed for the neonicotinoids than for their metabolites.

Metallo-oxidase Inhibitor Surveys. The neonicotinoids were poor inhibitors of these enzymes with IC_{50} values (μM) of 1740 for ACE with TYR and 1860 for CLO with XO; the other neonicotinoids were less active and enzymes less sensitive. TYR, XO, and AOX were generally more sensitive to the carboxaldehydes than to the carboxylic acids (Tables 1 and 2). The three carboxaldehyde metabolites of the neonicotinoids were therefore compared with two standards (kojic acid for TYR and allopurinol for XO) and PhCHO with each of the three enzymes (Table 1, Figure 4). The metallo-oxidases as assayed responded to the

Table 2. Relative Sensitivity of Four Metallo-oxidases to Neonicotinoids and Related Carboxaldehydes and Carboxylic Acid[a]

		IC_{50} 1000 μM or less	
enzyme	chemotype	active/total compd	amt (%)
TYR	neonicotinoids	0/7	0
	carboxaldehydes	8/11	73
	carboxylic acids	4/8	50
XO	neonicotinoids	0/7	0
	carboxaldehydes	6/11	55
	carboxylic acids	0/8	0
AOX	neonicotinoids	0/7	0
	carboxaldehydes	11/11	100
	carboxylic acids	0/8	0
CAT	neonicotinoids	0/7	0
	carboxaldehydes		
	carboxylic acids	0/8	0

[a] Data from Table 1 for TYR, XO, and AOX plus those for CAT (not tabulated elsewhere) with all of the neonicotinoids and carboxylic acids. The carboxaldehydes were not assayed for CAT inhibition because they react directly with Purpald.[35]

standard inhibitors in the expected manner, with IC_{50} values (μM) of 9 for kojic acid (**20**) with TYR, 17 for allopurinol (**21**) with XO, and 1533 for raloxifene (**22**) with AOX (Table 1; see footnote *d* relative to species differences). Cyanide (**23**) gave IC_{50} values (μM) of 24 for TYR, >1000 for XO, and 69 for AOX (Table 1).

Structural Aspects of TYR Inhibitors (Table 1, Figures 4 and 5). Twelve of the 19 carboxaldehydes and carboxylic acids inhibited TYR more than 50% at 1000 μM and gave IC_{50} values of 7−770 μM. The most potent inhibitor was 5-ThCHO (**4**) (IC_{50} = 7 μM), with 9-fold lower potency for its 2-chloro analogue (**5**). Three other heterocyclic analogues (3-PyCHO (**1**), 2-FuCHO (**6**), and 3-FuCHO (**7**)) gave IC_{50} values of 15−43 μM followed by PhCHO (**9**) at 177 μM. The 2-chloro substituent of compound **2** and 6-chloro substituent of compound **3** strongly reduced the potency of **1** (nicotinic aldehyde) from 28 μM for **1** to >1000 μM for **2** and 770 μM for **3**. The potency of benzaldehyde (**9**) was lowered by 2- and 4-hydroxy substituents (**10** and **11**). Two mercaptopyridinylcarboxylic acids (**14** and **15**) were very potent, with IC_{50} values of 42−80 μM. Other inhibitors were benzoic acid (**18**) (IC_{50} = 270 μM) and 4-chlorosalicylic acid (**19**) (IC_{50} = 520 μM). On an overall basis, several of the test chemicals here fall in the potency range of the most effective compounds from an earlier survey of 648 candidate TYR inhibitors.[23]

Structural Aspects of XO Inhibitors (Table 1, Figure 4). XO was less sensitive than TYR, with six carboxaldehydes giving IC_{50} = 35−630 μM and the other five carboxaldehydes and eight carboxylic acids being almost noninhibitory at 1000 μM (Table 1). The most potent carboxaldehydes for XO were benzaldehyde (**9**) and its 2- and 6-chloro derivatives (**2** and **3**) (IC_{50} = 147−470 μM) and its 2- and particularly 4-hydroxy analogues (**10** and **11**) (IC_{50} = 5−340 μM). 2- and 4-hydroxybenzaldehyde (**10** and **11**) were 3−22-fold selective for XO in comparison with TYR, while almost all of the other compounds highly active with TYR were at least 25-fold less potent on XO.

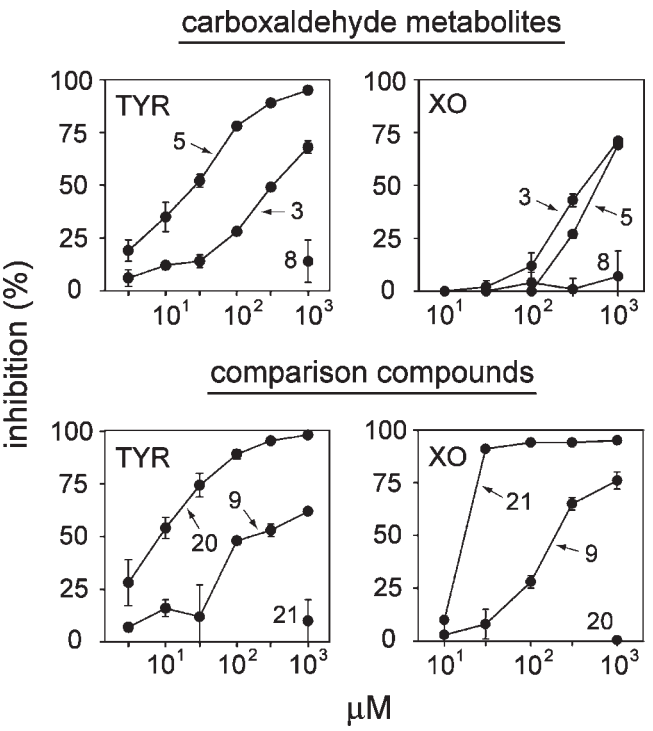

Figure 4. Inhibition of TYR and XO by three neonicotinoid carboxaldehyde metabolites (**3**, **5**, and **8**) and three comparison compounds (**9**, **20**, and **21**) identified in Table 1.

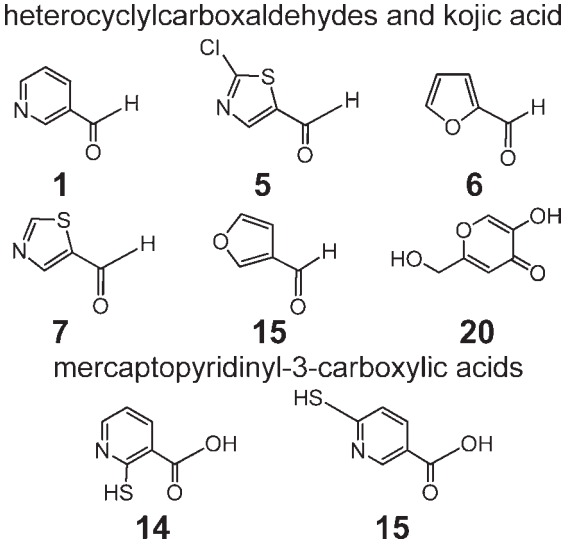

Figure 5. Eight tyrosinase inhibitors with IC_{50} values of 9−80 μM.

Structural Aspects of AOX Inhibitors (Table 1). The neonicotinoids and carboxylic acids (**12**−**19**) were not inhibitory at 1000 μM, while the active carboxaldehydes (**1**−**11**) differed only 4-fold in potency (IC_{50} = 260−1140 μM).

Structural Aspects of CAT Inhibitors. CAT was not sensitive to the neonicotinoids, carboxylic acids (**12**−**19**) (IC_{50} > 1000 μM) (Table 2), or guanidine cleavage products referred to above, except for aminoguanidine with an IC_{50} value of about 500 μM. As a standard inhibitor, CAT was completely inhibited by sodium azide at 300 μM.

DISCUSSION

Neonicotinoid Effects on Plant Growth, Redox Systems, and Oxidative Stress. IMI, independent of its insecticidal properties, improves the growth and yield of cotton, melons, and pepper[6] and also has beneficial physiological effects on woody plants.[36] IMI in cotton does not significantly affect CAT, peroxidase, and glutathione reductase activities 2 days after treatment at 30−36 °C, but at 39 °C it significantly lowered glutathione reductase activity, leading to the proposal that it has antioxidant properties and reduces oxidative stress by decreasing antioxidant enzyme activity.[37] Many pyridinyl-containing compounds such as nicotinic acid (3-PyCOOH) have scavenging activity against hydroxyl radicals,[38] but the reported studies did not include chloropyridinyl-containing compounds such as neonicotinoid metabolites. IMI is not genotoxic to human lymphocytes in vitro and does not induce ROS (a potential cause of DNA damage) detected by the 2′,7′-dichlorofluorescein diacetate assay.[39] However, IMI induces oxidative stress in female rats at 20 but not 10 mg/kg/day for 90 days on the basis of assays of antioxidant enzymes and lipid peroxidation.[40] A portion of the neonicotinoid effects in *Arabidopsis* are attributable to elevated salicylic acid (SA) levels and associated plant responses,[8] but lesion formation was not SA-dependent, since it occurred equally in SA-biosynthetic mutant *ics*1−2 compared to wild-type treated with IMI metabolite **13** (see ref 8 and unpublished results).

We report here that five (IMI, ACE, THI, TMX, and CLO) of the seven commercial neonicotinoids induce peroxidative damage and foliar lesions in soybeans, the most sensitive of the crops examined. The finding that NIT and DIN did not cause oxidative damage may be related to unique structural features such as the THF substituent of DIN and nitromethylene moiety of NIT, influencing their primary pathways or kinetics of metabolism in plants.[4,5] It might also be related in part to physical properties such as partitioning and solubility. Thus, the log *P* values are much lower and the water solubilities are much higher for NIT and DIN compared with those of the other five neonicotinoids examined.[41]

Effects of Neonicotinoids and Metabolites on Metallo-oxidases. In our earlier study, global expression profiling for IMI-, CLO- and SA-treated *Arabidopsis* revealed a number of metallo-oxidases with altered expression compared to untreated controls. This included aldehyde oxidases, xanthine oxidase, catalase and tyrosinases (see ref 8 and the Supporting Information therein). The sensitivity of these metallo-oxidases was therefore examined to the seven neonicotinoids, the CHO and COOH metabolites, and the guanidine cleavage products. Although the parent neonicotinoids were poor inhibitors or were inactive, some CHO and COOH metabolites were potent inhibitors of TYR and XO, adding to a large array of structurally related known inhibitors.[23,42,43] The metallo-oxidases assayed were from mushroom (TYR) or mammals (XO, AOX, CAT) rather than plants and therefore might have a different sensitivity, although standard inhibitors for each of these enzymes are active in both plants and mammals.[44−47] These enzymes were inhibited by several neonicotinoid-derived carboxaldehydes and carboxylic acids in vitro at relatively high concentrations, but the extent to which this carries over to in planta at critical sites to result in the observed physiological effects remains to be determined. The effective inhibitors were not the neonicotinoids themselves but instead their carboxaldehyde and carboxylic acid metabolites: e.g., 2-Cl-5-ThCHO (**2**), 6-Cl-3-PyCHO (**3**), and 6-HS-3-PyCOOH (**15**). The 6-mercapto compound (**15**) is an intermediate in mouse metabolism of the four chloropyridinyl neonicotinoid insecticides and 6-Cl-PyCOOH (**13**), since the 6-CH_3S derivative is a urinary metabolite of each of these chemicals.[18] However, it is not known whether compound **15** is formed in plants as a contributor to neonicotinoid effects. Different mechanisms are possibly involved in the phytotoxic effects of guanidine thiocyanate[48] and nitroguanidine.[49]

The heterocyclylcarboxaldehydes are relatively nonspecific as to the aryl moiety, with potencies for phenyl, thiazolyl, pyridinyl, and furanyl in the 7−177 μM range. The 2- and 6-isomers of mercaptonicotinic acid (**14** and **15**) are also potent TYR inhibitors (IC_{50} = 42−80 μM). The hydroxypyrone kojic acid (**20**) is a potent and selective inhibitor of TYR and is used as a preservative to prevent TYR-catalyzed darkening of food.[50] With XO, in contrast to TYR, the chloro substituent enhances potency with 3-PyCHO and 5-ThCHO. The aldehydes are active but not the acids with 4-HOPhCHO (**11**) optimal and almost as effective as the standard allopurinol (**21**). PhCHO and its 2-HO and 4-HO analogues are known substrates for both AOX and XO.[51] AOX with *N*-methylnicotinamide reduces the nitro group of IMI, CLO, and DIN[28,29] and is also inhibited by nitroso-IMI[52] and their heterocyclylcarboxaldehyde metabolites (this study).

Neonicotinoid-Induced Oxidative Stress in Planta and Metallo-oxidase Inhibition (Figure 2). Neonicotinoids vary in their in planta effects, depending on the compound and the plant species. Cell death and peroxidative staining does not appear to be due to direct inhibition of the metallo-oxidases examined by the parent neonicotinoids. Interestingly, the CHO and COOH analogues that are plant metabolites can inhibit metallo-oxidase activity but are relatively poor inducers of cell death/ROS damage. Although metallo-oxidase inhibition does not correlate overall with lesion formation, it may play an indirect and as yet unknown role in the plant response to neonicotinoids and their metabolites.

AUTHOR INFORMATION

Corresponding Author
*E-mail: ectl@berkeley.edu.

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ABBREVIATIONS USED

ACE, acetamiprid; AOX, aldehyde oxidase; CAT, catalase; CHO, carboxaldehyde; CLO, clothianidin; COOH, carboxylic acid; DAB, 3,3′-diaminobenzidine; Fu, furanyl; IMI, imidacloprid; NIT, nitenpyram; PGR, plant growth regulator; Ph, phenyl; Py, pyridinyl; ROS, reactive oxygen species; Th, thiazolyl; THF, tetrahydrofuranyl; TMX, thiamethoxam; TYR, tyrosinase; XO, xanthine oxidase; SOD, superoxide dismutase

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