Phytoestrogen Signaling and Symbiotic Gene Activation Are Disrupted by Endocrine-Disrupting Chemicals

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Some organochlorine pesticides and other synthetic chemicals mimic hormones in representatives of each vertebrate class, including mammals, reptiles, amphibians, birds, and fish. These compounds are called endocrine-disrupting chemicals (EDCs). Similarly, hormonelike signaling has also been observed when vertebrates are exposed to plant chemicals called phytoestrogens. Previous research has shown the mechanism of action for EDCs and phytoestrogens is as unintended ligands for the estrogen receptor (ER). Although pesticides have been synthesized to deter insects and weeds, plants produce phytoestrogens to deter herbivores, as attractant cues for insects, and as recruitment signals for symbiotic soil bacteria. Our data present the first evidence that some of the same organochlorine pesticides and EDCs known to disrupt endocrine signaling through ERs in exposed wildlife and humans also disrupt the phytoestrogen signaling that leguminous plants use to recruit *Sinorhizobium meliloti* soil bacteria for symbiotic nitrogen fixation. Here we report that a variety of EDCs and pesticides commonly found in agricultural soils interfere with the symbiotic signaling necessary for nitrogen fixation, suggesting that the principles underlying endocrine disruption may have more widespread biological and ecological importance than had once been thought. *Key words:* ecosystem, endocrine-disrupting chemicals, endocrine disruption, environmental signaling, estrogen receptor, nitrogen fixation, *Rhizobium*, symbiosis. *Environ Health Perspect* 112:672–677 (2004). doi:10.1289/ehp.6456 available via *http://dx.doi.org/* [Online 29 January 2004]

Endocrine-disrupting chemicals (EDCs) represent one subset of a more general phenomenon we have termed environmental signals (McLachlan 2001). Although most studies of endocrine disruption have focused on endocrine-signaling effects within vertebrates (Bennetts et al. 1946; Donohoe and Curtis 1996; Fry and Toone 1981; McLachlan 2001; Tyler et al. 1998), here we show that endocrine disruption also occurs in organisms that lack an estrogen receptor (ER). Synthetic compounds found in the environment mimic estrogen, testosterone, and other steroids by disrupting steroid receptor-signaling (Kelce et al. 1995; Longnecker et al. 1997). Given that hormonally active chemical signals are also produced by plants, fungi, and other natural sources (Collins-Burow et al. 2000; Kuiper et al. 1998; Kurzer and Xu 1997), we have hypothesized that parallels exist between these ecosystem signaling systems and the endocrine system of vertebrates. Thus, the concept of EDCs as agents that are harmful only to organisms with recognizable steroid receptors, although useful for studying the deleterious effects of environmental chemicals on vertebrate reproduction and development, may limit our scope and lead us to overlook potential new and emerging targets of EDCs. We tested this hypothesis by evaluating whether EDCs block a critical phytoestrogen-signaling system regulating symbiosis between plants and bacteria.

Various natural and synthetic chemicals, including phytoestrogens, organochlorine pesticides, by-products of plastics manufacturing, and polychlorinated biphenyls (PCBs) (Bergeron et al. 1994; Collins-Burow et al. 2000; McLachlan 2001; Safe 2000), have the potential to mimic hormones and disrupt the endocrine system of exposed animals (Sonnenschein and Soto 1998; Tyler et al. 1998). *In vitro* and *in vivo* data have shown that EDCs disrupt estrogenic signaling by acting as or inhibiting the actions of 17β-estradiol (E_2) (Cheek et al. 1998; Korach et al. 1997; Zacharewski 1998). EDCs, in most cases, are thought to work either through modulating steroid hormone action at the receptor level or at the transcriptional level (Andersen et al.1999; Roy et al. 1997). *In vitro* evidence has shown that some EDCs can bind human ER-α and ER-β, although at a fraction (phytoestrogens 1/100, bisphenol A 1/100, hydroxylated PCBs 1/40) of the binding affinity of E_2 (Breinholt and Larsen 1998; Korach et al. 1979, 1988).

Exposure to endocrine-altering chemicals is not limited to synthetic pollutants. Phytoestrogens are also capable of antagonizing or mimicking the actions of E_2. A class of phytochemicals called flavonoids shares common characteristics with steroidal hormones, in that they are able to bind ERs and thereby modulate transcription of estrogen-responsive genes (Kuiper et al. 1998; Tham et al. 1998; Whitten and Patisaul 2001). Phytoestrogens, which are estrogenic in vertebrates, are produced by plants for many reasons, including as a recruitment signal for soil bacteria capable of living in symbiosis with leguminous plants (Schultze and Kondorosi 1998; Wynne-Edwards 2001). Although phytochemicals bind to and activate vertebrate ERs, the intended targets of phytoestrogen signaling, *Rhizobium* symbiotic soil bacteria, respond to phytoestrogen signaling via nodulation D (NodD) transcriptional activator proteins, which reportedly share homology with ERs (Gyorgypal and Kondorosi 1991). NodD proteins act as receptors for phytoestrogens in much the same way that vertebrate ERs are activated by these same phytoestrogens. Based on this analogous signaling, our experiments were designed to test whether EDCs that disrupt E_2-ER signaling also disrupt phytoestrogen-NodD signaling and determine which specific environmental chemicals or EDCs disrupt these signaling systems.

Leguminous plants such as soybean and alfalfa produce phytoestrogens to deter herbivores, to ward against fungal and bacterial pathogens, and as signaling agents to recruit soil bacteria to the plant's root system for nitrogen-fixing symbiosis (Koes et al. 1994; Wynne-Edwards 2001). Symbiosis occurs when host plants release small polyphenolic compounds known as flavonoids or phytoestrogens into the soil. Phytoestrogens act as specific attractants for symbiotic *Rhizobium* soil bacteria, which positively chemotax up the concentration gradient of phytoestrogen, enter the host plant root, and form nodules (Redmond et al. 1986). In exchange for the

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carbon source offered by the plant, the *Rhizobium* fix atmospheric nitrogen into a form (NH_3; ammonia) the host plant uses as a natural fertilizer. Host specificity between plants and *Rhizobium* is regulated by the unique profile of phytoestrogens produced by the host plant, which are recognized by species-specific NodD proteins within *Rhizobium* soil bacteria. For example, the leguminous plant *Medicago sativa* (alfalfa) secretes specific identifying flavonoids (luteolin and apigenin) into the soil to recruit the soil bacterium *Sinorhizobium meliloti* for symbiosis (Peters and Long 1988). Luteolin interacts with constitutively expressed rhizobial NodD receptors, leading to transcription of a suite of nodulation (*nod*) genes crucial for symbiosis (Peters et al. 1986). Therefore, luteolin-NodD signaling is both necessary and sufficient for initiating the events leading to nitrogen-fixing symbiosis beneficial to both plant and bacteria (Bladergroen and Spaink 1998; Spaink et al. 1987).

Phytochemicals produced by one species of host plant not only recruit their specific symbiotic bacteria but also antagonize the recruitment of symbiotic bacteria to competing host plant species. For instance, the symbiosis between alfalfa and *S. meliloti* bacteria, which is initiated when the alfalfa-produced phytochemicals luteolin and apigenin signal to *S. meliloti* NodD receptors, is antagonized by the soybean- or clover-produced phytochemicals chrysin and coumestrol (Peters et al. 1986; Peters and Long 1988; Redmond et al. 1986). Therefore, *S. meliloti* NodD receptors are ligand-dependent transcriptional activator proteins that are turned on or off by specific recognition of flavonoid ligands, and this NodD-ligand specificity regulates transcription of key *nod* genes (Spaink et al. 1987). Because symbiosis relies on the specificity of phytochemical signaling via NodD receptors, we hypothesize that natural and synthetic chemicals present in the environment that mimic or interfere with this phytochemical signaling to *S. meliloti* NodD receptors may disrupt *nod* gene expression crucial to symbiosis.

Materials and Methods

Chemicals. The insecticides and PCBs (> 99% pure) were purchased from AccuStandard (New Haven, CT); dichlorodiphenyltrichloroethane (DDT) and its metabolites (99% pure) from Aldrich (Milwaukee, WI); E_2 and diethylstilbestrol (DES) (98% pure) from Sigma Chemical Company (St. Louis, MO); and the phytochemicals (> 99% pure) from INDOFINE Chemical Co., Inc. (Belle Mead, NJ). All chemicals were obtained neat and dissolved in dimethyl sulfoxide (DMSO).

Bacterial strain. The bacterial strain used in this study was *S. meliloti* strain 1021 pRmM57, a wild-type *Rhizobium* strain containing a plasmid-borne *nodC-lacZ* gene fusion and an additional copy of the *nodD1* gene, which was donated by S.R. Long (Mulligan and Long 1985).

Bacterial growth assay. Overnight cultures of *S. meliloti* 1021 pRmM57 (5 mL) were grown at 30°C and used to inoculate 200 mL TY (tryptone/yeast extract) media plus 50 µg/mL spectinomycin. Each inoculated flask received 1 µM luteolin, to mimic the conditions of our *in vitro* β-galactosidase (β-gal) assay, as well as either vehicle (DMSO) or one EDC to be tested [50 µM chrysin, 50 µM *o,p´*-DDT, or 50 µM pentachlorophenol (PCP)] (Figure 1). Bacterial growth was monitored at time zero and at all subsequent time points by measuring the absorbance at 595 nm (A_{595}) (Sambrook et al. 1989).

HPLC-MS determination of cross-reactivity. To determine if secondary products are formed through interactions between the strongest inhibitor of *nod* gene induction (PCP) and luteolin, qualitative analyses of incubation medium was performed using HPLC-mass spectrometry (HPLC-MS) electrospray ionization. All analyses were performed on a ThermoFinnigan LCQ DUO using an ESI interface (Agilent Technologies, Palo Alto, CA) operating in the negative ionization mode. The 25-µL aliquots were injected on a 5 cm × 4.6 mm × 5 µm 300SB–C8 Zorbax reverse-phase HPLC column (Agilent Technologies, Palo Alto, CA) at a flow rate of 0.25 mL/min. The mobile phase was 30% acetonitrile in 10 mM ammonium acetate held isocratic for the first 3 min, followed by a linear gradient from 30 to 40% acetonitrile over 10 min, a second linear gradient from 40 to 50% over 20 min, then a constant gradient for 50–65% acetonitrile over 10 min before returning to the original composition.

Delivery of sample effluent into the 250°C heated ionized capillary was controlled using a sheath gas flow rate of 20 psi. The source voltage was set at 4.5 kV. Positive identification of PCP, luteolin, and possible intermediates were confirmed by performing three scan events. The first event was a full scan between 60 and 500 amu (atomic mass units), the second was an MS-MS scan of daughter peaks at 265.3 amu with 20% collision energy being applied to the parent ion, and the third was an MS-MS scan of daughter peaks at 285.3 amu with 20% collision energy being applied to the parent ion.

In vitro β-*galactosidase assay.* For β-gal assays, liquid cultures of *S. meliloti* 1021 pRmM57 were grown in TY media plus 50 µg/mL spectinomycin overnight at 30°C. For the assays, 50 µL of the overnight culture was added to 950 µL TY plus spectinomycin. To test dose-dependent induction of *nod* genes, increasing concentrations of luteolin (50 nm–50 µm) were added (Figure 2). On the basis of reports by Peters and Long (1988) and Spaink et al. (1989), the amount of *nod* gene expression elicited by 1 µM luteolin alone was chosen as 100% gene induction in all remaining experiments. As a control, vehicle (DMSO) alone was tested for induction and antagonistic effects. To test for possible agonistic activity, each environmental chemical was tested at each concentration alone for effects on *nod* gene expression (data not shown). In addition, each environmental chemical was tested at all concentrations in the presence of 1 µM luteolin to determine if any antagonistic effects on *nod* gene expression were caused by the presence of any of the environmental chemicals (Figure 3, Table 1). The solvent

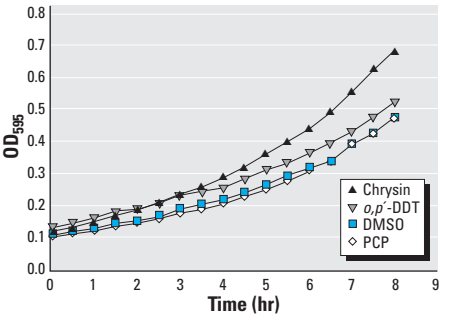

Figure 1. No inhibition of growth of *S. meliloti* cultures by EDCs and inhibitory phytochemicals at a concentration of 50 µM, the highest dose tested for effects on *nod* gene activation in all experiments. See "Materials and Methods" for details.

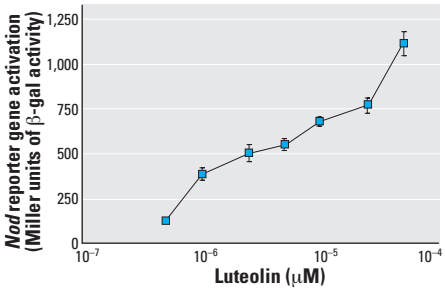

Figure 2. Luteolin activation of *nod* gene transcription determined by β-gal assay. See "Materials and Methods" for details.

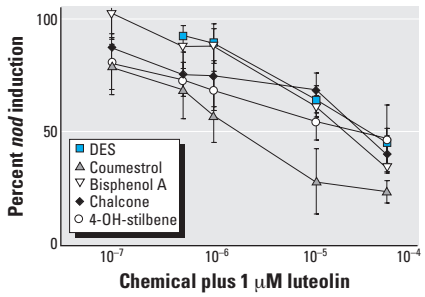

Figure 3. *nod* Gene induction antagonized by EDCs as determined by the β-gal reporter gene assay. Each of the chemicals significantly inhibited luteolin-NodD responsive *nod* gene activation in a dose-dependent manner from 100 nM to 50 µM.

concentration did not exceed 1% in the assays. In all cases, after a 3-hr incubation at 30°C, the bacteria were recovered by centrifugation at 15,000 × *g* for 5 min, and a β-gal assay was performed as described (Miller 1972; Mulligan and Long 1985). Briefly, the cell pellet was resuspended in 700 µL Z-buffer (60 mM Na_2HPO_4, 40 mM $Na_2H_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, and 35 mM β-mercaptoethanol) and permeabilized by the addition of 25 µL $CHCl_3$ and 25 µL 0.1% SDS followed by vortexing for 45 sec. The reaction was equilibrated at 30°C for 10 min, then 250 µL *o*-nitrophenyl β-D-galactopyranoside (4 mg/mL in Z-buffer) was added and the reaction returned to 30°C until the appropriate color was reached. The reaction was terminated by the addition of 500 µL 1 mM $NaCO_3$. The cell debris was removed by centrifugation, and absorbance was measured at A_{420}. Bacterial number was monitored by measuring the absorbance at A_{595}. Miller units were determined using the following formula: $A_{420}/(A_{595}$ of 1/10 dilution of cells × volume of culture × length of incubation) × 1,000. The data are representative of at least three independent experiments with three replicates.

BLAST protein homology analysis. We used the National Institutes of Health (NIH) Basic Local Alignment Search Tool (BLAST) program (NIH 2002) to compare *S. meliloti* NodD proteins (I, II, and III) with ER-α and ER-β, searching for any amino acid sequence homology between the NodD proteins and the ERs.

Results

EDCs do not significantly inhibit growth of* S. meliloti *soil bacteria. To determine if the EDCs used in our *in vitro* β-gal reporter assays were overtly toxic to *S. meliloti* at the concentrations tested, we compared bacterial growth in the presence or absence of the maximum dose (50 µM) of several EDCs used in our assays (Figure 1). Chrysin, the known phytochemical inhibitor of *nod* gene signaling, was also tested for effects on bacterial growth and had no deleterious effects on bacterial growth even at 50 µM (Peters and Long 1988). In addition, both the most potent synthetic inhibitor and a midrange synthetic inhibitor, PCP and *o,p′*-DDT, respectively, had no negative effects on growth of *S. meliloti*.

Cross-reactivity of EDC and agonist is not a mechanism of* nod *gene inhibition. To determine whether EDCs used in our *in vitro* β-gal assay were directly sequestering, binding, or altering the chemical composition of the agonist, luteolin, as a mechanism for inhibiting *nod* gene expression, we incubated luteolin and the strongest EDC inhibitor, PCP, and analyzed the products formed. HPLC-MS^2 analysis of incubated growth medium amended with PCP and luteolin did not show evidence of cross-reactivity or the production of a third intermediate during time-course incubation. Because the only products found at any time during incubation were PCP and luteolin, and no third intermediate or degradation products were detected, we conclude that the most potent inhibitor of *nod* gene expression, PCP, does not inhibit luteolin-signaling activity by direct substrate–inhibitor interaction.

A wide range of environmentally relevant EDC concentrations were tested. As a representative group of *nod* antagonists, DDT and its metabolites were tested in a full range of concentrations for a dose-dependent reduction of *nod* gene expression. Based on reported soil concentrations of EDCs, including those

Table 1. Many different classes of EDCs inhibit *nod* gene induction.

Chemical	Percent inhibition of *nod* expression (I_{max})	IC_{20}	IC_{50}
Insecticides			
PCP	90	2.1×10^{-7}	9.9×10^{-7}
Methyl parathion	89	1.2×10^{-7}	4.3×10^{-7}
Kepone	42	2.8×10^{-7}	
p,p′-DDT	45	7.6×10^{-8}	
p,p′-DDE	44	7.6×10^{-8}	
o,p′-DDT	43	3.4×10^{-7}	
o,p′-DDE	42	8.2×10^{-8}	
p,p′-DDD	35	1.0×10^{-7}	
o,p′-DDD	34	1.3×10^{-7}	
Hexachlorocyclohexane	24	3.7×10^{-6}	
Dicofol	22	4.2×10^{-6}	
Malathion	20	8.1×10^{-6}	
Lindane	13		
Toxaphene	7		
Methoprene	5		
Endosulfan	None		
Endosulfan sulfate	None		
Methoxychlor	None		
Aldrin	None		
Dieldrin	None		
Carbofuran	None		
S-Ethyl dipropylthiocarbamate	None		
Diazinon	None		
Dursban	None		
Herbicides			
2,4,5-T	37	6.8×10^{-6}	
2,4-D	32	7.0×10^{-6}	
Pendimethalin	16		
Trifluralin	12		
Atrazine	10		
Metolachlor	10		
Alachlor	None		
trans-Nonachlor	None		
Acetochlor	None		
Fungicide			
Vinclozolin	None		
Plasticizers			
Bisphenol A	66	2.9×10^{-6}	1.7×10^{-5}
tert-Octylphenol	25	8.7×10^{-6}	
4-Nonylphenol	20	7.0×10^{-6}	
Benzyl butylphthatlate	19		
PCBs			
4-OH-2′,3′,4′,5′-PCB	60	1.7×10^{-7}	5.4×10^{-6}
4-OH-2′,4′,6′-PCB	56	4.6×10^{-6}	3.2×10^{-5}
Arochlor	27	8.8×10^{-5}	
3,3′,4,5-PCB	23	5.9×10^{-5}	
2,3,4,5-PCB	15		
2,4,6-PCB	None		
PAHs			
6-OH chrysene	29	9.3×10^{-6}	
cis-Nonachlor	12		
Hormone-active compounds			
DES	55	5.0×10^{-7}	3.2×10^{-5}
4-OH-stilbene	53	3.1×10^{-6}	2.6×10^{-5}
Zearalenone (fungal)	33	2.1×10^{-6}	
Progesterone	17		
ICI 182,780	15		
Testosterone	10		
Estriol	7		
E_2	None		
Phytochemicals			
Genistein	86	9.4×10^{-8}	6.9×10^{-7}
Chrysin	85	1.5×10^{-7}	7.0×10^{-7}
Coumestrol	76	1.2×10^{-7}	8.8×10^{-6}
Chalcone	60	1.7×10^{-6}	6.7×10^{-6}
Kaempferol	59	3.6×10^{-6}	8.5×10^{-6}
Daidzein	None		
Apigenin	None		

Abbreviations: IC_{20}, concentration that inhibits 20%; IC_{50}, concentration that inhibits 50%; I_{max}, maximal inhibition. Each EDC was tested for the ability to significantly inhibit the amount of *nodC-lacZ* reporter gene transcription induced by 1-µM luteolin inducer (set as 100% induction) and measured by quantitative β-gal assay. See "Materials and Methods" for details. Results are the average of at least three independent experiments.

presented in Table 2, and a recent report of 40 different soils in the midwestern United States that found total DDT concentrations (DDT plus all metabolites) to be about 10 ppb (Aigner et al. 1998), we tested DDT and its metabolites at concentrations ranging from 50 nM to 50 μM (Table 1). Both isomers of DDT (*o,p´*-DDT and *o,p´*-DDT) significantly decreased luteolin-induced *nod* gene activation at all concentrations > 100 nM (Table 1). Other chemicals, tested at a range of concentrations, caused statistically significant inhibition of luteolin-NodD–induced *nod* gene expression at concentrations as low as 100 nM, including PCP, methylparathion, and the herbicides (2,4-dichlorophenoxy)acetic acid (2,4-D) and (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T) (Table 1).

One EDC induces nodulation gene expression. To determine whether various natural and synthetic chemicals could independently induce expression of *nod* genes, effects on reporter gene expression were measured in the presence of each chemical alone (no luteolin added) (Miller 1972; Mulligan and Long 1985). When the reporter strain was treated with the natural phytochemical agonists luteolin or apigenin alone, *nod* gene expression was induced 100% and 40%, respectively, which is consistent with previous reports of agonist activity in *S. meliloti* (Peters and Long 1988). Bisphenol A was the only synthetic chemical that, when added alone at a concentration of 50 μM, was able to induce *nod* gene expression 30% above control. None of the other synthetic chemicals tested significantly induced *nod* gene expression above control.

Many EDCs inhibit nodulation gene expression. Many different classes of synthetic environmental chemicals that affect estrogen-responsive gene expression in vertebrates were tested in our system for effects on luteolin-NodD signaling (Figure 3, Table 1). DDT and its metabolites dichlorodiphenyldichloroethane (DDD) and dichlorodiphenyldichloroethylene (DDE) inhibited luteolin-induced *nod* gene expression an average of 45% (Table 1). Other organochlorine pesticides inhibited *nod* gene expression, including PCP and methyl parathion, which both inhibited luteolin-induced *nod* gene expression by 90% (Table 1) (Fox et al. 2001). Although these pesticides had detrimental effects on *nod* gene expression, other EDCs, environmental chemicals, and organochlorine pesticides showed no appreciable effects (Table 1). Herbicides and polyaromatic hydrocarbons (PAHs) were also tested and had a lesser but statistically significant effect on *nod* gene expression controlled by luteolin-NodD signaling. PCBs inhibited luteolin-NodD signaling, resulting in as much as 85% inhibition of *nod* gene expression. Plastics by-products such as bisphenol A reduced *nod* gene induction by 66% (Figure 3, Table 1). As the only synthetic chemical shown to induce *nod* gene expression as well as inhibit NodD–induced *nod* gene expression, bisphenol A appears to act as a partial inducer/antiinducer, depending on the profile of chemicals present in the environment.

Natural and synthetic estrogens affect nodulation gene expression. Because of reported genetic homology between NodD and ER-α (Gyorgypal and Kondorosi 1991), the endogenous ER ligand E_2 was tested for effects on NodD-activated gene expression. E_2 alone caused no induction of *nod* gene expression, and E_2 did not inhibit luteolin-NodD activation of *nod* genes (Table 1). DES is a synthetic estrogen known to bind ER-α with 1,000 times greater affinity than E_2 (Korach et al. 1979, 1988). No effect was seen when DES was added alone, but DES inhibited luteolin-induced *nod* gene expression by 50% at 50 μM (Figure 3). Therefore, DES, which is derived from a stilbene plant product core, but not vertebrate steroids such as E_2, blocked the ability of luteolin-NodD–induced *nod* gene expression.

NodD and ER proteins do not share sequence homology. NodD and ER-α share affinity for many of the same phytoestrogen ligands and have been reported to share ligand-binding domain sequence homology (Gyorgypal and Kondorosi 1991). Using the BLAST program, we compared NodD to ER-α and ER-β and found no significant sequence homology at the nucleotide or amino acid level.

Table 2. Environmental data on pesticides and EDCs in agricultural soil.

	Pounds applied per year (U.S.)	Concentration detected in soil (μg/kg)	Half-life in soil (range)
Insecticides			
PCP	24 million[a]	< 1–590[a]	15–60 days[a]
Methyl parathion	6 million[b]	< 1–44[a]	5–30 days[c]
p,p´-DDT	[d]	< 1–68[e]	300 days–15 years[c]
p,p´-DDE	[d]	< 1–240[e]	2–16 years[c]
o,p´-DDT	[d]	< 1–42[e]	300 days–15 years[c]
o,p´-DDE	[d]	< 1–22[e]	2–16 years[c]
p,p´-DDD	[d]	< 1–130[e]	2–16 years[c]
o,p´-DDD	[d]	< 1–150[e]	2–16 years[c]
Hexachlorocyclohexane	200,000[f]	< 1–5[e]	25–100 days[f]
Dicofol	800,000[b]	< 1–26[a]	45–68 days[c]
Malathion	12.5 million[g]	< 1–690[g]	1–14 days[g]
Lindane	200,000[f]	< 1–500[f]	100–1,464 days[c]
Toxaphene	3.7 million[h]	< 100–630[e]	9–500 days[c]
Herbicides			
2,4,5-T	600,000[b]	< 1–380[a]	12–69 days[c]
2,4-D	41 million[b]	< 1–38[a]	2–15 days[c]
Pendimethalin	27 million[b]	< 1–30[a]	90–480 days[c]
Trifluralin	22 million[b]	< 1–860[a]	15–132 days[c]
Atrazine	75 million[b]	< 1–82[a]	18–402 days[c]
Metolachlor	67 million[b]	< 1–856[a]	12–292 days[c]
PCBs			
Total PCBs	[i]	< 1–13,000[f]	10 days–18 years[j]

[a]Data from the National Library of Medicine (2001). [b]Data from Gianessi and Silvers (2000). [c]Data from the Agricultural Research Service (2001). [d]U.S. production discontinued in 1972. [e]Data from the U.S. Geological Survey (1998). [f]Data from the Agency for Toxic Substances and Disease Registry (ATSDR 2003). [g]Data from the ATSDR (2001). [h]Data from the ATSDR (1996). [i]U. S. production discontinued in 1976. [j]Data from the United Nations Environment Program (2003).

Discussion

We tested 62 natural and synthetic environmentally relevant EDCs using a reporter gene assay to quantify any effects on symbiotic *nod* gene expression. After an expanded study, we now report that environmentally relevant concentrations of 45 of the 62 EDCs and organochlorine pesticides statistically significantly inhibited luteolin-NodD receptor signaling and symbiotic *nod* gene activation. Among other well-characterized endocrine-disrupting organochlorine pesticides, we also analyzed the effects of PCBs, PAHs, and plasticizers and found that many of these EDCs inhibit luteolin-NodD signaling and *nod* gene expression. We have shown that many EDCs exhibit dose–responsive, concentration-dependent inhibition of luteolin-NodD–induced *nod* gene expression. In addition, we have previously shown that EDC inhibition of *nod* gene expression can be overcome by increasing concentrations of luteolin, the natural agonist for the NodD receptor (Fox et al. 2001). Our *in vitro* studies tested concentrations of EDCs ranging from 50 nM to 50 μM and found no toxicity (Figure 1) or systemic effects on *S. meliloti* soil bacteria, which have been reported to survive up to 5-mM concentrations of such EDCs (Welp and Brummer 1999). Based on these observations and our data, we suggest that a competitive binding mechanism is responsible for EDC inhibition of luteolin-NodD–induced *nod* gene expression.

Symbiotic *Rhizobium* soil bacteria are found ubiquitously within the first 10 inches below ground in agricultural fields. Endocrine-disrupting pesticides routinely sprayed on agricultural crops are present in high concentrations

in this same soil environment in which phytoestrogen signaling and nitrogen-fixing symbiosis occur. For example, despite the suspension of DDT use in the United States in 1972, its extremely long half-life has made DDT and its metabolites among the most readily detectable contaminants in agricultural areas where it was formerly used (Aigner et al. 1998; Falconer et al. 1997). Quantities of DDT and other EDCs measured and reported by various U. S. government agencies are shown in Table 2. In addition, a recent sampling of 40 different soils in the midwestern United States found total DDT concentrations (DDT plus all metabolites) to be 10 ppb (Aigner et al. 1998). Wildlife exposure data have shown concentrations of *p,p′*-DDE as high as 20 μM in alligator eggs in Lake Apopka, Florida (Heinz et al. 1991). Similarly, agricultural soil concentrations of DDT, DDD, DDE, and other environmentally persistent compounds, such as PAHs, have been measured in the micromolar and millimolar range (Cooke and Stringer 1982; Falconer et al. 1997). Although detectable quantities of EDCs are measurable in the United States (Table 2), which has imposed limited-use restrictions or bans on many pesticides and EDCs, soil concentrations of these pesticides and EDCs are likely to be much higher in developing countries where many of these pesticides are still in use (Longnecker et al. 1997; U.S. Geological Survey 1998).

Many factors (solubility, concentration, sorption to soil particles, half-life) influence the bioavailability of pesticides to *Rhizobium* bacteria. Nevertheless, the routine application of high concentrations of pesticides to crops that rely on *Rhizobium* symbiosis results in transiently high soil concentrations of pesticides at levels we have shown to significantly antagonize symbiotic signaling. Pesticide-induced inhibition of symbiotic signaling, although not directly lethal to crops or *Rhizobium* bacteria, would produce a net result of delayed and/or suboptimal recruitment of bacteria to legume plants during the crucial seasonal window of crop growth, when the nitrogen-fixing abilities of rhizobia are needed the most.

Because the bacterial cascade of events regulating symbiosis is carried out by the *nod* genes, EDC inhibition of *nod* genes is a direct threat to nitrogen-fixing symbiosis and may have deleterious effects on soil nitrogen concentrations in many pesticide-treated agricultural fields (Schultze and Kondorosi 1998; van Rhijn and Vanderleyden 1995). In fact, interactions between symbiotic soil bacteria and synthetic EDCs that jeopardize nitrogen fixation would be expected to alter microbial species balance and reduce plant yields in heavily pesticide-treated or polluted areas (Leach and Givnish 1996; Zahran 1999). Our previous studies support this theory (Fox et al. 2001) by showing that EDC inhibition of phytoestrogen-NodD signaling *in vitro* resulted in fewer *S. meliloti* bacteria recruited to alfalfa roots *in vivo*. We have shown, both *in vitro* and *in vivo*, that some EDCs which disrupt vertebrate hormone signaling also inhibit plant-bacterial signaling necessary for symbiosis. When fewer bacteria are recruited to plant roots, nitrogen-fixing symbiosis is inhibited. A reduction in symbiotically produced natural nitrogenous results in reduced crop yields, which must be supplemented by adding costly synthetic nitrogenous fertilizer to affected fields.

Although our data demonstrate inhibition of symbiosis by pesticides *in vitro* or *in situ* in the laboratory, agricultural studies have shown negative effects of pesticides at the whole-crop level. Such studies have shown that synthesis of phenolic phytoestrogens, necessary for recruiting soil bacteria for symbiosis, is altered by the application of pesticides (Daniel et al. 1999). Herbicide application reduces the total amount of and alters the production levels of multiple phytochemicals in treated plants (Daniel et al. 1999). These findings are significant because the amount and exact profile of phytochemicals produced by a plant directly correlates with its ability to signal and recruit symbiotic soil bacteria (Daniel et al. 1999; Peters and Long 1988). As *Rhizobium*-host plant specificity is regulated by NodD receptor recognition of the particular phytochemical mixture or signature of the host plant, any alteration in the profile of phytochemicals produced may inhibit recruitment signaling necessary for nitrogen-fixing symbiosis. Other agricultural studies have shown that nodulation and nitrogen fixation are reduced in soybeans treated with a variety of herbicides and fungicides (Zahran 1999). In addition, PAHs induce a dose-dependent decrease in shoot length and nodule formation in alfalfa roots in symbiosis with *S. meliloti* (Wetzel and Werner 1995). Therefore, although many agricultural studies have noted negative effects of various EDCs (pesticides, herbicides, and PAHs) on nodulation and nitrogen-fixing symbiosis in treated crops, we have determined the genetic mechanism responsible for these deleterious effects: EDCs disrupt phytoestrogen recruitment of *Rhizobium* by competitively inhibiting phytoestrogen signaling to bacterial NodD receptors.

Both vertebrate ERs and bacterial NodD phytoestrogen receptors share affinity for phytoestrogen ligands, and phytoestrogen activation of these receptors results in transcription of responsive genes. Because certain structurally similar flavonoids activate both ERs and NodD proteins, we hypothesized that other phenolic or ring-structured compounds present in the environment, such as EDCs known to disrupt E_2-ER signaling, would also disrupt phytoestrogen-NodD receptor signaling (Djordjevic et al. 1987; Firmin et al. 1986; Peters and Long 1988). Here we report that 45 different EDCs statistically significantly inhibit phytoestrogen-NodD symbiotic signaling. EDC disruption of phytoestrogen-NodD signaling results in inhibition of symbiotic *nod* gene expression, which leads to reduced recruitment of soil bacteria and may result in a net loss of symbiotic nitrogen fixation and significantly reduced plant yields (Garry et al. 1999; Rawlings et al. 1998; Short and Colborn 1999). In addition to the possibly severe environmental consequences of EDC disruption of plant–*Rhizobium* symbiotic signaling, these findings also illustrate that new, unconventional targets of EDCs exist in the environment. Our data have outlined the previously unrecognized parallel disruption of vertebrate endocrine signaling and plant–bacterial symbiotic signaling by a group of EDCs. These results, as well as the recent description of an invertebrate ER (Thornton et al. 2003), strongly indicate that defining endocrine disruption as a phenomenon limited strictly to vertebrates that express ERs is a prohibitively narrow view.

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