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

Jennifer E. Fox, Marta Starcevic, Phillip E. Jones, Matthew E. Burow, and John A. McLachlan

1Environmental Endocrinology Laboratory, Center for Bioenvironmental Research at Tulane and Xavier Universities, New Orleans, Louisiana, USA; 2Center for Ecology and Evolutionary Biology, University of Oregon, Eugene, Oregon, USA; 3Department of Biology, Xavier University, New Orleans, Louisiana, USA; 4Section of Hematology and Medical Oncology, Department of Medicine, and 5Department of Surgery, Tulane University Medical School, New Orleans, Louisiana, USA; 6Department of Pharmacology, Tulane University Medical School, New Orleans, Louisiana, USA

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; Donehoo 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 (Sonneschein 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 (E2) (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 E2 (Breinholt and Larsen 1998; Korach et al. 1997, 1998).

Exposure to endocrine-altering chemicals is not limited to synthetic pollutants. Phytoestrogens are also capable of antagonizing or mimicking the actions of E2. A class of phytochemicals called flavonoids shares common characteristics with steroid 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 (Gyongyospal 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 E2-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...
carbon source offered by the plant, the Rhizobium fix atmospheric nitrogen into a form (NH₃; 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 Spanik 1998; Spanik 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 (Spanik 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); dichlorodiphenyltrichloroethylene (DDT) and its metabolites (99% pure) from Aldrich (Milwaukee, WI); E₂ 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 pRM57, 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 pRM57 (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 µL 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 (A595) (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 were 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 applied to the parent ion, and the third was an MS-MS scan of daughter peaks at 285.3 amu with 20% collision energy applied to the parent ion.

In vitro β-galactosidase assay. For β-gal assays, liquid cultures of S. meliloti 1021 pRM57 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 Spanik 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
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 Na2HPO4, 40 mM Na2H2PO4, 10 mM KCl, 1 mM MgSO4, and 35 mM β-mercaptoethanol) and permeabilized by the addition of 25 µL CHCl3 and 25 µL 0.1% SDS followed by vortexing for 45 sec. The reaction was equilibrated at 30°C for 10 min, then followed by vortexing for 45 sec. The reaction returned to 30°C until the appropriate color was reached. The reaction was terminated by the addition of 500 µL 1 mM Na2CO3. The cell debris was removed by centrifugation, and absorbance was measured at A420. Bacterial number was monitored by centrifugation, and absorbance was measured at A595. Miller units were determined using the following formula: \( A_{420} \times \text{bacterial number} \div \text{absorbance} \div \text{volume of culture} \times \text{length of incubation} = 1,000 \).

The data are representative of at least three independent experiments with three replicates.

**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). Chrysosin, 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*.

**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^{-6} | 4.3 × 10^{-7} |
| Kepone | 42 | 2.8 × 10^{-7} | |
| p,p′-DDT | 45 | 7.6 × 10^{-8} | |
| α,α′-DDE | 44 | 7.6 × 10^{-8} | |
| α,α′-DDE | 43 | 3.4 × 10^{-7} | |
| α,α′-DDE | 42 | 8.2 × 10^{-8} | |
| α,α′-DDE | 35 | 1.0 × 10^{-7} | |
| α,α′-DDE | 34 | 1.3 × 10^{-7} | |
| Hexachloro cyclohexane | 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 | | |
| Fungicides | | | |
| Fungicide | Vincluzolin | None | |
| Plasticsizers | | | |
| 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} | |
| Beryl butylphthalate | 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 | 4.8 × 10^{-6} | 3.2 × 10^{-5} |
| Aroclor | 27 | 8.8 × 10^{-6} | |
| 3′,3′,4′-PCB | 23 | 5.9 × 10^{-5} | |
| 2,3,4,5-PCB | 15 | | |
| 2,4,6-PCB | None | | |
| PAHs | | | |
| 6-OH chrysenol | 29 | 9.3 × 10^{-6} | |
| αs-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} |
| Zearaleone (fungal) | 33 | 2.1 × 10^{-6} | |
| Progesterone | 17 | | |
| ICI 182,780 | 15 | | |
| Testosterone | 10 | | |
| Estrol | 7 | | |
| E2 | None | | |
| Phytoc hemicals | | | |
| Genistein | 86 | 9.4 × 10^{-6} | 6.9 × 10^{-7} |
| Chrys | 85 | 1.5 × 10^{-6} | 7.0 × 10^{-7} |
| Coumestrol | 76 | 1.2 × 10^{-6} | 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-fucZ 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 a,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, mephathropain, 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.

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

| Insecticides | Pounds applied per year (U.S.) | Concentration detected in soil (µg/kg) | Half-life in soil (range) |
|--------------|-------------------------------|--------------------------------------|--------------------------|
| PCP          | 24 million³                   | <1–500³                             | 15–60 days²              |
| Methyl parathion | 6 million²                 | <1–44²                             | 5–30 days³               |
| p,p'-DDT     | d                             | <1–68d                             | 300 days–15 years³       |
| p,p'-DDE     | d                             | <1–240d                            | 2–16 years²              |
| o,p'-DDT     | d                             | <1–42d                             | 300 days–15 years³       |
| o,p'-DDE     | d                             | <1–22d                             | 2–16 years²              |
| p,p'-DOD     | d                             | <1–130d                            | 2–16 years²              |
| o,p'-DOD     | d                             | <1–150d                            | 2–16 years²              |
| Hexachlorocyclohexane | 200,000²            | <1–5²                             | 25–100 days²             |
| Dicofol      | 800,000³                      | <1–26³                             | 45–68 days²              |
| Malathion    | 12.5 million⁷                 | <1–650⁷                            | 1–14 days⁷               |
| Lindane      | 200,000³                      | <1–50³                            | 100–1,464 days³          |
| Toxaphene    | 3.7 million³                  | <1–60³                             | 9–500 days²              |
| Herbicides   |                               |                                     |                          |
| 2,4,5-T      | 600,000⁰                     | <1–300⁰                            | 12–69 days²              |
| 2,4-D        | 41 million³                  | <1–38³                            | 2–15 days²               |
| Pendimethalin | 27 million³                 | <1–30³                            | 90–480 days²             |
| Trifuralin   | 22 million³                  | <1–86³                            | 15–132 days²             |
| Atrazine     | 75 million³                  | <1–82³                            | 18–402 days²             |
| Metalachlor  | 67 million³                  | <1–85³                            | 12–292 days²             |
| Total PCBs   | i                             | <1–13,000i                         | 10 days–18 years²        |

*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 (1996). h U. S. production discontinued in 1976. i Data from the United Nations Environment Program (2003).

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 dichlorophenyl dichloroethane (DDD) and dichlorodiphenyldichloroethylene (DDE) inhibited luteolin-induced nod gene expression at an average of 45% (Table 1). Other organochlorine pesticides inhibited nod gene expression, including PCP and methylparathion, 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/antinducer, 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 E2 was tested for effects on NodD-activated gene expression. E2 alone caused no induction of nod gene expression, and E2 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 E2 (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 stillborn plant product core, but not vertebrate steroids such as E2, 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.

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–response, 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 (Schultz 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; Zarran 1999). Our previous studies support this theory (Fox et al. 2001) by showing that EDC inhibition of phytoestrogen-NodD signaling in *in vitro* resulted in fewer *S. meliloti* bacteria recruited to alfalfa roots in *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 *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 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 (Zarran 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 pholic or ring-structured compounds present in the environment, such as EDCs known to disrupt E2-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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