Pesticides reduce symbiotic efficiency of nitrogen-fixing rhizobia and host plants

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Unprecedented agricultural intensification and increased crop yield will be necessary to feed the burgeoning world population, whose global food demand is projected to double in the next 50 years. Although grain production has doubled in the past four decades, largely because of the widespread use of synthetic nitrogenous fertilizers, pesticides, and irrigation promoted by the “Green Revolution,” this rate of increased agricultural output is unsustainable because of declining crop yields and environmental impacts of modern agricultural practices. The last 20 years have seen diminishing returns in crop yield in response to increased application of fertilizers, which cannot be completely explained by current ecological models. A common strategy to reduce dependence on nitrogenous fertilizers is the production of leguminous crops, which fix atmospheric nitrogen via symbiosis with nitrogen-fixing rhizobia bacteria, in rotation with nonleguminous crops. Here we show previously undescribed in vivo evidence that a subset of organochlorine pesticides, agrichemicals, and environmental contaminants induces a symbiotic phenotype of inhibited or delayed recruitment of rhizobia bacteria to host plant roots, fewer root nodules produced, lower rates of nitrogenase activity, and a reduction in overall plant yield at time of harvest. The environmental consequences of synthetic chemicals compromising symbiotic nitrogen fixation are increased dependence on synthetic nitrogen fertilizer, reduced soil fertility, and unsustainable long-term crop yields.

In the past 40 years, synthetic nitrogen (N) fertilizer use has increased 7-fold, whereas pesticide use has increased 3-fold; if current practices continue unabated, application of both is expected to increase an additional 3-fold by 2050 (1–9). Paradoxically, as N fertilizer application has exponentially increased, yield potential of major staple food crops has become stagnant. To date, such diminishing returns have been explained by models showing the first addition of N fertilizer induces the largest gain in crop yield with efficiency declining at higher levels of application (10, 11). Additionally, agricultural intensification and continuous cropping can result in a loss of soil organic matter leaving soil less fertile, which then drives increased application of synthetic N fertilizer and pesticides.

Sustainable agriculture seeks to increase crop yields and nutrient-use efficiency while reducing the environmental costs associated with agricultural intensification. The goal of such strategies is to maximize the amount of crop output per unit of water, N fertilizer, and pesticide input. Certain farming practices have been found to increase efficiency and sustainability of crop production, including integrated pest management, improved drainage control, properly timed water and fertilizer application, and maximizing biological N fixation by incorporating legume crops and enriching soil with N-fixing rhizobia (Rhiz) bacteria (4, 10, 12–14). A well established practice for maintaining soil fertility has been the cultivation of legume crops, which replenish N in the soil via symbiosis with N-fixing Rhizobium bacteria that convert atmospheric N to ammonia and other sources utilizable by plants, in rotation with non-N-fixing crops (8, 15). The effectiveness of this strategy relies on maximizing symbiotic N fixation (SNF) and plant yield to resupply organic and inorganic N and nutrients to the soil. The vast majority of biologically fixed N is attributable to symbioses between leguminous plants (soybean, alfalfa, etc.) and species of Rhizobium bacteria; replacing this natural fertilizer source with synthetic N fertilizer would cost $10 billion annually (16, 17).

Effective SNF can significantly reduce the need for synthetic N fertilizers. An estimated $50–90 million net return could be realized by rotating alfalfa and corn crops in the Midwestern U.S. (17). In Brazil, soybeans inoculated with Rhiz are responsible for $1.3 billion per year savings in production costs (18). In addition to the economic benefits, using SNF to reduce dependence on commercial N fertilizer has environmental benefits. An understanding of factors controlling SNF will help to maximize symbiotic effectiveness for agricultural sustainability. Therefore, it is important to determine how addition of synthetic chemicals to the soil environment may affect SNF.

SNF is both initiated and maintained by an active exchange of chemical signals between host plant and Rhiz soil bacteria. Each species of Rhiz interacts only with a particular subset of host plant species. For example, the soil bacterium Sinorhizobium meliloti will form a symbiotic partnership with its host plant, alfalfa, but not with other legumes such as soybean or clover (19, 20). To establish host specificity, alfalfa exudes a unique mixture of flavonoid (luteolin and apigenin) phytochemical signals into the soil, which serve dual functions, to recruit S. meliloti and to inhibit or antagonize unfavorable species of Rhiz (21–23). Alfalfa phytochemical signals are specifically recognized by S. meliloti NodD receptors, which are transcriptional regulators that bind DNA response elements and induce transcription of rhizobial nodulation (nod) genes in a flavonoid-dependent manner. The end products of nod genes are Nod factors, which act as response signals sent by the S. meliloti back to the alfalfa host plant. S. meliloti Nod factors are recognized by specialized receptors in alfalfa roots, thus initiating development of root nodules where, in exchange for an energy source from the host plant, S. meliloti will fix atmospheric N to a usable fertilizer source for alfalfa (24). Therefore, symbiotic signaling is initiated by S. meliloti NodD receptors’ specific recognition of alfalfa-produced phytochemicals. Nonhost plant phytochemicals, such as genistein produced by soybeans and chrysins produced by...
clover, inhibit symbiotic signaling between alfalfa and *S. meliloti* by interfering at the level of the rhizobial NodD receptors effectively blocking communication and disrupting initiation of symbiosis (21–23).

Temporal and chemical specificity of symbiotic signaling is crucial for coordinating the actions of alfalfa and *S. meliloti* necessary for SNF. In the dynamic soil environment, *S. meliloti* is exposed to a mixture of agonistic and antagonistic phytochemicals, and the degree to which its NodD receptors are able to interpret these signals to locate its symbiotic partner, alfalfa, will most likely determine the efficiency of SNF in that particular soil environment. If crucial alfalfa phytochemical signaling is disrupted, then SNF will also be compromised (21). The importance of temporal specificity, timing of *S. meliloti* recruitment to alfalfa, to SNF has been demonstrated in studies in which early onset nodulation and N fixation has resulted in enhanced alfalfa growth (25) and by studies showing that *S. meliloti* that are recruited to alfalfa roots faster have a marked competitive advantage over their slower counterparts (26). Therefore, natural or synthetic chemicals in the soil environment that disrupt symbiotic signaling would not only delay symbiotic initiation but also decrease symbiotic efficiency.

A plant’s need for N increases progressively as the plant enters its exponential phase of growth and the demand for N outstrips the rate of supply from the soil (27). Crop legumes can fix 100–200 kg of N per hectare per year, but rates are often substantially lower, and over the past 25 years, soybeans and other legumes have shown a significant decline in N fixation (28, 29). To investigate what may be contributing to such a decline, we directly measured effects of exogenous factors, such as pesticides or environmental chemicals found in cattle feedlot effluent and irrigation water, on symbiotic signaling and SNF among legumes and Rhiz. In previous studies using in vitro reporter assays, we demonstrated that ~30 different pesticides and environmental contaminants specifically disrupted crucial symbiotic signaling between flavonoid phytochemicals and *S. meliloti* NodD receptors (30, 31). Here, we show previously undescribed in vivo evidence that a subset of organochlorine pesticides and pollutants inhibit symbiotic signaling between alfalfa and *S. meliloti*, resulting in delayed symbiotic recruitment, reduced SNF, and a decline in alfalfa plant yield.

**Results and Discussion**

To determine the effects of different pesticides and environmental contaminants on in vivo N-fixing symbiosis, alfalfa seeds were inoculated with *S. meliloti* and then treated with a subset of chemicals previously shown to specifically inhibit phytochemical–NodD symbiotic signaling in vitro (30, 31). The following natural and synthetic chemicals were tested: (i) chrysirin, a clover-derived phytochemical known to antagonize *S. meliloti* NodD activation in vitro (23); (ii) methyl parathion, an insecticide; (iii) dichlorodiphenyltrichloroethane (DDT), an insecticide; (iv) bisphenol A, a plasticizer and monomer used in the manufacture of polycarbonate plastic and a ubiquitous environmental contaminant; and (v) pentachlorophenol, an insecticide and wood preservative. Alfalfa seeds that were inoculated with *S. meliloti* and received no chemical treatment (no chemicals, +Rhiz) served as a positive control for SNF, whereas uninoculated alfalfa seeds [with chemicals (−Rhiz)] served as a negative control for effects of lack of SNF on nodule formation, N fixation activity, and plant yield.

Alfalfa seeds were inoculated with *S. meliloti* and then received a one-time treatment with the chemicals listed above. To measure whether each chemical alone affected plant growth, treatment groups were established that were not inoculated with *S. meliloti* but did receive the same chemical treatments as described above. Five independent replicate populations per treatment group were then assayed at 2, 4, and 6 weeks after inoculation for the following parameters: number of plants, number of root nodules per plant, nitrogenase activity, and reduce nitrogenase activity. Alfalfa seeds were inoculated with *S. meliloti* and treated with various environmental chemicals (at 5 × 10−5 M) at day 0. Alfalfa was harvested at 2, 4, and 6 weeks after inoculation, and the following were assayed: (A) The number of root nodules per plant was determined for each treatment group. At 2 weeks after inoculation, no nodules were present; (0) indicates no nodules present at weeks 4 and 6 for these treatment groups. At 4 and 6 weeks after inoculation, all treatment groups had significantly fewer nodules (as indicated by *) compared with the +Rhiz-positive control. (B) Nitrogenase activity was measured by using an acetylene reduction assay (32, 33). Five replicates per treatment per time point were assayed, and average nanomolar ethylene produced per minute per plant was calculated for each treatment group. At 3, 4, and 5 weeks after inoculation, nitrogenase activity was significantly reduced in all treatment groups (as indicated by *) compared with the +Rhiz-positive control. At 6 weeks after inoculation, nitrogenase activity of bisphenol A and pentachlorophenol treatment groups was significantly reduced (as indicated by #) compared with the +Rhiz-positive control. All results shown are mean values ± SEM for a minimum of three independent experiments. A one-way ANOVA with a Bonferroni correction for multiple tests was used to compare parameters of treatment groups (significant if *P* < 0.05).

**Fig. 1.** Pesticides inhibit recruitment of bacteria to host plant, delay nodulation, and reduce nitrogenase activity. Alfalfa seeds were inoculated with *S. meliloti* and treated with various environmental chemicals (at 5 × 10−5 M) at day 0. Alfalfa was harvested at 2, 4, and 6 weeks after inoculation, and the following were assayed: (A) The number of root nodules per plant was determined for each treatment group. At 2 weeks after inoculation, no nodules were present; (0) indicates no nodules present at weeks 4 and 6 for these treatment groups. At 4 and 6 weeks after inoculation, all treatment groups had significantly fewer nodules (as indicated by *) compared with the +Rhiz-positive control. (B) Nitrogenase activity was measured by using an acetylene reduction assay (32, 33). Five replicates per treatment per time point were assayed, and average nanomolar ethylene produced per minute per plant was calculated for each treatment group. At 3, 4, and 5 weeks after inoculation, nitrogenase activity was significantly reduced in all treatment groups (as indicated by *) compared with the +Rhiz-positive control. At 6 weeks after inoculation, nitrogenase activity of bisphenol A and pentachlorophenol treatment groups was significantly reduced (as indicated by #) compared with the +Rhiz-positive control. All results shown are mean values ± SEM for a minimum of three independent experiments. A one-way ANOVA with a Bonferroni correction for multiple tests was used to compare parameters of treatment groups (significant if *P* < 0.05).
activity of the five replicate populations from each treatment group was measured at 3, 4, 5, and 6 weeks after inoculation using an acetylene reduction assay, as described (32, 33). Briefly, nitrogenase activity is defined as the rate at which acetylene is converted to ethylene, acetylene reduction, as measured by real-time gas chromatography. Nitrogenase activity was significantly reduced in all chemical treatment groups compared with the +Rhiz-positive control at 3, 4, and 5 weeks after inoculation (Fig. 1B). At week 6, the pentachlorophenol and bisphenol A treatment groups still exhibited significantly reduced nitrogenase activity compared with the +Rhiz group. At all time points measured, the –Rhiz uninoculated control group exhibited essentially no nitrogenase activity.

In the absence of synthetic N fertilizer, legumes rely on SNF for growth. To determine the effects of chemical treatment on overall plant yield, we compared both the number of seeds germinated and dry plant biomass for all treatment groups at 2, 4, and 6 weeks after inoculation. Plant yield, as determined by dry weight of roots and shoots from all plants in a treatment group, was significantly reduced for the pentachlorophenol treatment group compared with the +Rhiz-positive control group beginning at week 2 (Fig. 2A). At 4 weeks after inoculation, all chemical treatment groups exhibited significantly lower plant yields compared with +Rhiz-positive control. At 6 weeks after inoculation, plant yields for all chemical treatment groups, except chrysin, remained significantly lower than the +Rhiz-positive control group (Fig. 2A). Treatment with synthetic environmental chemicals resulted in significantly reduced overall plant yields, as calculated by per seedling plant biomass (Table 1). The marked reduction in plant yield seen in the chemical treatment groups was not attributable to seed toxicity or adverse affects on germination, for all but one chemical tested (Table 1). Only pentachlorophenol treatment (both +Rhiz and –Rhiz) significantly reduced germination of alfalfa seeds, resulting in fewer plants compared with all other groups, an effect shared with the –Rhiz-uninoculated control group (Fig. 2B and Table 1).

Pentachlorophenol effectively negated the benefits of SNF for plant biomass production over the 6-week growth period (Fig. 2B and Table 1), as seen when comparing S. meliloti inoculated vs. uninoculated pentachlorophenol treatment groups. With the exception of pentachlorophenol, none of the chemicals tested was toxic to alfalfa or S. meliloti (31), yet each significantly inhibited nodule formation and nitrogenase activity. Although chrysintreated plants recovered nitrogenase activity comparable to +Rhiz-positive control plants by 6 weeks posttreatment, the synthetic pesticides and pollutants significantly inhibited nitrogenase activity throughout the course of the experiments. The greater persistence of inhibition by the synthetic chemicals may be because of S. meliloti bacteria more readily metabolizing the natural phytochemical chrysin than synthetic chlorinated chemicals (34).

At each time point measured, all parameters of SNF efficiency, including number of nodules, nitrogenase activity, and plant yields, were consistently highest for the +Rhiz-positive control group and lowest for the –Rhiz uninoculated negative control group. SNF efficiency of chemical treatment groups ranged from very low, the pentachlorophenol group was statistically equivalent to the –Rhiz uninoculated negative control, to midrange for the methyl parathion, DDT, and bisphenol A treatment groups, to nearly equivalent to +Rhiz-positive control levels for chrysin treatment group by week six. Synthetic chemicals, including methyl parathion, DDT, bisphenol A, and pentachlorophenol, which are present in the soil environment (Table 2), significantly inhibited in vivo establishment of symbiosis between S. meliloti and alfalfa, reduced SNF, and negatively impacted N fixation and plant biomass production.

Conclusions

The results of this study demonstrate that one of the environmental impacts of pesticides and contaminants in the soil environment is disruption of chemical signaling between host plants and N-fixing Rhiz necessary for efficient SNF and optimal plant yield. Organochlorine pesticides and other environmental chemicals are applied at high rates to agricultural soil and can enter the soil environment as components of cattle feedlot...
proposed that these pesticides are inhibiting symbiosis by and SNF to a similar or stronger degree than chrysin, which demonstrates that synthetic chemicals inhibit nodulation actions (30, 31, 40). Given the ligand, both hallmarks of competitive receptor–ligand interactions (30, 31, 40). We showed that the exception of pentachlorophenol, chemicals did not inhibit seed germination and showed no significant reduction in number of seedlings as compared with +Rhiz-positive control. Number of seedlings was significantly reduced for both inoculated (+Rhiz) and uninoculated (−Rhiz) pentachlorophenol treatment groups and −Rhiz uninoculated negative control compared with the +Rhiz-positive control. At dosage tested, chemicals did not adversely affect bacterial growth (31).

Because most commercial crops are treated with a mixture of pesticides multiple times during each growing season, and the average time until alfalfa harvest ranges from 28–35 days to obtain most protein per acre (42, 45), we propose that the significant degree and duration of pesticide-induced SNF inhibition demonstrated in this study is ecologically relevant and translates to an estimated one-third loss of plant yield per growing season according to our model (Fig. 3). Sustainable agricultural practices may be negatively impacted by pesticides, which, when applied to legume crops, disrupt SNF, decrease plant yield, and render legume crop rotations less effective for maintaining soil fertility. Pentachlorophenol, which is used as a pesticide, herbicide, antifungal agent, and wood preservative, is among the most ubiquitous chlorinated compounds found con-
Materials and Methods

Chemicals and Reagents. The following chemicals were purchased: methyl parathion and pentachlorophenol (>99% pure) from AccuStandard (New Haven, CT), DDT (99% pure) from Aldrich (Milwaukee, WI), bisphenol A (98% pure) from Sigma (St. Louis, MO), and chrysิน (>99% pure) from INDOLINE (Belle Mead, NJ). All chemicals were obtained neat and dissolved in DMSO. *S. meliloti* strain 1021 (46) was kindly provided by S. R. Long (Stanford University, Palo Alto, CA).

Inoculation, Treatment, and Growth of Plants. Alfalfa (*Medicago sativa*) var. Iroquois seeds were surface-sterilized and inoculated with *S. meliloti* strain 1021 (46). Five replicate populations were prepared for each time point tested (2, 4, and 6 weeks after inoculation) for each treatment group. Each replicate contained 1.0 g of surface-sterilized alfalfa (*M. sativa*) var. Iroquois seeds (~200 seeds by weight) suspended in either 25 ml of sterile water (−Rhiz, uninoculated negative control and chemical treatment-only groups) or dilute bacterial inoculate (all other treatment groups) prepared from overnight cultures of *S. meliloti* strain 1021 (46) that had been centrifuged and resuspended to a dilution of 10⁶ cells/ml in sterile water. Sets of uninoculated and inoculated seedlings then received a one-time treatment with either no chemicals (+Rhiz, positive control) or chrysิน, methyl parathion, DDT, bisphenol A, or pentachlorophenol at 5 × 10⁻⁵ M concentration. Plants were incubated at 21°C in glass containers (240-ml volume) containing 20 g of sterilized vermiculite, and soil moisture content was maintained at 60% of water-holding capacity using sterile water. Plants were exposed to a 12-h photoperiod with cool white fluorescent lights at 110.4 μmol m⁻²sec⁻¹ and watered with Jensen liquid media (25, 47). Five replicates from each treatment group were harvested at 2, 4, and 6 weeks after inoculation, and the number of plants, number of nodules, and dry biomass were determined for each replicate at each time point.

Acetylene Reduction Measurements. At 2, 3, 4, 5, and 6 weeks after inoculation, nitrogenase activities were determined for five replicate containers from each treatment group by using an acetylene reduction method as described (32, 33). Briefly, containers were sealed, and 10% of the headspace volume was replaced with atomic absorption-grade acetylene. Ethylene concentration in jar headspace was analyzed immediately after adding acetylene and subsequently at regular intervals for 4 h, by using a Shimadzu GC-14A gas chromatograph set up for direct headspace sampling (48) and equipped with a Valco E60 gas sampling valve, a 2-m stainless-steel column packed with Porapak N resin (Supelco, Bellefonte, PA), and a flame ionization detector. Rates of acetylene reduction were determined by monitoring the increase in the concentration of the reduction product, ethylene, in the headspace over time. Peak areas were quantified by using Shimadzu Class VP software. For statistical analysis, rates were averaged for five replicates per treatment per time point.

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