

Proving Allelopathy in Crop–Weed Interactions

Stephen O. Duke*

Nomenclature: Allelochemical; allelopathy; bioassay; competition; interference; soil effects.

This article is written to provide some directions on how to determine if a plant species might influence its neighboring plants by allelopathy. There is insufficient space for detailed instructions for individual experiments, so I will provide references for different general approaches and opinions on common pitfalls of those who have worked in this research area.

For the purpose of this review, I define *allelopathy* as the adverse effects of a plant on other plants by means of chemicals (allelochemicals) that it produces. To some, the definition of allelopathy is much broader, encompassing positive effects of plants on other plants by chemistry, as well as interactions with nonplant organisms. My more narrow definition is how the term allelopathy is interpreted most of the time, especially in weed/crop interactions. Allelopathy can be a component of plant/plant interference, the other component being competition that is covered in another article in this issue of *Weed Science* (Swanton et al. 2015). If allelopathy occurs, separating it from competition is usually challenging. There are many caveats for much of what I cover, in that allelopathy in the field is generally subtle and not easily teased out from competition, if it occurs at all. In contrast to herbicides, allelochemicals are generally weak phytotoxins that exert their effects at low, but constant or increasing concentrations over long periods.

Most articles that claim to demonstrate allelopathy do not prove that it occurs. They only demonstrate that a crude extract of a plant species suspected to be allelopathic, or one or more compounds from such a plant, are phytotoxic in unrealistic bioassays that maximize the effects of the phytotoxin. This can be a first step in the proof of allelopathy, but all plants produce compounds that are weakly phytotoxic in simple bioassays conducted in the absence of soil. Such “grind and squirt” approaches usually prove nothing with regard to allelopathy. One objective of this review is to give

the researcher a clearer view of what is required to implicate allelopathy in playing a role in plant/plant interference.

Many “allelopathy” articles are not really articles to prove allelopathy, but are efforts to discover phytotoxins that might have utility as herbicides or herbicide leads. Thus, proof of involvement of the discovered compound(s) in allelopathy is not provided, even though the paper may draw unfounded conclusions about the role of the compound in plant/plant interactions. Whether a compound is involved in allelopathy is dependent on many things, including the level of phytotoxicity in soil and the amount produced by the “allelopathic” plant (Hiradate 2006; Hiradate et al. 2010).

Fundamentally, to prove allelopathy, one must (1) identify one or more phytotoxins produced by the putative allelopathic plant or identify a compound(s) produced by the plant that is converted to a phytotoxin in the soil after leaving the producing plant, and (2) determine whether the compound(s) are found in sufficient quantity (in time and/or space) in the soil in which the plant grows or has grown to affect other plant species in that soil adversely. This can be reworded to exclude soil for aquatic plants, but the concepts are the same. Conceptually, this all seems simple, but few studies have done this in a credible fashion. Step two is complicated by potential interactions (additive, antagonistic, or synergistic) with other compounds in the soil, growth stage and physiological status of the receiving plant, soil microbes (especially those of the rhizosphere), soil moisture, temperature, etc. Finally, competition can influence allelopathy and vice versa, often making separation of the two processes extremely challenging. Thus, an absolute proof of the involvement of a particular compound in allelopathy is very difficult in cases of weak phytotoxins.

A potential complication is that the plant making the putative allelochemical (the donor plant) may only make sufficient amounts of allelochemicals for an allelopathic effect when in the presence of a targeted plant species (receiving plants). This is similar to the case of induction of phytoalexin

DOI: 10.1614/WS-D-13-00130.1

*Natural Product Utilization Research Unit, Agricultural Research Service, United States Department of Agriculture, University, MS 38677. E-mail: Stephen.Duke@ars.usda.gov

production in the presence of plant pathogens. Few studies have looked for enhanced production of putative allelochemicals by competing plant species or extracts from such species. Dayan (2006) found that the production of sorgoleone in grain sorghum [*Sorghum bicolor* (L.) Moench. Ssp. Bicolor] roots was not significantly affected by abiotic stresses, but was stimulated by a crude extract of velvetleaf (*Abutilon theophrasti* Medik). Similarly, Kong et al. (2004) reported that an allelopathic rice (*Oryza sativa* L.) variety exuded significantly more phytotoxins from its roots when grown with the rice weed barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) than when growing in monoculture. If this type of phenomenon is common, studies without the presence of chemicals from a receiving plant species may underestimate the amount of putative allelochemicals produced by the donor plant when in the presence of the receiving plant.

An alternative and more direct approach that has become available via molecular biology is to knock out the function of a gene needed for production of the putative allelochemical and then determine if there is less interference of the altered plant with other plant species. At this time, there is only one article of which I am aware in which this approach has been described (Xu et al. 2012). In this study, biosynthesis of the diterpene phytotoxin momilactone B of an allelopathic rice cultivar was blocked by a knockout mutation of a gene involved in its production. Plants with this alteration were not allelopathic. Interpretation of results from such studies must be made with the caveat that there could be pleiotropic effects of knocking out the gene that could alter the competitive capabilities of the putative allelopathic plant. Thus, the knockout gene should be as close to the synthesis of the putative allelochemical as possible. This article will not cover such approaches because of the lack of any further literature on the topic. However, I expect that this more direct approach will provide clearer proof of allelopathy in the future than the more indirect methods that I describe below.

There are previous reviews of various methods involved in allelopathy that will be mentioned in this short article. The seminal text on allelopathy by Rice (1984) contains considerable information on methods. I particularly recommend the recent book written by Udo Blum (2011), which details approaches and pitfalls of allelopathy research, with particular emphasis on phenolic acids as putative allelochemicals. In this short review, only general approaches to allelopathy research can be provided

that point the reader toward literature that contains more details than there is room for here. I would also like to point out that more rigorous methods need to be developed for proving allelopathy, especially for plant species that produce weakly phototoxic allelochemicals.

Clues to the Involvement of Allelopathy in Plant/Plant Interference

Several types of clues can indicate that a species is allelopathic. Plant species that are particularly aggressive in their interactions with other species may be allelopathic. Allelopathy has been invoked frequently to explain the success of invasive species. For example, spotted knapweed (*Centaurea stoebe* L., formerly *Centaurea maculosa* L.) often eliminates most native plant species where it invades (DiTomaso 2000). Allelopathy has been implicated as a component of its interference (Ridenour and Callaway 2001), although the identity of the allelochemical(s) involved remains unclear (Duke et al. 2009). The “novel weapons hypothesis” suggests that invasive plant species are more likely to be allelopathic to native vegetation because native vegetation has not evolved resistance to unique allelochemicals produced by the invader that are not found in the flora of the invaded area (Inderjit et al. 2006).

Sparse or no vegetation patterning around a particular species can indicate that it is allelopathic. An example of this is the long-observed difficulty in growing most plant species around black walnut (*Juglans nigra* L.) (Stickney and Hoy 1881), which led to the discovery of the allelochemical juglone (Davis 1928). The discovery of the phytotoxic effects of the phytochemical triketones was apparently due to the observation of vegetation patterning around the red bottlebrush plant (*Callistemon citrinus* (Curtis) Skeels) (Knudsen et al. 2000). However, other factors, such as competition for water in a dry environment, can be involved in vegetation patterning around species that are highly competitive for water. Mineral depletion by a species might also be detrimental to other species that are in close proximity.

Poor growth of a crop after other species or the same species has grown on that field is often attributed to buildup of allelochemicals in the soil. This occurs, but is sometimes hard to prove, as there may also be an accumulation of plant pathogens in the soil, soil nutrient depletion, or other effects on the soil unrelated to allelochemicals, especially if the same crop is grown year after year.

Knowledge that a plant species produces one or more potent cytotoxins can be a clue obtained from the phytochemical literature that the species might produce an allelochemical. For example, the fact that all species of *Sorghum* so far tested produce sorgoleone, a phytotoxic compound implicated in allelopathy of *Sorghum* spp. (Dayan et al. 2010), would suggest that any species of *Sorghum* might be allelopathic because of sorgoleone production. The finding of highly biologically active compounds used in medicine, such as artemisinin in annual wormwood (*Artemisia annua* L.) (Klayman 1985), can suggest that the compound might be a phytotoxin. Duke et al. (1987) found that artemisinin was highly phytotoxic, and this led to later studies that supported the view that annual wormwood is allelopathic, at least in part because of production of artemisinin (summarized by Jessing et al. 2014).

Preliminary Studies That Can Indicate Allelopathy

Armed with such clues, one can do preliminary studies that can support or refute the hypothesis that the suspected species is allelopathic. Ideally, such preliminary studies should eliminate or minimize competition from the interaction between species. In such studies the putative allelopathic species is termed the donor plant and the species that is affected is termed the receiver plant. Such studies include:

1. Growing the receiver plant with plant tissue residues (living material, dried biomass, or leaf litter) of the donor plant is commonly used to determine if a donor plant might produce allelochemicals (e.g., Batish et al. 2009). This approach should be used with caution, as it is usually very different from what occurs in the field. It is more appropriate for perennial donor plants for which significant litter accumulates. This experiment can be conducted with and without activated charcoal in the soil. Activated charcoal strongly binds many secondary products. So, a reduced inhibitory effect with a soil amendment of activated charcoal can be interpreted as support for the presence of an allelochemical (e.g., Bertin et al. 2009; Nilsson 1994; Ridenour and Callaway 2001; Swain et al. 2012). Such results are not conclusive, as not all charcoals give the same results (Keech et al. 2005), and activated charcoals can have influences other

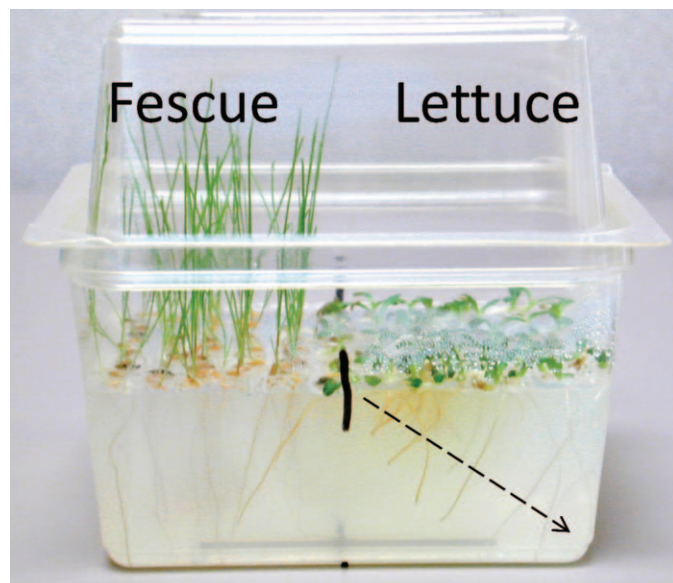


Figure 1. A highly allelopathic Chewing's fescue cultivar (Bertin et al. 2007, 2009) and lettuce growing side by side in agar.

those resulting from binding secondary compounds (Lau et al. 2008; Wurst et al. 2010).

2. A simple indicator of allelopathy is to grow the donor and receiver plants on agar or phytozel, side by side, so potential allelochemicals from roots can diffuse between species. When a strong allelochemical, such as *m*-tyrosine from a highly allelopathic red fescue (*Festuca rubra* L.) cultivar (Bertin et al. 2007, 2009), is produced, a gradient effect on root growth of the receiver plant can be observed, indicating diffusion of an allelochemical (e.g., Figure 1). A variation on this is to grow the plants in phytozel or agar with the shoots separated by an opaque separator (e.g., Wu et al. 2000) and/or the roots separated by a semipermeable membrane (e.g., Fujii et al. 2007).
3. Another approach is growing the receiver plant with the donor plant, with or without roots separated by a semipermeable membrane, such as a dialysis membrane (e.g., Hilt et al. 2012) or vinyl acetate fiber or teflon mesh (Fujii et al. 2007), that allows movement of molecules between donor and receiver plant, but does not allow root/root contact (Figure 2). For comparison, the roots of the two species can be also separated by a nonpermeable barrier in order to eliminate competition for the same nutrients and movement of allelochemicals between species (e.g., Nilsson 1994). This experiment can be done with and without an opaque shield to eliminate competition for light as long as the shield attenuates light from the growth chamber

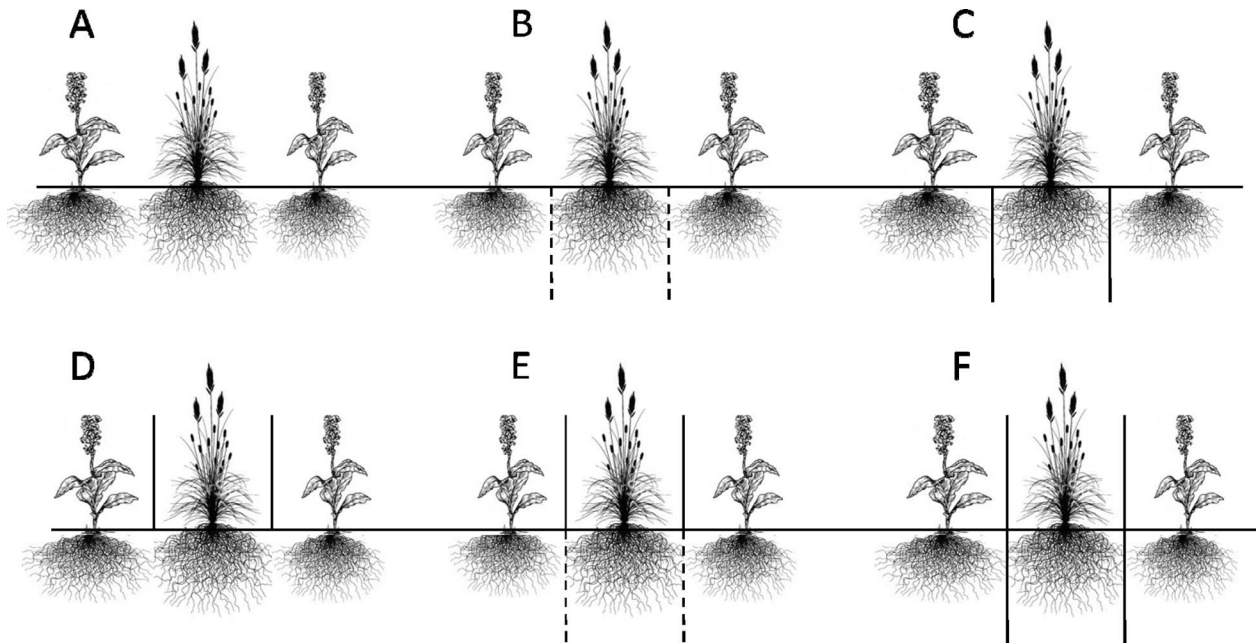


Figure 2. (A) Growth of donor (monocot) and receiver (dicot) species under conditions in which competition for all resources and allelopathy can occur. (B) Growth with semipermeable material separates roots, but allows allelochemicals to move freely (no root competition, but allelopathy can occur). (C) Growth with roots separated by a nonpermeable material so that roots do not compete and no chemicals move between soils of the two species. (A) to (C) Competition for light occurs. (D) to (F) Shoots are separated by opaque material. No competition for light occurs.

lighting or sun equally for both species. Lastly, activated charcoal can be mixed with the soil in some treatments to absorb allelochemicals for comparison without activated charcoal.

4. Comparison of the growth of the receiver plant in soil in which the donor plant has been grown with a control of the same soil that has not had the donor plant grown in it can provide an indication of whether allelochemicals are present without complications from competition. Such an experiment is shown in Figure 3. In this

experiment, growth of barnyardgrass planted in soil in which the allelopathic variety of rice (PI 31277 of Mattice et al. 1998) was grown is compared with its growth in soil in which a nonallelopathic, commercial variety (cv. Lemont) grew (Rimando and Duke 2003). A caveat is that the soil may also contain pathogens from the donor species. This possibility can be reduced by sterilization of the soil by autoclaving, but autoclaving may result in breakdown of some allelochemicals. Also, the planting density of the

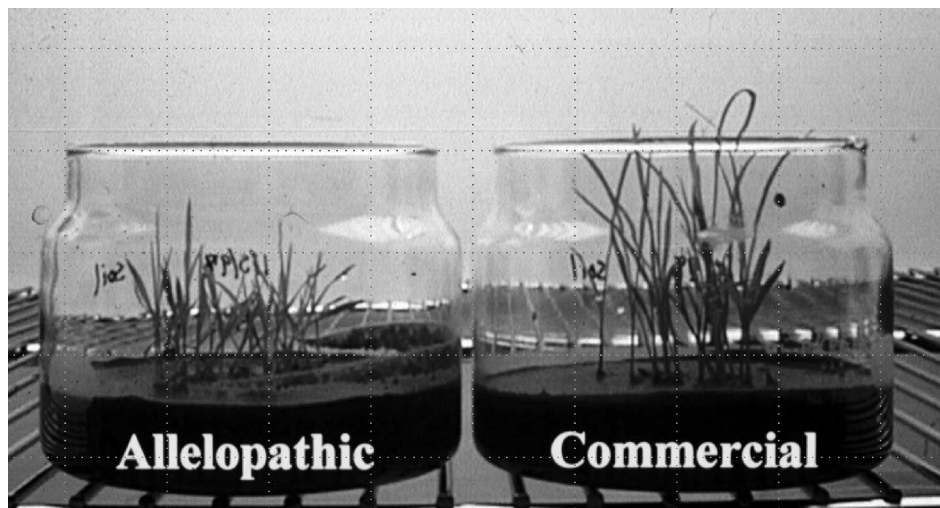


Figure 3. Growth of barnyardgrass in soil in which the allelopathic variety of rice (PI 31277 of Mattice et al. 1998) and a nonallelopathic, commercial variety (cv. Lamont) have grown is compared (Rimando and Duke 2003).

receiver plant species can greatly influence the result, with any effect of an allelochemical diminished as the planting density goes up, because there is less phytotoxin per plant. This phenomenon occurs with both soil-applied herbicides (e.g., Hoffman and Lavy 1978) and allelochemicals (e.g., Weidenhamer et al. 1989), so that the effect of the phytotoxin compared to resource competition can be irrelevant at high receiving plant densities (Thijs et al. 1994). This type of experiment can be done with and without activated charcoal as an amendment to the soil.

5. Growth of the receiver plant in leachate from containers of growing donor plants (e.g., Hagan et al. 2013) can provide evidence of allelochemicals. In some cases, leaves or other components of the donor plant shoot are soaked in water, and the receiver plant is watered with the resulting leachates (e.g., Chou and Muller 1972). Leaf litter leachate can also be used (e.g., Dietz et al. 1996).

An option is to have the pots with the donor plants drain to pots of the receiver plants that are at a lower level. With this method, both leachate and root exudates reach the receiver plants. This method may underestimate allelopathy if the allelochemical(s) is not very water soluble, reducing its leaching. The growth stage of the receiver plants can be seeds, seedlings, or more mature plants.

6. Simply growing the receiving plant in pots in the presence of different numbers of donor plants can indicate allelopathy if the receiver plant is grossly adversely affected more than one would expect from competition (e.g., Dayan et al. 2009). For comparison, different densities of the receiving species can be sown. But, as discussed for soil containing a phytotoxin, the effect of the phytotoxin will decrease with increasing receiver plant density if the donor plant density is kept constant. In this arrangement, competition might play a larger role as the effects of allelopathy decreases. If the receiving plant growth were to increase at any density greater than another, the best explanation would be loss of an allelopathic effect because of dilution of the phytotoxin (Weidenhamer 2006). Conversely, if the receiving plant density is a constant, the effects of both allelopathy and competition should both increase with increasing donor plant density. All of this is predicated in the assumption that chemical signals and/or competition from the receiving plant species do not influence allelochemical production

of the donor species (see discussion above). The results of this type of experiment can also be complicated by hormesis (stimulatory effect of a subtoxic dose of a toxin) effects at low densities of the donor species (Belz et al. 2005). Sampling time can also influence the results, so, ideally, several sampling times should be designed into the experiment. Doing this experiment with and without activated charcoal in the soil can provide further support for an allelopathy hypothesis.

Finding the Allelochemicals

For allelopathy, there must be allelochemicals, and to prove allelopathy they must be isolated and identified.

Pitfall. Finding a phytotoxic compound in plants does not mean that the compound is an allelochemical, even though the allelopathy literature is full of articles equating phytotoxicity of a compound found in a plant with allelopathy. Finding the phytotoxins in a suspected allelopathic plant species is only the first step in generating data to determine whether or not it might be an allelochemical.

There are two fundamental ways of finding allelochemicals. The first is simply to make extracts and identify known compounds in the extracts and then bioassay these compounds. As mentioned above, all plants produce secondary compounds that are at least moderately phytotoxic (e.g., simple, ubiquitous phenolic acids, such as ferulate and *p*-coumarate), so this approach guarantees that the investigator can report putative allelochemicals. New compounds or any compounds that are missed by the chosen analytical detectors (e.g., chemical ionization [CI], electron ionization [EI], mass spectrometry [MS]) or identification software associated with separation instrumentation (e.g., NIST or Wiley GC-MS databases) will not be found by this approach. Unfortunately, this simple approach has been the method for allelochemical discovery of most investigators. As a result, there are many articles on simple, phenolic acids as allelochemicals, although the fact that these compounds are ubiquitous in plants and have little activity in soil argues against them playing a strong role in allelopathy in most species (see Dayan and Duke 2009 for a discussion of this), unless the concen-

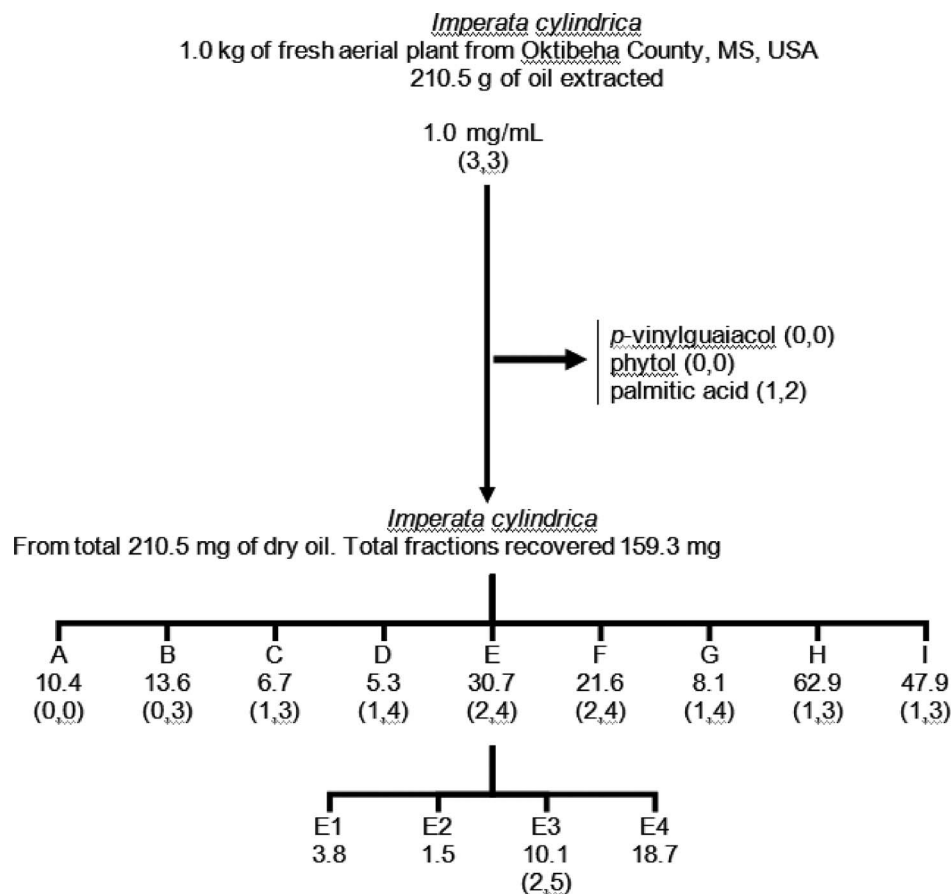


Figure 4. Bioassay guided fractionation of the essential oil of cogongrass, showing the amount (mg) of each fraction recovered and phytotoxicity. Parentheses denote toxicity at 1.0 mg/ ml⁻¹ to (lettuce, agrostis) unless noted otherwise. 0 = no effect; 5 = maximum effect. From Cerdeira et al. (2012).

trations are quite high. At least some truly allelopathic species for which simple phenolic acids have been claimed as the allelochemicals have later been found to have more plausible allelochemicals. For example, simple phenolic compounds and their derivatives and common fatty acids were once claimed as allelochemicals in a very allelopathic rice variety (Mattice et al. 1998), but the much more exotic terpenoid, momilactone B, was later found to be the primary allelochemical (Xu et al. 2012).

A more scientifically sound strategy is to use bioassay-guided isolation of phytotoxins, but this approach generally requires more chemical expertise and instrumentation. With this method, the tissues or exudates of the putative allelopathic plant must first be extracted with the use of the appropriate solvent to provide a crude extract with the highest level of phytotoxic activity.

Once extracted, these extracts are typically successively fractionated with solvents of varying polarity and the fractions bioassayed with the use of a rapid bioassay that does not require much material (see below). Active fractions are then further

fractionated, usually by a different chromatographic method, and the fractions reassayed. A typical procedure may use a combination of liquid/liquid partitioning (e.g., Kupchan partitioning), normal phase column chromatography (e.g., silica gel), reversed-phase column chromatography (e.g., C-18), and/or size-exclusion chromatography (e.g., LH-20) to produce a single phytotoxic constituent. Figure 4 provides an example of bioassay-guided isolation of phytotoxins from cogongrass [*Imperata cylindrica* (L.) Beauv.]. Our lab uses simple, miniaturized bioassays with a monocot and a dicot for bioassaying the usually small amounts of material from the different fractions (described in Dayan et al. 2000 and below). This process is continued until active fractions with almost pure compounds are obtained, at which time the individual active compounds can be identified. Structure elucidation of bioactive compounds typically involves the use of spectrophotometric (light, UV, IR) and spectrometric (MS and nuclear magnetic resonance [NMR]) techniques. Structural elucidation of complex molecules can be complicated and often involves a

combination of high-resolution liquid chromatography–mass spectrometry (LC-MS) techniques and one- and two-dimensional NMR methods, especially in the case of new compounds.

Starting with an aqueous extraction is not recommended, because oxidative enzymes such as polyphenol oxidase (PPO) and peroxidase (PO) are active in aqueous solutions. These enzymes are very promiscuous with substrates and can oxidize many secondary compounds to produce products that are not the same compounds that are released by the live plant or even the dead plant. When the cell is broken during extraction, these enzymes of the plastid (PPO) and cytoplasm (PO) are given access to secondary compounds that are stored mostly in the vacuole, oxidatively producing a witch's brew of compounds that are often observed as brown gunk in the extract, because of polymerization of reactive quinones formed by these enzymes. If one has a reason to start with an aqueous extraction, I recommend including 1 mM diethyldithiocarbamate (DETC) in the extraction solution. DETC is a strong copper chelator that inactivates all PPOs and most POs (Vaughn and Duke 1984).

Pitfall. A common misconception in much of the allelopathy literature is that only water-soluble compounds can be allelopathic, because highly lipophilic compounds would not be present at high enough concentrations in soil water to be active. If this were the case, most soil-applied herbicides would not work, as their water solubility is generally quite low (e.g., the very lipophilic dinitroanilines). Lipophilic compounds bind to soil particles reducing their leaching from the root zone. As the small amount of such compounds dissolved in soil water moves to lipophilic domains, such as membranes of the roots of target species, the compound in soil water is replenished from the reservoir bound to soil particles. Thus, there is mass flow from soil particles, through soil water, to lipophilic domains such as the roots of plant targets. Sorgoleone is a good example of a lipophilic allelochemical that would not be found by only aqueous extraction of the plant (Dayan et al. 2010).

If there is circumstantial evidence of allelopathy, as described earlier, and bioassay-guided isolation of plant tissues does not provide good evidence of sufficiently active and/or sufficiently abundant phy-

totoxins to support a hypothesis of allelopathy, examination of leachate and/or root-exuded compounds might provide better support. Some allelochemicals, such as sorgoleone (Dayan et al. 2010; Weston et al. 2013), *m*-tyrosine (Bertin et al. 2007), and scopoletin (Belz et al. 2005) are exuded by roots, and, as in the case of sorgoleone, may be exuded or secreted into the soil almost as quickly as they are produced. Furthermore, soil microbes can convert less-phytotoxic root exudates to more phytotoxic compounds, making the discovery of the true “allelochemical” even more difficult. An example of this is the formation of the highly phytotoxic 2-aminophenoxazin-3-one from 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(H)-one released from some Gramineae crop species such as barley (Macías et al. 2005).

Pitfall. A common problem with bioassay-guided isolation of bioactive compounds is that the parts do not always equal the whole. In other words, the activity of a mixture cannot be explained in terms of the activity of one or more of the components. This can be caused by loss of additive effects or to loss of a synergy. Degradation of the native compound(s) can also occur during extraction and purification if it is labile or prone to conversion via reactions that can occur with certain solvents, such as methanol. Reconstituting the activity of the extract by combining purified constituents is rarely successful, but is still worth pursuing.

Bioassays of Extracts and Compounds

The bioassay chosen must fit the question being asked and the materials available. As mentioned earlier, bioassays for bioassay-guided isolation of phytotoxins must be simple and quick, requiring minimal amounts of material. We use a microtiter plate-based (24- or 96-well) bioassay with a dicot (lettuce; *Lactuca sativa* L.) and a monocot (creeping bentgrass; *Agrostis stolonifera* L.) species (Dayan et al. 2000). Other small-seeded species with uniform germination would also be suitable for this type of bioassay. We normally place filter paper at the bottom of the plate wells. The initial bioassay on crude mixtures of unidentified compounds is generally conducted at 1 mg/ml. A visual rating of activity can be done, or effects on shoot or root length, and seedling weight can be determined.

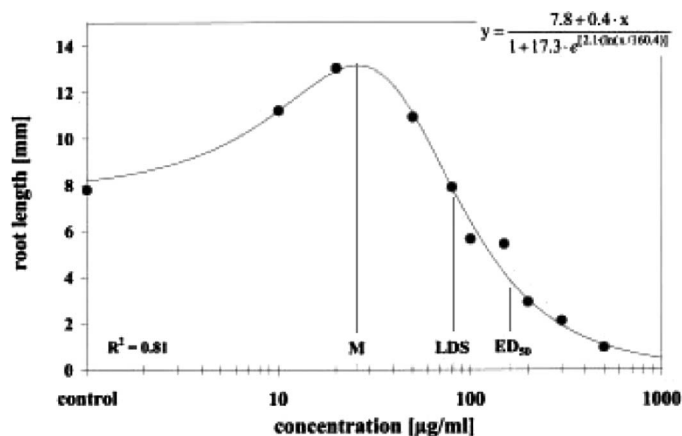


Figure 5. Stimulation of root growth of alfalfa (*Medicago sativa* L.) at low doses of the allelochemical benzoxazolin-2(3H)-one. ED₅₀ = effective dose causing 50% inhibition in root growth; LDS = limited dose for stimulation; M = dose giving maximum response. From Belz et al. (2005).

Once a compound is purified and identified, depending on the amount of the compounds, bioassays are conducted at half-log doses from 1 µM up to 1 or 3.33 mM. Strongly phytotoxic compounds are active at the low end of this dose/response range, whereas weakly phytotoxic compounds should have activity at the highest concentration. A good dose/response curve can allow calculation of the *I*₅₀ or EC₅₀ concentration (concentration required for 50% inhibition of the growth parameter measured). Hormesis (stimulation at subtoxic concentrations) is commonly found with allelochemicals (Belz et al. 2007). The dose range should ideally encompass the concentrations that have no effect to those that fully inhibit growth (e.g., Figure 5). Crude extracts or root exudates with more than one phytotoxic compound, each with its own hormetic range, can complicate dose/response curves (Belz et al. 2008).

The solution used to dissolve the material can be distilled water or a buffer solution. At high concentrations, care should be taken to determine if the effects are caused by pH, rather than a herbicidal effect if the material is in solution in unbuffered water. Likewise, at high concentrations, the osmotic effects of the tested compounds should be determined (e.g., Duke et al. 1983). Lipophilic compounds pose a problem in getting the material in solution. We usually make a stock solution of the test material in acetone, so that a 1% acetone in water solution will give the desired concentration of the compound in the test solution. This amount of acetone in water has no effect on germination or growth of the species that we have used. If the material is so lipophilic that it will not go into

solution by this method, it can be dissolved in an appropriate organic solvent and the proper amount pipetted onto dry filter paper in the well (Dornbos and Spencer 1990). After the filter paper has dried, water is added. The amount on the filter paper must be calculated to give a theoretical concentration as if it were in solution. With this method, the effect increases with the dose, just as the effect of a very lipophilic, soil-applied herbicide increases with the amount in the soil, even though the amount in the soil water is very small. For this dose/response phenomenon to occur, the flux of the phytotoxin from the substrate to the seeds through the water apparently increases as the amount on the substrate increases.

The ratio of seed weight to test solution can influence the results. The higher this ratio, the less phytotoxic the test material will be, apparently because the active compound(s) is diluted by more or larger seeds (Weidenhamer et al. 1987). This is one of the reasons that effects on small-seeded species are generally greater than on large-seeded ones.

Some labs use duckweed (*Lemna* spp.) bioassays to test the phytotoxicity of substances and compounds (e.g., Einhellig et al. 1985, Michel et al. 2004). This bioassay may have more relevance for studying aquatic plant allelopathy. A main advantage of this type of bioassay is that it lends itself to use of very small amounts of compounds or extracts because duckweed is such a small plant, and it is very sensitive to most phytotoxins. Furthermore, the effect of the compound or extract can be tested on whole, mature plants. Duckweed can be grown in microtiter plates or small petri dishes on nutrient solutions (recipe provided in Michel et al. 2004) with or without test materials or compounds. Fresh weight, frond number, and dry weight can be determined after a few days. Image analysis of the frond area can provide a daily determination of growth on the same plants over a period of several days (details in Michel et al. 2004) (Figure 6). Duckweed is more sensitive to most phytotoxins than are terrestrial plant species, so there is a large uncertainty factor in extrapolation of results from a duckweed bioassay to what happens in a terrestrial situation.

Bioassays that include soil are much more likely to indicate whether or not a particular compound is involved in allelopathy. Some compounds that are quite active in soil-free bioassays such as those discussed above are virtually or completely inactive in soil (e.g., Duke et al. 2009; Hiradate et al. 2010). Activity of a compound can also vary considerably between different soil types (Hiradate et al. 2010). The presence of other phytochemicals in the soil can

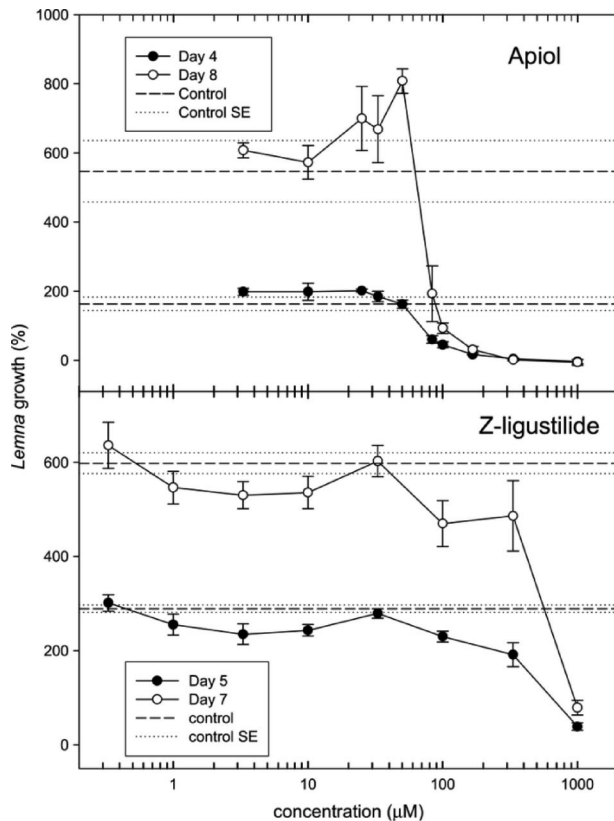


Figure 6. Evaluation of the effects of the putative allelochemicals Z-ligustilide and apiol isolated from California lomatium (*Lomatium californicum* (Nutt.) Mathias & Constance) and Hulthen's licorice root (*Ligusticum hulthenii* Fern.), respectively, on frond growth of *Lemna paucicostata*, as measured by image analysis at 5 and 7 DAT. This nondestructive method allows growth measurements of the same plants over a period of time. (From Meepagala et al. 2005).

increase the bioavailability of the putative allelochemical if they preferentially bind soil component sites that could otherwise bind the phytotoxin (Tharayil et al. 2006).

Simple, miniaturized bioassays can be done in microtiter plates, by wetting a small amount of soil with a solution of test material or treating dry soil with the compound in an organic solvent that is allowed evaporate from the soil before adding seeds and water. An alternative is to mix dry test material with soil before adding seeds and water. An example of such an assay is provided in Figure 7 with more details in Silva et al. (2014).

When allelopathy is not well supported by evidence of the activity of any one compound, some have invoked synergism to argue that mixtures of weak phytotoxins might be allelopathic when working together (e.g., Einhellig et al. 1983). However, synergism must be properly analyzed, and when this is done, antagonism or, at best, additive effects are more common (Duke et al.

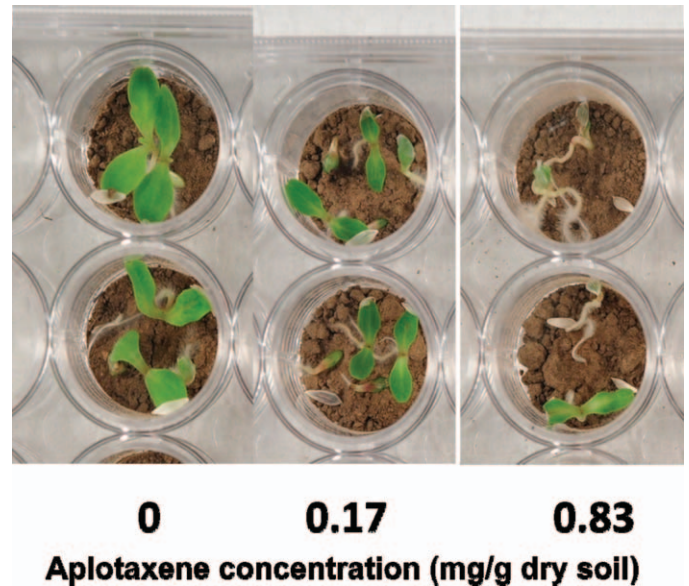


Figure 7. Effects of aplotaxene, a potential allelochemical found in high concentrations in the roots of a number of invasive species, on growth of lettuce in soil. Aplotaxene was added to the soil in acetone, allowed to dry and mixed in, and then seeds and water were added.

1983; Inderjit and Streibig 2002). Soil can influence the relative activity of a mixture of phytotoxins. For example, Tharayil et al. (2006) found that in combination, one compound can make the bioavailability and half-life of others greater in soil, because of cocompetitive sorption and preferential degradation, increasing the persistence of allelochemical mixtures in a soil matrix.

Relating Laboratory and Greenhouse Findings to the Field

Whether a compound acts as an allelochemical in the field depends on how phytotoxic it is to the vegetation that it comes in contact with in soil and how much of it is biologically available to these target plants. These two factors can be used to calculate the total activity of the compounds produced by a donor plant species (Hiradate 2006; Hiradate et al. 2010). This approach can also predict the relative contribution to allelopathy of each of the putative allelochemicals produced by a species. With the use of this simple approach, the molar concentration of the compound in the donor plant is divided by the specific activity of a compound in soil (the EC_{50} molarity) to generate total activity, a parameter with no unit of measure. For example, a compound found at 1 mM in the tissues of the donor species with an EC_{50} of 1 mM in soil would have a total activity of 1. The total

Table 1. Specific activity (EC₅₀), tissue concentration, and total activity in soils for selected compounds from suspected or confirmed allelopathic plants in terms of their candidate allelochemicals (adapted from Hiradate et al. 2010).

Species	Compound	Specific activity in soil (mM)	Tissue concentration (mM)	Total activity
<i>Juglans</i> spp.	Juglone	0.60 VS ^a	2	3.3
		0.29 CS ^a	2	6.8
<i>Leucaena leucocephala</i>	L- mimosine	> 15	20	< 2
<i>Fagopyrum esculentum</i>	(+)-catechin	> 7	0.35	< 0.05
<i>Xanthium occidentale</i>	<i>trans</i> -cinnamate	0.88	0.22	0.25

^a VS = volcanic ash soil, CS = calcareous soil.

activity value in soil will vary with soil type due to differences in the EC₅₀ values in different soils (Belz et al. 2005; Hiradate et al. 2010).

Comparative total activities of compounds provide an estimation of the relative importance of different compounds in the same soil or same plant. Table 1 provides some of these determinations by Hiradate et al. (2010) when comparing different putative allelochemicals from different donor plant species. We recently used this method to estimate the total activity of the abundant but weakly phytotoxic compound aplotaxene from the roots of the suspected allelopathic plumeless thistle (*Carduus acanthoides* L.) to be 3.08 (Silva et al. 2014), a value comparable to the more active compounds in Table 1. When doing bioassay-guided isolation, the relative activities of different phytotoxins can help in deciding which compound(s) are promising enough to determine their structures. A weakly phytotoxic compound that is a major component of the donor plant may be more important in allelopathy than a highly phytotoxic compound that is only present in minute quantities.

For an allelochemical to be available to a terrestrial receiver plant, it must be present in the soil. Determination of how much compound is in the soil, much less its bioavailability, is problematic, as those involved with analysis of pesticides in soil know well. Spiking the soil with known amounts of the allelochemical and determination of the recovery rate ([amount recovered from spiked soil – amount recovered from unspiked soil] ÷ amount used to spike) can help in estimating the actual amount of the allelochemical in the soil. But allelopathy is a more dynamic process than application of a synthetic herbicide to soil. There are fluxes of new allelochemicals from the donor plant over time. Approaches to determination of such fluxes are described by Weidenhamer (2005), Loi et al. (2008), and Mohney et al. (2009). One of these approaches is to use solid-phase sorbents in the soil to sample the allelochemical over time. The other approach by Mohney et al. (2009) used

tubing placed in the soil through which the allelochemical could diffuse, allowing periodic sampling with a solvent passed through the tubing without disturbing the soil. This method proved superior for measuring fluxes of lipophilic allelochemicals in soil and has been used to measure fluxes of the putative allelochemical artemisinin the root zone of annual wormwood (Jessing et al. 2013).

Using the Mode of Action of the Putative Allelochemical to Prove Allelopathy

If the mode of action of a putative allelochemical is known, this information can be used to provide a strong case for allelopathy, provided there is a way of monitoring a physiological parameter that is specifically associated with the mode of action of the compound and that this parameter is affected by levels of the phytotoxin that are found in the soil. I am aware of this approach being used only once. Sorgoleone is a potent photosystem II (PSII) inhibitor (Streibig et al. 1999), and PSII inhibition is easily monitored by its effects on variable chlorophyll fluorescence. Dayan et al. (2009) found that a sorghum cultivar that secretes sorgoleone from its roots (Dayan et al. 2010), when grown with redroot pigweed (*Amaranthus retroflexus* L.), caused a reduction in photosynthesis in the redroot pigweed as measured by fluorescence parameters. This approach is limited by our ignorance of the mode of action of most putative allelochemicals. Furthermore, a complication, even when the mode of action is known, is that secondary effects of phytotoxins are often mistaken for primary effects, especially when monitoring general physiological effects, such as photosynthesis (see Chapter 16 of Devine et al. 1993).

A variation on this approach would be to associate specific “omic” (e.g., transcriptome, proteome, metabolome, etc.) profiles in receiver plants exposed to donor plants with effects of a specific allelochemical produced by the donor plant (Duke et al. 2013). Such methods can provide detailed information on

the impacts of stress caused by competition or allelochemicals on plants, but the complexity and amount of information generated with these approaches makes it difficult to find definitive, unequivocal answers to specific questions.

Final Comments

Clearly, proof of allelopathy is usually quite difficult to obtain, especially when one is trying to extrapolate laboratory studies to the field. In the field, biotic (e.g., soil microflora, root exudates of competitors) and abiotic (e.g., temperature fluctuations, water stress) factors that are difficult to duplicate in the laboratory could enhance or reduce allelopathy. Thus, robust proof that allelopathy is not occurring is elusive, just as clear proof of an allelochemical interaction under realistic conditions is also difficult, especially if the effect is weak. New methods, such as gene knockout technologies, may be able to provide more robust evidence of allelopathy in the future.

Literature Cited

Batish DR, Kaur S, Singh HP, Kohli RK (2009) Nature of interference potential of leaf debris of *Ageratum conyzoides*. *Plant Growth Regul* 57:137–144

Belz RG, Cedergreen N, Sorensen H (2008) Hormesis in mixtures—can it be predicted? *Sci Total Environ* 40:77–87

Belz RG, Hurle K, Duke SO (2005) Dose–response—a challenge for allelopathy? *Nonlinearity Biol Toxicol Med* 3:173–211

Belz RG, Velini ED, Duke SO (2007) Dose/response relationships in allelopathy research., Pages 3–29, in Fujii Y, Hiradate S, eds. *Allelopathy: New Concepts and Methodology*. Enfield, NH: Science Publishers

Bertin C, Harmon R, Akaogi M, Weidenhamer JD, Weston LA (2009) Assessment of the phytotoxic potential of *m*-tyrosine in laboratory soil bioassays. *J Chem Ecol* 35:1288–1294

Bertin C, Weston LA, Huang T, Jander G, Owens T, Meinwald J, Schroeder FC (2007) Grass roots chemistry: *meta*-tyrosine, and herbicidal nonprotein amino acid. *Proc Natl Acad Sci U S A* 104:16964–16969

Blum U (2011) *Plant–Plant Allelopathic Interactions. Phenolic Acids, Cover Crops and Weed Emergence*. New York: Springer. 200 p

Cerdeira AL, Cantrell CL, Dayan FE, Byrd JD, Duke SO (2012) Tabanone, a new phytotoxic constituent of cogongrass (*Imperata cylindrica*). *Weed Sci* 60:212–218

Chou CH, Muller CH (1972) Allelopathic mechanisms of *Arctostaphylos glandulosa* var. *zacaensis*. *Am Midl Nat* 88:324–347

Davis EF (1928) The toxic principle of *Juglans nigra* as identified with synthetic juglone and its toxic effects on tomato and alfalfa plants. *Am J Bot* 15:620

Dayan FE (2006) Factors modulating the levels of the allelochemical sorgoleone in *Sorghum bicolor*. *Planta* 224:339–346

Dayan FE, Duke SO (2009) Biological activity of allelochemicals., Pages 361–384, in Osbourn A, Lanzotti V, eds. *Plant-Derived Natural Products—Synthesis, Function and Application*. Dordrecht, The Netherlands: Springer

Dayan FE, Howell J, Weidenhamer JD (2009) Dynamic root exudation of sorgoleone and its in planta mechanism of action. *J Exp Bot* 60:2107–2117

Dayan FE, Rimando AM, Pan Z, Baerson SR, Gimsing A-L, Duke SO (2010) Molecules of interest: sorgoleone. *Phytochemistry* 71:1032–1039

Dayan FE, Romagni JG, Duke SO (2000) Investigating the mode of action of natural phytotoxins. *J Chem Ecol* 26:2079–2094

Devine MD, Duke SO, Fedtke C (1993) *Physiology of Herbicide Action*. Englewood Cliffs, NJ: Prentice Hall. 441 p

Dietz H, Steinlein T, Winterhalter P, Ullman I (1996) Role of allelopathy as a possible factor associated with the rising dominance of *Bunias orientalis* L. (Brassicaceae) in some native plant assemblages. *J Chem Ecol* 22:1797–1811

DiTomaso JM (2000) Invasive weeds in rangelands: species, impacts, and management. *Weed Sci* 48:255–265

Dornbos DL, Spencer GF (1990) Natural products phytotoxicity: a bioassay suitable for small quantities of slightly water-soluble compounds. *J Chem Ecol* 16:339–352

Duke SO, Bajsa J, Pan Z (2013) Omics methods for probing the mode of action of natural and synthetic phytotoxins. *J Chem Ecol* 39:333–347

Duke SO, Blair AC, Dayan FE, Johnson RD, Meepagala KM, Cook D, Bajsa J (2009) Is (–)-catechin a novel weapon of spotted knapweed (*Centaurea stoebe*)? *J Chem Ecol* 35:141–153

Duke SO, Vaughn KC, Croom EM, ElSohly HN (1987) Artemisinin, constituent of annual wormwood (*Artemisia annua*), is a selective phytotoxin. *Weed Sci* 35:499–505

Duke SO, Williams RD, Markhart AH (1983) Interaction of moisture stress and three phenolic acids on lettuce seed germination. *Ann Bot* 52:923–926

Einhellig FA, Leather GR, Hobbs LL (1985) Use of *Lemna minor* L. as a bioassay in allelopathy. *J Chem Ecol* 11:65–72

Einhellig FA, Schon MK, Rasmussen JA (1983) Synergistic effects of four cinnamic acid compounds on grain sorghum. *J Plant Growth Reg* 1:251–258

Fujii Y, Pariasca D, Shibuya T, Yasuda T, Kahn B, Waller GR (2007) Plant-box method: a specific bioassay to evaluate allelopathy through root exudates., Pages 39–56, in Fujii Y, Hiradate S, eds. *Allelopathy: New Concepts and Methodology*. Enfield, NH: Science Publishers

Hagan DL, Jose S, Lin C-H (2013) Allelopathic exudates of cogongrass (*Imperata cylindrica*): implications for the performance of native pine savanna plant species in the southeastern US. *J Chem Ecol* 39:312–322

Hilt S, Beutler E, Bauer N (2012) Comparison of methods to detect allelopathic effect of submerged macrophytes on green algae. *J Phycol* 48:40–44

Hiradate S (2006) Isolation strategies for finding bioactive compounds: specific activity vs. total activity. *Am Chem Soc Symp Ser* 927:113–126

Hiradate S, Ohse K, Furubayashi A, Fujii Y (2010) Quantitative evaluation of allelopathic potentials in soils: total activity approach. *Weed Sci* 58:258–264

Hoffman DW, Lavy TL (1978) Plant competition for atrazine. *Weed Sci* 26:94–99

Inderjit, Callaway RM, Vivanco JM (2006) Can plant biochemistry contribute to understanding of invasion ecology?. *Trends Plant Sci* 11:574–580

- Inderjit, Streibig JC (2002) Joint action of phenolic acid mixtures and its significance in allelopathy research. *Physiol Plant* 114:422–428
- Jessing K, Cedergreen N, Mayer P, Libous-Bailey L, Strobel BW, Rimando A, Duke SO (2013) Loss of artemisinin produced by *Artemisia annua* L. to the soil environment. *Ind Crops Prod* 43:132–140
- Jessing K, Duke SO, Cedergreen N (2014) What is the ecological relevance of artemisinin production in *Artemisia annua*. *J Chem Ecol* 40:100–117
- Keech O, Carcaillet C, Nilsson M-C (2005) Adsorption of allelopathic compounds by wood-derived charcoal: the role of wood porosity. *Plant and Soil* 272:291–300
- Klayman DL (1985) Qinghaosu (artemisinin): an antimalarial drug from China. *Science* 228:1049–1055
- Knudsen CG, Lee DL, Michaely WJ, Chin H-L, Nguyen NH, Rusay J, Cromartie TH, Gray R, Lake BH, Fraser M, Cartwright D (2000) Discovery of the triketone class of HPPD-inhibiting herbicides and their relationship to naturally occurring β -triketones., Pages 101–111, in *Proceedings of the Third International Congress on Allelopathy in Ecological and Agriculture Forestry*
- Kong C, Xu X, Zhou B, Hu F, Zhang C, Zhang M (2004) Two compounds from allelopathic rice accession and their inhibitory activity on weeds and fungal pathogens. *Phytochemistry* 65:1123–1128
- Lau JA, Puliafico KP, Kopshever JA, Steltzer H, Jarvis EP, Schwarzländer M, Strauss SY, Hufbauer RA (2008) Inference of allelopathy is complicated by effects of activated charcoal on plant growth. *New Phytol* 178:412–423
- Loi RX, Solar MC, Weidenhamer JD (2008) Solid-phase microextraction method for in vivo measurement of allelochemical uptake. *J Chem Ecol* 34:70–75
- Macías FA, Marín D, Oliveros-Bastidas A, Castellano D, Simonet AM, Molinillo JMG (2005) Structure-activity relationships (SAR) studies of benzoxazinones, their degradation products and analogues, phytotoxicity on standard target species (STS). *J Agric Food Chem* 53:538–548
- Mattice R, Lavy T, Skulman B, Dilday R (1998) Searching for allelochemicals in rice that control duckweed., Pages 81–98, in *Olofsdotter M, ed. Allelopathy in Rice: Proceedings of the Workshop on Allelopathy in Rice, November 25–27, 1996. Manila, The Philippines: International Rice Research Institute*
- Meepagala KM, Sturtz G, Wedge DE, Schrader KK, Duke SO (2005) Phytotoxic and antifungal compounds from two Apiaceae species, *Lomatium californicum* and *Ligusticum hultenii*, rich sources of Z-ligustilide and apiol, respectively. *J Chem Ecol* 31:1567–1578
- Michel A, Johnson RD, Duke SO, Scheffler BE (2004) Dose-response relationships between herbicides with different modes of action and growth of *Lemna paucicostata*—an improved ecotoxicological method. *Environ Toxicol Chem* 23:1074–1079
- Mohney BK, Matz T, La Moreaux J, Wilcox DS, Gimsing AL, Mayer P, Weidenhamer JD (2009) *In situ* silicone tube microextraction: a new method for undisturbed sampling of root-exuded thiophenes from marigold (*Tagetes erecta* L.) in soil. *J Chem Ecol* 35:1279–1287
- Nilsson MC (1994) Separation of allelopathy and resource competition by the boreal dwarf shrub *Empetrum hermaphroditum* Hagerup. *Oecologia* 98:1–7
- Rice EL (1984) *Allelopathy*. 2nd ed. Orlando, FL: Academic Press. 422 p
- Ridenour WM, Callaway RM (2001) The relative importance of allelopathy in interference: the effects of an invasive weed on a native bunchgrass. *Oecologia* 126:444–450
- Rimando AM, Duke SO (2003) Studies on rice allelochemicals., Pages 221–244, in *Smith CW, Dilday RH, eds. Rice: Origin, History, Technology, and Production. Hoboken, NJ: Wiley*
- Silva FML, Donega MA, Cerdeira AL, Cantrell CL, Shea K, Duke SO (2014) Roots of the invasive species *Carduus nutans* L. and *C. acanthoides* L. produce the phytotoxin aplotaxene, a possible allelochemical. *J Chem Ecol* 40:276–284
- Stickney JA, Hoy PR (1881) Toxic action of black walnut. *Trans Wis State Hort Soc* 11:166–167
- Streibig JD, Dayan FE, Rimando AM, Duke SO (1999) Joint action of natural and synthetic photosystem II inhibitors. *Pestic Sci* 55:137–146
- Swain D, Paroha S, Singh M, Subudhi HN (2012) Evaluations of allelopathic effect of *Echinochloa colona* weed on rice (*Oryza sativa* L. ‘Vandana’). *J Environ Biol* 33:881–889
- Swanton CJ, Nkoa R, Blackshaw RE (2015) Experimental methods for crop-weed competition studies. *Weed Sci* 63:2–11
- Tharayil N, Bhowmik PC, Xing B (2006) Preferential sorption of phenolic phytotoxins to soil: implications for altering the availability of allelochemicals. *J Agric Food Chem* 54:3033–3040
- Thijs H, Shann JR, Weidenhamer JD (1994) The effect of phytotoxins on competitive outcome in a model system. *Ecology* 75:1959–1964
- Vaughn KC, Duke SO (1984) Function of polyphenol oxidase in higher plants. *Physiol Plant* 60:106–112
- Weidenhamer JD (2005) Biomimetic measurement of allelochemical dynamics in the rhizosphere. *J Chem Ecol* 31:221–236
- Weidenhamer JD (2006) Distinguishing allelopathy from resource competition: the role of density., Pages 85–103, in *Reigosa MJ, Pedrol N, González L, eds. Allelopathy: A Physiological Process with Ecological Implications. Dordrecht, The Netherlands: Springer*
- Weidenhamer JD, Hartnett DC, Romeo JT (1989) Density-dependent phytotoxicity: distinguishing resource competition and allelopathic interference in plants. *J Appl Ecol* 26:613–614
- Weidenhamer JD, Morton TC, Romeo JT (1987) Solution volume and seed number: Often overlooked factors in allelopathic bioassays. *J Chem Ecol* 13:1481–1491
- Weston LA, Alsaadawi IS, Baerson SR (2013) Sorghum allelopathy—from ecosystem to molecule. *J Chem Ecol* 39:142–153
- Wu H, Pratley J, Lemerle D, Haig T (2000) Laboratory screening for allelopathic potential of wheat (*Triticum aestivum*) accessions against annual ryegrass (*Lolium rigidum*). *Aust J Agric Res* 55:259–266
- Wurst S, Vender V, Rillig MC (2010) Testing for allelopathic effects in plant competition: does activated carbon disrupt plant symbioses? *Plant Ecol* 211:19–26
- Xu M, Galhano R, Wiemann P, Bueno E, Tiernan M, Wu W, Chung I-M, Gershenzon J, Tudzynski B, Sesma A, Peters RJ (2012) Genetic evidence for natural product-mediated plant-plant allelopathy in rice (*Oryza sativa*). *New Phytol* 193:570–575

Received September 5, 2013, and approved February 24, 2014.