

Chapter 2

Introduction to the Fundamentals of Laboratory Bioassays

Abstract This chapter describes and provides comments on the following basic features of laboratory bioassays: (a) biotic and physicochemical factors, (b) test materials, (c) measurements, hypotheses, experimental designs, and data analyses, and (d) basic information that should be provided by researchers for all bioassays.

2.1 Factors of Bioassay Systems

2.1.1 *Biotic Factors*

2.1.1.1 Bioassay Species

The species to be used in a bioassay really depends on the objective of the study. For example, if one is interested in understanding basic modes of action and processes of plant-plant allelopathic interactions, choosing sensitive species such as lettuce, tomato, clover, or cucumber as model plants may be quite appropriate. However, if the primary objective is to determine if seedlings of species A are stimulated or inhibited by allelopathic compounds released from mature plants of species B (putative promoters or aggressors) then the use of lettuce, tomato, clover, or cucumber seedlings by themselves as bioassay species is not appropriate. In this instance seedlings of species A should be the primary bioassay species being tested. The inclusion of frequently used sensitive species in addition to species A, however, is acceptable and in fact useful since these sensitive species can serve as a baseline for determining the relative sensitivity/resistance of species A and as a means of comparing results from the present bioassay to those of previously published bioassays as well as those that may be published in the future.

2.1.1.2 Stage of Life Cycle for Bioassay Species

The most vulnerable parts of a higher plant life cycle to environmental stressors, including allelopathic compounds individually or imbedded in complex mixtures of organic and inorganic compounds, (e.g., leachates, root exudates, litter, etc.) are likely to be germination, seedling emergence, early seedling development, and plant

reproduction (Blum and Heck 1980; Waters and Blum 1987). However, since plant species adjust (i.e., acclimate) to stresses differently due to genetic variation, stage of development, presence or absence of symbiotic relationships, and past and present environments, broad generalizations about sensitive life cycle stages (i.e., the most or least sensitive) must be viewed with some caution and skepticism (Levitt 1972; Cox and Conran 1996; Kozłowski and Pallardy 2002; Marshall et al. 2005). Thus, the responses of all the appropriate stages of the life cycle of a plant to allelopathic compounds must be studied. However, since growing seedlings to mature or reproductive stages is frequently time consuming and unwieldy under laboratory conditions, most researchers studying allelopathic interactions in the laboratory have chosen to utilize changes in germination or seedling physiology and growth as their main response indicators. In spite of the convenience of using seeds and seedlings in bioassays, it is important to recognize that responses of seeds and seedlings to allelopathic compounds are not necessarily (in fact unlikely to be) representative of (i.e., cannot be extrapolated to) the responses of mature or reproductive stages of plants.

The essential point is that the stage of the life cycle of a species used in a bioassay depends on the experimental objectives. For example, if the primary interest is germination or seedling emergence, then there is no need to study other parts of the life cycle. However, using germination or seedling emergence as an indicator of seedling or older plant responses is not appropriate.

2.1.1.3 Symbiotic Relationships

There are two structures resulting from microbial-plant symbiotic relationships that are of particular interest when it comes to laboratory bioassays, legume nodules and mycorrhizae (Fig. 2.1). Clearly when appropriate, roots should be inoculated with *Rhizobium*, *Bradyrhizobium* or mycorrhizal fungi. The presence of nodules and mycorrhizae will in most instances dramatically change the response of seedlings or older plants to allelopathic compounds (see Sects. 3.6 and 3.7). However, since:

- a. it takes approximately a week or more to develop functioning nodules and mycorrhizae and then an additional 4–9 weeks, depending on species and environment, for the extensive formation of nodules and the colonization of roots by mycorrhizal fungi (see Sect. 3.6),
- b. the target organisms are frequently seeds or very young seedlings, and
- c. most laboratory bioassays studying the effects of identified putative allelopathic (IPA) compounds last no more than a week or so, it could be argued that the inclusion of inoculums for the induction and development of nodules and mycorrhizae is not necessary.

In fact from a reductionist perspective, the inclusion of inoculums for the formation of nodules and mycorrhizae can at times make it much more difficult to sort out cause and effect. From a holistic perspective such inoculums should be included even when full development of the symbiotic relationships will not occur during

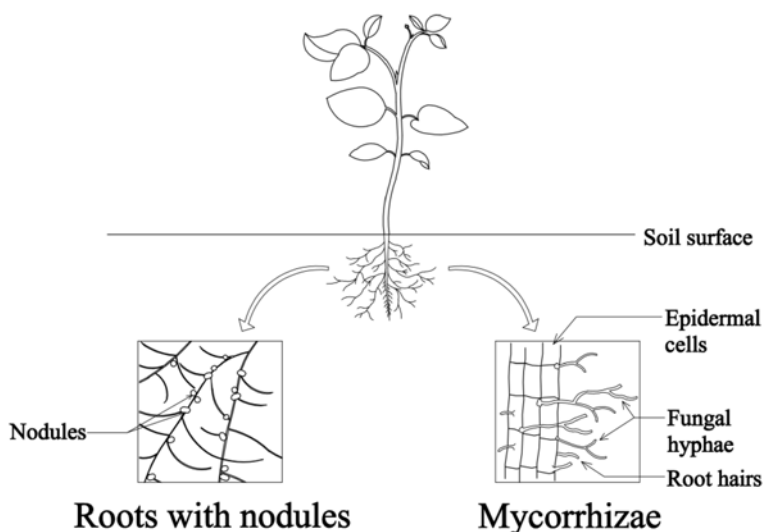


Fig. 2.1 Two symbiotic relationships, nodules and mycorrhizae. (Illustration by Amy Blum Grady, used with permission)

the time of the bioassay. After all there is considerable evidence that the presence of microbes, no matter their source, can and will modify and regulate, sometimes very dramatically, the observed effects of individual IPA compounds or IPA compounds imbedded in complex mixtures of organic and inorganic compounds (see Sect. 3.7). Furthermore, the absence of microorganisms involved in the formation of nodules and mycorrhizae will also prevent the detection of effects of allelopathic compounds on the induction, formation, and development of symbiotic relationships, important relationships that influence and regulate how seedlings or older plants respond to allelopathic compounds (see Sects. 3.6 and 3.7).

2.1.1.4 Treatment Surface Areas

The total or potential treatment surface area within a bioassay system is determined by seed, seedling, or older plant density, root initiation, rates of root growth, and the formation and development of mycorrhizae. The effective treatment surface area of a species is the proportion of the total surface area that actual interacts with allelopathic compounds. The effective surface area within a medium is determined by the distribution of the total surface area in relationship to the distribution of allelopathic compounds. In solution culture the total and effective surface areas are identical. In soil culture total and effective surface areas are not necessarily, in fact unlikely, to be identical.

Seed, seedling, or older plant effective treatment surface areas in bioassays may not be a major concern for systemic allelopathic compounds since the whole organism will be impacted even if only a small proportion of the surface area is contacted

by such compounds. However, one might expect that as the effective treatment surface area is increased, the speed of the response by a seed, seedling, or older plant to allelopathic compounds would increase at least initially.

For non-systemic allelopathic compounds the effects are local and, thus, the proportion of the total surface area in contact with such compounds will determine the ultimate observed effects both in speed and magnitude of response. Since most seeds and their initial radicles have such small surface areas, the proportion of their surface area contacted by allelopathic compounds are unlikely to be a major concern for even non-systemic allelopathic compounds. That, however, is not the case for seedlings and older plants (see Sect. 6.5) since a link between effective treatment surface area and seedling responses has been observed by Lyu and Blum (1990); Klein and Blum (1990); and Lehman et al. (1994). These authors found that for a given treatment concentration, inhibition increased as the proportion of the effective root treatment surface area was increased (see Sects. 5.2.3.1 and 6.5 for details).

Unfortunately most laboratory bioassays of seedlings or older plants ignore the potential variation in contact between allelopathic compounds and surface areas of roots and mycorrhizae. In fact, bioassays are frequently designed so that there is a high probability that the entire surface area of the roots and mycorrhizae are in direct contact with IPA compounds or sources. Data from such bioassays represent a worst-case scenario, a scenario that may or may not be consistent with what has occurred or is occurring in the field. The author suspects that in most instances, particularly for older seedlings and older plants, treating whole root and mycorrhizal systems is at odds with what occurs in the field. See Sects. 5.2.3.1 and 6.5 for ways to deal with this issue in laboratory bioassays.

2.1.1.5 Density

A quick perusal of the early literature will indicate that the densities of seeds and seedlings used in bioassays were extremely variable and frequently arbitrary; not until recently have researchers paid much attention to this factor of bioassays. The density used in a bioassay will, of course, depend on the experimental objectives but every effort should be made to use relevant densities or the artificial nature of the density used should be accounted for in the interpretation of the resulting data. The role of density in seed or seedling bioassays should not be underestimated since density can influence/determine the magnitude of effects of allelopathic compounds, the level of autotoxicity in some species, the total and effective treatment surface area of a bioassay species, and the levels of resource competition (e.g., Wilson and Rice 1968; Weidenhamer et al. 1987, 1989; Lyu and Blum 1990; Sinkkonen 2001, 2003, 2007; Huang et al. 2013; see Sects. 2.1.1.4, 3.2.2.6, and 3.5).

2.1.1.6 Microorganisms

The fact is that the populations of microorganisms present in laboratory bioassay systems will not be representative of the populations of microorganisms found in field systems. In spite of that the roles of microorganisms in laboratory bioassay

systems should not be underestimated (see Sparling and Vaughan 1981; Vaughan et al. 1983; Vaughan and Malcolm 1985; Heisey et al. 1985; Hoagland and Williams 1985; Siqueira et al. 1991; Schmidt 1991; Schmidt and Ley 1999; Barazani and Friedman 1999; Inderjit 2005; Blum 2006; Zhang et al. 2009). Their roles within bioassay systems must be identified, characterized, quantified, and understood, and when possible, controlled or regulated. Unfortunately, researchers have only limited options to control or regulate microbial populations within bioassay systems. The following are the only options:

- a. Eliminate the microbial populations for part or all of the components of a bioassay system by sterilization (Shay and Hale 1973; Prikryl and Vančura 1980; Dalton et al. 1983; Vaughan et al. 1983; Vaughan and Malcolm 1985; Blum et al. 1994, 1999; Pue et al. 1995; Wu et al. 2000a, b; Blum 2004; Kaur and Singh 2007).
- b. Inoculate bioassay systems that have been sterilized with cultures of individual microbial species, subsets of microbial species, or complex mixtures of microbial species such as soil extracts (Vaughan et al. 1983; Kossiak and Bohlool 1984; Vaughan and Malcolm 1985; Pue et al. 1995; Blum 2004; Kaur and Singh 2007; Zhang et al. 2009).
- c. Do nothing and live with whatever microbial populations are present within a bioassay system.
- d. The use of antibiotics, inhibitors, etc. to control or regulate microorganisms are usually not recommended unless their use is a specific part of an experimental design since such substances will not only lead to differential selection, amplification, and suppression of specific microbial species but can also function as promoters, modifiers, or inhibitors of bioassay species. However, the use of such compounds in selective media to enumerate populations of specific types of microorganisms (e.g., actinomyces, fungi, phosphatase-positive bacteria, gram-negative bacteria, phenolic acid utilizing microorganisms, etc.) can be very useful (see Blum and Shafer 1988; Shafer and Blum 1991).

When microorganisms are present in bioassay systems, the disparity between laboratory bioassay systems and field systems becomes even greater when laboratory bioassay systems are treated with IPA compounds, leachates, root exudates, extracts, or litter (see Sect. 3.2.2.8). Such treatments lead to intense modifications of microbial populations by differential selection, amplification, and suppression of specific microbial species (Blum and Shafer 1988; Shafer and Blum 1991; Blum et al. 1993; Pue et al. 1995; Blum 2006, 2011; see Sects. 3.6 and 3.7). The initial and induced differences in the nature and associated functions of microbial populations for laboratory systems are thus a major conundrum that desperately needs resolution.

2.1.2 Physicochemical Factors

2.1.2.1 Containers

There are a whole range of options ranging from Petri dishes to open or closed containers of various sizes constructed from a variety of materials (e.g., glass, plastic,

clay, metal), each with its own benefits and limitations. Ultimately the type and size of container used will be determined by the experimental objectives. The following are some of the factors that should be considered when choosing a container:

- a. the life-form characteristics, age, and density of the species to be used,
- b. root characteristics and their normal distribution with or without their symbiotic relationships,
- c. the desired type of growth medium (e.g., nutrient solution, sand, soil, levels of aeration, nutrition, and moisture retention),
- d. the desired control over environmental factors to be manipulated,
- e. the plant processes of interest (e.g., cellular processes, germination, seedling emergence and growth, nutrient uptake, water utilization, and transpiration),
- f. the duration of the bioassay (e.g., hours, days, weeks, or months),
- g. the actual growth environment (e.g., available space [e.g., container size and shape], aeration [e.g., open or closed container], light bank, growth chamber, or greenhouse),
- h. the potential release of chemical residues from container walls and the affinity of the container walls to the compounds being tested, and
- i. the normal growth environment and perceived requirements of the species in the field.

When the right type and size of container is chosen, day to day management (e.g., frequency of watering and fertilization) and the creation of inappropriate environments (e.g., pot-bound roots and mycorrhizae, or anaerobic media) will be minimized. Clearly if the containers used create a completely unrealistic or unrepresentative environment for the bioassay species, then the resulting data may have limited or little value to field systems recognizing that bioassays, particularly in the laboratory, represent simplified models of real systems and not the real thing (Blum 2007). However, the type and size of container chosen will not only affect the nature of the physicochemical growth environment of seeds, roots, and mycorrhizae but can also determine the dose of allelopathic compounds experienced by them. For example as the volume of a container and the associated medium containing a specific concentration of an IPA compound (mM/liter or $\mu\text{mol/g}$ of media) are increased the total number of molecules of that IPA compound that can potentially interact with seeds, roots, and mycorrhizae at a given density will also increase (see Weidenhamer et al. 1987; Hoffman and Lavy 1978).

2.1.2.2 Media

A variety of media have been used in bioassays. They include solutions (e.g., water, nutrient solutions, extracts, leachates, root exudates, etc.), filter paper, agar, sand, gravel, vermiculite, soil, and other substrates. What is used depends on the experimental objectives. Each has benefits and limitations that must be kept in mind when choosing a particular medium (see sect. 3.2.2.1). The choice of medium, however, should not be underestimated since the choice of media will directly influence the effective treatment concentrations required for observed allelopathic effects on cel-

lular processes, germination, growth, development, and reproduction of bioassay species (also see Sect. 3.2.2).

- a. **Solutions:** Solutions containing IPA compounds, extracts, leachates, and root exudates provide direct and continuous contact with seeds, roots, and mycorrhizae. For solutions, concentrations of both organic and inorganic compounds, pH, water potential, and aeration will change fairly rapidly over time. Changes will likely be due to microbial utilization and synthesis, uptake or losses from seeds, roots, and mycorrhizae, water loss due to transpiration and evaporation, and oxidation/reduction. Unless accounted for, these changes will make it difficult to identify cause and effect since such changes can also directly modify cellular processes, germination, and seedling growth and development of the bioassay species. However if experimental time intervals are short enough, then most of these changes will have only a limited impact. In addition the regulation by external means and inclusion of appropriate references/controls can potentially help to identify and quantify the impact of such changes. For example containers can be sterilized, solutions can be aerated, buffers can be added to slow pH changes, solutions can be filter sterilized, seeds and roots can be surface sterilized, water can be added back to compensate for transpiration and evaporation, chemically inactive osmotic compounds can be added to regulate water potential, and organic and inorganic compounds can be supplemented to minimize concentration changes. Ultimately, however, it may be simpler to include appropriate references/controls (e.g., a range of aeration, pH values, inorganic compounds [e.g., nutrients], and water potentials, etc.) in factorial designs that will allow for the identification of causes and effects or replace solutions completely at given time intervals to maintain a narrow range of solution changes. If done correctly, solution culture can provide an estimate of the potential stimulation (i.e., facilitation) or inhibition (i.e., phytotoxicity) of IPA compounds, extracts, leachates, and root exudates for the experimental conditions of the bioassay. Producing functional nodules and mycorrhizae in solution culture is possible but special conditions are required (Hawkins and George 1997; Mosse and Thompson 1984; Bollman and Vessey 2006; Mortimer et al. 2008).
- b. **Filter paper.** Filter paper is generally included in Petri dishes to provide for an even distribution and contact of treatment or reference/control solutions for seeds or seedling radicles in Petri dishes. Filter paper is generally assumed to be inert and, thus, have minimal effect on the solutions added to Petri dishes. Some adsorption and absorption, however, may be expected. Concerns regarding changes in pH, solute potential, inorganic and organic compounds, etc. for solution cultures apply here as well. Regulating these factors, however, is more cumbersome than for solution cultures.
- c. **Agar:** Very similar to solution culture (see a. and b.), with the exception that agar substrates (pure or with inclusions) tend to have more buffering capacity, provide support for seeds or seedlings, and adsorb and absorb organic and inorganic compounds on or within their matrix. The initial magnitude of sorption and fixation of IPA compounds, extracts, leachates, etc., and the pH and water potential of an agar matrix will vary with agar concentration, the presence of

other inclusions (e.g., inorganic and organic compounds, etc.), and the IPA compounds, leachates, etc. added to the matrix. Concerns regarding changes in pH, solute potential, etc. for solution cultures apply here as well. Regulating these factors, however, is much more cumbersome than for solution cultures. With proper care and conditions functional symbiotic relationships can be obtained in agar medium (Bago et al. 2004; Zhu et al. 2010; Shukla et al. 2012).

- d. Sand, gravel, etc.: These represent another form of solution culture and, thus, have very similar concerns since clean quartz sand and gravel are essentially inert (e.g., minimal adsorption or absorption). They do provide support for seeds and seedlings and aeration is generally much better than solution and agar cultures as long as these systems are open or flow-through systems. However, these media generate a resistance to root and mycorrhizal growth. The smaller the pore sizes, the greater the resistance. In addition, available water will very likely be much more limiting than for solution and agar media. Since such systems can be readily flushed, some of the concerns listed for solution and agar media can be minimized or controlled. Similar observations can be made for vermiculite and perlite which are, however, not inert. The use of peat moss or pine bark is generally not recommended because of their acidic nature and the high content of tannins, phenolic acids, etc. Finally inert materials such as sand and gravel, etc. have an additional advantage over solution cultures. They provide a support structure on and in which plant tissues, litter, and residues can be tested. Functional symbiotic relationships can readily be obtained in sand, gravel, etc. media as long as conditions are appropriate for their formation (Francis and Read 1984; Lynch et al. 1990; Medeiros et al. 1993; Gryndler et al. 2005; Zhu et al. 2010; Pang et al. 2011).
- e. Soil: The concerns associated with solution systems are more extensive and much more difficult to regulate or control in soils. Processes such as sorption, desorption, resorption, utilization by soil organisms (flora and fauna), movement, distribution, and contact of organic and inorganic compounds by seeds, roots and mycorrhizae, as well as water potential, nutrition, and the growth of roots and mycorrhizae are generally much more variable, dynamic, and unpredictable in soil systems than in solution, agar, sand, gravel, etc. systems. The physicochemical and biotic environments of field soils are even more complex in that every field soil sample is very likely to be different from every other field soil sample. (Note: Such difference in soil samples can be a particular concern for soil samples taken at various distances from a plant or row of plants.) To get around this problem soil samples are frequently sieved, mixed, air-dried, etc. (i.e., processed). Homogenizing soil samples by sieving, mixing, and air-drying leads to some soil uniformity for experimental units but this uniformity comes at a cost. The cost, structure, hydration, aeration, compaction, soil organism populations, and organic matter distribution of the resultant soil substrate are all modified in the process. The addition of clean quartz sand (or other inert substances with very low absorptive and adsorptive properties) or materials such as vermiculite and perlite to soil to reduce compaction, increase soil aeration and percolation, nodulation, formation of mycorrhizae, or reduce resistance to

growth of roots and mycorrhizae is another common practice. Homogenizing soils and adding such materials should only be made after carefully considering the experimental implications of such practices since these practices can modify soil processes and dramatically alter the responses of bioassay species to IPA compounds, leachates, root exudates, tissues, litter, and residues. Functional symbiotic relationships can readily be obtained in most soils as long as conditions are appropriate (Fitter et al. 1998; Arias et al. 1999; Vázquez et al. 2001; García et al. 2008; Jalonen et al. 2009; Hasbullah et al. 2011).

2.1.2.3 Environments

The first things that should be determined are the environmental limits (e.g., minimum and maximum) for growth and development of the chosen bioassay species. However if that is not possible or practical, then at least a normal-growth range for the bioassay species should be determined. Environmental conditions of interest within this range should then be used. The investigator could also just choose to use average or representative environmental conditions of the field in which the species is growing. However, since the field environment is constantly changing, it is important to remember that the effects observed for a laboratory bioassay at a given temperature, light intensity, humidity, pH, soil moisture content, etc. represent only a very small slice of the potential range of effects and physicochemical and biotic conditions that occur within the field. But no matter what environmental conditions are chosen, the actual environmental conditions of a laboratory bioassay must be clearly characterized or described.

Standard dose-response bioassays frequently are designed to have a single treatment factor, i.e., increasing or decreasing concentrations of allelopathic compounds. Researchers tend to ignore that these presumable single-treatment factor bioassays in reality turn out to be multi-treatment factor bioassays since the physicochemical (e.g., presence of organic and inorganic compounds other than those being tested, soil moisture, pH, compaction, aeration, solute potential, and soil temperature to name a few) and biotic (e.g., microbial populations) environments change, sometimes dramatically, as concentrations of IPA compounds, extracts, leachates, root exudates, litter, and residues are increased or decreased within bioassay systems. For them to function as single factor systems, requires that bioassay systems treated with increasing or decreasing concentrations of IPA compounds etc. be adjusted so that with the exception of the compounds of interest they are environmentally equivalent. That turns out to be an extremely tedious job particularly for complex mixtures of organic and inorganic compounds found in extracts, leachates, root exudates, litter, and residues since each system would have to be monitored for a variety of environmental factors and adjusted accordingly. But unless that is done, it will be impossible to characterize the effects of increasing concentrations of specific allelopathic compounds independently of other active (i.e., regulating and modifying) environmental factors.

Unfortunately it is not always possible to know and identify the entire range of environmental factors that will influence the results of a bioassay. However, that should not prevent researchers from characterizing the obvious factors (e.g., light intensity, temperature, moisture, nutrition, pH, aeration, water potential, composition and volume of substrate, presence or absence of microorganisms and other organisms, and symbiotic relationships). The importance of adequate monitoring and characterization of environmental factors for laboratory bioassays cannot be overemphasized since environmental factors will not only influence the bioassay species directly but also modify the actions and effects of the IPA compounds individually or imbedded in complex mixtures of organic and inorganic compounds (see Einhellig 1989; Lehman and Blum 1997; Gawronska and Golisz 2006; Pedrol et al. 2006; Blum 2011).

Most of the physical factors (e.g., light, temperature, moisture, nutrition, and soil compaction) can be readily regulated or controlled in laboratory bioassays. However the biotic factors, particularly the microorganisms, are another story. Soil microbial populations are a particular pernicious problem in laboratory bioassay systems since the enrichment or treatment of environmentally rich bioassay systems (e.g., adequate nutrients, moisture, and temperature) with carbon sources, such as IPA compounds, extracts, leachates, root exudates, litter, and residues can dramatically alter the qualitative and quantitative nature of microbial populations within the soil. Such changes are a concern because microorganisms not only utilize and modify organic compounds and in the process increase or decrease their potential allelopathic effects but also produce their own and sometimes unique allelopathic compounds. Furthermore, such enrichments or treatments can also modify potential symbiotic relationships (e.g., formation, development, and function of nodules and mycorrhizae) adding another level of complexity to our ability to identify causation in seedling or older plant bioassays. One could, of course, argue that microbial populations in field soils will also change with increasing and decreasing inputs of organic and inorganic compounds of leachates, root exudates, litter, and residues. However, the present evidence suggests that changes in microbial populations in laboratory bioassays are much more dramatic than in field soils. For a more detailed discussion of this problem see Sects 3.6 and 3.7. Clearly this is a subject that deserves much more attention.

2.1.3 Test Materials

2.1.3.1 Nature of Test Materials

Compounds tested in laboratory bioassays include identified putative allelopathic (IPA) compounds isolated and identified from living plant tissues (e.g., extracts of leaves, roots, mycorrhizae, and whole plants, leachates, root exudates, etc.), litter and residues (e.g., extracts or leachates of dead plant materials), soils (e.g., soil extracts, solutions or leachates), and complex unknown mixtures of compounds associated with the testing of extracts, leachates, and root exudates of living tissues,

litter, residues, and soils. IPA compounds can be purchased from a vender or isolated, purified, and collected from living tissues, litter, residues, and soils. However, locating, identifying, and collecting representative source materials (i.e., plants, tissues, residues, litter and soils) for testing or for obtaining extracts, leachates, or root exudates are another matter since for any given species, ecotype, cultivar, or soil these materials can be highly variable in time and with location.

Chemical Composition Since the chemical compositions of living plants, plant tissues, litter, and residues, and soils are highly variable their chemical composition should/must be characterized before they are used in laboratory bioassays. The reasons for this variability in chemical composition are as follows:

- a. Living plants or their tissues: The organic and inorganic composition of plants or their tissues at a particular time and location will vary with species, ecotype, cultivar, stage of plant development, stage of life cycle, time of the growing season, tissue type, presence or absence of symbiotic relationships, and past and present growth environments (Koeppel et al. 1969, 1970; Bates 1971; Hall et al. 1982, 1983; Rice 1984; Hocking 1994; Drossopoulos et al. 1996; Chaves et al. 1997; Lambers et al. 1998; Chaves and Escudero 1999; Zhao and Oosterhuis 1999; Bertran et al. 2010; also see Sects. 3.6 and 3.7). Since the allelopathic potential of living plants and their tissues varies with their inorganic and organic compositions, collecting them at the right time of the growing season, the right place (e.g., site where putative interactions are occurring), and under the right conditions (e.g., before or after major rain events or other disturbances) is essential. For example, Hall et al. (1982) found that total phenolic acid content of sunflower (*Helianthus annuus* L.) plants grown in sand culture varied with the nutrition and growth environment. As the concentrations of Hoagland's nutrient solution (Hoagland and Arnon 1950) supplied to sunflower plants growing in the greenhouse or in the out-of-doors were increased, the total phenolic acid content in chlorogenic acid equivalents of the plants declined. Total phenolic acid content ranged from 40 (1/4 strength) to 32 (full strength) mg/g dry weight for plants grown in the greenhouse and from 79 to 52 mg/g dry weight for plants grown outdoors. When sunflower plants were grown in a local garden, the total phenolic acid content was 19 mg/g dry weight. For additional environmentally induced variation and for developmental and tissue variation of phenolic acids in sunflower plants, see Koeppel et al. (1969, 1970) and Rice (1984). Hall et al. (1982) found that total phenolic acid content of freeze-dried and coarsely ground sunflower plant tissues obtained from sunflower plants grown under a range of environmental conditions (see above) was directly related to the inhibition of pigweed (*Amaranthus retroflexus*) seed germination when these sunflower tissues were mixed into soil. However, inhibition of pigweed seedling dry weight was not significantly related to total phenolic acid content unless NPK (total N, P and K determined on dry-ashed plant samples) content of the sunflower tissues was also included in the regression model (Hall et al. 1983). Total phenolic acid and N were directly related and P and K were inversely related to the inhibition of pigweed seedling dry weight. Inhibition

of germination was reduced and inhibition of seedling biomass was eliminated when NPK solution (in the form of NO_3^- , KNO_3 , and HPO_3) roughly equivalent to NPK content of sunflower tissue was also added to the sunflower tissue-soil bioassay system. These observations lead the authors to suggest that nutrients within tissues and nutrients within soils appear to act differently in modifying allelopathic interactions. The fact that increasing soil nutrients tends to reduce or eliminate the action of inhibitory allelopathic compounds by way of microbial utilization would tend to support this observation (Vaughan et al. 1983; Blum et al. 1993, 1999; Schmidt and Ley 1999).

Variations in organic and inorganic content of living plants and plant tissues are actually very common and can occur over a range of time intervals. For example, alkaloid content of hemlock fruits (*Conium maculatum* L.) changed not only weekly but also hourly during fruit development (Fairbairn and Wasel 1964). Hourly changes were particularly dramatic during times of pericarp development of fruits but were not obvious during early seedling development in the vegetative tissues. For 2 year old hemlock plants the alkaloid γ -coniceine (slightly water soluble) was found in the aerial parts and roots during March (spring growth) but only in the aerial parts during vigorous growth in June. Seasonal variation of organic compounds in plant tissue is actually a common occurrence (Witzell et al. 2003; Muscolo and Sidari 2006; Solar et al. 2006; Kotilainen et al. 2010). For examples of variations in inorganic compounds in plant tissues see Bates (1971); Hocking (1994); Drossopoulos et al. (1996); and Zhao and Oosterhuis (1999).

- b. Plant litter and residues: The organic and inorganic composition of plant litter and residues will vary with the:
 1. species, ecotype, and cultivar,
 2. tissue type (e.g., stems, leaves, or roots),
 3. stage of development and stage of life cycle,
 4. time of growing season,
 5. physicochemical and biotic growth environments of living plants and their tissues prior to senescence,
 6. timing and rates of senescence, mortality, and abscission (shedding),
 7. location of litter and residues (e.g., standing upright, lying on the soil surface, or located within the soil),
 8. length of time the litter and residues have been in the field (e.g., amount of fragmentation, weathering and decomposition), and
 9. precipitation events (e.g., rates, volume, frequency, and timing), and management (e.g., tillage and chemical applications).

That allelopathic potential of plant litter and residues will vary with their composition of organic compounds has been well established (Guenzi et al. 1967; Patrick 1971; Vaughan and Malcolm 1985; Coleman et al. 1989; Koch et al. 1992; Blum et al. 1997; Lehman and Blum 1997; Bonanomi et al. 2006). Circumstantial evidence would suggest that this is also the case for the composition of inorganic compounds within litter and residues (see a. Hall et al. 1983 above). Thus, collecting litter and residues for bioassays at the right time of the year, the

right place (e.g., site where putative interactions are occurring), and under the right conditions (e.g., before or after major rain events or other disturbances) is essential.

For example, total phenolic acid content in ferulic acid equivalents of crimson clover (*Trifolium incarnatum* L.), rye (*Secale cereale* L.), subterranean clover (*Trifolium subterraneum* L.), and wheat (*Triticum aestivum* L.) cover-crop residues taken at monthly intervals from a field after glyphosate desiccation, declined over time (Lehman 1993). However, the decline was not evident until 2 months after the glyphosate desiccation for wheat and rye residues. After 4 months the total phenolic acid content of the cover crop residues had declined by 31, 36, 38, and 56% for wheat, crimson clover, rye, and subterranean clover, respectively. Similar observations have been made for DIBOA (2,4 dihydroxy-1,4-benzoxazin-3(4H)-one) and two related compounds in rye (Yenish et al. 1995). For this study DIBOA and the related compounds, DIBOA glucoside and BOA (benzoxaolin-2-one), declined by 50% 10–12 days after clipping. Both phenolic acids and DIBOA and related compounds have been shown to have inhibitory properties under appropriate conditions at the appropriate concentrations (see Barnes and Putnam 1987; Blum 2011). For variation of inorganic compounds in litter and residues over time see Buchanan and King (1993); Schomberg and Steiner (1999); Lupwayi et al. (2006); and Ventura et al. (2010).

- c. Soils: The organic and inorganic content of soils and, thus, their allelopathic potential will vary with:
 1. soil type,
 2. the absence, or presence and nature of plant roots, mycorrhizae, and soil flora and fauna,
 3. the nature, state, and horizontal and vertical distribution of tissues, litter, residues, organic matter, and soil flora and fauna,
 4. the physicochemical and biotic environments of the soil and associated types and magnitudes of soil processes, and
 5. management. (Börner 1960; Buckman and Brady 1965; Patrick 1971; Alexander 1977; Brady 1984; Vaughan and Malcolm 1985; Coleman et al. 1989; Foth 1990; Blum et al. 1991, 1992; Hartel 1998; Lavelle and Spain 2001; Blum 2004, 2006, 2011).

Finally with the exception of pure IPA compounds, all other test materials (e.g., extracts, leachates, root exudates, tissues, litter, residues, and soil) are composed of complex mixtures of organic and inorganic compounds. In the past it was assumed, or at least implied by the experimental approaches taken, that plant-plant allelopathic interactions were primarily determined by specific organic compounds within such test materials. That was unfortunate since all active organic and inorganic compounds within a bioassay system, not just identified putative allelopathic organic compounds within the test materials, ultimately determine the stimulation or inhibition of sensitive species (see Sect. 1.2). Thus a few additional comments about allelopathic compounds, organic compounds, inorganic compounds, and the physical state of the test materials are warranted at this point.

Allelopathic Compounds As stated previously (see Sect. 1.3) the reader may have noticed some ambiguity in the way the term allelopathic compounds has been used in this volume. That stems from the fact that potentially any given organic compound can function as a promoter, a modifier, an inhibitor, or as a neutral compound depending on a variety of factors. Some determining factors are the sensitivity of the species and the process being studied, the concentrations of the compounds, the nature of other organic (and inorganic) compounds present, and the biotic and physicochemical environments (Blum and Rice 1969; Rice 1984, 1986; Blum et al. 1989, 1993; Gerig et al. 1989; Gerig and Blum 1991, 1993; Pandey 1994; Pue et al. 1995; Blum 1996, 2006, 2011; Belz et al. 2005, 2007; Duke et al. 2006; Belz 2008; Gianinazzi et al. 2010; also see Sects. 3.6 and 3.7). Therefore, in an absolute sense there is no such thing as a compound that is always an allelopathic compound. There are only organic compounds that are promoters or inhibitors under the appropriate conditions. Restated, under the appropriate conditions and at the appropriate concentrations, organic compounds function as allelopathic compounds or agents. Theoretically this is true but from a practical perspective, there clearly are organic compounds in nature which at very low concentrations readily affect (stimulate or inhibit) plant processes and there are other organic compounds that require very high or unnatural concentrations, to affect plant processes. Additional justification for the continued use of the term allelopathic compounds given this ambiguity can be found in Sect. 1.3. The reader, however, should be clear as to what is meant when the term is used.

Organic Compounds What is important to remember about organic compounds in the environment is the following:

1. That organic compounds can be reversibly or irreversibly sorbed to soil particles.
2. That organic compounds can be taken up by roots and mycorrhizae and detoxified, sequestered, and/or metabolized.
3. That microorganisms (flora and fauna) can modify and/or utilize organic compounds as a carbon and energy source.
4. That all active organic compounds can directly or indirectly promote or inhibit germination, growth, development, and reproduction of seeds, seedlings, and older plants, and promote or inhibit growth and reproduction of microorganism.
5. That the actions of individual active organic compounds within mixtures of organic compounds on seeds, seedlings, older plants, and microorganisms can be independent or similar joint action (synergistic, additive, or antagonistic) depending on the composition of the mixture, the bioassay system, and the physicochemical and biotic environments of the system (see Sects. 1.2, 1.4, 3.2, 5.3, and 6.2). That individual organic compounds within mixtures of organic compounds can also function as modifying agents of active organic compounds.
6. That the composition of organic (and inorganic) compounds within a bioassay system will be determined by the background levels of the system, the treatments added to the system, and the actions of the bioassay species, microorganisms, and medium present within the system (Sects. 1.3, 2.1.1, 2.1.2, and 2.1.3).

7. That the composition of the treatment materials will be determined by how and when the treatment materials were collected, processed, and applied to the bioassay systems (see Sects. 2.1.3.2 and 2.1.3.4).
8. That the concentrations required for specific effects of individual organic compounds (or even simple mixtures) in laboratory bioassays are not consistent with the concentrations required for individual compounds imbedded in complex mixtures of organic (and inorganic) compounds (see Sect. 3.2.2.3).

Inorganic Compounds As a general rule, the exception being nutrient and nutrient stress studies, researchers studying plant-plant allelopathic interactions have simply ignored or downplayed the importance of inorganic compounds. In fact, in some solution culture bioassays nutrients have been deliberately omitted. The general tendency has been to simply make sure that nutrition is adequate or more than adequate for seedlings and older plants. The initial presence of inorganic compounds in the media (e.g., solutions and substrates such as soil) and the subsequent inputs of inorganic compounds by extracts, leachates, root exudates, litter, residues, and nutrient solutions (e.g., Hoagland's solution; Hoagland and Arnon 1950), however, can have considerable impact on when and how potential allelopathic organic compounds will affect seeds, seedlings, older plants, and microorganisms (see Hall et al. 1982, 1983; Blum and Shafer 1988; Blum et al. 1993, 1999; Blum 2004, 2006, 2011). The types and concentrations of inorganic ions present will not only determine the availability of nutrients for plants and microorganisms but also determine pH, buffering capacity, and solute potential of test solutions, substrate sorption of organic compounds (e.g., multivalent-cation bridging and formation of polymers), salinity of substrates, and substrate structure (e.g., formation of aggregates), to name a few (see Blum 2006, 2011). Care should, therefore, be taken when designing bioassay systems to provide and maintain inorganic compounds (e.g., nutrients) at consistent and relevant levels (see Sect. 3.2.2.4).

Physical State of Test Materials Let us assume that the test materials have been properly collected, processes, stored, and applied to laboratory bioassay systems (see Sects. 2.1.3.2–2.1.3.4). Since the physical states of individual organic and inorganic compounds in solutions such as leachates and root exudates are ultimately determined by the physical and biotic environments of a bioassay systems creating and maintaining field-relevant bioassay systems and/or adequately characterizing other types of bioassay system are/is essential for interpreting and evaluating the relevance of the resulting data (see Sect. 3.2.2). For tissues, litter and residues the outcome of a bioassay can be, and likely will be, determined by the type, composition, and particle size (physical state) of tissue, litter, and residue added to the system (Ells and McSay 1991; Blum 1999). (Note: Effects are also determined by their location (see Sect. 6.5).) The smaller the size of the particles of a treatment sample, the faster the water soluble compounds will be released and decomposed. The larger the size of the particles of a treatment sample, the slower the water soluble compounds will be released and decomposed. The former will provide a pulse dose (higher concentration over a short time interval) and the latter a more phased or

chronic dose (lower concentration over an extended time interval although it is very likely that an initial pulse will also occur). Clearly actual particle size of the samples collected in the field should be maintained wherever and whenever possible.

Final Comments Given this range of variability for test materials, the importance of adequate and representative sampling is absolutely essential if meaningful (i.e., field relevant) information is to be obtained from bioassays. Otherwise the stimulation or inhibition observed in bioassays for the collected test materials may not be representative for the time period and location of interest. The bottom line, one of the important decisions a researcher studying plant-plant allelopathic interactions makes is when and where he or she collects test materials (i.e., the source) and then how those samples are processed and used in bioassays.

2.1.3.2 Collecting and Storing of Test Materials

Identified Putative Allelopathic (IPA) Organic Compounds For purchased, isolated, or synthesized pure compounds this is fairly straight forward since different concentrations of compounds can be mixed directly into a substrate, added in solution form to a substrate, (e.g., soil, sand, or gravel; substrate culture; Blum and Rice 1969; Blum et al. 1993) or used in solution form without a substrate (solution culture; Einhellig et al. 1970; Einhellig and Kuan 1971; Blum et al. 1984). A description of the synthesis, isolation, purification, and collection of sufficient quantities of pure putative allelopathic compounds is beyond the scope of this volume. For procedures regarding isolation, identification, purification, and collection of allelopathic compounds, the reader may wish to consult Waller et al. (1999); Yu and Dahlgren (2000); and Sampietro et al. (2009). Stocks of most pure liquids or dry compounds can be readily stored in their containers in the laboratory. Replacing air in storage containers with nitrogen may also provide a benefit for some compounds by reducing or eliminating oxidation. However, if liquid dilution series are created, they should be properly adjusted for pH and water potential and used immediately in bioassays. If there is a delay in their use, they should be filter sterilized or refrigerated for short delays and frozen for longer delays. However, freezing of such dilution series is generally not recommended since freezing and subsequent thawing are likely to alter the nature of the solutions.

Extracts For the extractions of tissue, litter, residue and soil samples, the following aspects should be considered before choosing and implementing a particular type of extraction procedure. Although tissues, litter, and residues extracts have frequently been tested in bioassays in the past, such bioassays are no longer recommended since they are not representative of what is lost from tissues, litter, and residues (for details see Sect. 2.1.3.3). For crude initial surveys of allelopathic potential they may, however, be useful as long confounding factors such as water potentials, pH, and potential anaerobic conditions are accounted for in the experimental design.

- a. Tissues: Organic solvent extracts of tissues cannot be used directly in bioassays for obvious reasons. Organic solvents must first be replaced by water. However, removal of organic solvents by freeze-drying, flash evaporation, or air-drying alters the nature of extracts as the organic and inorganic compounds in extracts are concentrated and precipitated. The bottom line, it is best to avoid organic solvent extracts altogether in bioassays. Water extractions are much more appropriate.

Cold, ambient or boiled distilled water extractions of ground or chopped living tissues have frequently been used in the past (Abdul-Wahab and Rice 1967; Wilson and Rice 1968; Rice 1984; Quayyum et al. 2000; Kumar et al. 2009). Boiled extractions are probably better than ambient or cold water extractions because the boiling reduces or eliminates degradation by enzymes associated with released by cold extraction procedures and reduces or eliminates microbial utilization and synthesis after extraction. However, since solubility of both organic and inorganic compounds is modified by temperature, the quantitative and qualitative composition of cold, ambient, and hot extracts will be different. Extraction of uncut, cut or ground freeze-dried, air-dried, or oven-dried tissues has also been a common practice (Guenzi et al. 1967; Leather 1983a; Liebl and Worsham 1983; Wardle et al. 1992; An et al. 2000; Wu et al. 2007). Freeze-drying maintains the chemistry of the living tissue. Air- or oven-drying changes the chemistry of the tissue during the drying process. The chemistry of air-dried tissue may, in fact, be more closely related to early stages of senescing tissues than living tissues. Oven-dried tissues have a highly modified chemistry, something that is to be avoided. The actual physical and chemical nature of extracts are determined by the source and type of the tissue (e.g., species, tissue type: leaves, roots, etc., health, acclimation, and growth environment of the plants from which the samples were taken, presence or absence of symbiotic relationships), tissue processing prior to extraction (e.g., freeze-drying, oven-drying, chopping, grinding, etc.), and the extraction procedure used (e.g., extraction time, temperature, solvent, solvent/tissue ratio, etc.).

- b. Residues: Most of the above comments about living tissue extracts are directly applicable to plant litter and residue extracts. However, extracts of litter and residues have generally not been tested. More commonly leachates of litter and residues or litter and residues have been tested directly in bioassays (see Sect. 2.1.3.3).
- c. Soil: A variety of extractants and extraction procedures have been utilized to recover a range of potential allelopathic compounds present in soil (see Whitehead 1964; Flaig 1971; Kaminsky and Muller 1977; Whitehead et al. 1981, 1982; Hartley and Whitehead 1985; Dalton et al. 1987; Cheng 1990; Blum 1997, 2011; Blum et al. 1991, 1992, 1994; An et al. 2000; Ohno et al. 2000). Extractants have ranged from water to organic (e.g., methanol, ethylenediamine tetraacetic acid [EDTA], sodium acetate) and inorganic (e.g., NaOH) solutions. Extraction procedures have ranged from soaking for various time periods to autoclaving with subsequent filtering or centrifugation. What is recovered (i.e., free, reversibly sorbed, or irreversibly sorbed) depends on soil type, the physicochemical nature

of the extractant (e.g., pH, temperature, and chemical composition), and extraction procedure (e.g., soaking, shaking, time, pressure, and solvent/soil ratio). However, only extraction procedures that recover compounds that are available to seeds, roots, mycorrhizae, and soil organisms (e.g., free and reversibly bound) are appropriate for field-relevant bioassays (Blum 2011). Furthermore, to test soil extracts in bioassays requires that the extracts be water based since both organic and inorganic extractants will/may be inhibitory/toxic to seeds, roots, and mycorrhizae and their associated microorganisms.

In general distilled water, rain water (real or simulated (see Sect. 4.3.3)), or irrigation water (when fields are irrigated) are recommended for the extraction of available (i.e., free and some reversibly sorbed) organic compounds from soil. However, the use of distilled water can be disruptive to algal, microbial, and plant cells and, thus, release contents of such cells into the soil during soil extractions. The length of time for water extractions, particularly at ambient temperatures, should be fairly short, since longer extraction times will likely be associated with the utilization and synthesis by microorganisms, sorption, desorption, and resorption of compounds to soil particles, oxidation/reduction, and polymerization. Utilization and synthesis by microorganisms can be eliminated by first sterilizing the soil (Dalton et al. 1983) or by using a water-autoclave extraction procedure (see Blum et al. 1991, 1992; Blum 2011). However, sterilizing soils directly or indirectly by the water-autoclave extraction procedure will modify the physicochemical nature of soils (e.g., modify sorption, desorption, and fixation of both organic and inorganic compounds present in the soil) and soil extracts (e.g., modify organic and inorganic composition).

Once collected, plant tissue, litter, residue, and soil extracts should be filtered or centrifuged to remove insoluble particles before testing and used immediately. As stated previously plant tissue, litter, and residue extracts are no longer recommended for use in bioassays (see Sect. 2.1.3.3). If plant tissue, litter, and residue extracts are to be used for crude initial surveys of allelopathic potential, they and soil extracts should be filter sterilized or refrigerated for short delays and frozen for longer delays. Extended refrigeration or freezing, however, may modify the overall chemical composition of extracts due to the precipitation of various components within the extracts. For those not filter sterilized, utilization and synthesis by microorganisms will modify those extracts over time.

Leachates A variety of approaches has been used to collect leachates from plants, litter, residues, and soils. Among them are the following procedures:

- a. Leaves: Throughfall and fog drip under vegetation canopies are the most representative of actual leachates in nature (del Moral and Muller 1969; Gallet and Pellissier 1997; Nilsen et al. 1999). However, the chemical and physical properties of throughfall and fog drip do vary with the type and nature of the canopy, sample location in and under the canopy, when and how samples are collected, and the physical (e.g., frequency, volume, and rates) and chemical nature of rain and fog events. Representative sampling locations and proper collecting and handling of samples are, therefore, essential.

Leachates of leaves (also petioles, stems, bark, flowers, and fruit leachates) have been collected by spraying plants with distilled water, rain water, simulated-rain water, or other types of solutions (Long et al. 1956; Tukey et al. 1957; Tukey 1966; Wilson and Rice 1968; Al-Naib and Rice 1971; Chou and Muller 1972; Cogbill and Likens 1974; Scherbatskoy and Klein 1983; Percy 1986; DuBay and Heagle 1987; Shafer et al. 1985; Shafer 1988, 1992). The physicochemical nature (e.g., composition, pH, etc.) of leachates collected will not only vary with species, leaf shapes and surface areas, amounts and types of leaf hairs and glands, nature of cuticles (e.g., thickness, cracks and crevices), angles and positions of leaves on plants and in the canopy, stages of maturity of the leaves, types and amounts of leaf injury, and past and present growth environments of plants and their leaves but also with the composition, temperature, pH, pressure, droplet size, volume, rate, frequency, retention time of the solution applied and time of day when samples are collected.

Leaf leachates have also been collected by immersing leaves in distilled water (Tukey and Mecklenburg 1964). Leaf leachates obtained with distilled water by either immersion or spraying are the least natural in their physicochemical nature (e.g., composition and pH, etc.) because the gradient generated between leaf content and distilled water is greater than what would be found with rain water. Distilled water can also be very disruptive to leaf cells because of its water potential (i.e., approximately zero).

Finally, no matter how collected, the composition of both organic and inorganic compounds in leaf leachates (also petiole, stem, bark, flower, and fruit leachates) will also be determined by the solubility (e.g., the hydrophobic or hydrophilic nature) of compounds being leached, the mobility of the compounds within and outside of leaves (e.g., bound or free, membrane and cuticle permeability), and the existing gradient between internal concentrations of leaves and the external concentrations of the leachate solution. For a given solution, however, the longer the droplets are on leaves (or the leaves are immersed in a solution) the greater will be the concentration of the compounds leached from those leaves. The highest concentrations will be recovered the first time leaves are leached and will decline with subsequent leaching events unless adequate time is provided for leaves attached to plants to recover. The first leachate will include materials on the surface of the leaves (e.g., dust, soil particles, microbe, insect remnants, etc.). A series of plants (leaves, etc.) should be used in rotation if bioassays are to be run over extended periods.

- b. **Litter and Residues:** In theory surface litter and residue leachates collected by properly preconditioned lysimeters (e.g., acid washed, aged in soil; see Dalton 1999) just underneath plant litter and residue layers in the field are the most representative of actual surface litter and residue leachates in nature. The chemical and physical properties of surface litter and residue leachates do, of course, vary with the type and nature of the litter and residue layer, the chemical and physical properties of rain, the frequency and volume of each rain event, the location of the lysimeter, the time samples are collected (e.g., during or after a rain event; length of time solutions were in lysimeter), and time of year. Simulated-rain

events may also be used with lysimeters in the field (see Sect. 4.3.3). Another option would be to bring undisturbed or minimally disturbed surface litter and residue samples (i.e., maintaining the profile of the litter and residue layer) into the laboratory. For these samples simulated-rain events could be used to collect leachates directly without lysimeters. Surface plant litter and residue leachates have also been collected by soaking plant litter or residues in water solutions for various periods of time at temperatures from below ambient to boiling (Nilsen et al. 1999; An et al. 2000; Bonanomi et al. 2006; Nakano et al. 2006). Ambient temperatures are probably the most realistic but degradation by microorganisms can be a problem if leachate times are too long. Placing residues in boiling water to obtain leachates is the least realistic particularly when the residues have been chopped or ground.

The author is not aware of any studies on belowground litter and residue leachates independent of soil and living roots and mycorrhizae. Studies of belowground root litter and residue leachates independent of living roots, mycorrhizae, and soils, however, could prove to be very useful in distinguishing their role from the role of surface litter and residue leachates in allelopathic interactions. Once collected and cleaned (i.e., soil is gently removed by brushing), simulated-rain events (see Sects. 2.1.3.2, 4.3.3, and 4.3.5.1) may be used to collect leachates from root and mycorrhizal litter and residues in the laboratory. When cleaning belowground plant samples, water should not be used unless absolutely necessary and then washing should be minimized as much as possible to limit the loss of both organic and inorganic compounds from the samples. Finally, litter and residue samples should never be chopped, ground or oven-dried if they are to be used to collect realistic leachates.

- c. Soils: Soil leachates and soil solutions have been collected with lysimeters (Grover and Lamborn 1970; Neary and Tomassini 1985; Debye et al. 1988; Hughes and Reynolds 1988; Dalton 1993, 1999; Zabowski and Ugolini 1990) in soils with adequate moisture or after sufficient rain or irrigation events (e.g., gravitational water). Lysimeters need to be properly preconditioned (e.g., acid washed, aged in soil; see Dalton 1999) before they are used to collect leachates and soil solutions. What is recovered from the lysimeters is determined by their location in the soil, physical and chemical nature of the soil, rate and extent of soil solution movement in the soil, volume of the soil solution, activity of microorganisms, tissues, litter, and residues present, etc. Soil solutions have also been collected from moist soils by centrifugation of soil samples. However, the volume collected is generally not sufficient for seed and seedling bioassays (Zabowski and Ugolini 1990).

Leachates, no matter their source, should be collected and filtered to remove insoluble particles and used immediately. If there is to be a delay in their use they should be filter sterilized or refrigerated for short delays and frozen for longer delays. Extended refrigeration or freezing, however, may modify the overall chemical composition of the leachates due to degradation, activity of microorganisms, and the precipitation of various components within the leachates.

Plant Tissues, Litter, and Residues Plant tissues, litter, and residues may be collected from plants grown in growth chambers, greenhouses, or field plots. Growth chamber and greenhouse environments can provide ample sources of living tissues while field environments are an excellent source of both living tissue and dead tissues (i.e., litter and residues). However, due to the differences in morphological, anatomical, and chemical characteristics of plant materials from these locations and, thus, their allelopathic potentials, it is generally best to collect these materials from field plots. This can be fairly easily done for aboveground shoot tissue, litter, and residues. Root and mycorrhizal tissues, litter, and residues are somewhat more difficult to obtain and are usually obtained with soil cores or from soil pits. The soil associated with root and mycorrhizal tissues, litter, and residues may then be removed by sorting, screening, or washing (see next paragraph). At that point recognizable root and mycorrhizal litter and residues may be separated from living roots and mycorrhizae. However, identifying and isolating root and mycorrhizal litter and residues from living roots and mycorrhizae is an onerous task since identifying belowground living and dead tissues is in most cases extremely difficult and time consuming (Böhm 1979; Schenk 1982). After plant tissues, litter, and residues are collected, cleaned, and sorted they may be used directly in bioassays, air-, oven-, or freeze-dried before being used in bioassays, or stored for future use (Abdul-Wahab and Rice 1967; Wilson and Rice 1968; Al-Naib and Rice 1971; Leather 1983a, b; Putnam et al. 1983; Weston et al. 1989; Lehman and Blum 1997; Kamara et al. 1999; Staman et al. 2001; Moonen and Bàrberi 2006; Kumar et al. 2009).

The chemical composition of plant tissues, litter, and residues collected will, of course, vary with species, tissue type, stage of development, stage of the life cycle, and growth environment. For litter and residues chemical composition will also vary with the cause of mortality (e.g., natural causes, herbicide desiccations, etc.), time of abscission or loss, weathering, and decomposition. Tissues, litter, and residues should not be washed unless absolutely necessary and then washing should be minimized as much as possible to limit the loss of both organic and inorganic compounds. Oven-drying of plant tissues, litter, and residues is not recommended since that will modify their chemistry excessively. Air-drying will also change the chemistry but one can argue that these changes in chemistry are somewhat similar to changes that occur in nature. This may be the case for aboveground litter and residues but is unlikely to be the case for root and mycorrhizal litter and residues except under extended drought conditions. Freeze-drying will maintain the chemistry at the time of collection. Dried plant tissues, litter, and residues can be stored for short periods in a cool dry place in the dark before they are used in bioassays but extended storage is not recommended. Dried plant tissues, litter, and residues should never be chopped or ground before storage because the increased surface area will expedite oxidation and associated chemical changes. Furthermore, chopped and ground living or dead tissue should not be mixed into soil for bioassays unless the researcher wishes to determine a worst-case scenario or doing so would be consistent with field management practices (Blum 1999).

Root Exudates The use of the term root exudates in root-root allelopathic interactions is somewhat misleading since exudates actually constitute only a part of the loss of organic compounds from roots (Rovira 1969; Rovira et al. 1979; Smith 1970, 1976; Moody et al. 1988; Bertin et al. 2003; Bais et al. 2006). Exudates according to Rovira et al. (1979) are composed of organic compounds of low molecular weight which leak (a passive process) from root cells. Roots also lose organic compounds by way of secretions, lysates and the production and release of mucilages. Secretions are composed of low and high molecular weight compounds which are released from root cells mediated by metabolic processes. Lysates are composed of low and high molecular weight compounds which are released by autolysis of epidermal and cortical cells. Mucilages are composed of a complex of high molecular weight compounds secreted by living epidermal cells, root hairs, and root cap cells. They are also a product of hydrolysis of primary cell walls and bacterial degradation of primary cell walls of old and dead epidermal cells. The resulting gelatinous matrix, a product of the root-microbial-soil complex, is referred to as mucigel. Roots also lose inorganic compounds to the soil and take up both organic and inorganic compounds from the soil. All of the above processes (e.g., exudation, secretion, uptake, etc.) will be determined and regulated by the species involved, their state of development, presence or absence of symbiotic relationships, and their biotic and physicochemical environments (Rovira 1969; Shay and Hale 1973; Barber and Gunn 1974; Barber and Martin 1976; Smith 1976; Přikryl and Vančura 1980; Bertin et al. 2003; Jones et al. 2004, 2009; Neumann and Römheld 2007; Carvalhais et al. 2011). Plants with mycorrhizae have additional pathways of uptake and of loss for both organic and inorganic compounds by the mycorrhizal hyphae in the rhizosphere and hyphosphere (Jones et al. 2004, 2009; Toljander et al. 2007; see Sect. 3.7.4). Given the actual multifaceted nature of what is lost from roots and mycorrhizae, root exudates from here on out will be referred to as “root exudates plus”. Furthermore, given:

- a. the complex chemistry of “root exudates plus”,
- b. the close association of microorganisms (e.g., actinomycetes, bacteria, fungi, etc.; see Sect. 3.7) and fauna with the rhizoplane and rhizosphere of roots, and the rhizoplane and mycorrhizosphere (i.e., rhizosphere, and hyphosphere, i.e., see Sect. 3.7.4) of mycorrhizae,
- c. the continuous turnover of organic and inorganic compounds released from roots and mycorrhizae (e.g., uptake, utilization, transformation, transport, sorption, desorption, oxidation/reduction, polymerization, etc.), and
- d. the variable and complex nature of soils (e.g., texture, structure, clay, organic, litter and residue content, etc.) surrounding roots and mycorrhizae of both producers and receivers, the collection of realistic “root exudates plus” samples from roots and mycorrhizae, as well as soils surrounding roots, will be extremely difficult under field conditions although some have tried (e.g., Smith 1970, 1976).

To get around this problem researchers studying allelopathic plant-plant interactions in the past have primarily used two approaches. “Root exudates plus” leachates have been tested by direct transfer from producing plants to receiving plants

(see leachate bioassays of Sects. 4.2.3 and 6.3) or “root exudates plus” samples have been extracted from media and tested in bioassays. However, the rhizosphere and mycorrhizosphere, per say, have been probed by much more sophisticated methods (see Narasimhan et al. 2003; Newmann et al. 2009; Badri et al. 2013).

Direct transfers of “root exudate plus” leachates between plants have been carried out in solution-sand/gravel culture (two or more container or stair-step systems; Bonner and Galston 1944; Martin and Rademacher 1960; Abdul-Wahab and Rice 1967; Wilson and Rice 1968; Bell and Koeppel 1972; Newman and Miller 1977; Kochhar et al. 1980) without and with XAD-4 resin columns (Tang and Young 1982; Tang 1986; Tang et al. 1989; Yu and Matsui 1994, 1997), and sterile agar culture (Wu et al. 2000a, b, 2007; Huang et al. 2003). In the absence of microorganisms, leachates consist primarily of the water-soluble (hydrophilic) compounds released by roots. (Note: Some chemical oxidation/reductions may occur after compounds are released from roots.) In the presence of microorganisms, leachates consist of water-soluble compounds released by roots and mycorrhizae, compounds modified by microorganisms, compounds synthesized by microorganisms, and compounds that have been oxidized or reduced during the transfer process. Few, if any, water insoluble compounds would be expected to be transferred between plants by “root exudates plus” leachates. The question of whether non-polar (hydrophobic) compounds may be partially solubilized or carried in water solution by mixtures with other amphiprotic compounds (having characteristics of both an acid and a base and acting as either) has really not been adequately determined. There is also the issue that some volatile compounds such as cineole which have been considered to be “insoluble” (hydrophobic) may in fact be fairly water-soluble. For example, oxygenated monoterpenes are soluble in the hundreds of ppm and which is much higher than the solubility of phenolic compounds like juglone (Weidenhamer, Personal Communications; also see Martin and Weidenhamer 1995). In addition since the rates and amounts of “root exudates plus” released from roots and mycorrhizae are to a large extent regulated by rates of leaching, activity of microorganisms, growth rates of shoots and roots, presence or absence of symbiotic relationships, type of substrate, nature of the substrate solution surrounding the roots and mycorrhizae, rates of substrate sorption/desorption, and the growth environment, the “root exudates plus” produced and tested by direct transfer in sand/gravel culture or that produced under sterile conditions in agar or solution culture will not be qualitatively or quantitatively the same as “root exudates plus” produced in soils in the laboratory and in the field (Shay and Hale 1973; Barber and Gunn 1974; Barber and Martin 1976; Přikryl and Vancura 1980; Hodge et al. 1996). The direct-transfer technique(s) can, however, provide some insight into potential plant-plant allelopathic interactions and how such interactions may be modified by various physicochemical and biotic factors that regulate the production of water-soluble components of “root exudates plus” by roots and mycorrhizae.

Samples from direct-transfer systems, sterile or non-sterile solution culture, or sterile or non-sterile agar culture have also been collected and tested for allelopathic activity. Frequently organic compounds from these samples have been isolated by a range of techniques (e.g., chromatographic techniques) or captured on and then

extracted from various types of sorption materials such as XAD resins (see Sect. 6.3 for other potential sorption materials; Börner 1960; Rovira 1969; Shay and Hale 1973; Smith 1970, 1976; Přikryl and Vančura 1980; Tang and Young 1982; Tang 1986; Tang et al. 1989; Moody et al. 1988; Pérez and Ormeño-Núñez 1991; Yu and Matsui 1997; Pramanik et al. 2000; Wu et al. 2001a, b; Hao et al. 2007; Yang et al. 2010; Zhang et al. 2010). Capillary mats and in situ tube microextractions have also been used to recover a range of organic compounds on root surfaces (i.e., rhizoplane) and within the rhizosphere and mycorrhizosphere and identified and quantified (Czarnota et al. 2001, 2003; Mohnhey et al. 2009; Weidenhamer et al. 2009; Barto et al. 2012). In case of delay in testing collected “root exudate plus” leachates or IPA compounds collected from “root exudate plus” they should be processed and stored the same way as leachates, solutions, and IPA compounds described in previous subsections.

2.1.3.3 Uses for Test Materials Collected

Identified Putative Allelopathic (IPA) Organic Compounds Purchased compounds or compounds that have been isolated from plant and soil extracts, leachates, and “root exudates plus” when used in solution culture (see Sect. 6.2), can provide considerable insight regarding the potential stimulation (facilitation) or inhibition (phytotoxicity) of individual compounds or mixtures of compounds and how such stimulation or inhibition may be modified by various environmental factors (e.g., pH, water potential, inorganic compounds, other organic compounds, temperature, light intensity, populations of microorganisms, and with or without the presence of symbiotic relationships; see Sect. 5.2.3). This can readily be achieved by manipulating these factors for solution cultures. Factors, however, change over time for solution cultures due to physical and biotic processes. The ability to completely replace solutions at various time intervals for solution-culture bioassays can help in maintaining the desired range of minimum and maximum concentrations of IPA compounds, pH, solute potential, etc. over time and to some extent controlling microbial populations. Solution-culture bioassays also provide an opportunity to gain insight into the modes of action by which IPA compounds affect bioassay species. The primary down side of using solution culture bioassays is the atypical environment (e.g., aeration, total immersion of seeds, root, and mycorrhizae, distribution of compounds being tested, and absence of modifying compounds, etc.) of solution cultures for seeds, roots, and mycorrhizae (see Sect. 7.2).

When added to substrate culture in solution form (see Sect. 6.2), they provide an opportunity to determine how substrate characteristics (e.g., pH, organic matter and clay content, inorganic compounds, microbial populations, moisture content, aeration, and compaction) modify the stimulation or inhibition of such compounds. Benefits of using treatment solutions containing IPA compounds in substrate bioassays are:

- a. the ability to modify, adjust, and maintain the composition and concentrations of identified putative allelopathic (IPA) compounds for the treatment solution,
- b. the ability to readily adjust treatment solutions for such things as pH, content of inorganic compounds, solute potential, etc. so that these remain within desired limits for all treatments,
- c. the ability to readily alter the nature of treatment solutions in order to determine how pH, inorganic compounds, other organic compounds, etc. can modify the effects of IPA compounds, and
- d. the ability to add treatment solutions repeatedly over time at predetermined intervals or continuously by drip irrigation to replenish and regulate maximum and minimum concentrations of IPA compounds under study.

The down side of using treatment solutions containing IPA compounds is:

- a. there is little control over the distribution patterns within a substrate system,
- b. the amount of solution that can be added per treatment to substrates can be limiting particularly for small volumes of substrate,
- c. depending on solubility, there are limits to the concentrations that can be added per treatment or over time,
- d. that for repeated additions, if substrate losses are slower than inputs, concentrations of the compounds of interest will increase in the soil with time, and
- e. the potential creation of atypical factors (e.g., intense modification of microbial populations by differential selection, amplification, and suppression of specific microbial species) by frequent additions of treatment solutions to environmentally enriched (e.g., adequate to excessive nutrition, soil moisture, etc.) substrate cultures.

IPA compounds in dry form can be directly mixed into a substrate (e.g., soil; Blum et al. 1993). The initial desired distribution pattern within the substrate for such additions can readily be achieved except for very low concentrations of IPA compounds. However, multiple additions to substrate once the bioassay is in progress are not possible without considerable disturbance of the bioassay system. They can be applied to the substrate surface at that point but, depending on the solubility of the compounds being tested, toxicity at or near the substrate surface and the resulting steep vertical gradients within the substrate will be a problem. Adjusting pH, solute potential, etc. of the substrate solutions for the different IPA concentrations added to a substrate in dry form will also be extremely cumbersome. In addition the amount of dry compounds added to the surface or mixed into substrate that is dissolved in the substrate solution is not predictable and, thus, available concentrations within a system are an unknown.

Organic Solvent Extracts of Plant Tissues, Litter, and Residues Organic solvents are best used to isolate, identify, and collect solvent-soluble organic compounds that may have promoter, inhibitor, or herbicidal properties. Organic solvent extracts cannot be used in bioassays unless the organic solvents are first removed and replaced with water. In the process of replacing organic solvents with water the physical and chemical properties of the extract will be modified.

Water Extracts of Plant Tissues, Litter, and Residues Water extracts are best used to isolate, identify, and collect water-soluble organic compounds that may have promoter, inhibitor, or herbicidal properties. They could be used in bioassays but their use is not recommended for even preliminary tests of potential allelopathic activity because composition and concentrations of both organic and inorganic compounds in tissue, litter, and residue extracts are totally inconsistent with what is released from living plants, plant litter, and plant residues under field conditions. In addition, effects of water potential, pH, potential anaerobic properties, and enzymatic degradation of extract are confounding factors that make the identification of cause and effect extremely challenging.

Organic and Inorganic Solvent/Extractant Extracts of Soils Some examples of solvents or extractants that have been used are methanol, sodium acetate, citrate, Melich III, ethylenediamine tetraacetic acid (EDTA), NaOH, etc. (see Dalton et al. 1987; Blum 1997; Dalton 1999). Such extracts are best used to isolate, identify, and collect compounds that may have promoter, inhibitor, or herbicidal properties. They are also excellent when used in combination with water extracts of soil for determining the states of the compounds in soil (free, reversibly or irreversibly sorbed) as well as for determining how various physicochemical and biotic factors may modify reversible and irreversible sorption to soil particles, uptake by seeds and roots (with or without symbiotic relationships), and utilization by soil organisms (see Blum 2011). These types of extracts cannot be used in bioassays unless the organic solvents (e.g., methanol) or the organic (e.g., EDTA, citrate, acetate) and the inorganic compounds (e.g., Na) of the extractants are first removed and replaced with water. Replacement of organic solvents may be achieved by evaporating or flash-evaporating extracts to dryness before dissolving the remaining organic and inorganic precipitates in water. Removal of organic and inorganic compounds (e.g., EDTA, citrate, acetate, Na) of extractants may be achieved with dialysis, anion and cation resin columns, or molecular sieves but these are very cumbersome and uncertain processes particularly for large volumes of extracts. In both instances the resulting solutions will be very different from the original soil solutions in pH, content of organic and inorganic compounds, water potential, etc. An additional reason for not using these extracts in bioassays is that in some instances they contain organic compounds that are not available to interact with seeds, roots, mycorrhizae, and soil organisms (e.g., compounds that are irreversibly sorbed to soil particles and the recalcitrant organic matter of the soil).

Water Extracts of Soils At ambient temperature and pressure water extracts recover what is in the soil solution and probably a small portion of what is reversibly sorbed to soil particles. This fraction has frequently been referred to as the free or soil solution fraction in the soil (compared to the reversible and the irreversible fractions) that is available to interact with seeds, roots, mycorrhizae, and soil organisms (Note: Both free and reversibly bound organic compounds are available to interact with seeds, roots, mycorrhizae, and soil organisms, thus ambient water extractions recover only part of what is available.). However, extraction times for these must be fairly short. For longer extraction times extracts will be modified

by the utilization, conversion, and synthesis of organic compounds by soil organisms, sorption, desorption, and resorption to soil particles, and oxidation/reduction of organic compounds.

Extractions using higher temperatures and pressures, assuming temperature and pressure are sufficiently high enough, will eliminate activity of soil organisms but will increase the probability that compounds that are normally not available to seeds, roots, mycorrhizae, and soil organisms will be recovered (e.g., compounds that are irreversibly sorbed to soil particles and the recalcitrant organic matter of the soil; see Blum 2011). When utilizing water extracts obtained with ambient or higher temperatures in bioassays, extract properties such as level of dilution, water potential, pH, and inorganic compounds must be accounted for in the experimental design (see Sects. 4.4.3 and 6.4.2). However with an appropriate experimental design, potential allelopathic effects of soil-water extracts for a given set of soil samples can be determined in the presence of other causative and modifying environmental factors (e.g., see Sect. 6.4.2). Water extracts of soil may also be used to isolate, identify, and collect compounds which may have promoter, inhibitor, or herbicidal properties although the author suspects that other more rigorous extraction procedures would be more effective in isolating and collecting such compounds.

When all is said and done, water extracts of soil at a given point or given points in time will provide only minimal, if any, information on turnover, flux, or flow rates of organic and inorganic compounds in soil systems and only a portion of the reversible sorbed fraction in the soil. Quantities of both organic and inorganic compounds recovered by water extraction of soil, if done properly, actually represent water extractable residuals or net concentrations left in the soil (i.e., what is left after reversible and irreversible sorption, microbial utilization, uptake by seeds, roots and mycorrhizae, etc. have taken place; see Sect. 1.4). If quantities of free organic and inorganic compounds recovered are expressed as units/unit of actual moisture in the soil [e.g., μM], then the resulting concentrations are approximately equivalent to soil solution concentrations at a given point in time. Estimating total available concentrations in soil will require not only data for the water extractable fraction but also data for the reversibly sorbed fraction not recovered by water extraction, and the flows, fluxes, or flow rates to seeds, roots, mycorrhizae, microbes, soil particles, etc. (i.e., source-sink relationships).

Throughfall and Leaf Leachates If carried out and collected properly, throughfall leachates contain what was actually lost from leaves, stems, fruits, etc. within the canopy and what was present in the initial rain water during a specific rain event in the field. Leachates generated by artificial or simulated-rain events in the field or laboratory using collected rain water, simulated-rain water (see Sect. 4.3.3), or irrigation water are an attempt to simulate throughfall leachates under more controlled conditions. The use of irrigation water is, however, not recommended unless overhead irrigation is part of the normal system management. Unfortunately the composition of leachates no matter how they are collected are highly variable both in the field and the laboratory for obvious reasons (e.g., composition and nature of canopy or plants, frequency of rain events, time between rain events, rates and length of rain events, etc.). The bottom line is that the chemical compositions of

throughfall and simulated leachates for any given event or location represent only a small fraction of all the possible chemical compositions of leachates that can occur in the field or in the laboratory. Therefore, the best that either can demonstrate is the potential for allelopathic effects (e.g., the difference in effects between rain water above the canopy and throughfall or leachate below the canopy) for the conditions under which the throughfall or leachates were collected and the nature and environment of the bioassay system. An upside for using collected rain water, simulated-rain water, or irrigation water to obtain leachates is that their physico-chemical nature (e.g., composition, pH, etc.) can be readily manipulated prior to application to leaves, plants, or canopies to determine how different physical and chemical factors of rain water, simulated-rain water, or irrigation water can modify the leachates recovered and subsequently how such modifications could potentially affect plant-plant allelopathic interactions. No matter the approach taken, with an appropriate experimental design, potential allelopathic effects of throughfall and simulated leachates for given points in time and for given places and systems can be determined in the presence of other causative and modifying environmental factors (see Sect. 4.2.2).

Throughfall and simulated leachates can also be used to isolate, identify, and collect compounds that may have promoter, inhibitor, or herbicidal properties. Finally, immersing leaves, plants, etc. in distilled water, rain water, simulated-rain water, or irrigation water results in poor imitations of actual leachates under field conditions and, thus, are of limited value. However, they may be useful as an initial screening tool.

Litter and Residue Leachates The comments for throughfall and simulated-leaf leachates are directly applicable to natural and simulated-surface litter and residue leachates. The primary differences are location, i.e., canopy vs. litter layer, and the ease with which they are collected. Collecting representative leachates from litter layers is much more difficult (e.g., defining what constitutes a litter layer, the need for lysimeters or similar tools, the proximity and potential interference of soil materials, etc.). As with throughfall and simulated leachates from canopies, the litter and residue leachates are also highly variable due to composition and nature of litter layer, frequency of rain events, time between rain events, rates and length of rain events, etc.

Immersions and soaking of or simulated-rain events for root and mycorrhizal litter and residues separated from living roots and mycorrhizae and soil are artificial but that may be the only way to obtain and test root and mycorrhizal litter and residue leachates independent of living roots, mycorrhizae, and substrates such as soil. The use of distilled water to collect leachates from root and mycorrhizal litter and residues should be avoided. Even the use of rain water or simulated-rain water (see Sect. 4.3.3) is questionable since the physical states and chemical compositions of simulated-rain solutions are very likely to be different from soil solutions or gravitational water.

No matter the approach taken, with an appropriate experimental design, potential allelopathic effects of litter and residue leachates for given points in time and for

given places and systems can be determined in the presence of other causative and modifying environmental factors (see Sect. 4.3.5). Litter and residue leachates can also be used to isolate, identify, and collect compounds that may have promoter, inhibitor, or herbicidal properties. However given the difficulties in obtaining representative litter and residue leachates, it is not surprising to find that most researchers have chosen to use litter and residues directly in soil bioassays instead of litter and residue leachates.

Soil Leachates and Soil Solutions For all intents and purposes soil leachates collected with lysimeters will most likely be a combination of gravitational water and soil solutions. The level of dilution, and thus the potential allelopathic effects, will be determined by the volume of rain water or simulated-rain water added to the soil system during natural or simulated-rain events, respectively. Those collected by centrifugation will likely represent soil solutions. Just like soil extracts (another potential way of collecting organic and inorganic compounds in soil and soil solutions), soil leachates and soil solutions represent residual concentrations and not total available concentrations. With an appropriate experimental design, potential allelopathic effects of soil leachates and soil solutions (assuming enough can be collected) for given points in time and for given places and systems can be determined in the presence of other causative and modifying environmental factors (see Sects. 4.4.3 and 6.4.2). Soil leachates and soil solutions can also be used to isolate, identify, and collect compounds that may have promoter, inhibitor, or herbicidal properties.

Plant Tissues, Litter, and Residues If tissues, litter, and residues are collected at the correct time, if litter and residues are collected at the right state of weathering and decomposition, if the collected materials are not altered dramatically by processing, and if they are added to field soil samples at the appropriate concentration (i.e., quantity, amount, or level) and location, then plant tissue, litter, and residue bioassays can provide reasonable insight regarding their potential stimulation or inhibition of bioassay species. Changes in soil moisture, temperature, pH, aeration, structure, inorganic compounds, and microbial populations with increasing levels of plant tissues, litter, and residues on the surface or within the soil will, however, make identification of the actual cause and effect challenging. Thus, when utilizing increasing or decreasing amounts of plant tissue, litter, and residues in bioassays, linked changes in soil pH, moisture, inorganic compounds, organisms, soil aeration, etc. must be accounted for in the experimental design. However, given an appropriate experimental design, it is possible to determine allelopathic effects of plant tissues, litter and residues for given points in time and for given places and systems in the presence of other causative and modifying environmental factors (see Sects. 4.3.4 and 6.4.1).

Soil cores with and without plant tissue, litter, and residues can also be used to determine effects of tissue, litter, and residues. However, actual causation will be extremely difficult, if not impossible, to determine for soil cores unless differences in the physiochemical and biotic environments for the soil cores can also be determined.

“Root Exudates Plus” “Root exudates plus” (see Sect. 2.1.3.2) collected with sterilized solution (e.g., in culture flasks) and from agar cultures are adequate for the isolation and identification of compounds released (active and passive processes) from roots. However, since the rates and amounts of “root exudates plus” lost or released from roots are to a large extent regulated by rates of leaching, activity of microorganisms, presence or absence of symbiotic relationships, the growth of shoots and roots, type of media, the nature of the solutions surrounding the roots and mycorrhizae, the rates of sorption and desorption, the rates of uptake of roots and mycorrhizae, etc., the “root exudates plus” produced in such closed sterile systems are not qualitatively and quantitatively representative of “root exudates plus” found under field conditions (Shay and Hale 1973; Barber and Gunn 1974; Barber and Martin 1976; Prikryl and Vančura 1980; Hodge et al. 1996; Neumann and Römheld 2007). Bioassays using such closed sterile system, thus, provide at best only limited insight regarding the actual production, modifications, and effects of allelopathic compounds released into natural soil systems.

The direct transfer in larger semi-closed or open systems with or without XAD resins or other sorbents (two container or stair step systems; see Sects. 2.1.3.2 and 4.2.3) should theoretically be more natural in the production of “root exudates plus” but the use of sand or a substrate (essentially porous inert materials), the frequent absence of symbiotic relationships, and the constant recycling of leachates within semi-closed systems or the collection of multiple leachates from the same plant or even different plants in open systems will generate different total and available concentrations than would be expected in field soils. Furthermore particularly for semi-closed systems used over extended periods, unless inorganic compounds, pH, and water levels of the solution re-circulating throughout the systems are carefully monitored and frequently adjusted, competition for inorganic compounds (i.e., nutrient), pH and solute potentials may be confounding factors. These systems also tend to be highly aerobic and, therefore, activities of microorganisms and oxidation/reduction are unlikely to be similar to those of soil environments. Finally, only the water-soluble compounds for “root exudates plus” are tested in these multi-pot systems since only those are leached and transferred from pot to pot. However, even with these limitations and if systems are properly managed, a potential role, if any, of “root exudates plus” leachates in allelopathic interactions may be determined (e.g., Sect. 4.2.3.1).

2.1.3.4 Application of Test Materials

The common procedure for solutions containing water soluble compounds (e.g., IPA compounds, extracts, and leachates) has been to apply these solutions directly to a bioassay species (e.g., Petri dish or solution culture) or to apply them to the surface of a medium containing a bioassay species (e.g., sand or soil). The application of test materials in recirculating systems (e.g., a “root exudates plus” stair step system) is, thus, unique since the source of the treatment materials comes directly from a putative allelopathic plant within the system. Fresh or dried tissues have

frequently been chopped or ground and placed on the surface of a medium or mixed throughout the medium. Unfortunately, in many instances such applications of test materials are not consistent (i.e., atypical) with what occurs under field conditions. Every effort should be made so that test or treatment materials applied to laboratory systems are consistent with the nature, location, and distribution of materials in the field. For more details regarding how such atypical applications may impact the behavior of bioassay species see Sects. 3.3 and 7.2.1.

2.1.4 Measurements

Both destructive and non-destructive measurements have been utilized to quantify how IPA compounds, extracts, leachates, litter, residues, etc. modify the behavior of bioassay species and system components and processes (e.g., physical, chemical, and biotic [predominately microbial] components and processes). Measurements range from the molecular level to the whole system level. What is measured and when, where, and how measurements are to be taken really depends on the experimental objectives of the research. On the surface it would appear that determining what to measure and when, where, and how to take measurements should be a simple matter. However, making these choices for a bioassay species and system should not be taken lightly since the measurements chosen can/will for example:

- a. determine the nature and complexity of the experimental design and the type of model system chosen for a study (e.g., solution culture, substrate culture, etc.),
- b. determine the level (e.g., molecular, cellular, tissue, organ, whole plant, or system) at which the effects of treatment materials are observed,
- c. determine the types of questions that can reasonably be answered, and
- d. determine how well measurements and observations made and conclusions reached for a chosen laboratory bioassay system will relate to the behavior of seeds, seedlings, plants, microorganisms, etc. in natural or field (managed) systems.

Most plant-plant allelopathic bioassays are designed to study how the behavior of a bioassay species (e.g., seeds, seedlings or older plants) is modified by promoters or inhibitors released from putative allelopathic plants or how various system components (e.g., media, biotic and physicochemical environment, etc.) and processes (e.g., sorption, leaching, uptake, and microbial utilization and transformation) may modify this behavior. When we add that the behavior of bioassay species and their associated systems are highly variable, at times ephemeral and extremely complex, it should not be surprising to find that most researchers have chosen measurements that integrate, summarize, or average all this behavior (i.e., represent some final end product). Thus, measurements such as rates of germination, radicle length, or changes in biomass of seedlings (actual size, weight, or growth rates) dominate the literature. In many ways this is logical since the processes of germination, seedling emergence, and growth, as well as tolerance/resistance, competitiveness, reproduction, and seed dispersal are or can be excellent indicators of the

success or failure of species in nature. For agricultural or horticultural species, the bottom line varies somewhat with the commercial and aesthetic interests of the producer and consumer. However, the processes of germination, seedling emergence, competition, survival, and vegetative and sexual reproduction (yield) are just as important as in natural systems. A lack of seed dispersal at maturity is a desirable characteristic for most agricultural and horticultural plants. In spite of their usefulness these measurements are far removed from the primary effects of stressors including allelopathic compounds. They have arisen from primary effects by way of a cascade of secondary, tertiary, etc. effects. Thus, these types of measurements are excellent for surveying or screening types of bioassays (i.e., are treatment materials effective as promoters or inhibitors) but not all that helpful for identifying primary sites and effects and modes of action (see Chaps. 3, 4, and 6).

For determining primary sites, effects, and modes of action lower levels (i.e., at cellular level and below) and frequently more ephemeral and elusive processes must be measured. For example, determining primary effects of phenolic acids would appear to require measurements of root membrane depolarization or alteration of membrane permeability since root membranes are a likely site of initial contact (i.e., primary sites; see Sect. 5.2.2). The other initial site of contact would be root cell walls. Changes in net water uptake or net nutrient uptake, nutrient efflux, metabolites, energy flow (i.e., carbon fixation, respiration, and translocation), cell wall lignifications, etc. resulting from the phenolic acid induced changes in membrane depolarization/permeability and cell walls already represent secondary, tertiary, etc. effects (i.e., the cascade of effects) that may or may not eventually lead to reductions in growth, yield, etc. Plants after all have considerable buffering capacity, the ability to acclimate, and a broad range of responses to biotic and physiochemical environments, (see Chaps. 3 and 7), thus, cellular-level measurements in laboratory bioassays may or may not be directly relevant to the final product of interest, the rates of germination, or seedling emergence, growth, and reproduction under natural or field conditions. For additional details see Sect. 3.11 and Chaps. 5 and 6.

To identify and characterize the primary sites and modes of action by which allelopathic compounds initiate and ultimately produce plant effects, however, requires much more comprehensive molecular and cellular methods than the type of measurements just described. A Group of tools that presently show considerable promise are the omics methods (e.g., genomics, proteomics, metabolomics, and physiomics). What omics methods can do is provide detailed information on genetic and biochemical impacts of allelopathic compounds (e.g., Gidman et al. 2003; Macías et al. 2007; Duke et al. 2008, 2013; Chen et al. 2009; Leão et al. 2009). For details regarding the potential use of omics methods in this way see Sect. 6.6.

There are three primary reasons for measuring other aspects of laboratory bioassay systems besides the behavior of the bioassay species.

- a. To characterize the biotic and physicochemical environment of the bioassay system.
- b. To determine how a range of biotic and physicochemical factors (i.e., modifiers) found in nature may influence or modify the behaviors of bioassay species to promoters and inhibitors (see Sect. 5.2.3).

- c. To determine cause and effect. Determining cause and effect requires that all other environmental factors (other than the treatment factor of interest) are initially constant, stay constant, or that changes occurring during the experimental period are measured and accounted for adequately before the final interpretations of the results are made by the researcher (see Sect. 6.4).

Measurements taken initially and over time could among other things include the following for solution and substrate bioassays.

Solution Bioassays

- a. types and concentrations of treatment compounds,
- b. types and concentrations of organic compounds other than treatment compounds present,
- c. types and concentrations of inorganic compounds present,
- d. types and population levels of microorganisms present,
- e. presence or absence of mycorrhizae,
- f. solute potentials, pH, temperature, and aeration of solutions, and
- g. photoperiod, light quality and quantity, temperature, and relative humidity of the growth environment.

Substrate Bioassays

- a. types, concentrations, and distribution of treatment compounds,
- b. types, concentrations, and distributions of organic compounds within the substrate other than treatment compounds,
- c. types, concentrations, and distributions of inorganic compounds within the substrate,
- d. types, population levels, and distribution (bulk soil, rhizosphere, rhizoplane, etc.) of microorganisms present,
- e. presence or absence of mycorrhizae,
- f. composition, texture, and structure of substrate,
- g. temperature, pH, moisture status, compaction, and aeration of substrate, and
- h. photoperiod, light quality and quantity, temperature, and relative humidity of the growth environment.

2.1.5 Hypotheses, Experimental Designs, and Data Analyses

Initial formulation of testable hypotheses before designing bioassays is essential. Once these have been formulated, experimental designs to test these hypotheses can be constructed. Afterwards hypotheses can be modified as appropriate and necessary or new hypotheses can be formulated. Hypotheses, thus, have a major role in determining the design of bioassay systems that will be used, the types of data that will be collected, when and how data will be collected, and how the resulting data will be analyzed. Hypotheses are tentative experimentally testable statements that provide potential explanations for phenomena, processes, or events and that

frequently include predictions as to the outcome if the hypotheses are true. Recall, however, that Science progresses not by trying to confirm hypotheses but by attempting to falsify hypotheses (Blum 2011). Examples of testable hypotheses can be found in Chaps. 4 and 6.

Examples of experimental designs for laboratory bioassays, methods and types of data to be collected, and general comments regarding data analyses can be found throughout the volume and in particular in Chaps. 4 and 6. In addition the benefits and limits of laboratory bioassays (Sect. 1.6), false assumptions and misconceptions of laboratory bioassays (Sect. 1.7), the fundamentals of laboratory bioassay systems (Chap. 2), issues and challenges when designing laboratory bioassays (Chap. 3), comparisons of field and laboratory systems (Sect. 7.2), and how to make laboratory bioassays more relevant to field systems (Sects. 1.5.2 and 7.4) are also provided in various sections and chapters of this volume.

Finally, it is beyond the scope of this volume to provide an in-depth description of statistical analytical procedures and techniques. Thus, the reader is encouraged to consult with a statistician and books on statistics for identifying and implementing appropriate experimental designs and to use the appropriate statistical techniques and tools to analyze and interpret their data (Steel and Torrie 1997; Summer et al. 2003; Cox and Donnelly 2011; Sokal and Rohlf 2012).

2.2 Basic Information Required for All Bioassay Systems

The following information should always be provided for the following types of bioassays:

- a. Germination and early seedling development in Petri dishes with filter paper or other substrates:
 1. size and type (e.g., plastic or glass) of Petri dish,
 2. size and type of filter paper or type (e.g. agar, sand, and soil), composition, amount, and volume of other substrates,
 3. presence or absence and source of microorganisms; in the absence of microorganisms, the type of sterilization procedure used,
 4. source and type of species being tested, number of seeds or seedlings per Petri dish, and location of seeds in reference to substrate (i.e., on surface or the depth below surface),
 5. type (e.g., IPA compounds, extracts, leachates [e.g., tissues, litter, residues, or “root exudates plus”], tissues, litter, or residues) and source of the materials being tested (e.g., How, when, and where was it collected? How was it processed and stored prior to use?),
 6. volume, concentration, pH, and solute potential of IPA compound solutions, extracts, or leachates added to Petri dish, or concentration, particle size, nature, and location (e.g., on top of substrate or mixed into substrate) of tissues, litter, or residues added to substrate in Petri dish,

7. volume, concentration, composition (e.g., nutrient solution, water, etc.) and nature (e.g., pH, solute potential, etc.) of reference or control solutions, or substrates,
 8. type and frequency of treatments and other additions (e.g., nutrients),
 9. incubation temperature, relative humidity if Petri dishes are not sealed; if sealed, type of seal, length of incubation, levels and type of lighting, and photoperiod,
 10. numbers of times solutions (e.g., water, treatments solutions) were added to compensate for evaporation if Petri dishes were unsealed and range of maximum and minimum volume of solution or moisture content of the substrate,
 11. method and frequency of measurements and length of experiment, and
 12. other relevant treatment and environmental factors.
- b. Seedling or older plant solution bioassays:
1. size (diameter and volume) and type of solution container (e.g., shape, plastic, glass, etc.)
 2. presence or absence and source of microorganisms; in the absence of microorganisms, the sterilization procedure used,
 3. presence and source of microorganisms for the formation of symbiotic relationships,
 4. source and type of species being tested, age of seedlings or plants, stage of development, and number of seedlings or plants per container,
 5. type (e.g., IPA compounds, extracts, or leachates [e.g., tissues, litter, residues, or "root exudates plus"]) and source of materials being tested (e.g., How, when, where was it collected or obtained? How was it processed and stored prior to use?),
 6. volume, concentration, pH, and solute potential of IPA compound(s) solutions, extracts, or leachates added to container
 7. volume, concentration, composition (e.g., nutrient solution, water, etc.) and nature (e.g., pH, solute potential, etc.) of reference or control solutions,
 8. frequency and timing of solution changes or additions and frequency and timing of water additions to compensate for evapotranspiration,
 9. type of growth environment before, during and after treatments (e.g., level of aeration, levels and type of lighting, photoperiod, and temperature),
 10. method and frequency of measurements and length of experiment, and
 11. other relevant treatment and environmental factors.
- c. Seed, seedling or older plant substrate bioassays other than Petri dishes:
1. size (diameter and volume) and type of container (e.g., shape, plastic, glass, etc.),
 2. composition, type, nature, amount, and volume of substrate,
 3. presence or absence and source of microorganisms; in the absence of microorganisms, the sterilization procedure used,
 4. presence and source of microorganisms for the formation of symbiotic relationships,

5. source and type of species being tested, age of seedlings or plants, number of seeds, seedlings or plants per container, and location of seeds in reference to substrate (i.e., on surface or the depth below surface),
6. type (e.g., IPA compound solutions, extracts, leachates [e.g., tissues, litter, residues, or “root exudates plus”], tissues, litter, or residues) and source of materials being tested (e.g., How, when, and where was it collected or obtained? How was it processed and stored prior to use?),
7. volume, concentration, pH, and solute potential of IPA compound(s) solutions, extracts or leachates added to substrate, or amount, particle size, nature, and location (e.g., on top of the substrate or mixed into the substrate) of tissue, litter or residues added to substrate,
8. controls, reference or baseline: volume, concentration, composition (e.g., nutrient solution, water, etc.) and nature (e.g., pH, solute potential, etc.) of reference or control solutions added to substrate or amount, particle size, nature, and location (e.g., on top of the substrate or mixed into the substrate) of inert material added to substrate,
9. if no nutrients are added to the substrate, then inorganic compound content particularly nutrient content of the substrate should be provided,
10. timing and frequency of solution or material additions to substrate, frequency and timing of water additions to compensate for evapotranspiration, and range of maximum and minimum moisture content of the substrate,
11. type of growth environment before, during and after treatments (e.g., levels and type of lighting, photoperiod, and temperature),
12. method and frequency of measurements and length of experiment, and
13. other relevant treatment and environmental factors.

In addition to the materials and methods, clear and precise information regarding location of the bioassay (e.g., light banks, growth chambers, or greenhouses), the distribution of the experimental units within a location (e.g., completely randomized, blocked, randomized and blocked, etc.), experimental design, and the tools and procedures used to analyze the resulting data collected must be provided. Background of the research topic, justification for the research, objectives of the research, and hypotheses tested should also be provided in clear and precise language.

References

- Abdul-Wahab AS, Rice EL (1967) Plant inhibition by Johnson grass and its possible significance in old-field succession. *Bull Torrey Bot Club* 94:486–497
- Al-Naib FA, Rice EL (1971) Allelopathic effects of *Platanus occidentalis*. *Bull Torrey Bot Club* 98:75–82
- Alexander M (1977) *Introduction to soil microbiology*, 2nd edn. Wiley, New York
- An M, Pratley JE, Haig T (2000) Phytotoxicity of *Vulpia* residues, IV: dynamics of allelochemicals during decomposition of *Vulpia* residues and their corresponding phytotoxicity. *J Chem Ecol* 26:2603–2617

- Arias HOR, De LaVega L, Ruiz O, Wood K (1999) Differential nodulation response and biomass yield of Alexandria clover as affected by levels of inorganic nitrogen fertilizer. *J Plant Nutr* 22:1233–1239
- Badri DV, Zolla G, Bakker MG, Manter DK, Vivanco JM (2013) Potential impact of soil microbiomes on the leaf metabolome and herbivore feeding behavior. *New Phytol* 198:264–273
- Bago B, Cano C, Azcón-Aguilar C, Samson J, Coughlan AP, Piché Y (2004) Differential morphogenesis of the extraradical mycelium of an arbuscular mycorrhizal fungus grown monoxenically on spatially heterogeneous bioassay media. *Mycologia* 96:452–462
- Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol* 57:233–266
- Barazani O, Friedman J (1999) Allelopathic bacteria. In: Inderjit, Daskshini KMM, Foy CL (eds) *Principles and practices in plant ecology: allelochemical interactions*. CRC Press, Boca Raton, pp 149–163
- Barber DA, Gunn KB (1974) The effects of mechanical forces on the exudation of organic substances by roots of cereal plants grown under sterile conditions. *New Phytol* 73:39–45
- Barber DA, Martin JK (1976) The release of organic substances by cereal roots in soil. *New Phytol* 76:69–80
- Barnes JP, Putnam AR (1987) Role of benzoxazinones in allelopathy by rye (*Secale cereale* L.). *J Chem Ecol* 13:889–906
- Barto EK, Weidenhamer JD, Cipollini D, Rillig MC (2012) Fungal superhighways: do common mycorrhizal networks enhance below ground communication? *Trends Plant Sci* 17:633–637
- Bates TE (1971) Factors affecting critical nutrient concentrations in plants and their evaluation: a review. *Soil Sci* 112:116–130
- Bell DT, Koeppel DE (1972) Noncompetitive effects of giant foxtail on the growth of corn. *Agron J* 64:321–325
- Belz RG (2008) Stimulation versus inhibition—bioactivity of parthenin, a phytochemical from *Parthenium hysterophorus* L. *Dose-Response* 6:80–96
- 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. In: Fujii Y, Hiradate S (eds) *Allelopathy: new concepts and methodologies*. Science Publishers, Enfield, pp 3–29
- Bertin C, Yang X, Weston LA (2003) The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* 256:67–83
- Bertran HC, Weisbjerg MR, Jensen CS, Pedersen MG, Didion T, Petersen BO, Duus JØ, Larsen MK, Nielsen JH (2010) Seasonal changes in the metabolic fingerprint of 21 grass and legume cultivars studied by nuclear magnetic resonance-based metabolomics. *J Agric Food Chem* 58:4336–4341
- Blum U (1996) Allelopathic interactions involving phenolic acids. *J Nematol* 28:259–267
- Blum U (1997) The benefits of citrate over EDTA for extracting phenolic acids from soils and plant debris. *J Chem Ecol* 23:347–362
- Blum U (1999) Designing laboratory plant debris-soil bioassays: some reflections. In: Inderjit, Daskshini KMM, Foy CL (eds) *Principles and practices in plant ecology: allelochemical interactions*. CRC Press, Boca Raton, pp 17–23
- Blum U (2004) Fate of phenolic allelochemicals in soils—the role of the soil and rhizosphere microorganisms. In: Maciás FA, Galindo JCG, Molinillo JMG, Cutler HG (eds) *Allelopathy: chemistry and mode of action of allelopathic chemicals*. CRC Press, Boca Raton, pp 57–76
- Blum U (2006) Allelopathy: a soil system perspective. In: Reigosa MJ, Pedrol N, González L (eds) *Allelopathy: a physiological process with ecological implications*. Springer, Dordrecht, pp 299–340
- Blum U (2007) Can data derived from field and laboratory bioassays establish the existence of allelopathic interactions in nature? In: Fujii Y, Hiradate S (eds) *Allelopathy: new concepts and methodologies*. Science Publishers, Enfield, pp 31–38
- Blum U (2011) Plant-plant allelopathic interactions: phenolic acids, cover crops, and weed emergence. Springer Science and Business Media, Dordrecht

- Blum U, Heck WW (1980) Effects of acute ozone on snap bean at various stages of its life cycle. *Environ Exp Bot* 20:73–85
- Blum U, Rice EL (1969) Inhibition of symbiotic nitrogen-fixation by gallic and tannic acid, and possible roles in old-field succession. *Bull Torrey Bot Club* 96:531–544
- Blum U, Shafer SR (1988) Microbial populations and phenolic acids in soils. *Soil Biol Biochem* 20:793–800
- Blum U, Dalton BR, Rawlings JO (1984) Effects of ferulic acid and some of its microbial metabolic products on radicle growth of cucumber. *J Chem Ecol* 8:1169–1191
- Blum U, Gerig TM, Weed SB (1989) Effects of mixtures of phenolic acids on leaf expansion of cucumber seedlings grown in different pH Portsmouth A₁ soil materials. *J Chem Ecol* 15:2413–2423
- Blum U, Wentworth TR, Klein K, Worsham AD, King LD, Gerig TM, Lyu S-W (1991) Phenolic acid content of soils from wheat-no till, wheat-conventional till, and fallow-conventional till soybean cropping systems. *J Chem Ecol* 17:1045–1068
- Blum U, Gerig TM, Worsham AD, Holappa LD, King LD (1992) Allelopathic activity in wheat-conventional and wheat-no-till soils: development of soil extract bioassays. *J Chem Ecol* 18:2191–2221
- Blum U, Gerig TM, Worsham AD, King LD (1993) Modification of allelopathic effects of *p*-coumaric acid on morning-glory seedling biomass by glucose, methionine, and nitrate. *J Chem Ecol* 19:2791–2811
- Blum U, Worsham AD, King LD, Gerig TM (1994) Use of water and EDTA extractions to estimate available (free and reversibly bound) phenolic acids in Cecil soils. *J Chem Ecol* 20:341–359
- Blum U, King LD, Gerig TM, Lehman ME, Worsham AD (1997) Effects of clover and small grain cover crops and tillage techniques on seedling emergence of some dicotyledonous weed species. *Am J Altern Agric* 12:146–161
- Blum U, Austin MF, Shafer SR (1999) The fate and effects of phenolic acids in a plant-microbial-soil model system. In: Macías FA, Galindo JCG, Molinillo JMG, Cutler HG (eds) *Recent advances in allelopathy I: a science for the future*. Cádiz University Press, Puerto Real, pp 159–166
- Böhm W (1979) *Methods of studying root systems*. Springer, Berlin
- Bollman MI, Vessey JK (2006) Differential effects of nitrate and ammonium supply on nodule initiation, development, and distribution on roots of pea (*Pisum sativum*). *Can J Bot* 84:893–903
- Bonanomi G, Sicurezza MG, Caporaso S, Esposito A, Mazzolenti S (2006) Phytotoxicity dynamics of decaying plant materials. *New Phytol* 169:571–578
- Bonner J, Galston AW (1944) Toxic substances from the bioassay media of guayule which may inhibit growth. *Bot Gaz* 106:185–198
- Börner H (1960) Liberation of organic substances from higher plants and their role in the soil sickness problem. *Bot Rev* 26:393–424
- Brady NC (1984) *The nature and properties of soils*, 9th edn. MacMillan Publishing Company, New York
- Buchanan M, King LD (1993) Carbon and phosphorus losses from decomposing crop residues in no-till and conventional till agroecosystems. *Agron J* 85:631–638
- Buckman HO, Brady NC (1965) *The nature and properties of soils*. The Macmillan Company, New York
- Carvalhais LC, Dennis PG, Fedoseyenko D, Hajirezaei M-R, Borriss R (2011) Root exudation of sugars, amino acids, and organic acids by maize as affected by nitrogen, phosphorus, potassium, and iron deficiency. *J Plant Nutr Soil Sci* 174:3–11
- Chaves N, Escudero JC (1999) Variation of flavonoids synthesis induced by ecological factors. In: Inderjit, Daskshini KMM, Foy CL (eds) *Principles and practices in plant ecology: allelochemical interactions*. CRC Press, Boca Raton, pp 267–285
- Chaves N, Escudero JC, Gutierrez-Merino C (1997) Role of ecological variables in the seasonal variation of flavonoid content of *Cistus ladanifer* exudates. *J Chem Ecol* 23:579–603
- Chen F, Liu C-J, Tschaplinski TJ, Zhao N (2009) Genomics of secondary metabolism in *Populus*: interactions with biotic and abiotic environments. *Crit Rev Plant Sci* 28:375–392

- Cheng HH (1990) Organic residues in soils: mechanisms of retention and extractability. *Int J Environ Anal Chem* 39:165–171
- Chou CH, Muller CH (1972) Allelopathic mechanisms of *Arctostaphylos glandulosa* var. *zacaensis*. *Am Midl Nat* 88:324–247
- Cogbill VC, Likens GE (1974) Acid precipitation in the Northeastern United States. *Water Resour Res* 10:1133–1137
- Coleman DC, Oades JM, Uehara G (1989) Dynamics of soil organic matter in tropical ecosystems. Dept. of Agronomy and Soil Science, College of Tropical Agriculture and Human Resources, University of Hawaii, Honolulu
- Cox JA, Conran JG (1996) The effects of water stress on the life cycles of *Erodium crinitum* Carolina and *Erodium cicutarium* (L.) L'Hérit. ex Aiton (Geraniaceae). *Aust J Eco* 21:235–240
- Cox DR, Donnelly CA (2011) Principles of applied statistics. Cambridge University, Cambridge
- Czarnota MA, Paul RN, Dayan FE, Nimbai CI, Weston LA (2001) Mode of action, localization of production, chemical nature, and activity of sorgoleone: a potent PSII inhibitor in *Sorghum* spp. root exudates. *Weed Technol* 15:813–825
- Czarnota MA, Rimando AM, Weston LA (2003) Evaluation of root exudates of seven sorghum accessions. *J Chem Ecol* 29:2073–2083
- Dalton BR (1993) Extraction and behavior of plant phenolic acids in soils. North Carolina State University Thesis, Raleigh
- Dalton BR (1999) The occurrence and behavior of plant phenolic acids in soil environments and their potential involvement in allelochemical interference interactions: methodological limitations in establishing conclusive proof of allelopathy. In: Inderjit, Daskshini KMM, Foy CL (eds) Principles and practices in plant ecology: allelochemical interactions. CRC Press, Boca Raton, pp 57–74
- Dalton BR, Blum U, Weed SB (1983) Allelopathic substances in ecosystems: effectiveness of sterile soil components in altering recovery of ferulic acid. *J Chem Ecol* 9:1185–1201
- Dalton BR, Weed SB, Blum U (1987) Plant phenolic acids in soils: a comparison of extraction procedures. *Soil Sci Soc Am J* 51:1515–1521
- Debyle NV, Hennes RW, Hart GE (1988) Evaluation of ceramic cups for determining soil solution chemistry. *Soil Sci* 146:30–36
- del Moral R, Muller CH (1969) Fog drip: a mechanism of toxin transport from *Eucalyptus globulus*. *Bull Torrey Bot Club* 96:467–475
- Drossopoulos B, Kouchaji GG, Bouranis DL (1996) Seasonal dynamics of mineral nutrients and carbohydrates by walnut tree leaves. *J Plant Nutr* 19:493–516
- DuBay DT, Heagle AS (1987) The effects of simulated acid rain with and without ambient rain on the growth and yield of field grown soybeans. *Environ Exp Bot* 27:401–395
- Duke SO, Cedergreen N, Velini ED, Belz RG (2006, February) Hormesis: is it an important factor in herbicide use and allelopathy? *Outlook Pest Manag* 17:29–33
- Duke SO, Baerson SR, Pan Z, Kagan IA, Sánchez-Moreiras A, Reigosa MJ, Pedrol N, Schultz M (2008) Genomic approaches to understanding allelochemical effects on plants. In: Zeng RS, Mallik AU, Luo SM (eds) Allelopathy in sustainable agriculture and forestry. Springer Science Business Media, New York, pp 157–167
- 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
- Einhellig FA (1989) Interactive effects of allelochemicals and environmental stress. In: Chou CH, Waller GR (eds) Phytochemical ecology: allelochemicals, mycotoxins, and insect pheromones and allomones, Academia Sinica Monograph Series, vol 9. Institute of Botany, Taipei, pp 101–118
- Einhellig FA, Kuan L (1971) Effects of scopoletin and chlorogenic acid on stomatal aperture in tobacco and sunflower. *Bull Torrey Bot Club* 98:155–162
- Einhellig FA, Rice EL, Risser PG, Wender SH (1970) Effects of scopoletin on growth, CO₂ exchange rates, and concentration of scopoletin, scopolin, and chlorogenic acid in tobacco, sunflower and pigweed. *Bull Torrey Bot Club* 97:22–23

- Ells JE, McSay AE (1991) Allelopathic effects of alfalfa plant residues on emergence and growth of cucumber seedlings. *HortScience* 26:368–370
- Fairbairn JW, Wassel G (1964) The alkaloids in *Papaver somniferum* L I: evidence for rapid turnover of the major alkaloids. *Phytochemistry* 3:253–258
- Fitter AH, Graves JD, Watkins NK, Robinson D, Scrimgeour C (1998) Carbon transfer between plants and its control in networks of arbuscular mycorrhizas. *Funct Ecol* 12:406–412
- Flaig W (1971) Organic compounds in soil. *Soil Sci* 111:19–33
- Foth HD (1990) Fundamentals of soil science, 8th edn. Wiley, New York
- Francis R, Read DJ (1984) Direct transfer of carbon between plants connected by vesicular-arbuscular mycorrhizal mycelium. *Nature* 307:53–56
- Gallet C, Pellissier F (1997) Phenolic compounds in natural solutions of coniferous forest. *J Chem Ecol* 23:2401–2412
- García I, Mendoza R, Pomar MC (2008) Deficit and excess of soil water impact on plant growth of *Lotus tenuis* by affecting nutrient uptake and arbuscular mycorrhizal symbiosis. *Plant Soil* 304:117–131
- Gawronska H, Golisz A (2006) Allelopathy and biotic stresses. In: Reigosa MJ, Pedrol N, González L (eds) Allelopathy: a physiological process with ecological implications. Springer, Dordrecht, pp 211–227
- Gerig TM, Blum U (1991) Effects of mixtures of four phenolic acids on leaf area expansion of cucumber seedlings grown in Portsmouth B₁ soil materials. *J Chem Ecol* 17:29–40
- Gerig TM, Blum U (1993) Modification of an inhibition curve to account for effects of a second compound. *J Chem Ecol* 19:2783–2790
- Gerig TM, Blum U, Meier K (1989) Statistical analysis of the joint inhibitory action of similar compounds. *J Chem Ecol* 15:2403–2412
- Gianinazzi S, Gollotte A, Binet M-N, van Tuinen D, Redecker D, Wipf D (2010) Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. *Mycorrhiza* 20:519–530
- Gidman E, Goodacre R, Emmett B, Smith AR, Gwynn-Jones D (2003) Investigating plant-plant interference by metabolic fingerprinting. *Phytochemistry* 63:705–710
- Grover BL, Lamborn RE (1970) Preparation of porous ceramic cups to be used for soil extraction of soil water having low solute concentrations. *Soil Sci Soc Am Proc* 34:706–708
- Gryndler M, Hršelová H, Sudová R, Gryndlerová H, Řezáčová V, Merhautová V (2005) Hyphal growth and mycorrhiza formation by the arbuscular mycorrhizal fungus *Glomus claroideum* BEG 23 is stimulated by humic substances. *Mycorrhiza* 15:483–488
- Guenzi WD, McCalla TM, Norstadt FA (1967) Presence and persistence of phytotoxic substances in wheat, oat, corn, and sorghum residues. *Agron J* 59:163–165
- Hall AB, Blum U, Fites RC (1982) Stress modification of allelopathy of *Helianthus annuus* L. debris on seed germination. *Am J Bot* 69:776–783
- Hall AB, Blum U, Fites RC (1983) Stress modification of allelopathy of *Helianthus annuus* L. debris on seedling biomass production of *Amaranthus retroflexus* L. *J Chem Ecol* 9:1213–1222
- Hao ZP, Wang Q, Christie P, Li XL (2007) Allelopathic potential of watermelon tissue and root exudates. *Sci Hortic* 112:315–320
- Hartel PG (1998) The soil habitat. In: Sylvia DM, Fuhrmann JJ, Hartel PG, Zuberer DA (eds) Principles and application of soil microbiology. Prentice Hall Inc, New Jersey, pp 21–43
- Hartley RD, Whitehead DC (1985) Phenolic acids in soils and their influence on plant growth and soil microbial processes. In: Vaughan D, Malcolm RE (eds) Soil organic matter and biological activity. Martinus Nijhoff/Dr W Junk Publishers, Dordrecht, pp 109–149
- Hasbullah, Marschner P, McNeill A (2011) Legume residues arbuscular mycorrhizal colonization and P uptake by wheat. *Biol Fertil Soils* 47:701–707
- Hawkins H-J, George E (1997) Hydroponic bioassay of mycorrhizal fungus *Glomus mosseae* with *Linum usitatissimum* L, *Sorghum bicolor* L, and *Triticum aestivum* L. *Plant Soil* 196:143–149
- Heisey RM, DeFrank J, Putnam AR (1985) A survey of soil microorganisms for herbicidal activity. In: Thompson AC (ed) The Chemistry of allelopathy: biochemical interactions among plants, ACS Symposium Series, vol 268. American Chemical Society, Washington DC, pp 337–349

- Hoagland DR, Arnon DJ (1950) The water-bioassay method of growing plants without soil. *Calif Agric Exp Sta Circ* 347
- Hoagland RE, Williams RD (1985) The influence of secondary plant compounds on the associations of soil microorganisms and plant roots. In: Thompson AC (ed) *The chemistry of allelopathy: biochemical interactions among plants*, ACS Symposium Series, vol 268. American Chemical Society, Washington DC, pp 301–325
- Hocking PJ (1994) Dry-matter production, mineral nutrient concentrations, and nutrient distribution and redistribution in irrigated spring wheat. *J Plant Nutr* 17:1289–1308
- Hodge A, Grayston SJ, Ord BG (1996) A novel method for characterization and quantification of plant root exudates. *Plant Soil* 184:97–104
- Hoffman DW, Lavy TL (1978) Plant competition for atrazine. *Weed Sci* 26:94–99
- Huang Z, Haig T, Wu H, An M, Pratley JE (2003) Correlation between phytotoxicity on annual ryegrass (*Lolium rigidum*) and production dynamics of allelochemicals within root exudates of allelopathic wheat. *J Chem Ecol* 29:2263–2279
- Huang L-F, Song L-X, Mao W-H, Shi K, Zhou Y-H, Yu J-Q (2013) Plant-soil feedback and soil sickness: from mechanisms to application in agriculture. *J Chem Ecol* 39:232–242
- Hughes S, Reynolds B (1988) Cation exchange properties of porous ceramic cups: implications for field use. *Plant Soil* 109:141–144
- Inderjit (2005) Soil microorganisms: an important determinant of allelopathic activity. *Plant Soil* 274:227–236
- Jalonen R, Nygren P, Sierra J (2009) Transfer of nitrogen from a tropical legume tree to an associated fodder grass via root exudation and common mycelia networks. *Plant Cell Environ* 32:1366–1376
- Jones DL, Hodge A, Kuzyakov Y (2004) Plant and mycorrhizal regulation of rhizodeposition. *New Phytol* 163:459–480
- Jones DL, Nguyen C, Finlay RD (2009) Carbon flow in the rhizosphere: carbon trading at the soil-root interface. *Plant Soil* 321:5–33
- Kamara AY, Akobundu IO, Sanginga N, Jutzi SC (1999) Effects of mulch from 14 multipurpose tree species (MPTs) on early growth and nodulation of cowpea (*Vigna unguiculata* L.). *J Agron Crop Sci* 182:127–133
- Kaminsky R, Muller WH (1977) The extraction of soil phytotoxins using a neutral EDTA solution. *Soil Sci* 124:205–210
- Kaur R, Singh RS (2007) Study of induced systemic resistance in *Cicer arietinum* L. due to non-pathogenic *Fusarium oxysporum* using a modified split root technique. *J Phytopathol* 155:694–698
- Klein K, Blum U (1990) Inhibition of cucumber leaf expansion by ferulic acid in split-root experiments. *J Chem Ecol* 16:455–463
- Koch HJ, Matthiessen A, Baeumer K (1992) Agronomical risks of straw mulch covers in reduced soil tillage systems 1. Influence of chopping intensity of wheat straw on the liberation of phytotoxins. (in German). *J Agron Crop Sci* 169:184–192
- Kochhar M, Blum U, Reinert RA (1980) Effects of O₃ and (or) fescue on ladino clover: interactions. *Can J Bot* 58:241–249
- Koepe DE, Rohrbach LM, Rice EL, Wender SH (1969) The effects of varying U.V. intensities on the concentration of scopolin and caffeoylquinic acids in tobacco and sunflower. *Phytochemistry* 8:889–896
- Koepe DE, Rohrbach LM, Rice EL, Wender SH (1970) Tissue age and caffeoylquinic acid concentration in sunflower. *Phytochemistry* 9:297–301
- Kosslak RM, Bohlool BB (1984) Suppression of nodule development on one side of a split-root system of soybeans caused by prior inoculation of the other side. *Plant Physiol* 75:125–130
- Kotilainen T, Tegelberg R, Julkunen-Tiitto R, Lindfors A, O'Hara RB, Aphalo PJ (2010) Seasonal fluctuations in leaf phenolic composition under UV manipulations reflects contrasting strategies of alder and birch trees. *Physiol Plant* 140:297–309
- Kozlowski TT, Pallardy SG (2002) Acclimation and adaptive response of woody plants to environmental stress. *Bot Rev* 68:270–334

- Kumar V, Brainard DC, Bellinder RR (2009) Suppression of powell amaranth (*Amaranthus powellii*) by buckwheat residues: roles of allelopathy. *Weed Sci* 57:66–73
- Lambers H, Chapin FS III, Pons TL (1998) Plant physiological ecology. Springer, New York
- Lavelle P, Spain AV (2001) Soil ecology. Kluwer Academic Pub, Dordrecht
- Leão PN, Vasconcelos LMTSD, Vasconcelos VM (2009) Allelopathy in freshwater cyanobacteria. *Crit Rev Microbiol* 35:271–282
- Leather GR (1983a) Sunflowers (*Helianthus annuus*) are allelopathic to weeds. *Weed Sci* 31:37–42
- Leather GR (1983b) Weed control using allelopathic crop plants. *J Chem Ecol* 9:983–989
- Lehman ME (1993) Effects of allelopathy on plant emergence and growth as modified by physical factors and root distribution. North Carolina State University Thesis, Raleigh
- Lehman ME, Blum U (1997) Cover crop debris effects on weed emergence as modified by environmental factors. *Allelopathy J* 4:69–88
- Lehman ME, Blum U, Gerig TM (1994) Simultaneous effects of ferulic and *p*-coumaric acids on cucumber leaf expansion in split-root experiments. *J Chem Ecol* 20:1773–1782
- Levitt J (1972) Responses of plants to environmental stresses. Academic Press, New York
- Liebl RA, Worsham AD (1983) Inhibition of pitted morning glory (*Ipomoea lacunosa* L.) and certain other weed species by phytotoxic components of wheat (*Triticum aestivum* L.) straw. *J Chem Ecol* 9:1027–1043
- Long WG, Sweet DV, Tukey HB (1956) Loss of nutrients from plant foliage by leaching as indicated by radioisotopes. *Science* 123:1039–1040
- Lupwayi NZ, Clayton GW, Donovan JT, Harker KN, Turkington TK, Soon YK (2006) Nitrogen release during decomposition of crop residues under conventional and zero tillage. *Can J Soil Sci* 86:11–19
- Lynch J, Epstein A, Läuchli A, Weigt GI (1990) An automated greenhouse sand bioassay system suitable for studies of P nutrition. *Plant Cell Environ* 13:547–554
- Lyu S-W, Blum U (1990) Effects of ferulic acid, an allelopathic compound, on net P, K, and water uptake by cucumber seedlings in a split-root system. *J Chem Ecol* 16:2429–2439
- Macias FA, Molinillo JMG, Valera RM, Galindo JCG (2007) Allelopathy—a natural alternative for weed control. *Pest Manag Sci* 63:327–348
- Marshall DL, Abrahamson NJ, Avritt JJ, Hall PM, Medeiros JS, Reynolds J, Shaner GM, Simpson HL, Trafton AN, Walsh S (2005) Differences in plastic responses to defoliation due to variation in the timing of treatments for two species of *Sesbania* (Fabaceae). *Ann Bot* 95:1049–1058
- Martin P, Rademacher B (1960) Studies on the mutual influences of weeds and crops. In: Harper JL (ed) *The biology of weeds. A symposium of the British Ecological Society*, Oxford. Blackwell, Oxford, pp 143–152
- Martin JJ, Weidenhamer JD (1995) Potassium deficiency increases thiopene production in *Tagetes erecta* L. In: Gustine DL, Flores HE (eds) *Phytochemicals and health. Current topics in plant physiology, an American Society of Plant Physiologists Series*, vol 15, pp 277–279
- Medeiros CAB, Clark RB, Ellis JR (1993) Effects of MES [2(N-morpholino)-ethanesulfonic acid] and pH on mineral nutrient uptake by mycorrhizal and nonmycorrhizal maize. *J Plant Nutr* 16:2255–2272
- Mohney BK, Matz T, LaMoreaux 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
- Moody SF, Clarke AE, Bacic A (1988) Structural analysis of secreted slime from wheat and cowpea roots. *Phytochemistry* 27:2857–1861
- Moonen AC, Bärberi P (2006) An ecological approach to study the physical and chemical effects of rye cover crop residues on *Amaranthus retroflexus*, *Echinochloa crus-galli* and maize. *Ann Appl Biol* 148:73–89
- Mortimer PE, Pérez-Fernández MA, Valentine AJ (2008) The role of arbuscular mycorrhizal colonization in the carbon and nutrient economy of tripartite symbiosis with nodulated *Phaseolus vulgaris*. *Soil Biol Biochem* 40:1019–1027

- Mosse B, Thompson JP (1984) Vesicular-arbuscular endomycorrhizal inoculum production I. Exploratory experiments with beans (*Phaseolus vulgaris*) in nutrient flow bioassay. *Can J Bot* 62:1523–1530
- Muscolo A, Sidari M (2006) Seasonal fluctuations in soil phenolics of a coniferous forest: effects on seed germination of different coniferous species. *Plant Soil* 284:305–318
- Nakano H, Morita S, Shigemori H, Hasegawa K (2006) Plant growth inhibitory compounds from aqueous leachate of wheat straw. *Plant Growth Regul* 48:215–219
- Narasimhan K, Basheer C, Bajic VB, Swarup S (2003) Enhancement of plant-microbe interactions using a rhizosphere metabolomics-driven approach and its application in the removal of polychlorinated biphenyls. *Plant Physiol* 132:146–153
- Neary AJ, Tomassini F (1985) Preparation of alundum/ceramic plate tension lysimeters for soil water collection. *Can J Soil Sci* 65:169–177
- Neumann G, Römheld V (2007) The release of root exudates as affected by the plant physiological status. In: Pinton R, Varanini Z, Nannipieri P (eds) *The rhizosphere: biochemistry and organic substances at the soil-plant interface*, 2nd edn. CRC Press, Boca Raton, pp 23–72
- Newman EJ, Miller MH (1977) Allelopathy among some British grassland species II: influence of root exudates on phosphorus uptake. *J Ecol* 65:399–411
- Newmann G, George TS, Plassard C (2009) Strategies and methods for studying the rhizosphere—the plant science toolbox. *Plant Soil* 321:431–456
- Nilsen ET, Walker JF, Miller OK, Semones SW, Lei TT, Clinton BD (1999) Inhibition of seedling survival under *Rhododendron maximum* (Ericaceae): Could allelopathy be a cause? *Am J Bot* 86:1597–1605
- Ohno T, Doolan K, Zibilske LM, Liebman M, Gallandt ER, Berube C (2000) Phytotoxic effects of red clover amended soils on wild mustard seedling growth. *Agric Ecosyst Environ* 78:187–192
- Pandey DK (1994) Inhibition of *Salvinia* (*Salvinia molesta* Mitchell) by parthenium (*Parthenium hysterophorus* L.) II: relative effect of flower, leaf, stem, and root residue on *salvinia* and paddy. *J Chem Ecol* 20:3123–3131
- Pang J, Tibbett M, Denton MD, Lambers H, Siddique KHM, Ryan MH (2011) Soil phosphorus supply affects nodulation and N:P ratio in 11 perennial legume seedlings. *Crop Pasture Sci* 62:992–1001
- Patrick ZA (1971) Phytotoxic substances associated with the decomposition in soil of plant residues. *Soil Sci* 111:13–18
- Pedrol N, González L, Reigosa MJ (2006) Allelopathy and abiotic stresses. In: Reigosa MJ, Pedrol N, González L (eds) *Allelopathy: a physiological process with ecological implications*. Springer, Dordrecht, pp 171–209
- Percy K (1986) The effects of simulated acid rain on germinative capacity, growth and morphology of forest tree seedlings. *New Phytol* 104:473–484
- Pérez FJ, Ormeño-Núñez J (1991) Root exudates of wild oats: allelopathic effect on spring wheat. *Phytochemistry* 30:2199–2202
- Pramanik MHR, Nagai M, Asao T, Matsui Y (2000) Effects of temperature and photoperiod on phytotoxic root exudates of cucumber (*Cucumis sativus*) in hydroponic bioassay. *J Chem Ecol* 26:1953–1967
- Pikryl Z, Vančura V (1980) Root exudates of plants VI: wheat root exudation as dependent on growth, concentration gradient of exudates and the presence of bacteria. *Plant Soil* 57:69–83
- Pue KJ, Blum U, Gerig TM, Shafer SR (1995) Mechanism by which noninhibitory concentrations of glucose increase inhibitory activity of *p*-coumaric acid on morning-glory seedling biomass accumulation. *J Chem Ecol* 21:833–847
- Putnam AR, DeFrank J, Barnes JP (1983) Exploitation of allelopathy for weed control in annual and perennial cropping systems. *J Chem Ecol* 9:1001–1010
- Quayyum HA, Mallik AU, Leach DM, Gottardo C (2000) Growth inhibitory effects of nutgrass (*Cyperus rotundus*) on rice (*Oryza sativa*) seedlings. *J Chem Ecol* 26:2221–2231
- Rice EL (1984) *Allelopathy*. Academic Press, London
- Rice EL (1986) Allelopathic growth stimulation. In: Putnam AR, Tang C-S (eds) *The science of allelopathy*. Wiley, New York, pp 23–42

- Rovira AD (1969) Plant root exudates. *Bot Rev* 35:35–57
- Rovira AD, Foster RC, Martin JK (1979) Note on terminology: origin, nature and nomenclature of the organic materials in the rhizosphere. In: Harley JL, Russell RS (eds) *The soil-root interface*. Academic Press, London, pp 1–4
- Sampietro DA, Catalan CAN, Vattuone MA (2009) Isolation, identification and characterization of allelochemical/natural products. Science Publishers, Enfield
- Schenk NC (1982) *Methods and principles of mycorrhizal research*. American Phytopathological Society, St Paul
- Scherbatskoy T, Klein RM (1983) Response of spruce and birch foliage to leaching by acid mists. *J Environ Qual* 12:189–195
- Schmidt EL (1991) Methods for microbial autecology in the soil rhizosphere. In: Keister DL, Cregan PB (eds) *Beltsville symposium in agricultural research*, vol 14. The rhizosphere and plant growth. Kluwer Academic Press, Dordrecht, pp 81–89
- Schmidt SK, Ley RE (1999) Microbial competition and soil structure limit the expression of allelopathy. In: Inderjit, Dakshini KMM, Foy CL (eds) *Principles and practices in plant ecology: allelochemical interactions*. CRC Press, Boca Raton, pp 339–351
- Schomberg HH, Steiner JL (1999) Nutrient dynamics of crop residues decomposing on a fallow no-till soil surface. *Soil Sci Soc Am J* 63:607–613
- Shafer SR (1988) Influence of ozone and simulated acid rain on microorganisms in the rhizosphere of *Sorghum*. *Environ Pollut* 51:137–152
- Shafer SR (1992) Responses of microbial populations in the rhizosphere to deposition of simulated acid rain onto foliage and/or soil. *Environ Pollut* 76:267–278
- Shafer SR, Blum U (1991) Influence of phenolic acids on microbial populations in the rhizosphere of cucumber. *J Chem Ecol* 17:369–389
- Shafer SR, Grand LF, Bruck RI, Heagle AS (1985) Formation of ectomycorrhizae on *Pinus taeda* seedlings exposed to simulated rain. *Can J For Res* 15:66–71
- Shay FJ, Hale MG (1973) Effects of low levels of calcium on exudation of sugars and sugar derivatives from intact peanut roots under axenic conditions. *Plant Physiol* 51:1061–1063
- Shukla A, Kumar A, Jha A, Ajit, Rao DVKN (2012) Phosphorus threshold for arbuscular mycorrhizal colonization of crops and tree seedlings. *Biol Fertil Soils* 48:109–116
- Sinkkonen A (2001) Density-dependent chemical interference—an extension of the biological response model. *J Chem Ecol* 27:1513–1523
- Sinkkonen A (2003) A model describing chemical interference caused by decomposing residues at different densities of growing plants. *Plant Soil* 250:315–322
- Sinkkonen A (2007) Modeling the effects of autotoxicity on density-dependent phytotoxicity. *J Theor Biol* 244:218–227
- Siqueira JO, Nair MG, Hammerschmidt R, Safir GR (1991) Significance of phenolic compounds in plant-soil-microbial systems. *Crit Rev Plant Sci* 10:63–121
- Smith WH (1970) Technique for collection of root exudates from mature trees. *Plant Soil* 32:238–241
- Smith WH (1976) Character and significance of forest tree root exudates. *Ecology* 57:324–331
- Sokal RR, Rohlf FJ (2012) *Biometry: the principles and practices of biological research*. WH Freeman, New York
- Solar A, Colarič M, Usenik V, Stampar F (2006) Seasonal variation of selected flavonoids, phenolic acids and quinones in annual shoots of common walnut (*Juglans regia* L.). *Plant Sci* 170:453–461
- Sparling GP, Vaughan D (1981) Soil phenolic acids and microbes in relation to plant growth. *J Sci Food Agric* 32:625–626
- Staman K, Blum U, Louws F, Robertson D (2001) Can simultaneous inhibition of seedling growth and stimulation of rhizosphere bacterial populations provide evidence for phytotoxin transfer from plant residues in the bulk soil to the rhizosphere of sensitive species? *J Chem Ecol* 27:807–829
- Steel RGD, Torrie JH (1997) *Principles and procedures of statistics: a biometrical approach*, 3rd edn. McGraw-Hill, New York

- Summer LW, Mendes P, Dixon RA (2003) Plant metabolomics: large-scale phytochemistry in their functional genomics era. *Phytochemistry* 62:817–836
- Tang C-S (1986) Continuous trapping techniques for the study of allelochemicals from higher plants. In: Putnam AR, Tang C-S (eds) *The science of allelopathy*. Wiley, New York, pp 113–131
- Tang C-S, Young C-C (1982) Collection and identification of allelopathic compounds from the undisturbed root system of bigalita limpograss (*Hemarthria altissima*). *Plant Physiol* 69:155–160
- Tang C-S, Komai K, Huang RS (1989) Allelopathy and the chemistry of the rhizosphere. In: Chou CH, Waller GR (eds) *Phytochemical ecology: allelochemicals, mycotoxins, and insect pheromones and allomones*, Monograph Series. Institute of Botany, Academia Sinica, vol 9. Taipei, pp 217–226
- Toljander JF, Lindahl BD, Paul LR, Elfstrand M, Finlay RD (2007) Influence of arbuscular mycorrhizal mycelial exudates on soil bacterial growth and community structure. *FEMS Microbiol Ecol* 61:295–304
- Tukey HB Jr (1966) Leaching of metabolites from above-ground plant parts and its implications. *Bull Torrey Bot Club* 93:385–401
- Tukey HB Jr, Mecklenburg RA (1964) Leaching of metabolites from foliage and subsequent reabsorption and redistribution of the leachate in plants. *Am J Bot* 51:737–742
- Tukey HB Jr, Wittwer SH, Tukey HB (1957) Leaching of carbohydrates from plant foliage as related to light intensity. *Science* 126:120–121
- Vaughan D, Malcolm RE (1985) *Soil organic matter and biological activity*. Martinus Nijhoff/Dr W Junk Publishers, Dordrecht
- Vaughan D, Sparling GP, Ord BG (1983) Amelioration of the phytotoxicity of phenolic acids by some soil microbes. *Soil Biol Biochem* 15:613–614
- Vázquez M, Barea J, Azcón R (2001) Impact of soil nitrogen concentration on *Glomus* spp.-*Sinorhizobium* interactions as affecting growth, nitrate reductase activity and protein content of *Medicago sativa*. *Biol Fertil Soil* 34:57–63
- Ventura M, Scandellari F, Bonora E, Tagliavini M (2010) Nutrient release during decomposition of leaf litter in a peach (*Prunus persica* L.) orchard. *Nutr Cycl Agroecosyst* 87:115–125
- Waller GR, Feng M-C, Fujii Y (1999) Biochemical analysis of allelopathic compounds: plants, microorganisms, and soil secondary metabolites. In: Inderjit, Daskshini KMM, Foy CL (eds) *Principles and practices in plant ecology: allelochemical interactions*. CRC Press, Boca Raton, pp 75–98
- Wardle DA, Nicholson KS, Ahmed M (1992) Comparison of osmotic and allelopathic effects of grass leaf extracts on grass seed germination and radicle elongation. *Plant Soil* 140:315–319
- Waters ER, Blum U (1987) The effects of single and multiple exposures of ferulic acid on the vegetative and reproductive growth of *Phaseolus vulgaris* BBL-290. *Am J Bot* 74:1635–1645
- Weidenhamer JD, Morton TC, Romeo JT (1987) Solution volume and seed number: often overlooked factors in allelopathic bioassays. *J Chem Ecol* 13:1481–1491
- Weidenhamer JD, Hartnett DC, Romeo JT (1989) Density-dependent phytotoxicity: distinguishing resource competition and allelopathic interference in plants. *J Appl Ecol* 26:613–624
- Weidenhamer JD, Boes PD, Wilcox DS (2009) Solid-phase root zone extraction (SPRE): a new methodology for measurement of allelochemical dynamics in soil. *Plant Soil* 322:177–186
- Weston LA, Harmon R, Mueller S (1989) Allelopathic potential of sorghum-sudangrass hybrid (Sudex). *J Chem Ecol* 15:1855–1865
- Whitehead DC (1964) Identification of *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids in soils. *Nature* 202:417–418
- Whitehead DC, Dibb H, Hartley RD (1981) Extractant pH and the release of phenolic compounds from soils, plant roots and leaf litter. *Soil Biol Biochem* 13:343–348
- Whitehead DC, Dibb H, Hartley RD (1982) Phenolic compounds in soil as influenced by the growth of different plant species. *J App Ecol* 19:579–588
- Wilson RE, Rice EL (1968) Allelopathy as expressed by *Helinathus annuus* and its role in old-field succession. *Bull Torrey Bot Club* 95:432–448

- Witzell J, Gref R, Näsholm T (2003) Plant-part specific and temporal variation in phenolic compounds of boreal bilberry (*Vaccinium myrtillus*) plants. *Biochem Syst Ecol* 31:115–127
- Wu H, Pratley J, Lemerle D, Haig T (2000a) Laboratory screening of allelopathic potential of wheat (*Triticum aestivum*) accessions against annual ryegrass (*Lolium rigidum*). *Aust J Agric Res* 51:259–266
- Wu H, Pratley J, Lemerle D, Haig T (2000b) Evaluation of seedling allelopathy in 453 wheat (*Triticum aestivum*) accessions against annual ryegrass (*Lolium rigidum*) by the equal-compartment-agar method. *Aust J Agric Res* 51:937–944
- Wu H, Haig T, Pratley J, Lemerle D, An M (2001a) Allelochemicals in wheat (*Triticum aestivum* L.): cultivar differences in the exudation of phenolic acids. *J Agric Food Chem* 49:3742–3745
- Wu H, Pratley J, Lemerle D, Haig T, An M (2001b) Screening methods for evaluation of crop allelopathic potential. *Bot Rev* 67:403–415
- Wu H, Pratley J, Lemerle D, An M, Lui DL (2007) Autotoxicity of wheat (*Triticum aestivum* L.) as determined by laboratory bioassays. *Plant Soil* 296:85–93
- Yang L, Wang P, Kong C (2010) Effects of larch (*Larix gmelini* Rupr.) root exudates on Manchurian walnut (*Juglans mandshurica* Maxim.) growth and soil juglone in a mixed-species plantation. *Plant Soil* 329:249–258
- Yenish JP, Worsham AD, Chilton WS (1995) Disappearance of DIBOA-glucoside, DIBOA, and BOA from rye (*Secale cereale* L.) cover crop residue. *Weed Sci* 43:18–20
- Yu Z, Dahlgren RA (2000) Evaluation of methods for measuring polyphenols in conifer foliage. *J Chem Ecol* 26:2119–2140
- Yu JQ, Matsui Y (1994) Phytotoxic substances in root exudates of cucumber (*Cucumis sativus* L.). *J Chem Ecol* 20:21–31
- Yu JQ, Matsui Y (1997) Effects of root exudates of cucumber (*Cucumis sativus*) and allelochemicals on ion uptake by cucumber seedlings. *J Chem Ecol* 23:817–827
- Zabowski D, Ugolini FC (1990) Lysimeter and centrifuge soil solutions: seasonal difference between methods. *Soil Sci Soc Am J* 54:1130–1135
- Zhang Z-Y, Pan L-P, Li H-H (2009) Isolation, identification and characterization of soil microbes which degrade phenolic allelochemicals. *J Appl Microbiol* 108:1839–1849
- Zhang Y, Gu M, Shi K, Zhou YH, Yu JQ (2010) Effects of aqueous root extracts and hydrophobic root exudates of cucumber (*Cucumis sativus* L.) on nuclei DNA content and expression of cell cycle-related genes in cucumber radicles. *Plant Soil* 327:455–463
- Zhao D, Oosterhuis DM (1999) Dynamics of mineral nutrient element concentrations in developing cotton leaves, bracts, and floral buds in relation to position in the canopy. *J Plant Nut* 22:1107–1122
- Zhu L-H, Wu X-Q, Qu H-Y, Ji J, Ye J (2010) Micropropagation of *Pinus massoniana* and mycorrhiza formation in vitro. *Plant Cell Tiss Organ Cult* 102:121–128

Plant-Plant Allelopathic Interactions II
Laboratory Bioassays for Water-Soluble Compounds
with an Emphasis on Phenolic Acids

Blum, U.

2014, XXI, 322 p. 27 illus., Hardcover

ISBN: 978-3-319-04731-7