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Plant Phenolics in Allelopathy

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I. Abstract

Phenolics are one of the many secondary metabolites implicated in allelopathy. To establish that allelopathy functions in a natural ecosystem, the allelopathic bioassay must be ecologically realistic so that responses of appropriate bioassay species are determined at relevant concentrations. It is important to isolate, identify, and characterize phenolic compounds from the soil. However, since it is essentially impossible to simulate exact field conditions, experiments must be designed with conditions resembling those found in natural systems. It is argued that allelopathic potential of phenolics can be appreciated only when we have a good understanding of 1) species responses to phenolic allelochemicals, 2) methods for extraction and isolation of phenolic allelochemicals, and 3) how abiotic and biotic factors affect phenolic toxicity.

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II. Introduction

Allelopathy, as defined by Rice (1984) is any direct or indirect beneficial or harmful effect of one plant (including microorganisms) on the other through release of chemicals into the environment. The term “allelochemicals” derives from “allelochemics,” coined by Whittaker and Feeny (1971), and was first used by Chou and Waller in 1983. Since then, the term has been used in literature dealing with interspecific chemical interactions between organisms. In order for a compound to be designated an allelochemical, its release into or its origin in the environment must be demonstrated. Allelochemicals are released into the environment through leaching of living plant parts, root exudates, volatilization, residue decomposition, microbial activity, and agricultural practices such as plowing of plant residues into the soil (Muller, 1966, 1969; Putnam & Tang, 1986; Inderjit & Dakshini, 1992b, 1994a, 1995b, 1996).

Many different secondary metabolites—e.g., phenolics, terpenoids, alkaloids, polyacetylenes, fatty acids, and steroids—can act as allelochemicals (Rice, 1984; Waller, 1987; Inderjit et al., 1995). These chemicals are present in various plant parts; however, their mere presence does not establish allelopathy (Putnam & Tang, 1986; Heisey, 1990). To demonstrate their involvement in allelopathy, it is important to establish 1) their direct release or indirect origin from plant-derived materials in the environment and 2) that the chemicals are present in sufficient quantities and persist for a sufficient time in the soil to affect plant species or microbes (Putnam & Tang, 1986).

The overwhelming evidence indicates that phenolics do play a significant role in allelopathy. Several researchers have reviewed the biochemistry and ecology of phenolics (Levin, 1971; Harborne, 1989a; Kuiters, 1990; Siqueira et al., 1991; Appel, 1993; Waterman & Mole, 1994). Phenolics have been implicated as having a role in allelopathic interactions among different groups of plants such as algae, fungi, lichens, bryophytes, pteridophytes, gymnosperms, and angiosperms (Rice, 1979; Fisher, 1987; Inderjit & Dakshini, 1994d; Lawrey, 1995). The objective of the present review is to describe how the role of plant phenolics in allelopathy may be characterized through bioassays and through extraction and isolation from soils.

III. Types of Laboratory Bioassays

A suitable bioassay is necessary in order to investigate the role of phenolic compounds in allelopathy. The purpose of bioassays could be 1) to demonstrate allelopathic potential, if any, of isolated phenolic compounds; 2) to determine at what concentration phenolic compounds are allelopathically active; and 3) to determine the percent of recovery and fate of phenolic compounds in soil. Generally, the concentration of phenolic compounds in soil is much less than that in plant parts. However, in most instances, phenolic mixtures of low individual concentrations are involved (Rice, 1987; Dalton, 1989; Einhellig, 1995a). The bioactive concentration of phenolics in soils largely determines the observed allelopathic effects.

Various types of laboratory bioassays have been conducted to investigate the allelopathic potential of phenolic compounds (Leather & Einhellig, 1986; Inderjit & Dakshini, 1995a). Seed germination is an important parameter for evaluating allelopathic potential of phenolic compounds (Rice, 1984; Waller, 1987). However, using seed germination as a bioassay parameter may be of little value (Stowe, 1979; Inderjit & Dakshini, 1995a). This is because allelopathic interactions include both promotory and inhibitory activities

of phenolic allelochemicals. In the case of 100% germination in control setups, it would not be possible to assess the stimulatory effects, if any, of phenolic compounds.

Shilling and Yoshikawa (1987) developed a bioassay to determine biological activity of allelochemicals quantitatively and qualitatively. They evaluated the allelopathic potential of *a*-phenylacetic and *p*-ethoxybenzoic acids using *Echinochloa crusgalli* and *Sesbania exaltata* as test species. It was suggested that seedling bioassay using shoot-plus-root fresh weights were an appropriate method for evaluating the phytotoxicity of biological material. However, in many studies, it was found that root growth was more sensitive than shoot growth (Inderjit & Dakshini, 1995a). Moreover, measuring fresh weights of roots and shoots separately should give a better evaluation of phytotoxicity of allelopathic compounds. Blum and Rebbeck (1989) discovered the inhibition and subsequent recovery of root elongation following removal from ferulic acid solution. Ferulic acid-treated roots were shorter and more delicate but were branched. The researchers suggested that ferulic acid may directly or indirectly stimulate the initiation of secondary roots. Allelopathic compounds are unlikely to affect the whole root system at a given point in time (Lyu & Blum, 1990). Blum and Rebbeck (1989) reported that influence of ferulic acid on net uptake of P, K, and water relations by cucumber seedlings was related to the portion of roots that came into contact with ferulic acid. Many studies have used lettuce as a bioassay species because of its sensitivity (Einhellig et al., 1985; Waller, 1987; Li et al., 1993). Chou and Patrick (1976) identified butyric, phenylacetic, 4-phenylbutyric, benzoic, *p*-hydroxybenzoic, vanillic, ferulic, syringic, *p*-coumaric, *t*-coumaric, and caffeic acids, resorcinol, phloroglucinol, and salicylaldehyde from decomposing corn residues; and ferulic, vanillic, phenylacetic, 4-butyric, *p*-coumaric, *p*-hydroxybenzoic, salicylic, and *o*-coumaric acids and salicylaldehyde from decomposing rye residues. They tested the allelopathic effects of these compounds on lettuce growth and found that phenylacetic, 4-phenylbutyric, salicylic, benzoic, and *o*-hydroxyphenylacetic acids were inhibitory to lettuce growth at the level of 25–50 ppm, whereas the rest of the phenolics were allelopathically active at ca. 100 ppm concentrations. Nicollier et al. (1983) isolated dhurrin from johnson grass (*Sorghum halepense*) rhizomes and used tomato (*Lycopersicon esculentum*) and radish (*Raphanus sativus*) as bioassay species. However, neither of the bioassay species was associated with johnson grass in natural systems. These types of bioassays are good for studying the mechanism of action of phenolic allelochemicals but are of little value in identifying allelopathic potential of phenolic compounds in natural systems. In the interest of selecting appropriate test species, artificially sensitive species should be avoided.

Dornbos and Spencer (1990) proposed an agar bioassay suitable for even small quantities of slightly water-soluble compounds. They evaluated germination and growth of alfalfa (*Medicago sativa*), annual ryegrass (*Lolium multiflorum*), and velvetleaf (*Abutilon theophrasti*) against known allelochemicals such as cinmethylin, coumarin, plumbagin, nigericin, juglone, and *trans*-cinnamaldehyde. Each compound was dissolved in 1 ml chloroform-hexane (1:99, v/v) mixture, and 1 ml of each solution was placed on water-agar in small tissue wells. After the solvent evaporated, seeds were placed on agar, and data on germination and seedling growth were collected after 3 days. Inoue et al. (1992) isolated two anthraquinones, emodin and physcion, from rhizomes of *Polygonum sachalinense*. However, they used TLC agar plate and seedling growth (0.5% agar gel) bioassays to evaluate allelopathic potential of test solutions. Li et al. (1993) studied interactions of *trans*-cinnamic, *o*-, *m*-, *p*-coumaric, and chlorogenic acids, and found that the phenolic compounds influence lettuce growth regardless of their concentration. These

studies have little ecological significance, however, because no soils were involved in the bioassays. On entry of phenolic allelochemicals into the soil, processes such as transport, retention, and transformation may influence the quantitative and qualitative availability of phenolic allelochemicals (Cheng, 1995). Furthermore, edaphic factors such as moisture regime, nutrient status, soil temperature, and organic matter content may also affect the availability and action of allelochemicals.

McPherson and Muller (1969) reported that *Adenostoma fasciculatum* exhibited an allelopathic influence over its associated plant species. Kaminsky (1981) found that the observed allelopathic interference of *Adenostoma* was due not to chemicals released by it but to associated microorganisms. He suggested that allelochemicals, mainly phenolics, released by *Adenostoma* may be responsible for the presence of microorganisms producing toxic allelochemicals. In a discussion of laboratory allelopathic bioassays, Inderjit and Dakshini (1994a) noted the significance of analyses of soil chemical characteristics—e.g., pH, electrical conductivity, and nutrients—in addition to phenolics. They suggested that addition of phytotoxic material to the soil, in differing concentrations, should be followed by chemical analyses of the soil. On addition of phytotoxic material, soil conditions will certainly be altered but should not be different from natural soils. This is important, for any difference in soil conditions can lead to quantitative and qualitative variations in the availability of phenolics (Dalton et al., 1983; Blum et al., 1987; Dao, 1987; Dalton, 1989).

The total phenolics are mainly the sum of soluble polymers (tannins) and monomers (phenolic acids and flavonoids). It is important to determine the total phenolic content of the soil associated with the donor plant in order to assess the probable involvement of phenolics in allelopathy. Allelopathic effects are mostly the result of additive or partially antagonistic activity of allelochemicals rather than of a single compound (Williams & Hoagland, 1982; Dalton, 1989). Under field conditions, additive or partially antagonistic effects become more influential even at low concentrations, as compared to effects of individual compounds (Einhellig et al., 1982; Williams & Hoagland, 1982). Since different phenolic compounds included in the mixture have different effects, total phenolic content is good in a relative sense rather than an absolute sense. That is why identification and characterization of each compound is desirable. Sorgoleone, a quinone oxidation product of a hydroquinone precursor from root exudates of sorghum, is highly lipophilic, and bioassays show sorgoleone to be more phytotoxic than phenolics (Netzly et al., 1988; Einhellig & Souza, 1992). However, no single compound was present in great enough bioactive concentration to cause inhibition in natural systems (Einhellig, 1995b). Ben-Hammouda et al. (1995) studied the chemical basis for differential allelopathic potential of *Sorghum* hybrids and reported that the concentration of *p*-hydroxybenzoic, vanillic, syringic, *p*-coumaric, and ferulic acids differed in seeds, glumes, leaves, culms, and roots of the three *Sorghum* hybrids. They concluded that the allelopathic potential of sorghum plants was positively correlated with total phenolic content. However, the relationship was more qualitative than quantitative within plant parts. It is important, therefore, to investigate mixtures of phenolic compounds in addition to individual phenolic compounds. While several phenolic compounds are present in plants, not all of them are necessarily allelopathically important. In spite of the presence of gallic, syringic, chlorogenic, vanillic, caffeic, ferulic, and coumaric acids in *Chenopodium album*, only chlorogenic acid was identified as a principle allelochemical (Mallik et al., 1994). However, this may be due to the selection of a particular bioassay procedure rather than to the allelopathic potential of a particular phenolic compound. Many important allelopathic responses

may have been overlooked simply because of the use of single phenolic compounds in determining allelopathic effects (Rice, 1987).

IV. Types of Field Studies

Certain bioassay techniques have been developed to isolate phenolic allelochemicals in natural systems. Tang and Young (1982) developed a continuous root-exudate-trapping system to isolate and identify allelochemicals from the root environment of undisturbed plants growing in pots. They isolated 3-hydroxyhydrocinnamic, benzoic, phenylacetic, and hydroxycinnamic acids from the undisturbed root system of *Hemarthria altissima*. Grodzinsky (1987) devised a method for the isolation of phenolics such as cinnamic, *p*-coumaric, and *p*-hydroxybenzoic acids by using ion-exchange resins under wheat, rye, and other cereals. This method helps isolate allelochemicals without destroying humus complexes. A comparative study of soils planted with allelopathic and non-allelopathic plants may indicate how the donor plants change soil phenolic content. Many field studies were carried out to study the effect of seasonal changes, natural disturbances, and soil and biotic factors on the quantitative and qualitative availability of phenolic allelochemicals (Lodhi, 1975, 1976; Turner & Rice, 1975).

Phenolics may also influence accumulation and availability of soil nutrients and rates of nutrient cycling, both of which ultimately affect plant growth (Lyu & Blum, 1990; Schlesinger, 1991; Inderjit & Dakshini, 1992b). For example, if phenolic compounds affect the accumulation and availability of PO₄, then the growth of PO₄-requiring plants would be adversely affected. Phenolics could compete for anion absorption sites on clay and humus and could also bind to soluble aluminium, iron, and manganese, which otherwise bind to phosphate; thus phenolics can increase phosphate availability (Tan & Binger, 1986; Kafkafi et al., 1988; Appel, 1993). Phenolics, by forming complexes with nutrients, affect rates of productivity and nutrient turnover in the substratum (Appel, 1993). Palm and Sanchez (1991) showed that the phenolic content of leaves of certain tropical legumes affect nutrient release from leaves. Northup et al. (1995) reported that *Pinus muricata* can influence the release of dissolved organic nitrogen in soils through production of polyphenol in leaf litter. Their results would help to explain the fact that plants on nutrient-poor soils often have high concentrations of phenolics (Chapin, 1995). Therefore, it is important to investigate the influence of phenolic compounds on soil nutrients in allelopathic studies.

V. Seasonal Changes and Natural Disturbances

Lodhi (1975) investigated ecological implications of allelopathic interference in terms of soil-plant interactions. He isolated ferulic, caffeic, and *p*-coumaric acids from soils under heckberry (*Celtis laevigata*) trees. Phytotoxicity of each phenolic acid was highest in January and April and lowest in September. However, he used NaOH to extract phenolic acids from soil, which may have also extracted tightly bound phenolics that could not be involved directly in allelopathic interactions. Lodhi (1976) found that soils collected in January, April, and August under sycamore, hackberry, red oak, and white oak inhibited seed germination and radicle growth of *Bromus japonicum* and *Elymus canadensis*. However, phenolic concentration in soils collected in August were not as high as those collected in January and April. The highest concentration of caffeic acid in humus of bilberry was recovered during snowmelt in May (Gallet & Lebreton, 1995). Kuiters and

Denneman (1987) reported that the phenolic acid content increased in autumn and declined in spring. They argued that the decline of phenolic acids in spring was the result of increased microbial activity due to high soil temperature and to the soil's adsorption of organic matter during humification. De Scisciolo et al. (1990) reported that the juglone concentration in soil was higher in spring and fall than in summer. However, they questioned the allelopathic effect of juglone under natural conditions. Lodhi (1978) suggested that phenolic content of soils may vary significantly from one stand to another of the same plant species. In order better to evaluate the allelopathic potential of donor plants, studies should be conducted at different sites and at different times of the year. Phytotoxicity of *Polygonella myriophylla* varied during the year due to certain environmental factors such as rainfall and temperature (Weidenhamer & Romeo, 1989). The actual allelopathic effect of the same volume of soil from the same site may differ according to the time of year (Jalal et al., 1982; Jalal & Read, 1983). Since seasonal changes play an important role in the availability and effectiveness of phenolics as allelopathic agents, it is important to monitor soil phenolic levels during different times of the year rather than sampling at just one time of year. In addition, the quantitative and qualitative changes in phenolic compounds may occur during succession (Rice, 1979).

Natural disturbances such as forest fire have been shown to increase significantly the bacteria:fungi ratio in soil and to alter proportions of *Streptomyces* spp. (Perry & Choquette, 1987). Forest fire further results in reduction in concentrations of the iron chelator hydroxymate siderophores, and iron oxides play an important role in breakdown of certain phenolic compounds (Perry & Choquette, 1987). Tanrisever et al. (1987) isolated a novel dihydrochalcone, ceratiolin, from *Ceratiola ericoides*. However, it was reported that, when exposed to light, heat, and acidic conditions, ceratiolin decomposed into the more potent allelochemical hydrocinnamic acid, which is highly active against grasses (Fischer et al., 1994). Aliotta et al. (1994) isolated 5-methoxypsoralen, 8-methoxypsoralen, and 4-hydroxy-coumarin from the aqueous extracts of *Ruta graveolens*, which are biologically active in the presence of light. Various other abiotic factors such as ozone, heavy metals, and herbicides are also known to enhance the allelochemical levels in plants (Rice, 1984). However, few attempts have been made to study the effect of these factors on the release of phenolic allelochemicals into the environment. Research along these lines would be worthwhile.

VI. Soil Factors

Soil is a very complex physical, chemical, and biological system. It affects quantitative and qualitative availability of phenolics and, hence, allelopathic responses of the plant (Haider & Martin, 1975; Blum & Shafer, 1988; Dalton et al., 1989a, 1989b; Cheng, 1995). When evaluating the involvement of phenolics in natural environments, it is important to consider the physiochemical properties of the soil and the role of soil microbes utilizing or converting phenolics to less toxic or more toxic forms (Dao, 1987). Once phenolic allelochemicals enter the soil system, processes such as retention, transformation, and transport may take place (Cheng, 1995). Retention processes retard the allelochemical movement in the environment. Ponder and Tadros (1985) found that juglone concentrations decrease with soil depth and distance from walnut trees. Huang et al. (1977) studied the retention of phenolic acids, *p*-hydroxybenzoic, *p*-coumaric, ferulic, syringic, and vanillic acids by non-crystalline hydroxy-aluminum and iron compounds and soil clay minerals. Catalytic polymerization of phenolic compounds by clay minerals was sug-

gested by Wang et al. (1978). Phenolic acids are known to be adsorbed by clay minerals and iron-hydroxides (Huang et al., 1977; Kogel & Zech, 1985). The ortho-substituted phenolics such as salicylic and *o*-coumaric acids, and dihydro-substituted phenolics such as protocatechuic and caffeic acids, are adsorbed by clay minerals by forming chelate complexes with metals (Shindo & Kuwatsuka, 1975). Vance et al. (1986) reported that the content of monomeric phenolics in soil is related to the stage of soil podzolization. Dalton et al. (1989b) studied the differential sorption of exogenously applied ferulic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids in Cecil, Portsmouth, and White Store soils. They found significant sorption of all compounds in all soils. The recovery of these phenolic acids varied with soil type, soil horizon, time, and type of functional group present on the aromatic ring of the phenolic compound. The transformation process changes the form of the compound, leading to its partial or complete degradation. The transport processes determine the way in which phenolic allelochemicals move in the soil environment. Soil variables such as pH, nutrients, organic matter, ion-exchange characteristics, and oxidation state play an important role in the fate of phenolics in soil.

Various workers have highlighted the significance of soil texture in explaining allelopathy (Del Moral & Cates, 1971; Inderjit & Dakshini, 1995a). Kuiters and Danneman (1987) found a larger amount of mild alkaline-extractable phenolics in sandy soil than in loamy soil. Therefore, in allelopathic bioassays, the texture of the soil used in laboratory experiments should be the same as that of the soil where the allelopathic plant occurs naturally.

Allelopathic effects of phenolic compounds may increase in nutrient-poor soils (Putnam, 1985; Einhellig, 1995a). Lehman and Rice (1972) found that the increased levels of caffeoylquinnic acids in sunflowers were caused by deficiencies in nitrogen, potassium, and sulphur. Koeppe et al. (1976) found that more phenolic allelochemicals were released from living roots, dried roots, and tops of phosphate-deficient plants than from those of phosphate-sufficient plants. Hall et al. (1982) found that the total phenolic content of *Helianthus annuus*, expressed as chlorogenic acid equivalent, increased as nutrient stress increased, and had a significant inhibitory effect on seed germination of *Amaranthus retroflexus*. However, when added to soil in a pure form, chlorogenic acid did not inhibit seed germination of *A. retroflexus* (Hall et al., 1982). Stowe and Osborn (1980) studied the influence of nitrogen and phosphorus on the phytotoxicity of phenolic compounds and found that phenolic allelochemicals were inhibitory at low nutrient concentration. Klein and Blum (1990) suggested that allelochemical activity of phenolic acids may play a significant role in limiting or not limiting soil nutrients for certain species.

The concentration of water-soluble phenolics, particularly polymers, is influenced by soil organic matter (Kuiters & Denneman, 1987). Kuiters and Denneman (1987) reported that the concentration of water-soluble phenolics was positively correlated to a soil's total carbon content. Ponder (1987) found that soil pH and soil organic matter were positively correlated to soil juglone concentration. The allelopathic activity of hydrocinnamic acid, a photochemical-degradation product of ceratiolin released from *Ceratiola ericoides*, was enhanced by low levels of nitrogen and potassium (Fischer et al., 1994). Blum et al. (1993) reported that the concentration of phenolic allelochemicals was correlated to soil pH, soil moisture, total soil carbon, and total soil nitrogen. They found that organic constituents such as glucose and methionine and inorganic constituents such as NO₃-N influenced the inhibitory activity of *p*-coumaric acid on biomass production of morning glory (*Ipomoea hederacea*) seedlings. Pue et al. (1995) found that non-inhibitory levels of glucose, an important carbon source, enhanced the inhibitory activity of *p*-coumaric acid on morning

glory (*Ipomoea hederacea*) seedling biomass in Cecil-B₁-horizon soil. According to the hypothesis proposed by Blum et al. (1993), glucose may reduce the microbial metabolism of *p*-coumaric acid and/or reduce the sorption of *p*-coumaric acid onto the soil particles. Blum et al. (1994) found that the sorption of phenolic acid to soil particles was positively correlated to the amount of phenolic acid added to the soil. Dalton et al. (1983) found that phenolic acid sorption increased with an increase in soil organic matter and that multiple-valent cation content and sorption were greatest under neutral to slightly alkaline conditions.

Phenolic compounds may undergo aerobic and anaerobic metabolism in soil (Dao, 1987). Haider and Martin (1975) reported that ¹⁴C-labeled benzoic and cinnamic acids were aerobically catabolized within two weeks of entering the soil. Under aerobic conditions, therefore, "free" phenolic acids may not occur in the soil (Dao, 1987). Free phenolic compounds may also undergo anaerobic degradation because of poor drainage or flooded soils (Dao, 1987). Dao (1987) suggested that microbial populations shift to facultative anaerobic organisms as soil water content increases, thus resulting in the breakdown of phenolic compounds through fermentation. In many allelopathic studies, phytotoxic plant parts are added to soils and then the amended soils are flooded (Rice, 1984). This technique may effect qualitative changes in phenolic profiles and quantitative changes in total phenolic content of the amended soils; the existence of such conditions in natural systems should be confirmed before designing an experiment that includes this type of amending. Bioassays using only synthetic reference compounds, in liquid culture or agar, indicate the probable allelopathic potential of the compound. In order to establish the expression of observed allelopathic potential of the compound under field conditions, however, it is necessary to use soil under actual or simulated field conditions.

VII. Biotic Factors

Biotic factors such as plant density, growth stage, microbial population, and age of donor plant affect the availability and activity of phenolics (Rice, 1984). Evans (1980) found that low-molecular-weight phenolics in the canopy increased as the proportion of coniferous to deciduous tree species increased. Whitehead et al. (1982) demonstrated that the growth of different plants is a significant factor influencing the concentration of soil phenolics. They studied 14 plots on which pteridophytes and graminaceous and dicotyledonous species had been grown individually for 16 years; using four extractants in the soil, the researchers found differences among the plots in the amounts released of phenolic compounds such as vanillin, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzoic, vanillic, *p*-coumaric, and ferulic acids. Weidenhamer et al. (1989) demonstrated that phytotoxicity decreases as plant density increases, since, at higher densities, each plant receives a smaller amount of each available allelochemical. Kimber (1973) found higher phytotoxicity in the green straw than in the mature straw of many legumes and grasses. Weston et al. (1989) isolated two phytoinhibitors, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde, from aqueous extracts of sudex (*Sorghum*-sudangrass hybrid) shoot tissue. Comparing the sudex tissue at 7 days and 28 days of age, they reported that as sudex tissue matured, both the two compounds and the allelopathic potential of shoot tissue significantly decreased. Studying Norway spruce, Gallet and Lebreton (1995) reported that only one-third of the monomeric phenolics recovered from the green foliage was recovered from brown foliage. They also reported that protocatechuic and *p*-coumaric acids in brown foliage were at 20–30% of the levels found in green foliage, that *p*-hydroxyacetophone

in brown foliage was at less than 5% the level found in green foliage, and that *p*-hydroxybenzaldehyde and catechol had totally disappeared from the brown foliage. Inderjit and Dakshini (1995c) reported no quantitative difference in phenolic content between soils with and without the annual weed *Polypogon monspeliensis*. However, *Polypogon* contributes phenolics, quantitatively and qualitatively, to the soils through incorporated straw. High concentrations of ferulic acid were detected under *Pinus nigra* and *Picea abies*, and high concentrations of benzoic acid were detected under *Quercus ruber* (Kuiters & Denneman, 1987). However, polyphenol concentrations were highest in *Picea* soils and lowest in *Pinus* soils, and concentrations were correlated positively to soil organic matter. Inderjit and Dakshini (1994c) reported that the total phenolic content of cultivated fields infested by *Pluchea lanceolata* was higher than that of uncultivated areas. Inderjit and Dakshini (1995b) found that quercetin concentrations in soils associated with *Pluchea lanceolata* were higher with regular cultivation. Compared to soils that are cultivated once a year, phenolic content of *P. lanceolata*-infested soils was higher in soils cultivated twice a year (Inderjit & Dakshini, 1996). It is important, therefore, to study phenolic availability in relation to agricultural practices, particularly in the case of crop residues and cropland weeds.

Microorganisms have been widely implicated in allelopathy (Rice, 1984; Blum, 1995). The fungus *Pullularia fermentans* var. *candida* is reported to degrade rutin into phloroglucinol, protocatechuic acid, and 2-protocatechuoyl phloroglucinol carboxylic acid (Hattori & Naguchi, 1959). Turner and Rice (1975) found that the microbes *Rhodotorula rubra* and *Cephalosporium curtipes* metabolized ferulic acid into vanillic acid, then into protocatechuic acid, and finally into β -keto-adipic acid. Microbial degradation of ferulic acid to a more toxic styrene derivative has been reported (Liebl & Worsham, 1983). Kaminsky (1981) discussed the microbial origin of allelochemicals that were mainly responsible for *Adenostoma* soil toxicity. Metabolites of many microorganisms play important roles in phytotoxicity of decomposing plant residues (Chapman & Lynch, 1983). Blum and Shafer (1988) found that in the presence of adequate nutrients, phenolic compounds present in the soils were utilized by microorganisms. Gallet and Lebreton (1995) reported rapid disappearance of *p*-hydroxyacetophone in spruce and of caffeic acid in bilberry. They reported the amount of compounds to be 5 mg g^{-1} in needles, $150 \text{ } \mu\text{g}^{-1}$ in litter, and $<0.3 \text{ } \mu\text{g}^{-1}$ in water-soluble humus. They hypothesized that this reduction resulted from the activity of specialized microbes. Rettenmaier et al. (1983) isolated the bacterium *Pseudomonas putida* from soils that convert juglone to 2-hydroxymuconic acid. Schmidt (1988) isolated a similar bacterium from the soil beneath black walnut trees. This bacterium could utilize juglone as its sole carbon source. Depending on abiotic and biotic soil factors, juglone might not persist in phytotoxic levels in the field (Schmidt, 1990).

VIII. Extraction and Isolation of Phenolic Allelochemicals

Most of the phenolics identified as allelochemicals have been extracted from plant material. Torti et al. (1995) compared the use of a conventional sonicator/shaker bath with the use of a homogenizer to extract phenolic compounds from fresh leaves of *Acomastylis rossii* and *Ouratea lucens*. They reported a higher yield of phenolic compounds with the homogenizer technique. However, there still remains the problem of relating plant extracts to allelopathic effects. Certain compounds present in plant components may show an inhibitory effect on test species but may not leach or exude from the plant under natural conditions. To establish the actual involvement of phenolics in allelopathy, it is desirable

to collect data on 1) bioactive concentration of phenolics in the medium (i.e., whether the concentrations at which they are active are actually present in the environment), 2) the residence time and the static and dynamic availability of the phenolics, and 3) the additive or partially antagonistic activity of the phenolic compound.

The quantitative concentration of phenolic compounds can be determined by two groups of methods: 1) chemical assays, which include redox assays, metal-binding assays, and assays based on specific chemical activity (Hagerman & Butler, 1991); and 2) protein-binding assays, which are used to determine tanning capacity of phenolic compounds (Mole & Waterman, 1987). Waterman and Mole (1994) discussed the various methods for determining total phenolics, such as the Folin-Denis, Folin-Ciocalteu, and Price-Butler methods. Because of the reactivity of non-phenolic compounds, these methods should not be used to determine the absolute concentration of total phenolics. Box (1983) suggested that the Folin-Ciocalteu method should be used to determine the variation in the concentration, rather than the absolute concentration, of humic substances, and the results depend on the standard used. The Folin-Denis method (1912) quantifies the concentration of easily oxidized phenolic compounds by color changes with redox reaction. The Folin-Ciocalteu method (1927) improved upon the Folin-Denis method by adding lithium sulfate to the reagent. This salt reduces the amount of precipitate that can form when high concentrations of reagent are used to enhance reactivity in the assay. However, both methods have the common problem of reacting with many oxidizable phenolic compounds (Van Alstyne, 1995). Gallet and Lebreton (1995) reported that, due to methodological problems, the amount of total phenolics in Norway spruce and bilberry (70 and 108 mg/g⁻¹, respectively) was not consistent.

The wavelength of measurement is 720 nm in the Price-Butler method. Harborne (1989b) and Swain and Hillis (1959) suggested a 725 nm wavelength in the Folin-Denis and Folin-Ciocalteu methods, while Waterman and Mole (1994) suggested 760 nm for these same methods. The other reagent methods for estimation of total phenolics are 1) vanillin-HCl, wavelength of 500 nm (Swain & Hillis, 1959); 2) titanium chloride, wavelength of 405–450 nm; and 3) Prussian-blue method, wavelength of 580 nm (Swain & Hillis, 1959). Van Alstyne (1995) compared three methods for quantifying phenolic compounds: 1) Folin-Ciocalteu assay for compounds dissolved in 80% methanol, 2) Folin-Ciocalteu assay for compounds dissolved in 75% methanol and 25% trichloroacetic acid, and 3) assay in which polyvinylpyrrolidene (PVPP) was used to remove phenolic compounds. He found that the first of these three assays yielded the most consistent results. In any assay, the investigator should select a suitable method depending on what she or he wants to detect (Waterman & Mole, 1994).

In allelopathic research, soils amended with phytotoxic material and natural soils have often been tested for their total phenolic content. Because of the variability in methods for determining total phenolics, care should be taken to select the most appropriate method of extraction of total phenolics. The relative concentration of phenolic pool after the addition of phytotoxic material to soil can then be compared with the concentration in soil before the addition of phytotoxic material.

Inderjit and Dakshini (1995a) discussed the important precautions that should be taken during isolation of allelochemicals. They suggested that a better understanding of allelochemicals is possible if one avoids the extraction of plant material with organic solvents and the grinding of plant material. Organic solvents can extract chemicals from soil organic matter, litter, humic acid, and microbe membranes (Schmidt, 1990); grinding of plant material results in the release of certain enzymes, salts, amino acids, and nitrogen

compounds that may not be released under natural conditions (Inderjit & Dakshini, 1995a).

Several workers have proposed techniques for the extraction of allelochemicals from soils (Rice, 1984). Del Moral and Muller (1970) suggested the significance of water-soluble compounds in situations where irrigation is frequent. Kaminsky and Muller (1977) suggested a technique for the extraction of allelochemicals from soils using water and a chelating agent. Kaminsky and Muller (1978), in the interest of obtaining ecologically significant results in allelopathy, advised against the use of alkaline soil extractions. In comparison to soil extractions with 0.5% $\text{Ca}(\text{OH})_2$ and 2 M NaOH, phenolics extracted with water are considered to be ecologically more important (Whitehead et al., 1981). However, water extractions do not account for reversibly bound phenolics. Blum et al. (1994) recommended water and EDTA for extractions of free and reversibly phenolic acids. Dalton et al. (1983) advocated neutral extraction procedures using water and Na_2EDTA (at pH 7.5) as a chelating agent; this method can be used to estimate the active fractions of phenolic acid in the soil system. Dalton et al. (1987) compared several procedures for extracting plant phenolic acids from soils. They recommended doing two soil extractions for phenolics, both times using either methanol or water: the first extraction is to estimate phenolics in soil solution and the second is to estimate the amount of reversibly bound phenolics that are potentially available to the soil solution (Dalton et al., 1983, 1987).

Dalton (1989) found that physiochemical and biological processes served to decrease the recovery of exogenously applied ferulic acid from tropical forest soils. The immediate recovery of ferulic acid was inversely related to soil organic matter. Dalton et al. (1989a) compared various extraction procedures for their ability to recover water-soluble phenolics from soil. They emphasized the significance of selecting the appropriate extraction procedure in order to obtain ecologically relevant results. Blum et al. (1994) suggested that EDTA- and water-extracted soils give allelopathically significant levels of free and reversibly bound phenolics. Therefore, absolute concentration of total phenolics in soils cannot be determined by any phenol reagent or HPLC analyses, and water and EDTA extractions recover only certain types, not all types, of phenolic compounds.

HPLC, gas chromatography, and mass spectroscopy have been successfully employed in quantitative and qualitative analyses of phenolics (Hartley & Buchen, 1979; Waller, 1987; Harborne, 1989a, 1989b; Inderjit & Dakshini, 1991a). J. D. Weidenhamer (pers. comm.) suggested the analysis of individual phenolics by appropriate chromatographic procedures such as HPLC, rather than or in addition to the analysis of total phenols. This is mainly because estimating total phenolic content with phenol reagents gives only relative concentration of phenolic compounds, particularly "free" phenolics. Furthermore, EDTA extractions cannot be used with phenol reagent, and EDTA-extracted phenolic acids are quantified using HPLC. However, Udo Blum (pers. comm.) is of the opinion that, at present, there is neither a satisfactory extraction procedure for soil total phenolic acids nor any procedure that estimates total phenolic acids in plant extracts. He believes that all values in the literature are crude approximations, and one can only assume that the resulting values are somehow related to actual values in the extracts and thus to the soil or plant tissue. In his view, for allelopathic studies, determining total phenolic acids in the soil is not meaningful, as most of the acids are in an unavailable form. He suggested, however, that 0.25 M EDTA at pH 7 can extract readily available phenolic acids, whereas water extractions do not recover all that is available to the seeds, roots, or soil microbes (Blum et al., 1994). Therefore, selection of extractant is one of the most important and difficult tasks in allelopathic research. Essentially, all of

the above-discussed procedures provide evidence of the complex mixtures of organics present in soil but are of limited ability to provide insights into allelopathic interactions.

IX. Concluding Remarks

Although phenolics have been widely implicated in allelopathy, conclusive proof of their involvement under natural conditions requires studies designed to be ecologically relevant. It is important to isolate, identify, and characterize compounds from the source and/or the sink or to identify the origin of compounds in the environment. It is necessary to collect data on residence time, mode of renewability, static and dynamic availability of phenolic compounds in the environment, and mode of action of phenolics. It is also necessary to demonstrate that compounds have bioactive concentrations great enough and persistent enough (or are produced at a faster-enough rate to compensate for loss) to affect other plant species. Furthermore, plant habit, physiochemical and biological properties of the substratum, edaphic and environmental conditions, and other abiotic and biotic factors—separately, sequentially, or simultaneously—determine the qualitative and/or quantitative availability of phenolic compounds. It is an important challenge to establish phenolic allelopathy as an ecological process and to determine the relationship between the phenolic measures in soils and the concentrations experienced by the roots, seeds, and soil microbes.

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