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BIOASSAY OF NATURALLY OCCURRING ALLELOCHEMICALS FOR PHYTOTOXICITY

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Abstract—The bioassay has been one of the most widely used tests to demonstrate allelopathic activity. Often, claims that a particular plant species inhibits the growth of another are based entirely on the seed germination response to solvent extracts of the suspected allelopathic plant; few of these tests are of value in demonstrating allelopathy under natural conditions. The veracity of the bioassay for evaluating naturally occurring compounds for phytotoxicity depends upon the physiological and biochemical response capacity of the bioassay organism and the mechanism(s) of action of the allelochemicals. The possibility that more than one allelochemical, acting in concert at very low concentrations, may be responsible for an observed allelopathic effect makes it imperative that bioassays be extremely sensitive to chemical growth perturbation agents. Among the many measures of phytotoxicity of allelochemicals, the inhibition (or stimulation) of seed germination, radicle elongation, and/or seedling growth have been the parameters of choice for most investigations. Few of these assays have been selected with the view towards the possible mechanism of the allelopathic effect.

Key Words—Allelopathy, bioassay, mechanism of action, seed germination, radicle growth, seedling growth, *Lemna* bioassay.

INTRODUCTION

We have recently reviewed the use of bioassays in the study of allelopathy (Leather and Einhellig, 1986). In that review, we discussed the nature and types of bioassays used by investigators to demonstrate the phytotoxicity of leachates, exudates, extracts, etc., from plants suspected of being allelopathic to other plants or microorganisms. Yopp's (1985) treatise, "Bioassays for Plant Hormones and Other Naturally Occurring Plant Growth Regulators," is an excellent source of information on assays for specific chemicals that control plant growth and function. Many of these bioassays may be useful in the study of allelopathy when the mechanisms of action are well understood.

Our purpose here is to evaluate the veracity of the most commonly used bioassays in allelopathy research, their value in determining allelochemical mechanism(s) of action, and the suitability of those bioassays to detect the presence and phytotoxicity of allelochemicals.

DISCUSSION

Mechanism of Action. Allelochemicals that are phytotoxic have been identified from many genera of plants and belong to many classes of chemical compounds (Rice, 1984). Einhellig (1986) and Putnam (1985) have reviewed the chemistry and mechanisms of action of allelopathic agents. Table 1 is a summary of our current knowledge of the possible mechanisms by which the allelochemicals inhibit plant growth and development. Proposed mechanisms

TABLE 1. MECHANISMS OF ACTION OF ALLELOCHEMICALS IN PLANT GROWTH AND DEVELOPMENT[a]

Mechanism	Allelochemical	Reference
Cell extension	Phenolic acids, tannins	Lee and Skoog (1965), Lee et al. (1982), Lee (1980), Ray et al. (1980)
Cell division	Volatile terpenes, coumarins	Muller (1965), Jankay and Muller (1976), Avers and Goodwin (1956)
Membrane permeability	Phenolic acids	Harper and Balke (1981)
Nutrient uptake	Phenolic acids	Harper and Balke (1981), Kobza and Einhellig (1987)
Chlorophyll synthesis	Coumarins Phenolic acids	Einhellig et al. (1970), Einhellig and Rasmussen (1979)
Photosynthesis	Phenolic acids	Nyberg (1986), Scholes (1987)
Protein synthesis	Phenolic acids, coumarins	Van Sumere et al. (1971)
Enzyme activity	Phenolic acids	Jain and Srivastava (1981), Sato et al. (1982), Schwimmer (1958)
Respiration	Juglone, volatile terpenes, phenolic acids	Scholes (1987), Koeppe (1972), Muller et al. (1969)
Water relations	Phenolic acids	Einhellig et al. (1985), Blum and Dalton (1985), Blum et al. (1985a)

[a] Condensed from Einhellig (1986), and Putnam (1985).

encompass most major plant functions, including regulation of growth by interfering with cell division or cell extension either directly or through interaction with hormones, effects on respiratory metabolism, photosynthesis, and altered water balance.

It can be noted from Table 1 that a great deal of research has involved phenolic acids and that these compounds act on a number of plant processes. It is unlikely that these compounds would have such a wide range of primary action, and it is probable that the results observed may be secondary and/or tertiary responses to the compounds. This range of responses could be due to the lack of veracity from improper bioassays. Indeed, most purported allelopathic agents have not been tested in bioassays with the goal of determining their mechanisms of action.

The biochemical and/or physiological response of a bioassay organism to allelochemicals is not always linear over a range of concentrations. Many allelochemicals are inhibitory at millimolar concentrations but stimulate the measured parameters in bioassays at micromolar concentrations. This anomaly confounds efforts to determine mechanisms of action in bioassays and to relate the results to in situ concentrations of allelochemicals.

Seed Germination Bioassays. The inhibition (or stimulation) of seed germination has been the most widely used bioassay for the determination of allelopathic activity. However, the seed germination process is probably the least understood of all plant functions (Leather, 1987). Seed germination begins with imbibition of water and ends with the protrusion of the radicle through the testa. Radicle elongation is by cell extension only and does not involve cell division. The biochemical events associated with germination are not well defined and may only be preparatory for the mobilization of reserves for seedling growth. Thus, definitive conclusions of allelopathic mechanisms in seed germination bioassays are limited but may involve membrane alteration, resulting in loss of metabolites and the ability to establish the necessary osmotic potential for cell elongation (Koller and Hadas, 1982). Other processes, such as alteration of the phytochrome control of germination, may also be effected. We found that some naturally occurring volatile compounds stimulated the dark germination of *Rumex* sp. that normally require postimbibitional light (French and Leather, 1979). Other perturbations from allelochemicals of seed germination processes may be involved, but we must await further knowledge of the biochemical events that occur during seed germination that are directly related to the germination process.

The greatest problem that affects the veracity of seed germination bioassays results from the manner in which the bioassay is conducted. Anderson and Loucks (1966) emphasized the importance of the solution osmotic potential when testing plant extracts; however, few reports on allelopathic effects consider this precaution (Leather and Einhellig, 1986). Weidenhamer et al. (1987)

reported that the number of seeds relative to the solution volume used in a seed germination bioassay was a factor in the results obtained. They found that the amount of ferulic acid available to each seed influenced the germination, rather than the concentration of chemical in the test solution. In conducting this research, care was taken to prevent anaerobic conditions by submersion of the seeds in water. We have found reports in the literature of allelopathic action where the investigators germinated the test species in volumes of solution that were 20 times the amount required for optimum germination without anaerobiosis.

Blum et al. (1984) offer recommendations for the standardization of germination bioassays. However, their results are based on radicle growth subsequent to germination and perturbations at any of the stages of germination and growth may have effects on any subsequent stage. Nonetheless, their observations regarding pH, microbial contamination, photolability of allelochemical, and loss of test compound are very important when conducting germination bioassays. Our recent review on this subject outlines additional precautions that should be observed when conducting seed germination bioassays (Leather and Einhellig, 1986).

Radicle Elongation Bioassays. Radicle elongation is a more sensitive assay for allelochemicals than seed germination (Leather and Einhellig, 1985; Einhellig, 1986). Like seed germination, radicle elongation is extremely sensitive to high (100 mosmol) osmotic potentials of solutions, and concentrations of purified extracts must be evaluated prior to assay (Bell, 1974). Generally, the radicle is completely dependent upon the seed (cotyledon) reserves for growth in the dark, and precautions must be taken to separate effects upon the seed and the mobilization of storage material by the allelochemical during or immediately following germination. Blum et al. (1984) reported that surface sterilization of seeds with sodium hypochloride modified radicle growth. Thus, as previously noted, care must be taken to minimize early effects of the allelochemical upon the seed that may alter subsequent radicle growth.

The accuracy of results obtained from the measurement of radicles elongating in Petri dishes is questionable. Few such radicles elongate on a straight course, and precise measurements are difficult. We have found that removing the radicle from seed germinated in Petri dishes and thoroughly drying to a constant weight gives accurate results with small statistical error (Leather and Hurtt, unpublished). Parker (1966) described a method to determine herbicide uptake and effects on radicle elongation. We modified this method to use pregerminated seed placed between chromatography sandwich plates that are maintained at a 45° angle, thus having straight radicles for measurement. Additionally, this method allows only the radicle to be in contact with the test chemical solution (Leather and Einhellig, 1985).

Radicle elongation does afford greater possibilities for mechanism studies than seed germination. It is particularly suited for determining effects of allelochemicals on hormones responsible for cell growth. Radicle elongation also allows evaluation of allelochemical effects on respiration and cell division.

Seedling Growth Bioassays. Seedling growth bioassays are extremely versatile but require a greater quantity of chemical than is usually available during initial isolation and identification of allelochemicals. These bioassays usually have greater sensitivity and provide the basis for a variety of mechanism studies, such as nutrient uptake, water relations, and photosynthesis, but here again, it is difficult to determine the primary sites and mechanisms of action of the allelochemical.

Blum and colleagues (Blum and Dalton, 1985; Blum et al. 1985a,b), used leaf expansion of cucumber seedlings grown in nutrient culture to determine the mechanism of action of ferulic acid and its microbial metabolic products. In these reports, they stress the importance of monitoring the loss of allelochemical through absorption, microbial breakdown, or other mechanisms, including dissociation of the chemical in solutions of changing pH values.

Using a sorghum (*Sorghum*) seedling bioassay, we found that [^{14}C]salicyclic acid was rapidly taken up from the nutrient solution in which the seedling was growing, and it was distributed throughout the seedling within 24 hr after treatment (unpublished results). Such rapid, widespread translocation severely limits the utility of seedling growth bioassays for pinpointing primary sites or mechanisms of allelochemical action.

Lemna *Bioassays.* The *Lemna* bioassay developed in our laboratories is a versatile and extremely sensitive assay for allelochemical phytotoxicity (Einhellig et al., 1985). It meets many of the criteria for the assay of naturally produced phytotoxins (Duke, 1986) and can be used in the study of mechanisms of allelochemical action (Leather and Einhellig, 1985). Although only recently reported (Einhellig et al., 1985), this bioassay is now used by numerous laboratories for the detection of phytotoxic natural substances, including those produced by microorganisms. A drawback of this bioassay may be in relating the results obtained to the allelochemical effect on terrestrial plants. This relationship needs to be further investigated.

Lemna species (duckweeds) are angiosperms and offer a variety of parameters, including flowering (Cleland and Tanaka, 1982), which can be used as indicators of phytotoxicity. We measure the effects of allelochemicals on the dry weight, frond reproduction, chlorophyll content, and anthocyanin production. Anthocyanin production by *L. obscura* is the most sensitive to inhibition by selected allelochemicals (Leather and Einhellig, 1985). *Lemna gibba* affords other parameters such as chlorophyll content and flowering as indicators of allelochemical phytotoxicity, but because it is among the largest of the duckweeds, it is not suitable for low-volume assays (Ramirez-Toro et al., 1988).

Photosynthesis and overall respiratory metabolism of *Lemna* plants are particularly sensitive to perturbation and can thereby provide insight to the potential mechanisms of allelochemical action. Nyberg (1986) used *L. minor* to evaluate the effects of 22 different allelochemicals on chlorophyll content, photosynthesis, and respiration. Using a photorespirometer in conjunction with the 24-well assay plate, the tests could be conducted using very small quantities of allelochemical. Scholes (1987) evaluated the effects of compounds from six classes of allelochemicals on photosynthesis and respiration in *L. minor*. She found, for example, that juglone depressed the photosynthesis of duckweed at very low concentrations; this is in contrast to previous reports (Koeppe, 1972) that this allelochemical acted by interfering with respiration of isolated corn (*Zea mays* L.) mitochondria. However, caution must be observed when comparisons of two different systems are made.

CONCLUSIONS

Bioassays are essential tools for the identification of allelochemicals with phytotoxic properties. Because these chemicals are generally produced in small amounts and probably exert their effects in concert with other allelochemicals, it is desirable that the selected bioassay be extremely sensitive and that some indication of the mechanism of action of the allelochemical(s) can be determined. Nearly all reports of allelopathic activity indicate the response of the suspect leachate, exudate, or extract from a plant in some bioassay. With a better understanding of the perturbations elicited, advances in identification technology, and extremely sensitive bioassays, we can now begin to evaluate the integrity of bioassays that have formed the basis for the growing science of allelopathy.

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