ALLELOPATHIC EFFECTS OF FERULIC, GALLIC, AND VANILLIC ACIDS ON CORN (*Zea mays* L.)

by

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(ABSTRACT)

Studies on the activity of ferulic, gallic, and vanillic acids on germination and growth of corn (*Zea mays* L.), radish (*Raphanus sativus* L.), and peanut (*Arachis hypogaea* L.) showed that the inhibitory effects of these acids were concentration and growth variable dependent. Ten days after treatment, significant reduction in percent germination of the three species occurred with higher phenolic acid treatments, except that gallic acid did not significantly inhibit peanut germination. Among the growth parameters investigated, root elongation and dry weight were more affected than either germination or shoot length and dry weight. Radish and corn were more sensitive than peanut.

In two-combination experiments, the interactive effects of phenolic acids on corn germination and shoot growth were generally not significant, indicating an additive effect. Ferulic acid, generally, antagonized higher concentrations of vanillic or gallic acids on corn root length and dry weight, suggesting a differential uptake of phenolic acids by corn roots or a limited uptake of gallic and vanillic

acids in the presence of ferulic acid. In a soil system, higher and repeated phenolic acid treatments were required to bring about inhibition of corn growth than those which were effective in petri dishes.

All levels of the synthetic auxin, 2,4-D (2,4-dichlorophenoxyacetic acid) were effective in reversing the inhibitory effects of 1 mM ferulic acid on corn root length when these two acids were applied in combination. No 2,4-D treatment counteracted 10 mM of ferulic acid. All levels of 2,4-D combined with 1 mM ferulic acid and the mixture of 0.1 nM 2,4-D with 10 mM ferulic acid were antagonistic for corn shoot length. No significant interactions were obtained on corn germination or seedling growth when 2,4-D was combined with gallic acid.

Using manometric techniques, no inhibitory effects of ferulic or gallic acids observed on O_2 consumption of germinating corn seeds. Ferulic acid did not interfere with water uptake of corn seeds during imbibition and germination. These findings indicate that the phytotoxicity of these acids observed on corn germination and seedling growth are not due to their interference with water uptake and respiratory activity of germinating seeds.

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DEDICATION

الحمد لله الذي به نستعين والصلاة والسلام على
خاتم الأنبياء والمرسلين

إلى والدتي الحنونة، ...

إلى أختي الشقيقة، ...

إلى جدتي العزيزة، ...

لأنكم أسررتم وأكملتم على تعليمي وثقافتي...

لأنكم شجعتموني دائماً على إنهاء وإتمام دراستي، ... لأنكم مهدتم طريقي وأزحتم منها الأشواك بتربيتكم الصالحة لي، ... ولأنكم أوقدتم في مشعال العلم، ... لأنكم وفرتم لي أحضاناً دافئة، ... ولأنكم وضعتم فيّ ثقتكم الكاملة، ...

واعترافاً مني بجميلكم الذي لا يحصى، ... وتعبيراً مني عن محبتي لكم المتزايدة، ... فلولا عون الله جل جلاله، ولولا دعواتكم الطيبة المباركة، وعونكم المعنوي والمادي لما وصلت إلى غايتي هذه، ولما حققت حلمي هذا...

فتقبلوا أرجوكم هديتي هذه مع شكري وامتناني لكم الجزيلين، واحترامي وتقديري الدائمين، وحفظكم الله ورعاكم وأدام عليكم فضله الذي لا يعد، ورحمته التي لا تحصى.

ابنتكم المطيعة

فاطمة العبيدي

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INTRODUCTION

Allelopathic agents or allelochemicals are substances produced by living plants or released from decaying plant parts by microbial degradation or leaching which affect seed germination and plant growth and development. They are present in virtually all plant tissues including leaves, flowers, fruits, stems, roots, rhizomes, and seeds. The number and diversity of compounds implicated in allelopathic interactions are rapidly growing. Most of the currently identified allelochemicals are secondary products that arise from the shikimate and acetate pathways. Numerous phenolic compounds have been implicated in allelopathy, and derivatives of cinnamic acid, benzoic acid, and coumarin are among those most often identified from soils and higher plants.

The possibility of exploiting allelopathy and allelochemicals for beneficial purposes in agriculture has increased considerably in the last decade. The hope is that allelochemicals, sometimes referred to as "nature's own herbicides", can either aid in increasing crop yields, reduce production expenses, or prevent harm to the environment that is implicated in some current production practices. Different strategies have been proposed to capitalize on allelopathy and can be categorized as 1) avoidance of negative impacts, 2) exploitation of stimulatory effects, 3) management and development of allelopathic crops to suppress weeds, 4) development of allelochemicals as pesticides or growth regulators, and 5) combination of these approaches.

The allelopathic potential of diverse compounds has typically been evidenced by plant growth following residue amendments to growth medium in bioassays and/or in soil using extracts, leachates, or exudates. Emphasis has been on the effects of residues of weeds on crops, crops on weeds, and crops on crops. The major drawback with studies of plant residues and their decomposition involves the identity of the chemicals present, their concentrations, and biological activities. Recently, research efforts have been directed towards isolation and characterization of potential or alleged allelochemicals. The data necessary for the future exploitation of allelopathy and allelopathic agents are only now being collected. Much of the information required to understand the biological activity of the identified allelochemicals on plant growth dynamics is lacking. Studies involving the elucidation of dose/response relationships and determination of threshold levels for growth inhibition are needed in order to assess negative impacts that these chemical compounds may exert on crop plants.

The objectives of this research were 1) to study and compare the activity of a cinnamic acid derivative, ferulic acid, and two benzoic acid derivatives, vanillic and gallic acids, on germination and seedling growth of corn (*Zea mays* L.), radish (*Raphanus sativum* L.), and peanut (*Arachis hypogaea* L.); 2) to investigate and characterize the nature of two-way combinations of these phenolic compounds on corn germination and root and shoot growth in bioassays and in soil environment; 3) to determine and compare threshold levels of these acids for growth inhibition in laboratory and greenhouse studies; and 4) to elucidate the

interaction of ferulic and gallic acids with the synthetic auxin 2,4-dichlorophenoxyacetic acid on corn in the laboratory. Other experiments were also conducted to investigate the effects of ferulic acid on imbibition of germinating corn seeds and of both ferulic and gallic acids on respiration during the time course of corn germination.

CHAPTER I

LITERATURE REVIEW

The word allelopathy, when used in its broad sense, refers to biochemical interactions, inhibitory as well as stimulatory, between all types of plants including microorganisms (Putnam, 1986; Rice, 1984). Implicit in this concept is the suggestion that the allelopathic effect depends on a chemical compound being added to the environment in contrast to competition, which involves the removal or reduction of some factor that is required by other plants sharing the habitat (Rice, 1984). The biochemical substances involved in allelopathic interactions are termed allelochemicals (Einhellig et al., 1988; Patrick, 1986; Balandrin et al., 1985; Rice, 1984)

SOURCES OF ALLELOPATHIC CHEMICALS

Allelochemicals are released from plant tissues in a variety of ways, including volatilization, root exudation, leaching, and the decomposition of plant residues. The importance of each of these varies with the specific case. In general, release through volatilization of compounds has been reported from arid

and semi arid regions (Rice, 1984). Release through leachates and exudates requires water solubility, and a broad range of allelochemicals are involved (Einhellig, 1985).

Volatilization. In a few cases, allelopathic agents may volatilize and either be absorbed directly from the atmosphere by neighboring plants, or they may reach the soil and be taken up by plant roots (Putnam, 1985; Einhellig, 1985). Apparently, several terpenoids transfer in these ways (Neill et al., 1971; McCahon et al., 1973; Weaver et al., 1977; Muller, 1965; Muller et al., 1966)

Leaching. Tukey (1971) defines leaching as the removal of substances from plants by the action of aqueous solvents such as rain, dew, mist, and fog. According to the same author, all plants seem to be leachable, although the degree is influenced by different factors such as type of tissue, stage of maturity, and type and amount of precipitation. Leaching is an important avenue of release of allelochemicals; and a large number of compounds, both organic and inorganic are leached. They include known allelopathic agents such as phenolic acids, alkaloids, and terpenoids (Rice, 1974; Tukey, 1971, 1966).

Root exudation. According to Rovira (1969), root exudates are those substances synthesized in the plant and released into the surrounding medium by healthy and intact living plant roots. While the volume of exudation from roots is

generally small, these exudates are often significant in allelopathy (Young, 1986; Einhellig, 1985; Putnam, 1985; Rice, 1984; Rovira, 1971, 1969). Much of the evidence for root mediated-allelopathy has come from studies where solutions passing by root systems of one plant are recycled into media containing indicator species. Often these experiments are set up in staircase systems so that solutions may move by gravity through a series of interconnected pots (Bell et al., 1972). Other studies use an adsorptive column to selectively trap organic and hydrophobic root exudates while allowing nutrient ions and other hydrophilic compounds to pass through (Tang, 1986; Tang et al., 1982). These researchers were able to identify several compounds, most of which were phenolic acids, with derivatives of cinnamic and benzoic acids being the major rhizosphere compounds (Tang, 1986).

Residue decomposition. The decomposition of plant residues potentially provides the largest quantity of allelochemicals that may be added to the rhizosphere. Debris of important field crops that have been studied include wheat, barley, rye, corn, sorghum, and rice (Lodhi et al., 1987; Shilling et al., 1985; Barnes et al., 1983; Putnam et al., 1983; Chou et al., 1976; Patrick, 1971). Many noncrop species from a variety of plant communities have also been implicated in allelopathy due to residue decomposition (Kuiters et al., 1987; Rice, 1984, 1974). Investigations using aqueous plant extracts show that water-soluble inhibitors may be present in either crop plants, weeds of agronomic fields, or

plants of natural communities (Hegazy et al., 1990; Abdul-Rahman et al., 1989; Jain et al., 1989; Chou et al., 1989; Porter et al., 1986; Casal et al., 1985). Among the compounds released, phenolic acids are the substances most often identified from residue decomposition (Kuiters et al., 1987; Einhellig, 1985).

In many parts of the United States and the world, phytotoxicity associated with crop residues is a potential problem facing conservation tillage systems (Putnam, 1985; Elliott et al., 1977; McCalla et al., 1974). Residues left on or near the soil surface for soil and water conservation frequently reduce growth and yields of the next crop as compared with fields where residues are removed or when conventional tillage is used (Lodhi et al., 1987; Elliott et al., 1978; Cochran et al., 1977; McCalla et al., 1974; Davidson et al., 1973; Kimber et al., 1973). Some studies indicate that the yield production problem associated with stubble-mulch tillage is residue related because yields were decreased more frequently as annual rainfall increased (McCalla et al., 1974; Davidson et al., 1973; Kimber, 1973). While the poor crop growth under this system resembles nitrogen deficiency, it is not corrected by nitrogen applications (Davidson et al., 1973; Kimber, 1973). Reduced yields have been attributed to toxic compounds released from crop residues and/or microbial production of phytotoxic substances during residue decomposition. Not only can the residues have an influence on crop emergence, growth, and productivity, but also they can influence similar aspects of weed growth. Management of selected crop residues can greatly reduce weed germination and growth (Shilling et al., 1985; Liebl et al., 1983; Leather et al.,

1983).

CHEMICAL NATURE OF ALLELOCHEMICALS

Most chemical inhibitors are compounds that have been characterized as secondary natural products, because they are of sporadic occurrence and thus do not appear to play a role in the primary metabolism of organisms (Harborne, 1988; Whittaker et al., 1971). There are many thousands of such compounds, but only a limited number of them have been identified as toxins involved in allelopathy (Rice, 1984). Rice (1984) classified allelochemicals into 14 categories plus a miscellaneous group. Most arise either from acetate or from amino acids which are products of the shikimic acid pathway. These categories are 1) cinnamic acid derivatives, 2) coumarins, 3) simple phenols, benzoic acid derivatives, 4) flavonoids, 5) condensed and hydrolyzable tannins, 6) terpenoids and steroids, 7) water soluble organic acids, straight chain alcohols, aliphatic aldehydes, and ketones, 8) simple unsaturated lactones, 9) long chain fatty acids, 10) naphthoquinones, anthraquinones, and complex quinones, 11)amino acids and peptides, 12) alkaloids and cyanohydrins, 13) sulfides and mustard oil glucosides, and 14) purines and nucleosides, and 15)miscellaneous group.

According to Einhellig (1985), these different categories are not equally important. Derivatives of cinnamic acid, benzoic acid, coumarin, and the terpenoids are the allelochemicals most often reported in the literature (Classen

et al., 1990; Jain et al., 1989; Duke et al., 1988; Mandava, 1985). The terpenoids are of limited distribution and are produced in small quantities, whereas the first three are regarded as phenolic compounds and are present in fair abundance (Einhellig, 1985; Rice, 1984; Robinson, 1980; Mandava, 1979). Phenolic compounds have been identified as allelopathic inhibitors more often than any class of chemicals (Einhellig, 1985; Putnam, 1985). They are also of direct importance in several other types of interactions in the ecosystem, such as protection against pathogens and insects (Classen et al., 1990; Van Sumere, 1989; Harborne, 1988; Klocke, 1987; Patrick, 1986; Lynn, 1985; Swain, 1979). Important cinnamic acid derivatives functional in allelopathy are ferulic, *p*-coumaric, caffeic, and chlorogenic acids. In a similar listing of benzoic acid compounds, vanillic, *p*-hydroxybenzoic, and gallic acids have been repeatedly reported (Rice, 1984; Mandava, 1985; Einhellig, 1985).

MECHANISMS AND MODES OF ACTION OF ALLELOCHEMICALS

Rice (1987) stated that "the surface has just been scratched in determining the mechanisms by which the different kinds of allelopathic compounds exert their actions". According to Einhellig (1986), a clear insight into the precise physiological perturbations caused by these substances has not been obtained. The major difficulty in defining mechanisms of actions of allelochemicals is that a

specific compound may affect several metabolic processes, and as a result, it has seldom been possible to separate primary from secondary effects (Putnam, 1985; Mandava, 1985). Another difficulty arises from the uncertainty in translating the observed effects on isolated enzymes and other biochemical systems to whole plant. (Mandava, 1985). Published reports on allelopathic agents, however, indicate that they interfere with many of the primary metabolic processes of higher plants. These include cell division and elongation, interactions with hormones, respiration, photosynthesis, protein and lipid synthesis, and membrane permeability (Einhellig, 1986, 1985; Putnam, 1985; Mandava, 1985; Rice, 1984).

The effects of allelopathic phenolics on cell division/elongation, on respiration, and their interactions with phytohormones have been discussed in the following chapters of this research. In the following sections of this chapter discussion will focus on the action of phenolic acids on other plant processes. Whenever it is possible, specific examples will be given where ferulic, vanillic, and/or gallic acids were investigated.

Effects on photosynthesis. Patterson (1981) reported that 1 mM concentrations of ferulic, gallic, vanillic and other phenolic acids caused marked reductions in concentrations of chlorophyll in soybean leaves. The same treatment severely reduced the net photosynthesis rate and stomatal conductance of single fully expanded leaves. Similar results were obtained by Einhellig et al.; (1979). Using manometric techniques, it was shown that an array of coumarins

and cinnamic and benzoic acids derivatives suppressed photosynthesis of *Lemna minor* L. (Einhellig et al., 1985). Moreland et al. (1987) reported that several cinnamic and benzoic acid derivatives, coumarins, and flavonoids inhibited CO_2-dependent oxygen evolution in isolated chloroplasts. However, this occurred at fairly high concentrations. In the thylakoids, the compounds tested primarily affected photophosphorylation, but they neither acted like uncouplers nor classical energy transport inhibitors.

Effects on protein and lipid syntheses. Results of the few studies on action of allelochemicals on protein synthesis using labeled amino acids and acetate suggest that some allelopathic agents inhibit germination and growth by reducing amino acid and acetate uptake and incorporation into cell proteins and lipids (Cameron et al., 1980; Danks et al., 1975; Van Sumere et al., 1971). For instance, ferulic and coumaric acids at 0.01 mM inhibited the incorporation of ^{14}C phenylalanine into proteins in lettuce and barley seeds and embryos (Van Sumere et al., 1971). Danks et al. (1975) found that incubation of rose-cells with glucose-[U-^{14}C] plus ferulic acid (0.1 mM) or cinnamic acid (0.01 mM) reduced carbon flow into proteins, but these two compounds acted in different ways. Ferulic acid promoted incorporation of the label into soluble lipids and decreased organic acids and soluble amino acid fractions. Cinnamic acid, however, elevated soluble amino acids and did not promote lipids. Similar results were reported for these two compounds by Cameron et al. (1980), who indicated that inhibition of

protein synthesis by cinnamic and ferulic acids was a reliable indicator of their inhibitory action on lettuce growth.

Effects on membrane permeability. Glass et al. (1974) reported that membranes of barley root cells were rapidly depolarized by the addition of 0.5 mM of salicylic acid to the buffer medium. Other benzoic and cinnamic acids tested caused the same effect with the extent of disruption correlated with their lipid solubility. These authors concluded that the phenolic tested caused an increase in membrane permeability to both cation and anions, allowing a nonspecific efflux of ions.

EXPLOITATION OF ALLELOPATHY IN AGRICULTURE

Recently, there has been a considerable interest in developing strategies for exploiting allelopathy in agricultural production either by avoiding negative impacts of allelochemicals or by exploiting allelopathic mechanisms for additional pest control and in approaches to growth regulation (Einhellig et al., 1988, Einhellig, 1986, 1985; Putnam, 1986, 1985, 1983; Patrick, 1986). Different approaches have been proposed and include genome enhancement, manipulation of crop metabolism to increase allelochemical production, utilization of allelopathic rotational crops, cover crop and residue management, use of

allelochemicals as complements to herbicides, and as structural templates for novel pesticides (Einhellig et al., 1988; Lydon et al., 1988; Canal et al., 1987; Duke, 1987, 1986; Patrick, 1986; Einhellig, 1985; Putnam, 1986, 1983).

Different crop germplasm collections have been found to contain superior weed suppressing types. Crop collections investigated include as sunflowers (Leather, 1983), soybeans (Massantini et al., 1977), oats (Fay et al., 1977), and cucumbers (Putnam et al., 1974). In field tests, one allelopathic accession of cucumber reduced total weed fresh weight to approximately one third of the weed biomass found with Pioneer, a commonly grown cucumber cultivar (Lockerman et al., 1979). The allelopathic effect, however, was less under periods of increased rainfall. Some authors have speculated that altering the genome of crops to enhance herbicidal activity could be approached through classical breeding programs or genetic recombination techniques (Einhellig et al., 1988; Putnam, 1986). The use of the latter, however, requires identification of allelochemicals that may achieve a desired change, information about regulation of their production, and the development of a genetic engineering approach for moving a controlling gene element into an agronomic crop. Cover crops of wheat, barley, oats, rye, grain sorghum, and sudangrass have been used effectively to control weeds (Barnes et al., 1983; Putnam et al., 1983; Shilling et al., 1985; Schumacher et al., 1983). Barnes et al. (1986) reported that weed biomass in a cover crop of living rye was reduced 90% over unplanted controls and that even a mulch of 40-day-old spring planted rye gave a 69% reduction. According to Shilling et al.

(1985) soybeans and sunflowers planted without tillage into desiccated rye mulch gave over 90% reduction in the biomass of common lambsquater, red root pigweed and common ragweed, compared to tillage or no rye mulch.

Putnam et al. (1983) found that better weed control in vegetable crops was obtained from herbicide desiccated residue in late spring compared to winter killed residue. Einhellig et al. (1988) suggested that a reduced level of herbicide may be feasible when used in combination with an allelopathic agent. The authors advised, however, that caution should be exercised that dual herbicide-allelopathic methods do not cause crop damage or have other environmental hazards. It has been shown that when a level of atrazine that did not inhibit oat seedlings was applied in combination with ferulic acid, the net inhibition was greater than from ferulic acid alone. Moreover, combination of 100 ppb alachlor with 0.2 mM ferulic acid reduced sorghum growth more than 50% (Einhellig, 1987; Einhellig et al., 1988).

The use of allelochemicals as structural templates is obviously illustrated by the benzoic acid type herbicides such as dicamba, and other plant benzoic compounds are frequently implicated in allelopathy (Einhellig et al., 1988). Other examples include the herbicide cinmethylin and the insecticide chlorinated camphene. Both are derived from terpenoids isolated from the plant genus *Artemisia* (Duke et al., 1988). According to the same authors, the most important group of insecticides derived from natural products are the pyrethroids. This insecticide class was derived from the chemistries of insecticidally active

terpenoids from various *Chrysanthemum* species. Shilling et al. (1985) reported that salicylic acid, *p*-hydroxybenzoic acid, hydroquinone, and umbelliferone suppressed the growth of several weeds when applied as spray. Unfortunately, they were not selective and the rates necessary to inhibit weeds were high.

Phytotoxins isolated from bacteria and fungi appear to be more important sources of pesticides than those of higher plant origin (Duke, 1986; Cutler, 1986). Advantages of these compounds over those isolated from higher plants include their ease of isolation, the larger quantities obtainable for study, potential for specificity, efficacy at low rates, and opportunities for fermentation production (Duke, 1986; Cutler 1985).

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CHAPTER II

ALLELOPATHIC EFFECTS OF PHENOLIC ACIDS ON CORN, RADISH, AND PEANUT

Abstract. Gallic, ferulic, and vanillic acids were evaluated for their phytotoxic effects on corn (*Zea mays* L.), radish (*Raphanus sativus* L.), and peanut (*Arachis hypogaea* L.) germination and seedling growth. Phenolic acid concentrations ranged from 0 to 10 mM. Germination was monitored in petri dishes and germination counts were made at 12-h intervals for 60 h for corn and radish and 96 h for peanut and at 5 days after treatment for radish and 10 days after treatment for corn and peanut. Results showed that phenolic acid concentrations of 1 mM and lower delayed germination of the species tested, while higher concentrations, 5 and 10 mM, significantly inhibited germination 10 days after treatment. No gallic acid concentration significantly affected peanut germination at 10 days after treatment. Phenolic acids were more inhibitory to radish and corn seedlings than peanut. Radicle elongation was more affected by phenolic acid treatment than either germination or shoot elongation. Ferulic acid caused more reduction in both root and shoot length than other growth parameters evaluated, whereas gallic acid was more inhibitory to root and shoot dry weight.

INTRODUCTION

Over the past three decades, extensive research has documented the production of allelochemicals by agricultural weeds and crops, and that associated microbial metabolism further contributes to diversity of the compounds produced (Putnam, 1986; Cutler et al., 1985; Rice, 1984). Most research efforts have been directed towards isolation and identification of potential allelopathic compounds. As a result, phenolic acids have been shown to be common in a variety of plant species and soil types (Singh et al., 1989; Vance et al., 1985; Chou et al., 1976; Hennequin et al., 1976). Ferulic, vanillic, and gallic acids are among the most commonly identified phenolics with important allelopathic potential (Abdul-Rahman et al., 1989; Jain et al., 1989; Chang, 1989; Kuiters et al., 1987; Kuiters et al., 1986; Porter et al., 1986; Chang, 1985; Casal et al., 1985).

Recently, research emphasis has shifted to the development of allelochemicals as herbicides through the alteration of their chemistry (Duke, 1987, 1986; Patrick, 1986). The potential use of allelopathic agents as complements to herbicides has been explored (Einhellig, 1988; Wegher, 1986; Hamm, 1986). Other alternative ways to exploit allelochemicals for weed management have been suggested. One proposes altering the genome of crop plants to enhance herbicidal activity (Einhellig et al., 1988; Putnam et al., 1974). The second is based on the manipulation of crop metabolism to achieve an increase in allelochemical production (Lydon et al., 1988; Canal et al., 1987;

Duke, 1986, 1984).

Studies on biological activity of identified allelochemicals on plant growth, elucidation of dose/response relationships, and determination of threshold levels for growth inhibition are needed to assess negative impacts that these chemicals may exert on crop plants. The objectives of this study were to: 1) determine the effects of ferulic, vanillic, and gallic acids on germination and radicle and hypocotyl growth of corn, peanut, and radish; 2) establish the differential sensitivity of these species to the three phenolics examined; and 3) determine threshold levels at which significant growth reduction occurs.

MATERIALS AND METHODS

Chemicals and Seeds. Ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid), gallic acid (GA) (3,4,5-trihydroxybenzoic acid), vanillic acid (VA) (4-hydroxy-3-methoxybenzoic acid), and MES buffer [2-(N-morpholino) ethanesulfonic acid] were purchased from Sigma Chemical Company, St. Louis, Missouri. Seeds of corn (*Zea mays* L.)(cv. Southern States 727), radish (*Raphanus sativus* L.) (cv. Cherry Belle), and peanut (*Arachis hypogaea* L.) (cv. Flora Giant) were stored at 4 C until used.

Experimental Procedure. Each phenolic acid stock solution was prepared by dissolving the predetermined amount in 3 mM MES buffer. A few drops of 0.1 N NaOH were added to achieve complete dissociation of the acids. Final phenolic concentrations of 0.05, 0.1, 0.5, 1.0, 5.0, and 10 mM were obtained by appropriate dilution of stock solution with 3 mM MES. One control consisted of distilled water, while another control consisted of 3 mM MES adjusted to pH 5.8 using 0.1 N NaOH. The pHs of phenolic acid solutions were also adjusted to 5.8.

Germination experiments were conducted in petri dishes (10, 15, and 2.5 cm diameter for corn, peanut, and radish, respectively). Twenty seeds of each species were placed in a petri dish containing Whatman No 1 filter paper moistened with 7, 15, and 3 ml of test solution for corn, peanut, and radish, respectively. Petri dishes were wrapped with aluminum foil and placed in an incubator at 25 +/- 1 C. Germination percentage was determined at 24, 36, 48,

and 60 h after treatment for corn and radish and at 48, 60, 72, 84, and 96 h after treatment for peanut. Seeds were considered to be germinated if the radicle protruded from the seed coat by at least 2 mm. Germination counts were also made five days after treatment for radish and 10 days after treatment for corn and peanut. Five days after treatment, 3 and 5 ml of the test solution were added to corn and peanut seedlings, respectively. Root and shoot length were measured 10 days after treatment for corn and peanut and 5 days after treatment for radish. Roots and shoots were oven dried at 55 C for 48 h, and the dry weights were recorded.

Experimental Design and Analysis. Experiments were arranged in a completely randomized design with six replications. Data were subjected to single degree of freedom contrast analysis where the two controls were compared, and the acid treatments were compared to each of the two controls. All experiments were repeated at least once.

RESULTS

Contrast analysis did not reveal any significant differences between the distilled water and the 3 mM MES buffer controls. Therefore, results reported in this study are those obtained for comparisons of acid treatments to the 3 mM MES buffer control.

Effects of Ferulic, Gallic, and Vanillic Acids on Corn.

Germination. Effects of different concentrations of individual acids on corn germination are shown in Figure 2.1. As acid concentration increased, corn germination decreased. The amount of decrease varied with the phenolic compound. FA significantly depressed corn germination at concentrations greater than 0.05 mM at all intervals of germination counts. Significant reductions in germination were obtained with GA concentrations of 0.5 mM and higher at 24 h and with all concentrations at the 36-h germination count. At 48 and 60 h intervals, significant germination inhibition was observed only with concentrations of 1 mM and higher. Corn germination was significantly reduced by VA concentrations of 0.5 mM and higher.

A pronounced inhibition of germination was observed with all phenolic acids at 24 h, but the effect diminished over the time course of germination. For example, 53, 29, and 56% germination reductions were obtained at 24 h with the 0.5 mM concentration of FA, VA, and GA, respectively. This same concentration caused only 17, 17, and 14% germination reduction for the respective acids at 60 h. Similar trends were observed for other concentrations. All the three phenolic acids at 5 and 10 mM caused a significant inhibition on corn 10 days after treatment (Fig. 1.7). The reductions obtained were 26, 13, and 9% at 10 mM of FA, GA, and VA respectively.

Root and Shoot Length. Individual phenolic compounds showed similar effects on corn seedling growth as those observed on germination (Figure 2.2. A).

In general, root and shoot length were inversely related to phenolic acid concentration, with radicle length being more sensitive than shoot length. FA significantly reduced corn radicle elongation with all concentrations used in this experiment, whereas shoot elongation was significantly inhibited by concentrations of 1 mM or higher. VA at 0.05 mM did not significantly affect root length while shoot length was significantly inhibited by this and higher concentrations. GA concentration of 1 mM was the threshold level for inhibition of root and shoot length.

FA was the most inhibitory phenolic acid on radicle elongation. FA at 10 mM caused 87% reduction in radicle elongation compared to 40 and 57% inhibition caused by VA and GA respectively, at the same concentration. Shoot elongation, however, although significantly inhibited at higher concentrations, appeared to be less sensitive to the phenolic compounds than root length. At 10 mM, 45, 15, and 45% reductions in shoot length were obtained for FA, VA, and GA, respectively.

Root and Shoot Dry Weight. The inhibitory effects on germination and root and shoot elongation were also observed for corn root and shoot dry weight (Figure 2.2. B). Significant root dry weight reduction occurred at 0.1 mM FA and 1 mM VA and GA. All concentrations of FA and VA significantly reduced shoot dry weight, while the effective GA concentrations were 0.5 mM and higher.

At 10 mM, GA caused more inhibitory effects to both root and shoot dry weight than either FA or VA. Reductions obtained with this concentration of the

phenolic acids were 67, 59, and 30% for root dry weight and 47, 32, and 31% for shoot dry weight for GA, FA, and VA, respectively.

Effects of Ferulic, Vanillic, and Gallic Acids on Radish.

Germination. Effects of phenolic acids on radish germination were concentration dependent (Fig 2.3). Significant inhibition of germination was caused by FA at 1 mM and higher at 36, 48 and 60 h after treatment. VA caused significant reduction in germination at 0.1 mM at 24 h, but at 36, 48, and 60 h, germination was significantly different from the control only at 1 mM or greater. GA at 0.5 mM and higher significantly depressed radish germination at 24 and 60 h. As with corn, phenolic acid inhibition of radish germination was most evident at 24 h. Five days after treatment, all three acids caused significant inhibition of germination at 1 mM and higher concentrations (Fig. 1.7).

Root and Shoot Length. All FA concentrations except 0.05 mM significantly reduced radish root length while shoot length was inhibited at all FA concentrations tested (Fig 2.4 A). VA at 0.5 mM and higher depressed root length. Shoot length, however, was significantly affected only at 10 mM. All concentrations of GA significantly inhibited radish root length, while shoot length was significantly affected at 0.1 mM or higher.

The magnitude of root and shoot length reduction increased with increasing concentrations of individual phenolics. Root length was more sensitive than shoot length. The 1×10^{-2} M concentration of FA, VA, and GA

caused 53, 41, and 32% root length inhibition and 31, 12, 24% shoot length reduction, respectively. ·FA was more inhibitory to both radish root and shoot elongation than either VA or GA.

Root and Shoot Dry weight. Radish radicle dry weights were significantly inhibited by FA and GA only at higher concentrations (Figure 2.4. B). FA and GA at 5 mM caused 34 and 25% reduction, respectively, while 10 mM caused 42 and 30% reduction in root dry weight, respectively. Shoot dry weight was not significantly affected by FA and VA concentrations, whereas all GA concentrations caused significant reductions.

Effects of Ferulic, Vanillic, and Gallic Acids on Peanut.

Germination. Peanut germination occurred over a longer period than either corn or radish germination (Figure 2.5.). Whereas an average of 79% of the corn and 89% of the radish germinated in controls by 48 h, only 44% of peanut seeds had germinated at the same time interval. Moreover, inhibition of peanut germination required higher concentrations. Like corn and radish germination, the inhibition was most pronounced at earlier germination periods. The 10 mM concentration of FA, VA, and GA solutions caused 79, 74, and 29% reduction, respectively, in percent germination at 48 h. At 96 h acids at the same concentration caused only 22, 45, and 6% germination reduction, respectively. At 10 days after treatment, both FA and VA significantly reduced peanut germination at 5 and 10 mM (Fig. 2.7).

Root Length and Dry Weight. Both peanut root length and dry weight were more sensitive to the phenolic acids than was germination (Figure 2.6.). All concentrations of GA significantly reduced root elongation, while a significant reduction was caused by concentrations of 0.1 mM FA and 0.5 mM VA. The magnitude of this inhibition was equal for all acids. At 10 mM, FA, VA, and GA caused 30, 31, and 29% reduction in root length, respectively. Inhibition of peanut root length was less than that observed for corn and radish.

Significant reduction in root dry weight occurred at 0.1 mM (Figure 2.6. B). At this concentration, the activity of these compounds on root dry weight was equivalent, as illustrated by the 27, 25, and 26% reductions caused by FA, VA, and GA, respectively. VA at 10 mM was more inhibitory than either FA or GA, giving 52% reduction in root dry weight versus 39 and 46%, respectively.

DISCUSSION

The phenolic acids used in these experiments were more inhibitory to corn and radish than to peanut. These results are in agreement with those reported by other authors and support the concept of species specific allelochemical effects (Kuiters, 1989; Singh et al., 1989; Einhellig et al., 1979, 1978; Van Sumere et al., 1971). Williams et al. (1982) reported that some phenolics only delay and do not substantially inhibit germination. This is supported by results obtained in this study, where 1 mM and lower concentrations of FA, VA, and GA delayed

germination (Figure 2.1, 2.3, and 2.5). In our results, however, germination was still significantly inhibited at 10 days after treatment, at 5 and 10 mM of FA, GA, and VA for corn and the at same concentrations of FA and VA only for peanut (Fig. 2.7). Williams et al. (1982) pointed out other factors affecting activity of exogenous allelochemicals such as seed size, seed coat permeability, differential uptake, and metabolism. Differential sensitivity of species to phenolic acids was also related to seed size by Blum et al. (1984), who concluded that this may be due to radicles of larger seeds not always being in direct contact with the test solution. This was true in this study for peanut. Due to its seed size, a large portion of the radicle was kept above the filter paper, while the root tip was generally and continuously in contact with the filter paper and thus with the acid solution.

Among the growth parameters investigated, root elongation and dry weight were more affected than either germination or hypocotyl length and dry weight (Figures 2.2, 2.4, and 2.6). Moreover, FA was more active in reducing both root and shoot length, while GA was more inhibitory to both root and shoot dry weight. Several authors have reported sensitivity of radicle growth of different plant species to phenolic acids. (Kuiters, 1989; Singh et al., 1989; Blum et al., 1989; Wondimagegnehu et al., 1988; Lodhi et al., 1987, 1985; Einhellig et al., 1978, 1979).

Threshold concentration of phenolics that significantly reduced the growth parameters investigated ranged from 0.1 to 1 mM. Exceptions were inhibition of

peanut germination (Figure 2.5), which required higher concentrations, and radish shoot dry weight (Figure 2.4. B), which was not affected by any of the FA or VA concentrations. These effective concentration levels agreed with other findings that growth is often inhibited at a threshold value of 0.25 mM (Williams et al., 1982). According to Einhellig (1986), the threshold for inhibition of postemergence growth and plant physiological functions for many phenolics is in the range of 0.1 to 1 mM.

Phenolic compounds have been shown to interfere with several metabolic processes in plants, and a single acid can target one or more physiological systems to block growth processes (Einhellig, 1986, 1985; Putnam, 1985; Rice, 1984). Their effects on plant root growth could be explained by at least two mechanisms, interference with plant hormones, action on cell division or expansion, or by both mechanisms. Recent reviews on the mode of action of allelochemicals pointed out to the key role of phenolic acids in the regulation of IAA (indole-3-acetic acid) oxidase in plants (Einhellig, 1986; Putnam, 1985; Rice, 1984).

Early work has shown that a variety of allelochemicals inhibit mitosis in plant roots. Unfortunately, these reports tested either volatile inhibitors (Muller et al., 1964) or phenolic compounds other than those used in these experiments (Jensen et al., 1962; Avers et al., 1956; Cornman, 1946). A more recent work on cell division used crude plant extracts with no indication as to the nature of the allelopathic agents and demonstrated that there was a gradual increase in the percentage of cells in prophase and a decrease in other mitotic stages, as well as

a decrease in the mitotic index with increasing extract concentrations (Hegazy et al., 1990).

In conclusion, the results of this study showed that 1) there was a sensitivity differential among the three crop species tested to FA, VA, and GA. In order of decreasing sensitivity, species can be ranked as radish > corn > peanut. 2) The growth parameters investigated were inhibited in a concentration dependent relationship. 3) Lower phenolic acid concentrations delayed germination, while 5 and 10 mM caused significant germination inhibition. 4) Significant growth reduction generally occurred at a threshold concentration ranging between 0.1 to 1 mM depending on the growth variable measured. 5) Radicle elongation was more affected by the individual acids than either germination or shoot elongation. 6) FA was more inhibitory to both root and shoot length, whereas GA caused more reduction in root and shoot dry weight.

Figure 2.1. Effects of ferulic (FA), vanillic (VA), and gallic (GA) acids on corn germination. Within measurement time, data points followed by an asterisk indicate the greatest value which was significantly different from the control at 0.05 probability level as determined by contrast analysis.

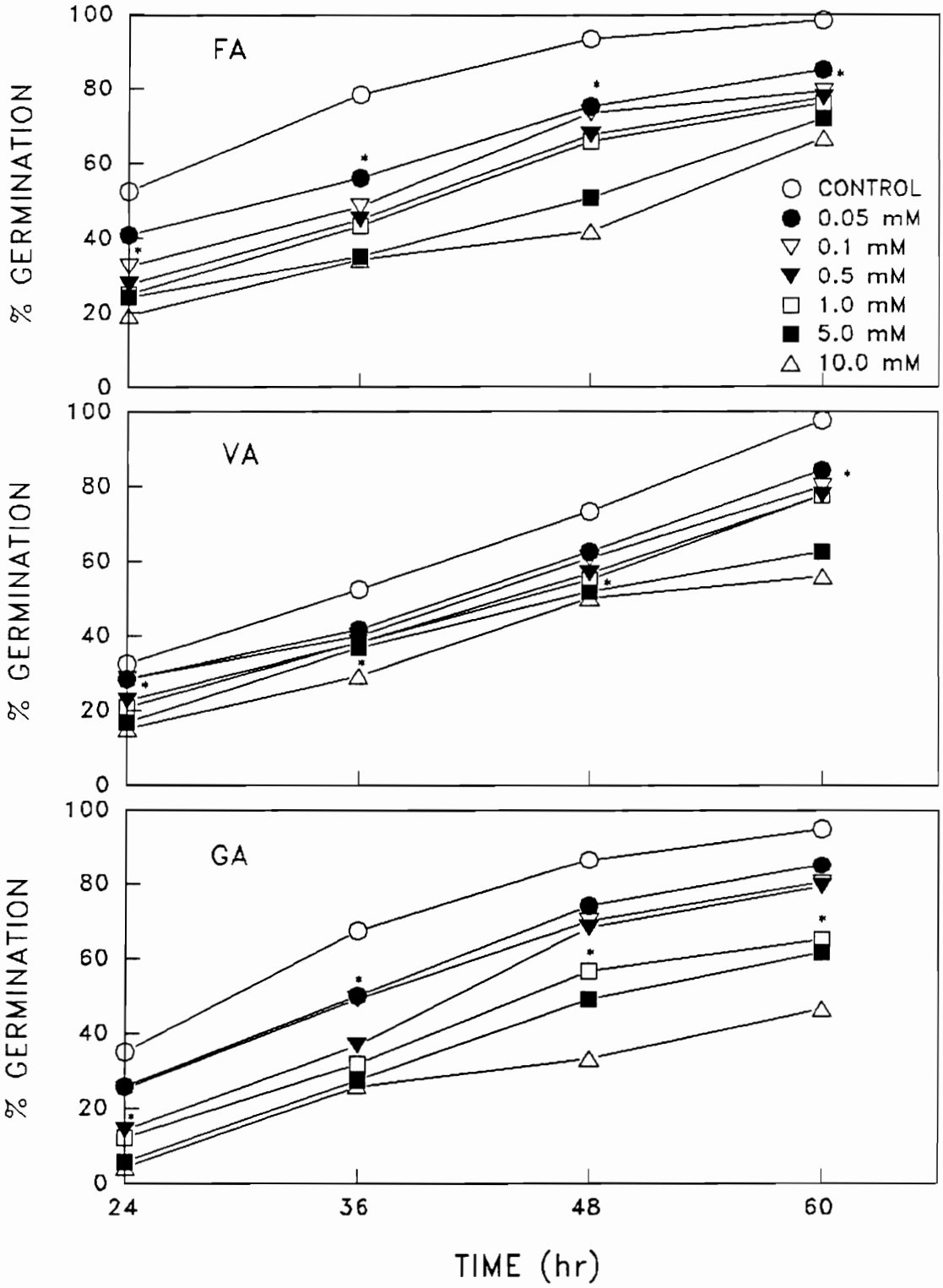

FA
VA
GA
% GERMINATION
TIME (hr)
CONTROL
0.05 mM
0.1 mM
0.5 mM
1.0 mM
5.0 mM
10.0 mM
0
20
40
60
80
100
24
36
48
60

Figure 2.2. Effects of ferulic (FA), vanillic (VA), and gallic (GA) on corn root and shoot length (A) and dry weight (B). Within acid concentrations at which significant differences were observed, data points followed by an asterisk indicate the greatest value which was significantly different from the control at 0.05 probability level as determined by contrast analysis.

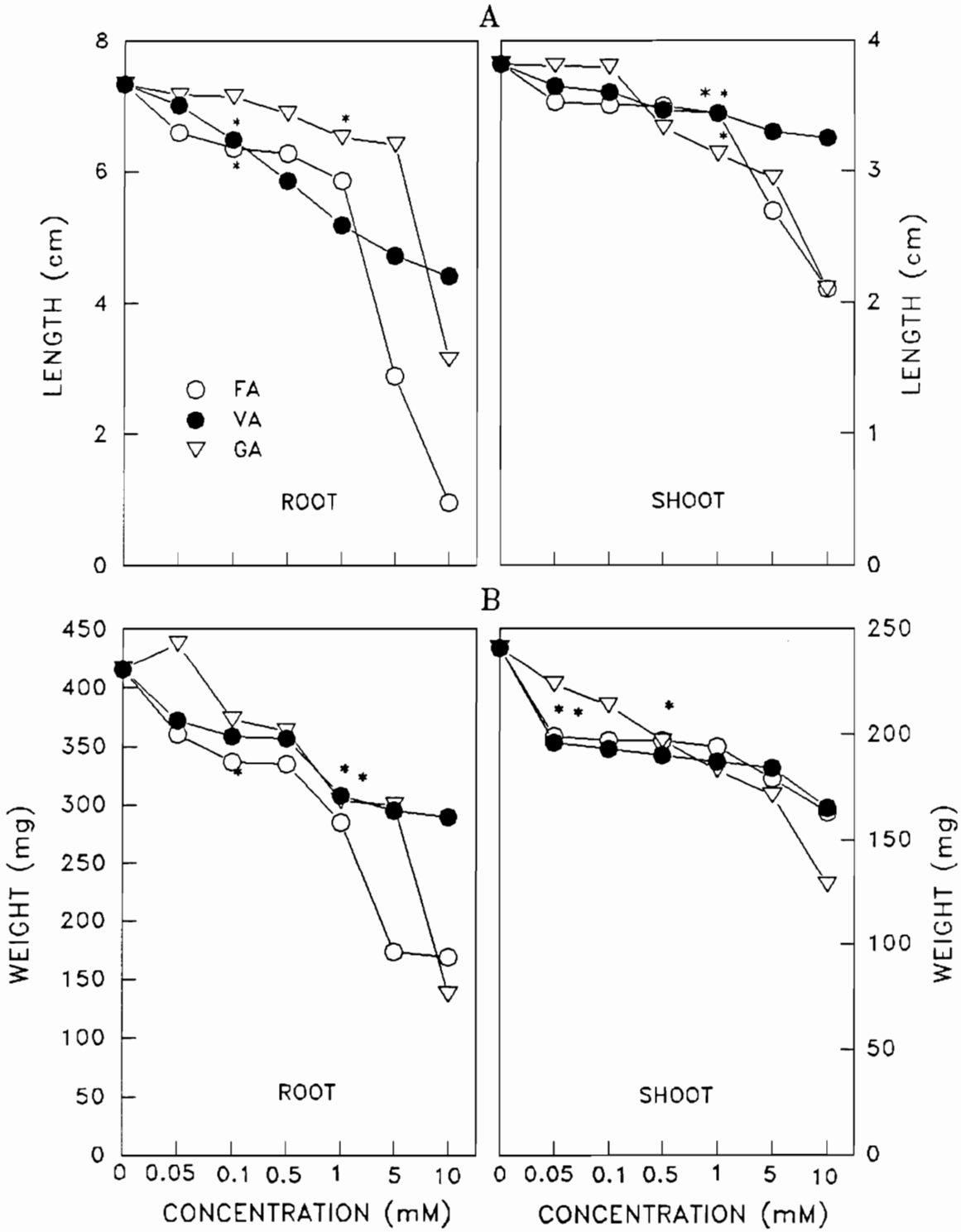

A
B
LENGTH (cm)
WEIGHT (mg)
FA
VA
GA
ROOT
SHOOT
CONCENTRATION (mM)
0
0.05
0.1
0.5
1
5
10

Figure 2.3. Effects of ferulic (FA), vanillic (VA), and gallic (GA) acids on radish germination. Within measurement time, data points followed by an asterisk indicate the greatest value which was significantly different from the control at 0.05 probability level as determined by contrast analysis.

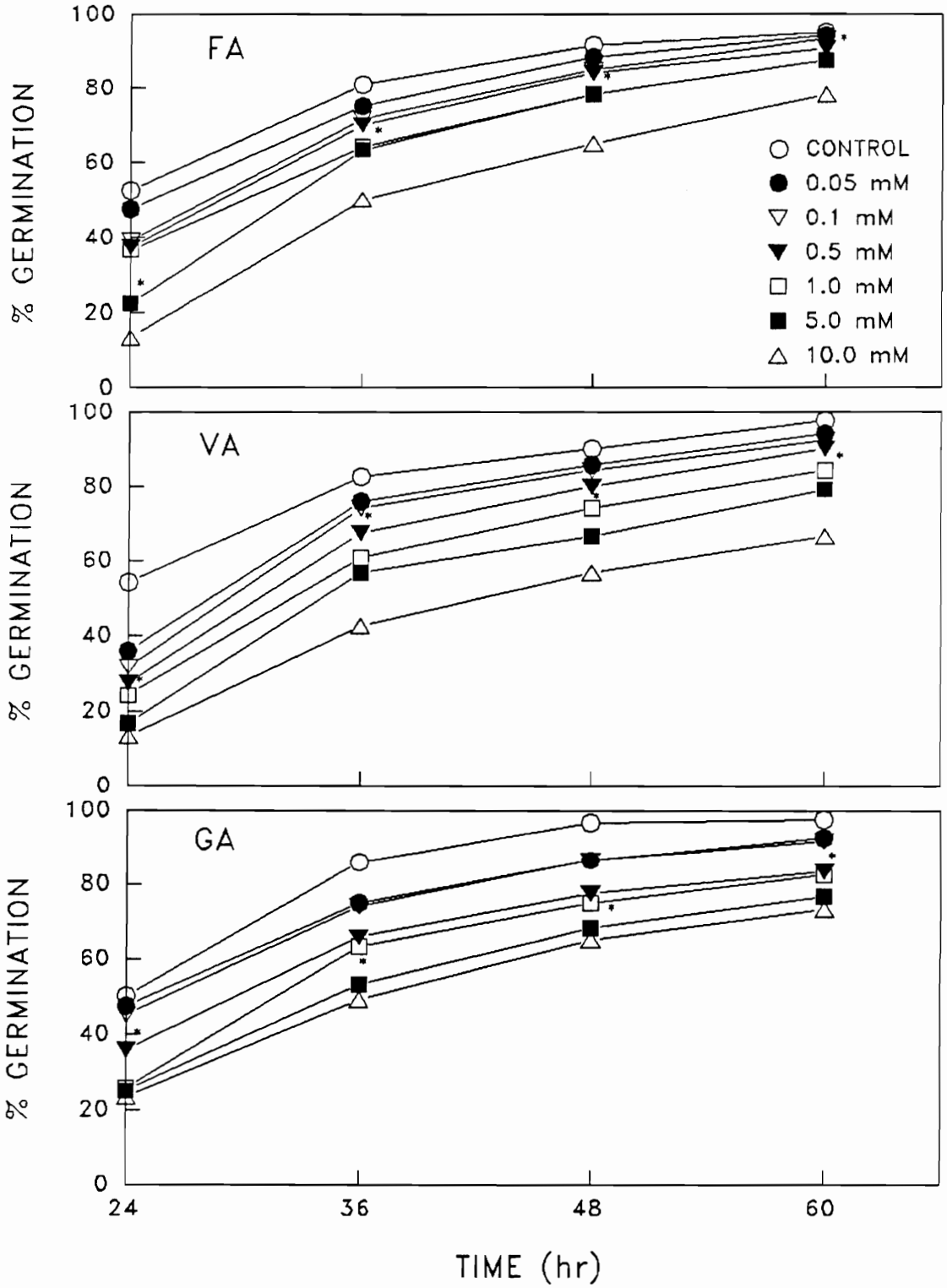

FA
VA
GA
% GERMINATION
TIME (hr)
CONTROL
0.05 mM
0.1 mM
0.5 mM
1.0 mM
5.0 mM
10.0 mM
0
20
40
60
80
100
24
36
48
60

Figure 2.4. Effects of ferulic (FA), vanillic (VA), and gallic (GA) on radish root and shoot length (A) and dry weight (B). Within acid concentrations at which significant differences were observed, data points followed by an asterisk indicate the greatest value which was significantly different from the control at 0.05 probability level as determined by contrast analysis.

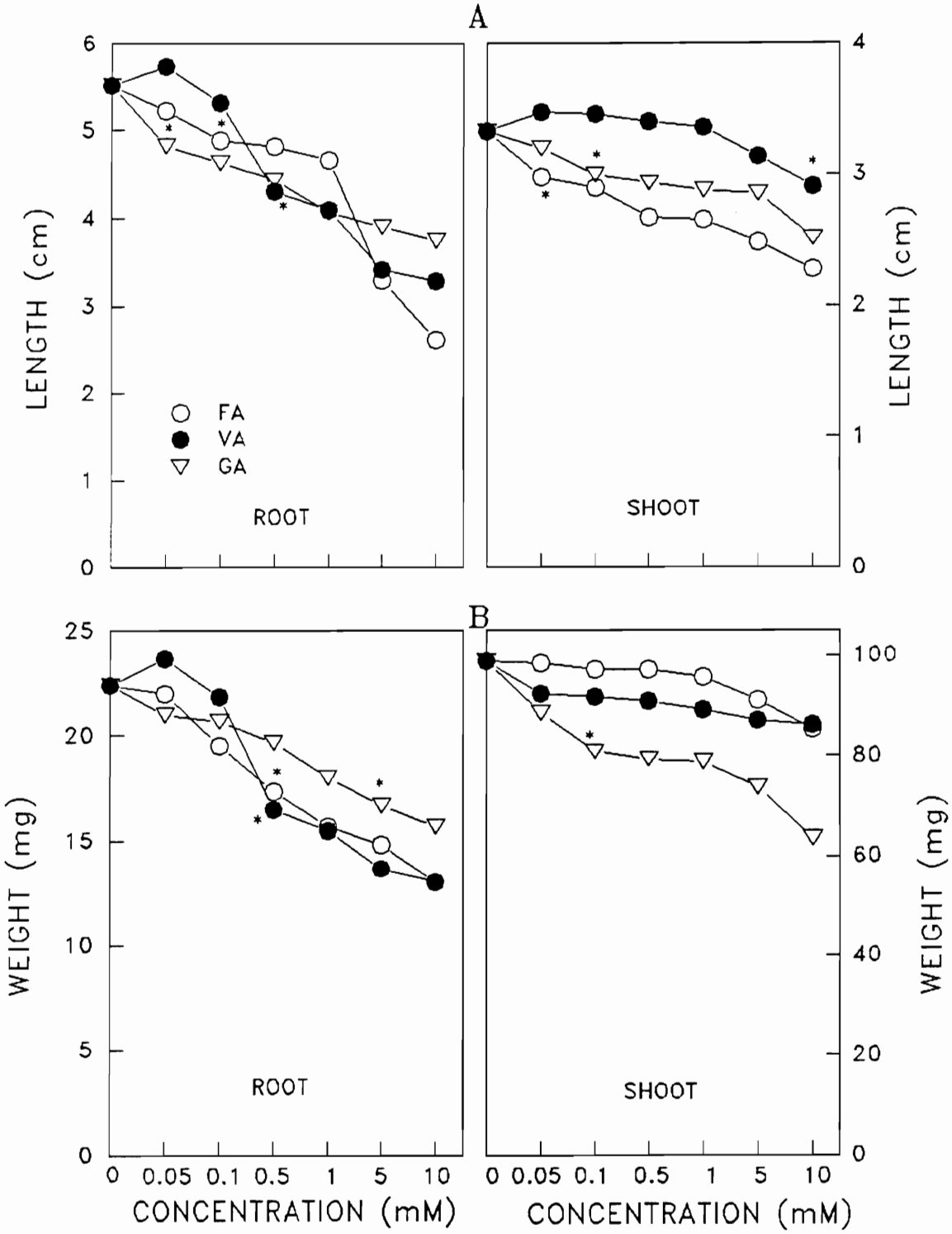

A
LENGTH (cm)
FA
VA
GA
ROOT
SHOOT
B
WEIGHT (mg)
ROOT
SHOOT
0 0.05 0.1 0.5 1 5 10
CONCENTRATION (mM)

Figure 2.5. Effects of ferulic (FA), vanillic (VA), and gallic (GA) acids on peanut germination. Within measurement time, data points followed by an asterisk indicate the greatest value which was significantly different from the control at 0.05 probability level as determined by contrast analysis.

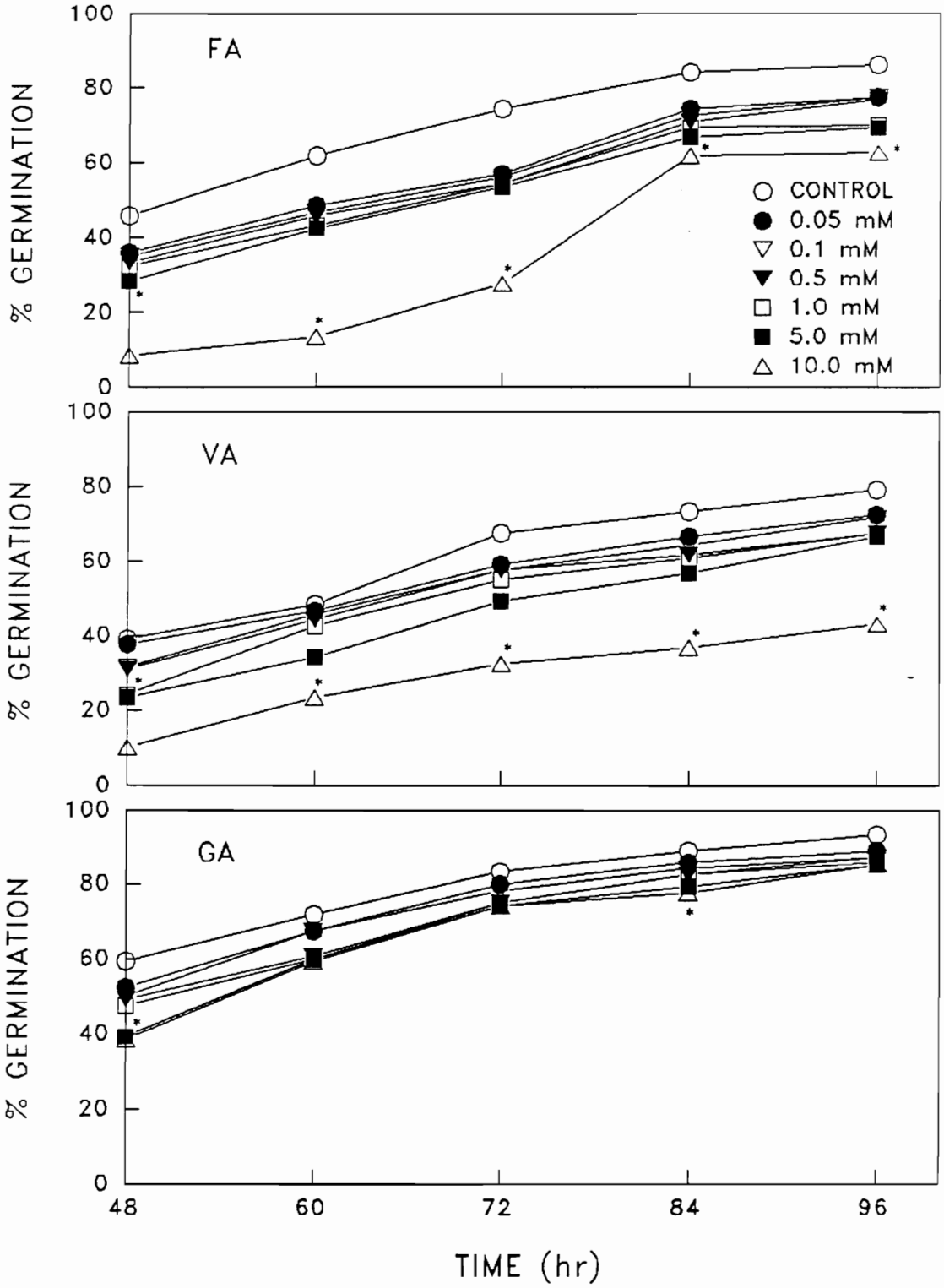

FA
VA
GA
% GERMINATION
TIME (hr)
CONTROL
0.05 mM
0.1 mM
0.5 mM
1.0 mM
5.0 mM
10.0 mM
0
20
40
60
80
100
48
60
72
84
96

Figure 2.6. Effects of ferulic (FA), vanillic (VA), and gallic (GA) on peanut root length (A) and dry weight (B). Within acid concentrations at which significant differences were observed, data points followed by an asterisk indicate the greatest value which was significantly different from the control at 0.05 probability level as determined by contrast analysis.

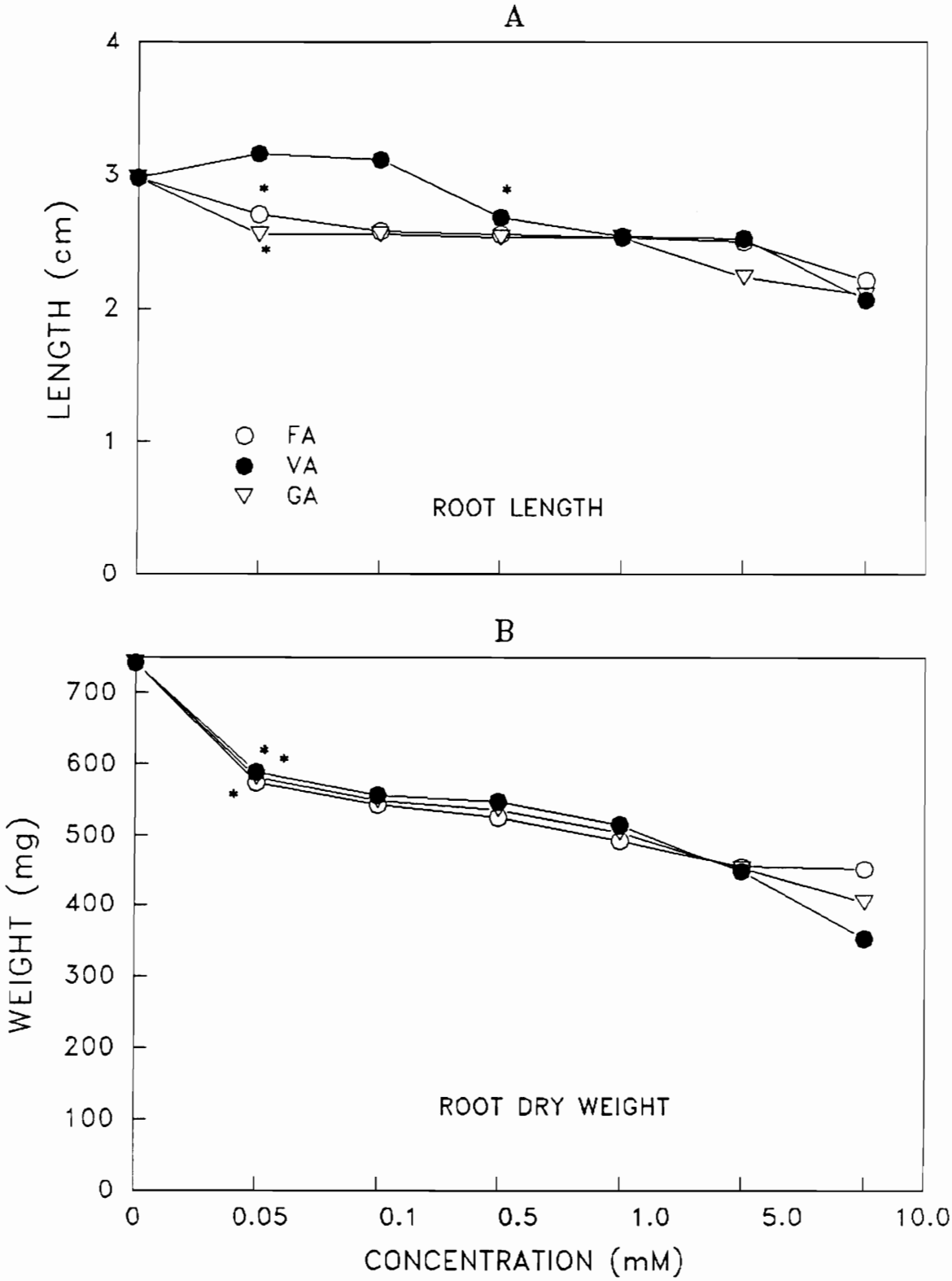
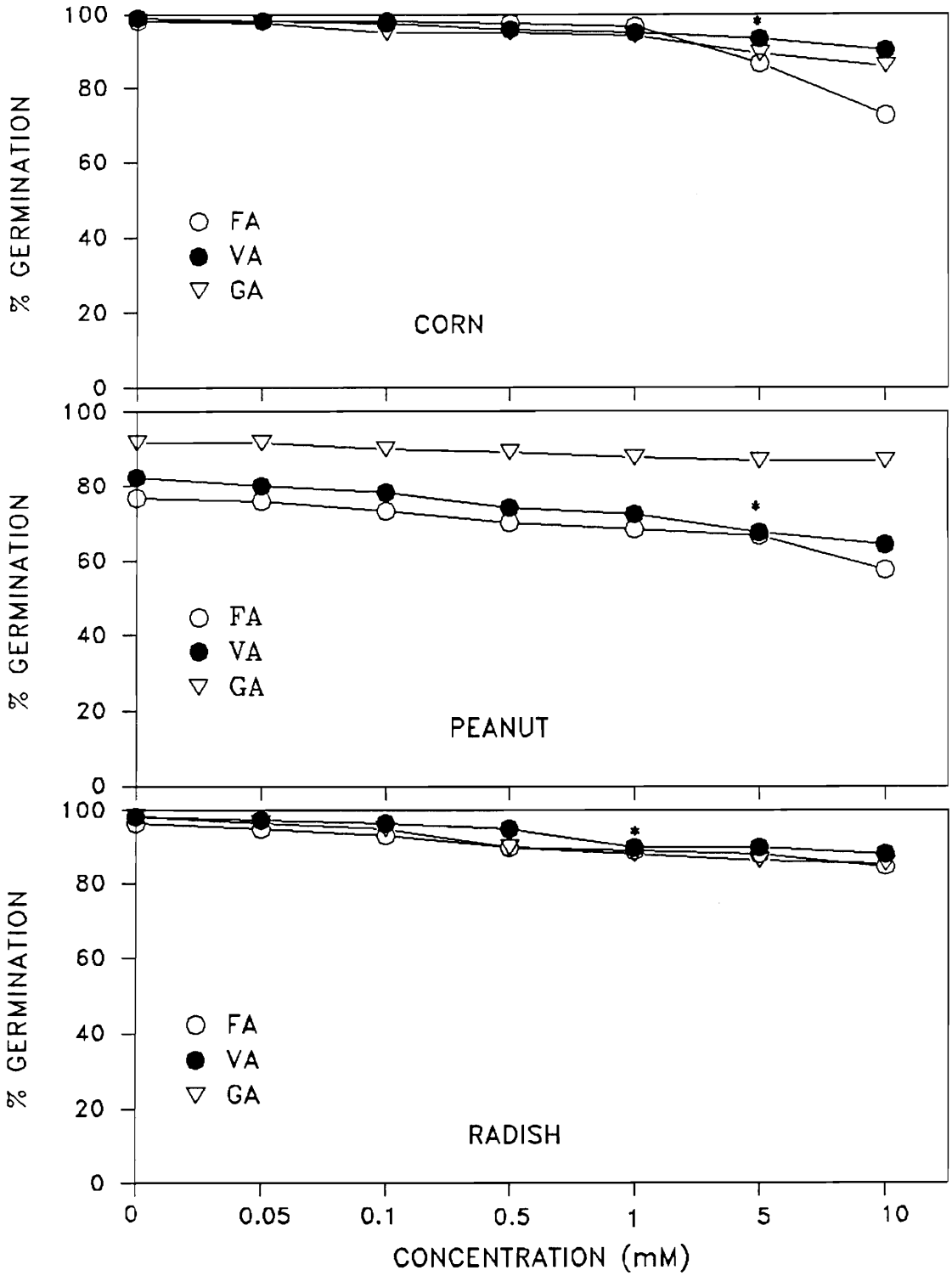

A
LENGTH (cm)
4
3
2
1
0
FA
VA
GA
ROOT LENGTH
B
WEIGHT (mg)
700
600
500
400
300
200
100
0
ROOT DRY WEIGHT
0
0.05
0.1
0.5
1.0
5.0
10.0
CONCENTRATION (mM)

Figure 2.7. Effects of ferulic (FA), vanillic (VA), and gallic (GA) acids on germination of corn, peanut at 10 days after treatment, and radish at 5 days after treatment. Within acid concentrations at which significant differences were observed, data points followed by an asterisk indicate the greatest value which was significantly different from the control at 0.05 probability level as determined by contrast analysis.


% GERMINATION
100
80
60
40
20
0
FA
VA
GA
CORN
PEANUT
RADISH
0
0.05
0.1
0.5
1
5
10
CONCENTRATION (mM)

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CHAPTER III

EFFECTS OF PHENOLIC ACID COMBINATIONS ON CORN GERMINATION AND SEEDLING GROWTH

Abstract. The allelopathic effects of two-way combinations of gallic acid, ferulic acid, and vanillic acid were evaluated on corn (*Zea mays* L.) germination. Root and shoot length and dry weight were used to determine the effects of these acids on the growth of corn seedlings. Phenolic acid concentrations ranged from 0 to 10 mM. Germination was monitored in petri dishes and the number of germinated seeds was recorded at 12-h intervals from 24 to 60 h. Root and shoot length of 10 randomly selected seedlings was measured 10 days after treatment. Root and shoot tissue was then oven dried and dry weights recorded. Results showed that phenolic acids in mixtures interacted significantly for root length and dry weight but not for shoot growth. FA and VA combinations showed significant interactions on corn germination at 24 and 60 h after treatment. Statistical characterization of the interactions revealed that all levels of FA antagonized higher concentrations of either VA or GA. Few interactions were synergistic and they occurred only when GA was present in the combination.

INTRODUCTION

Phenolic acids are among the secondary plant metabolites that have received considerable attention due to their protective functions against plant pests and their involvement in allelopathic situations (Klocke, 1987; Putnam, 1985; Einhellig, 1985; Rice, 1984). Allelopathic effects of several crops and weeds have been attributed to the presence of phenolic compounds in their residues (Shilling et al, 1985; Tang et al., 1982, Chou et al, 1975). The benzoic acid derivatives gallic and vanillic acids, as well as the cinnamic acid derivative ferulic acid, have been among those most commonly identified from higher plants (Jain et al, 1989; Fisher, 1987; Saggese et al., 1985, Mandava; 1985) and from different soil systems (Kuiters et al, 1987; Vance et al., 1985). Mixtures of these acids have been isolated from soybean (Porter et al, 1986; Hardin et al., 1980), corn, rye, and rice (Chou, 1976), wheat (Lodhi et al., 1987), trees (Kuiters et al, 1987, 1986; Chou et al, 1986), and several other plants (Chou, 1989; Lydon et al, 1986; Shilling et al., 1985).

Bioassay studies on the effects of individual compounds showed that the threshold level of most phenolic acids that causes inhibition of germination and postemergence growth is, generally, about 1 mM or above (Einhellig, 1987). However, it has been argued that in natural environments, allelopathic effects are the results of the collective action of complex mixtures of allelochemicals including phenolic compounds (Einhellig, 1987; Einhellig et al., 1982). Studies on

the combined effects of phenolic acids are very limited and the results are conflicting. While there is a general agreement that phenolic acids act additively, some interactive effects of phenolic mixtures have been shown to be synergistic (Einhellig et al., 1982, 1978; Rasmussen et al, 1977), and others have been demonstrated to be antagonistic (Blum et al, 1989; Duke et al., 1983; Williams et al., 1982).

An understanding of cooperative action of different mixtures of phenolics is an important aspect of allelopathic investigations. Characterizing the interaction effects of phenolic combinations may help to further clarify the actual role, impact, and expression of allelopathic phenomena associated with phenolic acids and their combinations.

This study was undertaken to investigate and characterize the nature of two-way combinations of ferulic, vanillic, and gallic acids on corn germination and seedling growth.

MATERIALS AND METHODS

Experimental procedure. Corn (*Zea mays* L.) (cv. Southern States 727) was used in bioassays of phenolic acid mixtures. Phenolic acids as well as MES buffer [2-(N-morpholino)ethanesulfonic acid] were obtained from Sigma Chemical Company, St. Louis, Missouri.

Two-way combinations of ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid), vanillic acid (VA) (4-hydroxy-3-methoxybenzoic acid), and gallic acid (GA) (3,4,5,-trihydroxybenzoic acid) were evaluated. The six different concentrations used for each of the phenolic acids included 0, 0.1, 0.5, 1, 5, and 10 mM. Each two-way combination was structured as a matrix of six levels (including the 0 mM control) of each of the acids present in the combination. The control treatment consisted of 3 mM MES, which was used in preparing all concentrations of the acids. Complete dissociation of acids was achieved by adding a few drops of 0.1 N NaOH, which was also included in the control. The pH of all solutions and mixtures was adjusted to 5.8 with 0.1 N NaOH.

Twenty corn seeds were placed in 10 cm petri dishes on a disk of Whatman No 1 filter paper moistened with 7 ml of test solution. Germination proceeded in the dark in an incubator at 25 +/- 1 C and germinated seeds were counted at 24, 36, 48, and 60 h after treatment. Seeds were considered germinated when the radicle extruded at least 2 mm. Five days after initial treatment, 3 ml of the same test solution were added to the petri dishes. Ten

days after initial treatment, roots and shoots of 15 randomly selected seedlings were harvested and their length measured. Dry weights were obtained after oven drying roots and shoots at 55 C for 48 h.

Experimental design and analysis. All experiments were analyzed as a two-way (6 by 6) factorial in a randomized complete block design with 6 replications. Data were subjected to analyses of variance, and significance was determined by partitioning the effects of treatments into main effects (single acid) and interactions (acid combinations). Statistically significant interactions were further characterized by the statistical treatment of Colby's method developed by Flint et al., (1988). All experiments were repeated twice.

RESULTS

Analyses of variance revealed that phenolic acids in mixtures interacted significantly for root length and dry weight but not for shoot growth. FA and VA combinations showed significant interactions on corn germination at 24 and 60 h after treatment (Table 3.1). Since only significant interactions were further statistically characterized, other interactions are not reported.

Combination of FA with VA. The combinations of FA and VA interacted significantly on both corn germination and radicle growth (Table 3.1). A significant interaction was not detected on shoot growth. Although some antagonistic interactions involving the highest rate of either acid in mixture were

evident on germination, most of the combined treatments of FA and VA interacted additively (Figure 3.1 A). Moreover, some of the antagonistic interactions that occurred on germination at 24 h were not evident at 60 h after treatment (Figure 3.1 B). At this time, all FA concentrations except 5 mM decreased the inhibitory effects of 1 x 10 mM VA, resulting in antagonistic interactions.

Of the twenty five combinations characterized for root length, one-third were shown to be antagonistic (Figure 3.2. A). They involved all concentrations of FA, which statistically reduced the effects of 5 and 10 mM VA. Similar antagonistic trends between FA and VA were observed on root dry weight (Figure 3.2. B).

Combination of GA with VA. Root and shoot lengths and root dry weight were the only growth parameters where significant interactions between GA and VA treatments occurred (Table 3.1). Most of these interactions, however, were additive. GA at 5 mM antagonized the effects of VA on root length at this and lower concentrations (Figure 3.3. A). At the same concentration, VA decreased the action of GA at 0.5 and 1 mM. At equimolar concentrations of 10 mM, these acids caused a synergistic reduction of root length. All levels of GA, however, counteracted the effects on root dry weight of VA at 0.5 and 5 mM (Figure 3.3 B).

Two synergistic interactions of GA and VA occurred on shoot length

(Figure 3.4. A). They involved the combination of the highest GA concentration with either 5 or 10 mM VA. Two other interactions were identified and characterized as being antagonistic: concentrations of 5 mM of both acids and the combination of 10 mM GA with 0.1 mM VA. The interactive actions of these two acids at the remaining concentrations were additive.

Combination of FA with GA. Interactive effects of FA and GA were significant for root length only (Table 3.1). Although most of these effects were additive, some antagonistic as well as synergistic actions were also observed (Figure 3.4 B). All levels of FA except 10 mM significantly reduced the inhibitory effects of 10 mM GA, resulting in greater root length than expected. Lower concentrations of GA, however, significantly increased the action of FA at 10 mM, causing less root elongation than expected.

DISCUSSION

The results of this study have demonstrated that both FA and GA, when applied alone, significantly reduce the growth parameters investigated. VA alone, however, did not significantly inhibit corn shoot growth. However, when these acids where applied in a two-way combination, their interactive effects on germination and shoot growth were, generally, not significant (Table 3.1).

Statistical characterization of interactions revealed that they ranged from

antagonism to synergism depending on the phenolic acids combined, their concentrations, and on the growth parameter measured (Figures 3.1, 3.2, 3.3 and 3.4). While some of the interactive effects were antagonistic for all combinations of acid pairs used in these experiments, synergism occurred only when GA was present in the combination (Figure 3.4 A and B). These synergistic effects involved the highest concentration of at least one of the acids. However, all levels of FA used in mixtures generally counteracted the inhibitory effects of either VA or GA at 0.1 mM and higher concentrations. This may be explained by the fact that FA is more inhibitory than either VA or GA. For instance, at 5 mM, FA caused 67% reduction in root length compared to 37 and 46% obtained with the same concentration of VA and GA, respectively (data not shown).

Antagonism, synergism, and additivity have been shown for various mixtures of phenolic acids. Einhellig et al.(1977) reported that equimolar concentrations of 1 mM VA and p-hydroxybenzoic acids caused synergistic effects on both radish and sorghum germination and sorghum root length (Einhellig et al., 1978). Similar results were reported for combinations of FA with p-coumaric acid (Einhellig et al., 1982). Although, in our study, no synergistic interactions were shown on corn germination, some did occur on corn length. Duke et al.(1983) demonstrated, however, that while, the interactive effects of FA and *p*-coumaric acid where additive, those of FA and caffeic acid were antagonistic. The authors concluded that caffeic acid may competitively inhibit the effects of FA. Recent

investigations, however, have demonstrated interference in the uptake of one phenolic acid by another (Shann et al., 1987). These authors observed a 30% reduction in uptake of *p*-hydroxybenzoic acid when FA was present in the mixture compared to the uptake of p-hydroxybenzoic acid when applied alone. Similarly, Lyu et al. (1990) reported that the uptake of VA was decreased 52 to 66% in the presence of FA. They also noted that, when applied alone, the uptake of VA was 1.5 to 4.6 times lower. These findings may explain the results obtained in this study. The antagonistic interactions that occurred may be due to a differential uptake of these acids by corn roots or to an interference of one of the acids with the other present in the mixture.

In summary, this research has demonstrated that radicle growth was more sensitive to the combined effects of phenolics than either corn germination or shoot growth. Although most of the interactive effects were additive, all levels of FA generally antagonized higher concentrations of either VA or GA. Few interactions were synergistic, and they occurred only when GA was present in the mixture.

TABLE 3.1. SIGNIFICANCE OF INTERACTIONS IN TWO-WAY COMBINATIONS OF PHENOLIC ACIDS ON CORN[a].

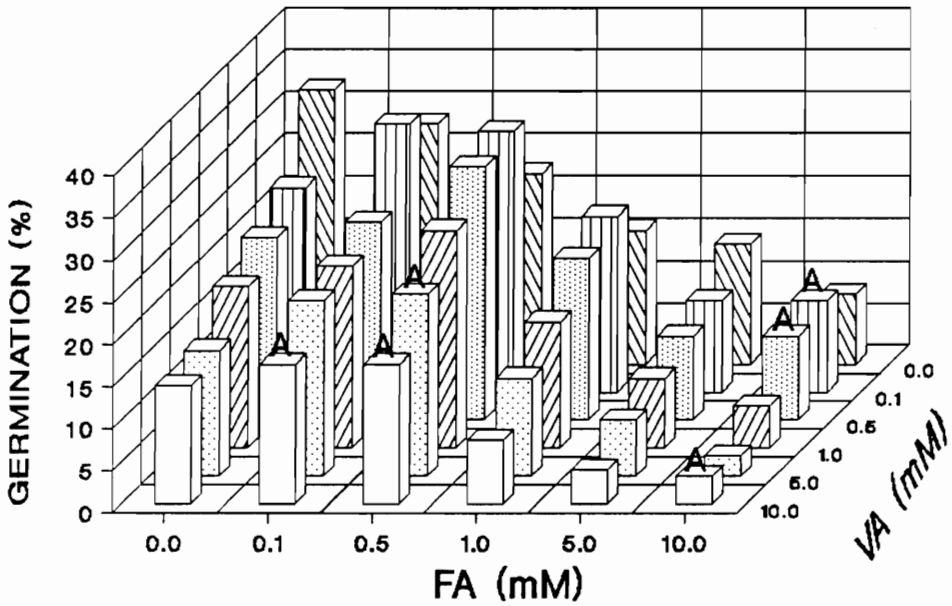
Source of	Germination				Length		Weight	
Variation	24 h	36 h	48 h	60 h	Root	Shoot	Root	Shoot
FA	*	*	*	*	*	*	*	*
VA	*	NS	NS	*	*	*	*	NS
FA x VA	*	NS	NS	*	*	NS	*	*
FA	*	*	*	*	*	*	*	*
GA	*	*	*	*	*	*	*	*
FA x GA	NS	NS	NS	NS	*	NS	*	NS
GA	*	*	*	*	*	*	*	*
VA	*	*	*	*	*	*	*	NS
GA x VA	NS	NS	NS	NS	*	*	*	NS

[a]Significance determined by ANOVA test. * indicates significance at 0.05 probability level.

NS = not significant at 0.05 probability level.

Figure 3.1. Effects of FA and VA combinations on corn germination at 24 h (**A**) and 60 h (**B**) after treatment. **A** indicates antagonism at 0.05 level of significance according to the statistical treatment of Colby's method. Bars with no letter represent additive interactions.

A


GERMINATION (%)
40
35
30
25
20
15
10
5
0
0.0
0.1
0.5
1.0
5.0
10.0
FA (mM)
VA (mM)
A

B

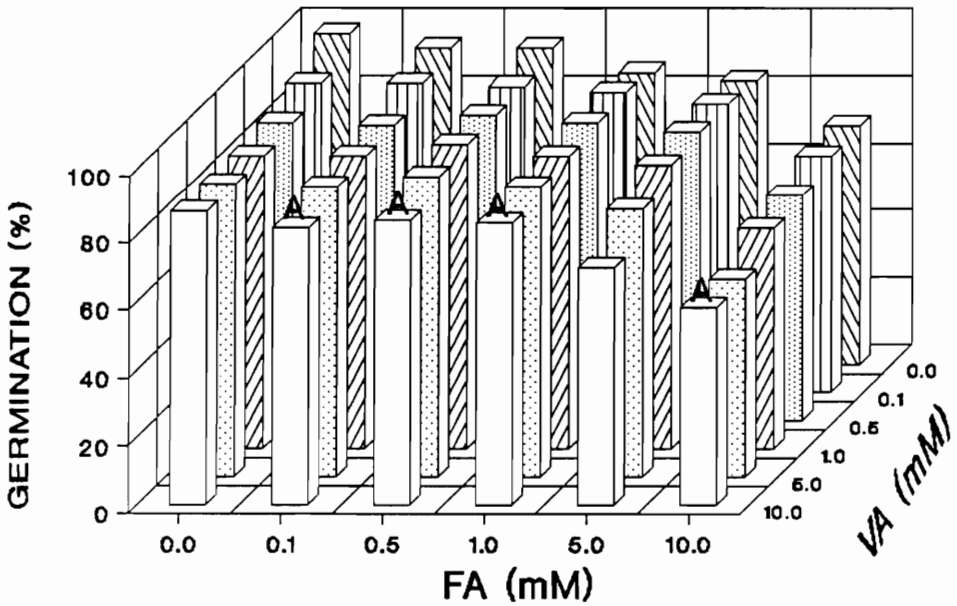
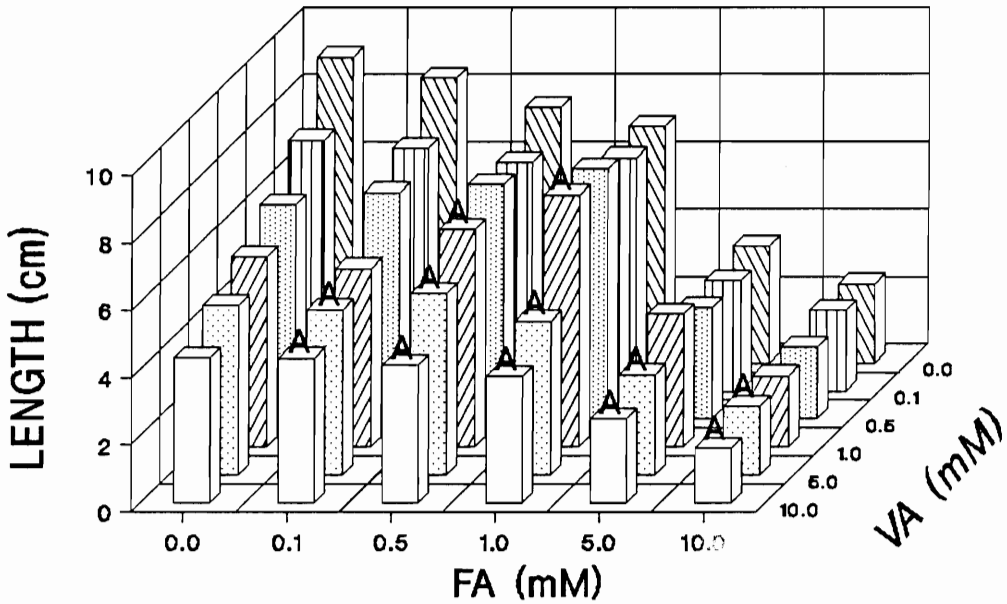

GERMINATION (%)
100
80
60
40
20
0
0.0
0.1
0.5
1.0
5.0
10.0
FA (mM)
VA (mM)
A

Figure 3.2. Effects of FA and VA combinations on corn root length (**A**) and dry weight (**B**). **A** indicates antagonism at 0.05 level of significance according to the statistical treatment of Colby's method. Bars with no letter represent additive interactions.

A


LENGTH (cm)
0
2
4
6
8
10
0.0
0.1
0.5
1.0
5.0
10.0
FA (mM)
VA (mM)

B

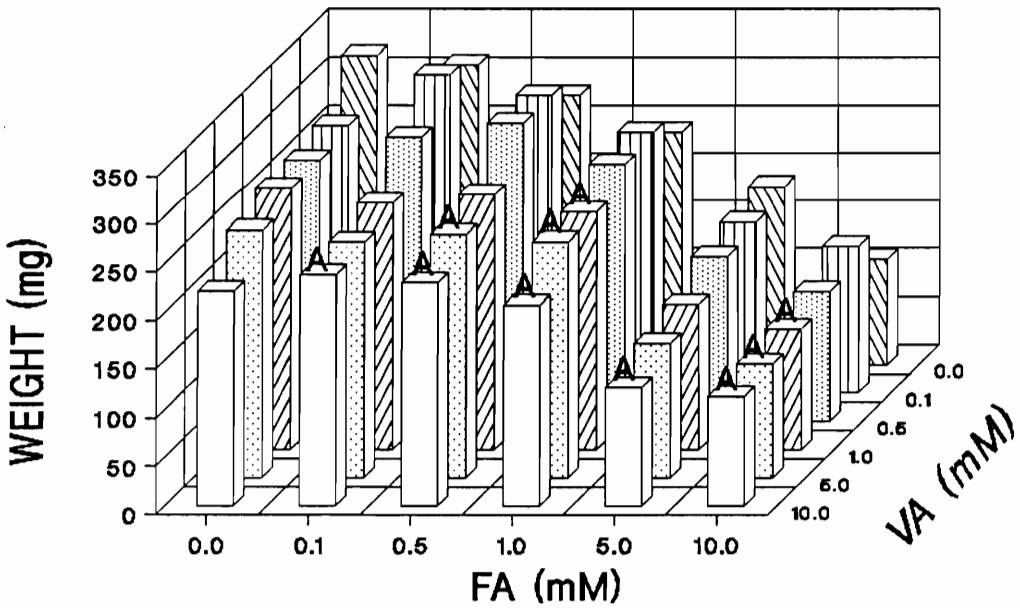
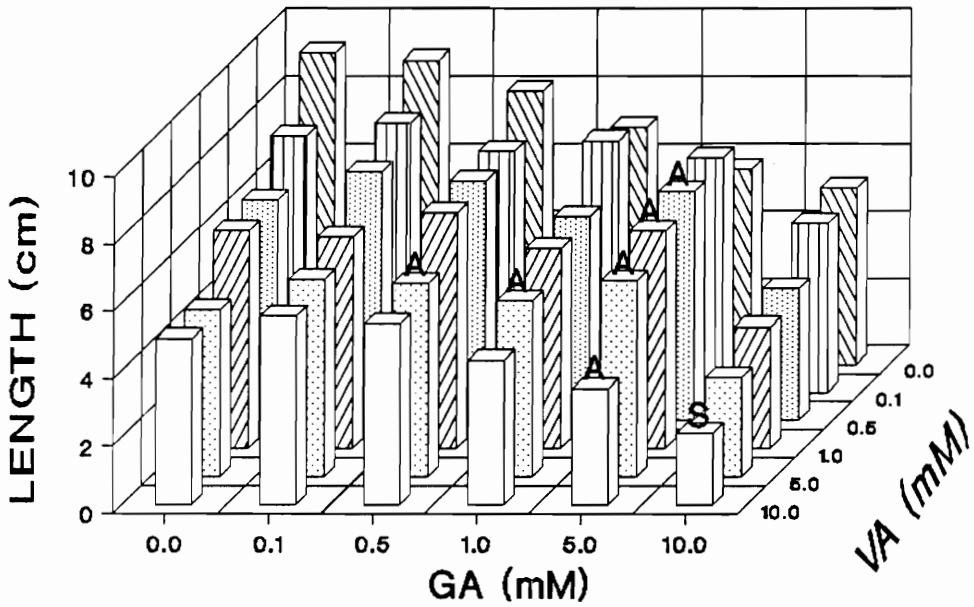

WEIGHT (mg)
0
50
100
150
200
250
300
350
0.0
0.1
0.5
1.0
5.0
10.0
FA (mM)
VA (mM)

Figure 3.3. Effects of GA and VA combinations on corn root length (**A**) and dry weight (**B**). **A** indicates antagonism and **S** indicates synergism at 0.05 level of significance according to the statistical treatment of Colby's method. Bars with no letter represent additive interactions.

A


LENGTH (cm)
0
2
4
6
8
10
0.0
0.1
0.5
1.0
5.0
10.0
GA (mM)
VA (mM)
A
S

B

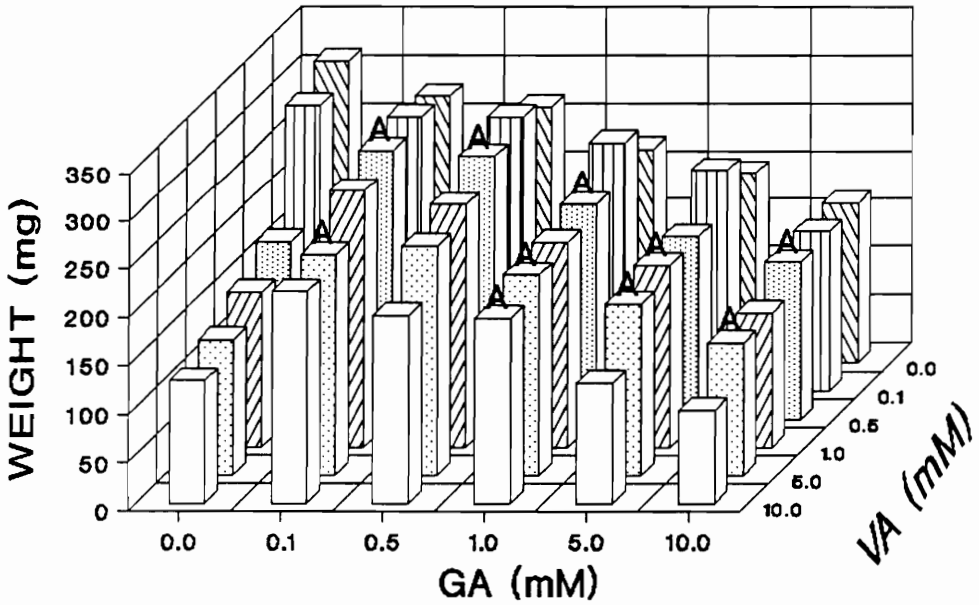
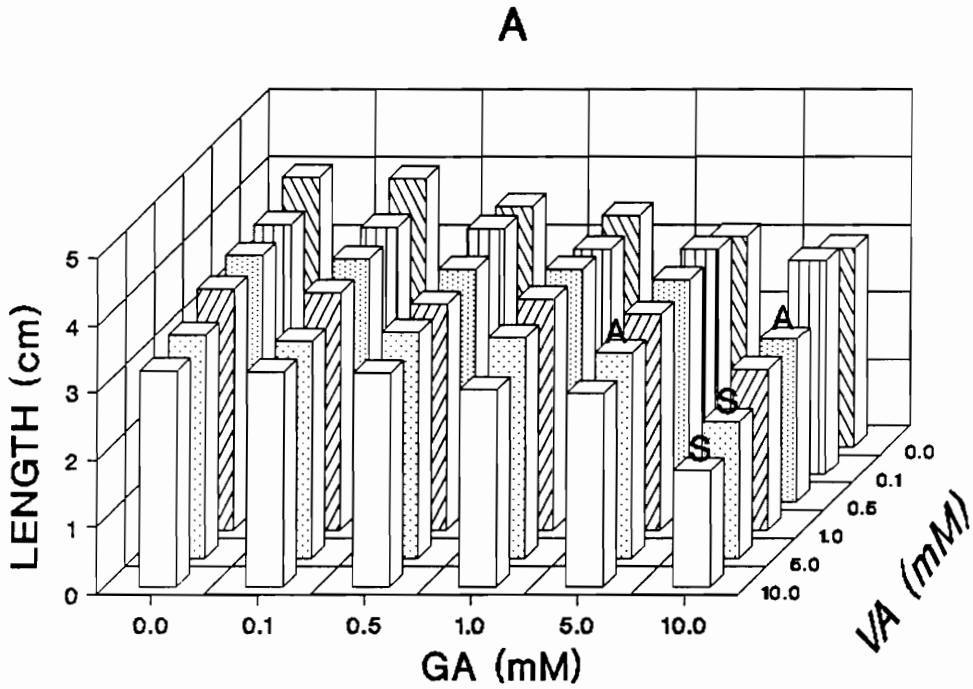
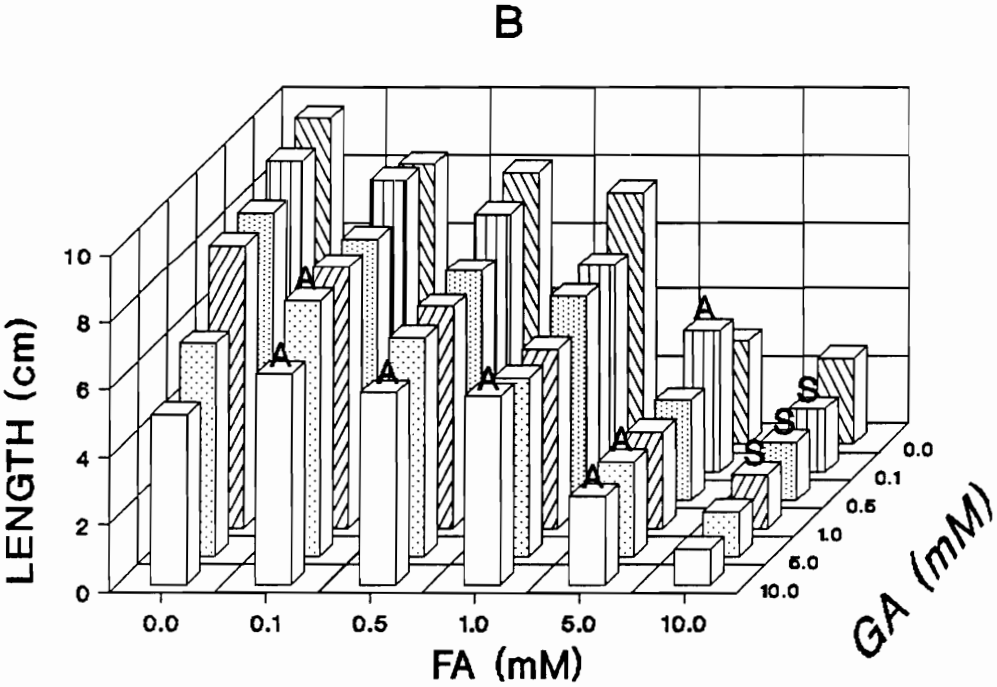

WEIGHT (mg)
0
50
100
150
200
250
300
350
0.0
0.1
0.5
1.0
5.0
10.0
GA (mM)
VA (mM)
A

Figure 3.4. Effects of GA and VA combinations on corn shoot length (**A**) and of FA and GA on corn length (**B**). **A** indicates antagonism and **S** indicates synergism at 0.05 level of significance according to the statistical treatment of Colby's method. Bar with no letter represent additive interactions.


A
LENGTH (cm)
0
1
2
3
4
5
0.0
0.1
0.5
1.0
5.0
10.0
GA (mM)
VA (mM)
A
S


B
LENGTH (cm)
0
2
4
6
8
10
0.0
0.1
0.5
1.0
5.0
10.0
FA (mM)
GA (mM)
A
S

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CHAPTER IV

EFFECTS OF SOIL APPLIED PHENOLIC ACIDS ON GROWTH OF CORN SEEDLINGS

Abstract. Gallic, vanillic, and ferulic acids, alone and in combination, have been shown to inhibit corn germination and radicle and hypocotyl growth in petri dishes. This study was undertaken to examine the allelopathic effects of these acids on corn seedlings grown in soil. Phenolic acids were applied alone or in two-way combinations. Amounts of phenolic acids added to soil ranged from 8.3 to 832 ug/g dry soil. Fourteen days after treatment, the length of seedling roots and shoots was measured and their dry weight was obtained after oven drying at 55 C for 48 h. Results showed that ferulic acid alone at 83 ug/g dry soil and higher levels inhibited root and shoot length and dry weight. Gallic acid alone at 364 ug/g dry soil was effective in reducing shoot length and dry weight. Vanillic acid did not cause any significant reduction in the corn growth parameters investigated. The interactive effects of pair mixtures of these acids were shown to be not statistically significant, suggesting additive effects.

INTRODUCTION

Phenolic compounds including ferulic, vanillic, and gallic acids have been shown to occur widely in soils (Kuiters et al., 1987; Vance et al., 1986; Flaig, 1971; and Whitehead et al., 1964, 1981, 1983). They are mainly released during decomposition of plant materials (Abdul-Rahman et al., 1989; Lodhi et al., 1987; Kogel et al., 1985; Jalal et al., 1983; Whitehead et al., 1982) and are also synthesized by soil microorganisms (Harborne, 1989; Shilling et al., 1895; Liebel et al., 1983; Vaughan et al., 1983; Turner et al., 1975; Martin et al., 1971). Both free and bound forms of ferulic and vanillic acids have been shown to exist in soils (Whitehead et al., 1983; and Katase, 1981).

Because of various extractants and extraction procedures, information on soil phenolic concentrations has varied widely from one report to another. As a consequence, the amount of individual compounds reported in soils ranges from less than 10 ppm to 1000 ppm (Jalal et al., 1983; Einhellig et al., 1982; Lodhi 1978; Chou et al., 1976; Whitehead et al., 1982, 1981; Whitehead, 1964). Some researchers argue that the levels of phenolic acids in soil are too low to account for any inhibitory effects; but others suggest that phenolic substances are not evenly distributed in soil and concentrations that reach toxic levels are probably present in localized pockets of decomposing residues (Rice, 1984, Whitehead et al., 1983; Liebel et al., 1983).

Many studies that have dealt with the effects of decaying plant residues in

soils have implicated phenolic acids as inhibiting compounds (Menges, 1987; Shilling et al., 1985; Tang et al., 1982; Drost et al., 1980; Toai et al., 1979; Hartley et al., 1979; Chou et al., 1976). Problems with studying plant residues and their decomposition involve the identity of the chemicals, their concentrations in soil, and their biological activities. Very limited research has been done on the effects of exogenously applied ferulic acid (Blum et al., 1987; Liebel et al., 1983) and ferulic-vanillic combination (Blum et al., 1989). To the best of our knowledge, no research has examined the effects of exogenous gallic acid in the soil.

Results reported in the previous two chapters have shown that ferulic, vanillic, and gallic acids applied alone and in combination inhibit corn germination and growth in bioassays. These inhibitory effects, however, were acid, concentration, and growth variable dependent.

The objectives of this study were to 1) investigate the biological activity on corn growth of soil-applied ferulic, vanillic, and gallic acids alone and in two-way combinations, and 2) determine the threshold concentrations of phenolic acids on corn growth inhibition in a soil system.

MATERIALS AND METHODS

Experimental procedure. Experiments were conducted in a greenhouse on a Ross silt loam soil (fine-loamy, mixed, mesic Cumulic Hapludolls) collected from an uncultivated site on the Whitethorne-Kentland farm near Blacksburg, Virginia. Soil was air dried and sieved through a 2-mm mesh screen, and the undecomposed plant residues were discarded. The soil had a pH of 6.9 and contained 2.6% organic matter and 18, 36, 1200, and 120 ppm of P, K, Ca, and Mg, respectively, according to analysis by the Virginia Polytechnic Institute and State University Soil Testing Laboratory. Plastic pots (9.6 cm diameter) were filled with air-dried soil (350 g/pot).

Phenolic compounds consisted of ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid), gallic acid (GA) (3,4,5-trihydroxybenzoic acid), and vanillic acid (VA) (4-hydroxy-3-methoxybenzoic acid). Each acid was dissolved in 3 mM MES [2-(N-morpholino)ethanesulfonic acid] buffer, and appropriate dilutions of the stock solution were made to obtain final concentrations of 0.1, 0.5, 1, 5, and 10 mM. When expressed on soil dry weight basis these concentration were equivalent to 8.3, 41.6, 83, 416, and 832 ug/g dry soil for FA, 7.2, 36, 72, 360, and 720 ug/g dry soil for VA, and 7.3, 36.4, 72.9, 364, and 729 ug/g dry soil for GA. Controls consisted of buffer treated soil. The effects of each phenolic acid alone and in two-way combinations were examined. Phenolic acid combinations consisted of ferulic-vanillic, ferulic-gallic, and gallic-vanillic.

Six corn (*Zea mays* L.) seeds (cv. Southern States 727) were planted in each pot, and 150 ml/pot of test solution were added at planting. After emergence (3 to 4 days after planting) plants were thinned to 4 seedlings/pot. Pots were subirrigated every other day with 50 ml distilled water except on the fifth and tenth days, when test solution was added. Both individual acid and two-way phenolic combination experiments were conducted in a greenhouse with an average day light of 600 $uE/m^2/s$ and average day/night temperatures of 35/25 +/- 2 C. Fourteen days after treatment the soil was carefully washed from the roots. Seedling roots and shoots were harvested separately, and their length and fresh weight measured. Dry weights were obtained after oven drying roots and shoots at 55 C for 48 h.

Experimental design and analysis. Single acid studies were arranged in a randomized complete block design. Two-way acid combination experiments were set up as a 6 x 6 factorial in a randomized complete block design. All experiments had four replications and were repeated twice.

Data from single acid experiments were analyzed by single degree of freedom contrast analysis, where each acid concentration was compared to the control. Data of two-way combination experiments were subjected to analysis of variance, and significance was determined by partitioning the effects of treatments into main effects (single acids) and interactions (acid combinations).

RESULTS

Analyses of variance of two-way acid combination data did not reveal any significant interactions between any acid pairs. There were, however, significant individual acid effects. These effects were acid and growth variable dependent (data not shown). Because of non-significant interactions, means of one acid were averaged over levels of the other acid present in the combination. LSD was then used to separate means of the main effects. This analysis showed similar trends as those obtained with individual acids. Therefore, only the results obtained with single acid application experiments are reported in this study.

The effects of different concentrations of each phenolic acid applied alone on corn root and shoot length are given in Figure 4.1, 4.2, 4.3 A. Whereas neither GA nor VA affected root length, FA caused significant reduction at 83, 416, and 832 *u*g/g dry soil. The magnitude of this reduction (13%) was equal for the three FA concentrations. FA significantly inhibited shoot length at 83 *u*g/g dry soil and higher concentrations. GA concentrations of 364 and 729 *u*g/g dry soil; however, caused more inhibition than FA at 416 and 832 *u*g/g dry soil. Although not significant, stimulation of root and shoot length was observed with lower concentration of VA. No significant effects on root and shoot length were observed with higher concentrations of this acid.

Both FA and GA significantly inhibited root and shoot fresh weight (Figure 4.1 and 4.2 B). Significant shoot fresh weight reduction occurred at

concentrations five times higher than that at which root fresh weight was reduced. Moreover, both root and shoot fresh weight were more inhibited with FA than with GA. FA at 832 ug/g dry soil caused 35 and 38% reduction in root and shoot fresh weight, respectively, compared to 23 and 25% reduction for GA at 729 ug/g dry soil. There was no significant effect with any VA concentration on either root or shoot fresh weight.

Effects of the individual phenolic acids on corn root and shoot dry weight were similar to those obtained for root and shoot length (Figure 4.1, 4.2, and 4.3. C). While shoot dry weight was inhibited by both FA and GA at 83 and 72.9 ug/g dry soil and higher concentrations, respectively, root dry weight was effectively inhibited by these FA levels only. VA had no significant effect on either root or shoot dry weight.

DISCUSSION

The results of this study show that FA and GA inhibit corn seedlings at 83 and 72.9 ug/g dry soil and higher concentrations, respectively. FA was more inhibitory than GA. VA, however, did not show any significant activity. When these phenolic acids were applied in two-way combinations, their effects on the corn growth parameters examined interacted additively (data not shown). These results differ from those obtained in bioassays (chapters I and II), where some of the combined actions of acid pairs were shown to be antagonistic. Moreover, the

magnitude of phenolic inhibition was much lower in soil than in petri dishes. For example, three applications to the soil of 832 ug/g soil FA caused only 13% reduction in root length compared to 26% reduction obtained with a single application of the same concentration of FA in petri dishes. Finally, threshold concentrations for inhibition of soil grown corn seedlings were five to ten times higher than those causing significant reduction in petri dishes.

Inhibitory effects of soil-applied FA have been reported by other researchers (Blum et al., 1987; Liebel et al., 1983). Blum et al. (1987) reported that leaf expansion and shoot dry weight of cucumber seedlings were significantly reduced by FA concentrations ranging from 10 to 70 ug/g dry soil. It has also been reported that the interactive effects of FA and VA mixtures were antagonistic on cucumber seedlings, when added to the soil every other day for a total of five applications (Blum et al., 1989). In our case, however, a mixture of these two acids applied three times to the soil caused no significant effects on the growth of corn seedlings. This may be due to the fewer acid applications used in this study, to differential sensitivity between corn and cucumber seedlings, and/or to chemical and physical differences inherent to soil type used in each study.

The ecological significance of the results obtained here cannot be discussed without consideration of concentration and fate of these acids in soils. Soil concentrations of 2.6 to 14 ug/g dry soil and 2.2 ug/g dry soil to 23 ug/g dry soil have been reported for VA and FA, respectively, (Jalal et al., 1983; Whitehead et al., 1964, 1983). These soil concentrations are below those

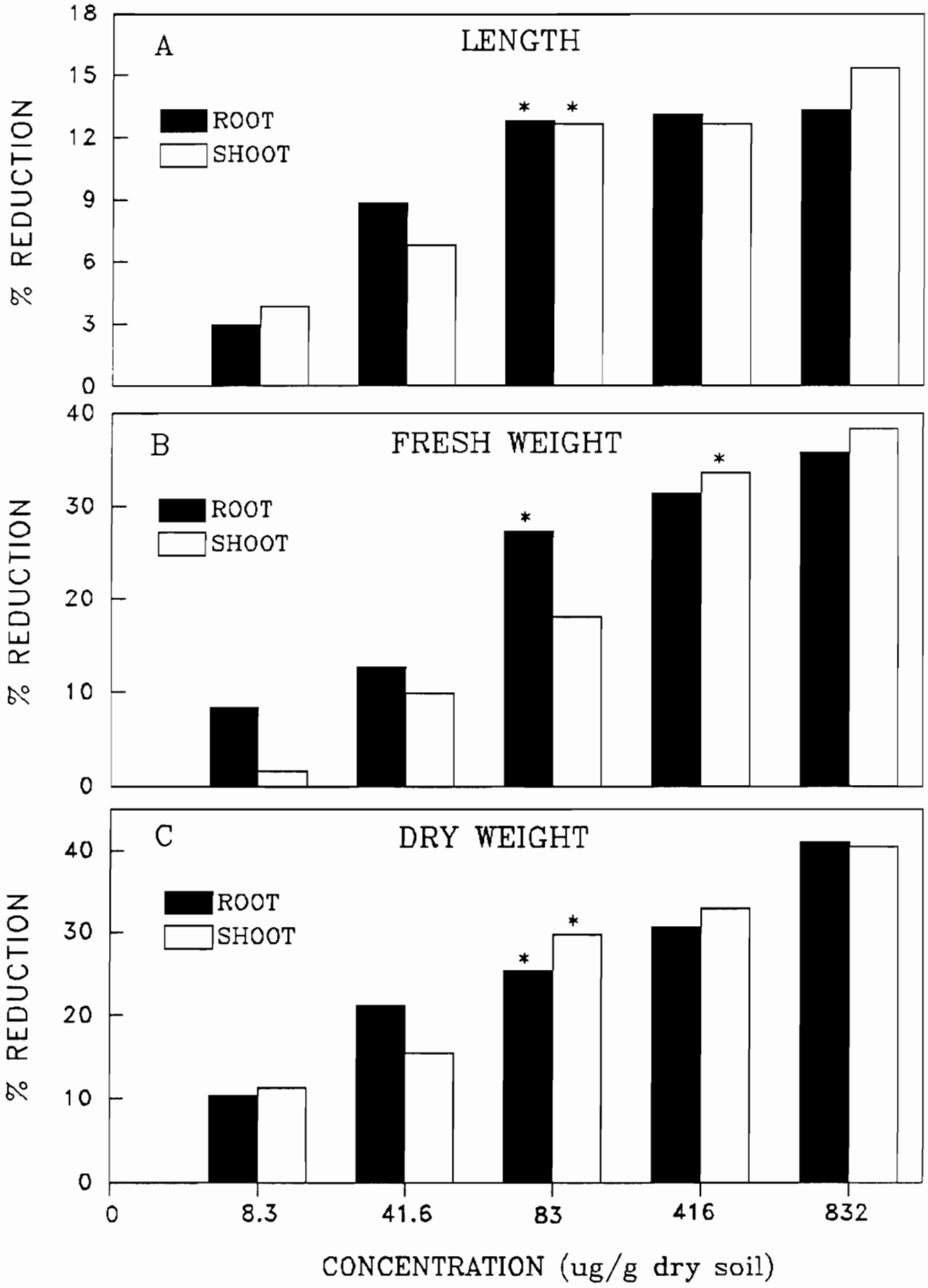
required for the growth inhibition observed here, however, other authors have reported higher soil concentrations for both FA and VA (Lodhi et al,1987; Katase, 1981). For instance, FA concentration of 941 kg/ha (equivalent to 420 *u*g/g soil, assuming even distribution of FA on the top 6" of a hectare of dry soil) was recently reported (Lodhi et al., 1987) and a total amount of 520 *u*g/g soil of VA was extracted from forest soil by Katase (1981).

Attention needs to be drawn to the fact that the action of microorganisms has not been eliminated through sterilization of the soil used in this study, and microorganisms certainly play an important role in the dynamics of soil phenolics (Blum et al., 1988; Vance et al., 1986; Shilling et al., 1983; Vaughan et al., 1983; Sparling et al., 1981). Some researchers argue that microbial transformation of soil phenolics does not necessarily result in a lessening in toxicity; degradation by-products might still be biologically active (Williamson et al.; 1990, Lodhi et al., 1987; Einhellig 1986). Blum et al. (1987) demonstrated that depletion of FA from the soil solution was due primarily to root uptake and microbial activity, and that adsorption and polymerization of FA with organic matter and clay particles were less important. Besides microorganisms, other soil properties such as pH, moisture content, temperature, and nutrient status influence the action of soil phenolics (Stowe et al., 1980; Einhellig et al., 1983; Taoi et al., 1979).

Although the results of this study have demonstrated an inhibitory effect of soil-applied FA and GA on the growth of corn seedlings, the levels required to produce phytotoxic effects are not commonly found in soils. Certainly, more

research involving fate and behavior of soil phenolics and their interactions with different soil components and properties is warranted before all aspects of phenolic allelopathy can be clearly understood.

Figure 4.1. Effects of ferulic acid (FA) on corn root and shoot length (**A**), fresh weight (**B**), and dry weight (**C**). Length and weight are expressed as percent reduction relative to the control. * indicates lowest concentration at which significant inhibition occurred compared to the untreated control at the 0.05 level of significance according to contrast analysis. Concentrations represent the amount of phenolic acid added to 350 ug/g dry soil.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LENGTH
ROOT
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% REDUCTION
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12
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B
FRESH WEIGHT
ROOT
SHOOT
% REDUCTION
40
30
20
10
0
C
DRY WEIGHT
ROOT
SHOOT
% REDUCTION
40
30
20
10
0
0
8.3
41.6
83
416
832
CONCENTRATION (ug/g dry soil)

Figure 4.2. Effects of gallic acid (GA) on corn root and shoot length (A), fresh weight (B), and dry weight (C). Length and weight are expressed as percent reduction relative to the control. * indicates lowest concentration at which significant inhibition occurred compared to the untreated control at the 0.05 level of significance according to contrast analysis. Concentrations represent the amount of phenolic acid added to 350 ug/g dry soil.

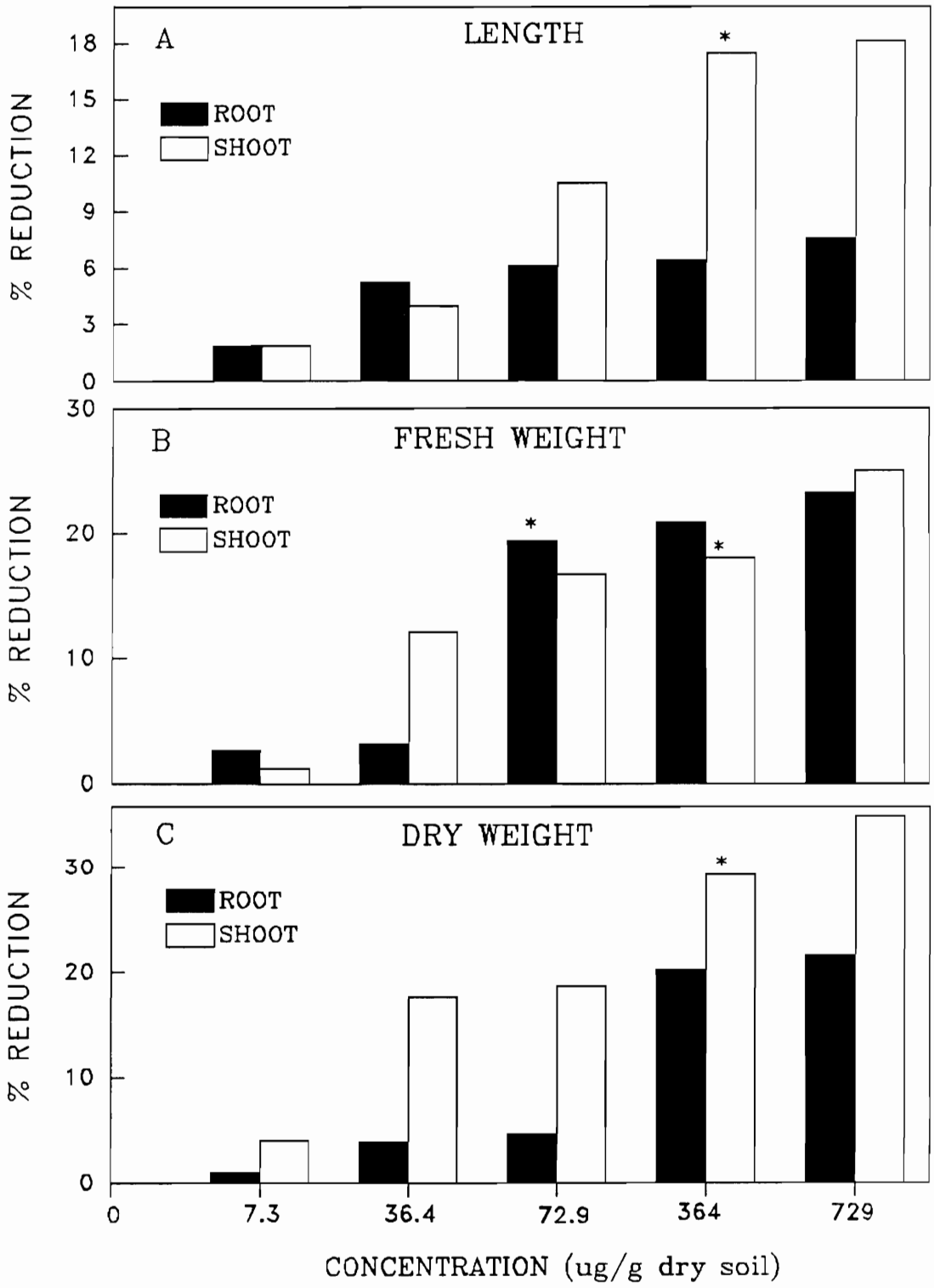
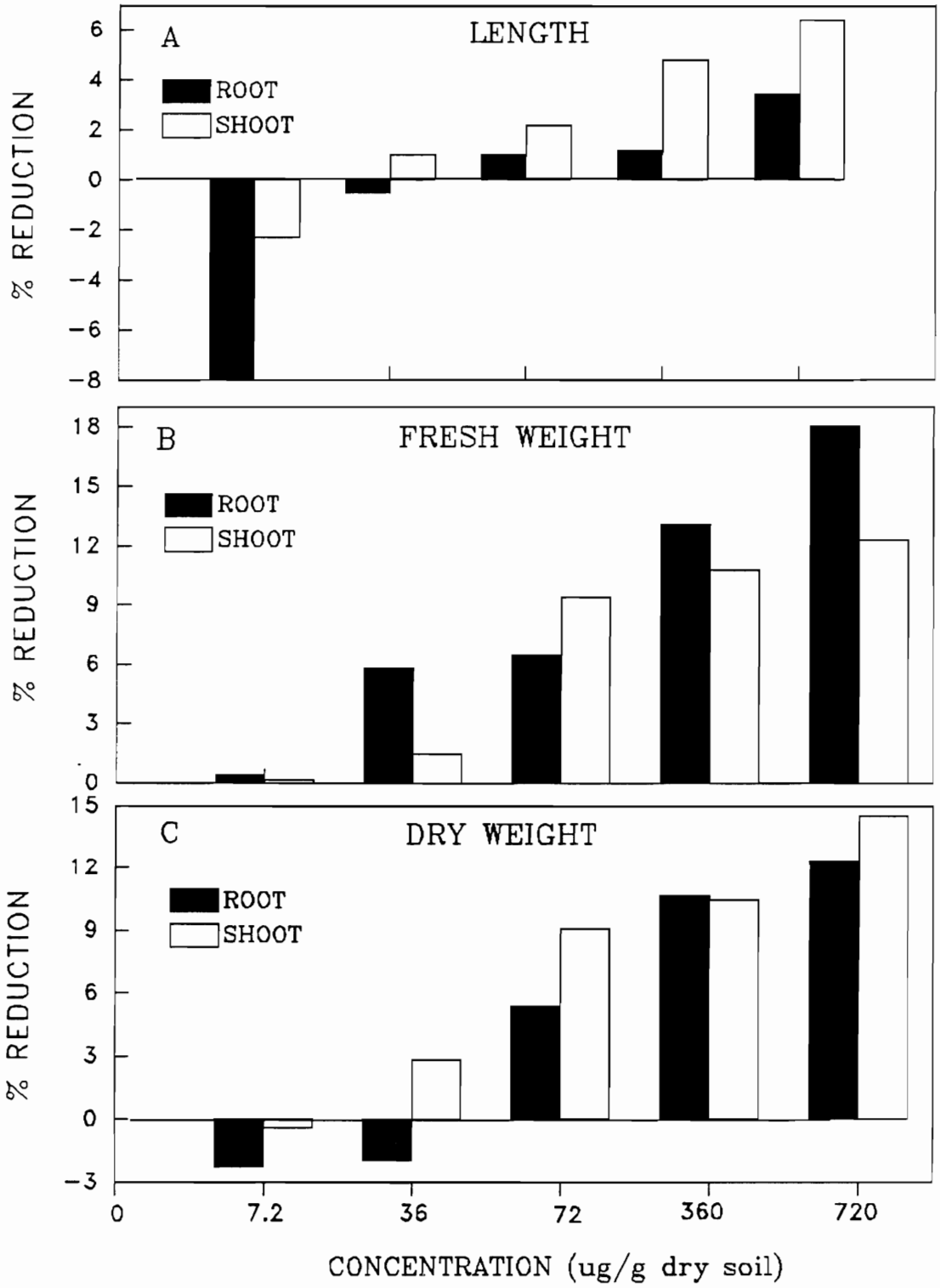

A
LENGTH
ROOT
SHOOT
% REDUCTION
18
15
12
9
6
3
0
B
FRESH WEIGHT
ROOT
SHOOT
% REDUCTION
30
20
10
0
C
DRY WEIGHT
ROOT
SHOOT
% REDUCTION
30
20
10
0
0
7.3
36.4
72.9
364
729
CONCENTRATION (ug/g dry soil)

Figure 4.3. Effects of vanillic acid (VA) on corn root and shoot length (**A**), fresh weight (**B**), and dry weight (**C**). Length and weight are expressed as percent reduction relative to the control. * indicates lowest concentration at which significant inhibition occurred compared to the untreated control at the 0.05 level of significance according to contrast analysis. Concentrations represent the amount of phenolic acid added to 350 ug/g dry soil.


A
LENGTH
ROOT
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FRESH WEIGHT
ROOT
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% REDUCTION
18
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3
0
C
DRY WEIGHT
ROOT
SHOOT
% REDUCTION
15
12
9
6
3
0
−3
0
7.2
36
72
360
720
CONCENTRATION (ug/g dry soil)

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CHAPTER V

EFFECTS OF 2,4-D AND PHENOLIC ACID COMBINATIONS ON CORN GERMINATION AND SEEDLING GROWTH

Abstract. Experiments were undertaken to examine the interaction of ferulic acid and gallic acid with 2,4-dichlorophenoxyacetic acid (2,4-D) on corn (*Zea mays* L.) germination and seedling growth. Ferulic and gallic acid concentrations of 0, 0.01, 1, and 10 mM were used. 2,4-D treatments included 0, 0.01, 0.1, and 1 nM. Both ferulic acid and 2,4-D, individually, affected germination and root dry weight, however, their interaction was not significant. Significant interaction of these acids was evident for root and shoot length. While all levels of 2,4-D reversed the inhibitory effects of the lower concentration of ferulic acid on root length, only the higher levels of 2,4-D counteracted the effects of 1 mM ferulic acid. Four antagonistic interactions were characterized on shoot length and involved all levels of 2,4-D combined with 1 mM ferulic acid and the mixture of 0.1 nM 2,4-D with 10 mM ferulic acid. No significant interactions were observed for any of the corn growth variables studied when 2,4-D was combined with gallic acid.

INTRODUCTION

The inhibitory action of phenolic acids on germination and seedling growth has been demonstrated in previous chapters as well as in the literature (Mayer et al., 1989; Kuiters, 1989; Blum et al., 1984; Patterson, 1982). The biochemical mechanisms through which these allelochemicals exert their inhibitory effects, however, are largely unknown (Putnam, 1985; Einhellig, 1985; Rice, 1984). In his recent review on the modes of action of allelochemicals, Einhellig (1986) suggested that membrane perturbations and phytohormone interactions may be two primary modes of actions of phenolic acids.

Ferulic acid has been reported to have dual, contrasting roles in the enzymatic degradation of indole acetic acid (IAA) in vitro (Lee et al., 1981; Tomaszewski et al., 1966; Zenk et al., 1963). On one hand, the phenolic acid has been shown to cause a temporary inhibition in peroxidase-catalyzed IAA oxidation, by introducing a lag period before the onset of the reaction (Machackova et al., 1975; Schaeffer et al., 1967). On the other hand, it may function as a cofactor for both IAA peroxidase (Lee et al., 1982; Machackova et al., 1975) and IAA oxidase (Gortner et al., 1958). The generally accepted theory for the mechanism of phenolic acid inhibition of IAA oxidation is that these acids trap free radical intermediates which would otherwise contribute to this IAA oxidation process (Lee et al., 1981; Lee 1977; Gelinas 1973; Tomaszewski et al., 1966; Ray et al., 1956). Other investigators, however, have demonstrated that

IAA and ferulic acid compete for the binding site of peroxidase involved in IAA oxidation (Machackova et al., 1975; Gelinas 1973).

Most of the studies on phenolic acids and plant hormone interactions have been done in vitro on sections of either hypocotyl or coleoptile, and the effects on growth have been observed only after the application of either a phenolic acid or a phytohormone alone (Ray et al., 1980; Wolf et al., 1976).

The objectives of this study were to 1) determine the effects of simultaneous applications of either ferulic or gallic acid combined with 2,4-dichlorophenoxyacetic acid on corn germination and seedling growth and 2) characterize the nature of two-way interactions of the synthetic auxin with the two phenolic acids.

The synthetic auxin 2,4-D was chosen because it is more stable than IAA and also because it is used extensively in place of IAA in plant physiology studies (Marumo, 1986; Shannon et al., 1981; Loos, 1975; Key, 1963; Henderson et al., 1962).

MATERIALS AND METHODS

Experimental Procedure. Corn (*Zea mays* L.) (cv. Southern States 727), previously shown to be sensitive to phenolic acid treatments, was used in experiments involving 2,4-dichlorophenoxyacetic acid (2,4-D) combined with either ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid) or gallic acid (GA) (3,4,5-trihydroxybenzoic acid). A preliminary study was conducted to determine the highest 2,4-D concentration which, when combined with different FA concentrations, was not inhibitory to corn. Concentrations of 2,4-D ranged from 1 to 100 nM. Due to the inhibitory effects obtained with these 2,4-D levels, lower concentrations were used in subsequent experiments. They were 0.01, 0.1 and 1.0 nM. Phenolic acid concentrations consisted of 0.1, 1, and 10 mM of either FA or GA. The control consisted of 3 mM MES [2-(N-morpholino)ethanesulfonic acid], which was used in preparing individual and combination treatment of acids. The pH of solutions was adjusted to 5.8 with 0.1 N NaOH.

Twenty corn seeds were placed in 10-cm petri dishes on a disk of Whatman No 1 filter paper moistened with 7 ml of test solution. Germination proceeded in the dark at 25 +/- 1 C, and germinated seeds were evaluated at 24, 36, 48, and 60 h after acid treatment using a 2 mm radicle extrusion as a criterion. Five days after treatment, 3 ml of test solution were added to each petri dish. Ten days after initial treatment, length of roots and shoots of 15

randomly selected seedlings was measured. Dry weights were obtained after oven drying roots and shoots at 55 C for 48 h.

Experimental Design and Analysis. The three experiments contained a two-way (4 x 4) factorial arrangement of treatments in a randomized complete block design with four replications. Factors included four levels (including 0 mM) of 2,4-D and either FA or GA. Data were subjected to analyses of variance and significance was determined by partitioning the effects of treatments into main effects and interactions. All means were averaged over the levels of the other factor present in the combination when significant interactions did not occur. Means were then separated using Fisher's Protected Least Significant Difference (LSD) test at 0.05 significance level. Statistically significant interactions were further characterized by the statistical treatment of Colby's method developed by Flint et al.(1988). All experiments were repeated at least once.

RESULTS

Analyses of variance showed that the higher rates of 2,4-D and FA did not interact on either corn germination or shoot and root dry weight (data not shown). When lower 2,4-D concentrations were combined with different levels of FA, the interactive effects were significant on both root and shoot length (Table 5.1). No interaction was evident with 2,4-D (at the lower concentrations) and GA (Table 5.1).

FA and 2,4-D combination. Because of the inhibition observed with high levels of 2,4-D (data not shown), another experiment was developed to investigate the effects of combinations of the same concentrations of FA with lower concentrations of 2,4-D. Results showed that these two acids interacted on root and shoot length (Table 5.1). Both individual acids significantly affected germination and root growth. No significant 2,4-D effects were observed on shoot dry weight. All 2,4-D concentrations stimulated germination as well as seedling growth (Figures 5.1, 5.2, and 5.3 B). For all growth parameters investigated, higher stimulation occurred at 0.1 nM 2,4-D than at either 0.01 or 1 nM. A 32% stimulation of germination was obtained with 0.1 nM 2,4-D at 24 h, compared to 17 and 20% stimulation caused by 0.01 and 1 nM, respectively (Figure 5.1 B). Similar stimulatory effects of 2,4-D concentrations were also observed for root dry weight (Figure 5.2 B). Stimulation of root growth increased dry weight 28, 42, and 29% by 0.01, 0.1, and 1 nM of 2,4-D, respectively. Although significant, 2,4-D stimulation of germination and root growth did not counteract the inhibitory effects of FA, as demonstrated by the nonsignificance of the interaction of these two acids (Table 4.1). Their interactive effects, however, were significant on both root and shoot length.

The statistical characterization of the interactions of 2,4-D and FA on root and shoot length are given in Figure 5.3. While all levels of 2,4-D antagonized the effects of lower concentrations of FA on root length, only the higher levels of 2,4-D counteracted the effects of 1 mM of FA. None of the 2,4-D concentrations

significantly reduced the inhibitory effects of 10 mM FA (Figure 5.3 A). Four antagonistic interactions were characterized on shoot length (Figure 5.3 B) and involved all concentrations of 2,4-D with 1 mM of FA and the combination of 0.1 nM 2,4-D with 10 mM FA.

GA and 2,4-D combination. No significant interactions were observed for any of the corn growth variables studied when 2,4-D was combined with GA (Table 5.1). LSD separation of the means of main effects revealed that 2,4-D concentrations of 0.1 and 1 nM significantly stimulated corn germination at 24 h after treatment as well as root and shoot length and root dry weight. As was the case in FA and 2,4-D combination, greater stimulation was induced by the middle than by the lower and higher 2,4-D concentrations. For instance, 0.01, 0.1, and 1 nM of 2,4-D caused 18, 35, and 26% stimulation, respectively, in germination 24 h after treatment (Figure 5.4 A). Similar stimulatory trends were seen on root and shoot growth (Figure 5.5 B). Stimulation was greater on root growth than on shoot growth or germination.

While all concentrations of FA were inhibitory to corn growth, only the two higher GA concentrations caused significant inhibition (Figures 5.4, 5.5, and 5.6). Similar to FA action, GA inhibition was concentration and growth variable dependent.

DISCUSSION

The results of the combination of FA with 2,4-D demonstrated that, while concentrations of 2,4-D greater than 1 nM were inhibitory to early corn germination and root growth (data not shown), 2,4-D concentrations of 1 nM and below were stimulatory. Similar stimulatory effects of 2,4-D were also evident on corn seedling growth when the synthetic auxin was combined with GA. Except for the antagonistic effects observed with FA combinations for root and shoot length, 2,4-D stimulation did not overcome the inhibition caused by either phenolic acids.

The results of this study also showed that the synthetic auxin did not act similarly with both phenolic acids. In the presence of FA, 2,4-D stimulated germination, but did not when combined with GA. Furthermore, although 2,4-D alone was stimulatory, it did not overcome GA inhibition on root and shoot length, while it did in combination with FA. From this it can be suggested that the two phenolics may have different modes of action in inhibiting corn germination and seedling growth. The fact that 2,4-D counteracted the effects of FA on root and shoot length but not on germination points to the possibility that FA may have inhibited germination by a mechanism other than that involved in root and shoot length inhibition.

One of the suggested mechanisms of action of phenolic acids is their interference with the operation of plant hormones (Einhellig, 1986; Putnam,

1985; Rice, 1984). Both GA and FA have been shown to inhibit gibberellin and IAA-induced growth (Corcoran et al., 1972; Wolf et al., 1976) and to reverse the inhibition of hypocotyl growth caused by abscisic acid (ABA) (Ray et al., 1980). Although a synthetic auxin was used in this study, it was shown that the magnitude of 2,4-D stimulation was less than the magnitude of inhibition caused by either phenolics present in the combination. Furthermore, the interactive effects of FA and 2,4-D were antagonistic for both root and shoot length (Figure 5.3).

Although the results of this study have shown a negative interaction of FA with the synthetic auxin, the inhibitory effects of FA cannot be explained solely on the basis of this interaction. Besides their interaction with phytohormones, phenolic acids including FA and GA have been shown to interfere with several other metabolic processes in plants including protein and lipid synthesis (Cameron et al., 1980; Danks et al., 1975; Van Sumere et al., 1971), respiration (Moreland et al., 1987; Van Sumere et al., 1971; Demos et al., 1975,), water and mineral uptake (Einhellig, 1987; Danks et al., 1975; Glass, 1974; Glass, 1973), enzymes systems, (Putnam, 1985; Rice, 1984; Lee et al., 1982; Jacobson et al., 1977) and photosynthesis (Moreland et al., 1987; Einhellig, 1987, and Einhellig et al.; 1979). It seems appropriate, therefore, to conclude this chapter by the following statement of Einhellig (1986). "Considerable speculation is still involved in any attempt to draw together the various inferences and interrelationships about allelochemical functions".

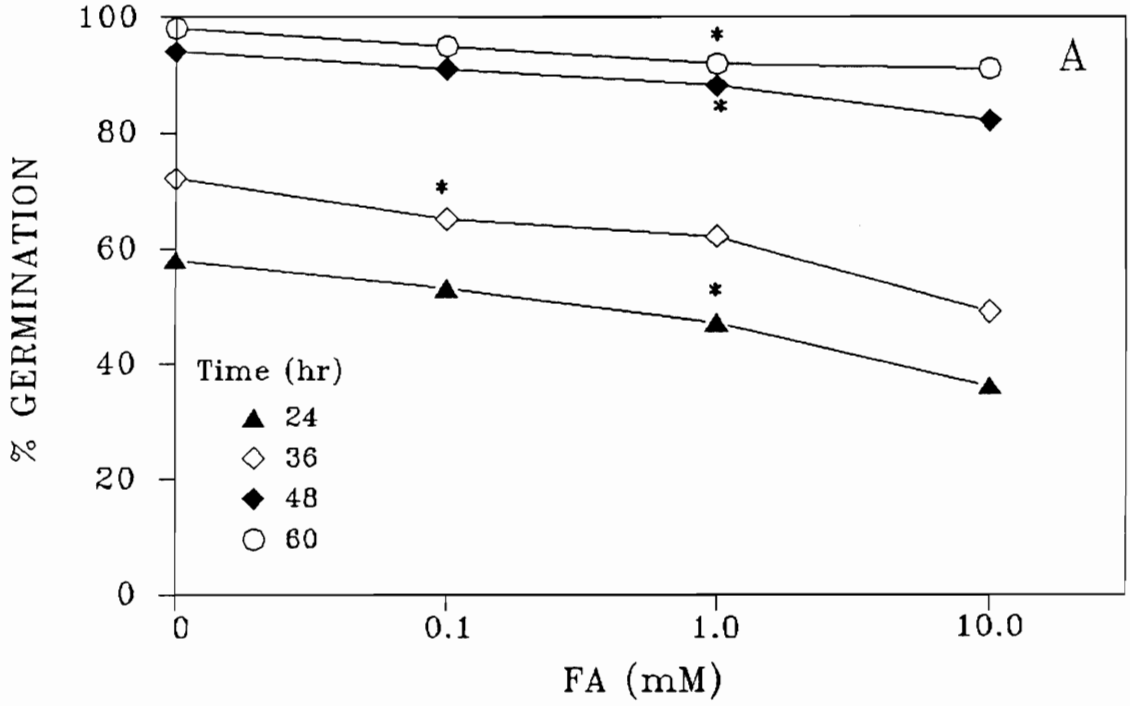
TABLE 5.1. SIGNIFICANCE OF INTERACTIONS IN TWO-WAY COMBINATIONS OF PHENOLIC ACIDS AND 2,4-D ON CORN[a].

Source of	Germination				Length		Weight	
Variation	24 h	36 h	48 h	60 h	Root	Shoot	Root	Shoot
FA and 2,4-D combination								
FA	*	*	*	*	*	*	*	*
2,4-D	*	NS	*	*	*	*	*	NS
FA x 2,4-D	NS	NS	NS	NS	*	*	NS	NS
GA and 2,4-D combination								
GA	*	*	*	NS	*	*	*	*
2,4-D	NS	NS	NS	NS	*	*	*	*
GA x 2,4-D	NS	NS	NS	NS	NS	NS	NS	NS

[a]Significance determined by ANOVA test. * indicates significance at 0.05 probability level.

NS = not significant at 0.05 probability level.

Figure 5.1. Effects of ferulic acid (FA) (**A**) and 2,4-D (**B**) on corn germination. Within time intervals, * indicates lowest concentration at which either significant inhibition (**A**) or stimulation (**B**) occurred at the 0.05 level of significance as determined by the LSD test.


100
80
60
40
20
0
% GERMINATION
A
Time (hr)
24
36
48
60
0
0.1
1.0
10.0
FA (mM)

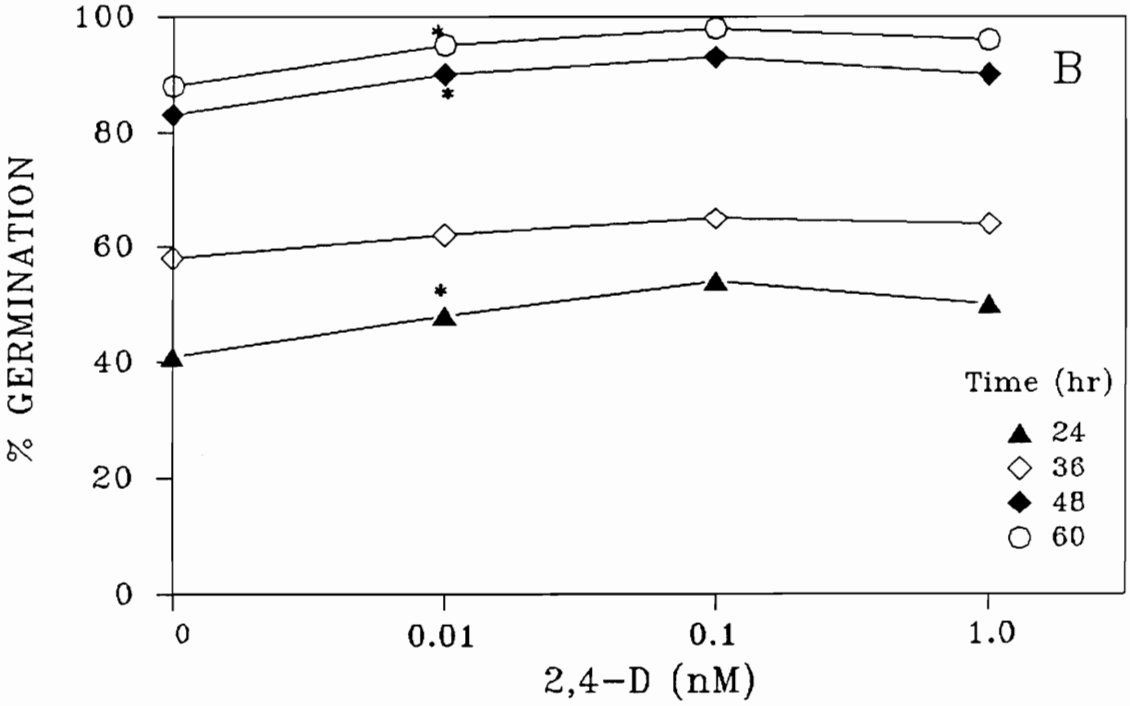
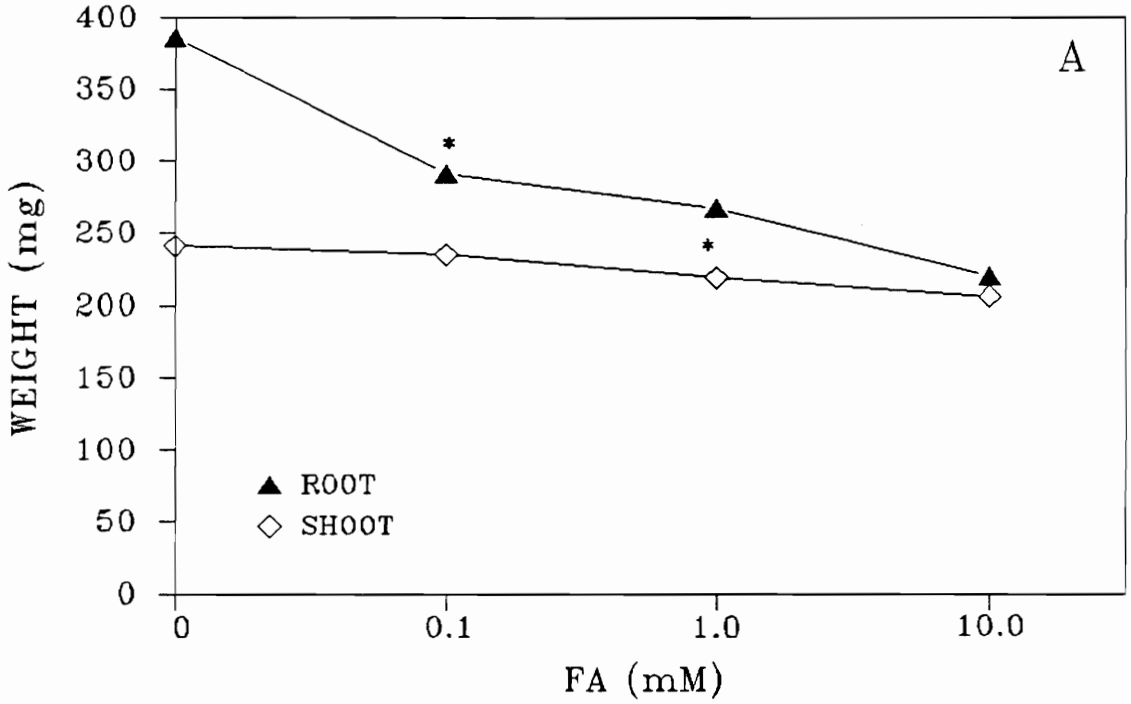

100
80
60
40
20
0
% GERMINATION
B
Time (hr)
24
36
48
60
0
0.01
0.1
1.0
2,4-D (nM)

Figure 5.2. Effects of ferulic acid (FA) (**A**) and 2,4-D (**B**) on corn root and shoot dry weight. * indicates lowest concentration at which either significant inhibition (**A**) or stimulation (**B**) occurred at the 0.05 level of significance as determined by the LSD test.


A
WEIGHT (mg)
400
350
300
250
200
150
100
50
0
*
*
ROOT
SHOOT
0
0.1
1.0
10.0
FA (mM)

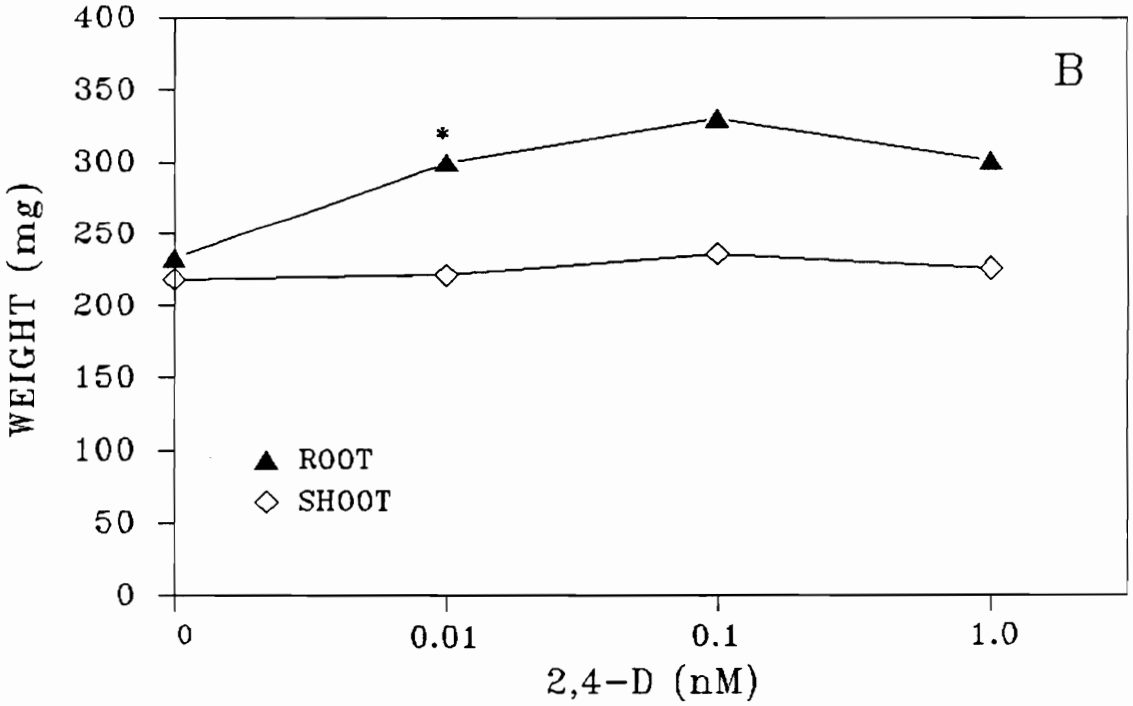
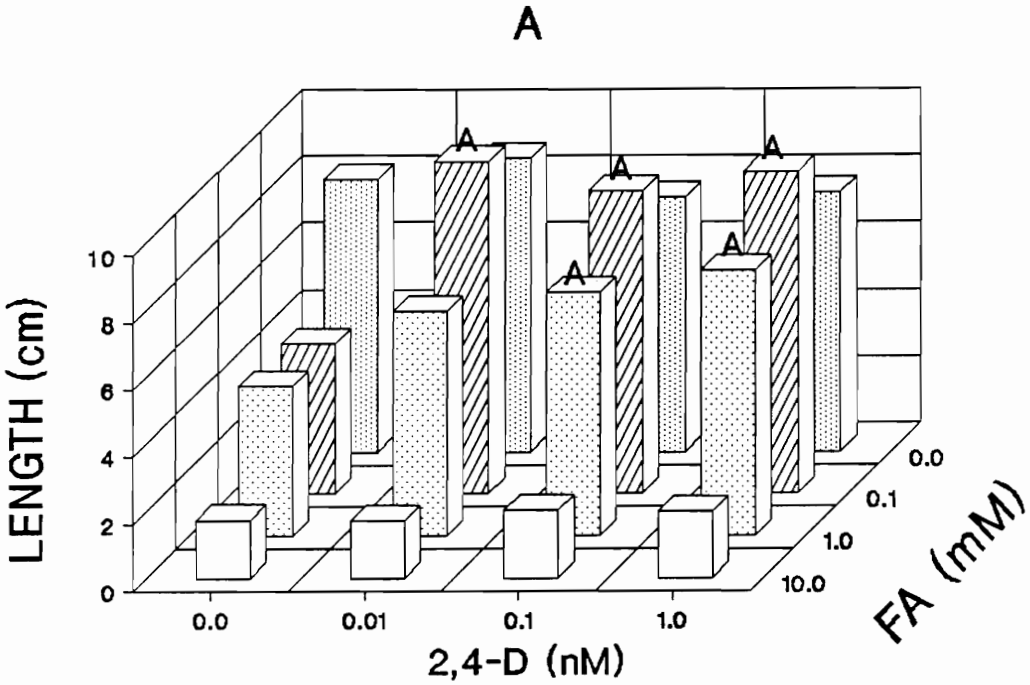

B
WEIGHT (mg)
400
350
300
250
200
150
100
50
0
*
ROOT
SHOOT
0
0.01
0.1
1.0
2,4-D (nM)

Figure 5.3. Interactive effects of ferulic acid (FA) and 2,4-D on corn root (A) and shoot (B) length. **A** indicates antagonism at 0.05 level of significance according to the statistical treatment of Colby's method. Bar portions not followed by a letter represent additive interactions


A
LENGTH (cm)
0
2
4
6
8
10
0.0
0.01
0.1
1.0
2,4-D (nM)
0.0
0.1
1.0
10.0
FA (mM)
A
A
A
A
A

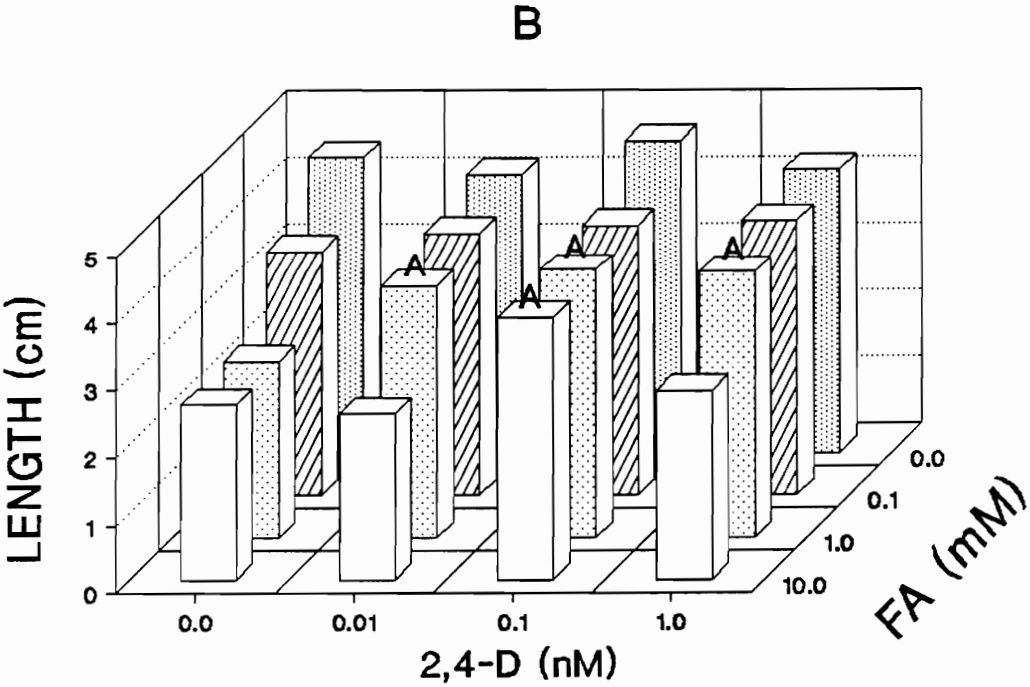
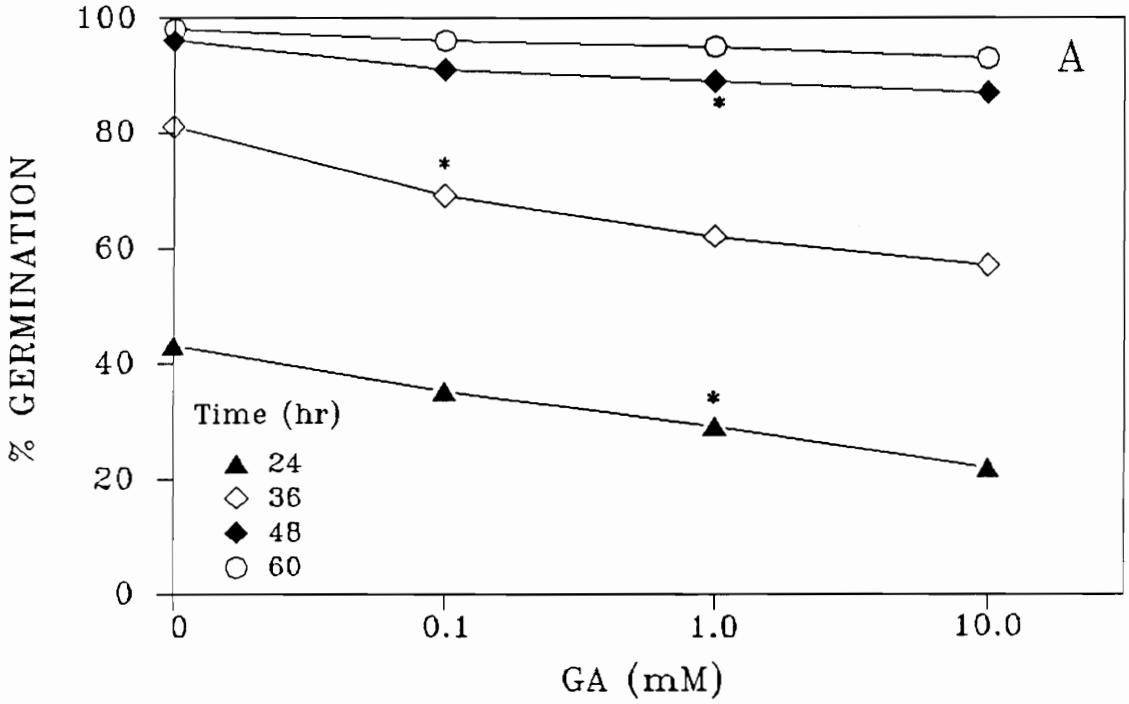

B
LENGTH (cm)
0
1
2
3
4
5
0.0
0.01
0.1
1.0
2,4-D (nM)
0.0
0.1
1.0
10.0
FA (mM)
A
A
A
A
A

Figure 5.4. Effects of gallic acid (GA) (**A**) and 2,4-D (**B**) on corn germination. Within time intervals, * indicates lowest concentration at which significant inhibition occurred at the 0.05 level of significance as determined by the LSD test.


100
80
60
40
20
0
% GERMINATION
A
Time (hr)
24
36
48
60
0
0.1
1.0
10.0
GA (mM)

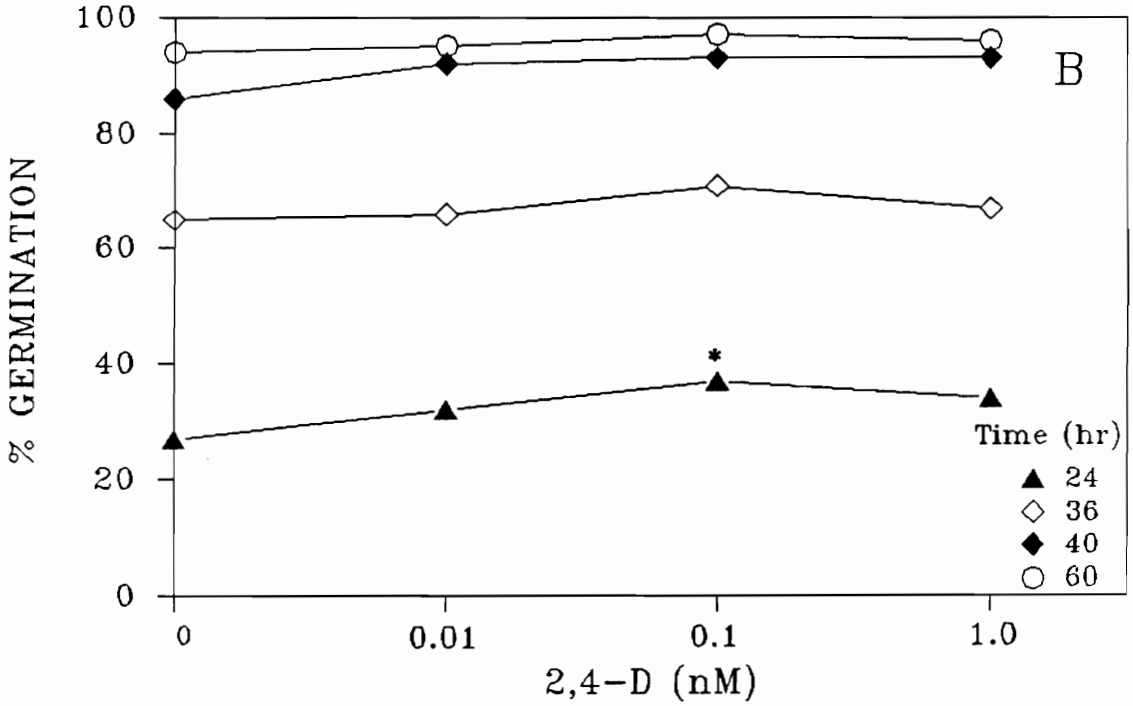
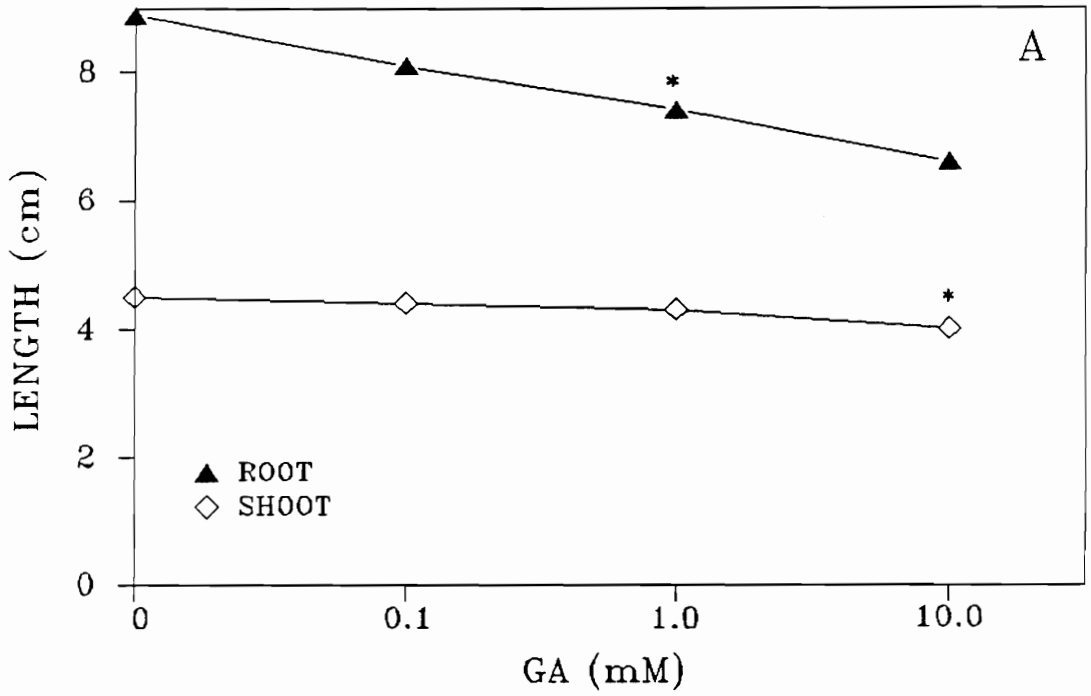

100
80
60
40
20
0
% GERMINATION
B
Time (hr)
24
36
40
60
0
0.01
0.1
1.0
2,4-D (nM)

Figure 5.5. Effects of gallic acid (GA) (**A**) and 2,4-D (**B**) on corn root and shoot length. * indicates lowest concentration at which either significant inhibition (**A**) or stimulation (**B**) occurred at the 0.05 level of significance as determined by the LSD test.


A
LENGTH (cm)
8
6
4
2
0
*
*
ROOT
SHOOT
0
0.1
1.0
10.0
GA (mM)

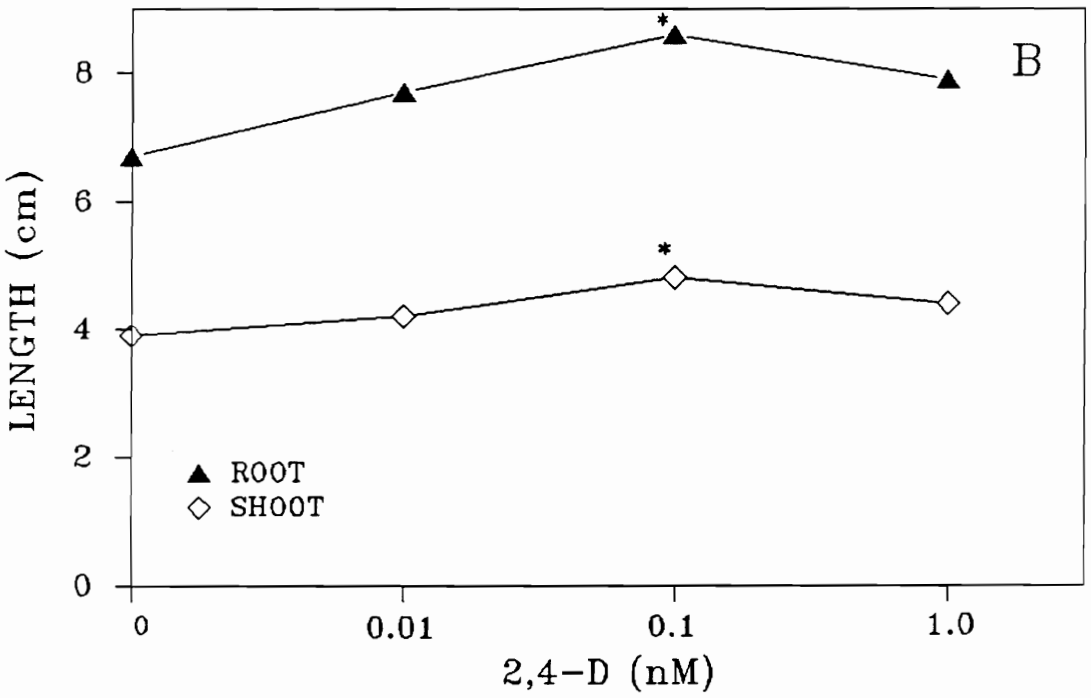
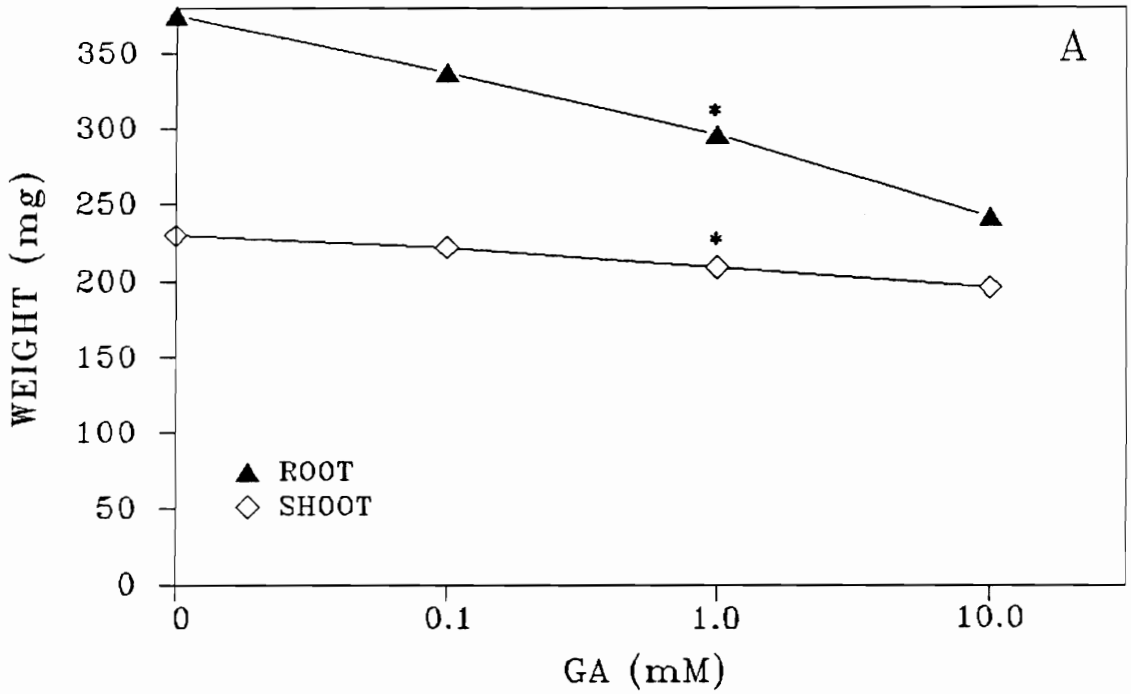
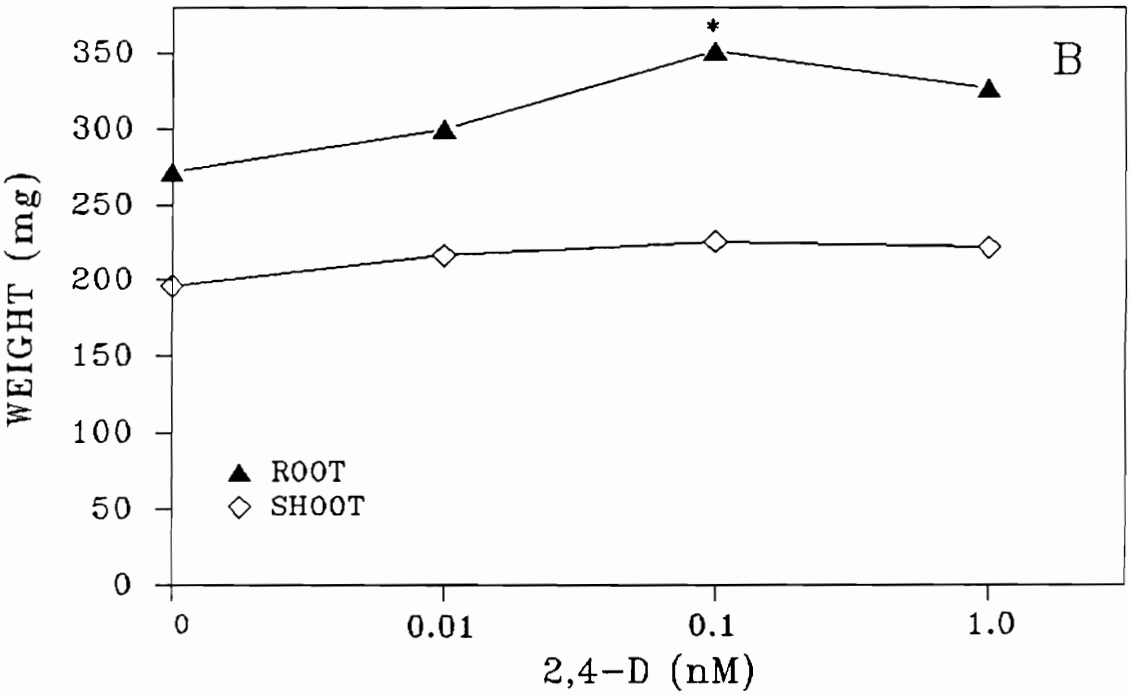

B
LENGTH (cm)
8
6
4
2
0
*
*
ROOT
SHOOT
0
0.01
0.1
1.0
2,4-D (nM)

Figure 5.6. Effects of gallic acid (GA) (**A**) and 2,4-D (**B**) on corn root and shoot dry weight. * indicates lowest concentration at which either significant inhibition (**A**) or stimulation (**B**) occurred at the 0.05 level of significance as determined by the LSD test.


A
WEIGHT (mg)
350
300
250
200
150
100
50
0
ROOT
SHOOT
0
0.1
1.0
10.0
GA (mM)


B
WEIGHT (mg)
350
300
250
200
150
100
50
0
ROOT
SHOOT
0
0.01
0.1
1.0
2,4-D (nM)

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CHAPTER VI

EFFECTS OF PHENOLIC ACIDS ON CORN IMBIBITION AND RESPIRATION

Abstract. Studies were conducted to determine the effects of ferulic acid on water uptake of corn (*Zea mays* L.) seeds during germination and to investigate the action of both ferulic and gallic acids on respiration of germinating corn seeds. Results showed that no ferulic acid levels interfered with water uptake at early stages of imbibition or during the time course of germination. Respiratory activity of germinating seeds as measured by manometric techniques was not affected by either ferulic or gallic acids. These results suggest that the inhibition of corn germination by these phenolic acids cannot be due to adverse effects on water uptake or O_2 consumption of germinating seeds.

INTRODUCTION

Although information has developed in previous chapters and in the literature on the inhibitory activities of different phenolic acids on germination and seedling growth (Lyu et al., 1990; Kuiters, 1989; Jain et al., 1989; Williams et al., 1982; Patterson, 1981), the physiological and biochemical aspects of how these compounds affect growth is still obscure (Putnam, 1985; Mandava, 1985; Rice, 1984). Information gathered from the literature shows that phenolics can alter several aspects of plant metabolism (Einhellig, 1986, 1985; Cameron et al., 1980; Glass, 1974).

Only a limited number of studies have examined the effects of phenolic compounds on energy metabolism including respiration and oxidative phosphorylation (Moreland et al., 1987; Makovec et al., 1985; Demos et al., 1975; Van Sumere et al., 1971), and effects reported for ferulic acid differed from one study to another. Ferulic and gallic acids were reported in one study to inhibit respiration in mitochondria isolated from potato tubers (Tissut et al., 1980); ferulic acid did not have any effect on respiration or on phosphorylation activities of mitochondria isolated from potato tubers (Makovec et al., 1984) or mung bean hypocotyl (Demos et al., 1975). Moreover, ferulic acid was shown to stimulate O_2 uptake in yeast mitochondria and algae cells (Dedonder et al ., 1971; Van Sumere et al., 1971). Other studies with isolated mitochondria reported that ferulic acid and other phenolics did not act as uncouplers or inhibit ATP

synthesis (Moreland et al., 1987). Similar results were shown from a study with germinating seeds (Van Sumere et al., 1971).

With the exception of one study (Van Sumere et al., 1971), all studies were carried out in mitochondria isolated from root or hypocotyl and did not involve germinating seeds. Moreover, while some information exists in the literature on the effects of ferulic acid on respiration, to the best of our knowledge, no report exists on the effects of this acid and other phenolics on water uptake by germinating seeds.

The first objective of the present study was to investigate the effects of ferulic acid on water uptake of germinating corn seeds in early stages of imbibition and during the time course of germination. The second was to examine the effects of ferulic and gallic acids on respiration of germinating corn seeds.

MATERIALS AND METHODS

Experimental Procedure. Both ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid) and gallic acid (GA) (3,4,5-trihydroxybenzoic acid) were used in respiration experiments. Only FA was used in the imbibition study. FA, GA, and MES [2-(N-morpholino)ethanesulfonic acid] were obtained from Sigma Chemical Company, St. Louis, Missouri. Stock solutions of each phenolic acid were prepared by dissolving the appropriate amount in 3 mM MES buffer. A few drops of 0.1 N NaOH were added to achieve dissociation of the acids. Final phenolic concentrations of 0.1, 1.0, and 10 mM were obtained by appropriate dilution of stock solution with 3 mM MES.

Imbibition study. Twenty corn (cv. Southern States 727) seeds were used in three different experiments. Seed moisture content, determined by drying at 55 C for 48 h, was 8 to 9%. To determine the time course of imbibition in the presence of different FA treatments, air dried seeds were weighed, placed for a time in 40 ml beakers containing 10 ml of the phenolic acid solution, removed, blotted, weighed, and returned to the test solution. In addition to the FA concentrations mentioned above, the experiments contained three controls consisting of: 1) distilled water, 2) 3 mM MES, and 3) 3 mM MES adjusted to pH 5.8 using 0.1 N NaOH. In the first experiment, water uptake during early stages of imbibition was determined every 10 min for 180 min. In the second experiment, the weighing of seeds was done every 2 h over a period of 12 h. In

the third experiment, seeds were weighed every 6 h for 60 h in order to monitor water uptake during the time course of germination used in the previous bioassays.

Respiration Experiments. Seeds were placed in a 2.5 cm petri dish over a disk of Whatman No 1 filter paper moistened with 5 ml of test solution containing either FA or GA. Five seeds/treatment were used. At 12, 24, 36, 48, and 60 h, seeds were transferred to the main chamber of 15 ml reaction flasks for O_2 uptake measurements. In order to absorb the CO_2 produced during respiration, a piece of filter paper was immersed in 0.3 ml of KOH which was placed in the center well of the reaction flask. O_2 uptake readings were taken every 30 min over a period of 150 min. O_2 consumption was measured at 30 C with a differential respirometer (Gilson Medical Electronics, Inc., Middleton, Wis.). The manometer system was allowed to reach thermal equilibrium before it was closed. Due to the limited number of manometers on the respirometer, the 3 mM MES control was not included in these experiments

Experimental Design and Analysis. All experiments were arranged in a randomized complete block design. Except for experiments involving GA, which contained three replications, other experiments had four replications. Data were subjected to single degree of freedom contrast analysis, in which controls were compared, and phenolic treatments were compared to each of the controls.

RESULTS AND DISCUSSION

Imbibition Study. Results of FA application on imbibition of water by corn seeds are given in Figures 6.1, and 6.2. None of the FA levels interfered with seed water uptake at early stages of imbibition or during the time course of germination. It is difficult to validate the results of this study by comparing them with other published results, because we have been unable to find any other data on the effects of naturally occurring phenolics on imbibition by seeds. The results reported here, however, demonstrate that the inhibition of germination by FA is not due to an interference by this acid with water uptake. Other inhibition mechanisms may have been involved, and mention has already been made of possible interference with cell membranes and other metabolic processes.

Respiration Experiments. In order to examine the effects of FA and GA on seed respiration over the time course of germination, O_2 consumption was monitored at each germination interval every 30 min over 150 min. Within germination intervals, and for each reading time, comparison of treatments to controls was performed using contrast analysis. Results are shown in Figures 6.3 and 6.4 for FA and in Figures 6.5 and 6.6 for GA. No significant differences were detected for any FA or GA treatments.

These results are in agreement with those reported by Van Sumere et al. (1971) who showed that FA did not inhibit respiration of barley or lettuce seeds. They also agree with some studies on isolated mitochondria. For instance, no

inhibitory effects on respiration were found for FA with isolated mung bean mitochondria (Demos et al., 1975) or for GA with isolated potato tuber (Makovec et al., 1984). Tissut et al.(1980), however, reported that both these acids inhibited respiration in mitochondria isolated from potato tubers. In other studies, respiration was stimulated by FA in mitochondria of *Saccharomyces cervisiae* (Van Sumere et al., 1971) and in *Chlorella vulgaris* cells (Dedonder et al., 1971). These diverse results suggest that there is no consistent effect from FA and GA on respiratory metabolism.

From the results obtained in this study, it is evident that the inhibition of corn germination by FA and GA cannot be due to their adverse effects on water uptake or O^2 consumption by germinating seeds. This is further supported by the finding of Van Sumere et al. (1971) that FA, although inhibitory to lettuce and barley germination, did not affect respiration or oxidative ATP formation in the germinating seeds. The same study, however, showed that FA inhibited the accumulation and incorporation of phenylalanine-1^{14}C in seed embryos. The authors suggested that FA may inhibit germination by inhibiting the transport of amino acids and synthesis of proteins in the germinating seeds.

Figure 6.1. Effects of ferulic acid on early stages of corn imbibition. Imbibition is expressed as percentage of initial dry weight of seeds. Seeds were weighed every 10 min. No significant difference between treatments was shown by contrast analysis.

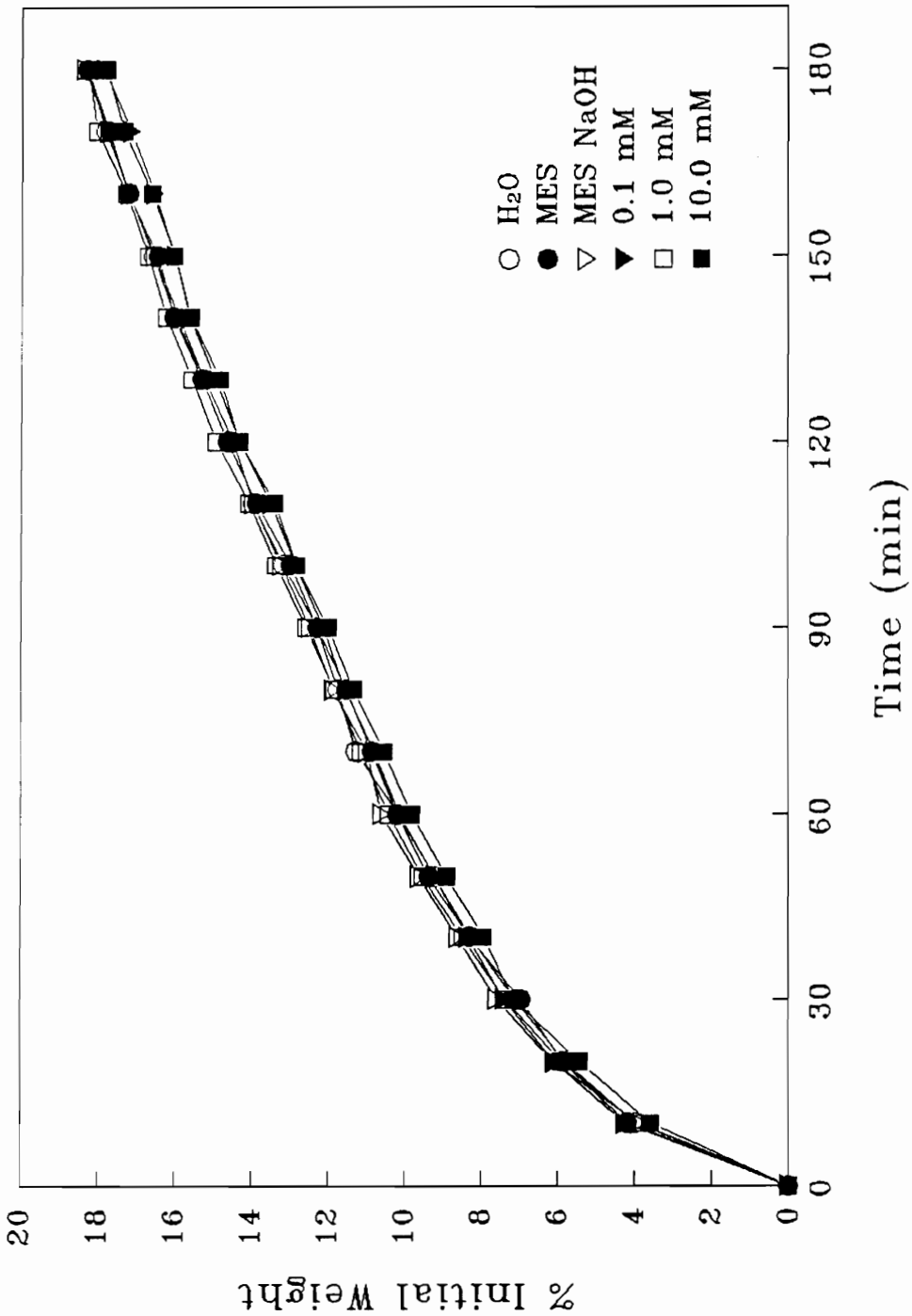

% Initial Weight
20
18
16
14
12
10
8
6
4
2
0
0
30
60
90
120
150
180
Time (min)
H2O
MES
MES NaOH
0.1 mM
1.0 mM
10.0 mM

Figure 6.2. Effects of ferulic acid on water uptake by corn seeds. Imbibition is expressed as percentage of initial weight of seeds. Seeds were weighed every two h (**A**) and every 6 h (**B**). No significant difference between treatment was shown by contrast analysis.

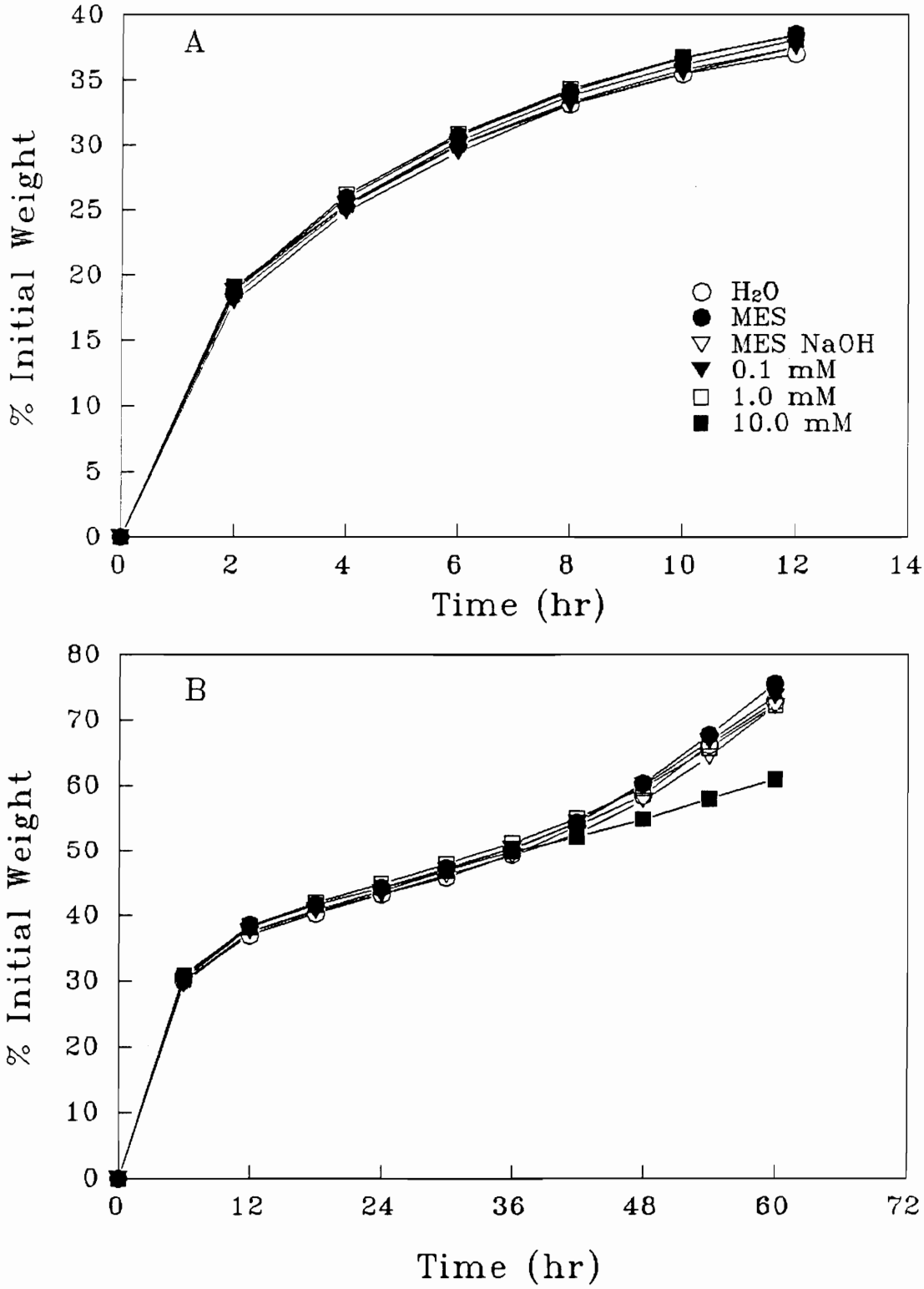

A
% Initial Weight
Time (hr)
H2O
MES
MES NaOH
0.1 mM
1.0 mM
10.0 mM
B
% Initial Weight
Time (hr)

Figure 6.3. Effects of ferulic acid on respiration of germinating corn seeds at 12, 24, and 36 h after treatment. For each time interval, O_2 uptake readings were done every 30 minutes over a period of 150 minutes. No significant difference between treatments was shown by contrast analysis.

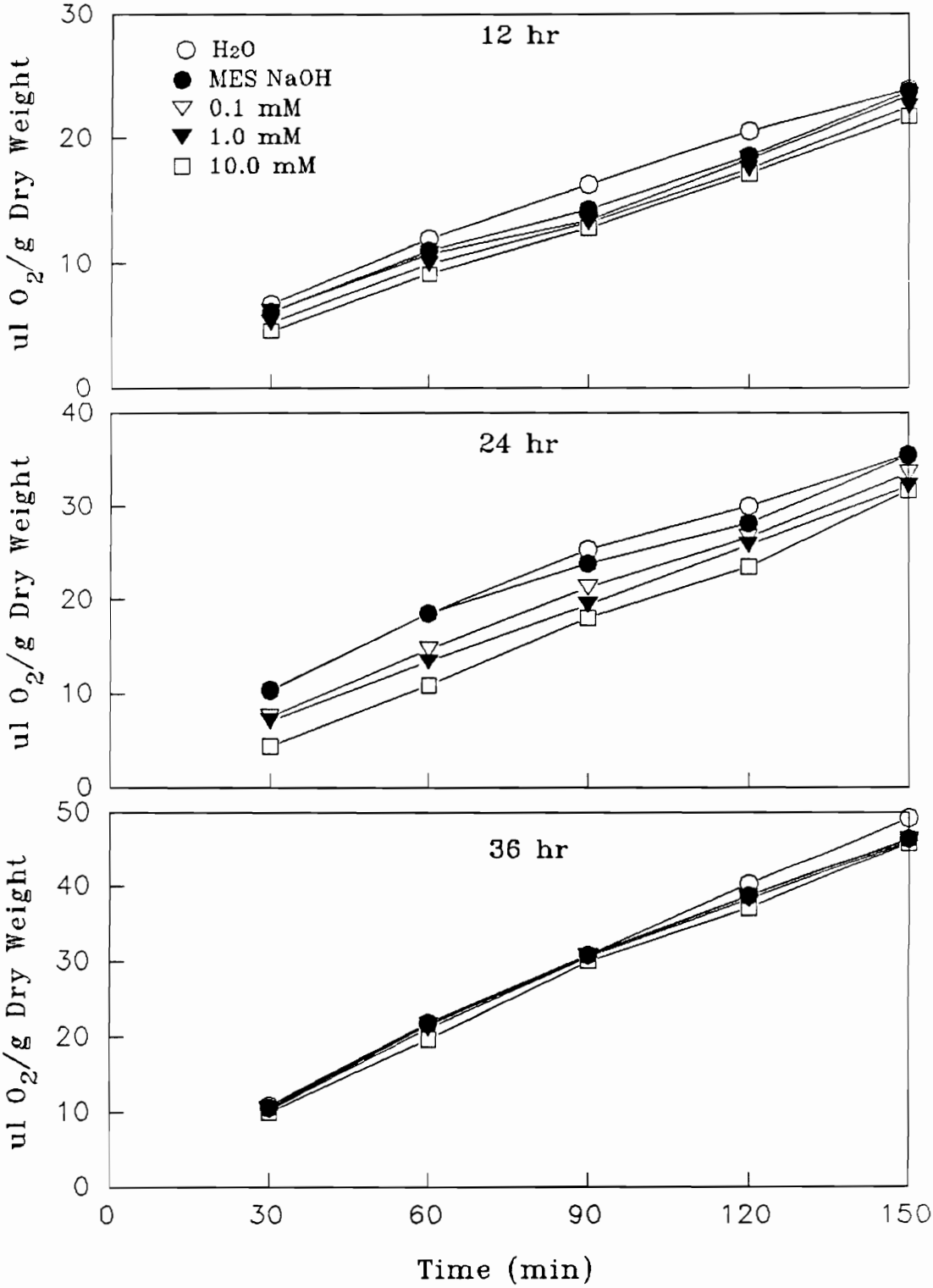

12 hr
24 hr
36 hr
H2O
MES NaOH
0.1 mM
1.0 mM
10.0 mM
ul O2/g Dry Weight
Time (min)
0
30
60
90
120
150
10
20
40
50

Figure 6.4. Effects of ferulic acid on respiration of germinating corn seeds at 48 and 60 h after treatment. For each time interval, O_2 uptake readings were done every 30 minutes over a period of 150 minutes. No significant difference between treatments was shown by contrast analysis.

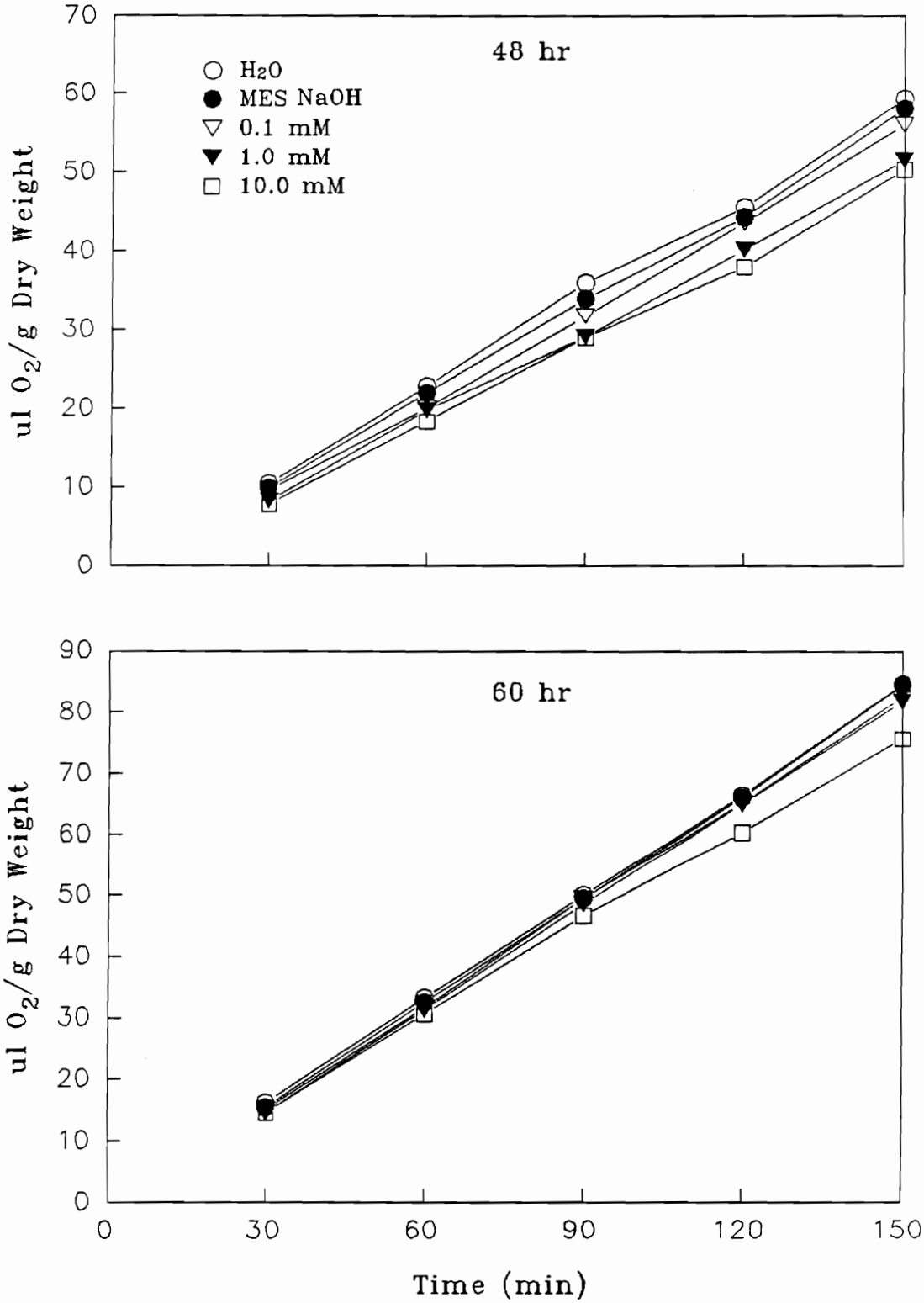

48 hr
H2O
MES NaOH
0.1 mM
1.0 mM
10.0 mM
ul O2/g Dry Weight
0
10
20
30
40
50
60
70
60 hr
ul O2/g Dry Weight
0
10
20
30
40
50
60
70
80
90
0
30
60
90
120
150
Time (min)

Figure 6.5. Effects of gallic acid on respiration of germinating corn seeds at 12, 24, and 36 h after treatment. For each time interval, O_2 uptake readings were done every 30 minutes over a period of 150 minutes. No significant difference between treatments was shown by contrast analysis.

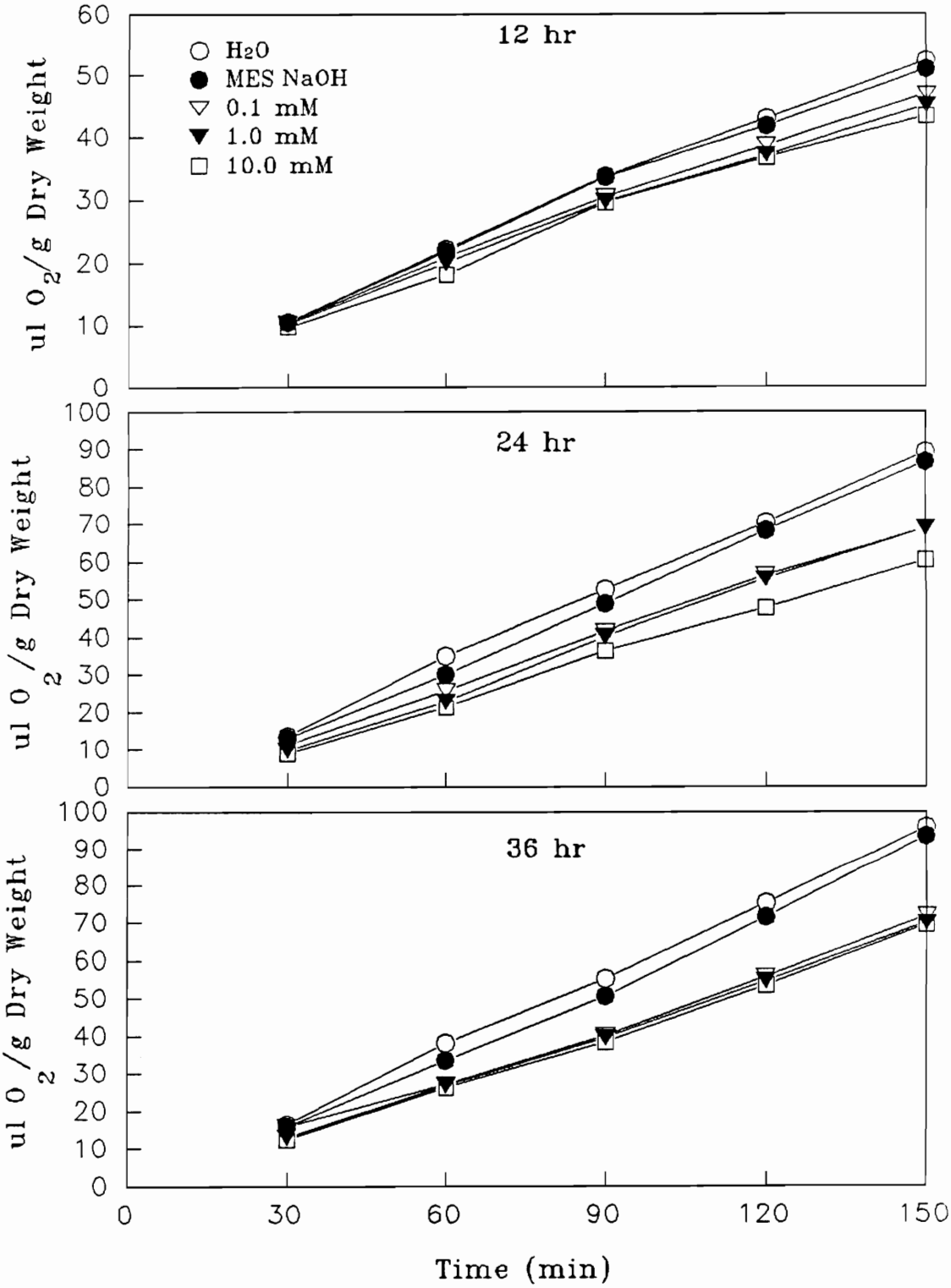
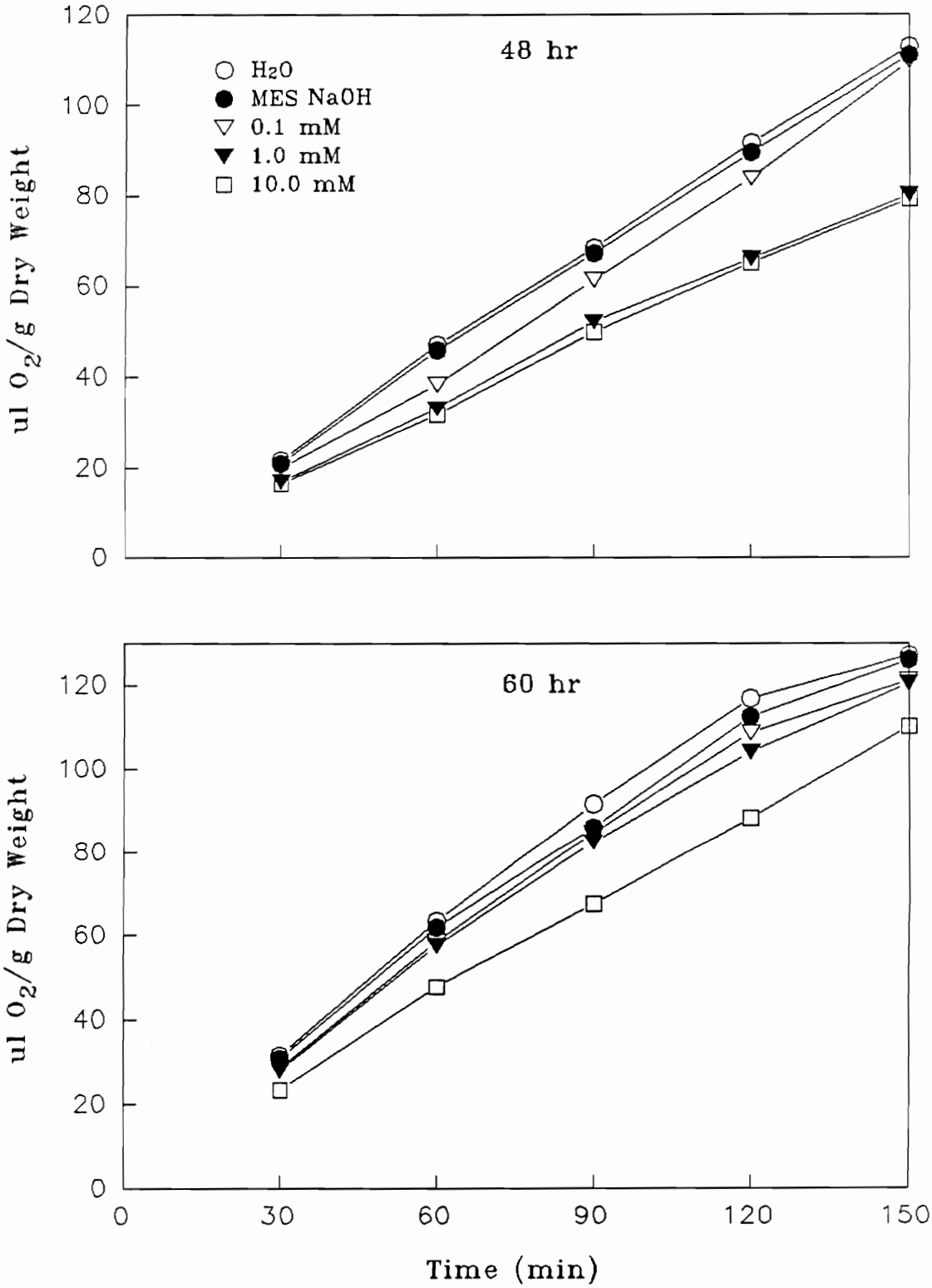

12 hr
24 hr
36 hr
H2O
MES NaOH
0.1 mM
1.0 mM
10.0 mM
ul O2/g Dry Weight
Time (min)
0
30
60
90
120
150

Figure 6.6. Effects of gallic acid on respiration of germinating corn seeds at 48 and 60 h after treatment. For each time interval, O_2 uptake readings were done every 30 minutes over a period of 150 minutes. No significant difference between treatments was shown by contrast analysis.


48 hr
60 hr
H2O
MES NaOH
0.1 mM
1.0 mM
10.0 mM
ul O2/g Dry Weight
Time (min)
0
20
40
60
80
100
120
0
30
60
90
120
150

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SUMMARY AND CONCLUSIONS

The main objectives of this research were to investigate the potential of three of the most commonly occurring phenolic acids to inhibit corn (*Zea mays* L.) germination and seedling growth and to characterize the nature of their combined effects in bioassays in petri dishes and soils. Other objectives included comparison of the activity of the acids on three different crop species, their interaction with the synthetic auxin 2,4-D, and their effects on corn respiration during germination.

Ferulic, gallic, and vanillic acids were not equally inhibitory to corn, radish (*Raphanus sativus* L.), and peanut (*Arachis hypogaea* L.), suggesting a sensitivity differential among these plant species. Sensitivity of the three species can be ranked as radish > corn > peanut. The phytotoxic activity of phenolic acids tested was concentration dependent and was more pronounced on radicle growth than on either germination or shoot growth. The threshold levels for significant growth inhibition generally occurred in the range of 0.1 and 1 mM depending on the growth variable investigated.

When used in two-way combinations in bioassays, the interactive effects of phenolic acids on corn were generally found to be additive. However, all levels of ferulic acid, generally antagonized higher levels of either gallic or vanillic acids suggesting that ferulic acid may have decreased root uptake of the other two

phenolic acids. Another explanation may be that the acids were not taken up equally by corn roots. A limited number of the significant interactions were synergistic and occurred only when gallic acid was present in the combination.

Higher phenolic acid concentrations were required to inhibit growth of corn grown in soil than those which were phytotoxic in bioassays. Moreover, the importance of inhibition was much lower in soil than in petri dishes. These results suggest that phenolic acids may not be a problem in soil systems or may only be a problem in soils where they are not inactivated. An assessment of the rates and duration of phenolic acid production and accumulation in soil is certainly necessary to ascertain these conclusions. In assessing the dynamics of phenolic acids in soils, consideration should be taken of soil properties, nutrient status of the system, microbial biomass inter-relationships, moisture, temperature, pH and etc.

The synthetic auxin 2,4-D (2,4-dichlorophenoxyacetic acid) did not interact similarly with ferulic and gallic acids. Whereas lower 2,4-D levels stimulated germination when combined with ferulic acid, they did not cause this stimulation when gallic acid was present in the mixture. Moreover, 2,4-D did not overcome the inhibition of corn root and shoot length caused by gallic acid but antagonized the phytotoxic effects of ferulic acid. These findings point to the possibility that these two phenolic acids may have different mode of action in inhibiting corn germination and seedling growth. This is further supported by the fact that ferulic acid alone was more inhibitory to root and shoot length while gallic acid

alone caused more reduction in root and shoot dry weight; and that, in combination, some interactions of ferulic and gallic acids were synergistic.

Neither ferulic nor gallic acids inhibited water uptake or respiration of germinating seeds of corn. Thus the inhibitory effects of these acids observed in bioassays cannot be explained by their adverse effects on these two processes. Surely some other process has been altered, and more research on the mode of action of the phenolic acids tested ie therefore warranted.

VITA

Fatima El Abdaoui was born in Ait Brahim, a small town of the Province of Meknes in the Mid-Atlas region of Morocco, on September 21, 1953. She is the eldest of two children of Itto bent Moha El Abdaoui and Mohamed ben Driss. She attended elementary and higher secondary schools in Meknes, Morocco and obtained the Baccalaureat Diploma in July, 1975. She received the Agricultural Engineer Degree from the National Agricultural School of Meknes in July, 1979 and the Phytochemistry Certificate from the State University of Agronomic Sciences of Gembloux, Belgium in July, 1981. She then worked for the Moroccan government as head of experimental station until September, 1985, when she came to the United States. In September, 1986 she joined the Department of Plant Pathology, Physiology, and Weed Science at Virginia Polytechnic Institute and State University as a Graduate Research Assistant to complete her doctoral program under the direction of Dr. E. S. Hagood.

Fatima

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